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Eversa® Transform 2.0 lipase: Immobilization strategies and performance in FAME and phospholipids synthesis

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Bruno Ricardo Facin

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O presente trabalho em nível de doutorado foi avaliado e aprovado por banca examinadora composta pelos seguintes membros:

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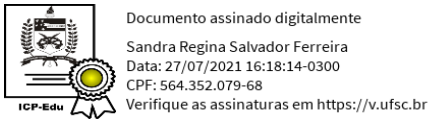

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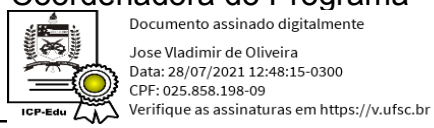

Prof. José Vladimir Oliveira, Dr.
Orientador

Florianópolis, 2021.

Este trabalho é dedicado aos meus queridos pais, irmãos, esposa e ao fiel amigo Nino.

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Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no mar. Mas o mar seria menor se lhe faltasse uma gota. (CALCUTA, Madre Teresa)

RESUMO

Os biocatalisadores vêm ganhando espaço na química orgânica como uma alternativa aos processos químicos convencionais visto o grande número de vantagens que são capazes de conferir. Esses fatores impulsionaram a produção e a comercialização de enzimas em geral, mas o alto custo ainda é considerado uma barreira à sua difusão em processos comerciais, mesmo com seus aspectos tecnológicos conhecidos e aprovados. Em vista disso, novos suportes e técnicas de imobilização enzimática têm sido utilizados a fim de promover estabilidade enzimática e garantir uma catálise economicamente eficiente por meio da recuperação e reutilização destes biocatalisadores. Desta forma, este trabalho investigou a imobilização da recente lipase comercial Eversa® Transform 2.0 solúvel (ET2) em diferentes suportes por meio de diversas técnicas e validou a eficiência dos derivados enzimáticos na síntese de ésteres metílicos de ácidos graxos (FAME) a partir de resíduos graxos industriais e na síntese de fosfolipídios em sistemas anidros. A ET2 imobilizada por aprisionamento em espuma flexível de poliuretano (EI-PU) apresentou carga máxima de 9,13 mg/g (enzima/suporte), 72,1% de atividade residual em relação à enzima livre, comportamento semelhante à enzima livre em estudos de estabilidade à temperatura e pH, valores acima de 91% para conversão em FAME e pôde ser reutilizada por até quatro ciclos de síntese desses compostos. O planejamento fatorial revelou que as melhores condições operacionais para a síntese de FAME foram 2% em massa de água, 2,0 eqv de metanol, 300 ppm de NaOH e 500 ppm do cofator enzimático. A ET2 imobilizada por interação hidrofóbica em suporte comercial *Immobead* ADS-3 (ADS3) apresentou carga máxima de 160 mg/g (enzima/suporte) e excelentes resultados na síntese de fosfolipídios mantendo rendimentos acima de 63% após 6 ciclos de reação. Além disso, apresentou estabilidade tanto em meio contendo 30% de butanona quanto em sistema livre de solvente. Assim, esses resultados sugerem uma via econômica do uso desse biocatalisador imobilizado sendo excelentes as possibilidades para, inclusive, novas aplicações.

Palavras-chave: Biocatalisador. Lipase. Imobilização enzimática. Biodiesel. FAME. Esterificação. Fosfolipídio.

RESUMO EXPANDIDO

Introdução

Os biocombustíveis têm sido intensamente estudados nos últimos anos como uma alternativa viável para a crescente demanda energética global. Dentre eles, o biodiesel surge com a vantagem de poder ser adicionado ao diesel de petróleo. Na última década, atenção especial foi dedicada ao uso de lipases como biocatalisadores para a produção de biodiesel, já que permite o uso de matéria-prima de baixo custo proveniente de indústrias de petróleo ou recuperada de estações de tratamento de efluentes. Essa percepção levou ao lançamento da lipase Eversa® Transform 2.0, uma formulação de lipase líquida desenvolvida especialmente para essa finalidade. No entanto, a aplicação industrial de lipases livres é comumente dificultada por sua fácil desativação, baixa estabilidade operacional, baixa recuperação e reutilização e, principalmente, pelo custo do biocatalisador. Nesse sentido, uma imobilização adequada pode melhorar as propriedades enzimáticas e contribuir para a viabilidade econômica do processo, principalmente por facilitar os ciclos de reuso. Além disso, um derivado imobilizado pode apresentar uma nova gama de aplicações onde o biocatalisador original poderia não performar, como na esterificação de fosfolipídios em sistemas anidros. A síntese de fosfolipídios via processo enzimático geralmente ocorre em condições mais amenas e, devido à alta estereosseletividade dos biocatalisadores, permite a obtenção de produtos que os métodos químicos não conseguem. Assim, por se tratar de uma enzima ainda recente no mercado, poucos trabalhos são encontrados na literatura reportando a imobilização da lipase Eversa® Transform 2.0 ou ainda sua aplicação em sistemas que não envolvam a produção de biodiesel.

Objetivos

O objetivo deste trabalho foi imobilizar a lipase Eversa® Transform 2.0 em diferentes suportes por meio de diferentes técnicas e avaliar a eficiência dos derivados enzimáticos na síntese de ésteres metílicos de ácidos graxos (FAME) a partir de um resíduo graxo industrial e na síntese de fosfolipídios em sistemas anidros.

Metodologia

A lipase comercial Eversa® Transform 2.0 (ET2) de *Aspergillus oryzae* foi fornecida pela Novozymes (Araucária, PR, Brasil). Os monômeros poliol poliéter e difenilmetano diisocianato (MDI) para síntese de poliuretano (PU) foram gentilmente doados pela Purcom Company (fabricante industrial de espuma flexível de PU no Brasil). Resíduos graxos industriais foram gentilmente cedidos pelas empresas Prisma Comercial Exportadora de Oleoquímicos LTDA (Sumaré, SP, Brasil) e Transfertech (Erechim, RS, Brasil). Immobead IB-ADS-3 e Immobead IB-COV-3 foram adquiridos da Chiral Vision (Den Hoorn, Holanda) e SP Sepharose Fast Flow da GE Healthcare (Madrid, Espanha). O p-nitrofenil butirato (pNPB), a polietilenimina, MW 25000 (PEI) e o ácido oleico foram adquiridos a Sigma-Aldrich (Darmstadt, Alemanha). Sn-glicero-3-fosfocolina (GPC) foi adquirido da Bachem AG (Bubendorf, Suíça). ET2 foi imobilizada em espuma de PU por aprisionamento durante a síntese do suporte. A atividade enzimática foi determinada por meio da atividade de hidrólise em óleo de soja determinado por titulometria. A síntese de FAME a partir de resíduos graxos industriais foi realizada a 45 °C usando um incubador horizontal (250 rpm) em um sistema em batelada alimentada usando um

frasco selado de 1,5 L e seguiu um planejamento fatorial 24, sendo água, NaOH, cofator enzimático e metanol as variáveis. A conversão em FAME for determinada por titulometria seguindo a ISO 660:2009. Os derivados enzimáticos foram caracterizados por densidade aparente, microscopia eletrônica de varredura (MEV) e FTIR. As estabilidades ao pH, temperatura e armazenamento foram determinadas pelo desempenho da atividade enzimática após a exposição dos derivados em condições pré-estabelecidas. Os ensaios de reuso na síntese de FAME foram realizados na melhor condição encontrada pelo planejamento fatorial. A ET2 foi também imobilizada em Immobead IB-ADS-3 por interação hidrofóbica e em Immobead IB-COV-3 (recoberto com PEI) e SP Sepharose por interação iônica por meio de simples imersão dos suportes em solução enzimática. A atividade enzimática foi determinada espectrofotometricamente utilizando pNPB. A síntese de fosfolipídios foi realizada em sistema composto por GPC e solução de ácido oleico e butanona a 40 °C e em agitador horizontal (150 rpm). A formação dos produtos oleoil-lisofosfatidilcolina (oleoil-LPC) e dioleoil-lisofosfatidilcolina (dioleoil-PC) foi determinada por cromatografia em camada delgada utilizando solução de metanol-clorofórmio-hidróxido de amônio (65:25:4) como fase móvel. A estabilidade dos derivados for verificada em diferentes concentrações de butanona no mesmo sistema de síntese de fosfolipídios. Os ensaios de reuso na síntese de fosfolipídios foram realizados para o derivado mais promissor dentre os avaliados.

Resultados e discussão

ET2 foi imobilizada com sucesso em espuma flexível de poliuretano (PU) pela técnica de aprisionamento e o derivado enzimático (EI-PU) foi avaliado para a síntese de FAME a partir de resíduos graxos industriais. O planejamento fatorial revelou que a condição 2% em peso de água, 2,0 eqv de metanol, 300 ppm de NaOH e 500 ppm de cofator enzimático foi a que melhor performou para essa matéria-prima. EI-PU foi capaz de catalisar a síntese de FAME sob essas condições, atingindo conversão acima de 91% em 24 h de reação. O EI-PU não apresentou separação de fases, boa distribuição de poros (macroporos bem distribuídos) e homogeneidade. O derivado enzimático não apresentou inativação durante o processo de imobilização e foi capaz de reter até 72,1% da atividade inicial. Em relação à temperatura, pH e estabilidade de armazenamento, foram observados resultados semelhantes aos da enzima livre. Apesar de não ter sido capaz de garantir melhorias na estabilidade da enzima frente a temperatura e pH, o processo de imobilização permitiu a reutilização do biocatalisador por quatro ciclos de síntese de FAME. Este trabalho mostrou pela primeira vez um relato bem-sucedido de imobilização da lipase Eversa® Transform 2.0 solúvel em espuma de PU. A ET2 foi também imobilizada em outros suportes comercialmente disponíveis por diferentes técnicas visando aplicação na síntese de fosfolipídios (lisofosfatidilcolina, LPC). Os melhores resultados foram encontrados para a estratégia de imobilização por adsorção física por interação hidrofóbica em Immobead ADS-3. O derivado obtido (ADS3) apresentou alta carga enzimática (até 160 mg/g, enzima/suporte) e excelentes resultados na síntese de LPC, além de ser estável em 30% de butanona e sistema livre de solvente. Para o estudo de reutilização, rendimentos acima de 63% após seis ciclos de reação na síntese de LPC foram alcançados. Os resultados aqui obtidos demonstram, pela primeira vez, a performance satisfatória da lipase Eversa® Transform 2.0 para a síntese de LPC.

Considerações finais

Mesmo que a lipase Eversa® Transform 2.0 tenha sido originalmente projetada para ser utilizada como enzima livre, uma imobilização adequada permitiu ainda mais eficiência na produção de FAME, principalmente pela possibilidade de reutilização. Além disso, outra nova aplicação pode ser vislumbrada na qual a enzima original não havia sequer sido empregada. Portanto, a imobilização da lipase Eversa® Transform 2.0 solúvel pode ser considerada economicamente atrativa com perspectivas de aplicações não apenas na síntese de biodiesel, mas em outras reações de interesse, uma vez que investigações adequadas possam ser desenvolvidas para este objetivo.

Palavras-chave: Biocatalisador. Lipase. Imobilização enzimática. Redução de custo. Biodiesel. FAME. Esterificação. Fosfolipídio.

ABSTRACT

Biocatalysts have been outstanding in organic chemistry as an alternative to conventional chemical processes due to a large number of advantages they are capable of conferring. These factors boosted the production and commercialization of enzymes in general, but the high cost is still considered a barrier to their diffusion in commercial processes, even with their known and proved technological aspects. Thus, new supports and techniques for enzyme immobilization have been used to promote enzyme stability and guarantee economically efficient catalysis through the recovery and reuse of these biocatalysts. This work explored the immobilization of the commercial soluble Eversa® Transform 2.0 lipase (ET2) on different supports through different techniques and proved the efficiency of enzyme derivatives on the synthesis of fatty acid methyl esters (FAME) from industrial fatty waste and on the synthesis of phospholipids in anhydrous systems. The ET2 immobilized by entrapment in flexible polyurethane foam (EI-PU) had a maximum enzyme loading of 9.13 mg/g (enzyme/support) and up to 72% of residual activity, in relation to the free enzyme. In addition, from temperature and pH stability studies, the EI-PU was able to provide a very similar behavior to the free enzyme. For FAME conversion, values above 91% were found, and the EI-PU could be reused for four cycles. Factorial design revealed that the best operating conditions for the FAME synthesis were 2 wt% of water, 2.0 eqv of methanol, 300 ppm of NaOH, and 500 ppm of the enzymatic cofactor. ET2 immobilized by hydrophobic interaction on commercial support Immobead ADS-3 (ADS3) had a maximum enzyme loading of 160 mg/g (enzyme/support), excellent results in phospholipid synthesis, maintaining yields above 63% after 6 reaction cycles, and was stable either in 30% butanone or in a solvent-free system. Thus, these results suggest an economical route for these immobilized biocatalysts, with excellent possibilities for even new applications.

Keywords: Biocatalyst. Lipase. Enzyme immobilization. Biodiesel. FAME. Esterification. Phospholipid.

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SUMMARY

CONCEPTUAL DIAGRAM

Why?

- ✓ Lipases play a very important role in the technological processes in several industrial segments;
- ✓ Eversa® Transform 2.0 lipase is a recent low expensive liquid lipase formulation;
- ✓ Enzymatic biodiesel has received increasing attention due to the high global energetic demand;
- ✓ Phospholipids are a important class of bioactive compounds.

Who did?

- ✓ Few reports were found in the literature for the immobilization of soluble Eversa® Transform 2.0 lipase;
- ✓ There are few reports of lipases immobilization on polyurethane foams;
- ✓ No reports were found in the literature for the application of Eversa® Transform 2.0 lipase for the synthesis of phospholipids.

Hypotheses

- ✓ The immobilization of soluble lipase Eversa® Transform 2.0 on polyurethane polymeric support can be performed efficiently?
- ✓ The soluble Eversa® Transform 2.0 lipase can be immobilized on commercially available supports?
- ✓ How does the immobilization process affect the properties of the immobilized derivatives?
- ✓ Is it possible to apply the immobilized derivatives on reactions of industrial interest, such as biodiesel and phospholipids production?

Methodologies

- ✓ Immobilization of Eversa® Transform 2.0 lipase by diverse techniques in several supports;
- ✓ Immobilized derivatives characterized regarding pH, temperature, solvent, and storage stabilities;
- ✓ Reactions of interest performed at lab scale.

Responses

- ✓ Promote the effective immobilization of Eversa® Transform 2.0 lipase;
- ✓ Optimize the experimental conditions that lead to greater conversion of biodiesel production ;
- ✓ Determine the behavior of immobilized derivatives on phospholipids synthesis in anhydrous media.

1 INTRODUCTION

Biofuels have been intensively studied in recent years as a viable alternative fuel for the rising energy demand currently supplied by fossil sources. Among them, biodiesel has the advantage of can be blended with petroleum-based diesel (MEHER; VIDYA SAGAR; NAIK, 2006; MIBIELLI et al., 2019; REMONATTO et al., 2016). Over the last decade, particular attention has been dedicated to the use of lipases as biocatalysts for biodiesel production (NIELSEN et al., 2016; PRICE et al., 2016; REMONATTO et al., 2016). The enzyme-based biodiesel production allows the use of low-cost feedstock from oil industries or recovered from effluent treatment plants. Moreover, it is considered a green route since it requires less energy and also is a highly selective catalyst (MEHER; VIDYA SAGAR; NAIK, 2006). This insight had drawn attention to one of the great global enzyme manufacturers, Novozymes, to launch the Eversa® Transform 2.0 lipase, a liquid lipase formulation specially designed for biodiesel production (MONTEIRO et al., 2021).

Lipases (triacylglycerol hydrolases, EC 3.1.1.3) are the most often used enzymes for biocatalysis applications due to their broad specificity associated, in many instances, to a high regio- and enantioselectivity (KUMAR et al., 2016; REIS et al., 2009a). Nevertheless, the industrial application of free lipases is commonly hindered by their easy deactivation, low operational stability, low recuperation and reuse, and especially, the biocatalyst cost. In this sense, proper immobilization can improve the enzymatic properties and contribute to the economic viability of the process mainly by favoring the reuse cycles (ALI KHAN; ALZOHAIRY, 2010; REMONATTO et al., 2018). Moreover, an immobilized derivative may present a new range of applications where the original biocatalyst could not perform itself, such as the esterification of phospholipids in anhydrous systems. The synthesis of phospholipids via enzymatic process usually occurs under milder conditions and, due to the high stereoselectivity of the biocatalysts, allows the obtaining of products that chemical methods cannot (MNASRI et al., 2017).

Interfacial adsorption onto hydrophobic supports is the most common method for lipase immobilization (ALVES et al., 2017; SUESCUN et al., 2015; URRUTIA et al., 2018). This is related to the catalytic mechanism of lipases which involves a polypeptide lid that blocks the active center, but when in contact with hydrophobic

surfaces shifts and exposes the active center (ARANA-PEÑA; LOKHA; FERNÁNDEZ-LAFUENTE, 2018; FUENTES et al., 2008). In this sense, polyurethanes (PU) are a promising class of hydrophobic polymers used as supports for enzyme immobilization (CUI et al., 2013; LI; DYER; GREENWELL, 2018; NYARI et al., 2017) considering they have attractive properties such as mechanical strength and solvent resistance, as well as high durability and low cost (CIPOLATTI et al., 2015; VALÉRIO et al., 2014; VALÉRIO; ARAÚJO; SAYER, 2013; ZANETTI-RAMOS et al., 2006, 2008). On the other hand, whenever a new enzyme is discovered or becomes available, a series of immobilization studies are initiated involving the wide variety of supports currently available. That is because each enzyme interacts with the support in a distinct way, making the support suitable for one enzyme not necessarily suitable for all (ZDARTA et al., 2018).

Thus, the present work aimed to evaluate different strategies to immobilize the Eversa® Transform 2.0 lipase. The immobilized derivatives were proved on the synthesis of fatty acid methyl esters (FAME) from an industrial fatty waste and on the synthesis of phospholipids in anhydrous systems. In addition, the immobilized biocatalysts were evaluated in terms of stability at different ranges of temperature, pH, solvent, and storage conditions. To the best of our knowledge, there are no reports on the open literature about the immobilization of this lipase (soluble) in the supports investigated in this study. It is expected that this work might contribute to the development of the biodiesel field by exploring the use of a soluble, low-cost commercial lipase immobilized on an inexpensively support and using non-edible, waste raw materials as substrates. Moreover, this work is expected to encourage other researchers to explore the use of this enzyme in other applications than that proposed by the manufacturer to expand the range of Eversa® Transform 2.0 lipase applications.

1.1 OBJECTIVES

1.1.1 Main objective

The main goal of this work is to immobilize the soluble Eversa® Transform 2.0 lipase on several supports through different techniques and then to evaluate the efficiency of enzyme derivatives on the synthesis of fatty acid methyl esters (FAME) from an industrial fatty waste and on the synthesis of phospholipids in anhydrous systems.

1.1.2 Specific objectives

- Immobilize soluble Eversa® Transform 2.0 lipase on PU support by entrapment;
- Immobilize soluble Eversa® Transform 2.0 lipase in other commercially available supports by different techniques;
- Evaluate the enzyme activity of all immobilized derivatives;
- Characterize the structure of the PU supports with the Eversa® Transform 2.0 lipase immobilized;
- Evaluate the stability of the immobilized derivatives regarding pH, temperature, solvent, and storage condition;
- Evaluate the efficiency of the PU supports with the Eversa® Transform 2.0 lipase immobilized for FAME synthesis;
- Evaluate the efficiency of the others immobilized derivatives on the synthesis of phospholipids in anhydrous systems.

In order to better present the key issues and the obtained results, this thesis was divided into five chapters.

Chapter 2 describes, concisely, the enzymes, with emphasis on the lipases and their peculiar enzymatic mechanism, techniques for lipase immobilization, and available supports. Moreover, it includes the immobilization of the recent Eversa® Transform 2.0 lipase and, finally, the biodiesel field and phospholipids synthesis are discussed.

Chapter 3 involves the immobilization of soluble Eversa® Transform 2.0 lipase in polyurethane foam and its application for the evaluation of the parameters for the FAME synthesis. In this section, the effect of various operating parameters on FAME synthesis as water, methanol, NaOH, and cofactor are deeply discussed.

Chapter 4 includes discussions around the improvement of the immobilized biocatalyst obtained in Chapter 3 aiming for better kinetics on FAME synthesis. The characterization of the new enzyme derivative regarding structure, and pH, thermal, and storage stabilities is also presented. Finally, the reuse cycles on FAME synthesis are estimated.

Chapter 5 was developed in cooperation with *Instituto de Investigación en Ciencias de la Alimentación* (Spain) through a *Sandwich* period funding by PRINT-CAPES (Brazil). This Chapter comprises the immobilization of soluble Eversa® Transform 2.0 lipase in several supports by different strategies. Moreover, the synthesis of phospholipids in anhydrous media catalyzed by the immobilized derivatives is discussed.

Finally, **Chapter 6** summarizes important conclusion of the thesis and contemplates possible future perspectives.

2 LITERATURE REVIEW

This chapter presents a brief review of the pertinent subjects to this work. Firstly, the main information about enzymes with emphasis on lipases and some prospects in the immobilization techniques are introduced. Then, important aspects about the choice of the support for lipase immobilization are described and the current state of the art of immobilized lipases is presented. Finally, the biodiesel field and the synthesis of phospholipids are discussed.

Part of this chapter was published in Industrial & Engineering Chemistry Research as "**Driving immobilized lipases as biocatalysts: 10 years state of the art and future prospects**". "Reprinted with permission from FACIN et al. (2019). Copyright 2019 American Chemical Society."

2.1 ENZYMES

Enzymes are organic substances of protein or, in some cases, glycoproteic nature. They act by reducing the activation energy necessary for biochemical reactions that may occur without distorting their equilibrium since they are not consumed in the process. This function places enzymes as biological catalysts since they are able to accelerate, or even promote biochemical reactions of substrates in products that probably would not happen or would be excessively slow without its presence (NELSON; COX, 2004).

The enzymes may be obtained from animal, vegetable or microbial origin. The preference from the economic and industrial point of view is by microbial sources due to the relative cost of your isolation. According to the literature, it is estimated about 35% of the current biochemical processes be occupied by the class of lipases (DALLA-VECCHIA; NASCIMENTO; SOLDI, 2004; PAQUES; MACEDO, 2006).

Biocatalysts have been reaching attention in organic chemistry as an alternative to conventional chemical processes due to a large number of advantages, such as the employ of milder process conditions, compatibility with synthetic substrates, catalysis of two-way reactions, in some cases, and great selectivity as to the type of reaction they catalyze. These factors have boosted the production and commercialization of enzymes in general, so new applications in the industrial sector

emerge requiring a continuous flow of innovative products from the sector itself (PAQUES; MACEDO, 2006).

The high cost of enzymes was considered for a long time as an obstacle to their use in commercial processes, even with their known and proven technological aspects. These catalysts were not economically viable or could not compete with synthetic products. However, due to the countless and incessant researches developed for overcoming this barrier, that scenario has changed (SANTOS, 2016).

The enzymes can be classified into seven groups: oxidoreductases, transferases, lyases, isomerases, ligases, hydrolases, and translocases. For organic chemistry, the most investigated are the hydrolases, such as proteases, cellulases, amylases, and lipases. Among the several reasons that make this class particularly attractive are wide availability, low cost, mild synthesis conditions, high specificity for substrates, and no need for cofactors. Special attention is given to lipases, which are used for the hydrolysis of fatty esters in nature as well as to act in polycondensation and transesterification reactions, ring-opening polymerizations, and polymer modification reactions (DALLA-VECCHIA; NASCIMENTO; SOLDI, 2004; DE GEUS, 2007).

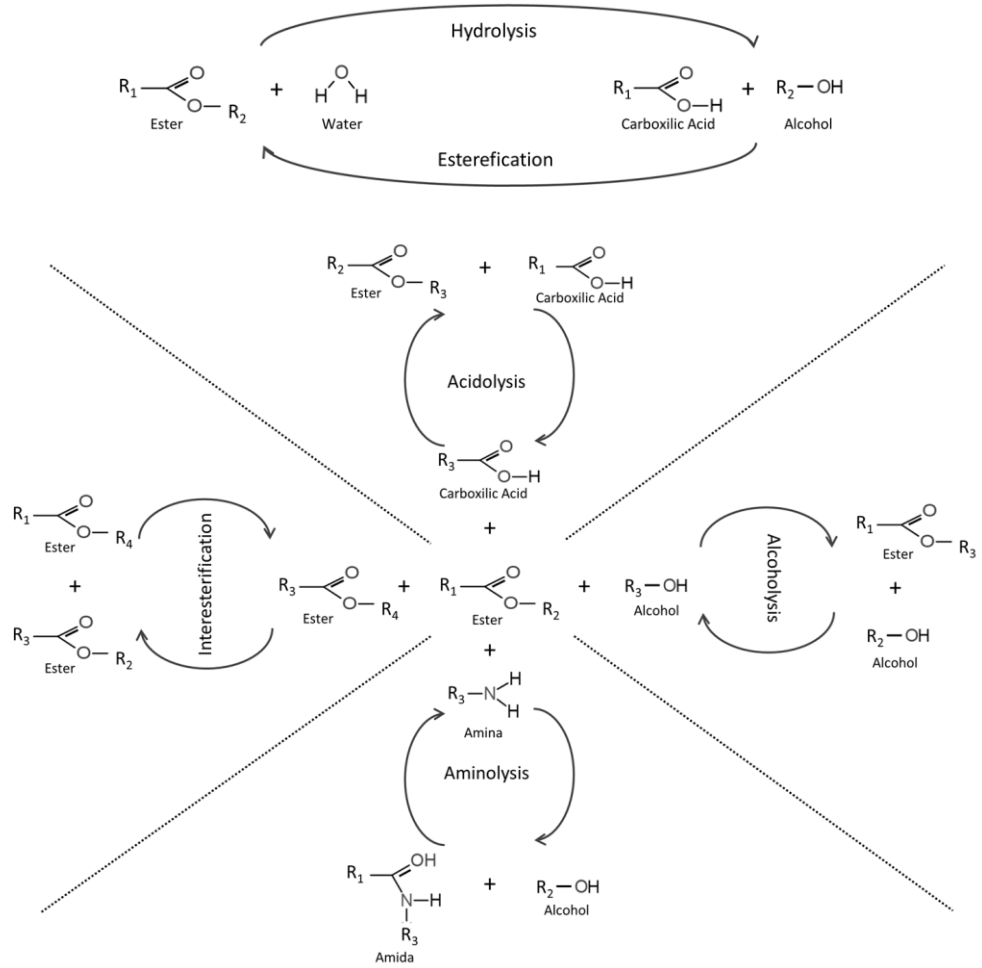
2.1.1 Lipases

Lipases (triacylglycerol hydrolases, EC 3.1.1.3) are the most important enzymes employed in organic synthesis due to the ability to carry out bioconversion reactions in a wide range of industrial applications such as biofuels, detergents, food, cosmetics, personal care, and drugs (DE SOUZA et al., 2018; GUPTA et al., 2015; MALCATA et al., 1992; NUNES et al., 2011; STERGIOU et al., 2013; TRAN et al., 2013). The natural substrates for lipases are fat and oils, which are hydrolyzed to fatty acids and glycerol, but under suitable conditions, these enzymes are able to catalyze other reactions such as esterification, acidolysis, alcoholysis, interesterification, aminolysis, and glycerolysis (PAIVA; BALCÃO; MALCATA, 2000; PATEL; NAGARAJAN; KILARA, 1996; ZAKS; KLIBANOV, 1985).

