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DEPARTAMENTO DE ENGENHARIA QUÍMICA
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ENGENHARIA DE BIOCATALISADORES APLICADA À SÍNTESE DE ÉSTERES GRAXOS DE XILOSE OBTIDA DA FRAÇÃO C-5 DE RESÍDUOS LIGNOCELULÓSICOS

SÃO CARLOS -SP
2026

FEDERAL UNIVERSITY OF SÃO CARLOS
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BIOCATALYST ENGINEERING APPLIED TO THE SYNTHESIS OF FATTY XYLOSE ESTERS DERIVED FROM THE C5 FRACTION OF LIGNOCELLULOSIC RESIDUES

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Doctoral thesis submitted to the Graduate Program in Chemical Engineering at the Federal University of São Carlos in partial fulfillment of the requirements for the Doctoral degree in Chemical Engineering.

Supervisor: Prof. Dr. Paulo Waldir Tardioli

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DEDICATION

I dedicate this thesis to my family, especially to my mother, Berenice Maria de Souza, my example of strength, support, and inspiration, and to my wife, Suellen, for her encouragement, unconditional support, and motivation throughout this journey.

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"*Whoever watches the wind will not plant; whoever looks at the clouds will not reap*".

Ecclesiastes 11:4

RESUMO

A crescente demanda por surfactantes sustentáveis e de baixo impacto ambiental tem impulsionado o desenvolvimento de rotas biotecnológicas capazes de converter matérias-primas renováveis em produtos de alto valor agregado. Nesse contexto, a síntese enzimática de ésteres de ácidos graxos de xilose destaca-se como uma alternativa promissora, sobretudo por permitir a valorização da fração C5 da biomassa lignocelulósica, ainda pouco explorada em aplicações industriais. Entretanto, desafios associados à baixa solubilidade da xilose em solventes orgânicos, à limitada estabilidade dos biocatalisadores e à eficiência catalítica em meios não convencionais ainda restringem a implementação desses processos. Esta tese teve como objetivo desenvolver e avaliar estratégias integradas para a produção enzimática de ésteres de xilose, abordando lacunas relacionadas à engenharia do biocatalisador, à escolha do meio reacional e ao uso de matéria-prima derivada de biomassa lignocelulósica. Inicialmente, foi avaliado o estado da arte da síntese enzimática de ésteres de xilose, com aplicação dos princípios do mapeamento sistemático da literatura, a fim de identificar estratégias recorrentes, limitações e oportunidades associadas aos catalisadores, aos doadores de grupo acil, aos solventes e às etapas de purificação. Os resultados da busca mostraram que 87,18% dos estudos utilizavam catálise enzimática e que, entre esses, as lipases constituíam os biocatalisadores preferenciais para a síntese de ésteres de xilose. Adicionalmente, observou-se que a escolha do solvente é uma etapa crucial nesse processo e que os doadores de grupos acil mais utilizados foram o ácido oleico, o ácido láurico e ésteres metílicos e vinílicos, correspondendo a 16%, 16% e 47% dos casos reportados, respectivamente. Em seguida, foram investigadas estratégias de pós-tratamento do biocatalisador comercial Novozym® 435. Os resultados demonstraram que o revestimento com polietilenimina, seguido de reticulação com glutaraldeído, resultou em biocatalisadores mais robustos, promovendo aumento de 2,3 vezes na conversão de ácido oleico quando um solvente eutético profundo foi utilizado para solubilizar a xilose, e de 1,5 vez quando se empregaram metiletilcetona e terc-butanol. O pós-tratamento também conferiu maior estabilidade operacional ao biocatalisador em ciclos sucessivos de reação, com retenção de aproximadamente 75% da atividade catalítica após cinco ciclos consecutivos de reutilização. Um dos avanços centrais desta tese foi a utilização de um solvente eutético profundo à base de cloreto de colina e xilose, o qual proporcionou elevada solubilidade da xilose, atingindo concentrações superiores a 600 mM, valor

expressivamente superior ao observado em solventes orgânicos convencionais, como metiletilcetona (7 mM) e terc-butanol (60 mM). O aumento da solubilidade da xilose refletiu-se diretamente no desempenho da síntese enzimática, permitindo maiores taxas de conversão e elevada seletividade para monoésteres de xilose. Paralelamente, foi avaliada a influência de diferentes protocolos de imobilização e da metalização da lipase Eversa® Transform 2.0, evidenciando-se variações na atividade catalítica, na estabilidade térmica e na especificidade, com incrementos de até 3 vezes na atividade específica em comparação com os biocatalisadores não modificados. Técnicas espectroscópicas confirmaram alterações conformacionais nas enzimas imobilizadas, possibilitando correlacionar o pH de imobilização com a atividade hidrolítica e a especificidade catalítica observadas. Por fim, demonstrou-se a viabilidade da produção de ésteres de xilose a partir do bagaço de cana-de-açúcar, integrando etapas de pré-tratamento da biomassa. O pré-tratamento com ácido acético e peróxido de hidrogênio promoveu a liberação de quantidade de açúcares quatro vezes maior durante a hidrólise enzimática com xilanases, em comparação com o bagaço não tratado. Biocatalisadores obtidos pela imobilização da lipase Eversa® Transform 2.0 em pH 5, na presença de NaCl 100 mM, e posteriormente metalizados com $MgCl_2$ foram avaliados, sendo que o biocatalisador não metalizado apresentou os melhores resultados, alcançando 15% de conversão de ácido oleico e 63% de conversão de xilose, valores aproximadamente 55% superiores obtidos com o biocatalisador preparado na ausência de NaCl. De modo geral, os resultados obtidos quanto à modulação das características dos biocatalisadores por métodos de imobilização e pós-tratamento, ao uso de solvente eutético profundo na esterificação enzimática da xilose e ao aproveitamento de xilose proveniente do bagaço de cana contribuem significativamente para o avanço do conhecimento em biocatálise aplicada à síntese de ésteres de açúcares. Os resultados evidenciam o papel dos solventes eutéticos e das estratégias de engenharia de biocatalisadores na intensificação de processos, além de oferecerem perspectivas concretas para o desenvolvimento futuro de rotas industriais sustentáveis para a produção de biossurfactantes.

Palavras-chave: ésteres de xilose; solventes eutéticos profundos; síntese enzimática; imobilização de lipases; biocatálise; biomassa lignocelulósica; biossurfactantes.

ABSTRACT

The growing demand for sustainable, low-environmental-impact surfactants has driven the development of biotechnological routes capable of converting renewable feedstocks into high-value products. In this context, the enzymatic synthesis of xylose fatty acid esters stands out as a promising alternative, particularly due to its potential do valorize the C5 fraction of lignocellulosic biomass, which remains underexploited in industrial applications. However, challenges related to low solubility of xylose, the limited stability of biocatalysts, and reduced catalytic efficiency in non-conventional media still hinder the implementation of these processes. This thesis aimed to develop and evaluate integrated strategies for the enzymatic production of xylose esters, addressing key gaps in biocatalyst engineering, reaction medium selection, and the use of biomass-derived feedstocks. Initially, the state of the art in the enzymatic synthesis of xylose esters was assessed through systematic literature mapping, enabling the identification of recurring strategies, limitations, and opportunities related to catalysts, acyl group donors, solvents, and downstream processing. The results indicated that lipases are the preferred biocatalysts for xylose ester synthesis. Additionally, solvent selection was identified as a crucial factor, with oleic acid, lauric acid, and methyl and vinyl esters being the most commonly employed acyl donors. Subsequently, post-treatment strategies were investigated for the commercial biocatalyst Novozym® 435. The results demonstrated that coating with polyethyleneimine followed by crosslinking with glutaraldehyde yielded more robust biocatalysts, resulting in a 2.3-fold increase in oleic acid conversion when a deep eutectic solvent was used to solubilize xylose, and a 1.5-fold increase when methyl ethyl ketone and tert-butanol were employed. In addition, post-treatment improved operational stability over successive reaction cycles, with approximately 75% retention of catalytic activity after five reuse cycles. A key advancement of this work was the use of a chloride–xylose deep eutectic solvent, which significantly enhanced xylose solubility, reaching concentrations above 600 mM, substantially higher than those observed in conventional organic solvents (7 mM in methyl ethyl ketone and 60 mM in tert-butanol). This increased solubility directly improved enzymatic performance, enabling higher conversion rates and high selectivity toward xylose monoesters. In parallel, the effects of different immobilization protocols and metal modification of the lipase Eversa® Transform 2.0 were evaluated, revealing variations in catalytic activity, thermal stability, and specificity. Spectroscopic analyses confirmed conformational changes in the immobilized enzymes, allowing correlation between immobilization pH

and the observed hydrolytic activity and catalytic specificity. Finally, the feasibility of producing xylose esters from sugarcane bagasse was demonstrated through the integration of biomass pretreatment and enzymatic conversion steps. Pretreatment with acetic acid and hydrogen peroxide resulted in a fourfold increase in sugar release during enzymatic hydrolysis with xylanases, compared to untreated bagasse. Biocatalysts prepared by immobilizing Eversa® Transform 2.0 at pH 5 in the presence of 100 mM NaCl, followed by metal modification with $MgCl_2$, were evaluated. The non-metal-modified biocatalyst exhibited superior performance, achieving 15% oleic acid conversion and 63% xylose conversion. Overall, the modulation of biocatalyst properties through immobilization and post-treatment strategies, the use of deep eutectic solvents in xylose esterification, and the application of xylose derived from sugarcane bagasse significantly advance the field of applied biocatalysis for sugar ester synthesis. This findings provide concrete perspectives for the development of sustainable industrial routes for biosurfactant production.

Keywords: xylose esters; deep eutectic solvents; enzymatic synthesis; lipase immobilization; biosurfactants; lignocellulosic biomass.

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LIST OF ABBREVIATIONS AND ACRONYMS

ANOVA - Analysis of variance

CALB - *Candida antarctica* lipase B

C5-SEs -Five-carbon sugar esters

C6 - Polymeric chains containing 6 carbon atoms

C8 - Polymeric chains containing 8 carbon atoms

C10 - Polymeric chains containing 10 carbon atoms

C12 - Polymeric chains containing 12 carbon atoms

C13 - Polymeric chains containing 13 carbon atoms

C18 - Polymeric chains containing 18 carbon atoms

C20 - Polymeric chains containing 20 carbon atoms

C22 - Polymeric chains containing 22 carbon atoms

DES - Deep eutectic solvents

DMF – Dimethylformamide

DMSO – Dimethyl sulfoxide

EI - Emulsification index

ETL - Lipase Eversa® Transform 2.0

FAEE - Fatty acid ethyl esters

FFA – Free Fatty Acids

FID - Flame ionization detector

FTIR - Fourier transform infrared spectroscopy

GA - Glutaraldehyde

HBA - Hydrogen bond acceptor

HBD - Hydrogen bond donor

HPLC - High-performance liquid chromatography

ILs - Ionic liquids

IUPAC - International Union of Pure and Applied Chemistry

MEK - Methyl ethyl ketone

MOPS - 3-(N-Morpholino) propanesulfonic acid

NADES - Natural deep eutectic solvents

N435 - Commercial biocatalyst Novozym® 435

NMR - Nuclear magnetic resonance

PEI - Polyethyleneimine

rpm - Revolutions per minute

SDS-PAGE - Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

SDGs - United Nations Sustainable Development Goals

SE - Sugar ester

TERT - tert-Butyl alcohol

TLC – Thin-layer chromatography

TBU - Unit of tributyrin hydrolysis activity

TLL - *Thermomyces lanuginosus* lipase

TRIS - Tris(hydroxymethyl)aminomethane

U_{trans} - Transesterification activity unit

U_{est} - Esterification activity unit

LIST OF CHEMICAL COMPOUNDS

$CaCl_2$ – Calcium chloride

$CoCl_2$ – Cobalt(II) chloride

$CuCl_2$ – Copper(II) chloride

$MgCl_2$ – Magnesium chloride

$MnCl_2$ – Manganese(II) chloride

NaCl – Sodium chloride

$NiCl_2$ – Nickel(II) chloride

$ZnCl_2$ – Zinc chloride

LIST OF SYMBOLS

f_0	Number of observed agreements
f_E	Number of agreements expected by chance
FIA	Flow injection analysis
H_e	Emulsion height
H_t	Total height
R_f	Retention factors
U	Enzymatic unit
N	Total number of observations
k	Kappa coeficiente

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CHAPTER I
INTRODUCTION

1.1. BROAD CONTEXT

Surfactants are amphipathic organic compounds composed of distinct hydrophilic and hydrophobic moieties, which confer them the ability to reduce surface and interfacial tension between immiscible phases, such as water and oil, as well as to promote micelle formation in aqueous solutions [1–3]. These properties favor processes such as wetting, emulsification, solubilization, and dispersion, and are therefore widely exploited in a variety of industrial and domestic applications, including detergents, cosmetics, and cleaning products [2,4].

In this context, biosurfactants have emerged as biologically derived surface-active compounds produced by microorganisms, such as bacteria and yeasts, or through enzymatic processes [5–8]. These molecules exhibit amphiphilic structures and surface-active properties comparable to those of conventional synthetic surfactants [5–8]. According to their chemical nature, biosurfactants can be classified into glycolipids, such as rhamnolipids, sophorolipids, and trehalolipids, lipopeptides and lipoproteins, such as surfactin and iturin, phospholipids, fatty acids and neutral lipids, as well as polymeric biosurfactants, including emulsans and lipopolysaccharides [2,9].

Among these classes, sugar esters (SEs) stand out as glycolipids that can be obtained via enzymatic catalysis, representing a promising and sustainable alternative for biosurfactant production [10–13]. Sugar esters are formed through an ester linkage between the hydroxyl groups of sugars and the carboxyl groups of fatty acids [10–13], resulting in molecules that combine a polar sugar moiety with a nonpolar fatty acid chain, enabling interaction with both polar and nonpolar interfaces. In addition to their surface-active properties, sugar esters are odorless, tasteless, exhibit low toxicity, and present a reduced irritation potential, which has attracted increasing industrial interest [14–17].

The enzymatic synthesis of SEs offers significant advantages, particularly due to the possibility of operating under mild and environmentally friendly reaction conditions [14–17]. Moreover, the use of enzymes such as lipases, which exhibit high specificity and regioselectivity, minimizing substrate degradation and the formation of undesired by-products [11,18–20]. The global sugar esters market is expected to grow from US$ 76 million in 2020 to approximately US$ 120 million over the next decade, driven by broad applicability and increasing demand for biodegradable and natural products [3,21]. From a sustainability

perspective, five-carbon sugar esters, particularly xylose esters, are especially attractive, since they can be produced from xylose obtained from renewable lignocellulosic biomass, enabling the valorization of residual streams from biorefineries [3,13,14,22].

Despite these advantages, the enzymatic route still faces challenges related to the high cost of enzymes, their sensitivity to physicochemical conditions, and difficulties associated with biocatalyst recovery and reuse, as enzymes are often soluble in aqueous media [23,24]. In this context, enzyme immobilization and post-treatment strategies have been explored as effective approaches to enhance catalytic stability, modulate enzyme properties, and improve process robustness [25–27]. Enzyme immobilization has proven to be an effective strategy for obtaining biocatalysts with different stability, activity, and specificity profiles, even when the same enzyme and support are used, simply by modifying the immobilization conditions or applying post-treatment steps [10,25,26,28]. Changes in the immobilization parameters such as pH, ionic strength, and the presence of desorbing agents can induce conformational changes in the enzyme, enabling its immobilization in conformations different from those obtained under standard conditions [26,29]. Enzymes immobilized in distinct conformations may exhibit significant variations in catalytic activity, stability, and selectivity, allowing the selection of biocatalysts with properties better suited to specific applications [26,29,30].

Post-treatment is frequently applied to increase the robustness of the biocatalyst under process conditions. In general, this strategy is adopted to prevent enzyme desorption from the support or its inactivation under adverse physical conditions, such as variations in pH, temperature, and ionic strength, thereby enabling greater operational stability, resistance to thermal degradation, and reuse of the biocatalyst in reaction environments prone to enzyme desorption [10,25,28,31]. Post-treatments primarily aim to increase the rigidity of the enzyme molecule on the support or to promote the formation of cross-links that interconnect immobilized enzymes. The main post-treatment methods applied to immobilized enzymes include polymer coating, cross-linking, and metallization [10,25,28,31].

The enzymatic synthesis of sugar esters presents as its main challenge the selection of an appropriate solvent for sugar solubilization that meets four key requirements: the ability to solubilize the sugar; low water activity to shift the reaction equilibrium toward esterification and prevent hydrolysis; non-denaturing behavior toward the enzyme; and the absence of hydroxyl groups that may be recognized as substrates by the enzyme [8,10,23]. In this context, environmentally friendly solvents, such as natural deep eutectic solvents, can be obtained from sugars, enabling their concomitant use as both solvent and substrate in enzymatic reactions for the synthesis of sugar esters [10,16,22].

Xylose is the second most abundant sugar in lignocellulosic biomass, representing a strategic raw material in the national context due to the large volume of biomass produced in the country [32–34]. Studies on the synthesis of xylose esters obtained from the enzymatic hydrolysis of different biomass sources have been widely reported, reinforcing the relevance of investigations that evaluate sugarcane bagasse as a feedstock for this type of product [13,16]

In this context, this thesis investigates the enzymatic production of xylose esters, focusing on the development of sustainable processes based on non-toxic solvents, immobilized biocatalysts, and renewable raw materials, with particular emphasis on xylose obtained from sugarcane bagasse.

1.2. OBJECTIVES

The aim of this thesis was to produce xylose esters from the C5 fraction of sugarcane bagasse through enzymatic catalysis, focusing on the development of sustainable and efficient biocatalytic processes. To achieve this aim, the following specific objectives were established:

i) To evaluate xylose-based natural deep eutectic solvents as alternative to conventional organic solvents, aiming to enhance xylose solubility during sugar ester synthesis;

ii) To develop and assess post-treatment strategies for immobilized biocatalysts in order to improve catalytic stability and activity and to reduce enzyme desorption during xylose ester synthesis;

iii) To investigate the influence of different enzyme immobilization conditions and protocols on the catalytic performance of biocatalysts applied to the synthesis of xylose esters;

iv) To produce xylose from sugarcane bagasse and evaluate its applicability as a substrate in the enzymatic synthesis of xylose esters.

1.3. THESIS OUTLINE

This thesis addresses key aspects related to the enzymatic synthesis of xylose esters. Chapter 2 presents a critical and systematic review of the state of the art in this filed, discussing the main advances, technological challenges, and future perspectives. In particular, the influence of the solvent, the type of biocatalyst (free or immobilized enzymes), acyl donor groups, and purification strategies is identified as critical, based on quantitative data reported in the scientific literature.

Chapter 3 presents two complementary strategies to overcome limitations associated with xylose ester synthesis: (i) the use of a xylose-based natural deep eutectic solvent as an alternative to conventional organic solvents, enabling enhanced xylose solubilization; and (ii) post-treatment of the biocatalyst with polyethyleneimine and glutaraldehyde to prevent enzyme desorption, increase operational stability, and enable a higher number of biocatalyst reuse cycles.

Chapter 4 reports the results obtained during the doctoral research period abroad and investigates the influence of different immobilization protocols and the metallization of the lipase Eversa® Transform 2.0 on catalytic activity, stability, and specificity. Metallization is based on the controlled formation of metal salt crystals around enzymes, a strategy originally applied in the production of enzymatic nanoflowers. This approach involves the incorporation of metal ions, typically in the form of metal phosphates, into the enzyme structure after immobilization on a solid support, resulting in enzyme-metal hybrid structures.

Chapter 5 analyzes the effect of pH during the immobilization of *Candida antarctica* lipase B and Eversa® Transform 2.0. The enzymes were immobilized in different media and at different pH values, and variations in hydrolytic activity and conversion during xylose ester synthesis were evaluated and correlated with infrared spectroscopy results, indicating conformational changes in the enzyme structures.

Finally, Chapter 6 presents the application of the biocatalyst developed in Chapter 5, which exhibited superior activity and stability, in the synthesis of xylose esters using both commercial xylose and xylose derived from sugarcane bagasse.

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CHAPTER II
ENZYMATIC SYNTHESIS OF XYLOSE FATTY ACID ESTERS CURRENT STATUS AND PROSPECTS

Sugar fatty acid esters have attracted considerable attention due to their biodegradability, low toxicity, and favorable surfactant properties, which make them valuable for application in the food, cosmetic, pharmaceutical, and agricultural sectors. Among this class of compounds, xylose fatty acid esters stand out as particularly promising bio-based surfactants, as they can be derived from renewable lignocellulosic biomass, thereby aligning with circular economy principles and sustainable development goals. Despite their potential, the enzymatic synthesis of xylose esters remains technically challenging. Key limitations include polarity differences between sugars and fatty acids, low solubility of xylose in conventional reaction media, and consequent reduction in reaction rates and overall conversion. These constrains continue to hinder process intensification and large-scale implementation. This review provides a comprehensive analysis of the current status of enzymatic xylose ester synthesis. A structured and systematic search across major scientific databases identified 20 relevant experimental studies, which were mapped and analyzed with respect to acyl donor groups, biocatalysts, reaction media, and downstream separation and purification strategies. The analysis reveal that lauric and oleic acids are the commonly used acyl donors, while activated substrates such as vinyl and methyl esters are frequently employed to enhance reaction conversion and shift equilibrium toward ester formation. Lipases remain the dominant class of biocatalysts, owing to their robustness and regioselectivity, with solvent selection playing a critical role in ensuring substrate compatibility, enzyme stability, and overall process performance. Keyword co-occurrence analysis further highlights emerging research trends and interconnections among substrates, reaction media, and downstream processes approaches. Overall, this review summarizes recent advances, identifies persistent technological gaps, and outlines future research directions for improving xylose ester production. In particular, it emphasizes strategies aimed at enhancing reaction efficiency, expanding the use of green and biomass-derived solvents, and enabling scalable and economically viable biocatalytic processes. The insights presented herein support the development of high-value xylose esters and contribute to the advancement of sustainable bio-based surfactants.

2.1. INTRODUCTION

The growing demand for sustainable products and processes reflects increasing awareness of global challenges such as climate change, environmental degradation, and food insecurity [1,2]. A critical step toward addressing these pressing issues is the shift from products derived from finite, fossil-based resources, such as petroleum, to those obtained from renewable, bio-based materials such as biomass [1]. In this context, sugar esters (SEs) emerge as highly promising alternatives to conventional petrochemical surfactants, offering not only environmental benefits but also superior physicochemical properties, including remarkable stability under extreme conditions [3,4].

SEs are amphiphilic molecules resulting from the esterification between a sugar moiety (polar portion) and an acyl group (non-polar portion) [5–7]. Both substrates can be derived from renewable and low-cost sources, such as biomass and agro-industrial residues [5,8–10]. The amphipathic nature of SEs imparts surfactant, emulsifying, lubricating, and stabilizing properties to these compounds [5,6,11–14]. Additionally, SEs are recognized as biodegradable [15,16], tasteless [17,18], odorless [17,18], non-irritating [11,19], and non-toxic [5–7,11,16,19], making them suitable for applications in the cosmetic, agricultural, food, and pharmaceutical industries [4–6,15,16,20]. SEs also stand out for their potential nutritional applications, as they can serve as carriers of essential fatty acids such as omega-3 [21–24] and omega-6 [21,23,25,26], as well as for therapeutic applications due to their antitumor [27,28], antimicrobial [6,29,30], and gene delivery properties [18,30,31].

In particular, five-carbon sugar esters (C5-SEs) have gained increasing attention due to their excellent lubricating and antimicrobial properties, positioning them as promising molecules for skin moisturizing formulations [11,32,33]. Furthermore, C5-SEs, especially xylose esters, show great potential as environmentally friendly and sustainable alternatives, since xylose can be sourced from renewable biomass such as wood, sugarcane bagasse, straw, and other lignocellulosic residues [8–10,34].

The production of xylose esters represents a sustainable strategy for the valorization of lignocellulosic residues derived from biorefineries or second-generation ethanol plants [8,32]. This approach aligns with the United Nations Sustainable Development Goals (SDGs), particularly goals 9 and 12, as well as with the principles of the bioeconomy. The growing global demand for biodegradable and natural products has also driven interest in xylose esters, with the sugar fatty acid ester market expected to increase from USD 76 million in 2020 to USD 120 million over the next decade [11,35].

The earliest reports describing the synthesis of sugar esters date back to the late 18th century, detailing the production of sucrose esters via chemical catalysis [36]. This process typically involves extreme temperature and pressure conditions [4,15,37], exhibits low specificity [6,7], and employs reagents and catalysts that are incompatible with the safety requirements of food, pharmaceutical, and cosmetic applications [5,15,21,38]. In contrast, enzymatic synthesis offers several advantages over chemical methods, including mild reaction conditions, selectivity, enantioselectivity, higher specificity, and one-step reactions [4,15,37]. Moreover, sugar esters produced enzymatically are perceived by consumers as "natural products" [5,34,39,40].

Enzymatic synthesis of SEs can be catalyzed by hydrolases such as esterases [41,42], proteases [43–45], and primarily lipases [15,21,46,47]. In lipase-catalyzed reactions, the water concentration in the reaction medium is a critical factor to prevent hydrolysis reactions [7,32,48,49] and to shift the thermodynamic equilibrium toward synthesis [15,21,47,50,51]. Therefore, non-aqueous media are widely employed in SEs synthesis [7,29,52,53]. Although the first studies on enzymatic synthesis of sugar esters date back to the late 19th century [7,36], the vast majority of commercially available SEs are still produced via chemical routes [6,7]. This is mainly attributed to the low reaction rates and productivity of enzymatic processes, which are primarily caused by the polarity difference between substrates, posing a challenge in identifying a solvent capable of solubilizing both [5,37,54–56]. Therefore, the selection of an appropriate solvent is a critical step for achieving successful enzymatic synthesis of sugar esters, influencing both substrate solubility and enzyme stability in the reaction medium [3,37,54–56].

Therefore, this study aims to provide a review of the synthesis of xylose fatty acid esters by applying systematic mapping principles and discussing the critical aspects and the various approaches used in the production of these esters.

2.2. METHODOLOGY

2.2.1 Database construction

The dataset was constructed through a systematic search of scientific databases using a predefined search string designed to identify studies related to the synthesis of sugar esters. The overall study selection procedure is illustrated in the flow diagram shown in **Figure 2.1**.

A literature review incorporating systematic mapping principles was conducted to compile articles retrieved from three bibliographic databases: Web of Science (Core

Collection: SCI-E and ESCI), Scopus, and ScienceDirect. The literature search was performed using the following search string to retrieve titles, abstracts, and keywords related to the topic: ("sugar ester*" OR "sugar fatty acid esters" OR "carbohydrate* fatty acid* ester*" OR "carbohydrate* ester*") AND (reaction OR synthes$ OR production).

The database was last updated on November 29, 2025, with no restrictions on publication date, encompassing all available content from the consulted scientific databases. Initially, after the removal of duplicate articles, 727 articles were retrieved. By restricting the dataset to experimental studies only, excluding reviews and book chapters, the number was reduced to 642 studies.

A screening process was then performed to exclude publications inadvertently unrelated to the search topic. Titles and abstracts were examined, and only studies meeting the following inclusion criteria were retained: i) articles addressing sugar ester synthesis via enzymatic routes and ii) articles in which xylose was used as the acyl acceptor in sugar ester synthesis. All articles retrieved from the search, along with the justification for their inclusion or exclusion in the set of studies synthesized in this review, are presented in **Table A2.1** of the appendix, available in: https://docs.google.com/spreadsheets/d/1QDoX0tKvucmxV_0mtCrnCotXTxg0dNSL/edit?usp=drive_link&ouid=112345131410579711542&rtpof=true&sd=true.

Among the screened studies, only 20 employed xylose as the acyl acceptor in the synthesis of sugar esters (**Table A2.2**, appendix, available in: https://docs.google.com/spreadsheets/d/141fU4FGkJOr7YQcYKh7ut2Jhh5lzQk3A/edit?usp=drive_link&ouid=112345131410579711542&rtpof=true&sd=true). Given the focus of this review on the production of xylose esters, the full texts of these publications were analyzed and constitute the final dataset used for all analyses and conclusions.

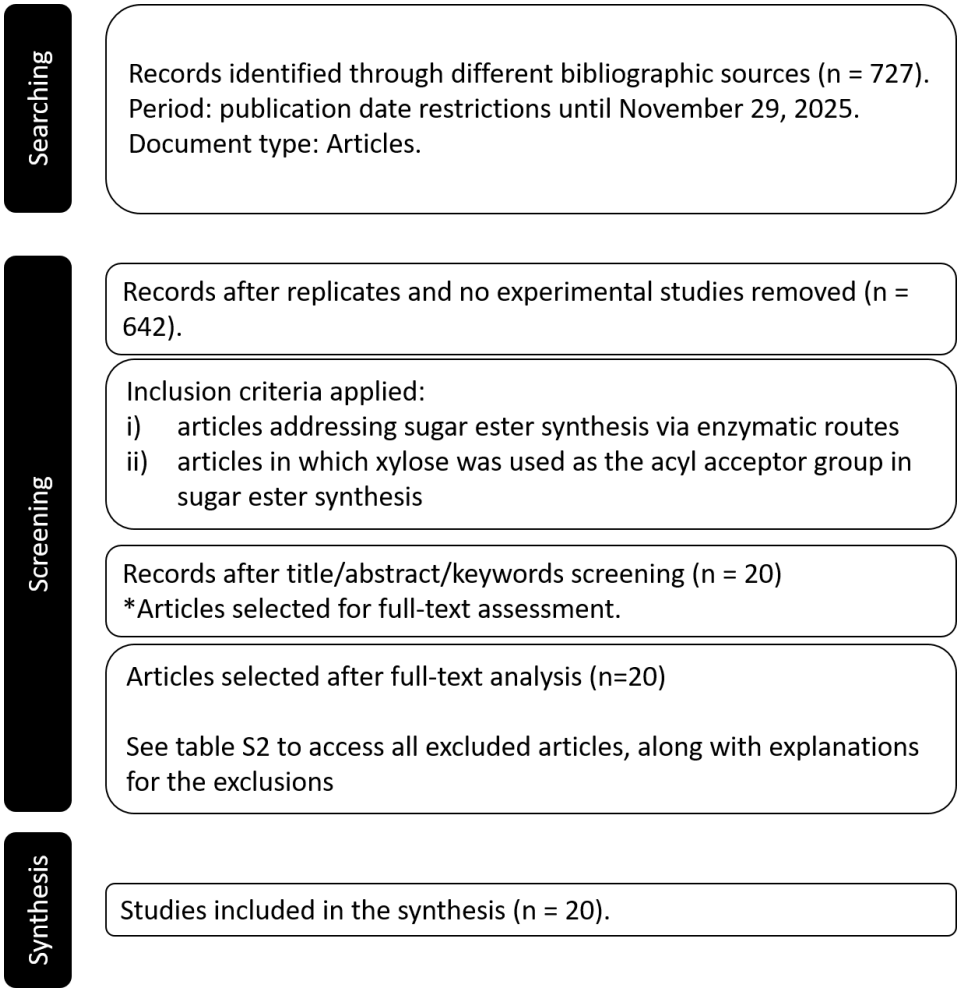

Figure 2. 1- Flow diagram of the study selection process (adapted from the ROSES Flow Diagram for Systematic Maps, Version 1.0).