Lipases have been originally designed to catalyze ester bonds via hydrolysis reactions with simultaneous consumption of water molecules. However, considering the principle of reversibility, the reverse reaction of ester synthesis also occurs

(PATEL; NAGARAJAN; KILARA, 1996). The equilibrium between both reactions depends on the water content of the reaction mixture and the combination of these two basic processes in a sequential mode can lead to other reactions usually namely transesterification (MALCATA et al., 1992). According to substrates available on the reaction mixture it may be termed acidolysis (ester + carboxylic acid), alcoholysis (ester + alcohol), and interesterification (ester + ester) (REIS et al., 2009a). In addition, lipases are able to catalyze other reactions with the consumption of specific substrates, such as aminolysis and glycerolysis (STERGIOU et al., 2013). In fact, in all such processes water is needed both for maintenance of the enzyme structural integrity and generation of the catalytic intermediate, being sequentially consumed and formed in the course of the reaction (PAIVA; BALCÃO; MALCATA, 2000). The reactions that can be catalyzed by lipases are schematically represented in Figure 1.

Figure 1 - General representation of lipase-catalyzed reactions.



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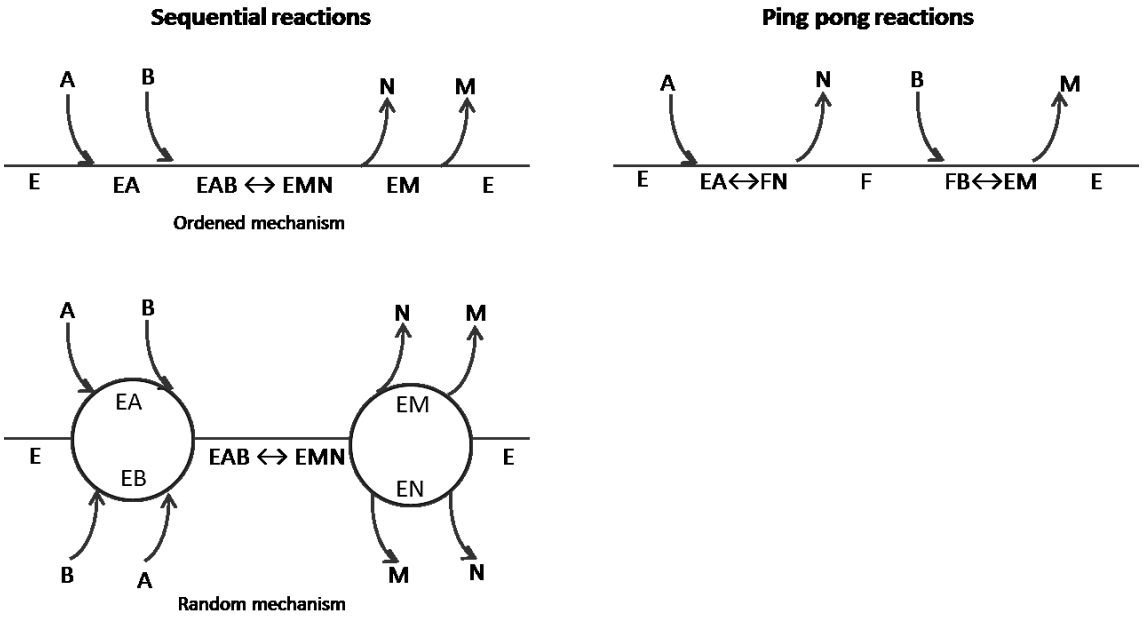
Independent of the reaction type, the most accepted description of the catalytic action of lipases is a Ping-Pong Bi-Bi mechanism (PAIVA; BALCÃO; MALCATA, 2000). This mechanism means two substrates generate two products (bi-bi) in a no-ordered (ping pong) way, i.e., the lipase releases the first product before binding all substrates (see Figure 2). Two main steps are known and describe the catalytic action of lipases: the first step consists of a nucleophilic attack of the serine hydroxyl group on the substrate ester bond resulting in the formation of an acyl-enzyme and release of the alcohol moiety of the original substrate; the second step is the hydrolysis of the acylated enzyme complex resulting in the formation of the product and the regeneration of the enzyme (MALCATA et al., 1992; PAIVA; BALCÃO; MALCATA, 2000).

Figure 2 - Kinetic mechanisms for enzyme-catalyzed reactions.

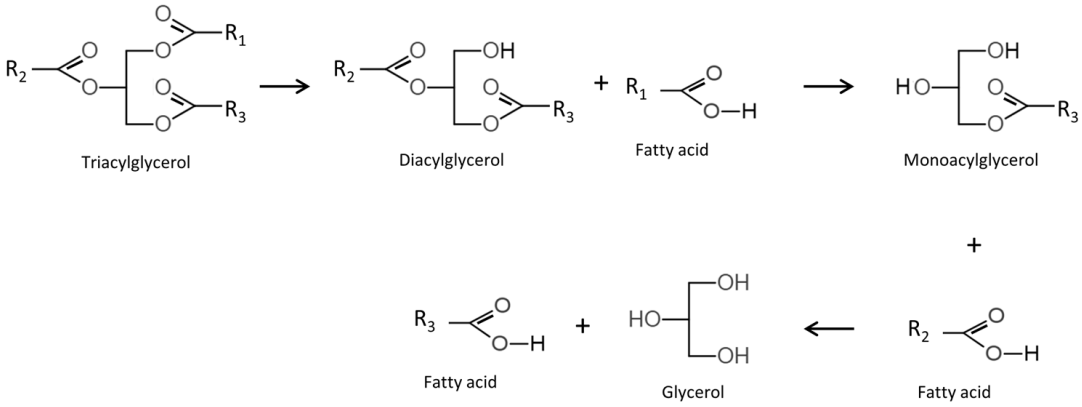


Source: Author.

As mentioned, the natural substrates of lipases are triacylglycerols (TAG) of long-chain fatty acids (fats and oils) which via hydrolysis reaction release diacylglycerols (DAG), monoacylglycerols (MAG), and glycerol, with fatty acids being released at each step (Figure 3) (PATEL; NAGARAJAN; KILARA, 1996). These fatty acids may be released either randomly from any position or preferentially from a specific position, depending on the origin of the lipase employed (TRAN et al., 2013). Lipases from *Chromobacterium oiscosum, Pseudomonas fluorescens, Candida*

cylindracea, Geotrichum candidum, and *Penicillium eyelopium* are considered as nonspecific and they release fatty acid in any position. *Rhizopus arrhizus* lipase, *Aspergillus niger* lipase, *Rhizopus delemar* lipase, and *Mucor miehei* lipase are known as *sn*-1,3 type since preferentially release the fatty acids from the terminal positions of the glycerol backbone. Meanwhile, sn-2 specificity is extremely rare and refers to preferential release from the center of the structure, such as lipase from *Geotrichum candidum* (MALCATA et al., 1992).

Figure 3 - Hydrolysis reaction of triacylglycerols catalyzed by lipases.



Source: Author.

The lipase B from *Candida antarctica* (CalB) is currently one of the most widespread lipases for industrial applications since it has high stability, stereoselectivity, broad substrate specificity, and enantio preference properties that make it superior to other lipases in biotransformation. The CalB was developed by the Novozymes S/A (Denmark) and it is produced from the B fraction of the *yeast Candida antarctica* (VALÉRIO et al., 2015).

The lipase Eversa® Transform has also been developed by Novozymes S/A (Denmark) and it has become commercially available in December 2014. In May 2016, the second generation of this lipase, the Eversa® Transform 2.0, more stable, was launched on the market. According to the Novozymes S/A, both lipases have high activity on transesterification of glycerides and esterification of free fatty acids having excellent potential to the application on industrial enzymatic biodiesel production. The soluble Eversa® Transform 2.0 lipase has an enzyme component from *Thermomyces lanuginosus* produced by the submerged fermentation of genetically modified *Aspergillus oryzae* (REMONATTO, 2017). As it is a product in

continuous evolution by the manufacturer, it has received diverse names such as Callera™ Trans and NS-40116. Even though this enzyme had been launched to be used in free form, some researchers have attempted its immobilization to improve its properties and explore its utility in other applications (MONTEIRO et al., 2021).

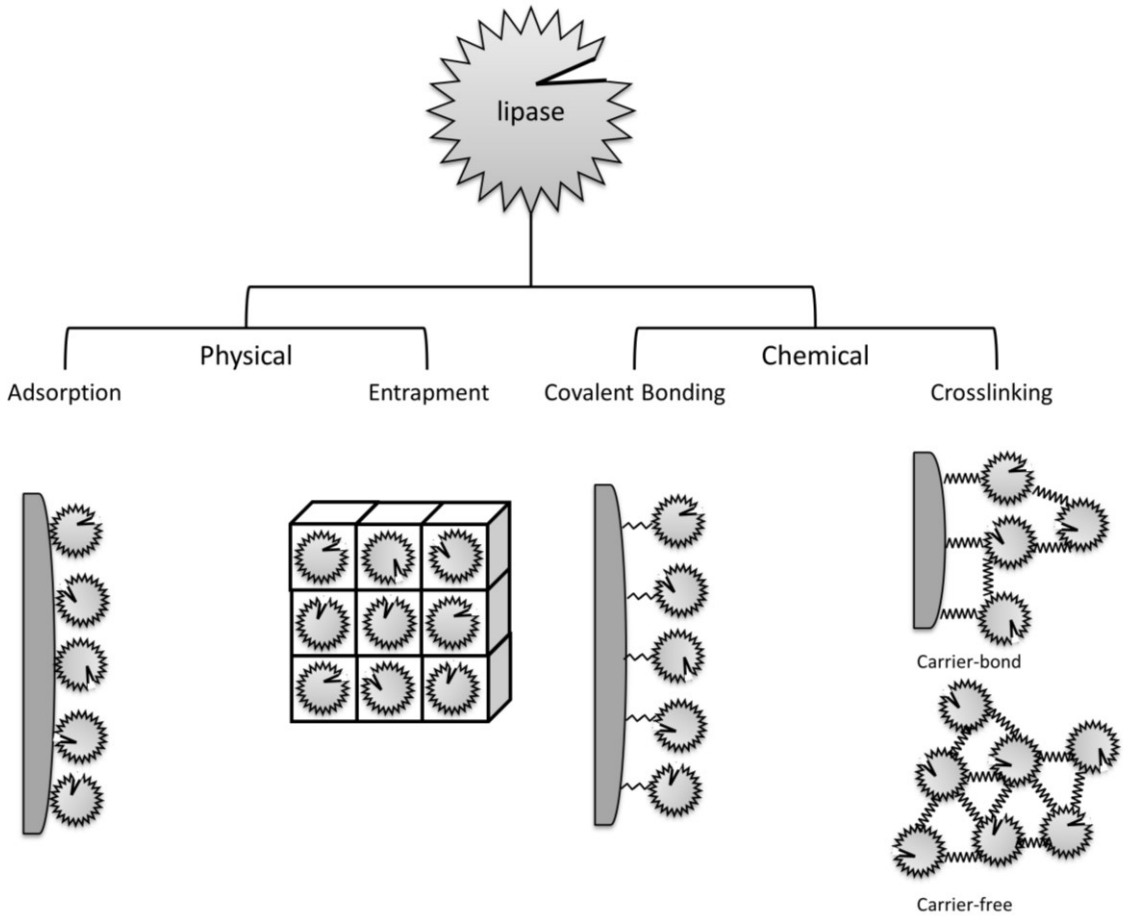
Although lipase-catalyzed reaction involves milder operational conditions than traditional reactions (chemical route), some misfortunes such as limited solubility, thermal, mechanical, and operational stability of the enzyme, as well as the impossibility of catalyst reuse, leads to high production costs thus hindering in large-scale applications (BORNSCHEUER, 2003; MOHAMAD et al., 2015; SANTOS et al., 2015). To overcome these disadvantages, immobilized enzymes on supports, especially in low-cost supports can offer some advantages such as increase activity, specificity and selectivity, improve structural stability, and ease of recovery (DATTA; CHRISTENA; RAJARAM, 2013; MERYAM SARDAR, 2015; XIE; ZANG, 2018).

2.2 ENZYME IMMOBILIZATION

The main goal of the immobilization process is to obtain a stable biocatalyst that can be reused several times with the minimal loss of initial activity. Thus, the immobilization process should be able to keep the original enzyme stability or allow the enzyme becomes highly stabilized during long periods of time (HOMAEI et al., 2013; MATEO et al., 2007).

Usually, the immobilization methods are divided into two major classes taking into account the enzyme interaction with support: chemical and physical methods (ADLERCREUTZ, 2013). Physical methods show normally physical confinement of enzyme within the support or weak and noncovalent interactions between enzyme and support such as hydrogen bonds, hydrophobic interactions, van der Waals forces, and ionic binding. On the other hand, chemical methods involve the formation of covalent bonds between enzyme and support such as ether, amide or carbamate bonds (see Figure 4) (MOHAMAD et al., 2015; SANTOS et al., 2015).

Figure 4 - General scheme of techniques for lipase immobilization.



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For the chosen immobilization method, some factors have been considered, such as lipase loading, relative enzymatic activity, cost of immobilization procedure, enzyme deactivation, the toxicity of immobilization reagents, and the final characteristics of the catalyst (HANEFELD; GARDOSSI; MAGNER, 2009; MOHAMAD et al., 2015). Since the physicochemical properties of both support surface and enzyme are known it can be used as an advantage to obtaining systems that outperform the free enzyme (CUI; JIA, 2015; HANEFELD; GARDOSSI; MAGNER, 2009). Thus, in the literature, there are different techniques for lipase immobilization. In this review, it will be explored physical adsorption, covalent bonding, cross-linked, and entrapment technique.

Table 1 presents some reports of enzyme immobilization techniques, highlighting the immobilization efficiency, the activity retained, lipase source, and support employed.

Table 1 - Summary of selected examples of immobilization techniques applied for lipase immobilization.

Immobilization technique	**Support**	**Lipase source**	**Immobilization efficiency (%)**	**Activity retention (%)**	**Ref.**
Adsorption	Fe_3O_4/ Chitosan	*Yarrowia lipolytica*	99	132	(LI; FENG; PAN, 2013)
Adsorption	Fe_3O_4 nanoparticles	*Thermomyces lanuginosus*	98	516	(HENRIQUES et al., 2018)
Adsorption	Core–shell nanoparticle Fe_3O_4/SiO_2	*Burkholderia* sp.	97	97	(TRAN; CHEN; CHANG, 2012)
Adsorption	Pullulan polysaccharide	*Burkholderia cepacia*	80	80	(XU et al., 2018)
Adsorption	Polyacrylonitrile (PAN) fibers	*Pseudomonas cepacia*	65	80	(SAKAI et al., 2010)
Adsorption	Core–shell nanoparticle styrene/divinylbenzene	*Rhizomucor miehie, Themomyces lanuginosus, Candida antarctica* B*, and phospholipase Lecitase Ultra*	-	377	(MANOEL et al., 2016)
Adsorption/Covalent bond	Porous carbon particles	*Thermomyces lanuginosus*	95	10	(REICHARDT et al., 2018)
Covalent bond	Multi-walled carbon nanotubes	*Thermomyces lanuginosus*	65	57	(VERMA et al., 2013)
Covalent bond	Glutaraldehyde-activated aminopropyl glass beads	*Candida rugosa*	98	166.9	(YILMAZ et al., 2011)
Covalent bond	Amino-Silane modified superparamagnetic Fe_3O_4	*Candida rugosa*	100	58.2	(CUI et al., 2010)
Covalent bond	Toyopearl AF-amino-650M resin	*Thermomyces lanuginosus* and *Pseudomonas fluorescens*	36.4 44	69.7 12.3	(MENDES et al., 2011)
Covalent bond	Chitosan	*Thermomyces lanuginosus*	97.3	-	(BONAZZA et al., 2018)

Covalent bond	Chitosan	*Candida antarctica* B	94.7	30.20	(DOS SANTOS et al., 2017)
Covalent bond	Octyl-glyoxyl agarose beads	*Rhizomucor miehei and Candida rugosa*	-	300	(FERNANDEZ-LOPEZ et al., 2016)
Covalent bond	Divinylsulfone-agarose beads	*Candida antarctica* B	-	150	(DOS SANTOS et al., 2015a)
Covalent bond	Divinylsulfone-agarose beads	*Pseudomonas flourescens, Rhizomucor miehei, Thermomyces lanuginosus, and Lecitase Ultra*	-	100	(DOS SANTOS et al., 2015b)
Crosslinked	Fe_3O_4/ Chitosan	*Candida rugosa*	-	55.6	(WU et al., 2009)
Crosslinked	Carrier-free	*Porcine pancreatic*	-	40	(RAMOS et al., 2018)
Crosslinked	SBA-15	*Candida rugosa*	-	80.5	(GAO et al., 2010)
Crosslinked	Octyl-silica-amino-glutaraldehyde, (OSGlu)	*Candida antarctica* B	98	71	(VESCOVI et al., 2016)
Crosslinked	Carrier-free	*Candida rugosa*	-	72.6	(SAMPATH; BELUR; IYYASAMI, 2018)
Crosslinked	Core–shell Fe_3O_4–MCM-41 nanocomposite	*Candida rugosa*	76	100	(XIE; ZANG, 2016)
Entrapment	Calcium alginate (Ca-Alg) gel beads	*Candida rugosa* and *Porcine pancreatic*	97.76 98.38	-	(OZYILMAZ; GEZER, 2010)
Entrapment	Activated carbon	*Candida rugosa*	95	100	(MORENO-PIRAJÀN; GIRALDO, 2011)
Entrapment	Celite 545	*Candida antarctica* B	94	100	(URSOIU et al., 2012)
Entrapment	Alginate	*Burkholderia cepacia*	98	92	(PADILHA; TAMBOURGI; ALEGRE, 2018)
Entrapment	β-cyclodextrin-based polymer	*Candida rugosa*	92	51	(YILMAZ; SEZGIN, 2012)
Entrapment	Polyurethane foam	*Thermomyces lanuginosus*	94.4	405	(BRESOLIN et al., 2019)
Entrapment	Chitosan-polyphosphate microspheres	*Candida antarctica* B	100	92	(MELO et al., 2017)

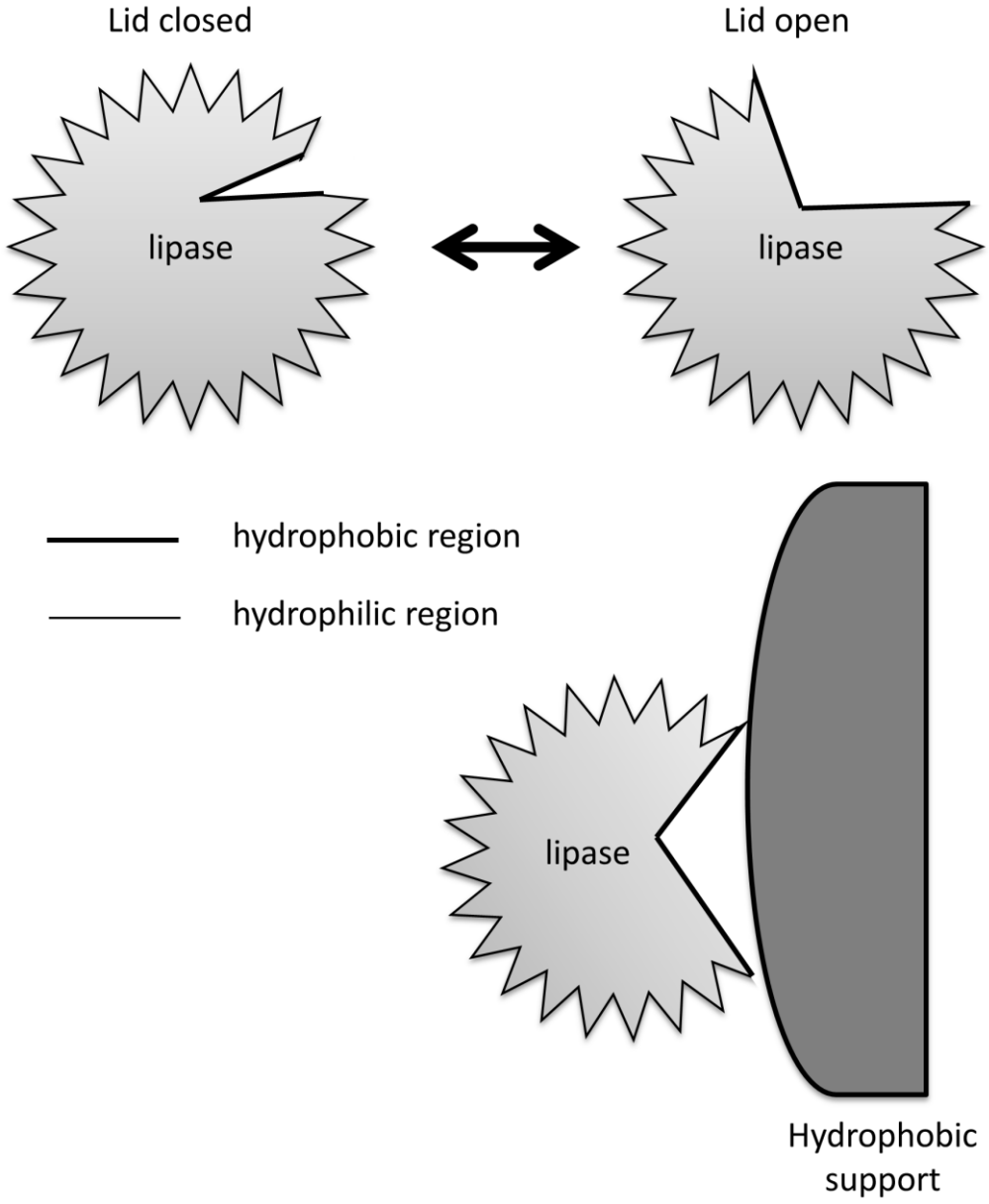
2.2.1 Physical adsorption

Physical adsorption is probably the simplest enzyme immobilization method and involves adsorbing physically enzymes or attaching onto the support material. This technique requires the contact between the support and an enzyme solution and, since the interaction enzyme-support is strong contact time dependent, the adsorption occurs by incubating or by drying the enzyme solution on the support material (ADLERCREUTZ, 2013; MOHAMAD et al., 2015).

The enzyme adsorption phenomenon implicates weak non-specific forces such as van der Waals, hydrophobic interactions, hydrogen bonds, and ionic bonds (MOHAMAD et al., 2015). In the case of lipases, hydrophobic interaction is most common because lipases are adsorbed spontaneously from aqueous solutions onto hydrophobic surfaces faster than most other proteins (ADLERCREUTZ, 2013; REHM; CHEN; REHM, 2016). Some research works (ADLERCREUTZ, 2013; HANEFELD; GARDOSSI; MAGNER, 2009; MATEO et al., 2007; RODRIGUES et al., 2013) suggested the lipase catalytic activity increase after physical adsorption, and this event may be related to the hydrophobic interaction support-lipase leading the opening of the polypeptide chain called lid what exposes the active site and changing the lipase to its active form, similar to interfacial activation motivated by hydrophobic substrate phase, as illustrated in Figure 5.

The major inconvenience of this technique is the reversibility of the immobilized enzymes by the fragile interaction with the support, being easily removed from support under mild conditions, especially in aqueous media (HANEFELD; GARDOSSI; MAGNER, 2009; MOHAMAD et al., 2015; REHM; CHEN; REHM, 2016). Generally, this is not a desirable characteristic, particularly when used in analytical assays and sensor devices (HOMAEI et al., 2013). However, it should be an attractive method when the cost of the support is significant, whereas as soon the enzymatic activity reduces due to the enzyme leaching, the support can be regenerated and reloaded with fresh enzyme (BARBOSA et al., 2015; MOHAMAD et al., 2015).

Figure 5 - Schematic representation of lipase in the closed and open form and the hydrophobic interaction between lipase and support.



Source: Author.

Related to the forces in the adsorption processes, when lipases are immobilized on hydrophobic supports, according to Hanefeld et al. (2009), van der Waals forces are responsible by the interaction support-enzyme leading to gain in the entropy. Conversely, when hydrophilic carriers are used, hydrogen bonds occur exclusively due to a large number of NH-groups of the polypeptidic chain which can readily interact with carbonyl groups of the polymers, for example (HANEFELD;

GARDOSSI; MAGNER, 2009; HOMAEI et al., 2013). The commercial immobilized lipase, Novozym® 435, is obtained by the adsorption method (YADAV; KAMBLE, 2018). This biocatalyst consists of *Candida antarctica* lipase B (CalB) adsorbed on hydrophobic macroporous polymer based on methyl and butyl methacrylic esters cross-linked with divinylbenzene to avoid or minimize the leaching effects (PÄIVIÖ; PERKIÖ; KANERVA, 2012).