2.2.3. Statistical tests

The repeatability of the screening process was assessed using Cohen's Kappa test. The test was conducted by two independent evaluators, given the potential for subjective decisions during the study selection process.

Cohen's Kappa test measures the level of agreement between reviewers on a scale from 0 to 1, as shown in Equation 2.1. The test evaluates whether the observed agreement exceeds that expected by chance alone. Values below 0.60 indicate inconsistency between reviewers, suggesting the need to redefine the inclusion criteria (Cohen, 1960). In this study, the Kappa score was 82.35%, indicating that the screening decisions are highly repeatable.

$$k = \frac{f_0 - f_E}{N - f_E} \qquad (2.1)$$

where f_0 is number of observed agreements, f_E is the number of agreements expected by chance, and N is the total number of observations.

2.2.4. Data analysis

Following the selection phase, 20 studies were included in the analysis. The extracted data were synthesized with the aim of identifying and highlighting key aspects and existing gaps in the enzymatic production of xylose esters. The synthesis focused on variables such as acyl donor groups, solvents, catalysts, and the separation and purification methods employed. Data organization and calculations were performed using Microsoft Excel (version 2016), while graphical representations were generated with OriginPro (version 8.5).

In addition, the synthesized data from the selected studies were used to construct network maps using the open-access software VOSviewer® (version 1.6.20). This stage involved bibliographic mapping through co-occurrence analysis of the studied terms, enabling the identification of relationships among acyl donor groups, solvents, and separation and purification methods. VOSviewer is a software tool specifically designed for the analysis of bibliometric data, allowing the visualization of co-occurrence networks that reveal the proximity and interconnections among the terms analyzed in the selected studies [32,58].

2.3. RESULTS AND DISCUSSION

2.3.1. Acyl donors

The diversity of possible sugar esters and their resulting properties arises from the wide range of substrate combinations and the different degrees of acylation of the synthesized esters. This diversity leads to a broad spectrum of characteristics, such as hydrophilic-lipophilic balance (HLB), emulsification capacity, foaming index, antimicrobial properties, among others [59,60]. Therefore, the acyl donor group used in the synthesis of xylose esters plays a crucial role in determining the final properties of the resulting ester. **Table 2.1** presents the of acyl donor groups employed in the synthesis of xylose esters compiled from the analyzed database.

Table 2. 1 - Proportion of acyl donors used in the enzymatic synthesis of xylose esters

Acyl donor	Occurrences	Source
Lauric acid	16%	[61–66];[67].
Oleic acid	16%	[8,62,68,69];[70];[9].
Vinyl laurate	9%	[13,41,64,71,72]
Methyl laurate	7%	[61,64,65]
Methyl myristate	5%	[34,61]
Methyl palmitate	5%	[34,61]
Methyl stearate	5%	[34,61]
Myristic acid	5%	[34,61]
Stearic acid	5%	[61,66]
Vinyl acetate	2%	[41]
Vinyl propionate	2%	[41]
Vinyl butyrate	2%	[41]
Vinyl decanoate	2%	[41]
Vinyl cinnamate	2%	[41]
Vinyl-octanoate	2%	[73]
Vinyl stearate	2%	[71]
Caprylic acid	2%	[68]
Butyric acid	2%	[68]
Linoleic acid	2%	[74]
Caproic acid	2%	[75]
Octanoic acid	2%	[73]

As shown in **Table 2.1**, the most frequently used acyl donors in the synthesis of xylose esters were lauric acid, oleic acid, and vinyl laurate, accounting for 16%, 16%, and 9% of occurrences in the database, respectively. These were followed by methyl laurate (7%), methyl myristate (5%), methyl palmitate (5%), methyl stearate (5%), myristic acid (5%), and stearic acid (5%). The remaining reagents listed each accounted for only 2% of occurrences in the database.

Lauric acid, also known as dodecanoic acid (C12), which showed the highest occurrence as an acyl donor in the synthesis of xylose esters, is a medium-chain fatty acid primarily sourced from coconut oil, which contains approximately 45-55% lauric acid [76,77]. Lauric acid exhibits antimicrobial activity, particularly against Gram-positive bacteria, as well as antiviral and antifungal effects, acting through disruption of microbial cell walls and interference with cellular processes [76,78]. Additionally, lauric acid has been reported to stabilize human cell membranes [76]. Its use as an acyl donor in sugar ester synthesis is advantageous because the antimicrobial properties of the fatty acid are also retained in the resulting ester [6,79,80].

Oleic acid (C18), a monounsaturated fatty acid (ω-9) known for its nutritional properties [81,82], was the second most frequently used acyl donor in the synthesis of xylose esters in the reviewed database. Its primary source is olive oil, which contains approximately 70-80% oleic acid [81,82]. Consumption of olive oil as part of the Mediterranean diet is commonly associated with reduced mortality from vascular diseases and cancer [82]. Beyond its nutritional benefits, oleic acid is recognized for its antioxidant and anti-inflammatory activities, as well as its role in regulating fat absorption [81].

Among the acyl donor groups applied in the synthesis of xylose esters listed in the database, 47% consist of methyl or vinyl esters of fatty acids. The use of fatty acids activated with vinyl or methyl groups is a common strategy to increase reaction rates and shift the thermodynamic equilibrium toward product formation [34,61,64,65,83]. The transesterification between methyl or vinyl fatty acid esters and sugars produces an alcohol as a byproduct, which is more volatile than the byproduct generated during esterification [84,85]. This alcohol can be readily removed during the reaction, thereby suppressing the reverse reaction. In the case of vinyl activation, the vinyl alcohol formed tautomerizes to acetaldehyde, which is highly volatile, as shown in **Figure 2.2** [84,85].

Martinez-Garcia *et al*. (2021) evaluated the conversion of xylose and lauric acid activated with methyl and vinyl groups, as well as the non-activated acid, during the synthesis of xylose esters. They observed that xylose conversion increased from 72% to 81% when

methyl laurate was used instead lauric acid, with a similar trend observed for acyl donor conversion, which increased from 88% to 98% [64]. However, reactions using vinyl laurate showed lower conversions compared to lauric acid, with xylose conversion decreasing from 72% to 66% and acyl donor conversion from 88% to 80% [64].

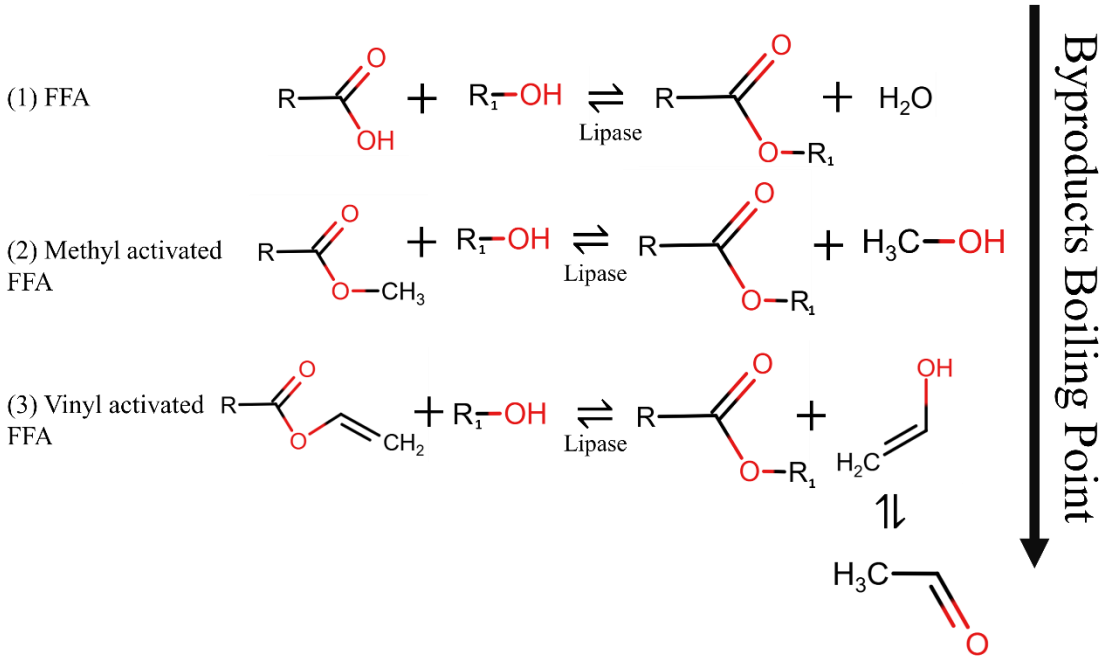

Figure 2. 2 - Activated fatty acids with vinyl or methyl groups as a strategy in the enzymatic synthesis of sugar esters

Studies focused on the production of sugar esters for food and pharmaceutical applications generally utilize medium-chain (C6–C12) or long-chain (C13–C20) fatty acids, since short-chain fatty acids or those with varying degrees of unsaturation often exhibit flavors or odors that limit their applicability [59]. Moreover, medium-chain esters typically exhibit low or negligible toxicity [60]. Zhu *et al*. (2022) evaluated the enzymatic synthesis of sucrose esters catalyzed by Lipozyme TLIM using acyl donors ranging from C12 to C22 and demonstrated that the hydrophilic-lipophilic balance (HLB), foaming capacity, and critical micelle concentration of the resulting esters tend to decrease with increasing fatty acid side-chain length [59]. Zhang *et al*. (2015) investigated sugar ester production catalyzed by Lipozyme TLIM using medium-chain fatty acids (C8, C10, and C12) and observed that the foaming capacity, foam stability, and antimicrobial activity varied with increasing fatty acid chain length [60].

In addition to the properties imparted by each fatty acid to the resulting ester, the choice of the acyl donor group must consider the specificity of the catalyst employed. Enzymes,

particularly lipases, exhibit varying substrate specificities [86]. Gonçalves *et al*. (2021) evaluated the specificity of Novozym® 435 (N435) and polyethyleneimine-coated N435 in the production of xylose esters, finding that uncoated N435 exhibited higher conversion of palmitic acid, whereas the coated form showed greater conversion of lauric acid [63]. Soultani *et al.* (2001) demonstrated that the synthesis of fructose esters catalyzed by *Candida antarctica* lipase was influenced by the fatty acid chain length, with the highest conversions observed for stearic acid (C18) [87]. Thus, it is evident that the acyl donor group significantly affects both the esterification reaction and the final properties of the sugar ester.

2.3.2. Biocatalysts

Various strategies have been employed in the synthesis of sugar esters, among which the development and application of different catalysts stand out [8,15,63,88–92]. Although the vast majority of commercially available sugar esters are produced via chemical synthesis [5,21], enzymes accounted for 87.18% of the catalysts used in the studies identified during the screening step. This reflects a strong trend toward environmentally friendly processes, as well as the use of more efficient and selective catalysts that ensure the safety of products intended for food and pharmaceutical applications [5,15,21,38]. Enzymatic synthesis of sugar esters enables mild reaction conditions and environmentally sustainable processes [4,15,37,93]. Moreover, the use of hydrolases, which exhibit high specificity and regioselectivity, helps prevent issues such as substrate degradation and the formation of undesired byproducts [4,15,37,93,94].

Figure 2.3 presents the percentage distribution of the types of catalysts used in the synthesis of xylose esters, as identified in the database compiled for this study.

Candida antarctica lipase B (CALB) was used as a catalyst in 68% of the studies involving the enzymatic synthesis of xylose esters. This enzyme, along with lipases from *Rhizomucor miehei* and *Burkholderia cepacia*, is among the most commonly applied lipases in esterification reactions carried out in organic media [7,32]. CALB has been widely used in the synthesis of sugar esters, both in its free and immobilized forms [32,53,89,95,96]. CALB exhibits high esterification activity, as well as enantioselectivity and high yields under mild reaction conditions [32,53,89,95,96].

Across the studies included in the database, the vast majority of enzymes applied in xylose ester synthesis were lipases, accounting for 95% of all enzymes used. Although other enzymes, such as proteases and esterases, can be employed in sugar ester synthesis, lipases are preferentially used due to their natural role in lipid metabolism and their ability to act at

hydrophilic-hydrophobic interfaces, which facilitates reactions involving substrates with differing polarities [21,21,38,47,97].

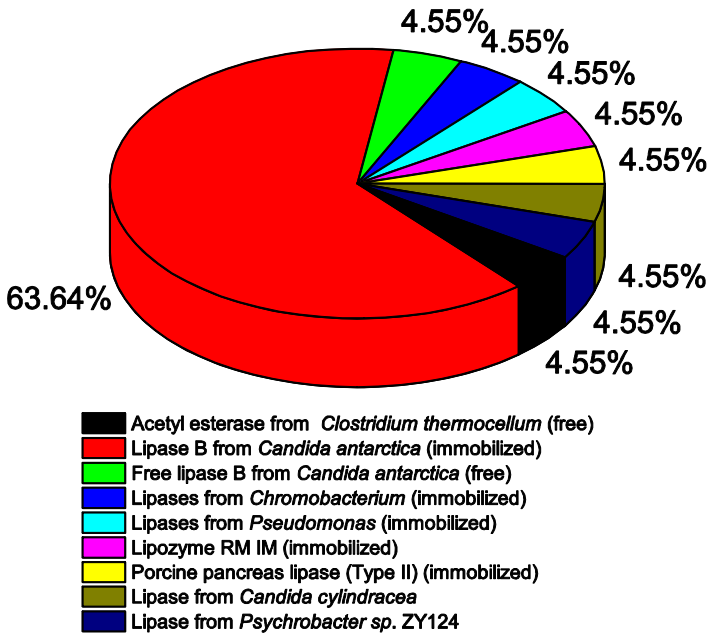

Figure 2. 3 - Proportion of enzymes used as catalysts in the enzymatic synthesis of xylose esters.

In the studies selected from the database, 82% employed immobilized enzymes as catalysts in the synthesis of xylose esters. Immobilized enzymes offer several advantages over free enzymes; in addition to enabling recovery and reuse of the biocatalyst, the immobilization process allows modulation of the enzyme stability, specificity, and activity depending on the immobilization conditions [70,86,98–101]. Among the immobilized lipases, the commercial biocatalyst Novozym® 435 (N435) stands out, accounting for 55% of the biocatalysts used in the synthesis of sugar esters in the selected studies. N435 consists of CALB physically immobilized on a Lewatit VP OC 1600 acrylic resin support via interfacial activation and is widely used due to its high thermal stability, especially at elevated temperatures [101,102].

The prominence of immobilized lipase use is largely due to the enzyme's interfacial activation mechanism, which allows for straightforward immobilization onto hydrophobic supports [98,103,104]. This immobilization approach relies on the adsorption of the enzyme onto hydrophobic carriers under mild conditions, typically involving low ionic strength and neutral pH [98,99,103,104]. In this process, the hydrophobic region surrounding the catalytic site plays a primarily role in adsorption, as the enzyme recognizes the hydrophobic support as analogous to its natural substrate [98,99,103,104]. This immobilization strategy, known as interfacial activation, often enhances catalytic activity. However, a critical limitation of this

method is the potential for enzyme desorption under harsh reaction conditions or in the presence of molecules with detergent-like properties [63,88].

2.3.3. Solvents in the Enzymatic Synthesis of Sugar Esters

The choice of solvent is a critical step in the enzymatic synthesis of SEs, as it must not only promote the solubility of substrates with differing polarities but also provide conditions that support high levels of enzymatic activity and stability [37,54–56,88,105]. The percentage distribution of solvents used in the synthesis of xylose esters, as identified in the selected studies from the database, is shown in **Table 2.2**.

Table 2. 2 - Solvents used in the synthesis of xylose esters.

Solvent	Occurrences	Source
Propanesulfonic acid (MOPS)-NaOH buffer pH 6.0	5%	[41]
tert-Butanol	15%	[62,68];[88]
Methyl ethyl ketone	25%	[8,63,66,69,71];[9].
Solvent free	5%	[61]
Deep eutectic solvent	15%	[34,73]; [88]
Dimethyl sulfoxide	10%	[72,75]
2-Methyl 2-butanol	10%	[13,64]
50% pentanol in water (v/v)	5%	[65]
Ionic liquid	5%	[67]
Hexane	5%	[74]

A variety of solvents were employed for the synthesis of xylose esters in the studies selected from the database. Methyl ethyl ketone was the most frequently used solvent, appearing in 25% of the selected studies, followed by *tert*-butanol and deep eutectic solvents (DES), each accounting for 15%, and dimethyl sulfoxide (DMSO) and 2-methyl-2-butanol, which appeared in 10% of the reported cases. These solvents are particularly noteworthy for their ability to dissolve substrates of differing polarity, their low viscosity, moderate volatility, and the absence of free hydroxyl groups that could be recognized as substrates by lipases [21,68,88,106–109].

Organic solvents such as tertiary alcohols (e.g., *tert*-butyl alcohol and 2-methyl-2-butanol), methyl ethyl ketone, and ionic liquids have been widely employed in the synthesis of

SEs [8,32,34,68,88,110]. Among the solvents used in the synthesis of xylose esters reported in the database, 65% were conventional organic solvents, 15% were deep eutectic solvents, 10% were water-containing systems, and 5% were solvent-free systems. In general, the solvents commonly used in the enzymatic synthesis of SEs can be classified into four main categories: conventional organic solvents, ionic liquids, deep eutectic solvents, and solvent-free systems.

2.3.4. Conventional Organic Solvents

A wide variety of solvents have been employed in the synthesis of SEs. The selection of an appropriate solvent is a critical step in SEs production due to the considerable polarity differences between the substrates [3,32,63]. Polar organic solvents such as tertiary alcohols, nitriles, and ketones are more commonly used, given the low solubility of carbohydrates and polyols in less polar solvents [7,93,94]. Tertiary alcohols, in particular, are widely applied because their hydroxyl groups are not recognized as substrates by enzymes [32,63,68,110]. In addition to sugar solubility, this enzymatic selectivity is a key factor in solvent selection, since the solvent itself may be recognized as a competing substrate, which can reduce product yield and lead to the formation of undesired by-products [69,111,112].

The octanol-water partition coefficient (log P), which reflects the hydrophobicity of a solvent, is an important parameter for evaluating potential solvents for use in the reaction medium [54,105]. Solvents with log P values below 2 tend to be harmful to enzymes, as they may disrupt the enzyme’s solvation layer. In contrast, solvents with log P values above 4 typically do not interfere with this layer and therefore less likely to compromise the enzyme's structural integrity [54,105]. However, this criterion alone is not sufficient to determine an optimal solvent.

This is because factors such as dielectric constant, dipole moment, hydrogen-bonding capacity, and viscosity, among others, also influence solvent-protein and solvent-substrate interactions, directly affecting substrate solubility, enzyme conformation, and the integrity of the catalytic site [54,105]. Additionally, enzymes generally exhibit low stability in polar organic solvents, as these solvents tend to strip away the residual water that is essential for maintaining the protein's active site [37,94,113]. When the sugar involved in SE synthesis is a di- or oligosaccharide, its solubility in organic solvents is even more limited. In such cases, it is often necessary to employ highly polar solvents such as dimethyl sulfoxide (DMSO), dimethylformamide (DMF), or pyridine, despite their-known detrimental effects on enzymatic stability [5,7,44,94,114].

One possible strategy to circumvent this issue is to use of a polar solvent as an adjuvant

or co-solvent: the sugar is first dissolved in the polar solvent and then blended with a less polar or non-polar solvent, such as *tert*-butyl alcohol or *tert*-amyl alcohol, to mitigate the negative impact on the enzyme [7,115–118]. CALB is frequently used in SE synthesis conducted in organic solvents, owing to its relatively high stability in polar media [39,87,91].

2.3.5. Ionic Liquids in Sugar Ester Synthesis

Ionic liquids (ILs) are organic salts with melting points below room temperature [5,119]. They are non-aqueous solvents capable of dissolving a wide range of compounds, including polar and non-polar substances, as well as organic, inorganic, and polymeric materials [93,120,121]. The physicochemical properties of ILs, such as melting point, polarity, hydrophobicity, viscosity, and miscibility, can be finely tuned through the selection and modification of their cationic and anionic components [5,121].

ILs have proven to be effective solvents for the solubilization of mono-, di-, oligo-, and polysaccharides [93,122–124]. In addition, ILs offer several advantages over conventional organic solvents, including non-volatility, non-flammability, and non-toxicity [97,119,121,125]. Nevertheless, their application also presents limitations. ILs are often expensive, exhibit poor biodegradability, and may result in prolonged reaction times, low product yields, as well as adverse effects on enzyme stability and recyclability [32,119].

2.3.6. Deep eutectic Solvents

Deep eutectic solvents (DES) are mixtures of two or three chemical compounds that, through hydrogen bonding interactions, form a eutectic mixture with a melting point lower than that of any of the individual pure components [126,127]. DES are typically prepared by combining an organic salt that acts as a hydrogen bond acceptor (HBA) with an organic compound that serves as a hydrogen bond donor (HBD). They can be synthesized from a wide range of low-cost reagents, which makes them particularly attractive for industrial applications [37,40,127–130].

DES can exhibit either hydrophobic or hydrophilic characteristics depending on their composition, and both types have been employed as solvents in sugar ester synthesis [37,131–135]. DES composed exclusively of natural reagents such as amino acids, organic acids, sugars, urea, or choline derivatives are known as natural deep eutectic solvents (NADES) [10,34,37,39,127,136–140]. NADES formulated with sugars as hydrogen bond donors are especially promising for lipase-catalyzed reactions, as they can simultaneously act as both solvent and substrate [10,34,39,88,133].

Souza *et al.* (2026) compared the synthesis of xylose oleate using methyl ethyl ketone, *tert*-butanol, and a xylose-based NADES, and observed that the xylose-based NADES provided higher xylose solubility and enhanced stability of the N435 biocatalyst compared with the other solvents [88]. Furthermore, DES composed of sugars as hydrogen bond donors are particularly advantageous in lipase-catalyzed reactions because they enable the use of an anhydrous reaction medium containing both sugars and fatty acids, thereby promoting efficient esterification catalysis by lipases [10,34,39,88,133,136,141].

2.3.7. Solvent-Free

The discussion up to this point emphasizes the crucial role of solvents in enzymatic reactions. In general, solvents exert a significant influence on biomolecules, either by providing a homogeneous environment that facilitates mass transfer or by inducing conformational changes that directly affect enzyme structure and function [54,94,142,143]. Enzyme activity and stability are closely are closely linked to the preservation of the enzyme's tertiary structure and catalytic site [54,143]. However, some enzymatic reactions are carried out in the absence of solvents, with the reaction medium consists primarily of the substrates and the enzyme. In certain cases, an aqueous enzyme solution is employed to provide the minimal water content necessary to maintain the enzyme's active conformation, whereas in others, enzymes are used in powdered form [61,144–147].

2.3.4. Product Separation and Purification

Downstream processing steps, including product separation and purification, account for approximately 50-80% of the total production costs of bioproducts and are therefore represent critical stages in their development [148,149]. This importance becomes even more pronounced when the final application is in the pharmaceutical or food industry, as is the case for SEs [150–152]. **Table 2.3** presents the separation and purification methods employed in the production of xylose esters, as reported in the studies included in the dataset.

The data presented in **Table 2.3** show that only 31% of the studies employed refined purification techniques, such as chromatography, while 27% did not report any separation or purification method for the xylose ester. Among the applied separation methods, 31% of the studies used precipitation techniques, and 12% employed solvent extraction methods. These two approaches are commonly reported in both scientific literature and patents as effective for the separation of sugar esters [34,63,69,71,73,153]. In precipitation-based methods, esters are precipitated by the addition of a solvent in which the ester is insoluble, often a mixture of

alcohol and water, and efficiency can be increased by repeating this step consecutively [63,69,153]. In turn, solvent extraction can be carried out using appropriate organic solvents, such as ethyl acetate or ketones, to selectively separate esters from unreacted sugars and other components [34,71,73].

Table 2. 3 - Overview of separation and purification methods used in the production of xylose esters.

Separation methods	Source
Precipitation with ethanol	[41]
No purification method was applied	[61,62,65,66,68,74,88]
Precipitation with a mixture of water and ethanol	[8,63,69];[9]
Extraction with distillated water	[71]
Extraction with ethyl acetate and purification of glycolipid extracts by flash chromatography	[73]
Precipitation with petroleum ether and purification by silica gel chromatography	[72]
Extraction with ethyl acetate and purification by silica gel chromatography	[34]
Precipitation due to temperature reduction (kept at 4 ˚C overnight) and purification by flash column chromatography on silica gel	[64]
Precipitation with tetrahydrofuran and purification by silica gel chromatography	[13]
Purification by silica gel chromatography	[67,75]

Chromatographic methods allow for product recovery with a higher degree of purity and are generally applied after initial separation steps. In the purification of SEs, different mobile phases, consisting of single solvents or solvent mixtures, are used to remove unreacted substrates and to separate esters with varying degrees of acylation [34,64,73,154,155].

The purification step plays a fundamental role in enabling accurate characterization and identification of the synthesized products. Gérard *et al.* (2020) precipitated the xylose and xylooligosaccharide esters during the reaction using petroleum ether, followed by purification through silica gel chromatography with ethyl acetate/methanol (9:1) as the eluent. Using more sensitive analytical techniques, such as nuclear magnetic resonance (NMR), the exclusive formation of monoesters was observed, suggesting potential regioselectivity of the biocatalyst toward the substrates employed [72].

Martinez-Garcia *et al.* (2021) employed temperature reduction to induce ester precipitation, followed by purification via flash column chromatography on silica gel using petroleum ether/ethyl acetate (7:3, v/v) as the mobile phase. This purification step revealed that, regardless of the acyl donor used, monoester formation was predominant, with minimal variation in the mono- to diester ratio as a function of the nature of the acyl donor [64].

However, the separation and purification of sugar esters remain challenging, as they depend strongly on the properties of the substrates and solvents employed. This complexity is reflected in the high number of studies that do not apply purification methods, highlighting the need for further research focused on the development and optimization of efficient purification strategies for these compounds.

2.3.5. Co-occurrence analysis of keywords

The co-occurrence analysis between acyl donor groups and solvents used in the synthesis of xylose esters was conducted based on the articles identified in the database. This approach allowed for the elucidation of the structure and distribution of terms within the network, as well as the frequency with which they co-occur. Based on these data, it was possible to highlight the main research focuses and identify underexplored areas in the literature.

The graph generated represents the co-occurrence of terms extracted from titles, abstracts, or keywords of the analyzed scientific articles. Each node corresponds to a term, and its size reflects its frequency in the dataset; larger nodes indicate higher occurrence. The connections (edges) between the nodes represent co-occurrence, that is, the simultaneous appearance of terms within the same article or context. The thickness of the lines indicates the strength of this co-occurrence, with thicker lines representing pairs of terms that co-occur more frequently. Additionally, the spatial proximity between terms in the graph suggests stronger relationships based on their co-occurrence frequency.

The presence of larger nodes in **Figure 2.4** is associated with the fact that many studies

in the dataset employed more than one acyl donor group in the synthesis of xylose esters. This strategy is commonly adopted to assess the biocatalyst's selectivity toward different acyl groups [63,87], to obtain esters with distinct properties [59,60], or to shift the reaction's thermodynamic equilibrium (in the case of vinyl and methyl esters) [64,84,85].

The acyl donor groups with the highest number of co-occurrences were caproic acid, stearic acid, butyric acid, vinyl acetate, and vinyl propionate, indicating the use of two or more of these groups within the same study.

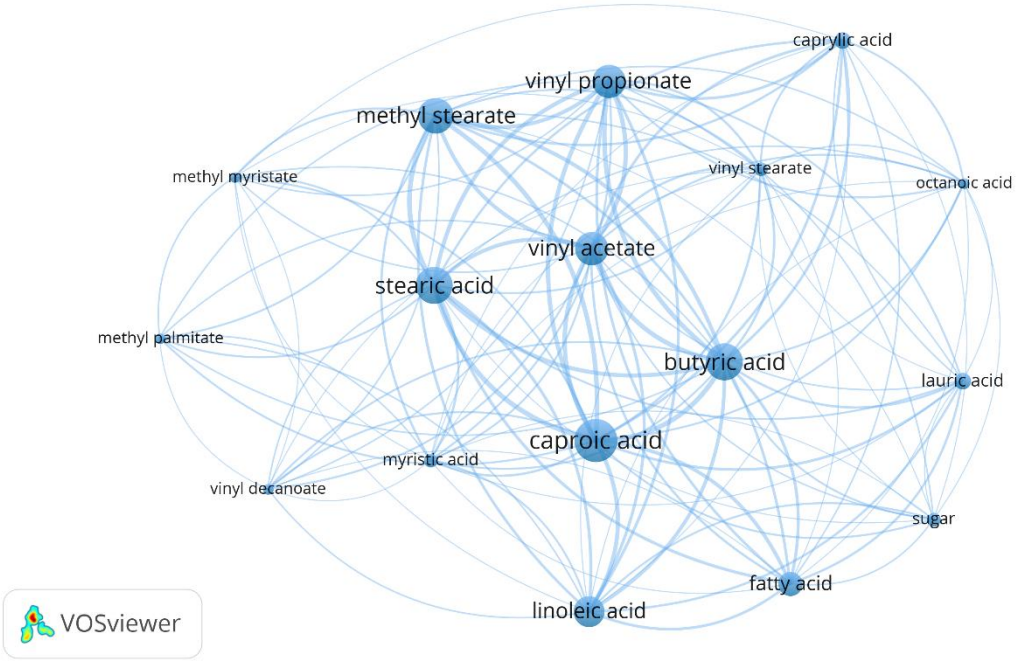

Figure 2. 4 - Bibliometric analysis of acyl donor groups applied in the synthesis of xylose esters. The node size is proportional to the number of occurrences, and the edge thickness represents the co-occurrences between items.

Figure 2.5 presents the co-occurrence analysis of the solvents used in the studies included in the database. Tert-butanol was among the solvents most frequently associated with other solvents, followed by methyl ethyl ketone, hexane, and dimethyl sulfoxide.

The use of multiple solvents in these studies typically involved solvent mixtures, a strategy adopted to enhance the solubility of substrates with differing polarities. Although less frequent, co-occurrence between DES and other solvents were also observed. However, in the studies included in the dataset, DES were not used in combination with other solvents directly in the synthesis of sugar esters; rather, the additional solvent was employed in a separate step of the process, such as the synthesis of methyl or vinyl fatty acids esters.

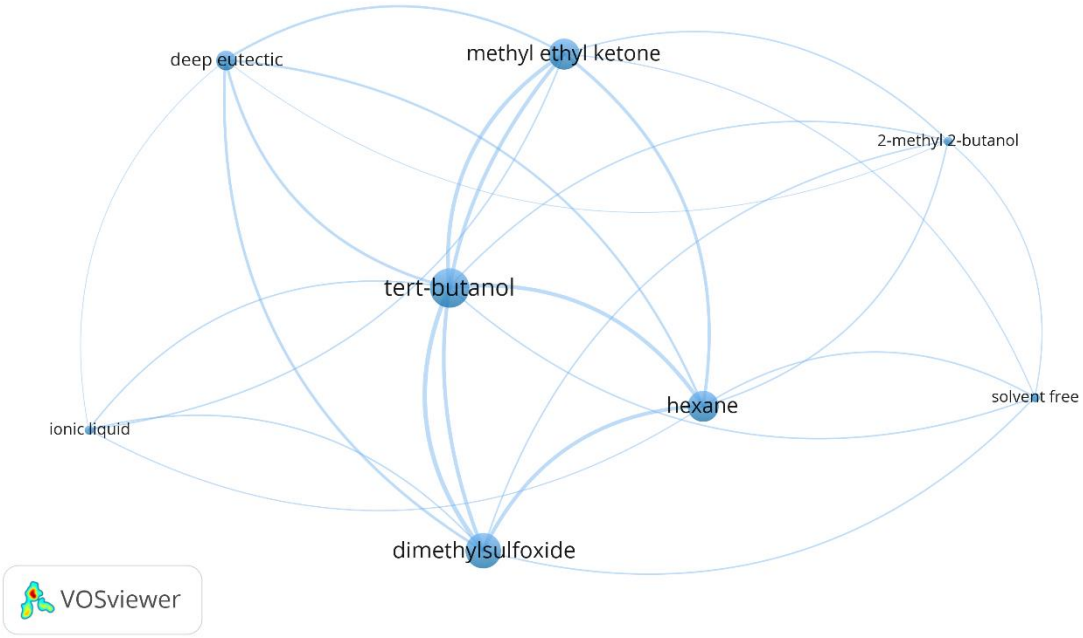

Figure 2. 5 - Bibliometric analysis of solvents applied in the synthesis of xylose esters. Node size is proportional to the number of occurrences, and edge thickness represents the co-occurrences between items.

2.4. FUTURE PERSPECTIVES

Current trends in the field of SEs focus on the production of high value-added esters with applications extending beyond their traditional use as surfactants and emulsifiers [156]. The synthesis of flavor esters enables the development of molecules that, in addition to the typical properties of SEs, exhibit desirable tastes and fragrances for the food and cosmetic industries [156–158]. Furthermore, flavor esters may exhibit lower toxicity compared to their aromatic acyl donor counterparts [158]. Generally, flavor esters are derived from natural sources and often display antioxidant and antimicrobial properties, making them attractive for food, pharmaceutical, and cosmetic applications in response to the growing demand for products with reduced synthetic content [157–162]. Moreover, products obtained via bioprocesses or biotechnological methods are considered natural by the U.S. Food and Drug Administration and by European regulatory agencies, provided that the raw materials employed are of natural origin [163,164].

2.5. CONCLUSIONS

The enzymatic synthesis of xylose fatty acid esters emerges as a sustainable and promising alternative for the production of high value-added molecules, driven by the global demand for renewable, biodegradable, and environmentally friendly products. Analysis of the 20 selected studies indicates that lipases, particularly *Candida antarctica* lipase B and its immobilized form, Novozym® 435 (N435), remain the most effective biocatalysts for this purpose. In addition, the choice of acyl donor and solvent plays a decisive role in both reaction performance and the functional properties of the resulting esters. Although conventional organic solvents still predominate in current studies, increasing attention has been directed toward greener alternatives, such as deep eutectic solvents, which can improve substrate solubility without severely compromising enzymatic activity. Nevertheless, several challenges remain unresolved, including the limited solubility of sugars, enzyme instability in highly polar media, and the lack of efficient and scalable separation and purification strategies, an aspect that is often underreported or overlooked in the literature. Future advances in this field will therefore depend on the integrated development of biocatalyst engineering, solvent innovation, reaction optimization, and downstream processing. Such combined efforts are essential to enable the transition of xylose ester production from laboratory-scale studies to industrial applications, particularly in cosmetic, food, pharmaceutical, and other bio-based sectors.

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2.7. APPENDIX

Table A2. 1 - The database was updated on November 29, 2025, with no publication date restrictions, and includes the reasons for inclusion or exclusion from the database used for the analyses. Available in: https://docs.google.com/spreadsheets/d/1QDoX0tKvucmxV_0mtCrnCotXTxg0dNSL/edit?usp=drive_link&ouid=112345131410579711542&rtpof=true&sd=true

Table A2. 2 - Database used for the analysis and main data obtained from the studies. Available in: https://docs.google.com/spreadsheets/d/141fU4FGkJOr7YQcYKh7ut2Jhh5lzQk3A/edit?usp=drive_link&ouid=112345131410579711542&rtpof=true&sd=true

CHAPTER III
POLYETHYLENIMINE-GLUTARALDEHYDE MODIFICATION OF NOVOZYM® 435 ENHANCES STABILITY AND SUGAR ESTER SYNTHESIS IN A XYLOSE-BASED NATURAL DEEP EUTECTIC SOLVENT

The synthesis of xylose oleate was investigated using an immobilized lipase (*Candida antarctica* lipase B, Novozym® 435), further modified with polyethylenimine and glutaraldehyde. Coating the lipase with polyethylenimine enhanced its stability in tert-butyl alcohol, methyl ethyl ketone, and xylose-based-natural deep eutectic solvent (NADES). Increasing the molecular weight of polyethylenimine improved enzyme stabilization, and biocatalyst stability was enhanced by subsequent treatment with glutaraldehyde. Notably, the lipase immobilized and coated with 750 kDa polyethylenimine and crosslinked with glutaraldehyde exhibited minimal enzyme leaching during desorption tests with Triton X-100 (20 mg/mL). In the enzymatic conversion of oleic acid to xylose oleate, the modified lipase outperformed the unmodified Novozym® 435, yielding 2.3-fold higher conversions in xylose-based NADES medium. The enhanced performance of the modified biocatalyst was also evident in operational stability assays, where it retained 75% of its relative conversion after five consecutive reaction cycles, demonstrating a threefold increase in stability compared with the untreated biocatalyst. The resulting xylose esters were purified and identified by mass spectrometry, confirming the formation of xylose mono-, di-, trioleate derivatives. The composition of the reaction products varied depending on both the solvent and the employed biocatalyst. Importantly, the synthesized esters exhibited superior emulsifying properties compared with certain commercial surfactants.

3.1. INTRODUCTION

Sugar fatty acid esters, or simply sugar esters (SEs), are amphiphilic molecules synthesized via esterification reactions between sugars and free fatty acids [1,2]. SEs are biodegradable [3,4] odorless [5], non-irritating [6,7], non-toxic [6–8], and non-anionic surfactants [8,9]. These properties make them highly applicable in the cosmetics, food, and pharmaceutical industries [8–11]. Furthermore, SEs have demonstrated therapeutic potential, including antitumor [12,13], antimicrobial activities [13–16], as well as serving as vectors for gene therapy [4,17,18]. Collectively, these features make SEs promising alternatives to petrochemical-based surfactants [19,20].

Glucose and sucrose esters are the most commonly produced SEs worldwide [9,19]. However, SEs derived from five-carbon sugars (C5-SEs), particularly xylose esters, exhibit remarkable lubricating and antimicrobial properties [7,20–22]. These esters can be obtained from lignocellulosic biomass hydrolysates, positioning xylose-rich residues from biorefineries as sustainable feedstocks for biosurfactant production [21–25].

Enzymatic catalysis is a suitable process for producing SEs, utilizing lipases as catalysts in a one-step esterification process [9,26,27]. This enzymatic route offers mild reaction conditions, along with high specificity and regioselectivity, thereby avoiding issues such as substrate degradation and, most important, the formation of unwanted by-products [9,10,28]. However, selecting an appropriate reaction medium remains a critical challenge in enzymatic SE production. Esterification requires low water activity to favor ester formation, and there is a significant difference in the polarities of the involved substrates. The significant polarity difference between substrates makes it difficult to solubilize both ones in the same solvent [9,14,19,24,28–30]. High-polarity solvents, such as hydrophilic organic solvents, can interact with the lipase and alter its structure, leading to reduced enzymatic activity [28,31–33]. Additionally, solvents containing hydroxyl groups may be recognized as substrates by the enzyme, thereby decreasing the yield of the desired product and promoting by-products formation, which may complicate the downstream processing of SEs [29,34,35].

Solvents such as tertiary alcohols (eg., tert-butyl alcohol and 2-methyl-2-butanol), methyl ethyl ketone, and ionic liquids are widely used in SEs synthesis **(Figure 3.1)** [7,24,25,36–40]. However, previous studies have shown that xylose exhibits moderate solubility in tert-butyl alcohol (68.94 mM) [41], *tert*-pentyl alcohol (52.75 mM) [41], and methyl ethyl ketone (7.16 mM) [29]. Additionally, ionic liquids are expensive, poorly biodegradable, and may adversely affect enzyme stability and recovery [24,42–44]. In this

context, natural deep eutectic solvents (NADES) have been proposed as suitable media for lipase-catalyzed reactions [23,28,45–50].

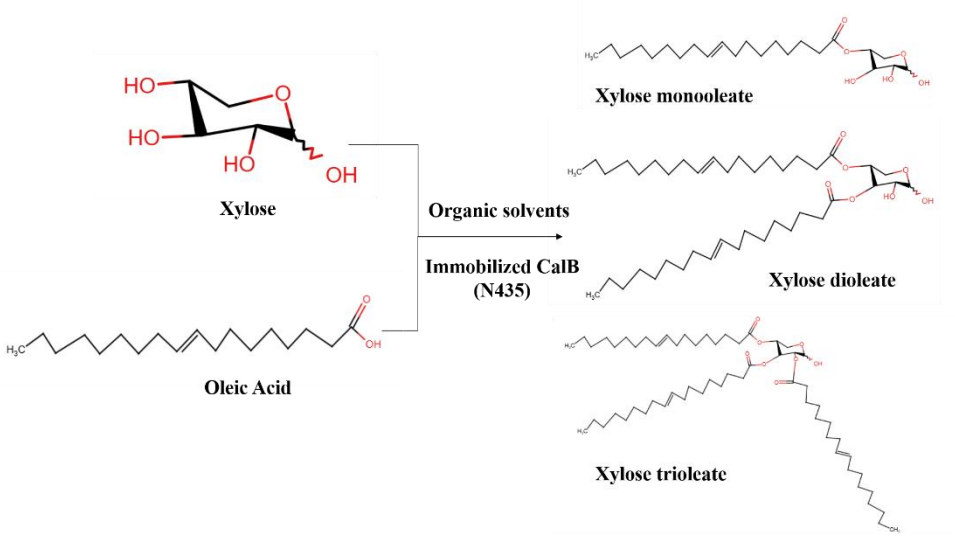

Figure 3. 1 - Lipase-catalyzed esterification of xylose esters using Novozym® N435 in conventional and unconventional solvents.

This new class of solvents consists of mixtures of two or three compounds that act as hydrogen bond donors and acceptors, resulting in a liquid medium with a melting point lower than that of the individual components [28,42,43,51–53]. NADES represent a promising alternative to conventional organic solvents owing to their non-toxicity, biodegradability, non-flammability, and non-volatility [7,45]. Compared with ionic liquids, which can exhibit toxicity and poor biodegradability despite their positive effects on reaction efficiency, NADES stand out for being composed of natural and biodegradable compounds, maintaining comparable performance without the associated environmental impact [24,42–44]. Furthermore, NADES formed with sugars as hydrogen bond donors can serve both as solvents and as substrates, providing an anhydrous reaction medium containing sugars and fatty acids that favors lipase-catalyzed esterification [47–49,54].

Immobilized lipases are preferred because they facilitate recovery from the reaction medium and enable enzyme reuse, provided the enzyme remains active [24,55,56]. Moreover, immobilization can enhance several enzyme properties, including stability, activity, selectivity, and specificity [57–64]. The commercial biocatalyst Novozym® 435 (N435) consists of *Candida antarctica* lipase B (CALB), immobilized on Lewatit VP OC 1600, an acrylic resin support, via interfacial activation [65]. It has demonstrated its ability to catalyze SEs synthesis

in various solvents, including NADES [24,25,29,38,44,49,66–70]. However, due to the reversible interactions between the enzyme and the support, this biocatalyst is susceptible to enzyme desorption under harsh conditions such as high temperatures, the presence of organic solvents or detergents [38,56,65,71–73]. That way, the surfactant properties of SEs can further promote enzyme desorption, leading to reduced catalyst activity and reusability [71].

To address these issues, researchers have proposed crosslinking enzymes physically immobilized on supports using physical and/or chemical agents to form protein aggregates that remain attached to the support [38,56,74–78]. Treatment of the biocatalyst with polyethylenimine (PEI) has been proposed as a strategy to prevent enzyme desorption and enhance biocatalyst stability under such conditions [38,56,75,79–81]. The PEI coating also creates a hydrophilic microenvironment that can facilitate the partitioning of hydrophobic compounds. This physical intermolecular crosslinking of lipases with ionic polymers presents a promising stabilization strategy. One advantage of this approach is that, if the enzyme is reversibly immobilized, it can be recovered after inactivation by incubating the biocatalyst under harsh conditions. However, a potential drawback exists: if the ionic interactions between PEI and the enzyme are disrupted by changes in the medium (e.g., pH or ionic strength), unwanted enzyme release may occur during operation [38,56,82–87].

To overcome this limitation, an alternative approach involves the chemical modification of the PEI-coated catalyst surface with glutaraldehyde [79]. This treatment renders the crosslinked enzyme irreversibly bound to the support, preventing its release under any reaction conditions and making the immobilization protocol permanent [75,88–90]. Glutaraldehyde, due to its small size, can form both intra- and intermolecular crosslinks between in one enzyme molecule or among different immobilized enzyme molecules, promoting enzyme rigidification and enhancing thermal stability [79,91–95]. When used to modify PEI-coated enzymes, glutaraldehyde enables the formation of long-distance covalent intermolecular crosslinks between PEI and the enzyme. Although this may have a smaller impact on enzyme stability compared to direct crosslinking, it effectively prevents enzyme desorption from the support [75,79,89,90].

Thus, in this study, we evaluate the performance of Novozym® 435 coated with PEI, and further modified with glutaraldehyde, as a biocatalyst for the synthesis of xylose oleate in xylose-based NADES, and compare its activity in *tert*-butyl alcohol and methyl ethyl ketone. The use of xylose as both a reaction medium and a substrate represents an interesting valorization route for one of the major components of hemicellulose, a principal constituent of lignocellulosic biomass.

3.2. MATERIALS AND METHODS

3.2.2 Materials

Novozym® 435 (N435) (flask activity 5,000 UI/g), tributyrin, butyric acid, glutaraldehyde (GA), Triton X–100, sucrose monolaurate (97%), and PEI with average molecular weights of 2 kDa, 25 kDa, or 750 kDa were purchased from Sigma-Aldrich (St. Louis, MO, USA). Xylose and heptane were obtained from Synth (São Paulo, Brazil). Methyl ethyl ketone (MEK), *tert*-butyl alcohol (TERT), and choline chloride were acquired from Neon (São Carlos, Brazil). Silica gel (0.063-0.2 nm) was purchased from Macherey-Nagel. All other reagents and solvents were of analytical grade and used without further treatment.

3.2.3. Coating of the N435 with PEI and glutaraldehyde

N435 was coated with PEI by adding 5 mL of PEI solution (100 mg/mL) with molecular weights of 2, 25, or 750 kDa to 25 mL of a 20% (w/v) N435 suspension in 5 mM sodium phosphate (pH 7.0). The suspension was incubated at 25 °C for 1 hour under stirring at 120 rpm. The immobilized PEI-coated derivative was washed with excess 5 mM sodium phosphate (pH 7.0) and distilled water, and recovered by vacuum filtration [38,96]. Hydrolytic activity of the coated lipase was subsequently measured as described in 3.2.13 Section.

For GA treatment, 1 g of the PEI-coated derivative was added to 50 mL of a 0.0025% (v/v) GA solution (0.265 mM) in 50 mM sodium phosphate (pH 7.0) and incubated at 25 °C with stirring at 120 rpm for 1 hour. The modified biocatalyst was washed with excess 5 mM sodium phosphate (pH 7.0) and distilled water, and recovered by vacuum filtration [93,97]. The hydrolytic activity was measured as described in 3.2.13 Section.

3.2.4. Determination of biocatalyst adsorption capability

The adsorption capability of the biocatalysts was determined by measuring the adsorption of the hydrophobic dye Rose Bengal per gram of biocatalyst. For this assay, 50 mg of the immobilized enzyme was incubated in 5 mL of a freshly prepared Rose Bengal solution (1 mg/mL in 25 mM sodium phosphate, pH 7.0), for 24 hours at room temperature. After incubation, the absorbance of the supernatant was measured at 549 nm to quantify dye adsorption [98].

3.2.5. Stability of the different N435 biocatalysts in the presence of solvents

The stabilities of the unmodified N435 and the PEI- and GA-treated derivatives were

evaluated in 50 mM TRIS (pH 7.0), TERT, MEK, and xylose-based NADES at a concentration of 0.5% (w/v) for 6 hours at 60 °C. Samples were collected before and after incubation, and the hydrolytic activity was measured.

3.2.6. Enzyme leaching in the in the presence of detergent

Desorption of N435 and modified derivatives was evaluated using 1% (v/v) Triton X-100 in 5 mM sodium phosphate at pH 7.0. Ten milligrams of biocatalyst were incubated in 1.5 mL of the buffer solution for 1h and 24h. Following incubation, the biocatalyst was washed with excess buffer and distilled water, recovered by vacuum filtration, and its hydrolytic activity was measured. SDS-PAGE analysis was performed by adding 0.1 g of each derivative to 1 mL of disruption buffer, boiling this suspension for 5 min, centrifuging at 10,000 rpm for 5 min, and loading 15 μL of the obtained supernatant on the gel, as well as 5 μL of marker proteins as standard. Electrophoresis was conducted at 100 V, and gels were stained with Coomassie Brilliant Blue [99].

3.2.7. Xylose solubilization in organic media

Xylose solutions were prepared in MEK and TERT at 7 mM and 60 mM, respectively, based on previously reported solubility limits [29,41]. Solutions were maintained at 60 °C for 24h under agitation at 200 rpm to ensure complete dissolution [29,41].

3.2.8. Preparation of the xylose-based NADES

Xylose-based NADES was prepared by mixing xylose and choline chloride in equimolar ratio under continuous stirring at 70 °C in a closed vessel for approximately 2h until a homogeneous, transparent liquid was formed [53]. This xylose-based NADES serve as both solvent and substrate.

3.2.9. Enzymatic synthesis of xylose oleate

Xylose oleates were synthesized by enzymatic esterification of xylose and oleic acid (1:5 molar ratio) in 20 mL total volume, using the different solvents describe above. Untreated N435 and PEI/GA-treated N435 were used as biocatalysts. Reactions were conducted in a jacketed reactor with flat-blade impeller at 60 °C, 550 rpm, and 500 U tributyrin hydrolysis activity (TBU)/g xylose. pH was not monitored or controlled. Samples were collected at 0, 1, 3, 6, 12, and 24 h, and oleic acid concentration was measured by gas chromatography (Section 2.13). All reactions were performed in duplicate.

3.2.10. Emulsion capacity assays

The final reaction mixtures were incubated at 70 °C for 24 h to remove organic solvents and water. Xylose-based NADES reaction mixtures were centrifuged at 10,000 rpm for 10 min to separate light (esters) and heavy phases. Fifty milligrams of the hydrophobic phase were diluted in 1 mL of distilled water, mixed with 2 mL of kerosene, homogenized for 2 min, and left for 24h at room temperature [100]. Emulsion height (H_e) and total height (H_t) were measured, and the emulsification index (EI, %) was calculated as:

$$EI\ (\%) = \left(\frac{H_e}{H_t}\right) \times 100 \quad (1)$$

3.2.11. Chromatographic separation of xylose esters

Mono-, di-, and triesters were separated using silica gel column chromatography (0.063-0.2 nm) following Ducret *et al*. (1995) [101]. The reaction mixture, diluted in minimum volume of chloroform, was applied to the top of a column (i.d. 1.5 cm, height 30 cm), pre-equilibrated with chloroform at 1.5 mL/min. Free fatty acids were eluted chloroform, monoesters with chloroform/methanol (90/10, v/v), and di-/triesters with chloroform/methanol/water (64/10/1, v/v/v). Fractions were monitored by thin-layer chromatography (TLC).

3.2.12. Operational stability

Operational stability of N435-PEI750-GA was evaluated in five successive 6-hour batches of xylose synthesis under previously reported conditions (60 °C, 550 rpm, 1:5 xylose-to-oleic acid molar ratio, and 500 TBU/g xylose). After each batch, biocatalysts were recovered by centrifugation (35 °C; 10,000 rpm, 2 min), washed with hexane, vacuum-filtered, and reused. Oleic acid concentration was measured by gas chromatography (2.13 Section).

3.2.13. Hydrolytic activity

Tributyrin hydrolysis activity of the biocatalyst was measured by mixing 1.5 mL tributyrin, 6 mL 100 mM sodium phosphate (pH 7.3), and 16.5 mL distilled water at 37 °C for 5 min. Released butyric acid was titrated with 20 mM KOH using a pH-Stat titrator (Titrando 907, Metrohm, Herisau, Switzerland). One unit of tributyrin hydrolysis activity (TBU) is defined as 1 µmol butyric acid release per min under the established experimental conditions [102].

3.2.14. Oleic acid determination

A 300 µL sample of the reaction supernatant was collected for analysis. Oleic acid concentration was measured using an Agilent 7890A gas chromatograph equipped with a flame ionization detector (FID) and an HP INNOWAX column (30 m × 0.25 mm × 0.25 µm; Restek Corporation, Bellefonte, PA, USA). The temperature program was: 150 °C for 1 min, ramped to 230 °C at 10 °C/min and held for 1 min, followed by a ramp to 250 °C at 5 °C/min, held for 5 min. Helium was used as the carrier gas at 1.8 mL/min, with a total run time of 14 min [38].

3.2.15. Thin Layer Chromatography (TLC)

TLC was used to monitor sugar ester production using an adaptation of the method described by Ducret *et al.* (1995) [101]. The method consisted of three steps: (1) a small drop of the reaction mixture was applied to a 6.5 cm pre-coated silica gel plate. A mobile phase of chloroform:methanol:water (64:10:1, v:v:v) was used until it reached 2 cm on the plate to separate monoesters; (2) after drying, a second mobile phase of chloroform:methanol:acetic acid (97.5:2.5:1, v:v:v) was applied until 4 cm on the plate to separate diesters; (3) after drying again, a third mobile phase of hexane:ethyl ether:acetic acid (70:30:1, v:v:v) was used until 5.5 cm to separate triesters and free fatty acids.

Retention factors (R_f) were as follows: xylose 0, monoesters 0.11, diesters 0.25, triesters 0.40, and free fatty acids 0.76 [101]. Components were visualized in an iodine chamber, and the visible spots were recorded.

3.2.16. Mass spectrometry

The reaction products were characterized by electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-QTOF-MS/MS) in negative ion mode using an Agilent 6545 instrument. The mass range was 100–1700 Da. Samples were dissolved in methanol and analyzed by flow injection analysis (FIA) at 0.35 mL/min, with a 5.0 µL injection volume at 40 °C. The mobile phase consisted of H_2O + 0.1% formic acid and MeCN + 0.1% formic acid (20:80, v:v). The total analysis time was 4.0 min. ESI source parameters were: capillary voltage 2500 V, nozzle voltage 500 V, gas temperature 350 °C, drying gas 12 L/min, nebulizer 35 psi, sheath gas 320 °C at 10 L/min, fragmentor 120 V, skimmer 80 V, and collision energies 20 and 22 V. Data were processed using Qualitative Navigator B.08.000 software.

3.2.17. Statistical analysis

Data were subjected to analysis of variance (ANOVA), and statistical significance was determined using the F-test at a 5% probability level ($p \leq 0.05$). Mean comparisons were performed using Tukey's test at the same significance level. All analyses were conducted using SISVAR software, version 5.6 [103].

3.4. RESULTS AND DISCUSSION

3.4.1. Effect of the different treatments on the adsorption capability and hydrolytic activity of N435

The effect of physical and/or chemical modification of N435 was evaluated by adsorption assays of aromatic and anionic Rose Bengal dye and by measuring the hydrolysis activity of tributyrin. Rose Bengal is a tetraiodo-substituted dye of the xanthene class of dyes containing hydrophobic regions (aromatic rings) and anionic groups [104,105]. In neutral or alkaline solutions, the dye is an anion due the deprotonation of carboxylic and phenolic groups [106]. Thus, hydrophobic surfaces in a neutral aqueous medium favor dye adsorption via hydrophobic interactions. However, on N435, the support surface is coated with enzyme molecules, which have ionized at neutral pH (e.g., carboxylates and amine groups). In this case, the dye can be adsorbed onto N435 through hydrophobic and ionic interactions, depending on the enzyme coating (whether coated or not with positively charged polymers, as such as PEI) [98,105], and on the enzyme isoelectric point.

Figure 3.2 shows that the amount of Rose Bengal adsorbed on N435 decreased approximately 25% after enzyme was treated with glutaraldehyde. This is not an expected results, as glutaraldehyde should reinforce the hydrophobic interactions between the enzyme and the dye [107] and just change the primary amino groups by secondary amino groups. Perhaps this reflected the lower accessibility of the dye to the support after enzymes intermolecular crosslinking. When N435 was coated with PEI, the amount of adsorbed dye decreased approximately 80%. This is a clear indicative that the adsorption of the dye is mainly by hydrophobic interactions, as the modification with this poly-cation instead of increase the adsorption of the dye, decreased it, as consequence of the hydrophilization of the biocatalysts. This behavior was observed for all evaluated PEI sizes.

Conversely, when N435 was coated with PEI (2, 25 and 750 kDa) and subsequently crosslinked with glutaraldehyde, the amount of adsorbed dye on the modified derivatives increased from approximately 80 to 160% compared to the amount adsorbed on the unmodified N435. This suggests that some hydrophobic interactions between the dye aromatic rings and the modified and more hydrophobic N435 [107]. These findings suggest that the biocatalysts underwent physical and/or chemical changes that modified their surface hydrophobicity. This can, in turn, contribute to substrate and product partitioning effects on the surface of the immobilized enzyme, particularly when substrates/products are hydrophobic (e.g., tributyrin, fatty acids, oils, fatty acid esters) or polar (e.g., glycerol, butyrate, xylose).

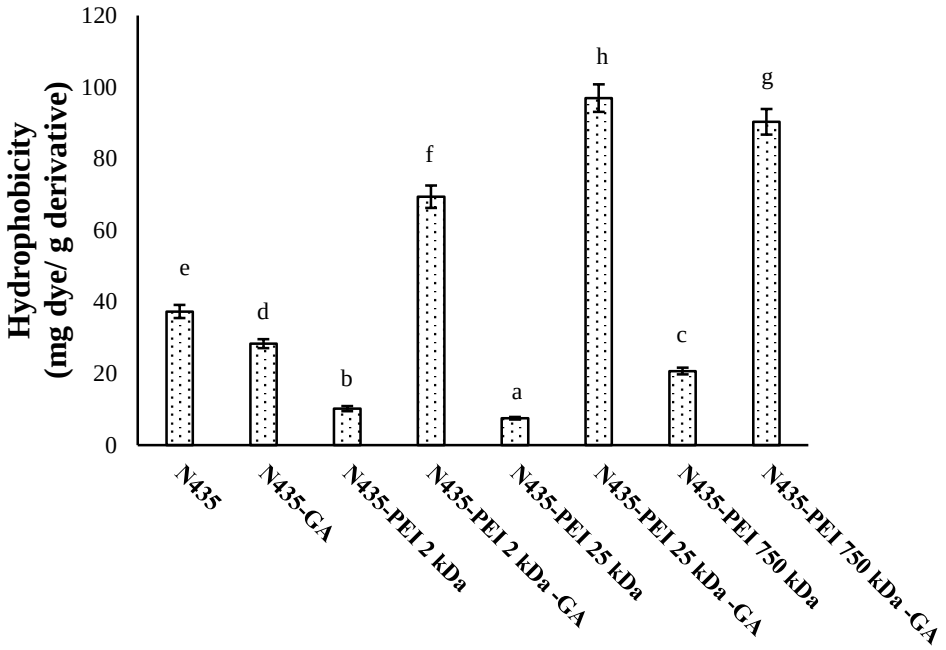

Figure 3. 2 - Comparative analysis of the adsorption capacity of the N435 biocatalysts: unmodified and modified by PEI coating and/or GA crosslinking, evaluated using Rose Bengal dye adsorption. The indices a-h indicate mean differences according to Tukey's test (5% significance), with h representing the highest significance level. Experimental conditions: 50 mg of biocatalyst in 5 mL of Rose Bengal (1 mg/mL in 25 mM sodium phosphate buffer, pH 7.0); incubation for 24 hours at room temperature; absorbance of the supernatant measured at 549 nm. Experiments were performed as described in the Methods section.

Figure 3.3 shows the hydrolytic activities of N435 before and after treatment with PEI of different molecular weights (2 kDa, 25 kDa, and 750 kDa) and/or crosslinking with GA.