2.2.2 Covalent bonding

Covalent bonding is a usual technique of irreversible enzyme immobilization and this method consists in the formation of covalent bonds between enzyme and support material (ZUCCA; SANJUST, 2014). These interactions involve side-chain amino acids, such as lysine, cysteine, aspartic and glutamic acids, and several functional groups, like carboxyl group, amino group, epoxy group, indole group, phenolic group, sulfhydryl group, thiol group, imidazole group, and hydroxyl group, which are not essential for the catalytic activity of the enzyme (MOHAMAD et al., 2015; SIRISHA; JAIN; JAIN, 2016). When compared with immobilization by adsorption, covalent immobilization exhibit, in general, some advantage since the enzyme can remain on supports even under strict conditions and can be used in any reaction media (HOMAEI et al., 2013; PARVULESCU et al., 2014).

Covalent bonding makes a robust support-enzyme link which may ensure the enzyme is tightly fixed preventing enzyme release (protein contamination of product) into the reaction media and, consequently, affording more reuse cycles. Thus, in general, covalently immobilized enzymes should be used in any reaction media, especially those aqueous, and when denaturing factors are present since the covalent bonds are strong enough to keep the enzyme linked, decreasing the conformational flexibility and thermal vibrations (MOHAMAD et al., 2015). Nevertheless, the major challenge around this technique is the chemical modification that the enzyme is submitted (HANEFELD; GARDOSSI; MAGNER, 2009).

Covalent bonding often leads to some degree of enzymatic inactivation and if critical functional groups to catalysis are modified this degree can become severe (LI et al., 2017). In order to prevent this inactivation, especially at the active site region, a

strategy is adopted which consists in carrying out the immobilization process in the presence of substrates or competitive inhibitors (PANESAR; KUMARI; PANESAR, 2010). It could be a valuable advantage when lipases are the targets of immobilization once they might be immobilized under their closed form protecting the active site and after process ending changed to open form (ADLERCREUTZ, 2013).

Some immobilization protocols report the employ of spacers between enzyme and support to improve the performance of the process. When long spacers are employed, higher conformational flexibility is expected for the enzyme. Lipases fit very well in this case for having this peculiar behavior (change their structural conformation) when interacting with the substrate. In contrast, short spacers do not allow too much mobility but can ensure more thermal stability and low risk of enzymes leaching under any distorting agent (heat, organic solvents, extreme pH values) (MATEO et al., 2007). Multipoint covalent attachment is commonly observed when short spacers are employed (BARBOSA et al., 2013; MATEO et al., 2007; SANTOS et al., 2015). Some research works report assistance of glutaraldehyde as a coupling agent due to its extraordinary capacity to build crosslinking (BARBOSA et al., 2014), but this mechanism will be more explored on the next topic.

In the same way as for physical adsorption, both hydrophilic and hydrophobic supports can be used for covalent immobilization. Jose et al.(2018) reported the use of functionalized chitosan, epoxy acrylic resin, and functionalized magnetite as supports for *Candida antarctica* lipase B covalent immobilization with subsequent application in the esterification of R/S-ibuprofen with ethanol. Another technique of immobilization was employed as well as others support, but some decrease in the specific activity was observed in those immobilized by covalent bonding. Miao et al. (2018) used superparamagnetic Fe_3O_4 nanoparticles to covalently immobilize lipase B from *Candida antarctica*. The obtained biocatalyst when compared to the free lipase was more stable, showed higher activity at several temperature, and pH, and also allowed five reuse cycles.

2.2.3 Crosslinking

Crosslinking is another technique of irreversible enzyme immobilization which uses a bifunctional agent to make the required bonds. It is quite common to see this

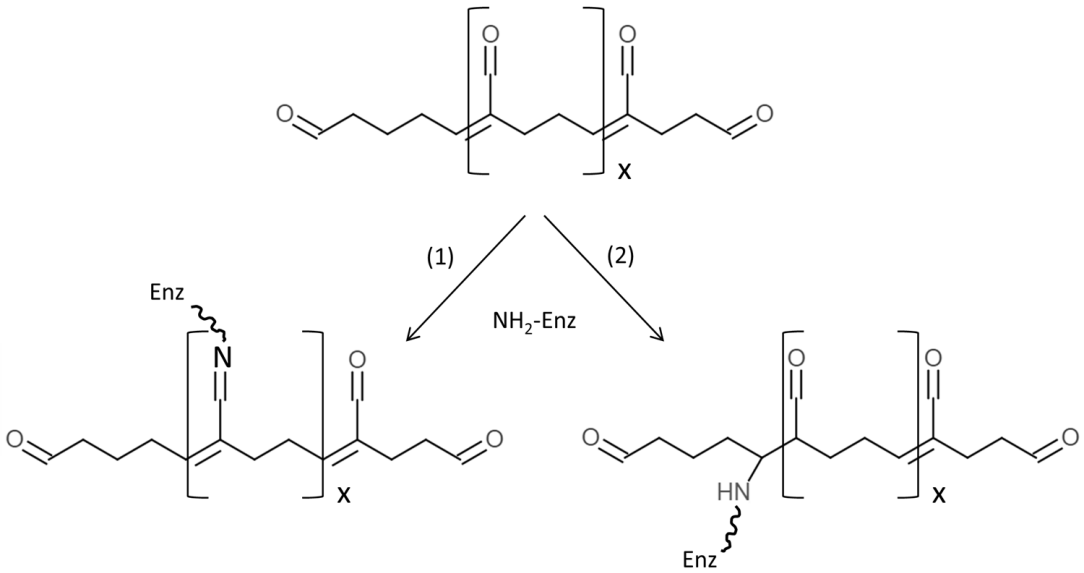
technique being used in combination with others, such as adsorption, to avoid enzyme leaching of carriers (ADLERCREUTZ, 2013). However, the main goal here is to form a carrier-free enzyme complex in order to eliminate most of the drawbacks associated with the employ of physical adsorption technique, as already discussed previously.

Glutaraldehyde is the most usual and popular crosslinking agent as it is economical and readily available in large quantities. However, others are also known, such as dextran polyaldehyde and divinyl sulfone, and can be used when glutaraldehyde shows unsatisfactory results (MOHAMAD et al., 2015; SHELDON; VAN PELT, 2013; ZUCCA; SANJUST, 2014). As in the case of covalent bonding to supports, the amino groups of the enzyme are strictly involved in the bond formation. The primary amino groups of lysine residues on the enzyme surface react with dialdehydes groups of crosslinking agent, leading to reversible Schiff's base formation. The subsequent reduction is required to turn this crosslinking irreversible. This second step is not necessary when the crosslinking agent employed is glutaraldehyde since hydration, oligomerization, and aldol condensations reactions occur and simple Schiff's base is not the dominating mechanism (ADLERCREUTZ, 2013). However, the exact mechanism of glutaraldehyde action still presents some controversies in the scientific community. The most accepted involves Michael-type addition of $–NH_2$ to α,β-double bonds, yielding a stable secondary amine (see Figure 6) (BARBOSA et al., 2014).

Crosslinking technique has been studied since the 1960s, but due to several limitations, such as poor reproducibility, low mechanical stability, and low residual activity, it was left out, and the focus switched to carrier-bound enzymes techniques. Only in the 2000s, this research took the next step and now it attracts growing attention. To date, carrier-free immobilization mainly includes a crosslinked enzyme (CLE), crosslinked enzyme crystals (CLECs), crosslinked spray-dried enzyme (CSDE), and crosslinked enzyme aggregates (CLEAs). The difference among them is basically the method of production, such as direct crosslinking, crystallization, spray-drying, and aggregation, respectively (CUI; JIA, 2015). Sheldon and van Pelt (2013) approached more deeply this topic in their review with a focus on the application of this technology to a variety of enzymes, including lipases. Cui and Jia

(2015) also published a review on this subject and discussed strategies and optimization processes for this technique.

Figure 6 - Schiff base (1) and Michael-type (2) reactions of glutaraldehyde with enzymes.



Source: Author.

Anand and Weatherley (2018) investigated the hydrolysis of sunflower oil catalyzed by immobilized lipase from the fungal yeast *Candida rugosa* OF360 in three different polyolefin supports. The authors carried out some reuse cycles and better enzymatic stability was found for the biocatalysts in which glutaraldehyde was used as a crosslinking agent in order to improve adsorption obtained results. Mbanjwa et al. (2018) produced *Pseudomonas fluorescens* lipase microspheres carrier-free using the enzymes in a water-in-oil emulsion and an oily suspension of glutaraldehyde and ethylenediamine as crosslinking fluid. The activity retention of the lipase after immobilization was 65% on hydrolysis of *p*-nitrophenyl butyrate (pNPB) and the microparticle biocatalyst was stable for at least 6 cycles. Manan et al. (2018) studied the immobilization of *Rhizomucor miehei lipase* (RML) on a hybrid support chitosan-chitin with 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDAC) as a crosslinking agent. The system was used in the esterification reaction of eugenyl benzoate and was able to reach higher conversion (56.3% in 5 h) than free RML (47.3%).

2.2.4 Entrapment

Entrapment is another technique of irreversible enzyme immobilization as long as the employed support is really insoluble in reaction media. This technique consists of the physical confinement of enzymes inside the support matrix (MOHAMAD et al., 2015). Usually, carrier and enzyme have not any chemical interaction, unlike the other techniques already discussed, which can avoid any negatives effects associated with the structure of the enzyme (HANEFELD; GARDOSSI; MAGNER, 2009). It is important to keep in mind that the support must act as a cage ensuring the passage of substrate and product but retaining the enzyme, therefore, support morphology and porosity are essential to the proper functioning of this system (SIRISHA; JAIN; JAIN, 2016).

In this technique, the support is not prefabricated. It is formed in the presence of the enzyme which becomes entrapped inside it (SHELDON; VAN PELT, 2013). Thus, the support formation conditions need to be compatible with the employed enzyme stability in order to avoid the early denaturation of the biocatalyst (REHM; CHEN; REHM, 2016). Nevertheless, the main disadvantage of this technique may be related to the mass transfer phenomena through the formed support. The diffusion rate of the substrate and the product is often the limiting parameter, and generally, high substrate concentrations are required to minimize its influence (MOHAMAD et al., 2015).

Rehman et al. (2017) immobilized lipase from *Penicillium notatum* (PNL) by entrapment in silicone polymeric films with efficiency higher than 92% and observed retention about 90% of its original activity after ten cycles of octyl octanoate synthesis reaction. Sodium alginate beads were used by Padilha et al. (2018) to immobilize lipase from *Burkholderia cepacia* also by entrapment technique. The immobilized catalyst provided a higher conversion yield (92% in 24 h) in the esterification of acetic acid and isoamyl alcohol to produce isoamyl acetate (banana flavor).

2.3 SUPPORTS FOR ENZYME IMMOBILIZATION

Supports or carriers to lipase immobilization can be obtained from different materials coming from many sources. Selected materials should be able to protect the enzyme structure against adverse reactions conditions keeping the structural integrity and catalytic activity; for example, hydrophobic carriers on lipase immobilization can raise the enzymatic activity (RODRIGUES; BERENGUER-MURCIA; FERNANDEZ-LAFUENTE, 2011; ZDARTA et al., 2018). In this sense, the choice of the support matrix, with the ideal characteristics to the desired purpose, is crucial for the immobilization process success (BAYRAMOĞLU, 2017; MOHAMAD et al., 2015; REHM; CHEN; REHM, 2016; SANTOS et al., 2015).

Enzyme supports can be classified according to the chemical composition in organic or inorganic materials, and the source can be natural or synthetic (SIRISHA; JAIN; JAIN, 2016). Inorganic supports, as silica (CAZABAN; WILSON; BETANCOR, 2016; KHARRAT et al., 2011), bentonite (DONG et al., 2013), montmorillonite (DÍAZ RAMOS; GIRALDO GÓMEZ; SANABRIA GONZÁLEZ, 2014), sepiolite (LUNA et al., 2014), hydroxyapatite (TRBOJEVIĆ IVIĆ et al., 2016), and activated carbons (ALAM; ASIH; SALLEH, 2014) exhibit good thermal and chemical stability and great mechanical resistance. These materials have also good sorption properties due to the well-developed porous structure which ensures high superficial area and multiple sites for enzymatic immobilization (ZDARTA et al., 2018).

Organic supports, on the other hand, especially polymers, are able to facilitate covalent binding without crosslinking agents due to a large number of functional groups (PARVULESCU et al., 2014). Besides that, these kinds of supports show high protein affinity, reducing possible negative effects of the lipase immobilization process. Among synthetic polymers it is important to highlight poly(vinyl alcohol) (PVA) (BERGAMASCO et al., 2013), polystyrene (PS) (LI et al., 2010), poly(methyl methacrylate) (PMMA) (NIELSEN et al., 2014), and polyurethane (PU) (FACIN et al., 2018b; NYARI et al., 2016); whereas for biopolymers it is possible to cite collagen (DEWEI; MIN; HAIMING, 2016), cellulose (GIRELLI; SALVAGNI; TAROLA, 2012), carrageenan (JEGANNATHAN; SENG; RAVINDRA, 2009), chitosan (URRUTIA et al., 2018), and alginate (ZHANG et al., 2014).

The main question during the support material selection is the process in which the biocatalyst will be used to focus on the reaction medium and operational conditions (JESIONOWSKI; ZDARTA; KRAJEWSKA, 2014). PMMA, a synthetic polymer, for example, has poor tolerance against some organic solvents and dissolves easily in such media (GUNATILLAKE; ADHIKARI, 2016). The idea about a universal appropriate support to lipase immobilization should not be considered since there are several parameters that may define a support: internal geometry, specific surface area, superficial activation degree, and pore diameter (MOHAMAD et al., 2015; SANTOS et al., 2015). However, some properties could be considered in the search of an ideal support, for example: hydrophilicity, inertness towards enzymes, biocompatibility, low cost, resistance to microbial attack, and high mechanical and chemical stability (DATTA; CHRISTENA; RAJARAM, 2013; MERYAM SARDAR, 2015; SIRISHA; JAIN; JAIN, 2016). Moreover, others important characteristics should be the high protein affinity, reactive functional group availability, not toxicity, and reuse viability (HANEFELD; GARDOSSI; MAGNER, 2009).

All these materials, independently of their source, are still extensively investigated, due to the abundance in nature, as biopolymers, for example, or due to the easy synthesis, as the inorganic oxides and synthetic polymers. However, particularly in the last decade, the attention has been focused on new supports morphologies, especially at nanoscale (HOMAEI et al., 2013; SONG; SHENG; ZHANG, 2013; VALÉRIO et al., 2015; WEISER et al., 2016), hydride (BADGUJAR; BHANAGE, 2017; CRUZ-ORTIZ; RÍOS-GONZÁLEZ, 2017), and composite supports (XIANG et al., 2018; XIE; ZANG, 2018) combining materials properties in order to improve the support characteristics such as high specific surface area and excellent mechanical properties. Moreover, a recent cost analysis suggests that the biggest cost involved in the commercial adoption of this biocatalyst is the cost of the support material and not the enzyme itself. This is because the available commercial carriers are made for laboratory quantities and are also not competitive in terms of costs (BUDŽAKI et al., 2018). Chiaradia et al. (2016) immobilized Lipase B from *Candida antarctica* on poly(urea-urethane) nanoparticles incorporated with iron oxide aiming to enhance the separation from the reaction medium by a magnetic field. Sóti et al.(SÓTI et al., 2016) used poly(lactic acid) (PLA) and poly(vinyl alcohol) (PVA)

nanofibers to successful immobilize Lipase PS from *Burkholderia cepacia* and Lipase B from *Candida antarctica* (CalB) and then obtain a biocatalytic complex with excellent stability even after ten reuse cycles of acylation and hydrolysis reactions.

2.3.1 Polyurethane

The first polymers based on poly(urea-urethane) were developed by the German company Bayer in 1937, but its discovery dates back to 1848 by Wurtz. In the early 1940s, the researches were already beginning to point the PU as a polymer that may be used as thermal and acoustic insulation, as well as support in the construction of ships and airplanes. However, it was only in the 1950s and 1960s that production on an industrial scale became widespread and eventually consolidated in the 1970s in many sectors, from civil to food (RANDALL; LEE, 2002; SZYCHER, 2013; VILAR, 2004).

Polyurethane (PU) has interesting properties such as durability, flexibility, biostability, and it can be synthesized in a way to make it biodegradable. These characteristics are increasingly exploited because of the globalized concern with health and the environment. In view of this, PU is attractive in the application as a drug carrier, tissue engineering, development of medical devices, and immobilization of enzymes (CIPOLATTI et al., 2015; VALÉRIO et al., 2014; VALÉRIO; ARAÚJO; SAYER, 2013; ZANETTI-RAMOS et al., 2006, 2008).

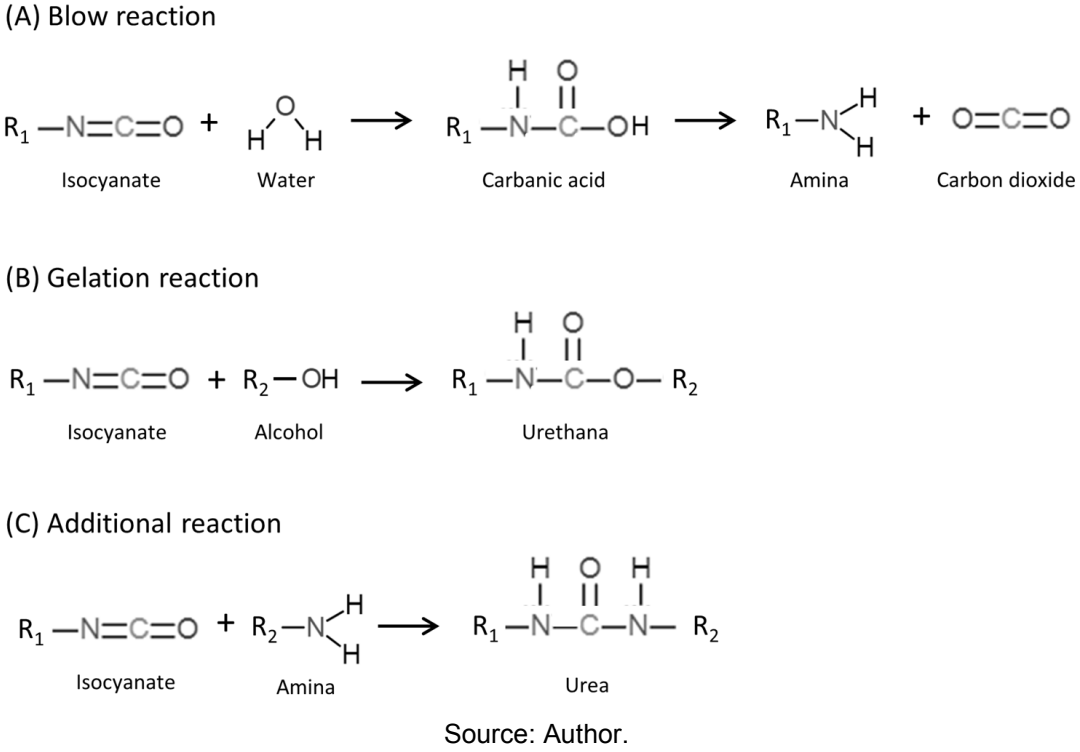
Polyurethanes encompass a broad class of chemically complex materials and may contain a variety of types of bonds, yet all have the polyurethane linkage in common. This bond is formed from the reaction between an isocyanate grouping with an alcohol grouping of another component. By controlling the composition of each component it is possible to adjust the morphology and performance of the final product as desired (KAUSHIVA, 1999).

Still about structure, unlike most polymers, whose monomer units are well defined, PU is a polymer that does not present repetitive units (urethane) regularly, thus not having an empirical formula representative for the macromolecule (SOARES, 2012).

The production of PU foams involves two main reactions: blow reaction (A) and gelation reaction (B) (Figure 7). During the blow reaction, there is the dispersion

of carbon dioxide gas by the reaction of the isocyanate with the water, causing the formation of bulbs or cells, which are connected in a three-dimensional structure. This gas is responsible for the growth of the polymer, acting as a blowing agent. In the gelation reaction, or crosslinking, occurs the reaction of the isocyanate with the alcohol forming the urethane group and a covalent network (KAUSHIVA, 1999; SOARES, 2012).

Figure 7 - Polyurethane foam synthesis.



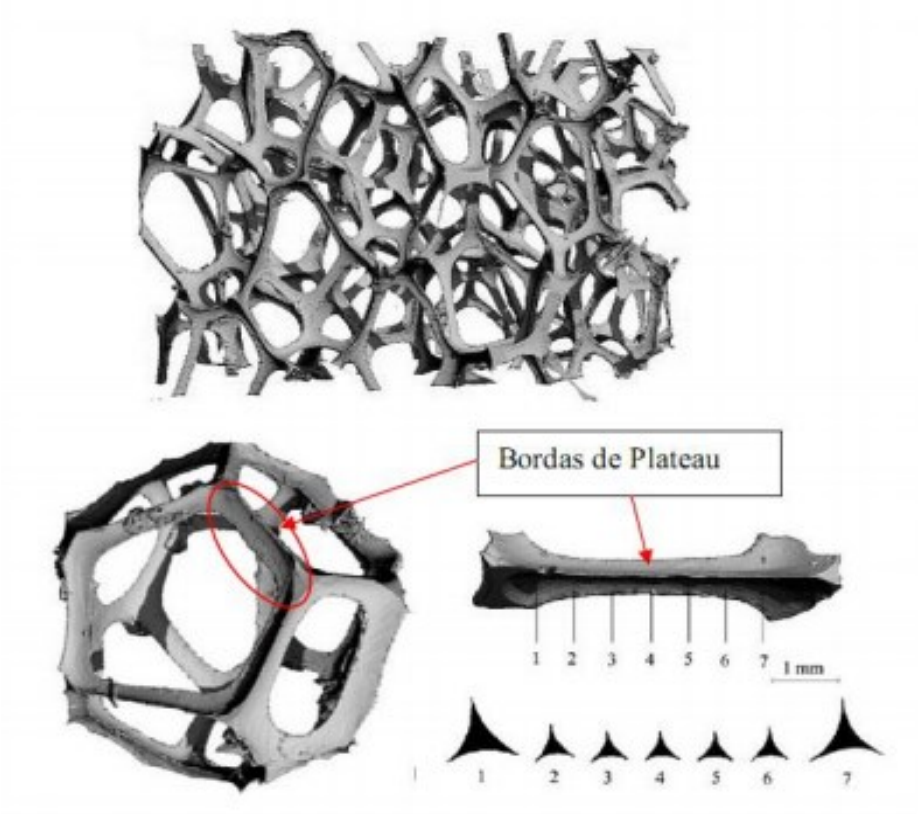
Source: Author.

There is also a third reaction occurring simultaneously to the process between the amine formed during the blow reaction and another isocyanate generating a urea component. This reaction may be a source of covalent crosslinks if the isocyanate has more than two functional groups or if polyfunctional amines are added. In addition, due to the strong interactions between the urea bonds promoted by the hydrogen bonds, aggregates of various sizes, called polyurea spheres, are easily formed. These aggregates interact with the rigid segments of the matrix contributing to the increase of the degree of polymer crosslinking and inducing the opening of foam cells (KAUSHIVA, 1999; LAN; HAUGSTAD, 2011; SOARES, 2012; YILGOR et al., 2006).

However, the balance between the two major reactions is the primary factor that will provide the open cell morphology in the foam reflecting directly on the physical properties of the polymer. If the blow or gas production reaction occurs too quickly, the cells may open before the polymer has enough strength to maintain the cell structure, resulting in foam collapse. On the other hand, if the gelation reaction, or crosslinking, occurs too quickly, it can result in a tight close-celled foam (KAUSHIVA, 1999).

During the process of PU foams synthesis, there is a step called nucleation. This occurs early in the process together with the homogenization of the components and consists of pre-forming the air bubbles during the stirring. These bubbles will receive the carbon dioxide gas generated later and will expand. There is no generation of new bubbles during foam growth, with the final cell number being at most the number of initial cells. It means that this step is directly linked to the porosity the material may display (KAUSHIVA, 1999; VILAR, 2004).

Figure 8 - Structure of the PU foam: a cell, the Plateau borders, and the respective transverse areas.



Source: Jang et al. (2008), modified by Antunes (2015).

The bubbles formed at the nucleation stage are very small and almost spherical, but as they expand, they tend to elongate towards the foam walls. When the volume of the reactive mixture becomes small in comparison to the volume of the expanding bubbles, the contact area between the adjacent bubbles becomes a triangular structure technically called Plateau edges (Figure 8). The liquid is transferred from the membranes to the edges of Plateau by capillary flow, leading to the formation of irregular polyhedral cells, which approach the shape of a truncated octahedron (ANTUNES, 2015; JANG; KRAYNIK; KYRIAKIDES, 2008; VILAR, 2004).

Regarding the use of PU as support for lipase immobilization in the different areas of knowledge, Table 2 presents some papers found in the literature relating the use of PU foam as support for lipase immobilization. Concerning the immobilization of Eversa® Transform 2.0 lipase on PU foam, just one paper was found in the open literature and it was developed by our own research group using this lyophilized enzyme (FACIN et al., 2018b).

2.3.2 Commercial supports

As discussed in section 2.3, new supports for enzyme immobilization are launched day after day, whether homemade or the result of extensive commercial investments. Each new support has new properties and brings new possibilities for the immobilization of enzymes which have often been extensively studied (ZDARTA et al., 2018). In the case of Eversa® Transform 2.0 lipase, a similar drive is expected, but with a focus on the enzyme itself. Due to the very short time since this enzyme is available, it is expected that shortly many new immobilized derivatives will be explored, owing to the wide variety of supports already developed. Table 3 shows the few works found in the literature reporting the immobilization of Eversa® Transform 2.0 lipase (or its correlated names).

Table 2 - Recent applications of immobilized lipases on polyurethane as polymeric supports.

Sector	Application	Lipase	Reference
Biofuel	Synthesis of fatty acid methyl ester	*Thermomyces lanuginosus*	(DIZGE; KESKINLER, 2008)
	Synthesis of fatty acid ethyl ester	*Candida antarctica* B	(NYARI et al., 2018b)
	Synthesis of fatty acid ethyl ester	*Candida antarctica* B	(SANTIN et al., 2014)
	Synthesis of fatty acid ethyl ester	*Pseudomonas cepacia*	(LI; DYER; GREENWELL, 2018)
Cosmetic	Synthesis of geranyl butanoate	*Candida antarctica* B	(SBARDELOTTO et al., 2018)
	Synthesis of lauryl laurate	*Yarrowia lipolytica* Lip2	(CUI et al., 2013)
Food	Hydrolysis of olive oil	*Burkholderia cepacia*	(NYARI et al., 2017)
	Hydrolysis of soybean oil	*Thermomyces lanuginosus*	(FACIN et al., 2018b)
	Synthesis of ethyl butyrate (flavor compound)	*Candida rugosa*	(PIRES-CABRAL; DA FONSECA; FERREIRA-DIAS, 2009)
	Synthesis of ethyl butyrate (flavor compound)	*Rhizopus oryzae*	(GROSSO; FERREIRA-DIAS; PIRES-CABRAL, 2013)
	Synthesis of *n*-butyl octanoate (flavor compounds)	*Pseudomonas cepacia*	(DHAKE et al., 2013)
	Synthesis of geranyl propionate (flavor compounds)	*Candida antarctica* B	(NICOLETTI et al., 2015)
	Synthesis of geranyl propionate, geranyl oleate, and ethyl oleate (flavor compounds)	*Candida antarctica* B	(NYARI et al., 2016)
	Synthesis of isoamyl acetate (flavor compounds)	*Candida antarctica* B	(NYARI et al., 2018a)
Pharmaceutical	Synthesis of 1,3-dicapryloyl-2-oleylglycerol (structured triglyceride)	*Bacillus stearothermophilus MC7*	(GUNCHEVA et al., 2009)

Table 3 - Summary of recent works on immobilization of Eversa® Transform 2.0 lipase.

Application	Immobilization technique	Support	Reference
Biodiesel production (FAEE)	Crosslinking	Silica magnetic nanoparticles	(MIRANDA et al., 2020)
Biodiesel production (FAME and FAEE)	Hydrophobic adsorption	Lewatit VP OC 1600, Sepabeads-C18, Purolite divinyl ECR1061 DVB, and Purolite ECR8804F	(REMONATTO et al., 2018)
Biodiesel production (FAME)	Entrapment	Polyurethane foam	(BRESOLIN et al., 2019)
Biodiesel production (FAME)	Entrapment	Polyurethane foam	(FACIN et al., 2020)
Biodiesel production (FAME)	Interfacial activation	Octyl agarose and octadecyl metacrylate matrixes	(MARTÍNEZ-SANCHEZ et al., 2020)
Esterification of adipic acid and isononyl in order to produce diisononyl adipate	Entrapment	Lewatit VP OC 1600	(LEE et al., 2019)
Glycerolysis of castor oil and the biopolyol	Entrapment	Poly(urea–urethane) nanoparticles	(BRESOLIN et al., 2020)
Hydrolysis of soybean oil esterification and transesterification	Interfacial activation	Non-porous polystyrene	(DANTAS et al., 2018)
Hydrolysis of soybean oil	Interfacial activation on the hydrophobic matrix	Polyurethane Foam	(FACIN et al., 2018a)
Hydrolysis reactions	Sol-gel technique	Tetraethyl orthosilicate	(HONAISER et al., 2019)

2.4 APPLICATIONS AND REACTIONS OF INTERESTING

2.4.1 Biodiesel production

Biodiesel is a biofuel, which is defined as a fuel comprised of mono-alkyl esters resulting from a chemical reaction between a short chain alcohol and a fatty substrate. The most common fatty acids esters (FAE) are those derived from the employ of ethanol and methanol as short chain alcohol, namely fatty acids ethyl esters (FAEE) and fatty acids methyl esters (FAME), respectively.

The production of biodiesel by the enzyme-catalyzed process has received increasing attention because it requires lower energetic demand than the conventional alkali-catalyzed technique and the substrates may be processed without any pretreatment (CANET et al., 2016; STERGIOU et al., 2013). Currently, China and Brazil are leading the industrial applications of lipase-catalyzed approach toward biofuels production (ZORN et al., 2016).

Raw materials for the production of biodiesel may have vegetable (soybean, castor bean, canola, palm, sunflower, and peanut) or animal (bovine, porcine, and poultry) origin. Reuse oils can also be used, such as used cooking oil discards from the oil industries or recovered from effluent treatment plants, and hence present relatively low-added value. The choice of the feedstock source is a fact that should be considered for reducing the manufacturing costs. It has been estimated that about 65-85% of the production cost of biodiesel comes from the cost of the raw material. To oilseeds, for example, factors such as content in vegetable oil, productivity, territorial adaptation, and the producing region are crucial. Another alternative is the employ of degummed oils which presents a lower cost than refined ones. The enzyme-catalyzed biodiesel allows the use of raw materials with high levels of water and FFA, avoiding the typical soap formation in the alkaline process. Table 4 summarizes the most common feedstock for the production of enzymatic biodiesel (AKGÜN; İŞCAN, 2007; RAMOS et al., 2003).

Table 4 - Common feed materials for enzymatic biodiesel.

Type	Pros	Cons	Pretreatment
Used cooking oil	Waste to fuel, feed collection systems in place, potentially high-quality glycerin	Increasing price, limited volume, content of polymeric glycerides	Filtration, water wash, mineral acid neutralization
Distillers corn oil	Bolt-on process for bioethanol plant, potentially high-quality glycerin	Waxes and sterylglycosides and phospholipid content, surfactant carryover from oil extraction	Setting/decantation* water wash, mineral acid neutralization
Animal/ rendered fat	Price, no food oil competition	High sulfur content, high protein content, high melting point	Water wash, mineral acid neutralization
Acid oil	Waste to fuel, bolt-on process for oil refining plant	Extreme mineral acid, very dark color, high unsaponifiable content, possible high sulfur	Water wash, mineral acid neutralization
Brown grease	Price, waste to fuel, fast reaction	High sulfur content, high protein content, high unsaponifiable content, limited volume avaliable	Setting/ decantation*, filtration, water wash, acid neutralization
FAD	No glycerolysis, low contamination, fast reaction, bolt-on process for oil refining plant	High melting point > fed batch/continuous process	Mineral acid neutralization, pre-heating
CPO	Large volume available, micronutrient recovery, high quality glycerin	High melting point, food oil competition	Filtration, mineral acid neutralization
Crude vegetable oil	High methyl ester yield	Price, food oil competition	Filtration, water wash enzymatic degumming, mineral acid neutralization
Reclaimed oil and slurries	Price, waste to fuel	Extreme variance in oil quality and nature and concentration of oil contaminants	Filtration, water wash/phosphoric acid wash**

FAD - Fatty acid distillates
CPO - Crude palm oil
* Setting or decantation can be useful to remove precipitates and macro particles
** Choice of water wash or enzymatic degumming depends on the specific feed oil
Font: reproduced from Novozymes (BURTON, 2015)

In Brazil, biodiesel is regulated by ANP (National Agency of Petroleum, Natural Gas and Biofuels) and it has already integrated with the energetic matrix since 2005 (Law Nº 11.097), with the mandatory addition of 2 wt% of biodiesel in all fossil diesel sold at the country. Since then, this percentage was gradually increased. Currently, the minimum mandatory addition in fossil diesel is 13 wt% (B13), up to a maximum of 15%. However, it is expected to reach the B15 in 2023 and B20 in 2028 ("Despacho Nº 621, de 6 de Agosto de 2019 do Ministério de Minas e Energia/Agência Nacional do Petróleo, Gás Natural e Biocombustíveis", 2019).

Thus, the increase in energy demand has already a reality and the search for alternatives to reduce the costs involved with biodiesel production becomes inherent. Therefore, the employ of non-edible feedstocks with a low-added value associated with alternative processes for biodiesel production may be an interesting way to solve this demand.

2.4.2 Other applications: phospholipids synthesis

Undoubtedly, Eversa® Transform 2.0 lipase has been mainly applied to biodiesel production, as seen in Table 3. However, as discussed, a proper immobilization may improve the enzyme performance and allow its application in many other reactions, such as the synthesis of phospholipids. Phospholipids (PLs) are amphiphilic molecules that are ubiquitous in nature. They have significant applications in the industry acting as emulsifiers, stabilizers, and antioxidants. Although a great variety of phospholipid (PL) structures have been found, they generally contain a glycerol or sphingosyl backbone (ANG et al., 2019; HAMA; OGINO; KONDO, 2015). The main type of PL is phosphatidylcholine (PC) which is commonly found in soybean or egg yolk lecithin. Among the PCs, the glycerophospholipids (GPC) occur naturally and are usually composed of saturated fatty acids, such as stearic or palmitic acid in the *sn*-1 position, while the *sn*-2 position is occupied by unsaturated acids (i.e., oleic, linoleic, and arachidonic) (GŁADKOWSKI et al., 2011).

The compositional variety of natural PLs are limited, furthermore, the separation of specific PLs has proven to be difficult as they rarely exist in nature (ANG et al., 2019). Therefore, the synthesis of structured PLs via enzymatic process

offers considerable advantages such as high selectivity and mild conditions, leading to the generation of products that cannot easily be obtained by chemical methods (HAMA; OGINO; KONDO, 2015). Preparation of structured phospholipids can be performed via esterification in anhydrous systems applying immobilized lipases and the reaction occurs between GPC and fatty acids to produce oleoyl-lysophosphatidylcholine (LPC) (VIKBJERG et al., 2006).

Interesting research about the synthesis of LPC by lipase-catalyzed esterification of GPC and free oleic acid in a reaction medium without solvent was published (MNASRI et al., 2017). The authors verified that the catalyzed reaction was more efficient with immobilized lipase from *Rhizomucor miehei* (Lipozyme RM-IM) than the immobilized lipases from *Thermomyces lanuginosus* (Lipozyme TL-IM), and *Candida antarctica* (Novozym 435). Other authors published a work in which LPC was successfully synthesized by enzyme-catalyzed esterification using a vacuum system (HONG et al., 2011). In another study, the production of LPC from purified soybean lecithin in two different reaction systems, water, and ethanol was evaluated (BAEZA-JIMÉNEZ et al., 2013). The authors evaluated the efficiency of a phospholipase versus two lipases and found the best conversions (70%) for the immobilized Lipase PS from *Burkholderia cepacia*.

2.5 FINAL CONSIDERATIONS REGARDING THE STATE OF THE ART

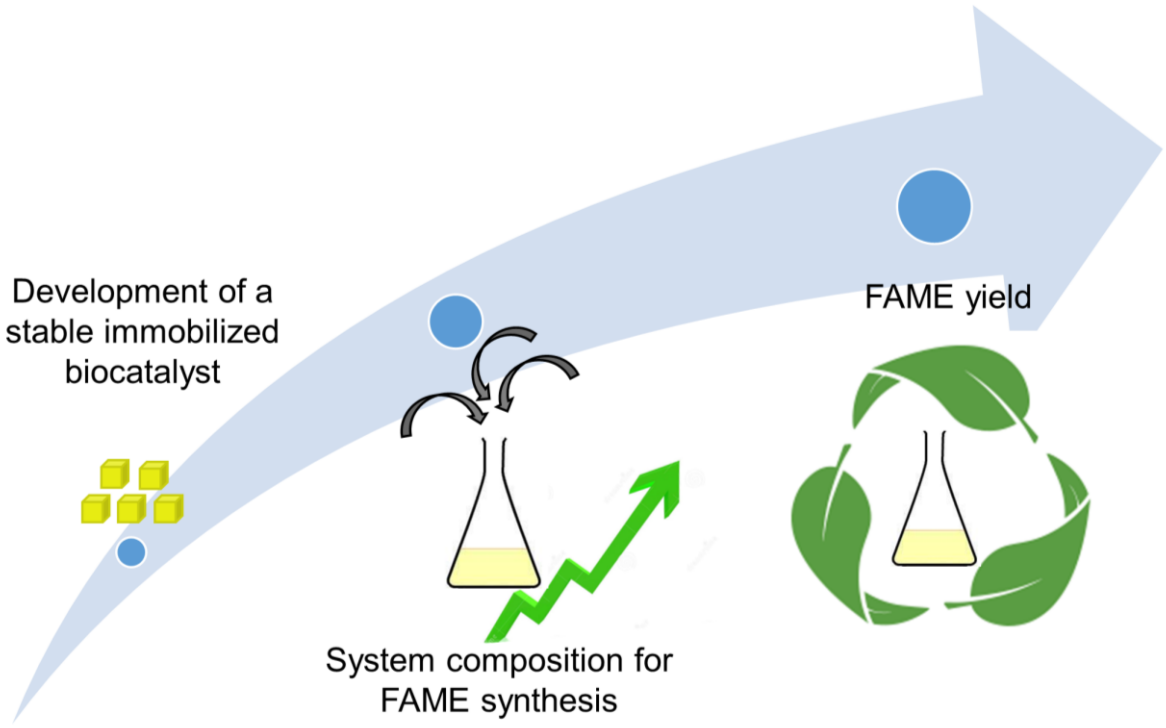
As discussed throughout this chapter, the interest in enzyme immobilization is increasing in view of its broad potential for industrial application. Allied to this, the great advantage in the use of immobilized enzymes is on the economic scope due to the possibility of easy recovery and reusability. In this sense, several materials are reported as supports for enzyme immobilization giving greater operational stability to the immobilized. Among these materials, polyurethane has been successfully used in the immobilization of several lipases. Moreover, the application of genetic engineering techniques in enzyme manufacturing is drastically boosting the exploration of new lipases and the development of new enzyme properties, opening a lack for further studies involving other supports. In addition, biocatalysis may be a good substitute for chemical synthesis in several industrial processes, especially in

economic and environmental aspects. It is the case of biodiesel production, where the employ of these biocatalysts allows the use of the diverse low-added value raw materials, and the synthesis of phospholipids, mainly favored by the high specificity of the biocatalysts.

3 DEVELOPMENT OF AN IMMOBILIZED BIOCATALYST AND PARAMETERS FOR FAME SYNTHESIS

The main objective of this chapter was to develop a stable immobilized biocatalyst that could be employed for the evaluation of the parameters involved in the FAME synthesis from an industrial fatty waste. The immobilization procedure was investigated and the parameters around the fatty acid methyl ester (FAME) synthesis was deeply discussed. Figure 9 shows the activities developed in this chapter.

Figure 9 - Scheme of the activities developed in Chapter 3.



Source: Author.

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3.1 MATERIAL AND METHODS

3.1.1 Material

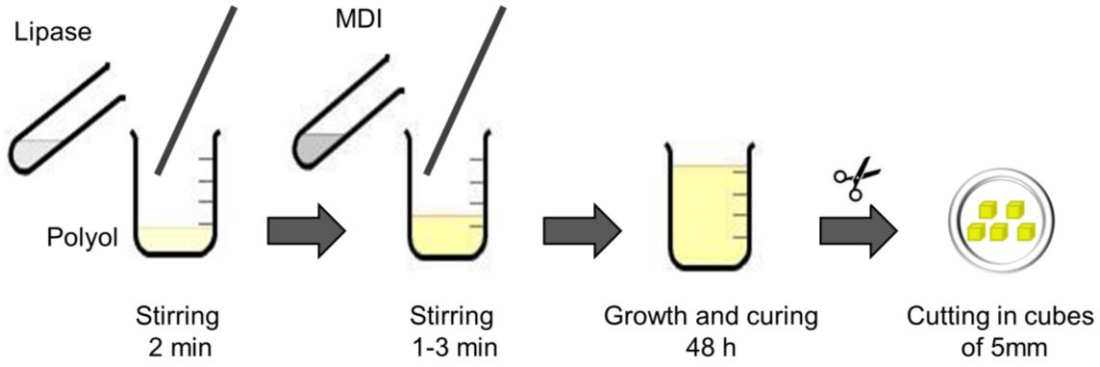
A commercial solution of Eversa® Transform 2.0 lipase (ET2) from *Aspergillus oryzae* (protein content 35 mg/mL) was provided by Novozymes (Araucária, PR, Brazil). The monomers polyol polyether and diphenylmethane diisocyanate (MDI) for polyurethane (PU) synthesis were kindly donated by Purcom Company (industrial manufacturer of PU flexible foam in Brazil). Soybean oil (Soya, Brazil), purchased from the local market (free fatty acid, FFA, content 0.3 wt%), was used as substrate for hydrolysis reaction without any further treatment. Oleic acid P.A., acquired from Neon Comercial Ltda (São Paulo, Brazil), was employed as a feedstock for the batch experiments without any further treatment. Industrial fatty waste, a blend of low-cost feedstocks, was kindly provided by Prisma Comercial Exportadora de Oleoquímicos LTDA (Sumaré, SP, Brazil) and Transfertech (Erechim, RS, Brazil) companies (FFA content 55.9 wt%, iodine value of 105, and water content of 2.4 wt%). All reagents and solvents used were of analytical grades.

3.1.2 Methods

3.1.2.1 Synthesis of polymeric support and lipase immobilization

ET2 immobilization was performed by entrapment technique (*in-situ*) in the synthesis of the PU support, following the methodology described in our previous work (FACIN et al., 2018b). Briefly, the procedure consisted of adding the soluble ET2 to the polyol polyether phase, followed by the addition of the MDI phase under stirring for 1-3 min until complete homogenization. After the polymerization process (5 min), the formed foam was kept at room temperature (~21 °C) for 48 h for complete cross-linking and curing of the structure (Figure 10).

Figure 10 - Scheme of support synthesis and simultaneous immobilization of soluble Eversa® Transform 2.0 lipase.



Source: Author.

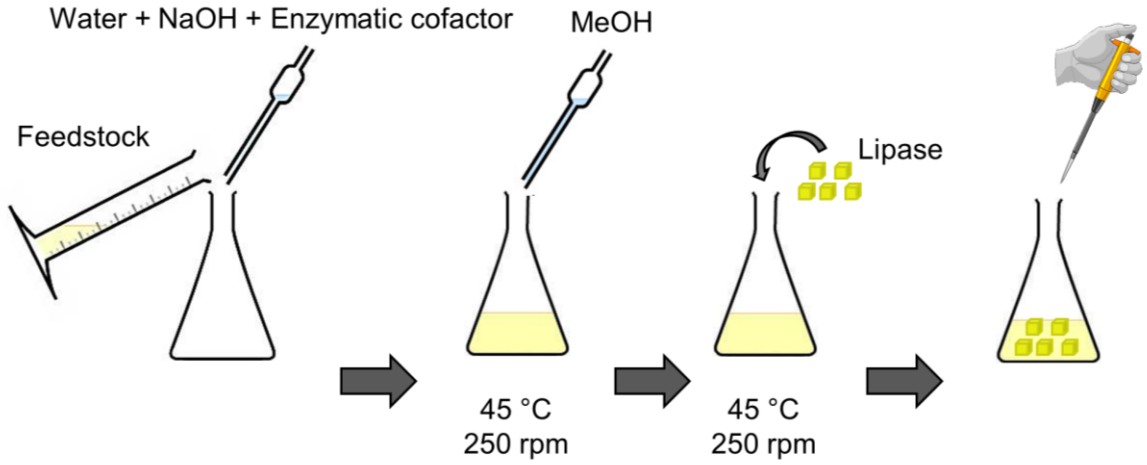
In order to investigate the possible influence of the heat generated by the PU polymerization reaction on the stability and, consequently, on the performance of the immobilized lipase, a second immobilization assay was performed using an ice bath (4 ºC) during the PU synthesis and ET2 immobilization. The procedure was the same described previously, but the plastic cups were kept immersed in an ice bath when the polymerization was performed.

3.1.2.2 Parameters for FAME synthesis

The enzymatic FAME synthesis followed the methodology proposed by Mibielli et al. (2019), with some modifications in the system composition and the variable ranges (Figure 11). The enzymatic reaction was carried out at 45 °C using a shaker incubator (250 rpm) in a fed-batch system using a 1.5 L sealed flask. The reaction system consisted of 100 g of feedstock, 0-2 wt% distilled water addition (by weight of feedstock), 0-300 ppm of NaOH (by weight of feedstock), 0-500 ppm of enzymatic cofactor (by weight of feedstock) (based on previous work of Bresolin et al. (2019) and not authorized to disclose by the manufacturer), and 0.3 wt% of free enzyme or immobilized enzyme (by weight of feedstock). Methanol was added in equal part stepwise from the first 10 h of reaction (0-10 h) in a total of 1.2-2.0 equivalent (eqv, on the basis of stoichiometric reaction) to prevent enzyme inhibition. Aliquots were taken out from the reaction mixture at given times for composition

analysis (FFA content and FAME conversion). The variables ranges were defined based on previous works (COPPINI et al., 2019; MIBIELLI et al., 2019; REMONATTO et al., 2016) and a 2^4 factorial design with triplicate at the central point was used to assess the influence of water, NaOH, enzymatic cofactor, and MeOH concentrations on FAME synthesis (Table 5).

Figure 11 - Scheme of FAME synthesis.



Source: Author.

Table 5 - Variables and respective levels of experimental design of FAME synthesis.

Variables	**Level**		
	-1	0	+1
Water (wt%)	0	1	2
NaOH (ppm)	0	150	300
Enzymatic cofactor (ppm)	0	250	500
MeOH (eqv)	1.2	1.6	2.0

3.1.2.3 Determination of FFA content

FFA amount in the reaction medium was determined by the titration technique according to ISO 660:2009 (INTERNATIONAL ORGANIZATION OF STANDARDIZATION, 2009), performed in triplicate (n = 3). For the acidity calculation, an aliquot of 0.5 g was taken out from the reaction mixture and diluted into 25 mL of neutral ethanol solution. Indicator (about three drops of 1 wt%

phenolphthalein in ethanol) was added, and the solution was titrated with 0.1 mol/L sodium hydroxide solution (NaOH) to purple color persistence under vigorous stirring. The FFA content was calculated by Equation 1:

$$FFAcontent(wt\%) = \frac{V_{NaOH} * M_{NaOH} * MM_{FFA}}{10 * m_s} \quad (1)$$

where: V_{NaOH} is the volume of NaOH solution consumed in the sample titration (mL); M_{NaOH} is the molarity of the NaOH solution (M); MM_{FFA} is the average molar mass of fatty acids (FA), 282 g/mol for the employed oil; m_S is the mass of titrated sample (g).

3.1.2.4 Determination of FAME conversion

To determine the conversion of raw material to FAME, the titration technique based on ISO 660:2009 (INTERNATIONAL ORGANIZATION OF STANDARDIZATION, 2009) was used and consists in determining the acid value of the sample, due to the presence of FFA. These acids can react with the potassium/sodium hydroxide solution, and the obtained result is expressed in terms of (mg) of KOH/NaOH per (g) of sample. For the calculation, an aliquot of 0.5 g was taken out from the reaction mixture and diluted in 25 mL of neutral ethanol solution. Indicator (two drops of 1 wt% phenolphthalein in ethanol) was added, and the solution was titrated with 0.1 mol/L sodium hydroxide solution (NaOH) to purple color persistence under vigorous stirring. The analysis was performed in triplicate (n=3). The acid value (AV) was calculated by Equation 2:

$$Acidvalue(mg\ KOH/g) = \frac{56.1 * V_{KOH} * M_{KOH}}{m_s} \quad (2)$$

where: V_{KOH} is the volume of KOH solution consumed in the sample titration (mL); M_{KOH} is the molarity of the KOH solution (M); m_S is the mass of titrated sample (g).

The FAME conversion was then calculated through the initial and final (or at specific time) acid value, as follows:

$$FAMEconversion(\%) = \frac{AV^o - AV^t}{AV^0} * 100 \quad (3)$$

where: AV^0 is the initial acid value (mg KOH/g); AV^t is the acid value at given time (mg KOH/g).

3.2 RESULTS AND DISCUSSION

3.2.1 Immobilization of soluble Eversa® Transform 2.0 lipase in polyurethane foam

The ET2 immobilization on PU foam resulted in a derivative with no phase separation and high pore homogeneity. After the immobilization process, the incorporation of lipase reached 12.5 wt% (by weight of polymeric support), leading to an enzyme loading of 4.4 mg/g (enzyme/support), based on the manufacturer's protein content (35 mg/mL). Figure 12 represents the PU support with soluble ET2 immobilized.

Figure 12 - Soluble Eversa® Transform 2.0 lipase immobilized on polyurethane polymeric support.

Source: Author.

Some authors also reported the successful immobilization of soluble lipases from *Candida antarctica* fraction B (SANTIN et al., 2014; NYARI et al., 2016;

SBARDELOTTO et al., 2018), *Candida rugosa* (FERREIRA-DIAS; CORREIA; BAPTISTA, 1999; PIRES-CABRAL; DA FONSECA; FERREIRA-DIAS, 2009), *Burkholderia cepacia* sp. (NYARI et al., 2017), *Rhizopus oryzae* (GROSSO; FERREIRA-DIAS; PIRES-CABRAL, 2013), and *Thermomyces lanuginosus* (DIZGE; KESKINLER, 2008) by entrapment methodology on PU supports with enzyme loading from 0.04 wt% to 75 wt%. This first immobilized biocatalyst was used as a model for the preliminary evaluation of the parameters for FAME synthesis.

3.2.2 Parameters for FAME synthesis

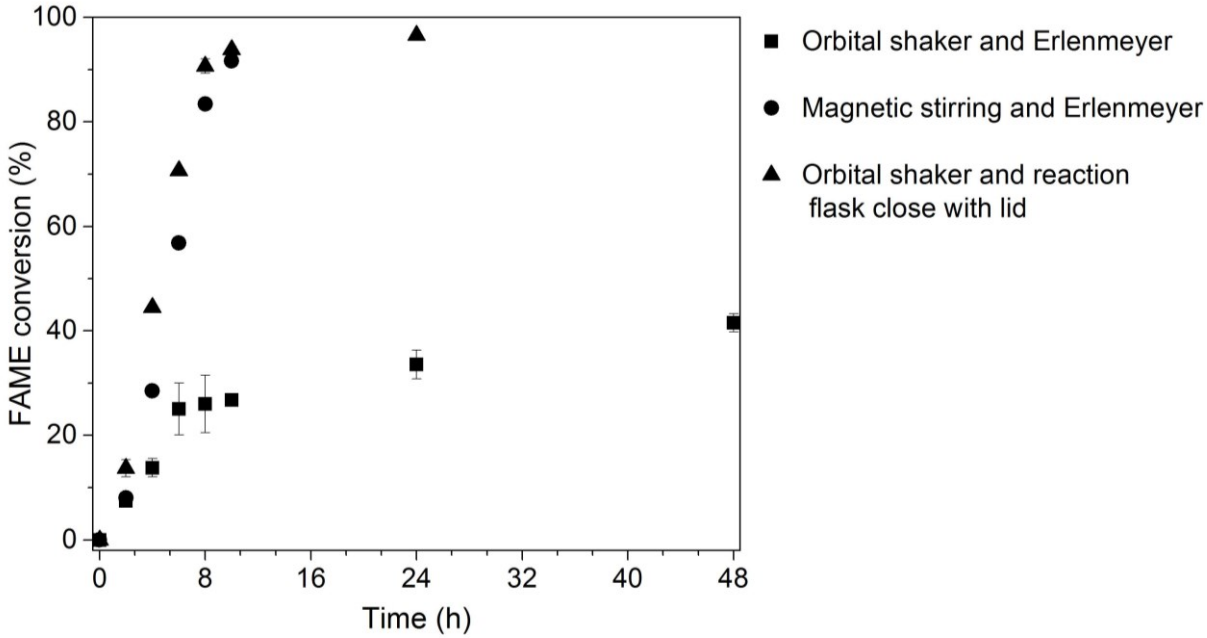
3.2.2.1 Stirring system and reactor design

The definition of the stirring system and the reactor design that will be used for the FAME synthesis is an important point that must be taken into account to provide a correct analysis of the biocatalysts performance. So, the first batch experiment was carried out with soluble free ET2 and oleic acid (feedstock) as the standard reaction due to its high acid content. Figure 13 shows the obtained results in terms of FAME conversion for the combination of those two factors.