Crosslinking the immobilized enzyme with GA resulted in a 60% decrease in hydrolytic activity compared to the untreated biocatalyst. This reduction is likely due to negative effects associated with chemical modification of the enzyme [94]. All treatments with PEI also reduced hydrolytic activity by at least one-third, except for the biocatalyst coated with 750 kDa PEI, which retained its original activity. These findings are consistent with previous reports describing either a slight reduction or a modest enhancement in hydrolytic activities depending on the molecular weight of the PEI used [74,79,80,108]. This behavior could be attributed to diffusional mass transfer limitations caused by the introduced polymer layer, as well as substrate/product partitioning effects within the hydrophilic microenvironment generated on the biocatalyst surface. When PEI-coated N435 was crosslinked with GA, hydrolytic activity decreased by up to approximately 60%, a value statistically similar to that of the biocatalyst treated only with GA **(Figure 3.3)**. In addition to enzyme modification, GA can also crosslink

PEI chains, leading to increased polymer rigidity. This rigidification may increase the tortuosity of the pathway that the substrate must diffuse to reach the enzyme active site [75,88–90]. Enzyme-PEI crosslinking may also occur and further contribute to this effect. **Table 3.1** summarizes the results of this study in comparison with previously reported findings.

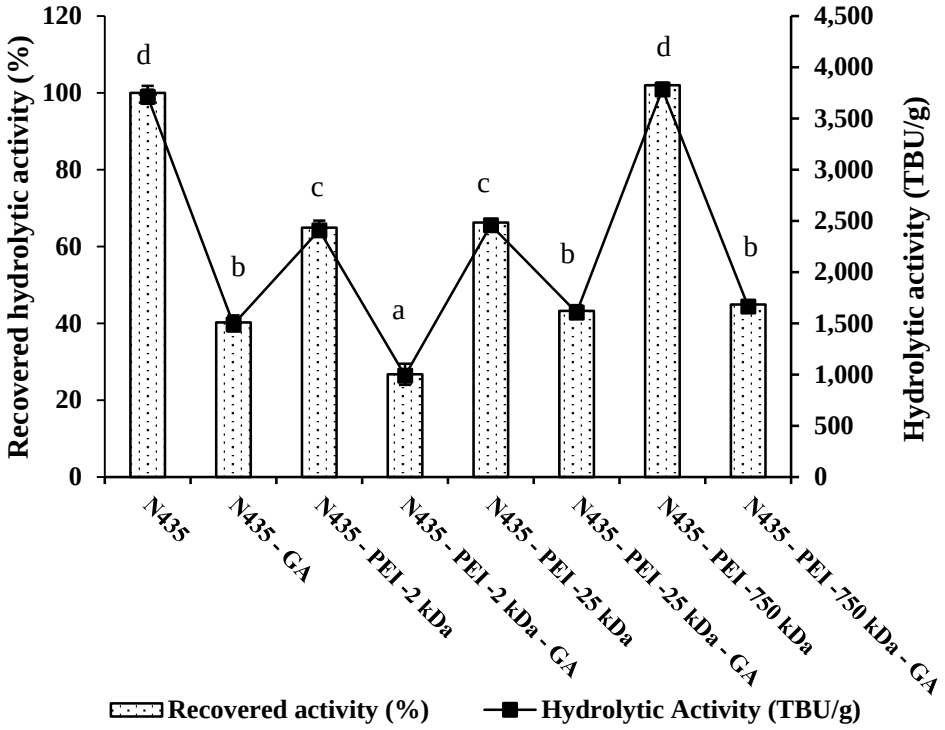

Figure 3. 3 - Effect of PEI and/or GA treatment on the hydrolytic activity of N435 biocatalysts (TBU/g). The indices a-d indicate mean differences according to Tukey's test (5% significance), with d representing the highest significance level. Hydrolytic activity was measured using a reaction mixture containing 1.5 mL tributyrin, 6 mL of 100 mM sodium phosphate buffer (pH 7.5), and 16.5 mL distilled water at 25 °C for 5 min. Experiments were performed as described in the Methods section.

Table 3. 1 - Summary of the effects of post-treatments with polyethylenimine (PEI) and glutaraldehyde (GA) on the hydrolytic activity of N435n435, and comparison with previous studies.

Treatment	Post-treatment effect on N435 hydrolytic activity (TBU)	Effect of treatment on previous work
GA	60% loss of activity	35% loss of activity of *Candida antarctica* lipase B immobilized on silica (SiO_2) nanoparticles and

		subsequently cross-linked with 0.01% GA (v/v) for 1 h [94]. 20% loss of activity of lipase from *Thermomyces lanuginosus* (TLL) immobilized on Octyl Sepharose CL-4B beads and cross-linked with 0.1% GA (v/v) for 1 h [79].
PEI-2 kDa	36% loss of activity	41% reduction in hydrolytic activity (TBU) of N435 coated with 10% (w/v) PEI (2 kDa) for 1 h [38].
PEI-2 kDa-GA	74% loss of activity	No previous reports describing the application of this post-treatment to biocatalysts were found.
PEI-25 kDa	34% loss of activity	20% loss of activity of lipase from *Rhizomucor miehei* immobilized on Octyl-agarose CL–4B beads and coated with 10% (w/v) PEI (25 kDa) for 8 h [74]. No change and 4% increase in activity of lipases from *Thermomyces lanuginosus* (TLL) and Lecitase Ultra immobilized on Octyl Sepharose CL-4B beads and coated with 10% (w/v) PEI (25 kDa) for 24 h, respectively [79]. 27% reduction in hydrolytic activity (TBU) of N435 coated with 10% (w/v) PEI (25 kDa) for 1 h [38].
PEI-25 kDa-GA	57% loss of activity	No previous reports describing the application of this post-treatment to biocatalysts were found.
PEI-750 kDa	9% increase in activity	43% reduction in hydrolytic activity (TBU) of N435 coated with 10%

		(w/v) PEI (750 kDa) for 1 h [38].
PEI-750 kDa-GA	56% loss of activity	No previous reports describing the application of this post-treatment to biocatalysts were found.

3.4.2 Stability in the presence of organic solvents.

Figure 3.4 shows the effect of different solvents (50 mM TRIS buffer, pH 7.0; MEK, TERT, and xylose-based NADES) on the activity of PEI-treated or/and GA-crosslinked biocatalysts after incubation at 60 °C for 6 hours.

The activity of untreated N435 and some biocatalysts treated with PEI and/or GA was reduced after incubation at 60 ºC in aqueous medium **(Figure 3.4a)** compared with the other solvents. In MEK **(Figure 3.4b)**, the biocatalysts modified with 2 and 25 kDa PEI exhibited lower activity than unmodified N435. On the other hand, treatment with 750 kDa PEI resulted in a 1.3-fold increase in activity after incubation at 60 ºC for 6 hours. Crosslinking these PEI-coated biocatalysts with GA further enhanced their recovered hydrolytic activity, increasing it by 1.32-, 1.47-, and 1.92-fold for the 2, 25, and 750 kDa PEI-treated biocatalysts, respectively.

In TERT **(Figure 3.4c)**, most biocatalysts treated with PEI, either alone or in combination with GA, maintained their initial activity. Notably, N435 treated with 25 kDa PEI showed increased activity after incubation at high temperature. In xylose-based NADES (Figure 3.4d), biocatalysts treated with PEI generally retained their initial activity. However, those modified with 25 and 750 kDa PEI and subsequently crosslinked with GA enhanced activity after 6 hours at 60 ºC. These findings demonstrate that each solvent interacts differently with biocatalyst, leading to distinct effects on enzymatic activity.

In thermostability tests conducted in organic media, the absolute hydrolytic activity of PEI-treated biocatalysts either remained unchanged or even increased by the end of the test. This behavior can be attributed to the hydrophilic microenvironment created by the PEI layer, which acts as a barrier to solvent penetration [56]. This protective effect is particularly relevant in the presence of hydrophilic organic solvents, which are known to damage enzymes by disrupting their solvation layer [109,110]. Conversely, the glutaraldehyde treatment, which involves covalent binding to the enzyme and induces molecular rigidification and structural alterations, resulted in a decrease in hydrolytic activity (solid line) and increase in residual activity (bars) [75,88–90].

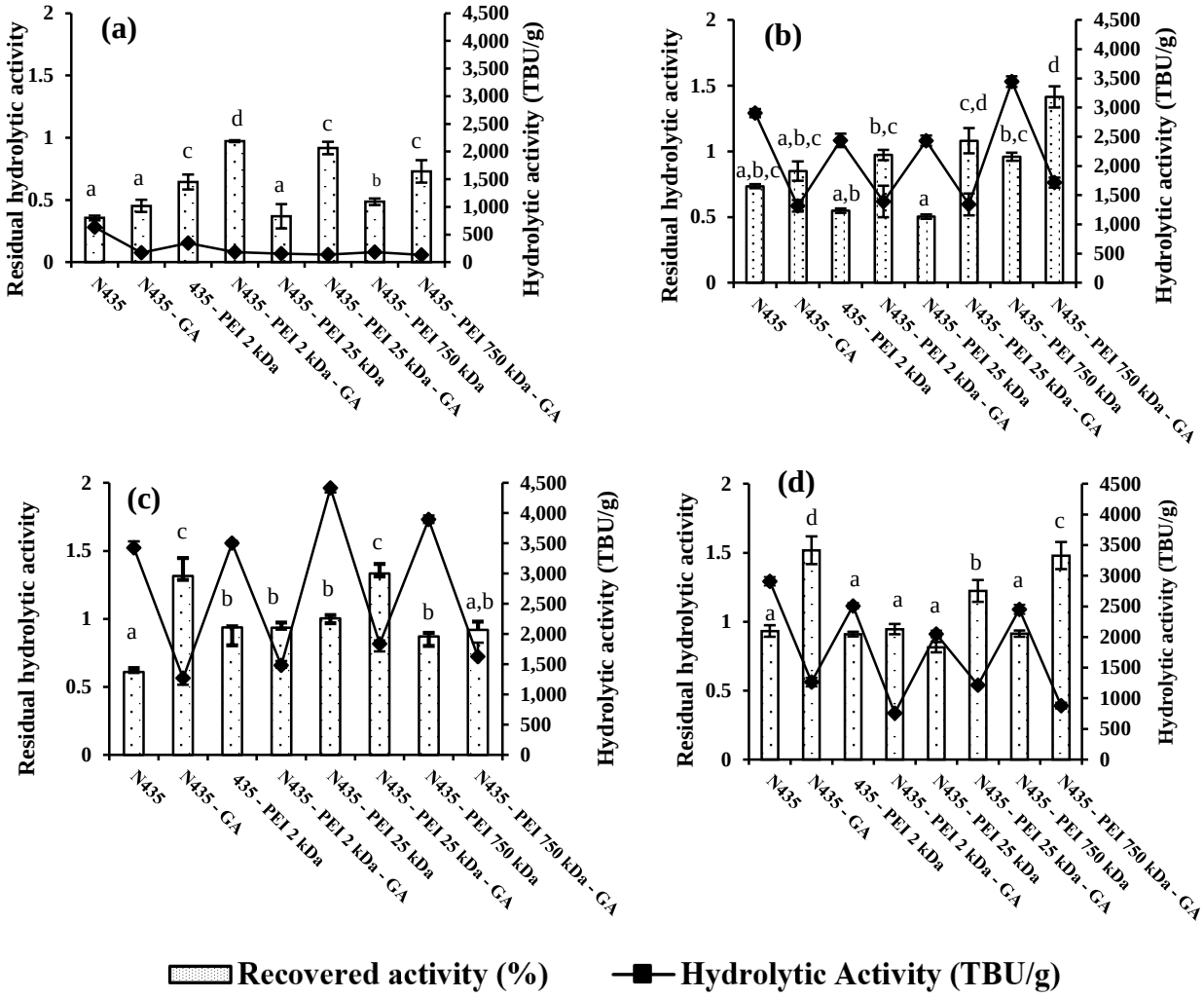

Figure 3. 4 - Influence of different solvents on the activity of biocatalysts modified with PEI and/or GA after incubation at 60°C for 6 h: (a) 50 mM TRIS buffer (pH 7.0), (b) MEK, (c) TERT, and (d) xylose-based NADES. The indices indexes a-d indicate mean differences according to Tukey's test (5% significance), with d representing the highest significance level. Experimental conditions: incubation of 0.5% (w/v) biocatalyst in the respective solvent at 60 °C for 6 h. Experiments were performed as described in Methods section.

3.4.3. Stability in detergent-containing medium and enzyme leaching

The stability of biocatalysts modified with PEI and/or GA in the presence of Triton X-100 was evaluated by measuring both residual enzymatic activity and desorption of CALB molecules from the support, as shown in **Figures 3.5** and **3.6,** respectively.

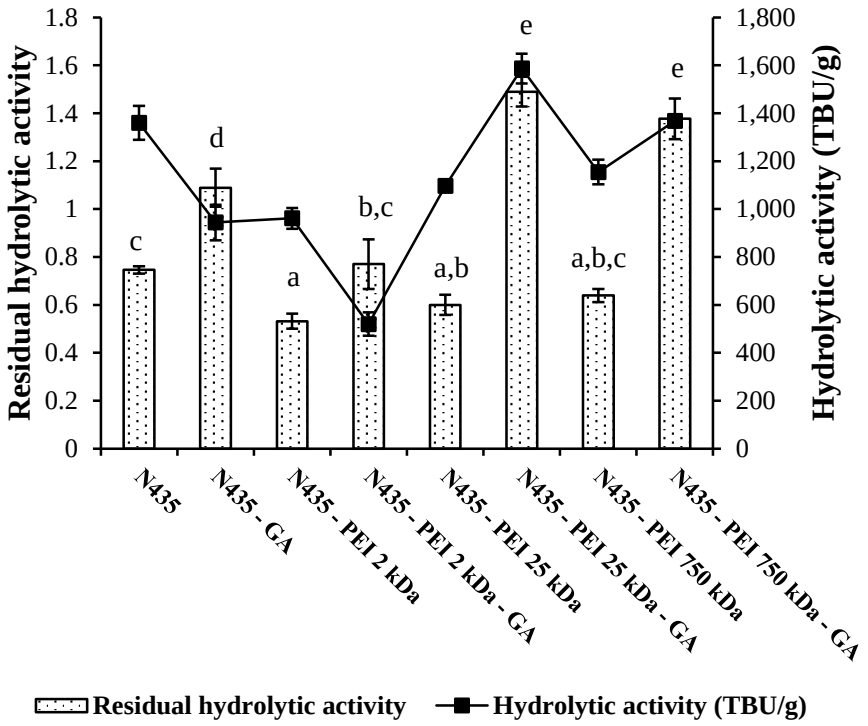

Figure 3. 5 - Effect of Triton X-100 on the activity of biocatalysts modified with PEI and/or GA. The indices a-d indicate mean differences according to Tukey's test (5% significance), with d representing the highest significance level. Experimental conditions: 10 mg of biocatalyst incubated in 1.5 mL of 1% (v/v) Triton X-100 prepared in 5 mM sodium phosphate (pH 7.0) for 1 h. Experiments were performed as described in Methods section.

It is worth noting that after 1 h of incubation, the biocatalysts were recovered by filtration and thoroughly washed before determining the residual activity, ensuring that any desorbed enzyme was removed prior to the assay. The unmodified N435 showed an approximately 30% reduction in enzymatic activity after 1 h of incubation **(Figure 3.5)**, which can be attributed to enzyme desorption from the support **(Figure 3.6)**.

Treatment of N435 with GA helped preserve enzymatic activity and significantly reduced CALB leaching from the support, suggesting the formation of intermolecular crosslinking. On the other hand, biocatalysts modified only with PEI, regardless of polymer molecular weight, exhibited remarkable enzyme desorption, resulting in about 40% loss of activity. These results indicate that the ionic interactions between PEI and the immobilized enzyme do not involve all enzyme molecules. Sequential modification with PEI followed by crosslinking with GA led to improved performance. Although the biocatalyst modified with 2 kDa PEI and GA exhibited reduced enzyme stability, enzyme desorption was minimal. Even with desorption prevented, the molecular rigidity provided by crosslinking the polymeric layer

was insufficient to inhibit molecular vibration at high temperatures, thereby disrupting the 3D structure Furthermore, biocatalysts treated with 25 or 750 kDa PEI followed by GA treatment exhibited enhanced stability without detectable protein release from support. This finding suggests that proper and extensive intermolecular crosslinking of most enzyme molecules was achieved by this protocol. The ability of the proposed PEI/GA post-treatment to prevent enzymatic desorption was further confirmed after 24 hours of incubation in detergent-containing media **(Figure A3.1-A3.2)**.

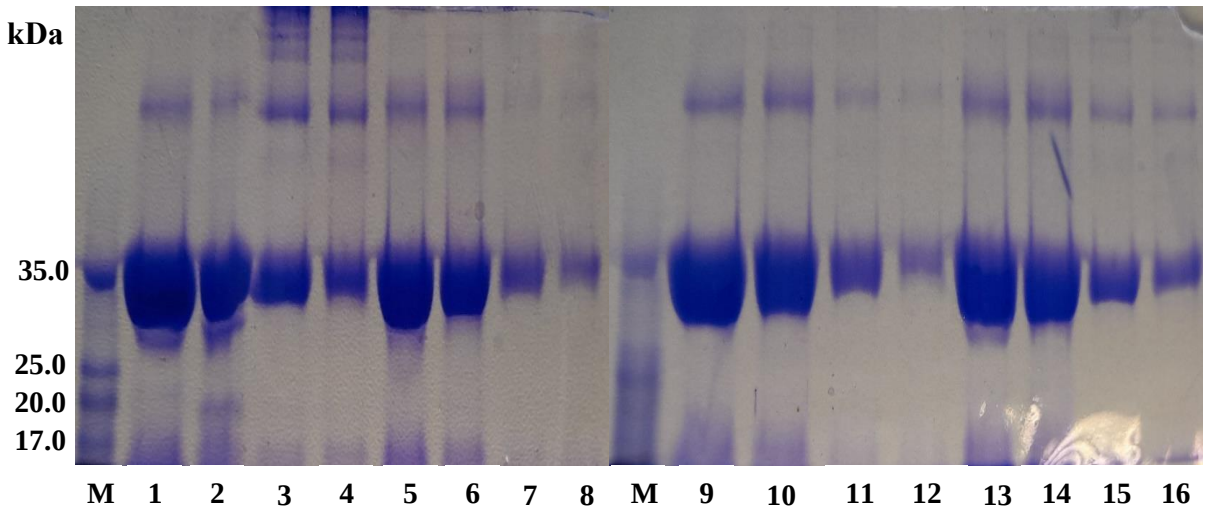

Figure 3. 6 - SDS-PAGE analysis of different N435 biocatalysts before and after incubation in detergent (see legend of Figure 3.5) Line M: molecular marker; Line 1: N435 without surfactant incubation; Line 2: N435 with incubation in surfactant; Line 3: N435-GA without incubation in surfactant; Line 4: N435-GA with incubation in surfactant; Line 5: N435-PEI2 without incubation in surfactant; Line 6: N435-PEI2 with incubation in surfactant; Line 7: N435-PEI2-GA without incubation in surfactant; Line 8: N435-PEI2-GA with incubation in surfactant; Line 9: N435-PEI25 without incubation in surfactant; Line 10: N435-PEI25 with incubation in surfactant; Line 11: N435-PEI25-GA without incubation in surfactant; Line 12: N435-PEI25-GA with incubation in surfactant; Line 13: N435-PEI750 without incubation in surfactant; Line 14: N435-PEI750 with incubation in surfactant; Line 15: N435-PEI750-GA without incubation in surfactant; Line 16: N435-PEI750-GA with incubation in surfactant. Other specifications are described in Methods section.

3.4.4. Oleic acid conversion profiles during the synthesis of xylose esters

The progress of oleic acid consumption during the esterification reaction between oleic acid and xylose was compared using untreated N435 and N435 coated with 750 kDa PEI and crosslinked with glutaraldehyde (N435-PEI750–GA) **(Figure 3.7)**.

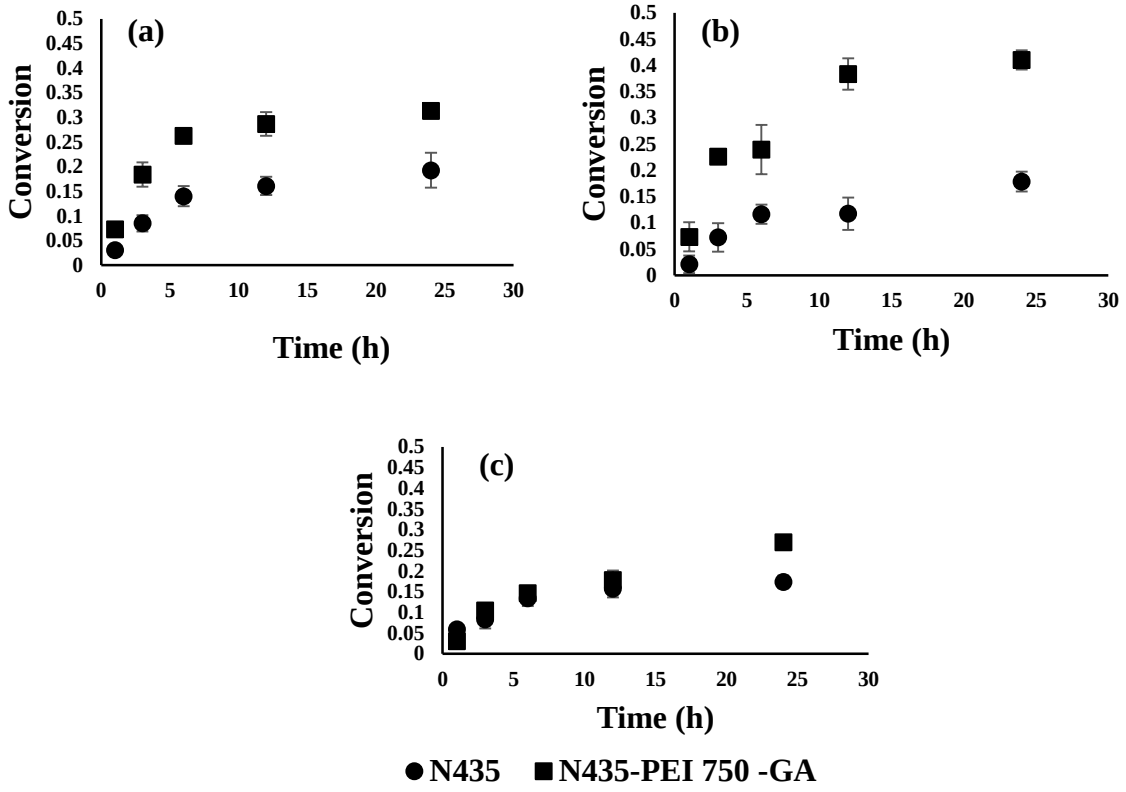

Figure 3. 7 - Oleic acid conversion profiles during the synthesis of xylose oleate catalyzed by untreated N435 and N435-PEI 750-GA using: (a) TERT, (b) MEK, and (c) xylose-based NADES. Circles represent untreated N435, and squares represent N435 treated with 750 kDa PEI and GA. Reaction conditions: xylose:oleic acid molar ratio of 1:5, enzyme load of 500 TBU/g xylose, 60 °C, 550 rpm.

The N435-PEI750–GA biocatalyst was selected for this stage because it exhibited superior thermal stability in the unconventional media used in the reaction (MEK, TERT, and xylose-based NADES), as well as operational stability in media containing non-ionic surfactant. This indicates that the product formed does not compromise its catalytic activity throughout the process.

The initial rate of oleic acid consumption was higher for N435-PEI750-GA than for untreated N435 when using TERT **(Figure 3.7a)** and MEK **(Figure 3.7b)**. However, no significant difference was observed between the two biocatalysts when xylose-based NADES was used **(Figure 3.7c)**. After 24 hours of reaction in TERT or xylose-based NADES, oleic acid consumption catalyzed by N435-PEI750-GA increased by approximately 1.6-fold compared with untreated N435. In MEK, this increase was even more pronounced, reaching a 2.3-fold difference.

Notably, the N435-PEI750-GA biocatalyst consistently promoted higher oleic acid conversion across all solvents tested. This improvement is likely attributed to the enhanced stability of the modified biocatalyst in unconventional reaction media and in the presence of the target product, a non-ionic surfactant.

Among the solvents, xylose-based NADES stands out as a promising and environmentally friendly medium. It can be synthesized from low-cost raw materials and provides excellent solubility for sugars, enhancing its solubility for biocatalytic applications [48,49,53].

Assuming that all xylose molecules were esterified to form only monoesters, the maximum theoretical conversion of oleic acid would be approximately 20%, given the 5:1 molar ratio of oleic acid to xylose. Complete peracylation of xylose, on the other hand, would result in up to 80% oleic acid conversion. Experimentally, untreated N435 achieved around 20% oleic acid conversion in MEK, TERT, and xylose-based NADES. In contrast, N435-PEI750-GA reached approximately 30% oleic acid conversion in TERT and xylose-based NADES, and 40% in MEK. These results suggest that more than one hydroxyl group of xylose was esterified, and that the products likely consisted of a mixture of mono-, di-, and triesters.

Product samples obtained from all three solvents were analyzed by TLC. As shown in **Figure A3.3**, bands corresponding to ester products were observed in the reaction mixtures but not in the enzyme-free controls (data not shown). Although the number and intensity of the bands varied depending on the solvent, no qualitative differences were observed between N435 and N435-PEI 750-GA, suggesting that xylose oleate was formed in all three systems. Given the possible variations in the degree of acylation, further product characterization was performed.

3.4.5. Characterization of the final product

The product was purified via preparative chromatography, and the eluent containing the separated components was monitored by TLC. **Figure A3.4** shows the TLC plates with the bands corresponding to xylose esters and oleic acid eluted during chromatography.

To confirm the presence of the product in the purified samples and to determine the degree of acylation, mass spectrometry analysis was performed **(Figures A3.5-A3.14)**. In reaction media containing xylose-based NADES, TERT, and MEK, the mass spectra of the $[M\text{-}H]^-$ ions with m/z values of 413.29, 677.54, and 941.78 confirmed the synthesis of xylose mono-, di-, and trioleates catalyzed by N435-PEI750-GA. Additionally, a mass spectra of the $[M\text{-}H]^-$ ions with m/z value of 367.35, corresponding to a choline ester, was detectable when

xylose-based NADES was used as the solvent. Buzatu *et al*. (2024) [44] previously reported the formation choline esters during the synthesis of glucose esters using glucose-based NADES as a solvent. Other studies using sugar-based NADES have also demonstrated the production of sugar esters using this unconventional medium **(Figure 3.8)** [43,47,49,111].

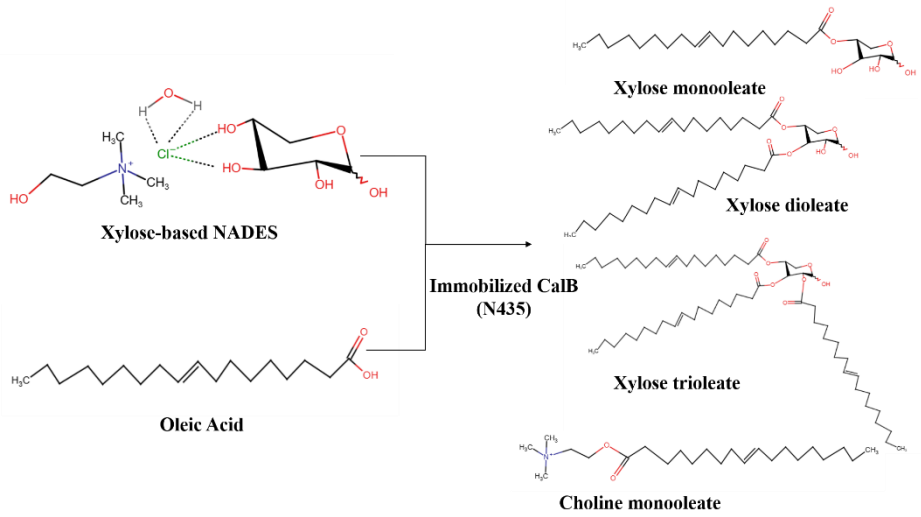

Figure 3.8 - Proposed pathways for the lipase-catalyzed esterification of a xylose-based NADES, leading to the formation of xylose oleate (mono-, di-, or tri-) and/or choline monooleate.

Previous studies using MEK as a solvent have shown that N435 can catalyze the formation of mono-, di-, and tri-esters [29,37,112]. In contrast, studies employing TERT as a solvent have emphasized the predominant production of monoesters [68,113,114].

The emulsification index (EI) of the reaction mixtures catalyzed by N435 and N435-PEI750-GA was also determined, and the results are presented in **Table 2**.

Table 3. 2 - Emulsification indices (EI) of the nonpolar phase in MEK-, TERT-, and xylose-based NADES after 24 hours of reaction, catalyzed by N435 and N435-PEI750-GA. The unreacted nonpolar phase (i.e., oleic acid) exhibited an EI of 0%.

Solvents	N435 (%)	N435-PEI 750 -GA (%)
MEK	3.19[a]	14.68[a]
TERT	44.70[b]	45.24[b]
Xylose-based NADES	3.22[a]	16.14[a]

* The letters *a* and *b* indicate statistically differences according to Tukey's test ($p \leq 0.05$); *b* corresponds to the highest significance index.

When MEK and xylose-based NADES were used as solvents, the EI of the reaction catalyzed by N435-PEI750-GA was approximately five times higher than that of N435. On the other hand, no significant difference was observed with TERT. Among the three solvents, the product obtained in TERT exhibited the highest EI compared to MEK and xylose-based NADES. These results highlight the potential of the xylose esters produced as surfactants, with some EI values surpassing that of the commercial surfactant sucrose monolaurate (EI = 12%). Furthermore, the choice of solvent was found to influence the EI in the synthesis reaction.

3.4.6. Biocatalyst operational stability in the synthesis of xylose oleates

The reusability of the N435 and N435-PEI 750-GA biocatalysts was assessed in the synthesis of xylose esters over five consecutive 6-hour cycles at 60 ºC **(Figure 3.9)**. Six-hour cycles were selected because conversion remained essentially constant during experiments up to 24 hours **(Figure 3.7)**.

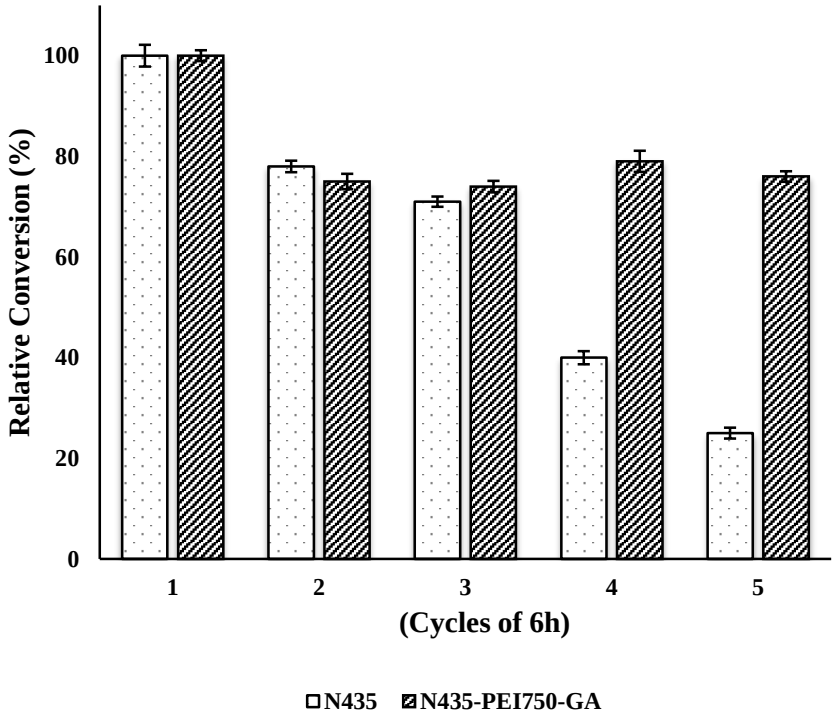

Figure 3. 9 - Operational stability of different N435 biocatalysts in the synthesis of xylose oleate. Each reaction cycle was carried out using a xylose:oleic acid molar ratio of 1:5, an enzyme load of 500 BTU/g xylose, in xylose-based NADES solvent at 60 °C and stirring at 550 rpm for 6-hour cycles.

N435-PEI750-GA retained 75% of its initial activity after five cycles, whereas

untreated N435 retained only 21%. These results demonstrate that the proposed treatments effectively stabilized the biocatalyst, preventing enzyme desorption under challenging conditions, including high temperature, organic solvents, and surfactant-like products and reagents. A simple strategy to mitigate the decline in activity of the treated biocatalyst could be to reduce the reaction temperature.

The capacity of Rose Bengal adsorption of the biocatalysts was also evaluated after five 6-hour reuse cycles. The untreated N435 showed a 1.8-fold increase in Rose Bengal adsorption compared with the initial biocatalyst, likely due to enzyme desorption exposing hydrophobic groups on the support or adsorption of oleic acid. On the other hand, the N435-PEI750-GA catalyst showed no change in dye adsorption after the five cycles, indicating that the proposed treatments effectively protected the catalyst surface under the reaction conditions used.

The findings of our work demonstrate that both the modified biocatalyst and the synthesis of xylose esters in a xylose-based NADES, used simultaneously as solvent and substrate, present promising potential for future studies focusing on process scale-up and different reactor configurations. However, a comprehensive assessment of technical, economic, and environmental feasibility is essential to provide insights into their potential advantages over the commercial biocatalyst Novozym® 435.

3.4. CONCLUSIONS

This study demonstrates the feasibility of using a modified immobilized enzyme, coated with a cationic polymer and subsequent crosslinked, for the synthesis of xylose oleate in unconventional media. Coating the Novozym® 435 biocatalyst with PEI and further covalent crosslinking with glutaraldehyde significantly enhanced its stability in reaction media containing solvents such as MEK, TERT, and xylose-based NADES. Additionally, these modifications reduced enzyme desorption in the presence of the nonionic surfactant Triton-X, leading to a substantial improvement in the conversion of oleic acid to xylose oleate. The treated biocatalyst exhibited higher activity retention after five 6-hour cycles compared to the untreated commercial biocatalyst, which can be attributed to the prevention of enzyme leaching in the presence of the surfactant product xylose oleate. The product reaction products obtained in all the media consisted of a mixture of xylose mono-, di-, and trioleates. Among the solvents tested, xylose-based NADES stood out as a promising environmentally friendly medium, derived from low-cost raw materials, offering excellent solubility for sugars and expanding its potential for biocatalytic processes. These results underscore the promising role of xylose, the main component of hemicellulose, not only as a reagent but also as a constituent of the reaction medium, opening possibilities for its use in other biocatalytic systems. The resulting reaction mixtures containing xylose oleates exhibited superior emulsifying properties compared to the commercial surfactant sucrose monolaurate.

3.5. REFERENCES

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APPENDIX

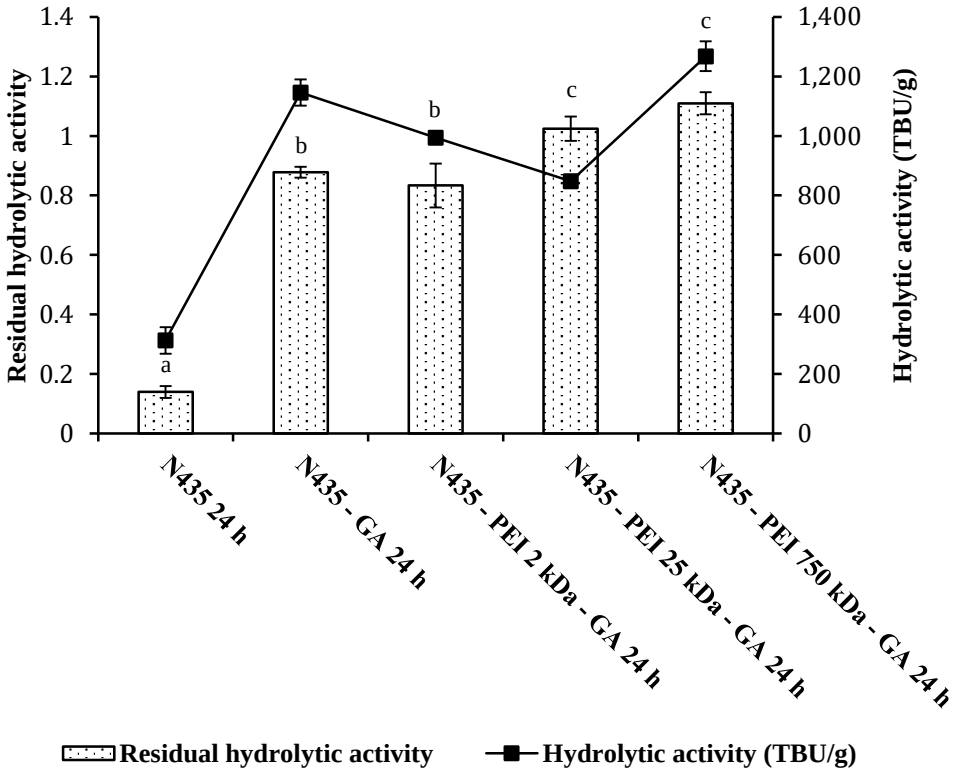

Figure A3. 1 - Effect of Triton X-100 on the activity of biocatalysts modified with PEI and/or GA. The terms a-c represent the coefficients assigned by Tukey's test, and differences between them indicate statistically significant differences at the 95% confidence level. Experimental conditions: 10 mg of biocatalyst incubated in 1.5 mL of 1% (v/v) Triton X-100 prepared in 5 mM phosphate buffer (pH 7.0) after 24 hours of incubation. Experiments were performed as described in Methods section.

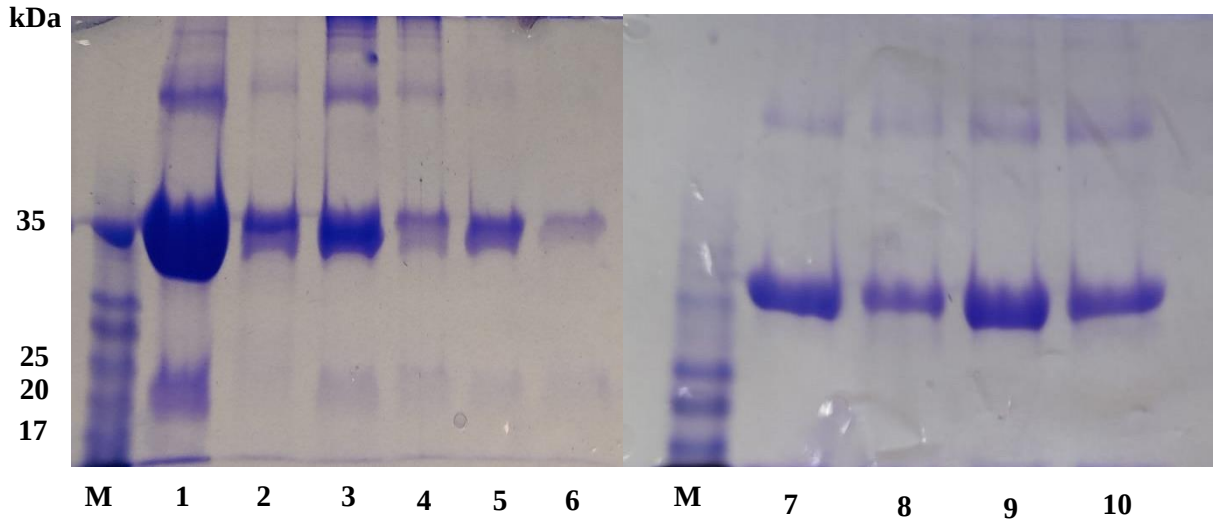

Figure A3. 2 - SDS-PAGE analysis of different N435 biocatalysts. Line M: molecular marker; Line 1: N435 without surfactant incubation; Line 2: N435 with incubation in surfactant; Line 3: N435-GA without incubation in surfactant; Line 4: N435-GA with incubation in surfactant; Line 5: N435-PEI2-GA without incubation in surfactant; Line 6: N435-PEI2-GA with incubation in surfactant; Line 7: N435-PEI25-GA without incubation in surfactant; Line 8: N435-PEI25-GA with incubation in surfactant; Line 9: N435-PEI750-GA without incubation in surfactant; Line 10: N435-PEI750-GA with incubation in surfactant. Other specifications are described in Methods.

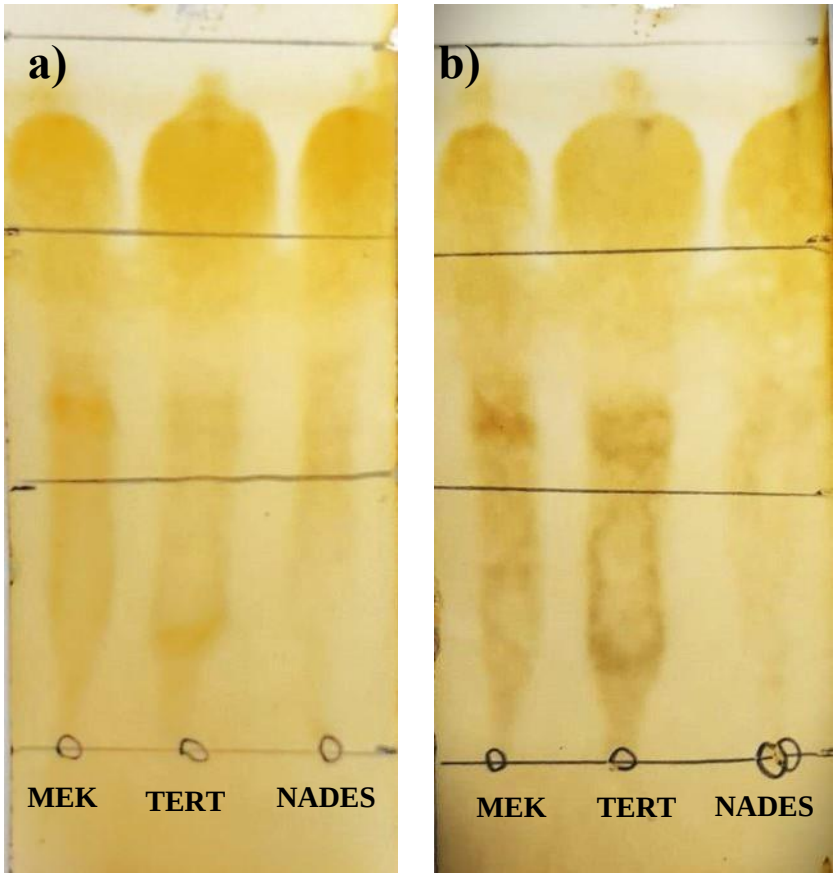

Figure A3. 3 - TLC of Nonpolar phase of the reaction medium after 24 hours of reaction. (a) reactions using N435 without treatment, (b) reactions using N435-PEI750-GA.

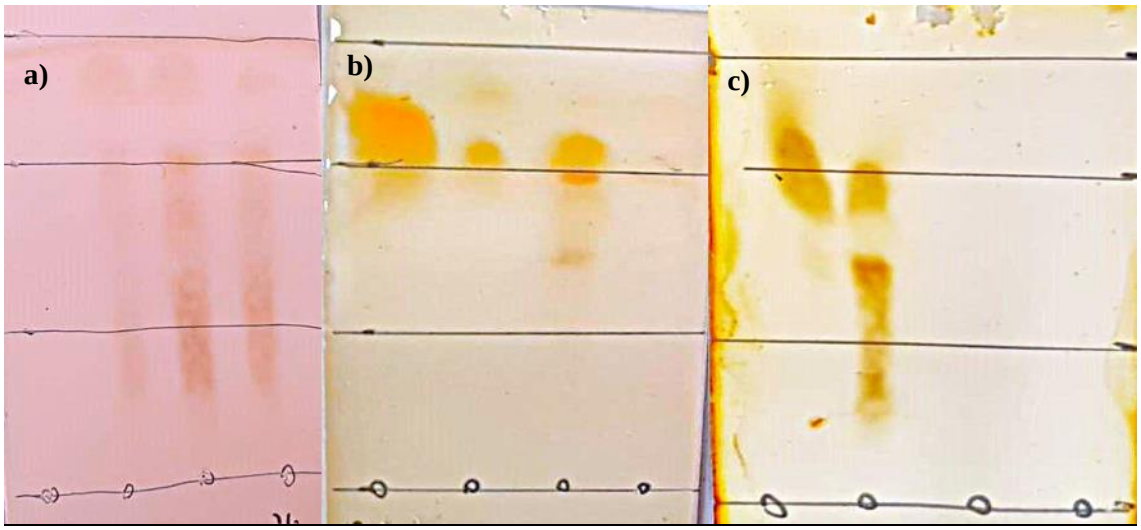

Figure A3. 4 - TLC of fractions collected during chromatography containing product bands. (a) chromatography of medium containing xylose-based NADES. (b) chromatography of medium containing MEK. (C) chromatography of medium containing TERK.

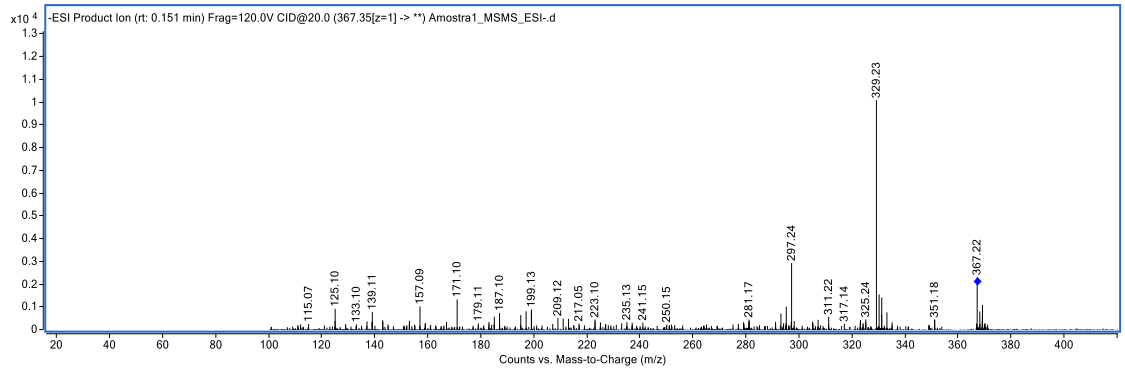

Figure A3. 5 - Mass spectrum of choline monooleate with m/z = 367.35 produced using the xylose-based NADES-containing medium.

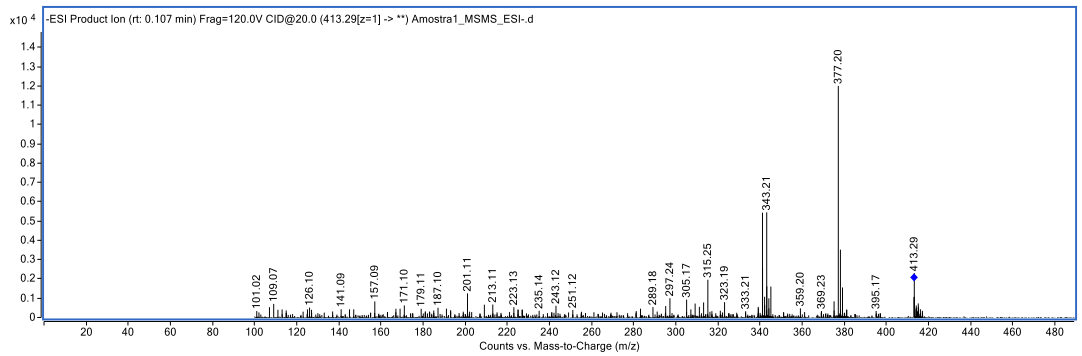

Figure A3. 6 - Mass spectrum of xylose monooleate with m/z = 413.29 produced using the xylose-based NADES-containing medium.

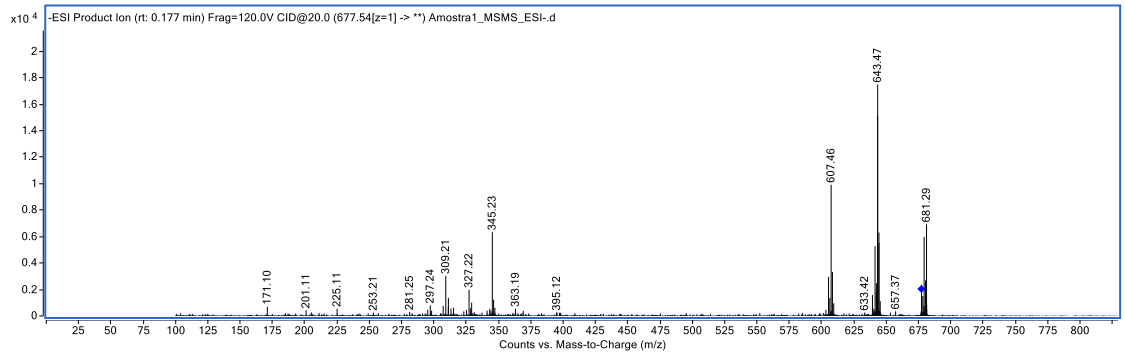

Figure A3. 7 - Mass spectrum of xylose dioleate with m/z = 677.54 produced using the xylose-based NADES-containing medium.

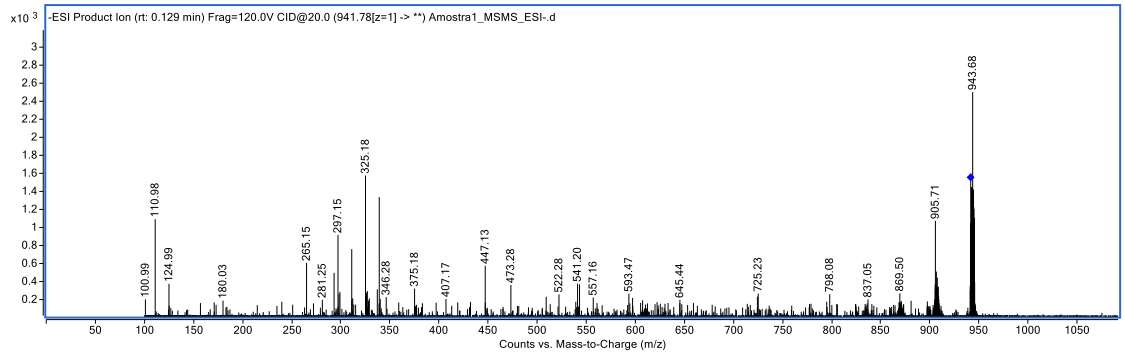

Figure A3. 8 - Mass spectrum of xylose trioleate with m/z = 941.78 produced using the xylose-based NADES-containing medium.

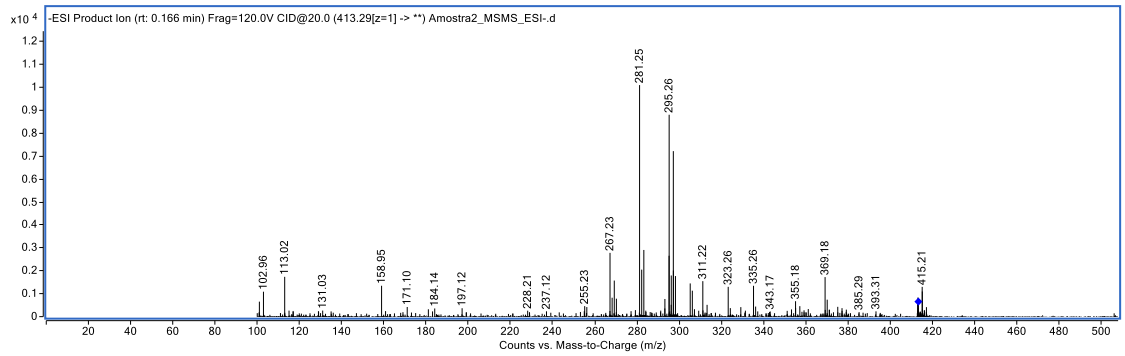

Figure A3. 9 - Mass spectrum of xylose monooleate with m/z = 413.29 produced using the TERC-containing medium.

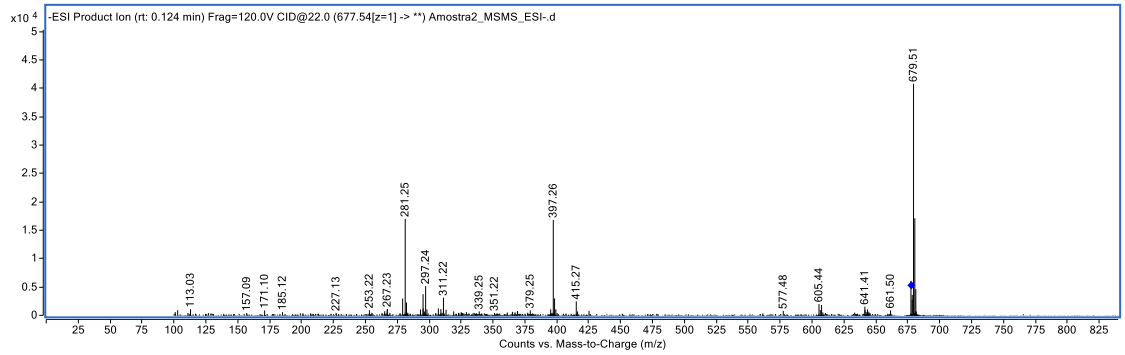

Figure A3. 10 - Mass spectrum of xylose dioleate with m/z = 677.54 produced using the TERC-containing medium.

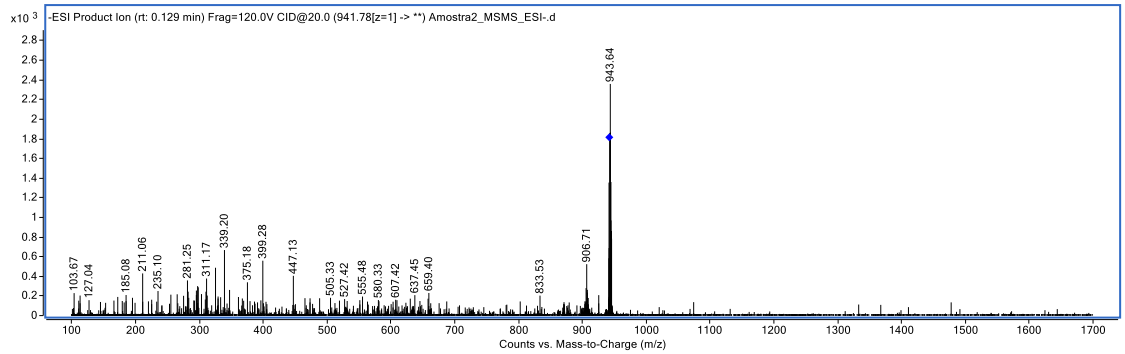

Figure A3. 11 - Mass spectrum of xylose trioleate with m/z = 941.78 produced using the TERC-containing medium.

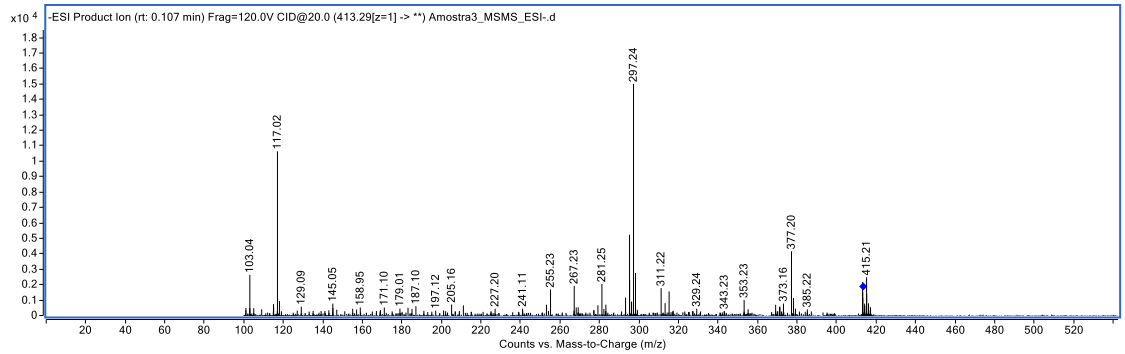

Figure A3. 12 - Mass spectrum of xylose monooleate with m/z = 413.29 produced using the MEK-containing medium.

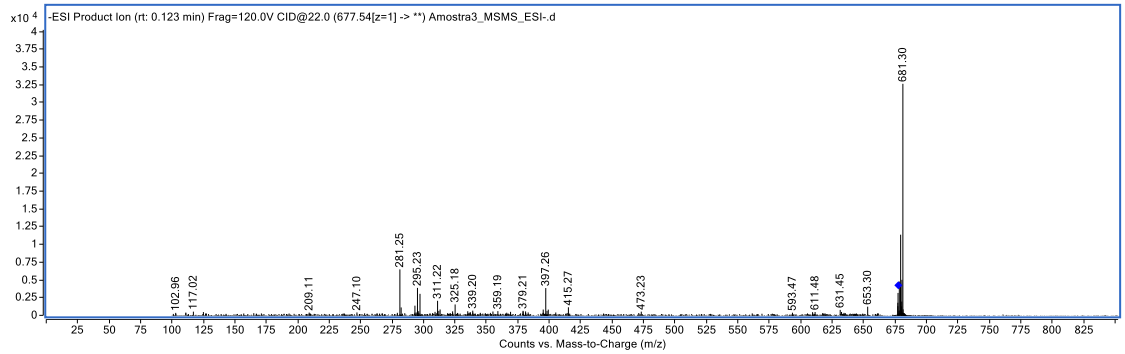

Figure A3. 13 - Mass spectrum of xylose dioleate with m/z = 677.54 produced using the MEK-containing medium.

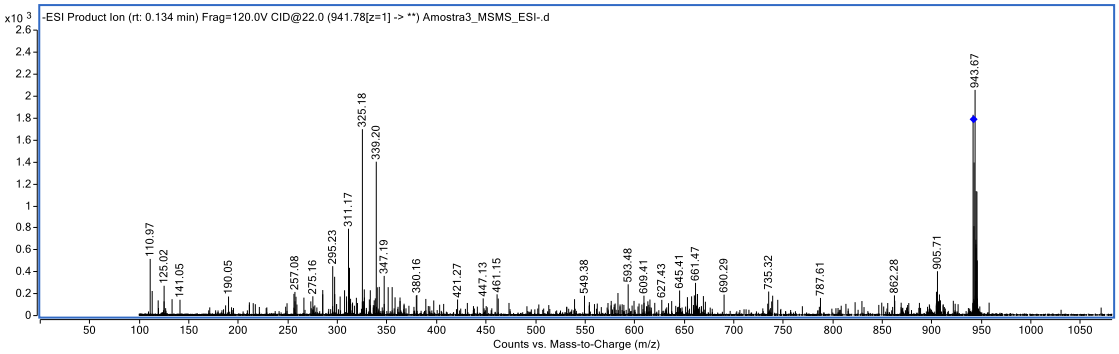

Figure A3. 14 - Mass spectrum of xylose trioleate with m/z = 941.78 produced using the MEK-containing medium.

CHAPTER IV

THE STRUCTURE OF THE IMMOBILIZED EVERSA TRANSFORM 2.0 DETERMINES THE ACTIVITY/STABILITY EFFECTS OF THE BIOCATALYST METALLIZATION

In this chapter, the human designed lipase Eversa Transform 2.0 (ETL) has been immobilized on octyl agarose beads using 4 previously published protocols that provided biocatalysts with very different properties. Then, the biocatalysts were submitted to incubation with 7 different metal cations in TRIS or buffer, with the objective of checking if the immobilized enzyme altered its properties after metallization and whether this modification has different qualitative and quantitative values when changing the immobilized enzyme protocol (that is, maintaining enzyme, support and enzyme orientation, only changing the enzyme structure). Enzyme activity versus nitro-phenol butyrate at different pH values using different buffers, enzyme activities versus this substrate and triacetin and *R* or *S* methyl mandelate and the enzyme stability under different conditions were studied. The results showed that the enzyme activity/ pH curve and specificity versus different substrates are drastically changed upon metallization, these changes depending on the presence of TRIS or phosphate during mineralization and very interestingly, depending on the biocatalyst that is submitted to this treatment. The same treatment could increase enzyme activity or stability for one biocatalyst while it could be negative for other biocatalysts.