The first combination was Erlenmeyer as the reactor and horizontal orbital shaker as the stirring system. As can be seen in Figure 13, the system did not perform as expected, and the final FAME conversion after 48 h of reaction was 41.6%. This result may be attributed to the vortex formation in the reactor which limits the action of the enzyme by reducing the mass transfer. Thus, for the second combined system, the same reactor was used, however, magnetic stirring was used to overcome the mass transfer limitation. It was observed an increasing reaction performance leading to 91.7% of FAME conversion and 8.3 wt% of FFA content in 10 h of reaction. The last combination attempted was a reactor that does not allow vortex formation (reaction flask close with lid) and an orbital horizontal shaker. In this combined system was possible to observe a significant reduction in the acidity after 10 h of reaction (6.2 wt% of FFA content) reaching a final acidity of 3.4 wt% and FAME conversion of 96.6% after 24 h of reaction, confirming the efficiency of this

combination. Therefore, the latter combined system was chosen as the standard for subsequent batch experiments with the immobilized lipases.

Figure 13 - Influence of the stirring system and the reactor design on the esterification reaction from oleic acid catalyzed by soluble free Eversa® Transform 2.0 lipase.



Experimental conditions: 0.3 wt% of free enzyme, 2 wt% distilled water, 2.0 eqv of methanol, 300 ppm of NaOH, and 500 ppm of enzymatic cofactor, at 45 °C and 250 rpm.
Source: Author.

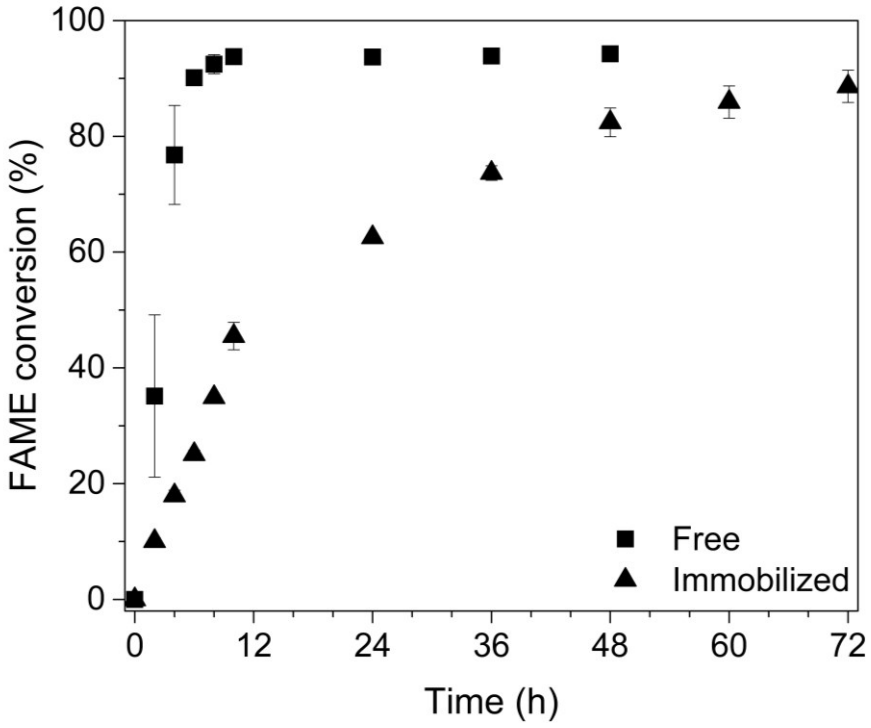
3.2.2.2 System composition for FAME synthesis

Batch experiments for the application of free and immobilized ET2 on the FAME synthesis from industrial fatty waste with high FFA content were carried out to evaluate the real efficiency of these biocatalysts in reactions of industrial interest, as shown in Figure 14. The experimental conditions employed for this first attempt were based on previous works (REMONATTO et al., 2016).

It was possible to verify in Figure 14 that for the free ET2 a faster reaction kinetic reaching 4.2 wt% of FFA content and 92.5% of FAME conversion was achieved in 8 h of reaction while for the enzyme derivative a slower reaction kinetic was observed reaching 6.4 wt% of FFA content and 88.6% of FAME conversion after 72 h of reaction. Even so, these values can be taken as very expressive if we evaluate the real amount of enzyme in the reaction mixture with enzyme derivative (8

times lower than the system with free enzyme). Therefore, it is possible to affirm that the lower reaction rate found for the immobilized biocatalyst may not be related to inhibition or deactivation due to the immobilization process, but a consequence of its low enzyme content. Moreover, it should be taken in mind that for immobilized enzyme systems there are mass transfer limitations that reduce the reaction rates due to the diffusional limitations of substrates and product transfer through the support pores (RODRIGUES et al., 2013).

Figure 14 – Kinetic curves for the FAME synthesis from industrial fatty waste catalyzed by free and immobilized soluble Eversa® Transform 2.0 lipase in polyurethane foam.



Experimental conditions: 0.3 wt% of enzyme, 2 wt% distilled water, 2.0 eqv of methanol, 300 ppm of NaOH, and 500 ppm of enzymatic cofactor, at 45 °C and 250 rpm.
Source: Author.

It is noteworthy that PU support had little or no fragmentation during the assays. This is quite important because one of the main purposes of enzyme immobilization is to ensure that the enzyme remains immobilized in the support allowing its reuse. Furthermore, we are evaluating the system as being immobilized and comparing them against the performance of the same enzyme in its free form. So, if there is the fragmentation of the support, there is a risk that the enzymes are

leaching from them to the reaction mixture, which may compromise the reliability of the obtained results.

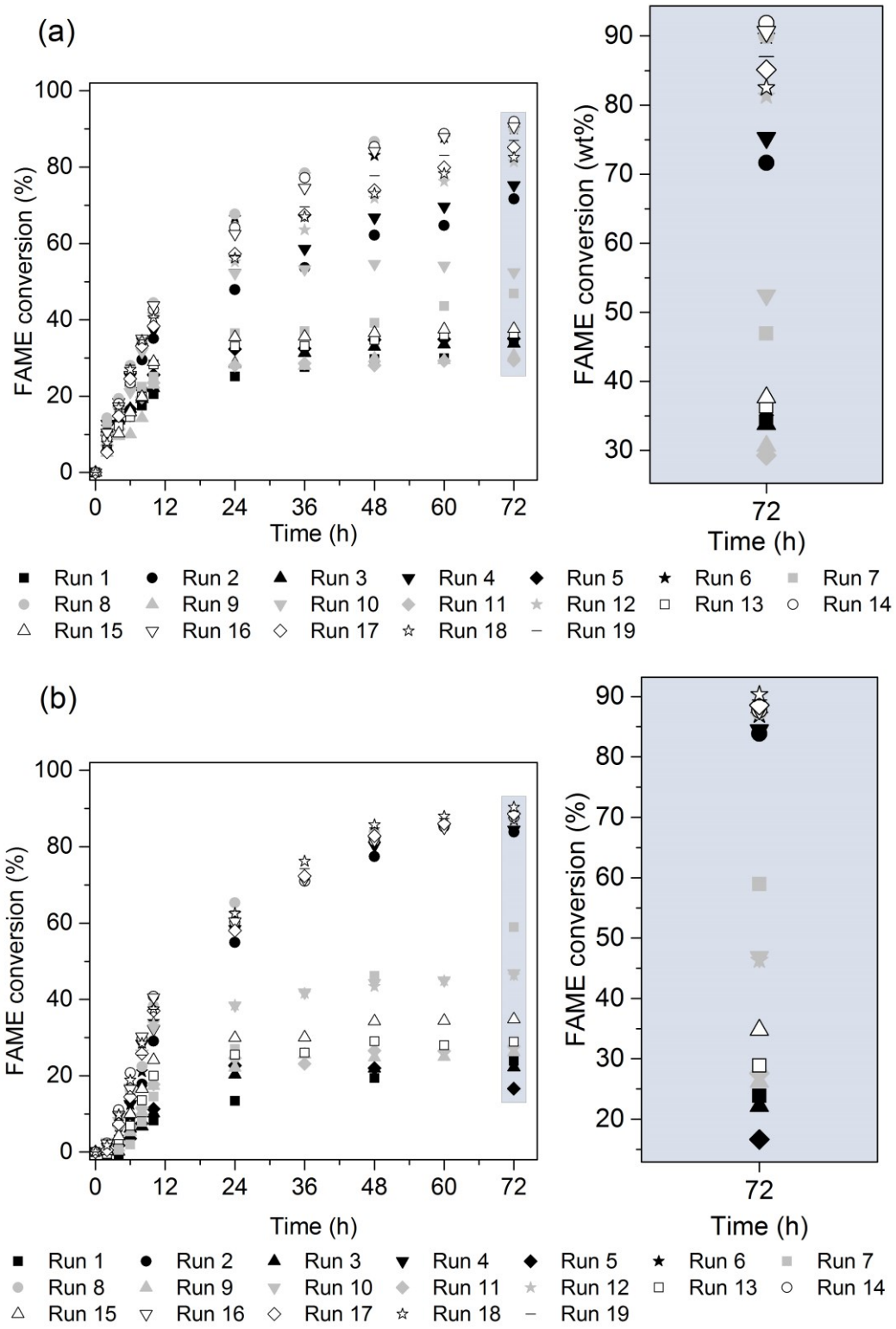
In view of the achieved, expressed, and discussed about the results from Figure 14 and, since the PU polymerization reaction is exothermic, the next step was to analyze the possible influence of such heat on the stability and, consequently, on the performance of the immobilized lipases. Thus, a novel immobilization assay was performed using an ice bath during the support synthesis and simultaneously ET2 immobilization.

These two enzyme derivatives (not-cooled and cooled) were employed on the FAME synthesis from industrial fatty waste with high FFA content, following an experimental 2^4 factorial design (Figure 15 and Table 6) to evaluate how the system composition affects the reaction kinetic. It was observed that the immobilized derivatives kept the structural integrity after all assays.

Lipases can convert both triacylglycerols and FFA into biodiesel. Among the thermodynamically possible reactions are i) transesterification of triglycerides to form methyl esters, ii) hydrolysis of triglycerides to form fatty acids and glycerol, and iii) esterification of fatty acids to form methyl esters. Thus, the standards recommend following the progress of the reaction by measuring the content of FAME using suitable techniques. The titration technique for FAME conversion is reported by several works (LEMAIRE et al., 2011; MARCHETTI; MIGUEL; ERRAZU, 2007; SANTIN et al., 2014; TRAN; CHEN; CHANG, 2012) and it is an efficient method, in this case, to replace the gas chromatography since no need the complete drying of the sample for water removal nor the evaporation of the alcohol excess. The FFA content variation is taken as complementary information to the FAME conversion and it allows more information on the different reactions involved.

Based on data from Table 6 it was possible to perform the statistical analysis (ANOVA, Table 7). The obtained R-squared value (R^2) was 0.81 and the F calculated for all variables and the interactions between them were lower than the F tabulated at 95% confidence level, except for the water content. This interpretation can be better observed by the p values shown in the Pareto charts (Figure 16). Based on that and the results shown in Table 6, it was verified that water had a positive significant effect (at 95% of confidence) for both enzyme derivatives; showing that at higher levels it could lead to higher results of conversion.

Figure 15 – Kinetic profiles of the experimental 2^4 factorial design for FAME synthesis from industrial fatty waste catalyzed by not-cooled (a) and cooled (b) soluble Eversa® Transform 2.0 lipases immobilized in polyurethane foam.



Source: Author.

Table 6 - Real and coded variables of experimental 2^4 factorial design for FAME synthesis from industrial fatty waste catalyzed by cooled and not-cooled soluble Eversa® Transform 2.0 lipases immobilized in polyurethane foam.

Assay	Water (wt%)	NaOH (ppm)	Enzymatic cofactor (ppm)	MeOH (eqv)	FFA content (wt%)[a]		FAME conversion (%)[a]	
					Not-cooled	Cooled	Not-cooled	Cooled
1	0 (-1)	0 (-1)	0 (-1)	1.2 (-1)	36.9	42.6	34.0	23.8
2	2 (+1)	0 (-1)	0 (-1)	1.2 (-1)	15.9	9.0	71.6	83.9
3	0 (-1)	300 (+1)	0 (-1)	1.2 (-1)	37.0	43.5	33.8	22.7
4	2 (+1)	300 (+1)	0 (-1)	1.2 (-1)	13.8	8.7	75.3	84.4
5	0 (-1)	0 (-1)	500 (+1)	1.2 (-1)	36.3	46.6	35.1	16.7
6	2 (+1)	0 (-1)	500 (+1)	1.2 (-1)	5.6	7.3	90.0	86.9
7	0 (-1)	300 (+1)	500 (+1)	1.2 (-1)	29.7	22.9	46.9	58.9
8	2 (+1)	300 (+1)	500 (+1)	1.2 (-1)	5.6	7.0	90.0	87.4
9	0 (-1)	0 (-1)	0 (-1)	2.0 (+1)	38.8	41.3	30.6	26.2
10	2 (+1)	0 (-1)	0 (-1)	2.0 (+1)	26.6	29.7	52.5	46.9
11	0 (-1)	300 (+1)	0 (-1)	2.0 (+1)	39.5	40.4	29.3	27.6
12	2 (+1)	300 (+1)	0 (-1)	2.0 (+1)	10.5	30.0	81.3	46.3
13	0 (-1)	0 (-1)	500 (+1)	2.0 (+1)	35.6	39.7	36.3	28.8
14	2 (+1)	0 (-1)	500 (+1)	2.0 (+1)	4.5	6.9	91.9	87.7
15	0 (-1)	300 (+1)	500 (+1)	2.0 (+1)	34.8	36.4	37.7	34.8
16	2 (+1)	300 (+1)	500 (+1)	2.0 (+1)	5.3	6.8	90.6	87.9
17	1 (0)	150 (0)	250 (0)	1.6 (0)	8.3	6.4	85.1	88.6
18	1 (0)	150 (0)	250 (0)	1.6 (0)	9.8	5.4	82.5	90.3
19	1 (0)	150 (0)	250 (0)	1.6 (0)	7.3	5.9	87.0	89.4

[a] 72 h of reaction

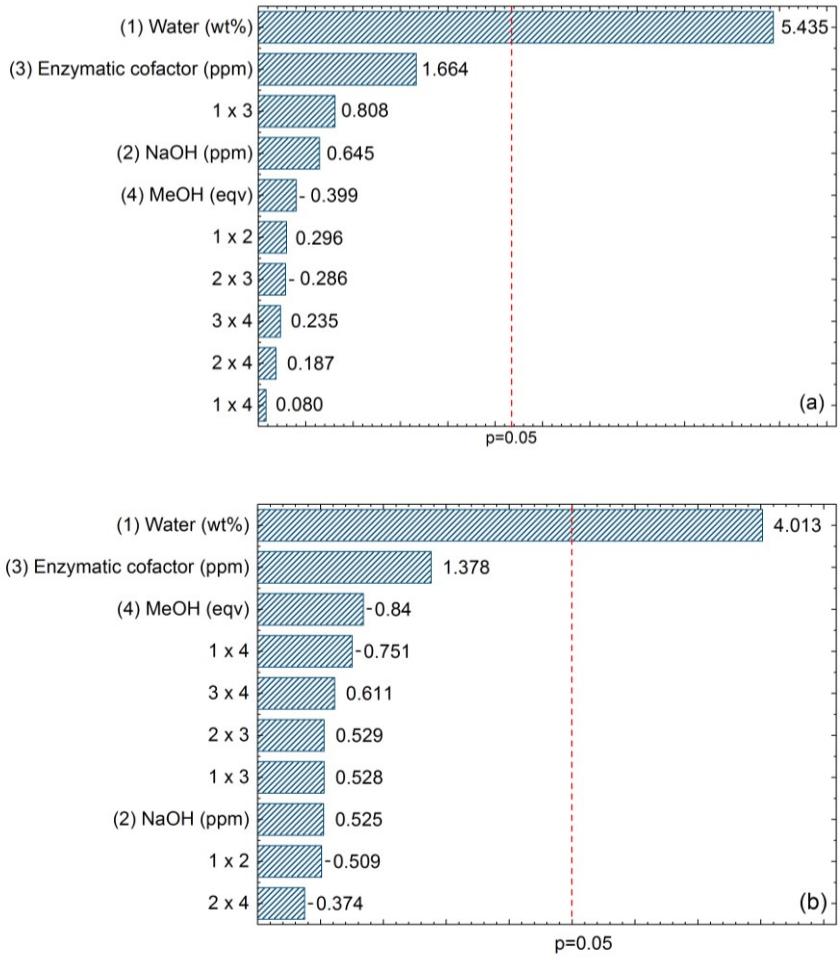
Table 7 - Analysis of variance (ANOVA) from Table 6.

Factor	SS	dF	MS	F	p
(1) Water (wt%)	8070.07	1	8070.065	29.54068	0.000620
(2) NaOH (ppm)	113.8	1	113.798	0.41656	0.536725
(3) Enzymatic cofactor (ppm)	756.82	1	756.816	2.77035	0.134593
(4) MeOH (eqv)	43.62	1	43.621	0.15968	0.699911
1 x 2	24.05	1	24.052	0.08804	0.774240
1 x 3	178.77	1	178.766	0.65438	0.441944
1 x 4	1.75	1	1.754	0.00642	0.938098
2 x 3	22.33	1	22.328	0.08173	0.782226
2 x 4	9.59	1	9.588	0.0351	0.856055
3 x 4	15.09	1	15.086	0.05522	0.820118
Error	2185.48	8	273.185		
Total SS	11421.35	18			

$R^2 = 0.81$.

The results confirmed the importance of water since its presence in the hydrolysis reaction of triglycerides prevents the enzyme molecule dehydration and promotes the interface layer needed for lipase mechanism (ORTIZ et al., 2019; REIS et al., 2009b). This effect can even be better visualized in Figure 15, where two reaction kinetic groups are observed: the first one with the presence of water and the other one with the absence. However, according to Remonatto et al. (2016), high contents of water should also be avoided. The authors observed the water effect by using 2.5–10 wt% of water in the FAME synthesis catalyzed by soluble Eversa® Transform and concluded that high content of water may have led to the reverse of esterification reaction, hence causing hydrolysis of some of the formed esters.

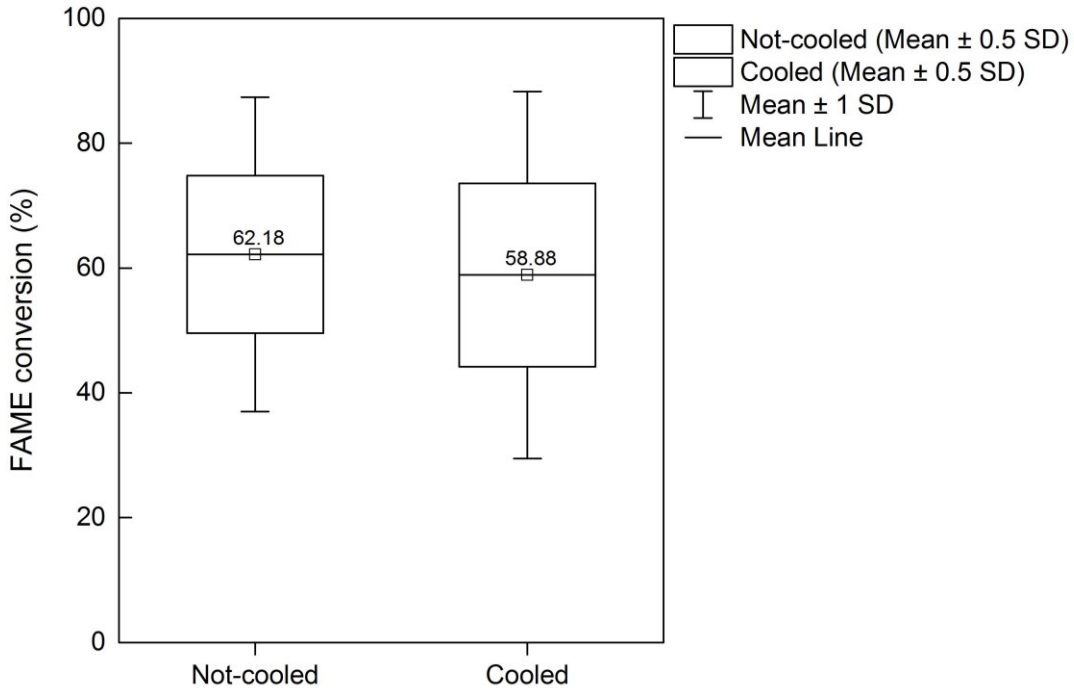
Figure 16 - Pareto chart with the effects of the variables ($p < 0.05$) for both experimental designs from Table 6 with the response in terms of FAME conversion (%) using: not-cooled (a) and cooled (b) soluble Eversa® Transform 2.0 lipases immobilized in polyurethane foam.



Source: Author.

Regarding the possible influence of the heat which is generated by the PU polymerization process and its influence on the stability and enzyme activity, the comparison between the data obtained from the two experimental designs (Table 6) can confirm that the proposed treatment (ice bath during the synthesis) did not show a significant effect according to the statistical analysis by Box Plot (Figure 17). Therefore, the following discussions will be based on a single experimental design (not-cooled ET2 immobilized in PU foam); however, they can be considered common for both.

Figure 17 - Box plot of FAME conversion (%) after 72 h of reaction for the experimental designs from Table 6.



Source: Author.

Related to methanol, no significant effect at 95% of confidence was observed. Thus, the ideal reaction condition can be kept at level -1 of the design (1.2 eqv) due to the economic aspect. However, in addition to the simple recovery process at the end of the process, at high concentration, methanol tends to shift the stoichiometric equilibrium of the reaction towards the formation of the product (HE et al., 2017; SHIBASAKI-KITAKAWA et al., 2015). On the other hand, the excessive

content of short-chain alcohols such as methanol may inactivate the lipase (MEHER; VIDYA SAGAR; NAIK, 2006; SHIMADA et al., 2002). The main factor responsible for the inactivation or loss of enzyme activity in organic solvents is the loss of water molecules. The low water content directly affects K_m and V_{max} values since it restrains conformation mobility. The loss of water can also weaken active site interactions in the interior of proteins and disrupt hydrogen bond formation between protein subunits on the exterior surface (KARAN; CAPES; DASSARMA, 2012). Therefore, the key to the process is to use an excess of MeOH sufficiently for the stoichiometric equilibrium shifts to ester formation without interfering in the enzyme activity.

Another parameter with similar behavior to methanol was NaOH content. The observed effect was also not significant at 95% confidence, and the level -1 (0 ppm) can be used aiming at the process cost reduction and a final product with lower impurities. However, the NaOH reacts with mineral acids present in the feedstock since even traces of those components might promote the inactivation of the enzymes. Moreover, note that the NaOH content employed in this study was not enough to promote soup formation. The same analysis may be done for the enzymatic cofactor parameter. The enzymatic cofactor acts protecting the enzyme against inhibitors in the medium, such as methanol (MIBIELLI et al., 2019) and it is constantly linked with an increase in activity of the enzymes (BRESOLIN et al., 2019; DANDAVATE et al., 2009; DUNBAR et al., 2015; KNAPE et al., 2015). Mibielli et al. (2019) evaluated the FAME synthesis from residual raw materials of a soybean oil industry using the free ET2 in a similar system to this study and observed higher FFA conversion to FAME when higher amounts of NaOH were added. Thus, aiming for a system more robust for a variation of the feedstock, these components may become crucial for the success of the reaction system.

The best result in terms of FAME conversion was achieved for assay 14 (Table 6), reaching 4.5 wt% of FFA content and 91.9% of FAME conversion after 72 h of reaction. However, assay 16 at almost the same experimental conditions, except for NaOH (+1), showed similar results (5.3 wt% of FFA content and 90.6% of FAME conversion) and was chosen for future experiments in Chapter 4, based on the previous discussion.