4.1. INTRODUCTION

Enzyme immobilization is an important step in the preparation of industrial enzyme biocatalysts [1,2]. This is an opportunity to tune the final enzyme features, as a proper immobilization system can increase enzyme stability due to different reasons [3,4,13–18,5–12]. Moreover, enzyme immobilization may alter enzyme conformation (in fact, it is almost impossible to prevent some random enzyme distortion caused by the enzyme-support interactions) [19] and, thus, different activity, specificity, selectivity and resistance to chemicals and inhibitors may be achieved by using different immobilization protocols [20]. Recently the enzyme immobilization techniques have been proposed to be divided in three categories: the enzymes self-attached to the cells *via* specific binding domains, their immobilization on pre-existing supports and the generation of *ex-novo* solids [21]. In the last category, nanoflowers stand up. In these nanoflowers, the enzymes become incorporated to the crystalline structure of the metal salts (usually phosphate), which in some instances acquire the form of rose petals (from where the name comes) [22–25]. This immobilization strategy permits, in many instances, to modulate enzyme stability and/or activity, and, in this sense, becomes a very interesting immobilization strategy [22–29]. However, the primary objective of enzyme immobilization is to facilitate enzyme recovery [1,2] and nanoflowers have two main problems: their nano-size makes it difficult to recover them and their mechanical rigidity causes them to break easily upon handling. This way, nanoflowers recovery in large (over 1000 L) reactors becomes a cumbersome task. Some authors have proposed to trap the nanoflowers in macro-porous entities with better mechanical properties (e.g., calcium alginate beads) or to bind them to magnetic nanoparticles, to facilitate their management [30,31,40,32–39]. Other authors recently proposed the possibility of taking advantage of the favorable effects of enzyme metallization in order to avoid these problems is the incubation in solutions of metal phosphate of previously immobilized enzymes, using pre-existing supports with the desired mechanical properties. Moreover, this strategy permits to couple the benefits of enzyme immobilization on preexisting supports with those of the nanoflowers. This strategy has been exemplified with some lipases and has permitted to improve the activities and/or stabilities of immobilized enzymes [41–43]. The researchers have shown that the use of the same immobilization technique but producing different final physical features of the support surface alters the effects of the mineralization on the features of the immobilized enzymes [44]. In this instance, this different effect of the enzyme mineralization on enzyme properties may be caused by the fact that the support surface is different or because the immobilized enzyme molecules presented

different structures on the different support surfaces. In fact, it has been shown how the different blocking agents produced different lipases structures [45,46].

In this new research effort, we intend to investigate if the mineralization of the same enzyme immobilized on exactly the same support with the same orientation but bearing different conformations may produce different results or the mineralization effects are independent from the immobilized enzyme conformation. That is, the objective is to analyze if the immobilized enzyme conformation is relevant to determine the effects of the mineralization on enzyme activity, specificity or stability. This possibility has been pointed by some researchers immobilizing the same free enzyme bearing different conformations using the nanoflowers strategy [47].

To reach this goal, we have utilized the artificial lipase called Eversa Transform 2.0 (ETL), designed by Novozym as an evolution from the lipase from *Thermomyces lanuginosus*, to be used in the synthesis of biodiesel in its free form [48–50]. However, it has been showed that its adequate immobilization greatly improves their features (stability, activity) enabling to expand their applications range [51–57]. Lipases present a catalytic mechanism called interfacial activation that enables them to act on the surface of drops of insoluble triglycerides (its natural substrate) [58–62]; similarly lipases can become immobilized on any hydrophobic support [63]. This immobilization technique also enables to purify (only lipases are fully immobilized on these supports at low ionic strength or even in the presence of moderate concentrations of solvents), stabilize (the open and absorbed open form of the lipase is more stable than the enzyme in conformational equilibrium) and hyperactivate (as the open form of the lipase is fixed) lipases, and all this based on a reversible immobilization protocol that enables the inactivated enzyme release and reuse of the support afterwards [64].

Moreover, it has been shown that the immobilization of some lipases on hydrophobic supports via interfacial activation under different conditions alters their features (activity, specificity and stability) [65,66], and this has been correlated to the existence of different immobilized enzyme conformations [67]. Eversa Transform 2.0 has been one of the enzymes where these different properties depending on the immobilization conditions have been found [68]. In this new research effort, we have immobilized ETL on octyl agarose under conditions where, following the previous results, the changes in enzyme activity/stability suggested different enzyme conformations: 50 mM sodium acetate pH 5 plus 100 mM NaCl, 50 mM sodium acetate pH 5, 50 mM sodium bicarbonate buffer pH 9 and TRIS buffer pH 7 plus 10% dioxane. An enzyme loading high enough to saturate the support surface with enzyme molecules (that is, using an excess of enzyme) was utilized to facilitate the mineralization of

the immobilized enzymes using many different metals (calcium, nickel, cobalt, copper, manganese, zinc and magnesium) and phosphate. This way, if the effects of these biocatalysts mineralization were similar for the 4 different biocatalysts, the effect of the enzyme conformation on the modification of the enzyme features by mineralization can be considered negligible, while if the effects of the mineralization greatly differed, it can be considered that the immobilized enzyme conformation is a critical feature to determine the effects of the mineralization.

4.2. MATERIALS AND METHODS

4.2.1 Materials

4B-CL octyl-Sepharose beads (octyl agarose beads), triacetin and *p*-nitrophenyl butyrate (*p*NPB) were purchased from GE Healthcare-Spain. Novozymes Spain kindly gifted Eversa Transform 2.0 (ETL), a liquid lipase preparation containing approximately 41 mg of protein per mL following Bradford method [69]. $CoCl_2$, $CuCl_2$, $ZnCl_2$, $NiCl_2$, $MgCl_2$, $MnCl_2$ and $CaCl_2$ were purchased from Sigma-Aldrich-Spain. All other chemicals used and solvents were of analytical grade.

4.2.2. Methods

4.2.2.1. Determination of enzyme activities

The results are supplied as averages and standard errors after performing the experiments at least 4 times.

4.2.2.1.1. Hydrolysis of *p*NPB

The absorbance increase at 348 nm due to *p*-nitrophenol (*p*NP) release was recorded using a Jasco V-730 spectrophotometer [70]. For each reaction, 50 μL of 20 mM *p*NPB in acetonitrile was added to 2.5 mL of 25 mM sodium phosphate at pH 7.0 and 25°C. The reaction started by adding 50 μL of enzyme solution or suspension and conducted under magnetic stirring and temperature control for 90 seconds. In some instances, the pH value in the measurement solution was changed using different 25 mM buffers: sodium acetate (pH 4-6), sodium phosphate (pH 6-8), or sodium bicarbonate (pH 8-9). Activity is expressed as micromoles of *p*NP produced per minute.

4.2.2.1.2. Hydrolysis of triacetin

A solution of 50 mM sodium acetate containing 50 mM triacetin was prepared, with the pH adjusted to 5. To initialize the reaction, immobilized enzyme was added to reach a final concentration of 0.42% w/v. The suspension was stirred continuously in a roller mixer (Tube Roller MXT6S, Scilogex, CT) at a controlled temperature of 25°C. The degree of conversion was quantified using a Waters 486 HPLC (Waters, Millford, MA, USA), with detection at 230 nm utilizing a Kromasil C18 column (15 cm × 0.46 cm, EKA Chemicals AB, Bohus, Sweden). The mobile phase consisted of 15% acetonitrile/85% Milli-Q water (v/v), operating at a flow rate of 1 mL/min. At pH 5, the produced 1,2-diacetin did not suffer acyl migration [71]. The

retention times were 18 minutes for triacetin and 4 minutes for 1,2-diacetin. Initial reaction rates were determined based on maximal triacetin conversions of 15% to 20%. Activity is given as micromoles of produced diacetin per minute.

4.2.2.2. Immobilization of ETL on octyl agarose beads

Octyl-agarose beads were employed to immobilize ETL through interfacial activation [63], offering 25 mg of enzyme per gram of support (exceeding the loading capacity of the support). Initially, the commercial enzyme solution was diluted in the appropriate volumes of the four different immobilization buffers described in the Introduction section [68]: 50 mM sodium acetate at pH 5, 50 mM sodium acetate /100 mM NaCl at pH 5, 50 mM TRIS-HCl plus 10% dioxane at pH 7, and 50 mM sodium bicarbonate at pH 9. Next, 10 g of support was added to 100 mL of the enzyme solution, and the mixture was continuously stirred at 25°C using a roller mixer. To track the immobilization process, we measured the enzymatic activities of a solution of the enzyme incubated under the immobilization conditions as a reference, the immobilization suspension, and the supernatant of the immobilization suspension using *p*NPB as described above [72]. After 2 h, the suspensions were filtered, and the immobilized lipase biocatalysts were rinsed thoroughly with distilled water. The biocatalysts were then stored at 4°C for future use. The ETL biocatalysts features description may be found in [68].

4.2.2.3. Modification of immobilized ETL with metals salts

The 4 immobilized ETL biocatalysts were treated with metallic salts following the procedure outlined by Guimarães *et al*. (2022) [41]. For this process, 1 g of the immobilized ETL was suspended in 10 mL of 10 mM sodium phosphate/125 mM NaCl at pH 7.4. After that, 400 μL of a 230 mM solution of the respective metallic chloride was added. Other immobilized lipase samples were incubated in 10 mL of 10 mM TRIS/125 mM NaCl at pH 7.4, and then the metal chloride was added. In both instances, the immobilized enzyme modification was carried out at room temperature under gentle stirring for 5 hours using an Agimatic-S (JP Selecta) (Barcelona, Spain). After the mineralization treatments, the biocatalysts suspensions were filtered, and the biocatalysts were rinsed five times with 10 volumes of distilled water, and then recovered and stored at 4°C.

4.2.2.4. Enzyme thermal inactivation at different pH values

Biocatalysts were incubated at pH 5 (10 mM sodium acetate), pH 7 (10 mM sodium phosphate) or pH 7 (10 mM TRIS-Cl). The inactivation temperatures were chosen to get a

reliable and yet rapid inactivation courses. Residual activity was evaluated periodically using *p*NPB and expressed as a percentage of the initial activity.

4.3. RESULTS AND DISCUSSION

4.3.1. Mineralization of the biocatalysts

As indicated in the Methods section, the 4 immobilized ETL biocatalysts were incubated in solutions containing TRIS or sodium phosphate and then, the metal chlorides were added. In the presence of TRIS, the biocatalysts did not take any apparent color when using colored metal salts, while when using phosphate, the biocatalysts acquired an intense color according to the used metal (see **Figure A4.1** and **Figures A4.2-A4.3** using one of these biocatalysts as example). This was an obvious difference: when using phosphate there are crystals of metal phosphate, while using TRIS, this did not occur. However, this lack of color is not enough to discard that the metal can interact with the immobilized enzyme molecules and alter their properties. That way, the effects of both modifications were analyzed.

4.3.2. Effect of the pH on the activity versus *p*NPB of the different immobilized ETL biocatalysts

Figure 4.1 shows the activity of the different unmodified biocatalyst versus *p*NPB in the pH range 4-9 (out of this range the spontaneous *p*NPB hydrolysis was too significant to get reliable results).

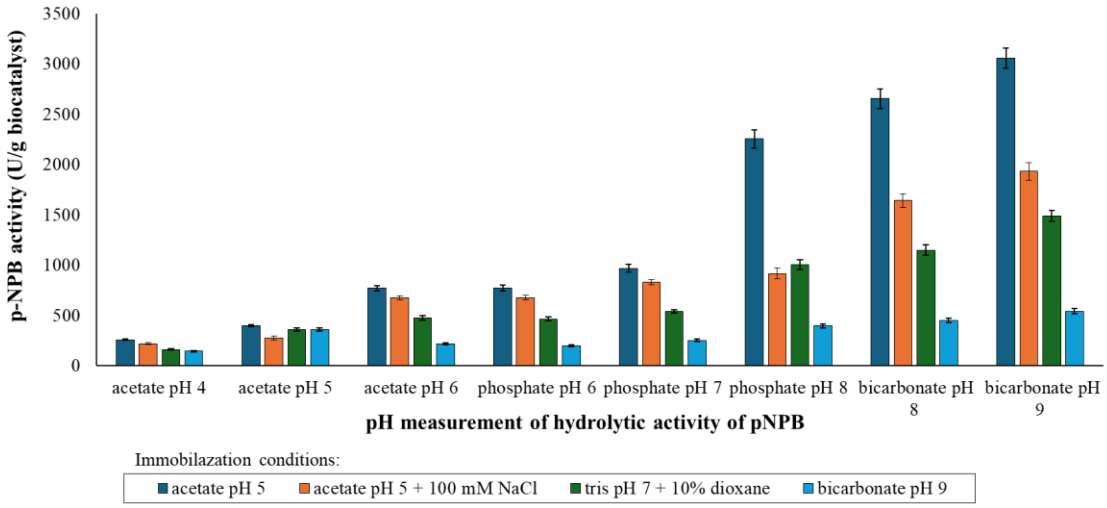

Figure 4. 1 – Effect of the pH in pNPB activity (U/g biocatalyst) at 25ºC of Eversa Transform 2.0 immobilized on octyl agarose at different pH values (using 50 mM buffers).

Figure 4.1 also shows in some instances the effect of changing the measurement

buffers. The highest activity was observed at pH 9 and the lowest at pH 4 in all cases. The most active biocatalysts at pH 9 was that prepared at pH 5 in 50 mM sodium acetate (3,057 U/g), followed by the biocatalysts immobilized in 50 mM sodium acetate plus 100 mM NaCl also at pH 5 (1,932 U/g), that immobilized in 50 mM TRIS plus 10% dioxane (1,489 U/g), being the least active one ETL immobilized at pH 9 (541 U/g). This exemplifies the great differences between the 4 different utilized biocatalysts [8]. The activities at pH 4 regarding the activities at pH 9 were 8.4%, 11.3%, 10.7% or 26.2% respectively for the different biocatalysts. At this pH value, even with the different preservation of the enzyme activity, the order of the biocatalysts as a function of their activities were maintained although differences become smaller than at pH 9. Differences in the relative activities could be observed comparing the activities at pH 5. Here the most active enzyme remained the one immobilized at pH 5, but the one immobilized at pH 5 in the presence of NaCl showed the lowest activity, while the enzyme immobilized at pH 7 and pH 9 showed similar activities. In general, at pH values under 7 the enzyme that was immobilized at pH 9 was the least responsible to the changes on the measurement pH values and the enzyme immobilized at pH 5 was the most sensible one.

The change of buffer in the measurements has diverse effects on the activity of the biocatalysts (this activity measurement at two different buffers was performed at pH 6, acetate and phosphate, and pH 8, phosphate and bicarbonate). At pH 6, the change of the buffer presented a negligible effect on the activity of both biocatalyst immobilized at pH 5 and in that immobilized at pH 7 in 10% dioxane, while the biocatalyst prepared at pH 9 presented 10% more activity when measuring in acetate. The change of the measuring buffer at pH 8 had more relevant effects, being the activity always higher in bicarbonate, but with diverse intensity: the enzyme immobilized at pH 7 plus 10% dioxane and the enzyme immobilized at pH 9 were the ones with a smaller activity change, increasing the activity by 14%-15%, the enzyme immobilized at pH 5 increased the activity by around 18%, while the enzyme immobilized at pH 5 plus NaCl increased the activity by 80%. Again, these data suggested the different properties of the used biocatalysts [68].

4.3.2.1. Effect of the pH on the activity versus *p*NPB of the biocatalyst prepared in 50 mM sodium acetate at pH 5 after mineralization

Next, we have analyzed the effects on the activity/pH curves of the mineralization of the biocatalyst prepared in 50 mM sodium acetate at pH 5, starting with the biocatalysts mineralized using phosphate **(Figure 4.2)**.

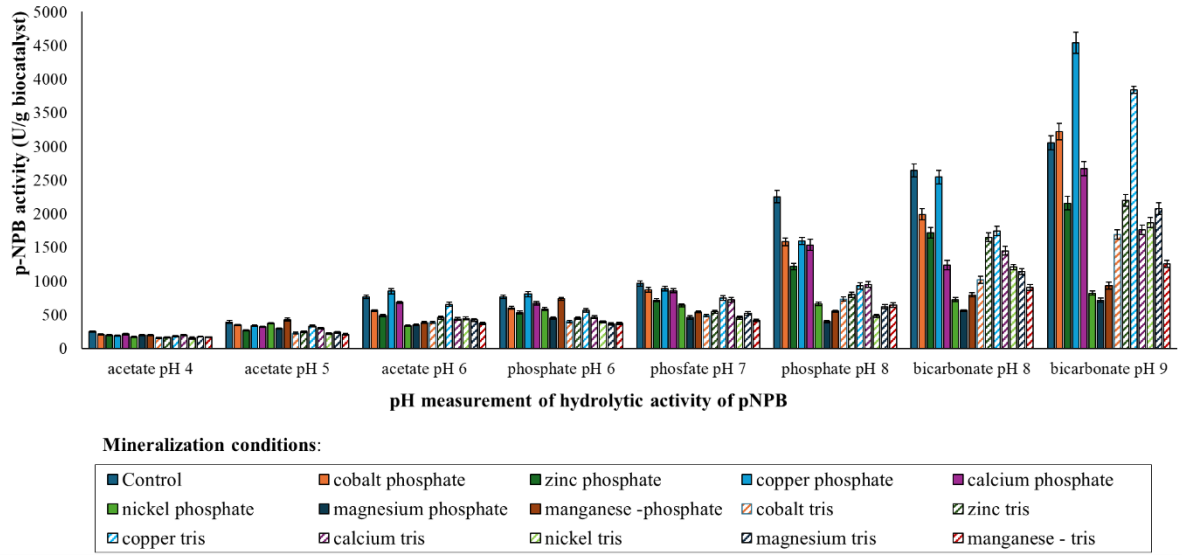

Figure 4. 2 – Effect of the pH in pNPB activity (U/g biocatalyst) at 25ºC of Eversa Transform 2.0 immobilized on octyl agarose at pH 5 (50 mM acetate) after mineralization under conditions described in methods.

In this instance, the mineralized biocatalysts maintained the maximum activity at pH 9 in all cases, although the shape of curves was significantly altered when comparing the biocatalysts among them and with the reference. That way, although in general a decrease in activity was found after the modification under most conditions, there are some exceptions. The most relevant example was the modification with copper when the activity was determined at pH 9 (increasing the activity of the unmodified biocatalyst by almost 50%), this modification gave the biocatalyst with the highest activity also at pH 6 in acetate (increasing the activity by 10%). The modification with Mn^{2+} gave a biocatalyst with the highest activity among all the biocatalysts of this group at pH 5 (increasing the activity of the initial biocatalyst by 8.5%), while the modification with Co^{2+} produced a slight improvement in the activity at pH 9 (5%). The least and the most active mineralized biocatalyst depended on the pH and buffer used to measure the activity. The modification with calcium and cobalt produced the most active mineralized biocatalysts if the activity was determined at pH 4, while the least active one was that produced using nickel. At pH 5, the most active biocatalyst was the one modified with manganese, while the least active one was that modified with zinc. When determining the activity at pH 6 in acetate, the least active biocatalyst was that mineralized using nickel and the most active was that modified with copper. The change of buffer to phosphate (with no effects using the non-mineralized biocatalyst) produced diverse effects on the activity depending on

the metal used in the treatment. The treatment with copper and calcium promoted no significant variations, a considerable change was observed for the biocatalyst modified with manganese, which increased the activity by approximately a 50%. Now, the least active biocatalyst was that modified with magnesium, while the most active was that modified with copper. When determining the activity at pH 7, the treatment with cobalt, copper and calcium produced the most active mineralized biocatalysts, while the least active one was that modified using magnesium (a 2 fold factor was the difference between the activities of these biocatalysts). The lowest decrease in activity at pH 8 in phosphate was obtained by mineralization using copper, cobalt or calcium, while the highest decrease in activity was observed after modification with magnesium. The change from phosphate to bicarbonate at pH 8 produced an increase in all the biocatalyst activity, but the intensity was not the same for all biocatalyst. The biocatalyst with the lowest activity in these conditions remained the one modified with magnesium and the most active was that modified using copper. Finally, at pH 9, the most active biocatalyst was that modified with copper (that even surpassed the activity of the unmodified biocatalyst as stated above), while the modification with magnesium produced the biocatalyst with the lowest activity. Thus, even the response of the mineralized biocatalysts to changes in buffer and pH value was different depending on the metal used in the mineralization.

The incubation of the biocatalysts in the presence of metal chlorides and TRIS, even without giving any color, clearly affected the activity of the biocatalysts. Using cobalt, the activity recovery is much lower in this instance (around 50% than using phosphate). Using zinc, the activity recovery is lower that using cobalt, but the difference with the mineralization in the presence of phosphate is lower (because this biocatalyst exhibited lower activity). Copper gives the highest activity, at pH 9 improving the activity of the unmodified biocatalyst, but the activity is lower than using phosphate, the differences decreasing at pH 4 and 5. Calcium modification decreased the biocatalyst activity compared to the same sample measured using bicarbonate, except at pH 8. The metallization with nickel gave mixed results, activity increase or decrease depending on the measure conditions. The biocatalysts activity is higher than when using phosphate at pH 8 and 9 in the measure buffer, and at pH 6 when the activity is determined in acetate (but not in phosphate), being lower under the other measurement conditions. Using magnesium, the activity is higher now than in the other case at pH 7-9 or at pH 6 using acetate to determine the activity (again, no using phosphate), being lower under the other conditions. Using manganese, the enzyme modification in TRIS gave more activity at pH 8 and 9 than if the biocatalyst is modified in phosphate.

The results suggest that the metal, even in the absence of phosphate, is able to modify

the enzyme features, usually promoting a more significant decrease in enzyme activity under acidic than at alkaline pH value. The metal modification with and without phosphate not only altered the activity and the effect of the pH, also the effect of the buffer on enzyme activity. This is more clearly visualized at pH 6, since some biocatalysts are more active after modification using phosphate metals in one buffer while other biocatalysts submitted to the same modification are less active in that buffer.

That way, metallization of the enzyme clearly affected the enzyme features using the biocatalyst prepared in 50 mM sodium acetate at pH 5, and the effect depended on the metal nature and the presence or not of phosphate during modification, and the measurement conditions.

4.3.2.2. Effect of the pH on the activity versus *p*NPB of the biocatalyst prepared in 50 mM sodium acetate plus 100 mM NaCl at pH 5 after mineralization

The biocatalyst prepared in 50 mM sodium acetate plus 100 mM NaCl at pH 5 was less active than in the previous case **(see Figure 4.1)**. **Figure 4.3** shows the results of the biocatalyst mineralization.

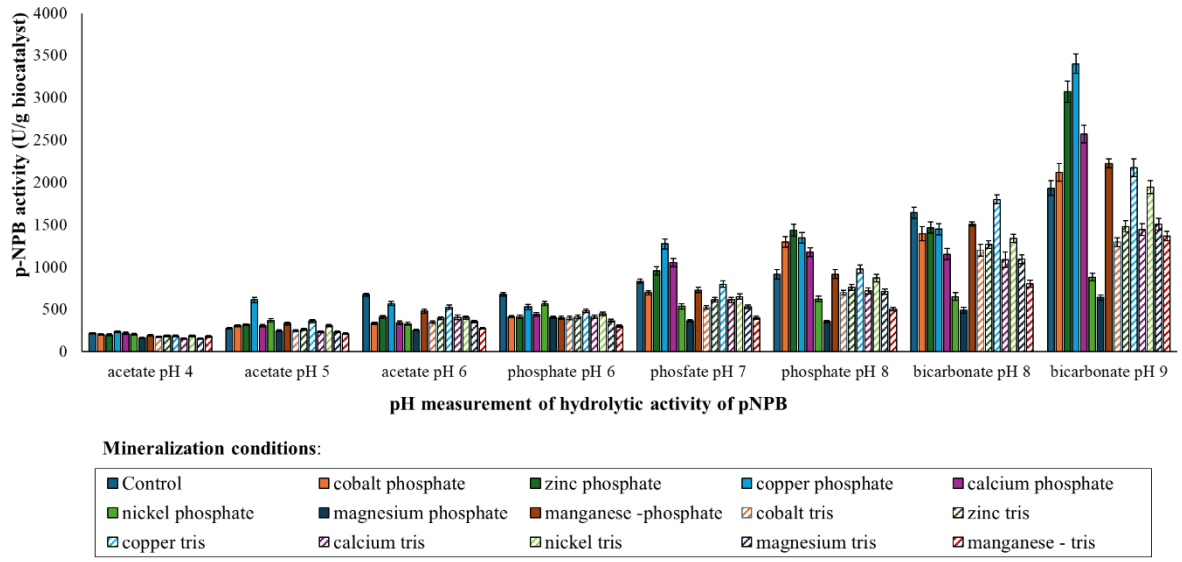

Figure 4. 3 – Effect of the pH in pNPB activity (U/g biocatalyst) at 25^{o}C of Eversa Transform 2.0 immobilized on octyl agarose at pH 5 (50 mM acetate) plus 100 mM NaCl after mineralization under conditions described in methods

Using phosphate in the modification, at pH 9 the activity usually increases even by 75%

using Cu^{2+}. Exceptions are the mineralization using nickel and magnesium (the activity decrease to 1/3 or its initial value). In TRIS, the effect of the incubation with the metals is negative in the *p*NPB activity of the biocatalyst, becoming manganese the metal that produced the lowest activity (down to 70%). Copper treatment again produces the best results, with a slight increase in enzyme activity (by around 12%), but become with only 64% of the activity of the biocatalyst metallized in the presence of phosphate. Measuring the activity at pH 8 in bicarbonate, the effect of the mineralization on enzyme activity was negative in most cases, using TRIS or phosphate, the only biocatalyst that increased the activity was that mineralized in TRIS using copper. The least active biocatalyst was that prepared in phosphate using magnesium (around 30% of the activity of the unmodified biocatalyst) and in TRIS using manganese (less than 50%). However, using phosphate at pH 8 to determine the enzyme activity, only the treatments with nickel or magnesium (to around 39%) using phosphate as buffer in the mineralization produced a decrease in activity. In this instance, the modification with zinc gave the biocatalysts with the higher activity (increasing the activity almost by 60%). Under these conditions to determine the enzyme activity, mineralization with TRIS also produced a decrease on enzyme activity (only copper given an increment in enzyme activity, by only 5%). Manganese gave the lowest activity (decreasing the activity to 55%). Using pH 7 to determine the enzyme activity, the results were mixed. The mineralization using phosphate and zinc, calcium, copper (that gave the highest activity, improving the biocatalyst activity by 55%), promoted an increase on enzyme activity while using cobalt, nickel, manganese or magnesium (the least active biocatalyst, with less than 45% of the unmodified biocatalyst) produced a decrease in enzyme activity. At this pH incubation with metals and TRIS produced a general decrease in enzyme activity, with manganese again producing the biocatalyst with the lowest activity (to least than 50%) and copper the one with the highest (decreasing the activity by only 5%). At pH 6 in the presence of phosphate, all biocatalysts incubated in metal and phosphate decreased the activity, with cobalt, zinc, magnesium and manganese giving similar results (to around 60%), giving the biocatalyst modified with nickel the highest activity (83%). Using TRIS in the mineralization, results were also negative in the *p*NPB activity under these conditions, manganese treatment gave the least active biocatalyst (44%) and copper the most active (more than 70%). Changing the buffer for acetate, the effect of the modification was also negative for the enzyme activity using both phosphate or TRIS in the mineralization. Copper gave the highest activity when using phosphate in the mineralization (almost 85%) or TRIS (almost 78%) the least activity biocatalyst being that prepared using magnesium and phosphate (37%) or TRIS and manganese (40%).

Determination of the enzyme activity at pH 5 showed a different picture. Now, the mineralization in the presence of phosphate increased the enzyme activity (with maximum increase using copper (increasing the activity 2.2-fold), only magnesium treatment decreasing the activity (to almost 90%). Using TRIS in the mineralization, only copper (by 30%) and nickel (by 11%) treatment increased enzyme activity, manganese giving the biocatalyst with the lowest activity (more than 75%). Finally, the determination of the activity at pH 4 shows that the biocatalyst activity is not very affected when using phosphate in the mineralization, being copper the only slightly increasing enzyme activity (by more than 7%) and magnesium giving the biocatalyst with the lowest activity (to around 75%). If the modification was performed in TRIS, the effect was negative for all biocatalysts, zinc, nickel and copper giving the highest activity (around 85%) and calcium the lowest (71%).

Again, metallization of the enzyme clearly affected the enzyme features using this biocatalyst, and the effect depends on the metal nature and the presence or not of phosphate during the modification, and the measure conditions. Although some similarities may be found with the results using the other biocatalyst, also many differences may be observed, suggesting that the effects of the mineralization also depend on the exact conformation of the enzyme.

4.3.2.3. Effect of the pH on the activity versus *p*NPB of the biocatalyst prepared in 50 mM TRIS plus 10% dioxane at pH 7 after mineralization

Then, the biocatalyst prepared at pH 7 in 10% dioxane was submitted to mineralization in phosphate and TRIS and the activity/pH curve analyzed **(Figure 4.4)**. The effect of the mineralization in phosphate on the activity at pH 9 is negative using all metals, cobalt is the one providing the highest activity (almost 85% of the activity of the unmodified biocatalysts) with magnesium being the lowest one (around 45%). If the modification was performed in TRIS, the results were mixed. While using zinc and cupper the activity increased slightly (by 5%), cobalt presented a low impact, and the other promoted a decrease in enzyme activity, with the biocatalysts incubated in manganese exhibiting the lowest activity at pH 9 (65% of that of the unmodified biocatalyst). Measuring the activity at pH 8 in bicarbonate, the decrease in activity after mineralization was more prominent, calcium permitted to maintain the highest activity (almost 85%) while magnesium was again the metal providing the higher losses in activity (to 45%). If the incubation with the metal was performed in TRIS, the situation was similar, a general decrease in enzyme activity was observed, the smallest using copper (slightly over 85%) and the highest using manganese (just over 35%). The change of buffer to phosphate at pH 8 in the activity determination change the picture. The modification with phosphate and

copper had almost no effect on enzyme activity, but in general, the decrease in enzyme activity was more important than the mineralization in the presence of phosphate than using bicarbonate in the activity determination, although now the lowest activity was exhibited by the biocatalyst modified with magnesium (40%), with a slightly higher retention of activity percentage than the least active biocatalyst measuring in bicarbonate. The metal modification in TRIS also produced a decrease in enzyme activity, copper modification permitted to maintain the highest percentage of activity (80%) and manganese modification gave the lowest activity (under 45%). Measuring at pH 7, the mineralization in the presence of phosphate produced a decrease in enzyme activity, the most active biocatalyst remained that incubated with copper, while the least active ones were those incubated with zinc, calcium and magnesium (around 65%), the most active was that modified with copper (over 95%). In the presence of TRIS during mineralization, the activity decreased also in all cases except when using copper (activity increased by 20%). The lowest activity was obtained after incubation in manganese (more than 60%). Measuring the activity at pH 6 in phosphate as buffer, the activity of the biocatalysts decreased after mineralization in both TRIS or phosphate (in a higher extension). In the presence of phosphate during mineralization, the activities of the biocatalysts ranged from 82% (using copper) to 54% (using magnesium). Using TRIS in the mineralization, the activities ranged from 90% (using copper) to 56% (using manganese). The use of acetate at pH 6 maintained the negative effect of the modification with metals in the presence of phosphate. In this instance, the highest activity was observed using the biocatalyst modified with manganese (almost 85%) while the lowest one was observed using cobalt in the modification (56%), followed by that modified with copper (that usually gave the highest activities). If metal incubation was performed using TRIS, the incubation with copper left the enzyme activity almost unaltered, while manganese produced a decrease in the enzyme activity to 60%). When determining the enzyme activity at pH 5, only the incubation of this biocatalyst in copper and TRIS maintained the initial activity, all the other metals and buffers decreased the activity, to 53% using phosphate and magnesium and to 44% using TRIS and magnesium. When determining the activity at pH 4 the situation becomes fully different. The incubation of the biocatalyst with phosphate and cobalt, copper, calcium, and mainly zinc (more than 1.5-fol) increase the enzyme activity, while nickel, magnesium and manganese had no effect on enzyme activity. situation was different if incubating the enzyme in metal and TRIS. Using copper, the enzyme activity increased by 23%, magnesium had no effect and only manganese (by 10%) and nickel (by less than 15%) produced a relevant decrease on enzyme activity.