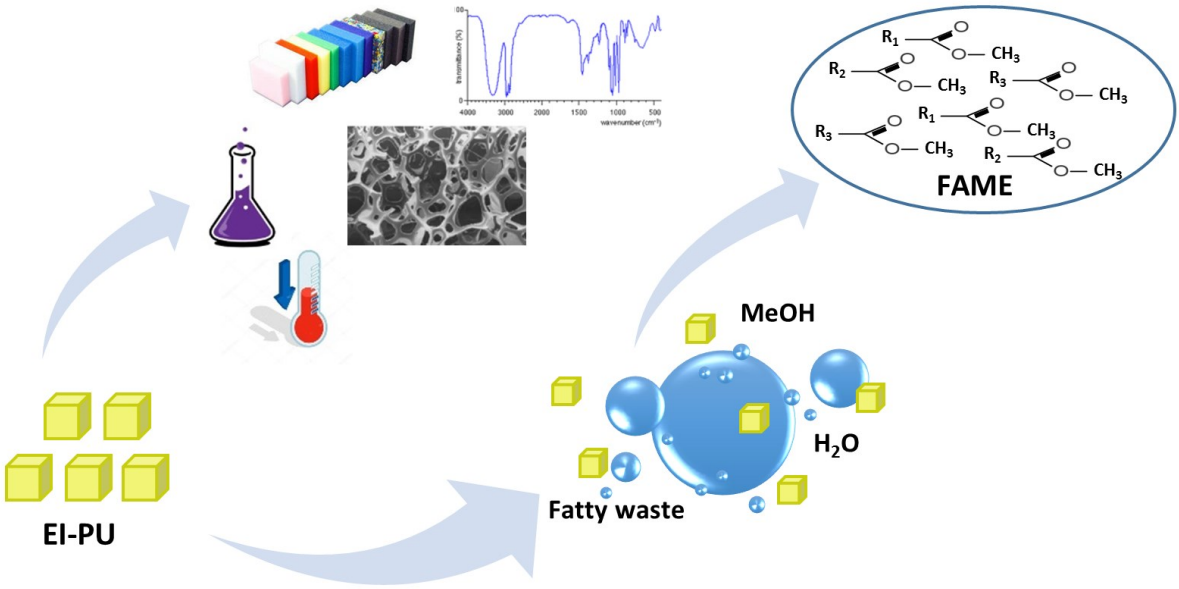
3.3 CONCLUSION

This chapter presented a simple immobilization methodology for commercial soluble ET2 on PU support. The enzyme derivative showed no inactivation during the immobilization process even without an ice bath during the process and it was able to further application on the reaction of interest. The immobilized biocatalyst was employed for FAME synthesis from industrial fatty waste reducing the FFA content from 55.9 wt% to values around 4 wt% and reaching FAME conversion above 90%. The experimental factorial design proposed to evaluate how the system composition affects the FAME synthesis revealed that the condition 2 wt% of water, 2.0 eqv of methanol, 300 ppm of NaOH, and 500 ppm of enzymatic cofactor was what better performed for that feedstock. This work showed for the first time a successful report of immobilization of soluble Eversa® Transform 2.0 lipase on PU foam.

4 CHARACTERIZATION OF LIPASE EVERSA® TRANSFORM 2.0 IMMOBILIZED ON POLYURETHANE FOAM AND SUCESSIVE CYCLES OF FAME SYNTHESIS

The main objective of this chapter was to improve the enzyme loading on the immobilized biocatalyst obtained in Chapter 3 aiming to a better kinetic of fatty acid methyl ester (FAME) synthesis from industrial fatty waste. Moreover, the enzyme derivative was fully characterized in terms of structure and thermal, pH, and storage stability. Lastly, successive cycles of reuse on FAME synthesis were performed. Figure 18 shows the activities developed in this chapter.

Figure 18 - Scheme of the activities developed in Chapter 4.



Source: Author.

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4.1 MATERIAL AND METHODS

4.1.1 Material

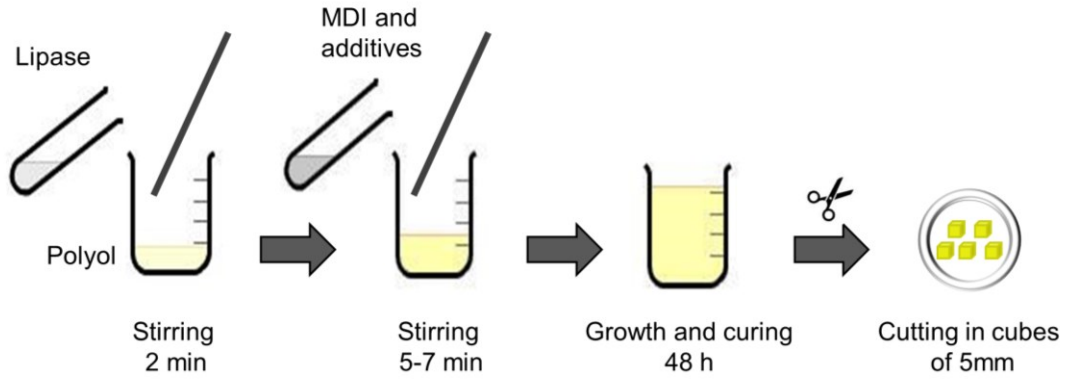
A commercial solution of Eversa® Transform 2.0 lipase (ET2) from *Aspergillus oryzae* (protein content 35 mg/mL) was provided by Novozymes (Araucária, PR, Brazil). The monomers polyol polyether and diphenylmethane diisocyanate (MDI) for polyurethane (PU) synthesis were kindly donated by Purcom Company (industrial manufacturer of PU flexible foam in Brazil). Soybean oil (Soya, Brazil), purchased from the local market (free fatty acid, FFA, content 0.3 wt%), was used as substrate for hydrolysis reaction without any further treatment. Industrial fatty waste, a blend of low-cost feedstocks, was kindly provided by Prisma Comercial Exportadora de Oleoquímicos LTDA (Sumaré, SP, Brazil) and Transfertech (Erechim, RS, Brazil) companies (FFA content 55.9 wt%, iodine value of 105, and water content of 2.4 wt%). All reagents and solvents used were of analytical grades.

4.1.2 Methods

4.1.2.1 Synthesis of polymeric support and lipase immobilization

ET2 immobilization was performed by entrapment technique (*in-situ*) in the synthesis of the PU support, following the methodology described in our previous work (FACIN et al., 2018b). Briefly, the procedure consisted of adding the ET2 to the polyol polyether phase, followed by the addition of the MDI phase under stirring for 1-3 min until complete homogenization. After the polymerization process (5 min), the foam formed was kept at room temperature (~21 °C) for 48 h for complete cross-linking and curing of the structure (Figure 19).

Figure 19 - Scheme of support synthesis and simultaneous immobilization of soluble Eversa® Transform 2.0 lipase.



Source: Author.

4.1.2.2 Determination of enzyme activity

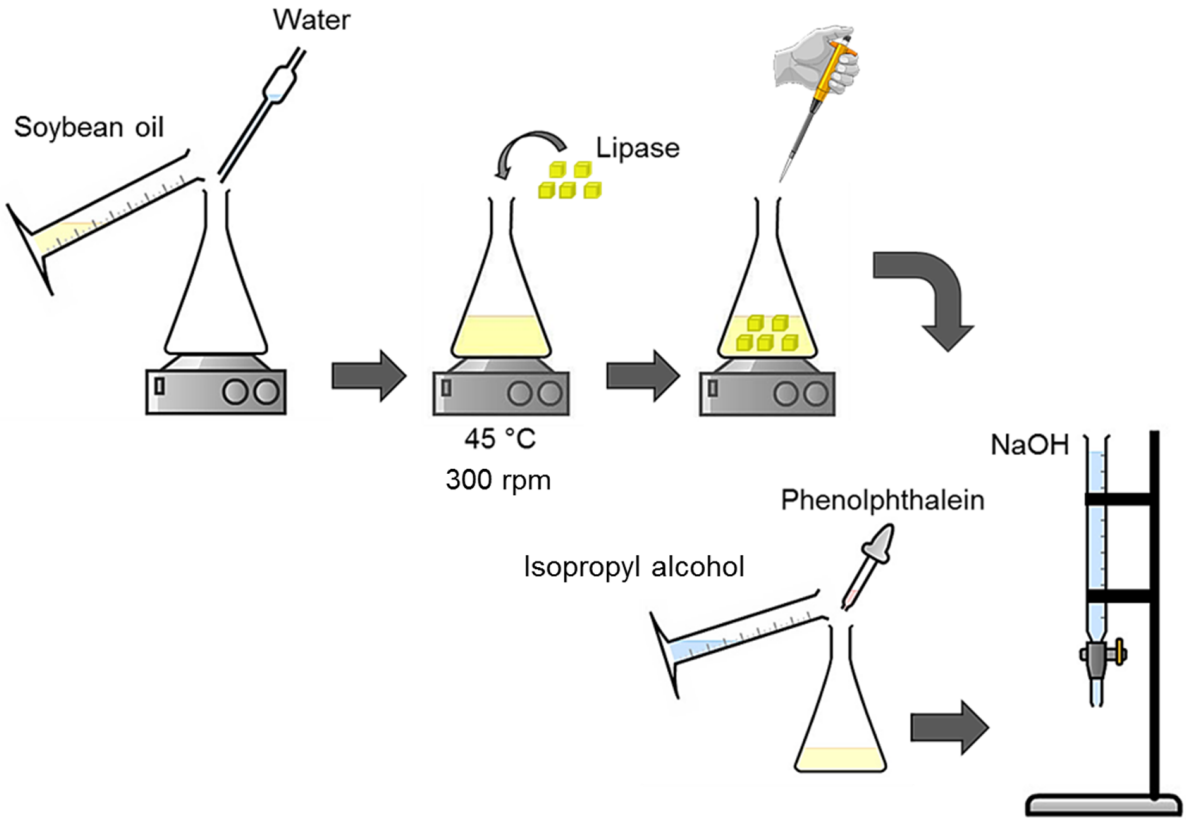
The hydrolytic enzyme activity was determined based on the adapted methodology of Zenevicz et al. (2016). In a jacket glass reactor, 100 g of soybean oil as substrate, 4 wt% of distilled water (by weight of substrate), and 0.5 wt% of free or immobilized enzyme (by weight of substrate) was added. The reaction was carried out under magnetic agitation (300 rpm), 45 ºC for 30 min. Then, a sample of 0.5 g was taken out and diluted in 25 mL of isopropyl alcohol. Indicator (two drops of 1 wt% of phenolphthalein in ethanol) was added, and the solution was titrated with 0.1 mol/L sodium hydroxide solution (NaOH) to purple color persistence under vigorous stirring (Figure 20). One unit of lipase activity (U) was defined as the amount of enzyme, which produces 1 µmol of fatty acids per minute under the assay conditions. The enzyme activity was expressed as a unit of lipase activity per mass of catalyst (U/g) and was determined by Equation 4. The residual activity was expressed as a percentage and defined as the ratio of (enzyme activity measured/enzyme activity of fresh free enzyme) × 100.

$$A_H = \frac{(V_{NaOH}^{30} - V_{NaOH}^{0}) * M_T * C_{NaOH} * 10^3}{m_S * t * m_E} \quad (4)$$

where: A_H is the enzyme activity of the lipase (U/g); $V_{NaOH}^{30}$is the volume of NaOH solution consumed in the sample titration after 30 minutes of reaction (mL); V_{NaOH}^{0} is

the volume of NaOH solution consumed in the sample titration at time zero (mL); M_T is the total mass of the reaction (g); C_{NaOH} is the NaOH molarity solution (M); m_S is the mass of the titrated sample (g); t is the reaction time (min); m_E is the catalyst mass employed on the reaction (g).

Figure 20 - Scheme for determination of enzyme activity.



Source: Author.

4.1.2.3 Characterization of support and enzyme derivative

The morphological characterization was performed by scanning electron microscopy (SEM, JEOL JSM-6390LV at 5 kV). The enzyme derivative was sliced, fixed with carbon tape on a stub, and covered with gold. The attenuated total reflectance with Fourier transform infrared spectroscopy (FTIR) was obtained from Agilent Technologies, model Cary 600 Series, in length range of 3900 to 584 cm^{-1} and resolution of 4 cm^{-1}. The density was determined according to ASTM D3574 (AMERICAN SOCIETY FOR TESTING AND MATERIALS, 2003) and by gas

pycnometer (Micromeritics, Accupyc II 1340). Protein concentration was determined by Bradford method (BRADFORD, 1976) with bovine serum albumin as standard to confirm the manufacturer information.

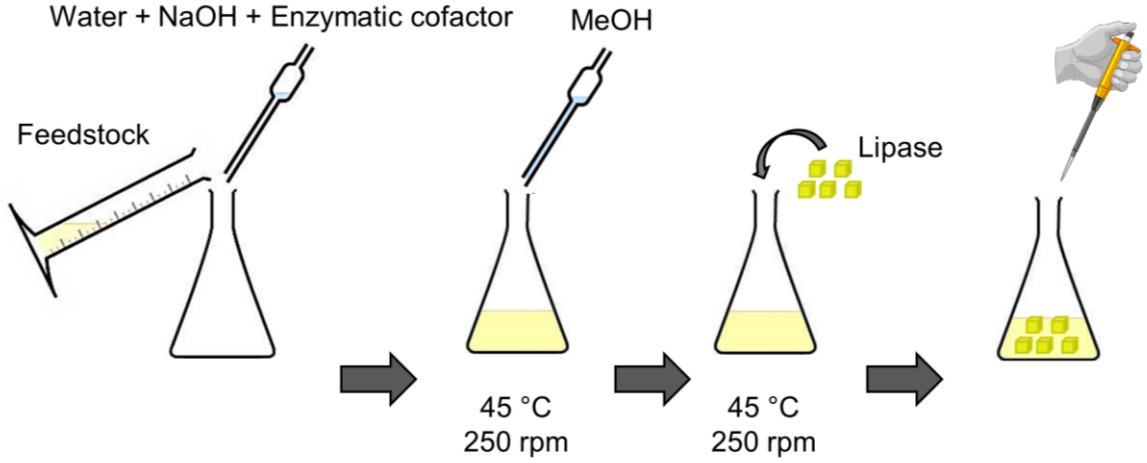
4.1.2.4 Thermal, pH, and storage stability studies

To evaluate the pH stability, free and immobilized enzymes were submitted to pH 3, 7, and 11, using 100 mM, 50 mM, and 25 mM sodium phosphate buffers, respectively, at room temperature (~21 °C), and the enzyme activity was determined by Equation 4 (see 4.1.2.2) using the buffers at different pH values. For all assays, the enzyme activity was measured when the enzyme was added to the buffer solution (t = 0 h) and after 6 h. Concerning the thermal stability, free and immobilized enzymes were incubated at 60 and 75 °C for 6 h. Storage stability was carried out keeping free and immobilized enzymes at 4 °C with the enzyme activity periodically determined. The enzyme activity was expressed as residual activity.

4.1.2.5 FAME synthesis

The enzymatic FAME synthesis followed the same methodology described at Chapter 3 (see 3.1.2.2) and was adapted from Mibielli et al. (2019) (Figure 21). The experimental conditions employed were that lead to the best results in terms of FAME conversion at that Chapter. The enzymatic reaction was carried out at 45 °C using a shaker incubator (250 rpm) in a fed-batch system using a 1.5 L sealed flask. The composition of the reaction system consisted of 100 g of feedstock, 2 wt% distilled water addition (by weight of feedstock), 300 ppm of NaOH (by weight of feedstock), 500 ppm of enzymatic cofactor (by weight of feedstock) (based on previous work of Bresolin et al. (2019) and not authorized to disclose by the manufacturer), and 0.3 wt% of free enzyme or 1.3 wt% of immobilized enzyme (by weight of feedstock). Methanol was added in equal part stepwise from the first 10 h of reaction (0-10 h) in a total of 2.0 equivalent (eqv, on the basis of stoichiometric reaction) to prevent enzyme inhibition. Aliquots were taken out from the reaction mixture at given times for composition analysis (FFA content and FAME conversion).

Figure 21 - Scheme of FAME synthesis.



Source: Author.

4.1.2.6 Determination of FFA content

FFA amount in the reaction medium was determined by the titration technique according to ISO 660:2009 (INTERNATIONAL ORGANIZATION OF STANDARDIZATION, 2009), performed in triplicate (n = 3). For the acidity calculation, an aliquot of 0.5 g was taken out from the reaction mixture and diluted into 25 mL of neutral ethanol solution. Indicator (about three drops of 1 wt% phenolphthalein in ethanol) was added, and the solution was titrated with 0.1 mol/L sodium hydroxide solution (NaOH) to purple color persistence under vigorous stirring. The FFA content was calculated by Equation 5:

$$FFAcontent(wt\%) = \frac{V_{NaOH} * M_{NaOH} * MM_{FFA}}{10 * m_s} \quad (5)$$

where: V_{NaOH} is the volume of NaOH solution consumed in the sample titration (mL); M_{NaOH} is the molarity of the NaOH solution (M); MM_{FFA} is the average molar mass of fatty acids (FA), 282 g/mol for the employed oil; m_S is the mass of titrated sample (g).

4.1.2.7 Determination of FAME conversion

To determine the conversion of raw material to FAME, the titration technique based on ISO 660:2009 (INTERNATIONAL ORGANIZATION OF STANDARDIZATION, 2009) was used and consists in determining the acid value of the sample, due to the presence of free fatty acids. These acids can react with the potassium/sodium hydroxide solution, and the obtained result is expressed in terms of (mg) of KOH/NaOH per (g) of sample. For the calculation, an aliquot of 0.5 g was taken out from the reaction mixture and diluted in 25 mL of neutral ethanol solution. Indicator (two drops of 1 wt% phenolphthalein in ethanol) was added, and the solution was titrated with 0.1 mol/L sodium hydroxide solution (NaOH) to purple color persistence under vigorous stirring. The analysis was performed in triplicate (n=3). The acid value (AV) was calculated by Equation 6:

$$Acidvalue(mg\ KOH/g) = \frac{56.1 * V_{KOH} * M_{KOH}}{m_s} \quad (6)$$

where: V_{KOH} is the volume of NaOH solution consumed in the sample titration (mL); M_{KOH} is the molarity of the NaOH solution (M); m_S is the mass of titrated sample (g).

The FAME conversion was then calculated through the initial and final (or at specific time) acid value, as follows:

$$FAMEconversion(\%) = \frac{AV^o - AV^t}{AV^0} * 100 \quad (7)$$

where: AV^0 is the initial acid value (mg KOH/g); AV^t is the acid value at given time (mg KOH/g).

4.2 RESULTS AND DISCUSSION

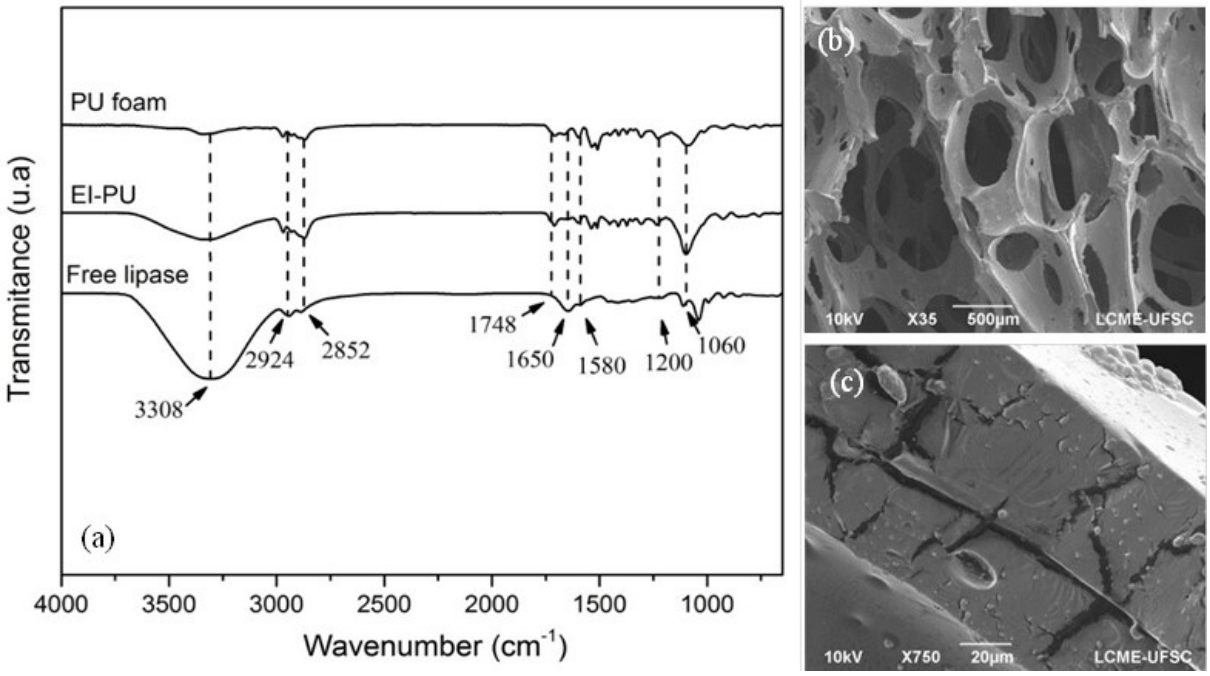
4.2.1 Improvement and characterization of enzyme derivative

To improve the enzyme loading in PU foam reached for the first immobilized derivative (Chapter 3) and increase the kinetic rates of FAME production, the

agitation time was raised to improve the CO_2 dispersion generated by the reaction of isocyanate with water during the PU formation. Water on the PU polymerization process can act as a blowing agent decreasing the foam density, and due to the formation of polyurea, increases hardness, elongation, and tensile strengths (KAUSHIVA, 1999). However, an excess of water may accelerate the growth of foam reaction formation causing the cells opening before the polymer to be strong enough to maintain its structure, hence resulting in foam collapse (VILAR, 2004).

The obtained immobilized biocatalyst (EI-PU) showed no phase separation, good pore distribution, and homogeneity (Figure 22b). The amount of lipase solution incorporated was improved from 12.5 wt% to 26.1 wt% (by weight of PU support) reaching an enzyme loading of 9.1 mg/g (enzyme/support) with an enzyme activity of 2826.8 ± 24.6 U/g that means a residual activity up to 72.1% (3919.6 ± 0.5 U/g for the free enzyme).

Figure 22 - FTIR spectrum (a) of polyurethane foam with immobilized soluble Eversa® Transform 2.0 lipase and scanning electron microscopy (b and c).



Source: Author.

EI-PU SEM images (Figure 22b-c) show a rough surface with well-distributed macropores. The presence of macropores was confirmed by low BET superficial area

(7.959 m^2/g), as also reported in works related to PU foam (GAMA et al., 2017; SILVA et al., 2013; TAVAKOLI; SOLEIMANI; ALAVI NIKJE, 2019).

The apparent density measured according to the ASTM D3574 was 0.071 ± 0.004 g/cm^3 and the gas pycnometer 1.192 ± 0.004 g/cm^3. The apparent density value obtained in this work was similar to those reported by Nyari et al. (2016) (0.029 ± 0.015 g/cm^3). On the other hand, Nicoletti et al. (2015) found higher values (0.627 ± 0.140 g/cm^3) may be associated with the monomers employed. Apparent density is the ratio between the mass and the geometric volume of the sample, which includes the polymeric material, and the gas within the foam cells (AMERICAN SOCIETY FOR TESTING AND MATERIALS, 2003). At this point, it is important to mention that at the industrial level, gas pycnometry is more acceptable since this technique is more accurate compared to apparent density.

FTIR analyses (Figure 22a) confirmed the PU formation and enzyme presence on PU foam. The spectrum of free enzyme shows a typical spectrum of proteins with absorption bands associated with primary and secondary amine with peak location at 1000-1200 cm^{-1} and 1580-1650 cm^{-1} (BARTH, 2007). After immobilization, these groups remained in the spectrum of the EI-PU confirming the presence of enzyme on the support. The stretching at 3308 cm^{-1} is associated with O-H bonds from water from enzyme solution (STUART, 2004) which is significantly reduced after the foam cures.

The stretching peak located at 1060 cm^{-1} is related to the C-NH-C bonds (BARNES; JUGDAOSINGH; KIAMIL, 2011) and the absorption band with peak location at 1748 cm^{-1} and 1600 cm^{-1} is attributed to stretching vibration of (C=O) urethane group, identifying PU in the support (CHIARADIA et al., 2016). The peak location at 2924-2852 cm^{-1} characteristic of C–H bond (STUART, 2004) was also observed. The absence of an absorption band with peak location at 2270 cm^{-1}, corresponding to the isocyanate group (N=C=O), indicates the total consumption of the isocyanate groups during the synthesis of support.

4.2.2 Enzyme stability: thermal, pH and storage

To evaluate the efficiency of PU foam as a support for the biocatalyst, studies related to the enzyme stability at different pH and temperature conditions were conducted as shown in Table 8.

As observed from the results, the EI-PU showed less thermostability than the free enzyme. Usually, enzyme thermostability increase after the immobilization process as reported in previous works (ARANA-PEÑA; LOKHA; FERNÁNDEZ-LAFUENTE, 2018; NICOLETTI et al., 2015).

Table 8 - Residual activity of EI-PU at different pH and temperature conditions.

Stability	Condition	Form	Residual Activity (%)*	
			Before treatment (time 0)	After treatment (6 h)
Temperature (°C)	60	Free	100 ± 0.1	99.1 ± 2.0
		EI-PU	72.1 ± 0.6	71.0 ± 0.6
	75	Free	100 ± 0.1	11.3 ± 1.1
		EI-PU	72.1 ± 0.6	0.3 ± 0.1
pH	3	Free	56.2 ± 0.7	57.3 ± 0.1
		EI-PU	24.6 ± 0.8	22.6 ± 2.6
	7	Free	105.8 ± 1.8	107.4 ± 0.2
		EI-PU	81.9 ± 0.3	95.2 ± 2.1
	11	Free	102.2 ± 1.7	113.1 ± 2.6
		EI-PU	80.2 ± 0.2	78.4 ± 1.1

* Mean values for measures in duplicate (n = 2).

It was verified that free and immobilized enzymes were able to keep the activity after 6 h at 60 ºC, but both were almost fully inactivated when the temperature was increased to 75 ºC for the same period. These results may be related to the water removal from the enzyme surface, essential to stabilize the three-dimensional structure of the enzyme.

For the pH evaluation, EI-PU followed the same behavior observed for the free enzyme. At pH 3, the activity dropped drastically for both free and immobilized enzymes immediately when put into the buffer (below 60% and 25%, respectively), although they remained practically unaltered after 6 h of treatment. These results

should be associated with the proximity of the isoelectric point of the enzyme what reduces its solubility and consequently its activity (CIPOLATTI et al., 2016).

When increasing the pH value, the activity of the free enzyme was increased, reaching up to 110% after 6 h at pH 11. This behavior was also verified for the EI-PU. However, the higher enzyme activity was observed after 6 h at pH 7 (up to 95% of the initial activity). Similar behavior was found by ARANA-PEÑA; LOKHA; FERNÁNDEZ-LAFUENTE (2018) for the same immobilized enzyme on octyl agarose beads. According to the authors, the difference in the effects of temperature and pH value on the activity of the enzyme preparations can be explained by the complexity of the lipase activity, which is extremely dependent on the active form. Furthermore, according to the authors, these results cannot be considered a negative or a positive result when only one specific condition is evaluated, but a combination of factors should be considered. Thus, the enzyme stability was also evaluated against the time at 4 ºC and the activity remained constant for the EI-PU over to 60 days.