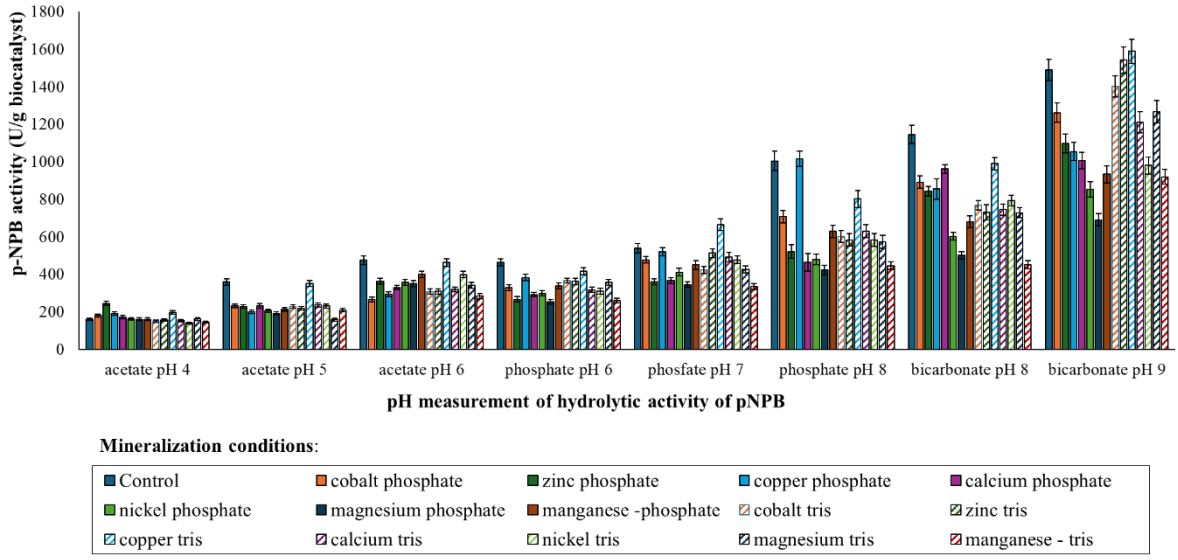

Figure 4. 4 – Effect of the pH in pNPB activity (U/g biocatalyst) at 25ºC of Eversa Transform 2.0 immobilized on octyl agarose at pH 7 (50 mM Tris) in presence of 10% dioxane after mineralization under conditions described in methods

4.3.2.4. Effect of the pH on the activity versus *p*NPB of the biocatalyst prepared in 50 mM bicarbonate at pH 9 after mineralization

Finally, we have analyzed the effects of the mineralization on the *p*NPB activity/pH profile of the biocatalyst prepared at pH 9 **(Figure 4.5).**

The mineralization in the presence of phosphate presented a mixed effect when determining the activity at pH 9, the cobalt, zinc, calcium and specially copper increased the activity (the last one by almost 70%), while magnesium, manganese and specially nickel (to 68%) decreased the activity. When decreasing the pH to 8, the effects were similar in the metals that promoted an increment on enzyme activity, while the 3 that decreased their activity at pH 9, also decreased the activity at pH 8, but with similar activities (ranging from 68% to 74%). The change of the buffer during the activity determination to phosphate produced that the mineralized biocatalyst decreased the activity compared to the reference, with the highest activity retention using copper and zinc (around 93%) and the minimal using nickel (65%). The activity determination at pH 7 changed the picture. Cobalt and nickel treatments had little effect on enzyme activity, copper treatment increased the activity by almost 35% while magnesium decreased the activity by almost 25%. Manganese treatment produced an increased activity (almost by 10%) in contrast with the previous results. Determining the activity at pH 6 in

phosphate, the mineralization produced a slight decrease in activity using cobalt, zinc, nickel (the least active biocatalyst, with a 90% activity), copper and magnesium almost produced no changes in activity, while manganese and calcium produced slight increases (by around 5%). The use of acetate at pH 6 as activity determination buffer changed the situation, now all mineralized biocatalysts were more active than the control, the copper treatment giving the highest activity (increasing the activity by 35%). The activity at pH 5 suffered a decrease in all cases for the mineralized biocatalysts (except for that modified with calcium) when compared with the activity at pH 6, while the control increased the activity. Thus, only the modification with calcium almost maintained the activity intact, while the treatments with nickel, magnesium and manganese gave the lowest activities (lower than 50%). At pH 4, nickel and magnesium maintained the activity, while all the others increased it (reaching an increase of over 30% with copper).

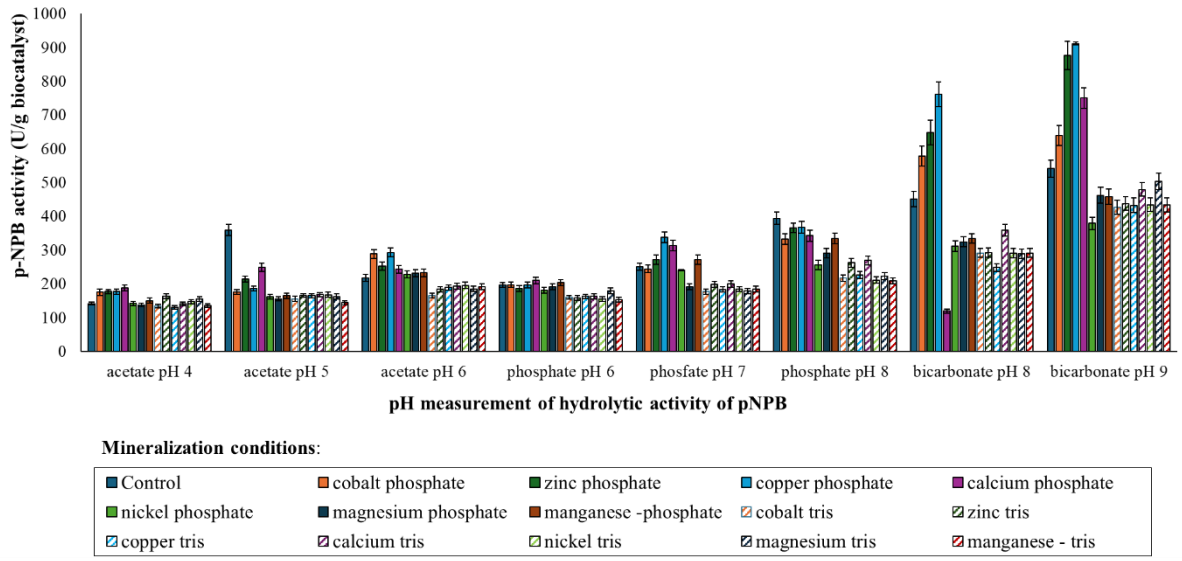

Figure 4. 5 – Effect of the pH in pNPB activity (U/g biocatalyst) at 25ºC of Eversa® Transform 2.0 immobilized on octyl agarose at pH 9 (50 mM bicarbonate) after mineralization under conditions described in methods

If during mineralization the buffer was TRIS, the situation was different. Measuring the activity at pH 9 all biocatalysts decreased their activity, ranging from 92% (using magnesium) to 80% (using copper, nickel and manganese). At pH 8 in bicarbonate, the activities decreased in a more significant way, moving from 64% (using cobalt, zinc, nickel, magnesium or manganese) to 55% (using copper). Calcium treatment was the exception, with more than 7% increase in the activity under these conditions. The change at pH 8 to phosphate produced a general decrease in the activity of the mineralized biocatalyst, while having almost no effect in

the control, with activates in the range 50-55%, except the biocatalyst treated with zinc, that maintained more than 65%. The activity at pH 7 also decreased after tis mineralization, with activities ranging from 80% (using zinc or calcium) to 70% (using magnesium or cobalt). At pH 6 in phosphate, the mineralization in TRIS also produced a decrease in activity for all biocatalyst, the change to acetate slightly reducing in general the decrease in activity. The use of pH 5 even accentuated the decrease in enzyme activity caused by the mineralization (to 40% for the biocatalyst treated with manganese) to 45% for the biocatalyst-modified calcium). The activity at pH 4 is the only that gave mixed results, with zinc improving the activity by 15% or by 10% using magnesium, while the lowest activities were obtained modifying this biocatalyst with cobalt or manganese (to 93%).

Taking together the results in all this heading, (section 4.3.2.), it seems that the biocatalysts altered their properties when using phosphate but also using TRIS (in some instances with more intensity), but in a dissimilar way. The effects of the mineralization on the enzyme activity depends on the buffer used in the incubation, on the metal nature, but also on the biocatalyst and on the measurement conditions. That is, it looks like these biocatalysts, prepared on the same support and that only differ on the enzyme structure, present a dissimilar response to the mineralization.

4.3.3. Effect of the metallization on the activity versus triacetin

Using triacetin as substrate, the differences in activity between the 4 initial biocatalysts were not so different as versus *p*NPB (ranging 11% between the most and the least active biocatalyst) **(Figure 4.6)**. Now, the least active preparations were both prepared at pH 5, being the other two biocatalysts more active and with similar activity (in contradiction with the results using pNPB). These results again pointed that the different enzyme features was quite different.

Next, we have analyzed the effects of the mineralization performed in phosphate and TRIS. The effects of these treatments were quite different depending on the used biocatalysts, but they were not as relevant as using *p*NPB.

Using the enzyme immobilized at pH 5 in 50 mM sodium acetate, the incubation in metal and phosphate produced scarce changes in enzyme activity (less than 10%) except using Cu^{2+}, that increase the activity by 18%, or Mn^{2+}, that decreased the activity by more than 40%. The incubation with metal in TRIS gave as the most significant results a decrease in activity using Zn^{2+} (87%) and Cu^{2+} (85%) (that usually was the metal giving the highest activates using *p*NPB, see section above) and an increase using Mg^{2+} (171%) (usually among the biocatalyst

with the lowest actives using *p*NPB, see section above).

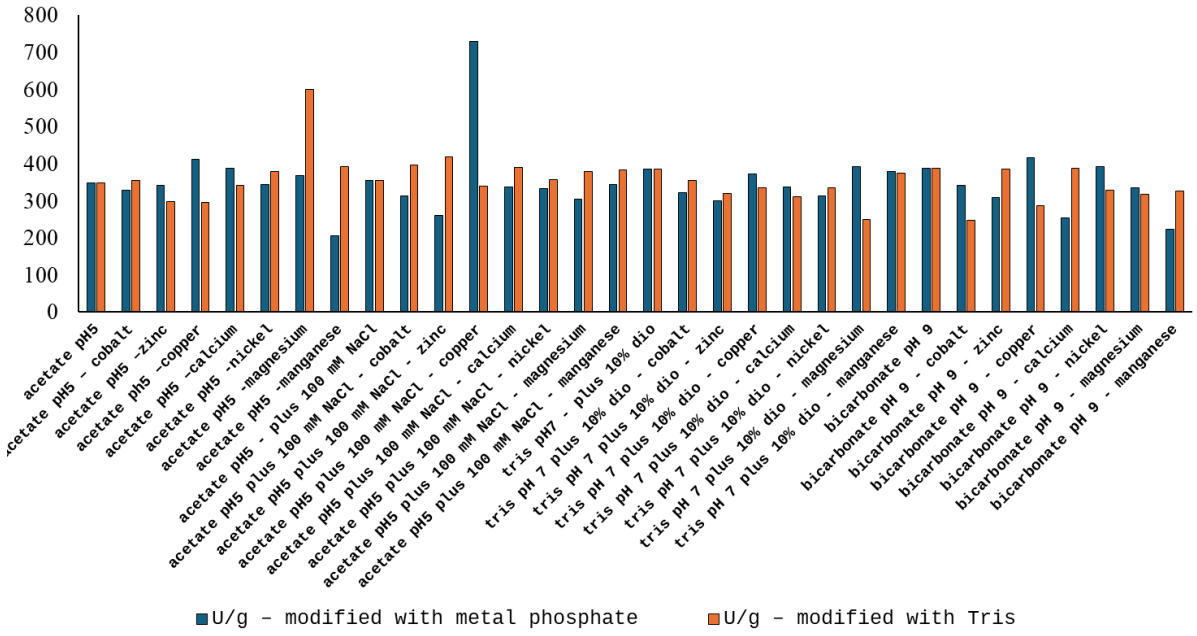

Figure 4. 6 - Mass activities versus 50 mM triacetin at pH 5 and 25 ºC of different ETL biocatalysts prepared as described in Methods. Activity is given in micromoles of transformed substrate/minute/ g of biocatalysts. Other specifications are described in Methods.

The result was fully different using different biocatalyst. If in the immobilization at pH 5 we added 100 mM NaCl, Co^{2+} (88%), Zn^{2+} (73%) and Mg^{2+} (86%) produced a decrease in activity if added in presence of phosphate, while Cu^{2+}produced an increase in enzyme activity (210%). The addition of the metal in TRIS, produced in most cases biocatalysts with higher activities, but not as high as when using Cu^{2+} in the presence of phosphate: Co^{2+} (112%), Zn^{2+} (117%), Ca^{2+} (110%) or Co^{2+} (108%). That way, not only it is clear the different results using TRIS or phosphate, but the different reaction of the diverse biocatalysts to metallization, the main objective of this paper, suggesting that the initial enzyme structure could be very important for the final effects of the mineralization.

Using the biocatalyst prepared in the presence of 50 mM TRIS plus 10% dioxane at pH 7, the treatments produced a general moderate decrease of enzyme activity except using Mg^{2+} (102%) or Mn^{2+} (97%) when the treatment was in the presence of phosphate or Mn^{2+} in TRIS (97%). The most significant decreases in activity when using phosphate were observed using Zn^{2+} (78%) Ni^{2+} (80%) or Co^{2+} (83%). If the treatment was performed using TRIS, the lowest activity was found using Mg^{2+} (64%), Ca^{2+} (80%) or Zn^{2+} (82%). Again, results were

different depending if crystals were formed or not, and different to the ones found using the other biocatalysts.

Finally, the biocatalyst immobilized at pH 9 when treated with metals decreased the activity except some exceptions. Using phosphate, the activity increased using Cu^{2+} (108%) and remained unchanged using Ni^{2+}, decreasing the activity in the other cases, being the decrease in activity more significant than that observed using Ca^{2+} (65%) or using Mn^{2+} 57%) . Using TRIS, the activity was maintained using Zn^{2+} and Ca^{2+}, and significantly decreased using Co^{2+} (63%) and Cu^{2+} (74%).

That way, it seems evident that the incubation of the enzymes in metals, in TRIS or in phosphate, can alter the enzyme features, although in different ways. These changes also differ of that observed using *p*NPB.

Most importantly in the context of our main goal, the effects of both treatments are seriously depended on the enzyme conformation, as the support and the enzyme are the same.

4.3.4. Inactivation of the different ETL biocatalysts under different conditions

The different biocatalyst were inactivated under diverse pH conditions, that could alter the ionization statutes of the different ionic groups of the enzyme and the metal salts altering the enzyme-salts interactions (pH 5 in acetate and pH 7 in TRIS). Moreover, the biocatalysts were inactivated at pH 7 in the presence of sodium phosphate, an anion described to have a negative effect on the octyl-ETL stability [73–76].

First, we have compared the stabilities of the 4 initial biocatalysts under these 3 inactivation conditions **(Figure A4.4)**. In all cases, the most stable biocatalyst was the biocatalyst prepared at pH 9, although the difference at pH 5 is smaller than at pH 7 in phosphate or TRIS (where the differences were maximal). The second most stable catalyst was that prepared at pH 7 in 10% dioxane (at pH 5 is near to the biocatalyst prepared at pH 9), while both biocatalysts prepared at pH 5 exhibited similar inactivation courses, being the least stable ones. This shows that also in terms of stability the different biocatalysts exhibited different properties, although that prepared at pH 5, which, as discussed above, presented very different activities, presented now similar stabilities.

Figure 4.7 shows the results of the inactivation at pH 5 of the 4 different biocatalysts after incubation with the different metals in the presence of phosphate.

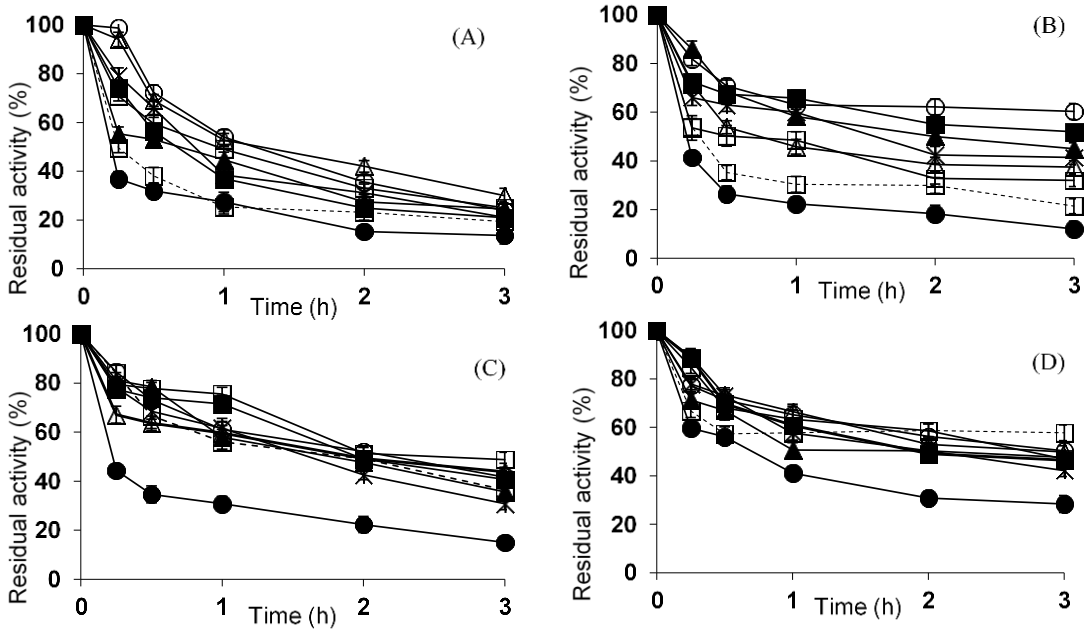

Figure 4. 7 - Inactivation courses of different octyl-ETL biocatalyst, modified with different metals in the presence of phosphate. The biocatalysts were inactivated in 25 mM acetate pH 5 and 75 °C. Other specifications are described in Methods. (A): octyl-ETL in 50 mM sodium acetate at pH 5; (B): octyl-ETL in 50 mM sodium acetate at pH 5 plus 100 mM NaCl; (C): octyl-ETL in 50 mM Tris at pH 7 plus 10% dioxane and (D): octyl-ETL in 50 mM sodium bicarbonate at pH 9. No mineralized (open square and dotted line); $CaCl_2$ (solid square); $NiCl_2$ (solid triangle); $CoCl_2$ (asterisk); $CuCl_2$ (solid circle); $MgCl_2$ (open square); $ZnCl_2$ (open triangle); $MgCl_2$ (open circle).

The effect of the metal treatments on enzyme stability is clear and depends on the initial biocatalyst. Eversa immobilized on 50 mM sodium acetate at pH 5 increased the stability after modification with all metal phosphate salts, except copper, that slightly decreased the stability. The most stable preparation was that obtained after modification with zinc. The situation changes when the treated biocatalyst was Eversa immobilized in 50 mM sodium acetate at pH 5 plus 100 mM NaCl. Now, the stabilizations caused by the mineralization are more relevant, the most stable biocatalyst is that modified with magnesium, while the biocatalyst modified with zinc was in the middle. Copper remained as the metal that produced the biocatalyst with the lowest stability. Eversa immobilized in 50 mM TRIS at pH 7 plus 10% dioxane gave different response to the mineralization, now the mineralization presented a scarce effect on enzyme stability, except copper that produced a series of decreases on enzyme stability. Eversa immobilized on 50 mM sodium bicarbonate at pH 9 was the biocatalyst with smaller changes

in enzyme stability, except using copper, which again promoted a destabilization. **Figure 5S** shows the comparison of the inactivation courses of the most and least stable metallized biocatalysts compared to the unmodified biocatalysts, where it is easier to appreciate the large differences in the effect of the mineralization of the different biocatalysts that only differs on enzyme conformation (enzyme, support and orientations are identical) [68].

Figure 4.8 shows the results of the inactivation at pH 5 of the 4 different biocatalysts after their mineralization by incubation with the different metals in TRIS.

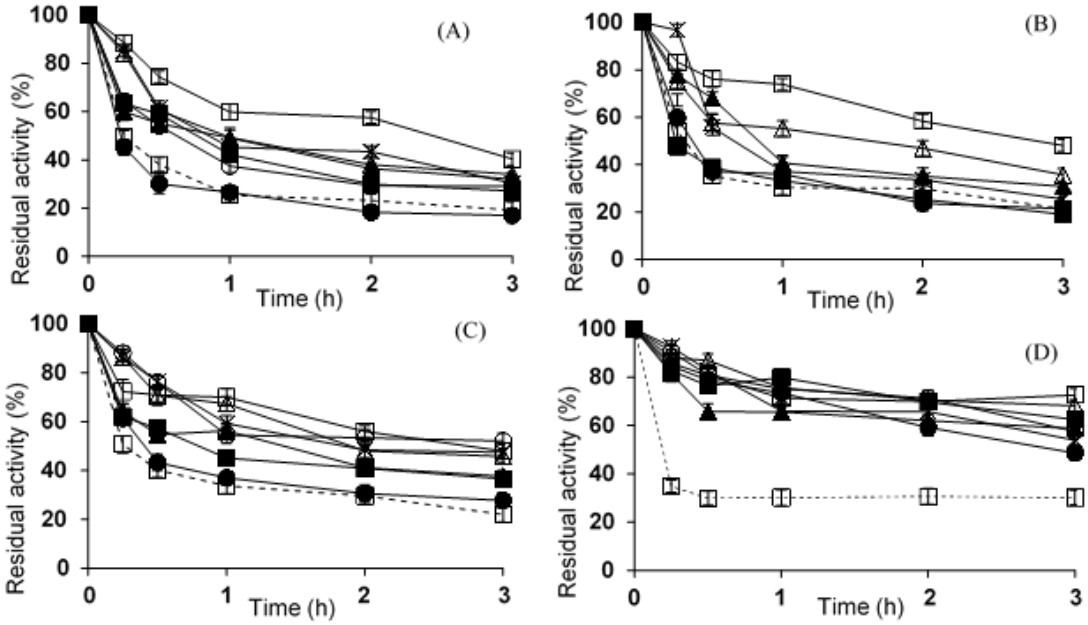

Figure 4. 8 - Inactivation courses of different octyl-ETL biocatalyst modified with different metals in the presence of Tris. The biocatalysts were inactivated in 25 mM acetate pH 5 and 75 °C. Other specifications are described in Methods. (A): octyl-ETL in 50 mM sodium acetate at pH 5; (B): octyl-ETL in 50 mM sodium acetate at pH 5 plus 100 mM NaCl; (C): octyl-ETL in 50 mM Tris at pH 7 plus 10% dioxane and (D): octyl-ETL in 50 mM sodium bicarbonate at pH 9 and modified. Control (open square and dotted line); $CaCl_2$ (solid square); $NiCl_2$ (solid triangle); $CoCl_2$ (asterisk); $CuCl_2$ (solid circle); $MgCl_2$ (open square); $ZnCl_2$ (open triangle); $MgCl_2$ (open circle).

The incubation of the biocatalysts in TRIS/metal solutions offers a fully different picture in some instances in the inactivation courses at pH 5 compared to the biocatalyst previously incubated in metal and phosphate. The negative effect of copper is now minimized, in fact the biocatalyst prepared at pH 9 improved its stability after copper mineralization, and the other biocatalysts presented similar stability after copper mineralization to the unmodified biocatalysts (leaving the copper modified biocatalysts as the one with the lowest stability),

while for the other 3 biocatalysts, the stability increased after metal incubation. It remained the least stable mineralized biocatalyst for all biocatalysts except that prepared at pH 5 plus 100 mM NaCl, in this case its stability was between the other biocatalysts stabilities. **Figure A4.6** illustrates the inactivation profiles of the most and least stable metallized biocatalysts in comparison to the unmodified biocatalysts for an easier visualization.

This shows that the incubation with metals, both in TRIS and phosphate, greatly alters enzyme stability, and these effects depended on the initial biocatalyst.

Figure 4.9 shows the inactivation in the TRIS at pH 7 of the 4 different biocatalysts after incubation with the different metals in the presence of phosphate.

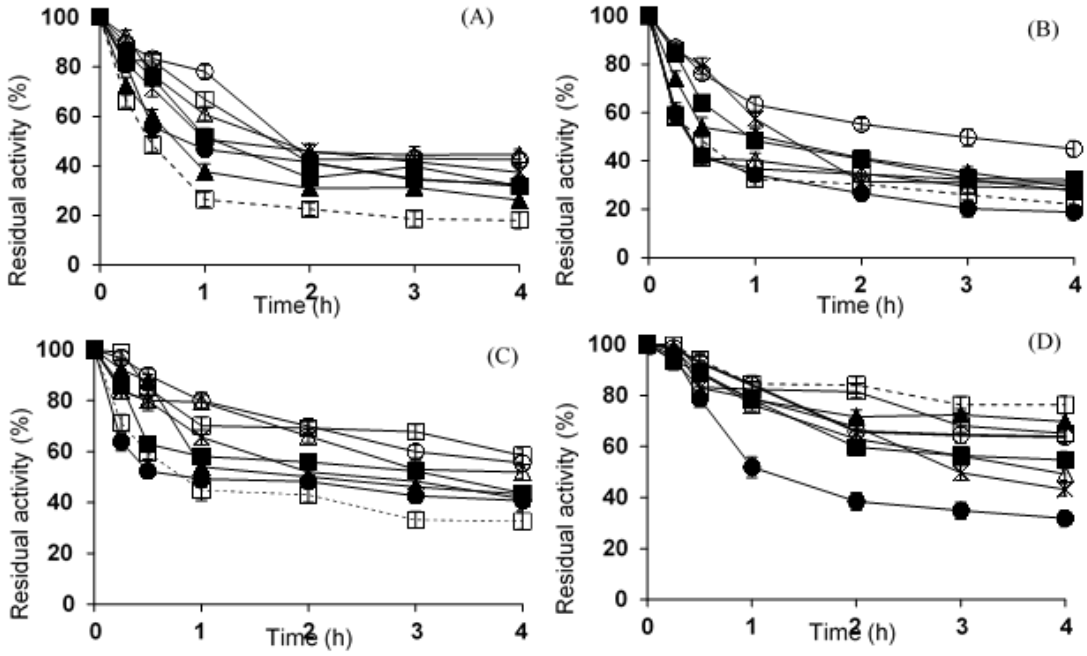

Figure 4. 9 - Inactivation courses of different octyl-ETL biocatalyst, modified with different metals in the presence of phosphate. The biocatalysts were inactivated in 25 mM Tris pH 7 and 75 °C. Other specifications are described in Methods. (A octyl-ETL in 50 mM sodium acetate at pH 5; (B): octyl-ETL in 50 mM sodium acetate at pH 5 plus 100 mM NaCl; (C): octyl-ETL in 50 mM Tris at pH 7 plus 10% dioxane and (D): octyl-ETL in 50 mM sodium bicarbonate at pH 9 and modified. Control (open square and dotted line); $CaCl_2$ (solid square); $NiCl_2$ (solid triangle); $CoCl_2$ (asterisk); $CuCl_2$ (solid circle); $MgCl_2$ (open square); $ZnCl_2$ (open triangle); $MgCl_2$ (open circle).

Results strongly differ from the inactivation at pH 5. The treatment with copper remained with a negative effect for the biocatalysts prepared at pH 5 plus 100 mM NaCl, and even with much more clarity for the biocatalyst immobilized at pH 9. The biocatalyst prepared

at pH 5 improved its stability after copper and phosphate incubation, while the nickel treatment provided the lowest stability. That way, the most stable mineralized biocatalyst depended on the used biocatalyst. Eversa immobilized in 50 mM sodium acetate at pH 5 was stabilized by all metallization treatments, with the highest stability after modification using zinc, the biocatalyst prepared at pH 5 in the presence of 100 mM NaCl offers a similar picture, except for copper, that produced a destabilization. The biocatalyst prepared at pH 7 plus 10% dioxane is also stabilized by all treatments, this time magnesium and manganese treatments providing the highest stability. The case of the biocatalyst prepared at pH 9 is fully different when compared to the other biocatalysts: all modified preparations decreased enzyme stability, with nickel offering the lowest decrease in enzyme stability and copper the lowest stability. **Figure A4.7** offers the comparison between the most and least stability biocatalysts for an easy visualization.

The incubation of the biocatalyst in metals/TRIS solutions produces some differences on the final stability of the biocatalysts inactivated in TRIS at pH 7 **(Figure 4.10)**.