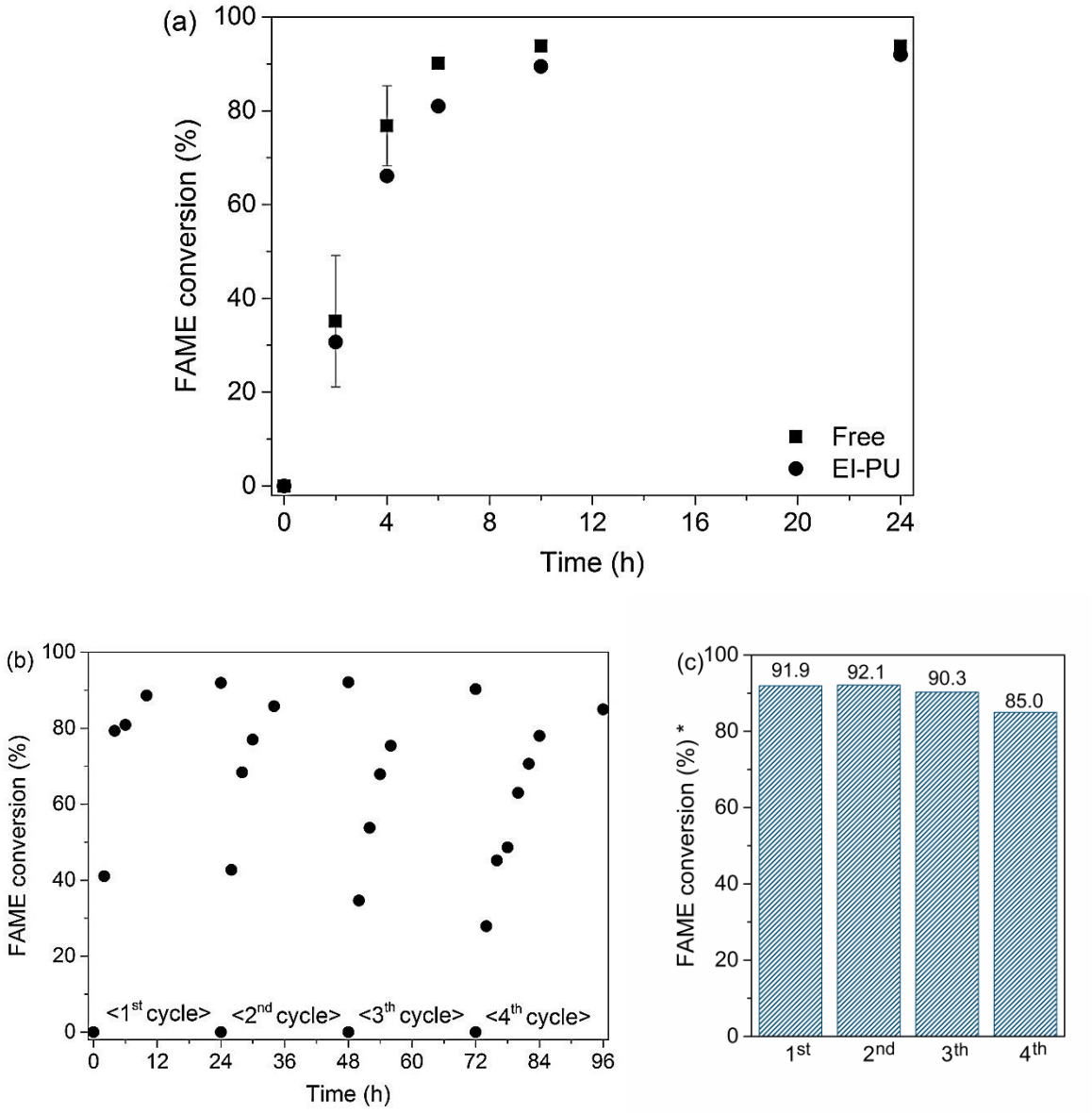
4.2.3 FAME synthesis: application and successive reuse cycles

The main goal of enzyme immobilization is the recovery possibility and ensure the enzyme activity after successive reuses (RODRIGUES et al., 2019). Also, it is important to choose support with high structural integrity, as observed in this work for the PU foam, once the enzyme may be leached from the support during the reaction process reducing the cycles of reuse and efficiency.

To investigate whether the enzyme loading could reflect a better performance of the enzyme derivative, the efficiency of the EI-PU was measured on the FAME synthesis using industrial fatty waste with high FFA content under the best conditions found previously (see Chapter 3) (Figure 23a). The amount of immobilized biocatalyst employed was improved (1.3 wt%) to equalize the amount of available enzyme in both systems (free and immobilized). From the results, it was observed that the EI-PU showed similar kinetics compared to the free enzyme achieving up to 4 wt% of FFA content and up to 91% of FAME conversion in 24 h of reaction. These results showed that the macroporous in the support structure were able to allow the reaction

between the enzyme and the substrate and did not reduce the diffusion and mass transfer offered by PU support.

Figure 23 – Kinetics profiles for FAME conversion from industrial fatty waste catalyzed by free Eversa® Transform 2.0 and EI-PU (a), reuse cycles of EI-PU (b), and final FAME conversion after each reuse cycle (c).



Experimental conditions: 0.3 wt% of free enzyme or 1.3 wt% EI-PU, 2 wt% of water, 2.0 eqv of methanol, 300 ppm of NaOH, and 500 ppm of enzymatic cofactor, at 45 °C and 250 rpm.
Source: Author.

Several reports are found in the open literature along the last years for FAME synthesis catalyzed by immobilized lipases. For example, RAITA; LAOSIRIPOJANA; CHAMPREDA (2015) employed *Thermomyces lanuginosus* lipase immobilized in

cross-linked protein-coated microcrystals for FAME synthesis from palmitic acid (PA), refined edible grade palm oil (RPO), and crude palm oil (CPO) reaching 97.6, 94.9, and 95.5% of conversion, respectively, after incubation at 50 ºC for 6 h with 20 wt% of enzyme loading. In another study, *Candida antarctica* lipase B (CALB) immobilized in polyporous magnetic cellulose beads (PMCBs) was used for FAME synthesis from Yellow horn seed oil reaching 92.3 wt% of conversion at 60 ºC for 2 h with 15 wt% of enzyme loading (ZHANG et al., 2020). What stands out in both works is the high amount of biocatalyst employed for a high FAME synthesis, which definitively hinders an industrial application. In our study, the EI-PU was able to reach high conversion on FAME at low biocatalyst loading (1.3 wt%).

A recent publication suggested that the cost of the support is higher than the enzyme price which can further hinder the commercial application (BUDŽAKI et al., 2018). Thus, biocatalyst reuse is an important parameter to be evaluated due to the high cost related to enzymes in industrial processes. In this work, the reuse assays showed that the EI-PU was able to be reused at least four times until reaching the endpoint defined (up to 5 wt% of FFA content or less than 90% of FAME conversion in 24 h of reaction) (Figure 23b-c). Regarding the cost of enzymatic processes, it is known they are notably higher than conventional processes. This contrast is even accentuated when immobilized biocatalysts are chosen since the cost of the support must be taken. Thereby, EI-PU has the potential to be commercially used due to the low-cost of the enzyme and also the polymer support. According to the supplier company of the feedstock employed in this study, the four reuse cycles reached in this study would be enough to reduce 35% of the cost related to the enzyme on the enzymatic-biodiesel synthesis and this cost reduction can reach up to 60% with seven reuse cycles.

4.3 CONCLUSION

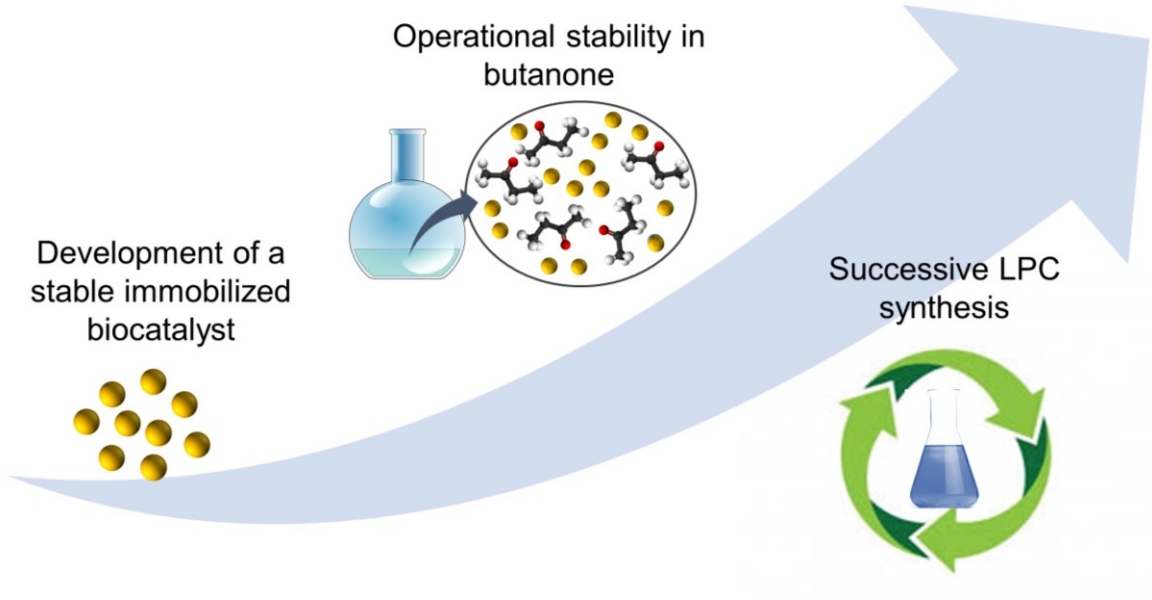
The EI-PU showed no inactivation during the immobilization process and was able to retain up to 72.1% of the initial activity. Regarding the temperature, pH, and storage stability, similar results compared to the free enzyme were observed. The immobilized biocatalyst was employed on FAME synthesis showing similar kinetic

profiles when compared to the free enzyme. EI-PU reached FFA content reduction from 55.9 wt% to values below 4 wt% and FAME conversion above 91% in 24 h of reaction. Despite it had not been able to ensure improvements in enzyme stability against temperature and pH, the immobilization process allowed the reuse of the biocatalyst for at least four cycles of FAME synthesis. This can certainly provide a significant cost reduction related to the enzyme in industrial processes, especially when large amounts of products are taking. Therefore, the immobilization of soluble Eversa® Transform 2.0 lipase on polyurethane foam for FAME synthesis can be taken as an interesting strategy and economically attractive.

5 DEVELOPMENT OF AN IMMOBILIZED BIOCATALYST FOR ESTERIFICATION IN ANYDROUS SYSTEMS

The main objective of this chapter was to develop a stable immobilized biocatalyst from soluble Eversa® Transform 2.0 lipase that could be employed for phospholipids synthesis in anhydrous systems. This system was chosen aiming to explore other alternatives than that proposed by the manufacturer, expanding the scope of applications for this enzyme. Different strategies and supports were evaluated, and the esterification reaction was deeply discussed. Figure 24 shows the activities developed in this chapter.

Figure 24 - Scheme of the activities developed in Chapter 5.



Source: Author.

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This chapter has already submitted as "**Strategies for the immobilization of Eversa® Transform 2.0 lipase: Just a biodiesel maker?**"

5.1 MATERIAL AND METHODS

5.1.1 Material

A commercial solution of Eversa® Transform 2.0 lipase (ET2) from *Aspergillus oryzae* (protein content 35 mg/mL) was provided by Novozymes (Araucária, PR, Brazil). Immobead IB-ADS-3 and Immobead IB-COV-3 were purchased from Chiral Vision (Den Hoorn, Netherlands), and SP Sepharose Fast Flow from GE Healthcare (Madrid, Spain). *p*-nitrophenyl butyrate (pNPB), polyethylenimine, MW 25000 (PEI), and Oleic acid were purchased from Sigma-Aldrich (Darmstadt, Germany). *Sn*-glycero-3-phosphocholine (GPC) was purchased from Bachem AG (Bubendorf, Switzerland). Protein concentration was determined by Bradford with bovine serum albumin as standard (BRADFORD, 1976).

5.1.2 Methods

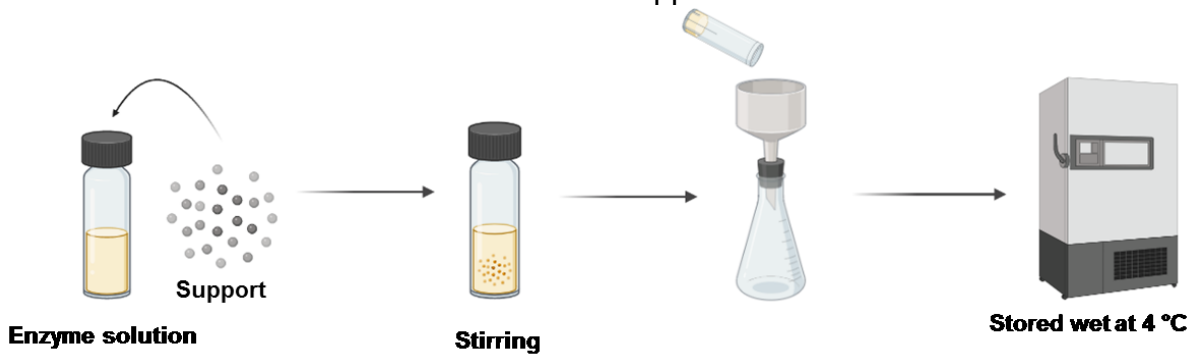
5.1.2.1 Lipase Immobilization

ET2 was immobilized in Immobeads IB-ADS-3 by hydrophobic adsorption employing 5-200 mg of protein per g of wet support. Briefly, 4 g of wet support was added to 40 mL of enzyme solution with appropriate concentrations. 10 mM sodium phosphate buffer pH 7.0 was used as solvent/diluent. Aliquots were taken at pre-established times for evaluating the immobilization progress. The decrease of enzyme content on supernatant was measured by the respective decrease in the enzyme activity employing pNPB. At the end of the immobilization process, the enzyme derivatives (ADS3) were filtered, washed with distilled water, and stored wet at 4 °C (Figure 25) (BASTIDA et al., 1998).

Immobead IB-COV-3 surface was modified by PEI before the immobilization. Briefly, 20 g of wet support was added to 300 mL of 10 mM PEI solution (MW 25k) for 24 h. 25 mM sodium bicarbonate buffer pH 8.5 was used as solvent/diluent. This step was necessary to modify the IB-COV-3 surface for ionic exchange since the original matrix has epoxide groups for covalent immobilization.

ET2 was also immobilized in Immobead IB-COV-3 covered with PEI and in SP Sepharose by ionic exchange employing 200 mg of protein per g of wet support. In synthesis, 4 g of wet support was added to the appropriate enzyme solution. 100 mM sodium phosphate buffer pH 8.5 and pH 6.0, respectively, were used as solvent/diluent. Aliquots were taken in determining times for evaluating the immobilization progress. The decrease of enzyme content on supernatant was measured by the respective decrease in the enzyme activity employing pNPB. At the end of the immobilization process, the enzyme derivatives (COV3 and SULF, respectively) were filtered, washed with distilled water, and stored wet at 4 °C (Figure 25) (FUENTES et al., 2008).

Figure 25 - Scheme of soluble Eversa® Transform 2.0 lipase immobilization in commercial supports.



Source: Author.

All the obtained derivatives were resuspended in distilled water for enzyme activity measurement and exhibited 100% of recovered activity, related to the free enzyme.

5.1.2.2 Determination of enzyme activity

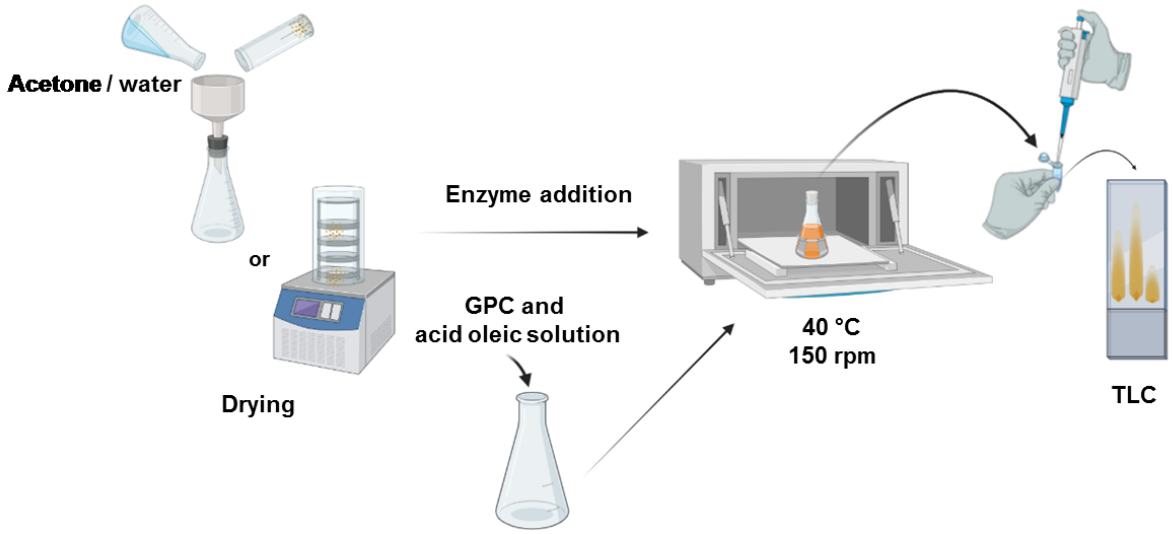
Enzyme activity was measured spectrophotometrically with *p*-nitrophenyl butyrate (pNPB) as substrate (MORENO-PÉREZ; GUISAN; FERNANDEZ-LORENTE, 2014). In a quartz cell, 20 µL of 50 mM pNPB were added into 2.5 mL of 25 mM sodium bicarbonate buffer pH 8.5. Then, 100 µL of enzyme solution in different concentrations were added and the reaction was monitored spectrophotometrically at 345 nm for 2 min. The increase in absorbance by the

release of *p*-nitrophenol in the hydrolysis of p-NPB was measured, and a unit of enzyme activity (U) was defined as the amount of enzyme that catalyzes the production of 1 µmol of *p*-nitrophenol per minute under experimental conditions.

5.1.2.3 Esterification of GPC in anhydrous system

The major reaction of GPC and oleic acid for lysophosphatidylcholine (LPC) synthesis in an anhydrous system was investigated (Figure 26). First, the derivatives were dried: ADS3 and COV3 were submitted to successive washing with acetone/water (progressive acetone concentration); SULF was lyophilized. Then, 60 mg of GPC was added to 5 mL of oleic acid solution (30% butanone v/v for GPC solubilization). After a brief period of homogenization, 0.05 g of derivative (ADS3, COV3, or SULF) was added, starting the reaction. Molecular sieves were added to absorb the water generated during the esterification reaction. The reaction was carried out at 40 °C in a horizontal shaker at 150 rpm. Aliquots were taken in determining times to follow the reaction progress. Thin Layer Chromatography (TLC) was used to evaluate the reaction products.

Figure 26 - Scheme of LPC synthesis.



Source: Author.

5.1.2.4 Thin-Layer Chromatography of the GPC esterification products

Analysis of the reaction products was obtained by thin layer chromatography (TLC) on silica plates DC-Alufolien Kieselgel 60 F254 (Merck, Darmstadt, Germany). The aliquots were diluted in butanone (1:1 v/v) and 5.0 µL were applied to a silica plate. The run was carried out with a mixture of methanol-chloroform-ammonium hydroxide (65:25:4 v/v). The visualization of the esterification products was performed by exposure to iodine vapor until the appearance of the corresponding bands (PEREIRA et al., 2017).

5.1.2.5 Effect of butanone and operational stability of the immobilized derivatives

To investigate the effect of butanone on the immobilized derivatives activity, 300 mg of GPC was added to 5 mL of oleic acid solution (0, 30, or 70% butanone v/v), and after a short period of homogenization, 0.2 g of enzyme derivative (ADS3 or COV3) was added starting the reaction. Molecular sieves were added to absorb the water generate during the esterification reaction. The reaction was carried out at 40 °C in a horizontal shaker at 150 rpm. Aliquots were taken at determined times to follow the reaction progress. Thin Layer Chromatography (TLC) was used to evaluate the reaction.

To study the operational stability of this system, 0.1 g of enzyme derivative (ADS3 or COV3) was added to 5 mL of oleic acid solution (0 and 30% of butanone v/v for ADS3, and 0 and 70% butanone v/v for COV3). The vessels were kept at 40 °C in a horizontal shaker at 150 rpm, and after determining periods (0, 1, 3, 7, and 9 days) 60 mg of GPC was added starting the reaction. Molecular sieves were added to absorb the water generate during the esterification reaction. Aliquots were taken in determining times to follow the reaction progress. Thin Layer Chromatography (TLC) was used to evaluate the reaction.

5.1.2.6 Successive reuse cycles on the LPC synthesis

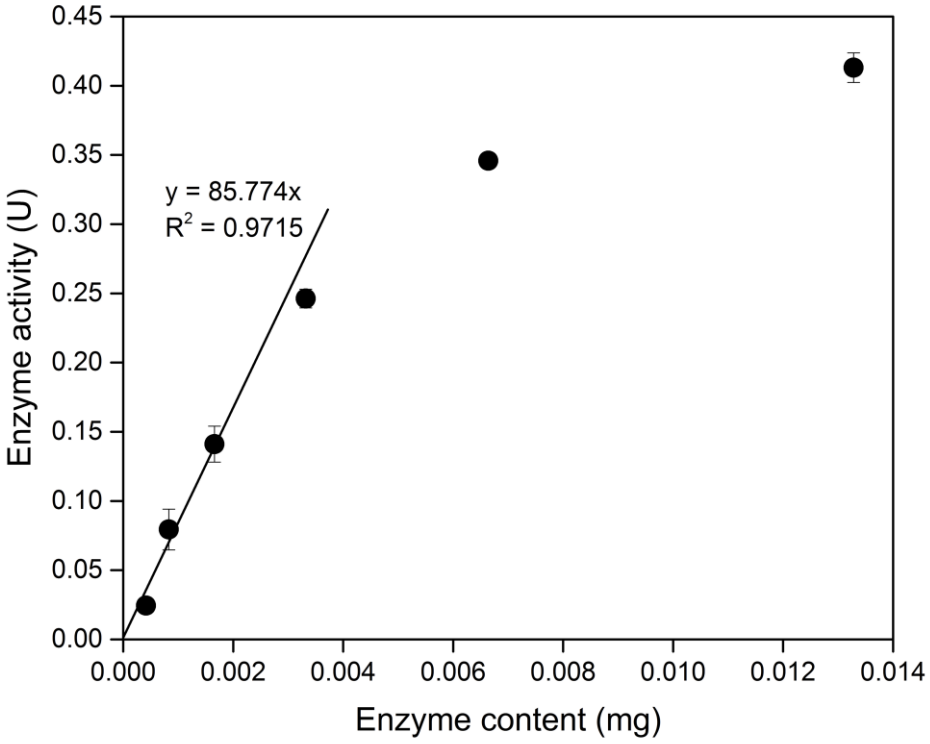
In a vessel, 60 mg of GPC was added to 5 mL of oleic acid solution (0 and 30% of butanone v/v). After a brief period of homogenization, 0.4 g of ADS3 was added, starting the reaction. Molecular sieves were added to absorb the water generate during the esterification reaction. The reaction was carried out at 40 °C in a horizontal shaker at 150 rpm for 24 h. At the end of each reaction cycle, the system was drained, and a new cycle began with the addition of new GPC and oleic acid solution. Aliquots were taken in determining times to follow the reaction progress. Thin Layer Chromatography (TLC) was used to evaluate the reaction.

5.2 RESULTS AND DISCUSSION

5.2.1 Enzyme fit to the enzyme activity method

It is known that some lipases may have a vast hydrophobic region where the active site is placed. These hydrophobic regions have a pronounced inclination to stabilize among themselves forming aggregates as dimers, for example. This phenomenon leads to a deceleration of reaction kinetics and inaccurate measurement of the enzyme activity for concentrations above the linear range. After that range, the addition of larger quantities of enzyme does not increase the enzyme activity and, as a consequence, the activity per mg of enzyme reduces (GUAUQUE TORRES; FORESTI; FERREIRA, 2013). The ET2 has a very high enzyme activity, and it was necessary several dilutions to find that linear range (Figure 27), i.e., enzyme concentration lower than 0.02 mg/mL (enzyme content 0.002 mg). Once most immobilization processes are monitored by the decrease of the supernatant enzyme activity over time, this insight becomes quite important otherwise may lead to an inaccurate value of immobilization yield and consequently misinterpretations.

Figure 27 - Hydrolytic activity versus the mass of enzyme curves found for pNPB hydrolysis using commercial Eversa® Transform 2.0 lipase.



Source: Author.

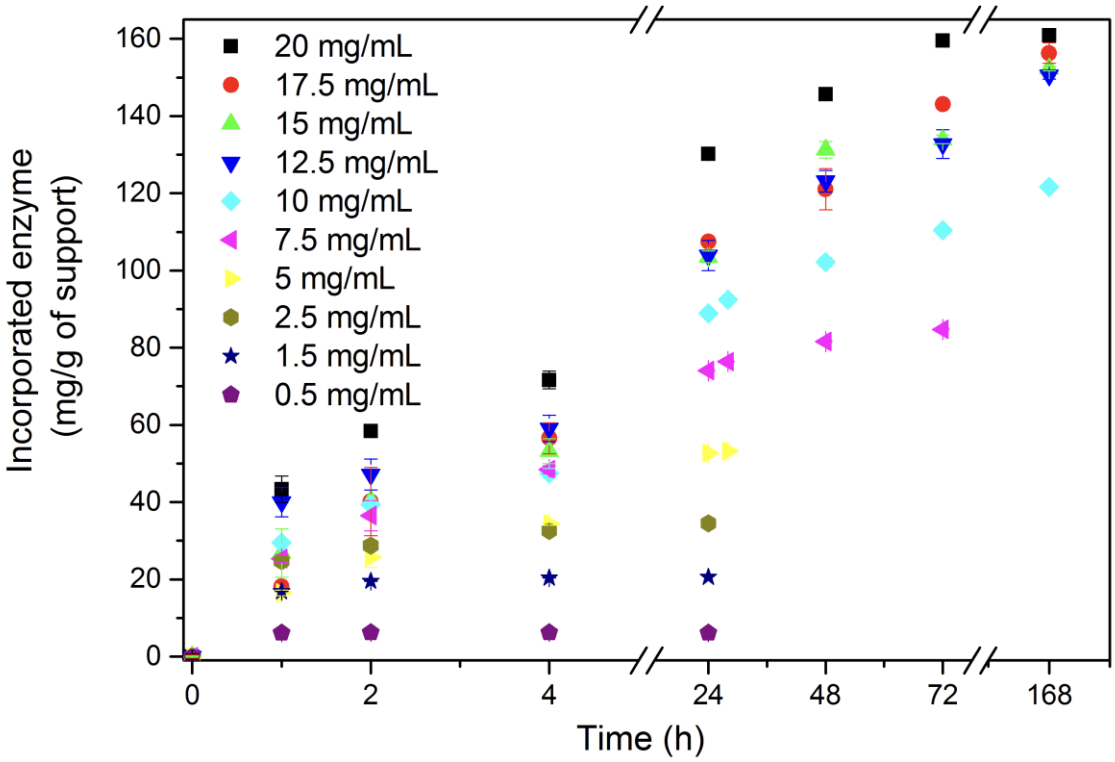
5.2.2 Immobilization of Eversa® Transform 2.0 lipase

Figure 28 shows the immobilization courses of ET2 on IB-ADS-3. The maximum enzyme loading (160.8 mg/g of support) was achieved at the highest concentration of enzyme solution (20 mg/mL) after 72 h of immobilization; which means 80.4% of immobilization yield. This condition was reproduced for the ET2 immobilization on IB-COV-3 and SP Sepharose. Table 9 shows a summary of the immobilized derivatives obtained.

After 72 h of immobilization, the ET2 immobilized on IB-COV-3 (COV3) reached 53.87% of immobilization yield and enzyme loading of 107.84 mg/g of support, whereas the ET2 immobilized on SP Sepharose (SULF) achieved 43.36% of immobilization yield and enzyme loading of 86.82 mg/g of support. The difference in max enzyme loading between the tested supports is expected since several parameters may define a support: internal geometry, specific surface area, superficial activation degree, and particle size. This way, each support has properties and

characteristics which made them unique. Additionally, it should be noted that different immobilization protocols may be employed, and accordingly, we will have a massive variety of possible immobilized derivatives, each one even more unique. For the ADS3, the ET2 was immobilized by hydrophobic interaction (Figure 29). This physical adsorption is related to the conformational modification of the enzyme that exposes its hydrophobic region containing the active site. The conformational transition is induced by the movement of the polypeptide chain (lid), which leads the lipase to its active form, similar to interfacial activation motivated by the hydrophobic substrate phase. The interaction support-lipase is maintained by Van der Waals forces (HANEFELD; GARDOSSI; MAGNER, 2009).

Figure 28 - Immobilization kinetics of Eversa® Transform 2.0 lipase on Immobead IB-ADS-3.

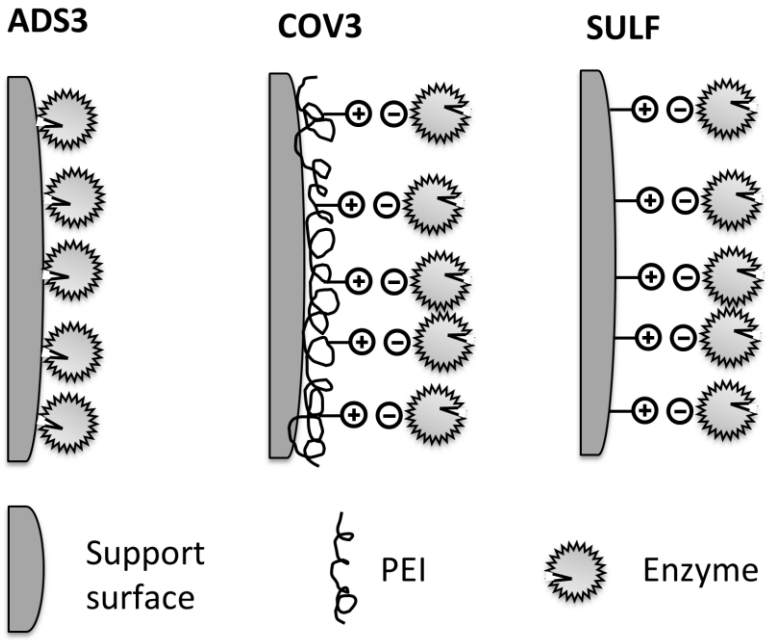


Source: Author.

Table 9 - Summary of immobilized derivatives.

Cod.	Support	Matrix	Group	Particle size (µm)	Buffer	Enzyme loading (mg/g support)	Immob. yield (%)
ADS3	IB-ADS-3	Methacrylate	Octadecyl	150-300	100 mM sodium phosphate pH 7.0	160.80	80.40
COV3	IB-COV-3	Polyacrylic	Epoxide modified with PEI	300-700	100 mM sodium phosphate pH 8.5	107.84	53.87
SULF	SP Sepharose	Agarose	Sulfopropyl	46-165	100 mM sodium phosphate pH 6.0	86.82	43.36

Figure 29 - Schematic representation of Eversa® Transform 2.0 lipase immobilization by hydrophobic interaction and ionic exchange.



Source: Author.

For the COV3 and SULF, the ET2 was immobilized by ionic exchange (Figure 29). Unlike immobilization by hydrophobic interaction, immobilization by ionic exchange occurs mainly through hydrogen bonds between NH-groups in the polypeptidic chain and the carbonyl groups of the matrix. In this case, very likely, the lipase open form should be maintained for steric reasons (HOMAEI et al., 2013). It is

worth noting that COV3 was covered by PEI before the immobilization, but the immobilization principle was the same.

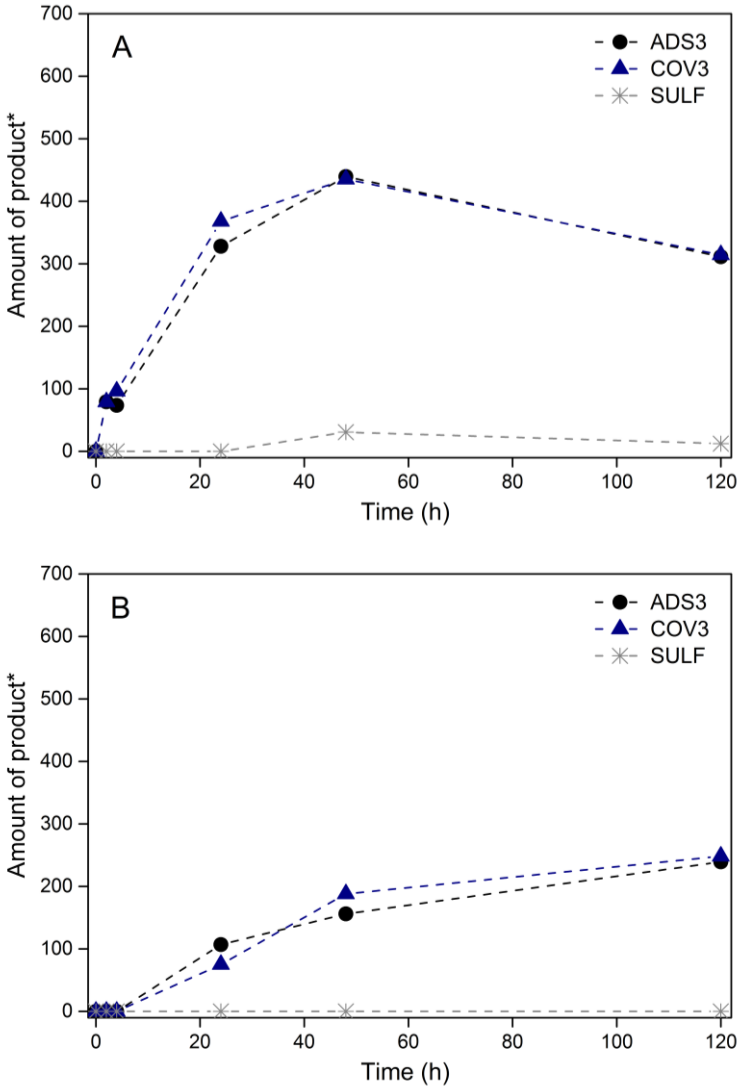
The major inconvenience for any immobilized enzyme either by hydrophobic or ionic exchange technique is the possibility of immobilization reversibility, consenting the enzymes to be desorbed from support under mild conditions, especially with detergent or ionic solution (REHM; CHEN; REHM, 2016). This way, the comprehension of the reaction system in which the derivatives will be performing becomes crucial. Anhydrous reaction systems usually do not share that nuisance and could be a great choice to prove the enzyme derivatives obtained from those protocols.

5.2.3 Esterification of GPC in anhydrous system

The three obtained immobilized derivatives were proved on the esterification of GPC. Figure 30 shows the two possible products for this esterification reaction: oleoyl-lysophosphatidylcholine (oleoyl-LPC) and dioleoyl-lysophosphatidylcholine (dioleoyl-PC). Some authors have suggested that when *sn*1 oleoyl-LPC is formed, the acyl group in the molecule may spontaneously migrate to the *sn*2 position. After migration, the 2-acyl becomes a new substrate for the enzyme that promotes the second acylation, forming the dioleoyl-PC. Furthermore, being the *sn*1 isomer more stable than the *sn*2 isomer, this phenomenon occurs slowly, explaining the quite low production of dioleoyl-PC (MNASRI et al., 2017; VIRTO; SVENSSON; ADLERCREUTZ, 1999). This behavior can be observed for ADS3 and COV3 derivatives.

In contrast, for the SULF derivative, the behavior was quite different, and it was barely able to produce some oleoyl-LPC. It may be related to the derivative surface charge that could be preventing the substrates to reach the active site of the enzyme (CAO, 2011). To check this, the SULF derivative was covered by PEI, and new reaction assays were performed, including with GPC or derivative in excess, but without success. Thus, further studies are demanded to put the SULF derivative in line with this application, and only the ADS3 and COV3 derivatives were kept for the next studies.

Figure 30 - Kinetic profiles for the synthesis of LPC from GPC catalyzed by ADS3, COV3, and SULF. (A) oleoyl-LPC and (B) dioleoyl-PC.



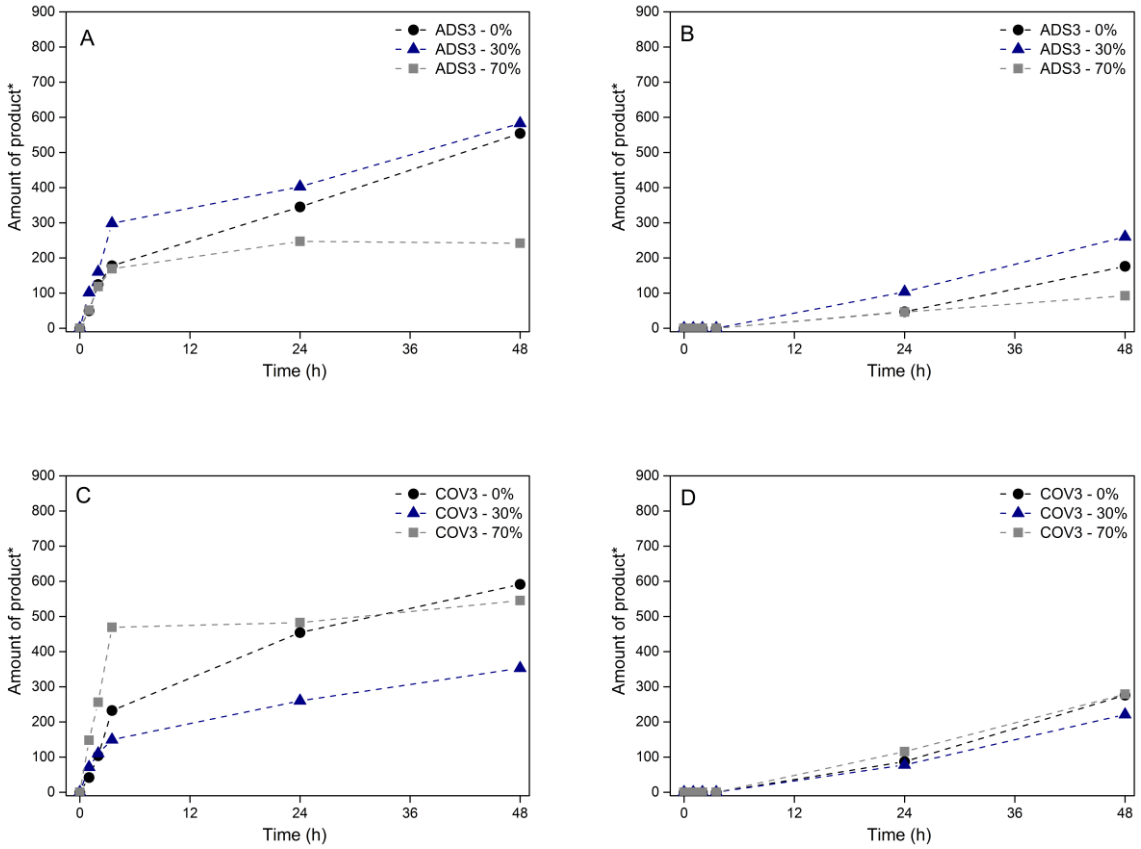
Experimental conditions: 0.05 g of enzyme derivative (ADS3, COV3, or SULF), 60 mg of GPC and 5 mL of oleic acid solution (30% butanone v/v), at 40 °C and 150 rpm. Dotted lines are presented to improve visualization. *Arbitrary scale.
Source: Author.

5.2.4 Butanone as solvent: effects on enzyme performance

Organic solvents are known to be very aggressive against enzymes and may lead to their inactivation, especially under long exposure periods (ADLERCREUTZ, 2013). In Figure 31, it is possible to notice the effect of butanone on the ADS3 and COV3 derivatives performance on LPC synthesis. For ADS3, the best results were achieved with 30% of butanone content, while the worst for 70%. On the other hand, for the COV3 the best results were achieved by the extremes of butanone content: 0

and 70%, while the worst by 30%. In fact, the same enzyme may have different behaviors and stabilities depending on its immobilization protocol and matrix employed.

Figure 31 - Kinetic profiles for the synthesis of LPC from GPC catalyzed by ADS3 and COV3 under different concentrations of butanone. (A)(C) oleoyl-LPC and (B)(D) dioleoyl-PC.



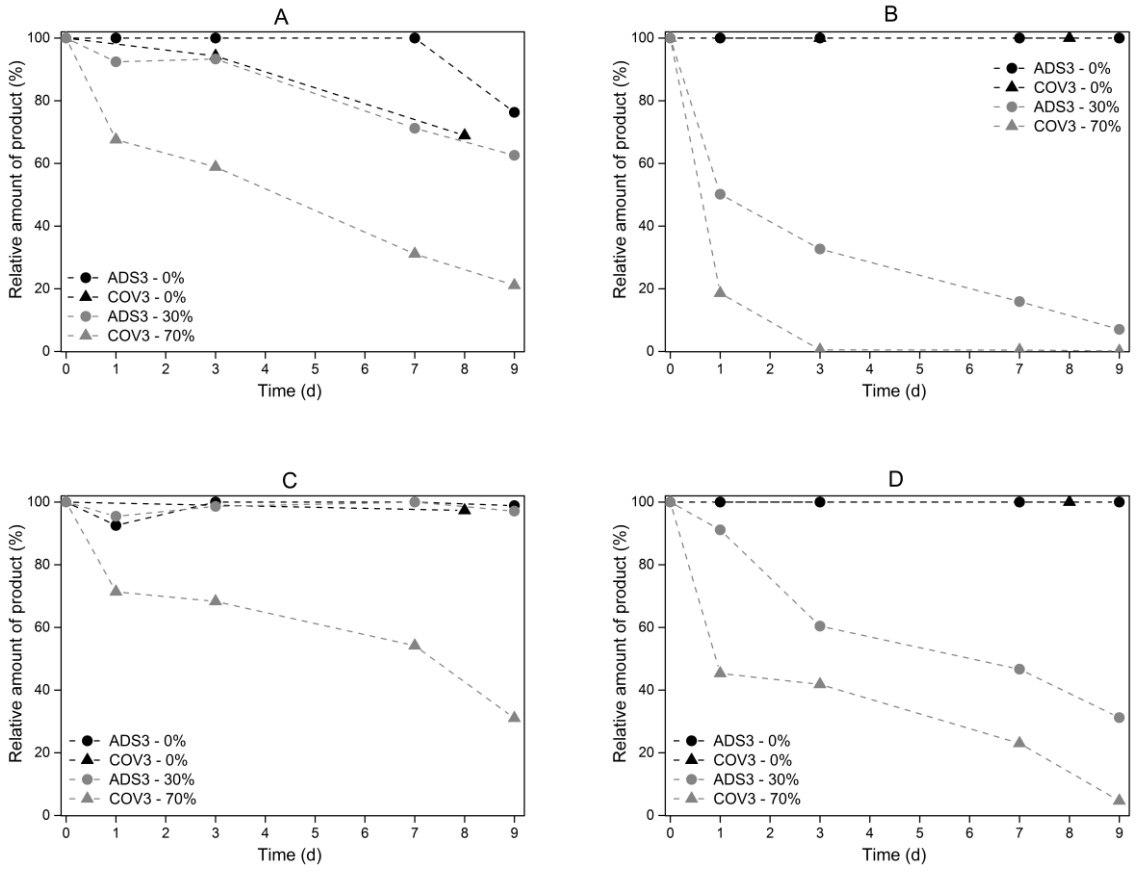
Experimental conditions: 0.2 g of enzyme derivative (ADS3 or COV3), 300 mg of GPC and 5 mL of oleic acid solution (0, 30, or 70% butanone v/v), at 40 °C and 150 rpm. Dotted lines are presented to improve visualization. *Arbitrary scale.
Source: Author.

For the absence of butanone, exciting outcomes were found for both immobilized derivatives. Initially, poor results were expected for this condition since the butanone was just added as a solvent to solve the GPC solubility issue (MNASRI et al., 2017). However, this enzyme seems to have no major problems with this and had given us excellent results. It is interesting because it allows us to think of a solvent-free system for the LCP synthesis, which would be more attractive in the view of green chemistry.

5.2.5 Stability study in LPC synthesis

Having defined the best concentrations of butanone for LPC synthesis for both immobilized derivatives, the next study was related to the stability of these derivatives under these conditions. For this study, two points of the kinetic reaction were analyzed: 24 h and 48 h of reaction. With 24 h of reaction, it was possible to evaluate mainly the formation of oleoyl-LPC since the amount of dioleoyl-PC until this moment is still low. For 48 h of reaction, it was possible to analyze the dioleoyl-PC. Figure 32 shows the results.

Figure 32 - Kinetic curves for the stability of ADS3 and COV3 on the synthesis of LPC from GPC under different concentrations of butanone. (A) oleoyl-LPC and (B) dioleoyl-PC after 24h of reaction; (C) oleoyl-LPC and (D) dioleoyl-PC after 48 h of reaction.



Experimental conditions: 0.1 g of enzyme derivative (ADS3 or COV3), 60 mg of GPC and 5 mL of oleic acid solution (0, 30, or 70% butanone v/v), at 40 °C and 150 rpm. Dotted lines are presented to improve visualization.
Source: Author.

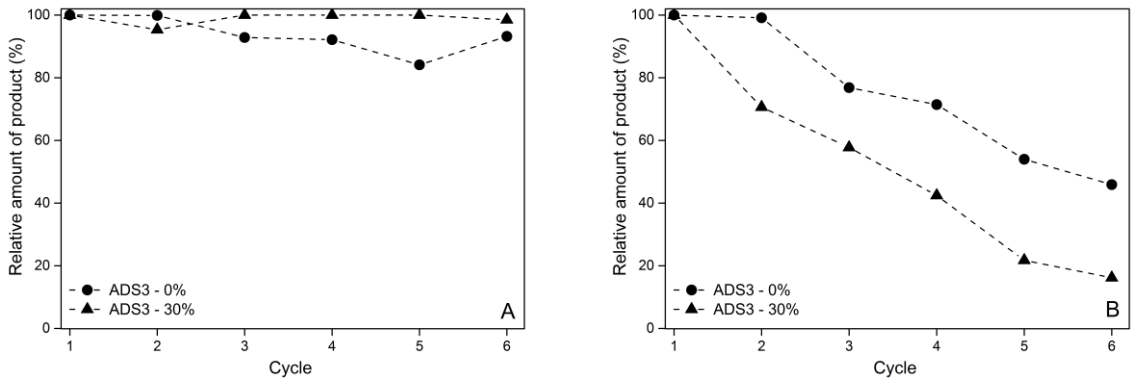
In general, the immobilized derivatives were stable in the solvent-free system (0% butanone), and only a significant decrease in oleoyl-LPC synthesis was detected after 8 days of incubation for COV3 (31.1%) and after 9 days for ADS3 (23.7%), see Figure 32A. However, for the systems with butanone, the results were quite different. COV3 exhibited poor stability in 70% of butanone, showing a gradual decrease in oleoyl-LPC and dioleoyl-PC synthesis (Figure 32D) as the incubation days were increased. After 9 days of incubation, the enzyme derivative had already lost more than 60% of stability.

For the ADS3 in 30% of butanone, a minor decrease was detected in oleoyl-LPC synthesis in the first days, reaching a reduction of 37.4% after 9 days of incubation (Figure 32A). For the dioleoyl-PC synthesis (Figure 32D), the decrease was higher than that observed for oleoyl-LPC.

5.2.6 Successive reuse cycles on the LPC synthesis

ADS3 proved to be the more stable among the investigated immobilized derivatives for the proposed system and then it was applied to a study of successive reuse cycles on the synthesis of LPC (Figure 33).

Figure 33 - Kinetic curves for successive reuse cycles on LPC synthesis catalyzed by ADS3. (A) oleoyl-LPC and (B) dioleoyl-PC.



Experimental conditions: 0.4 g of ADS3, 60 mg of GPC and 5 mL of oleic acid solution (0 and 30% butanone v/v), at 40 °C and 150 rpm. Dotted lines are presented to improve visualization.
Source: Author.

It is possible to note that the oleoyl-LPC synthesis (Figure 33A) has remained constant between the reuses, and only a minor decrease was detected for the solvent-free system (0% butanone). However, the same cannot be said about the dioleoyl-PC synthesis (Figure 33B). A high decrease in the dioleoyl-PC synthesis can be seen for both systems, slightly more pronounced for the system with 30% butanone. It implies that the enzyme derivative had its activity reduced due to some inactivation or diffusion issues, and it would take more time to reach the first conversion yields.

5.3 CONCLUSION

In this chapter, several supports and immobilization protocols were employed for ET2 immobilization. Promising results were found for the physical adsorption immobilization by the ionic exchange in Immobead COV-3. The obtained derivative COV3 showed high activity with remarkable results on GPC esterification, including systems with high solvent content (70% of butanone). On the other hand, it showed low stabilities results. The strategy of physical adsorption by hydrophobic interaction was used for the immobilization in Immobead ADS-3. The obtained derivative ADS3 showed high enzyme activity and excellent results on LPC synthesis, besides being stable in 30% of butanone and solvent-free system. ADS3 was also employed on successive reuse cycles on GPC esterification, reaching 63% of yield after 6 reaction cycles. Therefore, it is possible to conclude that ADS3 may be an excellent possibility for biocatalysis and reactions in anhydrous or solvent-free systems. Moreover, the high enzyme loading (up to 160 mg/g, enzyme/support) achieved in this work could be exceptionally attractive under the economic view.

6 FINAL CONCLUSION

In this work, several strategies for the immobilization of soluble Eversa® Transform 2.0 lipase (ET2) were investigated aiming at distinct applications. The ET2 was successfully immobilized onto flexible polyurethane foam (PU) by entrapment technique and the enzyme derivative (EI-PU) was evaluated for fatty acid methyl esters (FAME) synthesis from industrial fatty waste. The experimental factorial design proposed to evaluate how the system composition affects FAME synthesis catalyzed by EI-PU revealed that the condition 2 wt% of water, 2.0 eqv of methanol, 300 ppm of NaOH, and 500 ppm of enzymatic cofactor was what better performed for that feedstock. EI-PU was able to catalyze the FAME synthesis under these conditions reaching FFA content reduction from 55.9 wt% to values below 4 wt% and FAME conversion above 91% in 24 h of reaction.

The EI-PU showed no phase separation, good pore distribution (well-distributed macropores), and homogeneity. The enzyme derivative showed no inactivation during the immobilization process and was able to retain up to 72.1% of the initial activity. Regarding the temperature, pH, and storage stability, similar results compared to the free enzyme were observed. Despite it had not been able to ensure improvements in enzyme stability against temperature and pH, the immobilization process allowed the reuse of the biocatalyst for at least four cycles of FAME synthesis. This work showed for the first time a successful report of immobilization of soluble Eversa® Transform 2.0 lipase on PU foam.

ET2 was also immobilized in other commercially available supports by different techniques aiming for application in the synthesis of phospholipids (lysophosphatidylcholine, LPC). The best results were found for the immobilization strategy of physical adsorption by hydrophobic interaction in Immobead ADS-3. The obtained derivative (ADS3) showed high enzyme loading (up to 160 mg/g, enzyme/support) and excellent results on LPC synthesis, besides being stable in 30% of butanone and solvent-free system. For the reusability study, yields above 63% after six reaction cycles on LPC synthesis were achieved. To the best of our knowledge, it was the first time the Eversa® Transform 2.0 lipase was evaluated for LPC synthesis.

Thus, even that the Eversa® Transform 2.0 lipase was originally designed to be used as a free enzyme, a proper immobilization allowed even more efficiency on FAME production, especially by the reuse possibility. Moreover, other new applications were unlocked in which the original enzyme could not be employed. Therefore, the immobilization of soluble Eversa® Transform 2.0 lipase can be taken as economically attractive with prospects for applications not only in biodiesel synthesis but in other reactions of interest since suitable investigations can be developed for this goal.

6.1 SUGGESTIONS FOR FUTURE WORKS

Based on the results obtained in this work, the following suggestions for future works can be outlined:

- Evaluate the possibility for the scale-up of the FAME synthesis from industrial fatty waste catalyzed by the EI-PU, including a preliminary cost analysis;
- Evaluate the ADS3 derivative in other reactions at anhydrous systems, such as esters synthesis for pharmaceutical and food fields;
- Explore the ET2 immobilization in other supports to expand the scope of ET2 applications.

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