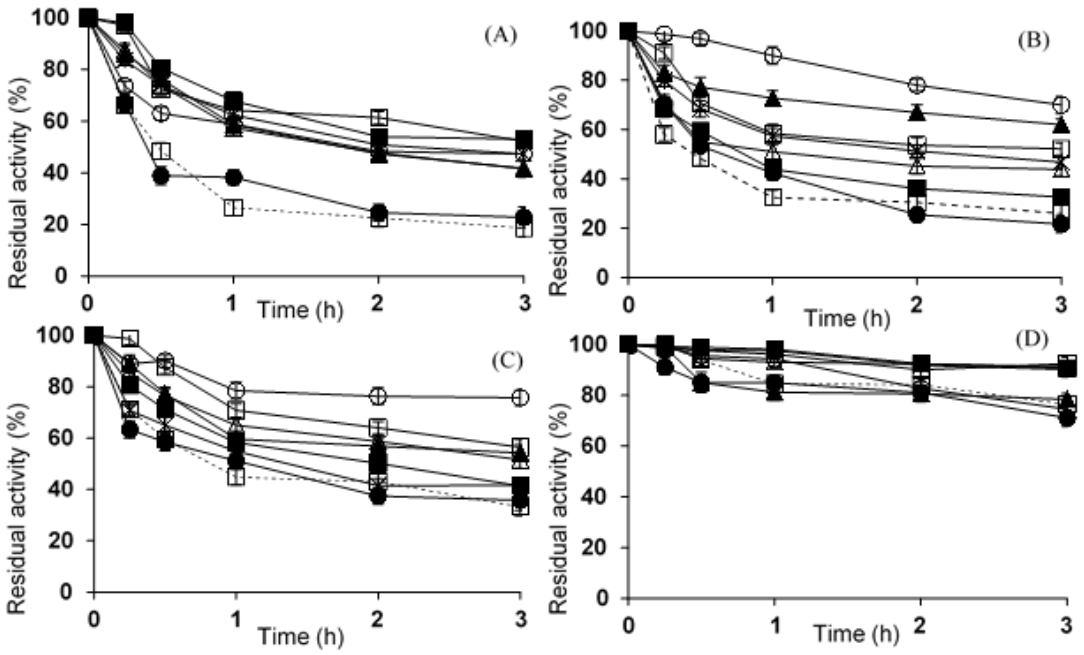

Figure 4. 10 - Inactivation courses of different octyl-ETL biocatalyst, modified with different metals in the presence of Tris. The biocatalysts were inactivated in 25 mM Tris pH 7 and 75 °C. Other specifications are described in Methods. (A): octyl-ETL in 50 mM sodium acetate at pH 5; (B): octyl-ETL in 50 mM sodium acetate at pH 5 plus 100 mM NaCl; (C): octyl-ETL in 50 mM Tris at pH 7 plus 10% dioxane and (D): octyl-ETL in 50 mM sodium bicarbonate at pH 9 and modified. Control (open square and dotted line); $CaCl_2$ (solid square); $NiCl_2$ (solid triangle); $CoCl_2$ (asterisk); $CuCl_2$ (solid circle); $MgCl_2$ (open square); $ZnCl_2$ (open triangle); $MgCl_2$ (open circle).

The modification with copper is again the treatment that produces the biocatalysts with the lowest stability, but the stability is similar to that of the unmodified biocatalyst. Magnesium treatment provides the highest stabilities for the biocatalyst immobilized at pH 5 plus 100 mM NaCl and that prepared at pH 7 plus 10% dioxane. However, the mineralization of the biocatalyst immobilized at pH 9 did not show a great impact on its stability. The effect of the metal modification is fully different for this biocatalyst using TRIS or phosphate (where the modification promoted strongly negative effects, **Figure 4.3)** during metallization. **Figure A4.8** provides a comparison between the most and least stable biocatalysts, facilitating a clearer visualization of their stability differences.

Again, the metal incubation of the biocatalysts in TRIS or phosphate induced great changes in enzyme stability, and, very interestingly in the context of the main objective of this research, this depended on the biocatalysts.

Finally, the enzyme stability at pH 7 in the presence of phosphate was analyzed. The previous incubation of the biocatalysts on metal/phosphate produced in general an increase on enzyme stability, with copper giving the lowest stabilities (in some instances even lower than the no modified enzyme) **(Figure 4.11).**

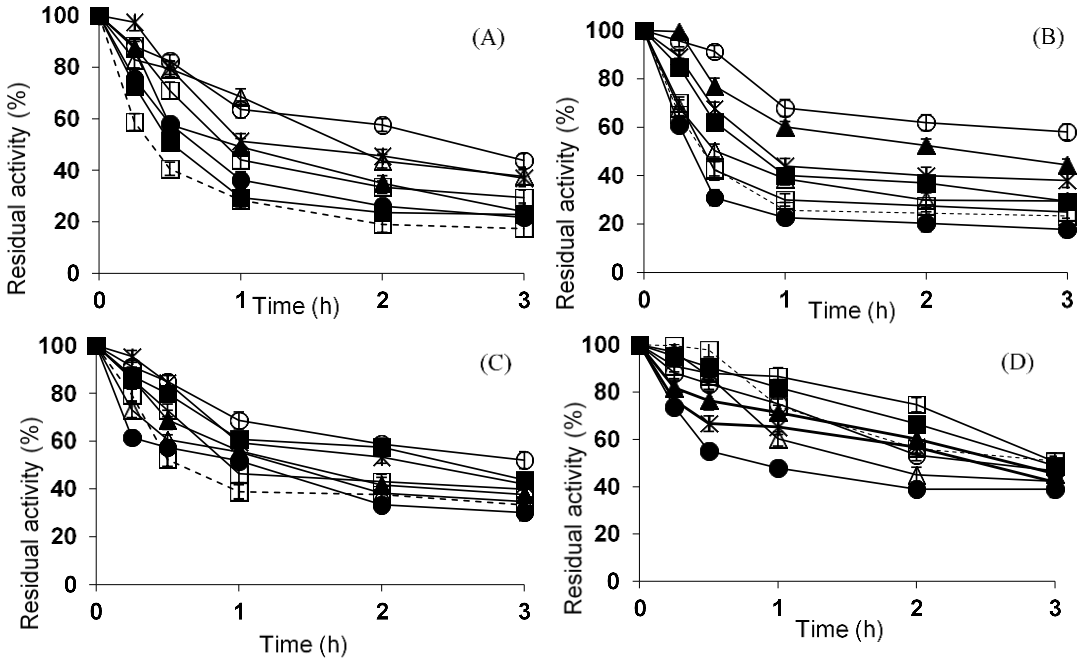

Figure 4. 11 - Inactivation courses of different octyl-ETL biocatalyst, modified with different metals in the presence of phosphate. The biocatalysts were inactivated in 25 mM phosphate at pH 7 and 75 °C. Other specifications are described in Methods. (A): octyl-ETL in 50 mM

sodium acetate at pH 5; (B): octyl-ETL in 50 mM sodium acetate at pH 5 plus 100 mM NaCl; (C): octyl-ETL in 50 mM Tris at pH 7 plus 10% dioxane and (D): octyl-ETL in 50 mM sodium bicarbonate at pH 9 and modified. Control (open square and dotted line); $CaCl_2$ (solid square); $NiCl_2$ (solid triangle); $CoCl_2$ (asterisk); $CuCl_2$ (solid circle); $MgCl_2$ (open square); $ZnCl_2$ (open triangle); $MgCl_2$ (open circle).

The exception is the biocatalysts prepared at pH 9, where many modifications led to a decrease in enzyme stability. Magnesium is the treatment that usually promoted the highest increase on stability, except for the biocatalyst prepared at pH 9, that is manganese. The treatment with metals in TRIS **(Figure 4.12)** offers some changes with the previous case.

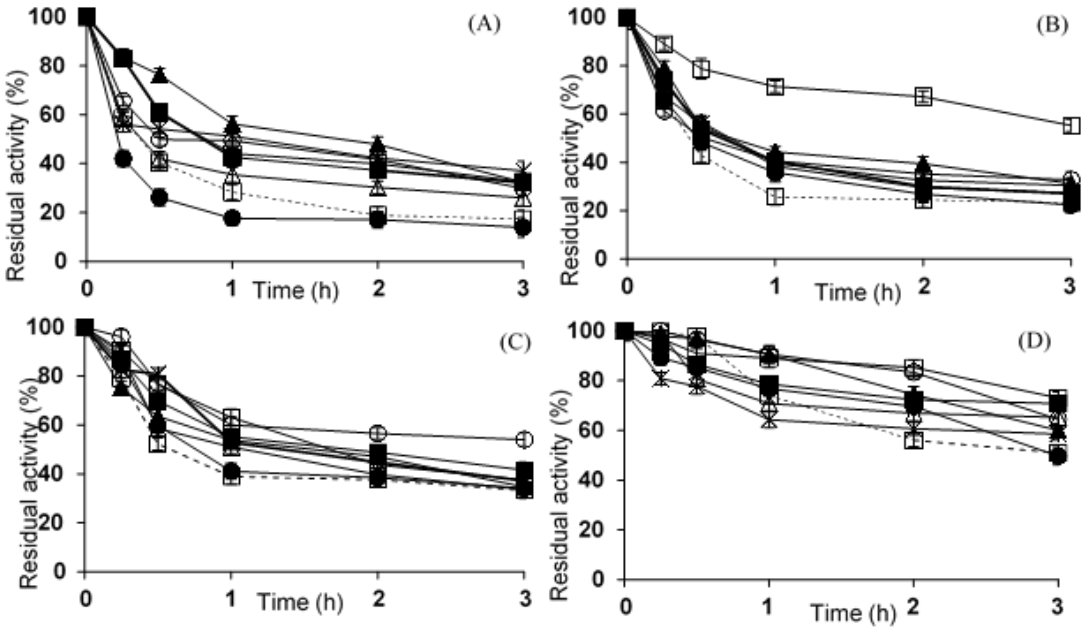

Figure 4. 12 - Inactivation courses of different octyl-ETL biocatalyst, modified with different metals in the presence of Tris. The biocatalysts were inactivated in 25 mM phosphate at pH 7 and 75 °C. Other specifications are described in Methods. (A): octyl-ETL in 50 mM sodium acetate at pH 5; (B): octyl-ETL in 50 mM sodium acetate at pH 5 plus 100 mM NaCl; (C): octyl-ETL in 50 mM Tris at pH 7 plus 10% dioxane and (D): octyl-ETL in 50 mM sodium bicarbonate at pH 9 and modified. Control (open square and dotted line); $CaCl_2$ (solid square); $NiCl_2$ (solid triangle); $CoCl_2$ (asterisk); $CuCl_2$ (solid circle); $MgCl_2$ (open square); $ZnCl_2$ (open triangle); $MgCl_2$ (open circle).

Nickel treatment enhanced the stability of the biocatalyst prepared at pH 5, whereas copper treatment had a detrimental effect, resulting in the lowest stability among all tested conditions. For the biocatalyst prepared at pH 5 plus 100 mM NaCl, an improvement in stability was observed following treatments with all metal treatments, with manganese exhibiting the

most pronounced stabilizing effect. In the case of the biocatalyst prepared in TRIS buffer pH 7 plus 10% dioxane, the highest stability was maintained following magnesium treatment. However, copper ion treatment resulted in increased stability compared to its effect on the biocatalyst prepared in the presence of metal phosphate. Furthermore, treatment with all metal salts led to enhanced stability of the biocatalyst prepared at pH 9. **Figures A4.1** and **A4.2** offers the comparison between the most and least stability biocatalysts for an easy visualization.

4.4. CONCLUSION

The results presented in this paper confirm the great effect of the metal incubation on the properties of immobilized lipases, in this instance Eversa transform 2.0. It has been established that the incubation with phosphate and the metals provide different changes to the incubation in TRIS and metals (in some cases improving the activity or decreasing the enzyme activity if the metal was used with TRIS or phosphate). These changes extend to activity/pH profile, enzyme substrate specificity (including enantiospecificity) and activity, or enzyme stability. Very interestingly, in this new research effort it has been confirmed that the enzyme structure has significant effect on the mineralization effects on the biocatalysts features (using TRIS or phosphate), as results were very different using the 4 biocatalysts, prepared using the same enzyme and support and where the only enzyme structure differed.

These results clearly exemplify how the optimal biocatalyst obtained by mineralization of a lipases should be performed exactly on the enzyme formulation that will be used in the reaction, and evaluated under the operation conditions where the biocatalyst will be utilized.

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APPENDIX

Figure A4. 1 - Biocatalyst octyl-ETL prepared in (1): octyl-ETL immobilized in 50 mM sodium acetate at pH 5; (2): octyl-ETL immobilized in 50 mM sodium acetate at pH 5 plus 100 mM NaCl; (3): octyl-ETL immobilized in 50 mM Tris at pH 7 plus 10% dioxane and (4): octyl-ETL immobilized in 50 mM sodium bicarbonate at pH 9. Other specifications are described in Methods.

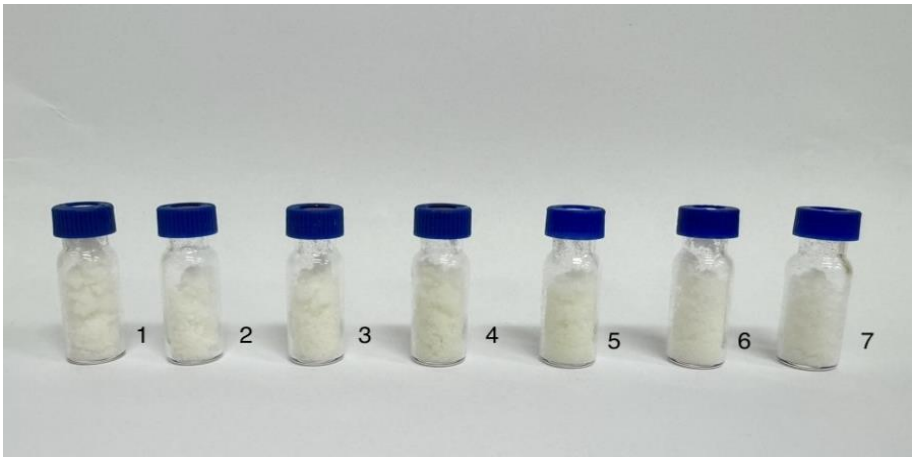

Figure A4. 2 - Biocatalyst octyl-ETL prepared in 50 mM sodium acetate buffer at pH 5, modified with different metals in the presence of Tris. (1) modified with $CoCl_2$; (2) modified with $ZnCl_2$; (3) modified with $CaCl_2$; (4) modified with $CuCl_2$; (5) modified with $NiCl_2$; (6) modified with $MgCl_2$ and (7) modified with $MnCl_2$. Other specifications are described in Methods.

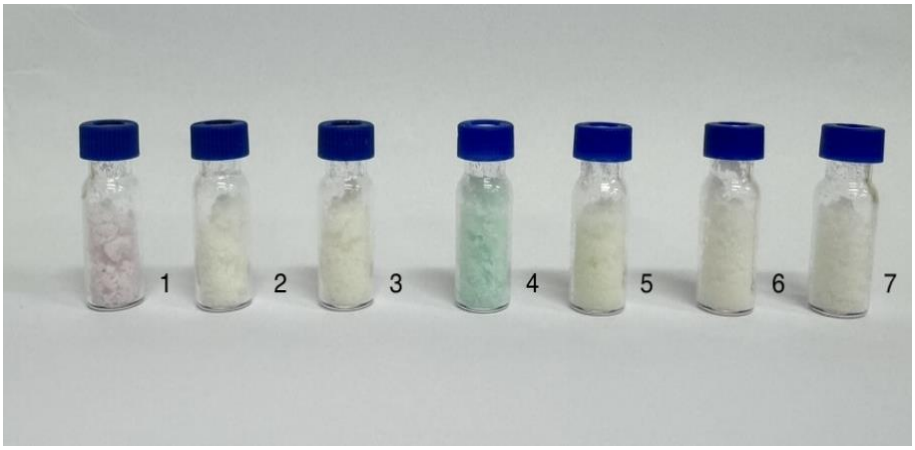

Figure A4. 3 - Biocatalyst octyl-ETL prepared in 50 mM sodium acetate buffer at pH 5, modified with different metals in the presence of phosphate. (1) modified with $CoCl_2$; (2) modified with $ZnCl_2$; (3) modified with $CaCl_2$; (4) modified with $CuCl_2$; (5) modified with $NiCl_2$; (6) modified with $MgCl_2$ and (7) modified with $MnCl_2$. Other specifications are described in Methods

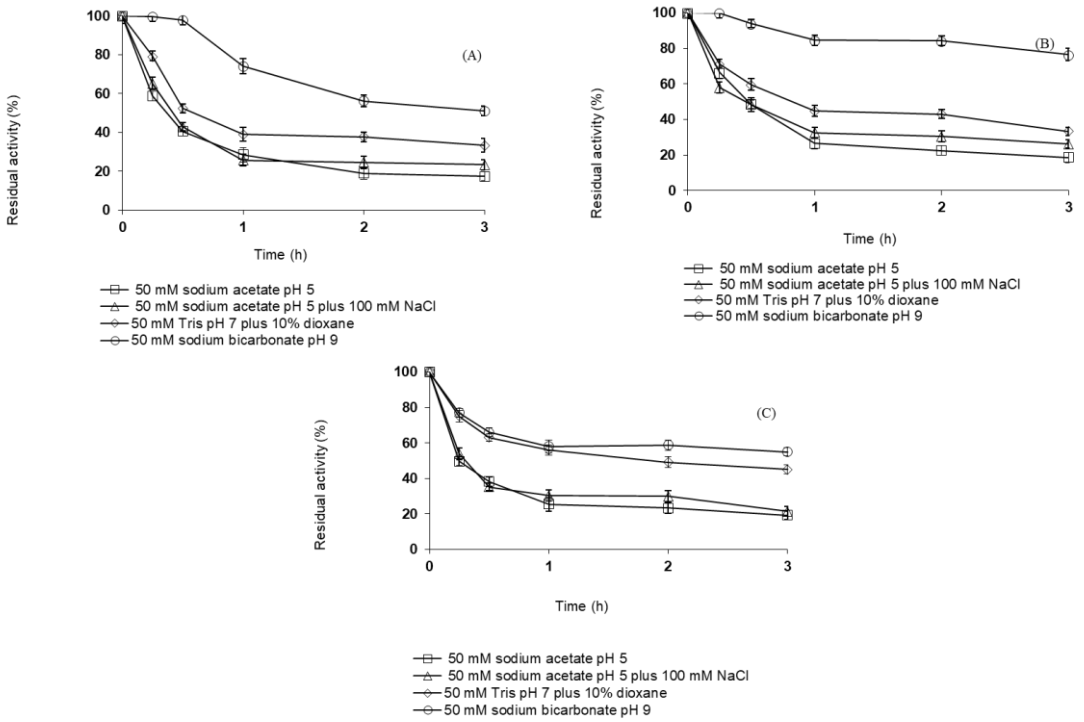

Figure A4. 4 - Inactivation course of untreated octyl-ETL. The biocatalysts were inactivated at 75 °C. Other specifications are described in Methods. (A): octyl-ETL inactivated in 25 mM phosphate pH 7 and 75 °C; (B): octyl-ETL inactivated in 25 mM Tris pH 7 and 75 °C and (C): octyl-ETL inactivated in 25 mM acetate pH 5 and 75 °C.

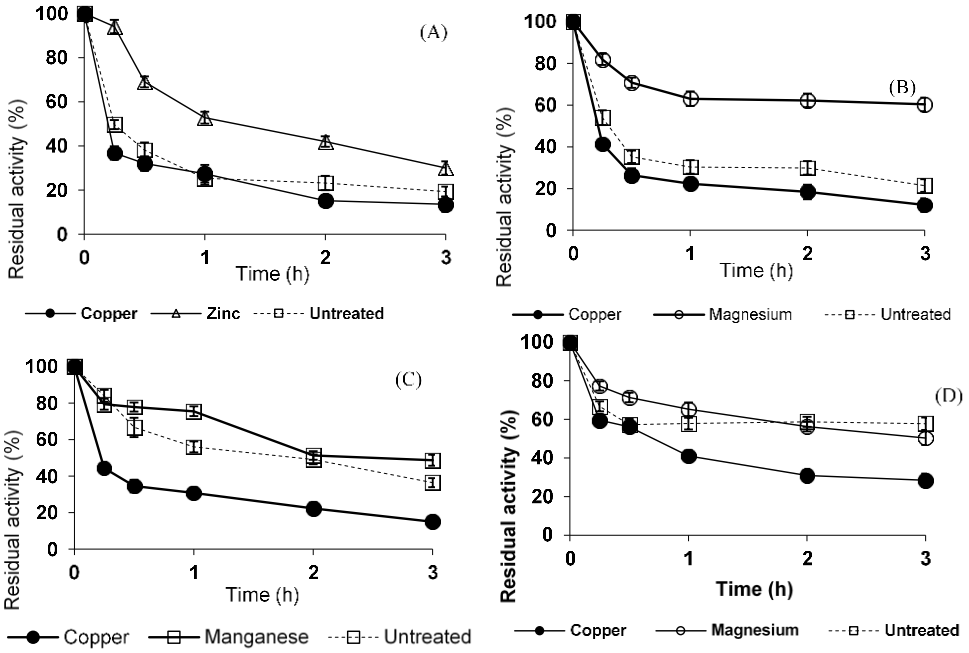

Figure A4. 5 - Inactivation courses of the most and least stable octyl-ETL biocatalyst modified with different metals in the presence of phosphate and unmodified biocatalysts. The biocatalysts were inactivated in 25 mM acetate pH 5 and 75 °C. Other specifications are described in Methods. (A): octyl-ETL immobilized at pH 5 and modified with different metallic salts; (B): octyl-ETL immobilized at pH 5 plus 100 mM NaCl and modified with different metallic salts (C): octyl-ETL immobilized in Tris at pH 7 plus 10% dioxane and modified with different metallic salts and (D): octyl-ETL immobilized in sodium bicarbonate at pH 9 and modified with different metallic salts.

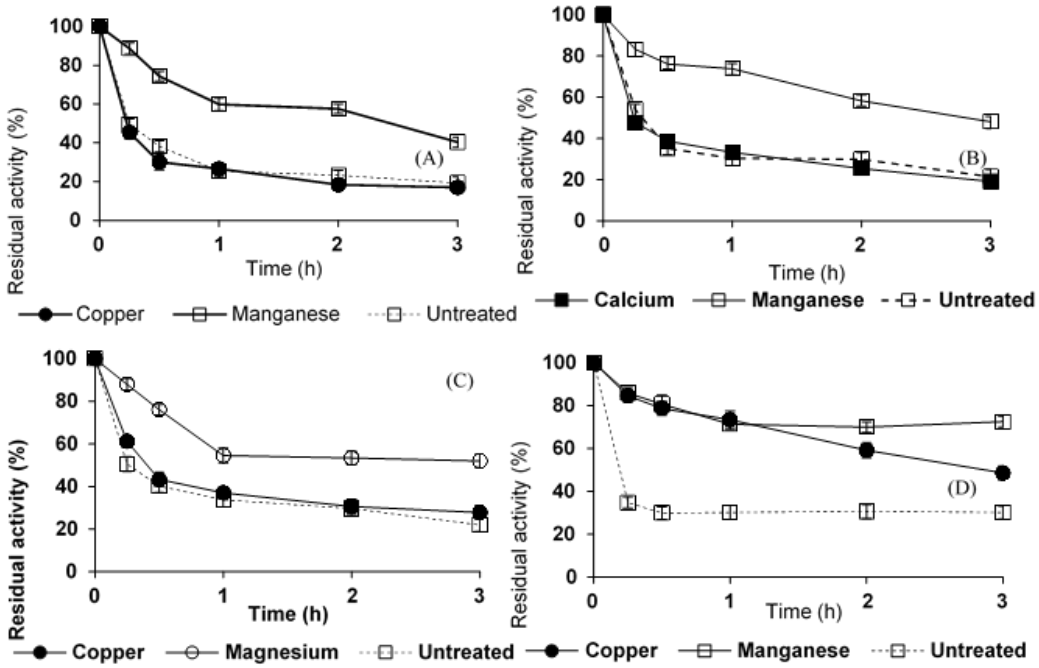

Figure A4. 6 - Inactivation courses of the most and least stable octyl-ETL biocatalyst modified with different metals in the presence of Tris and unmodified biocatalysts The biocatalysts were inactivated in 25 mM acetate pH 5 and 75 °C. Other specifications are described in Methods. (A): octyl-ETL immobilized at pH 5 and modified with different metallic salts; (B): octyl-ETL immobilized at pH 5 plus 100 mM NaCl and modified with different metallic salts (C): octyl-ETL immobilized in Tris at pH 7 plus 10% dioxane and modified with different metallic salts and (D): octyl-ETL immobilized in sodium bicarbonate at pH 9 and modified with different metallic salts.

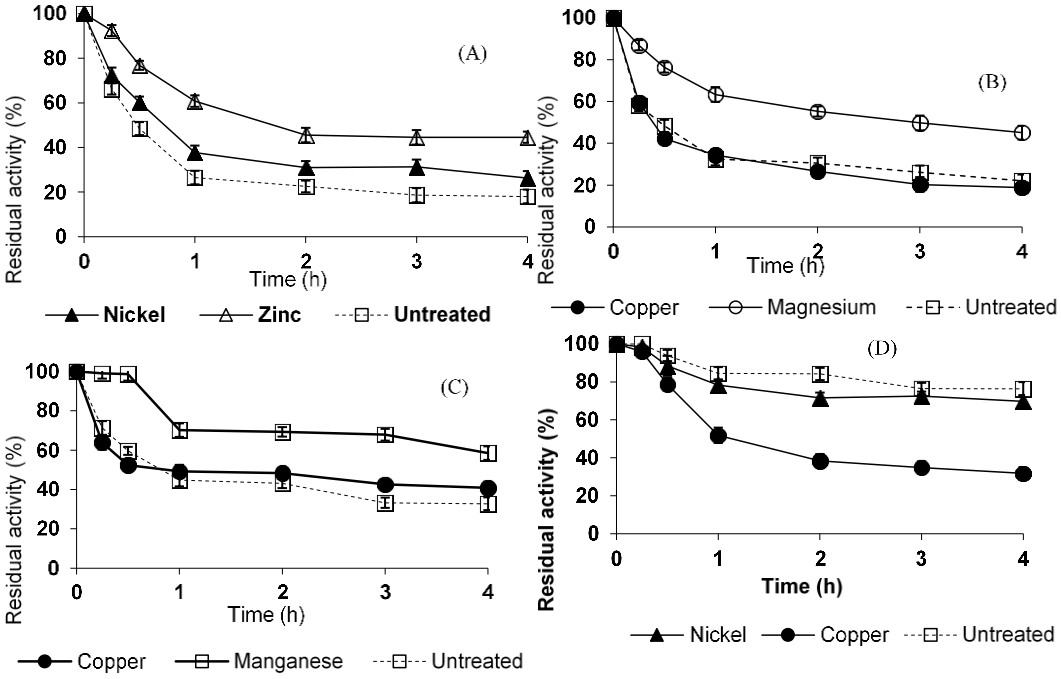

Figure A4. 7 - Inactivation courses of the most and least stable octyl-ETL biocatalyst modified with different metals in the presence of phosphate and unmodified biocatalysts. The biocatalysts were inactivated in 25 mM Tris pH 7 and 75 °C. Other specifications are described in Methods. (A): octyl-ETL immobilized at pH 5 and modified with different metallic salts; (B): octyl-ETL immobilized at pH 5 plus 100 mM NaCl and modified with different metallic salts (C): octyl-ETL immobilized in Tris at pH 7 plus 10% dioxane and modified with different metallic salts and (D): octyl-ETL immobilized in sodium bicarbonate at pH 9 and modified with different metallic salts.

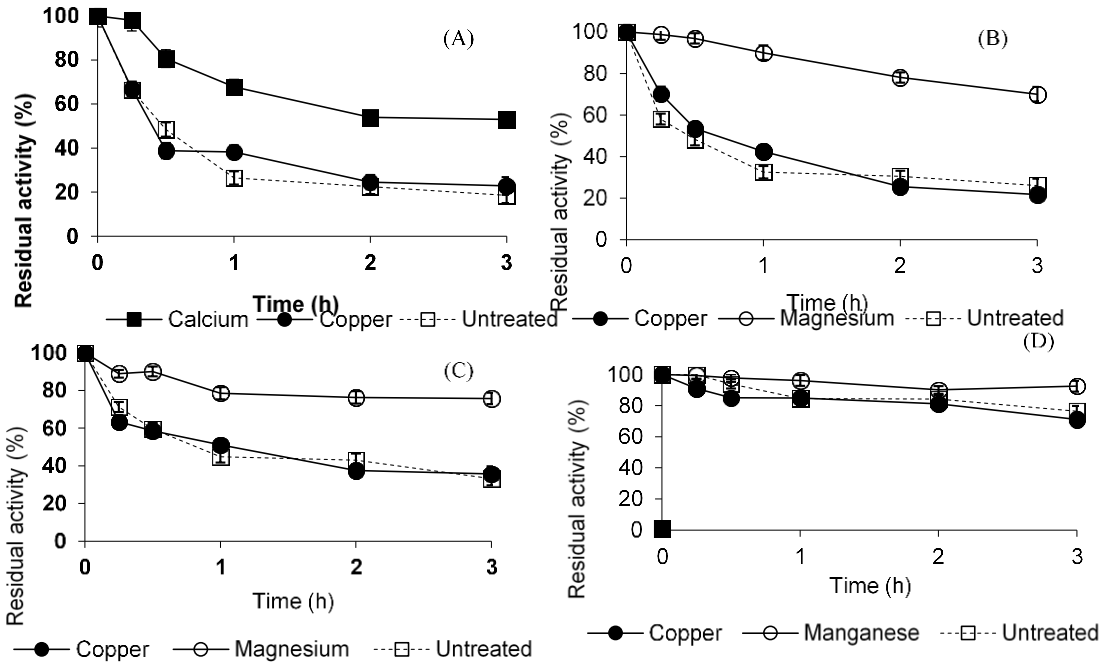

Figure A4. 8 - Inactivation courses of the most and least stable octyl-ETL biocatalyst modified with different metals in the presence of Tris and unmodified biocatalysts The biocatalysts were inactivated in 25 mM Tris pH 7 and 75 °C. Other specifications are described in Methods. (A): octyl-ETL immobilized at pH 5 and modified with different metallic salts; (B): octyl-ETL immobilized at pH 5 plus 100 mM NaCl and modified with different metallic salts (C): octyl-ETL immobilized in Tris at pH 7 plus 10% dioxane and modified with different metallic salts and (D): Eversa immobilized in sodium bicarbonate at pH 9 and modified with different metallic salts.

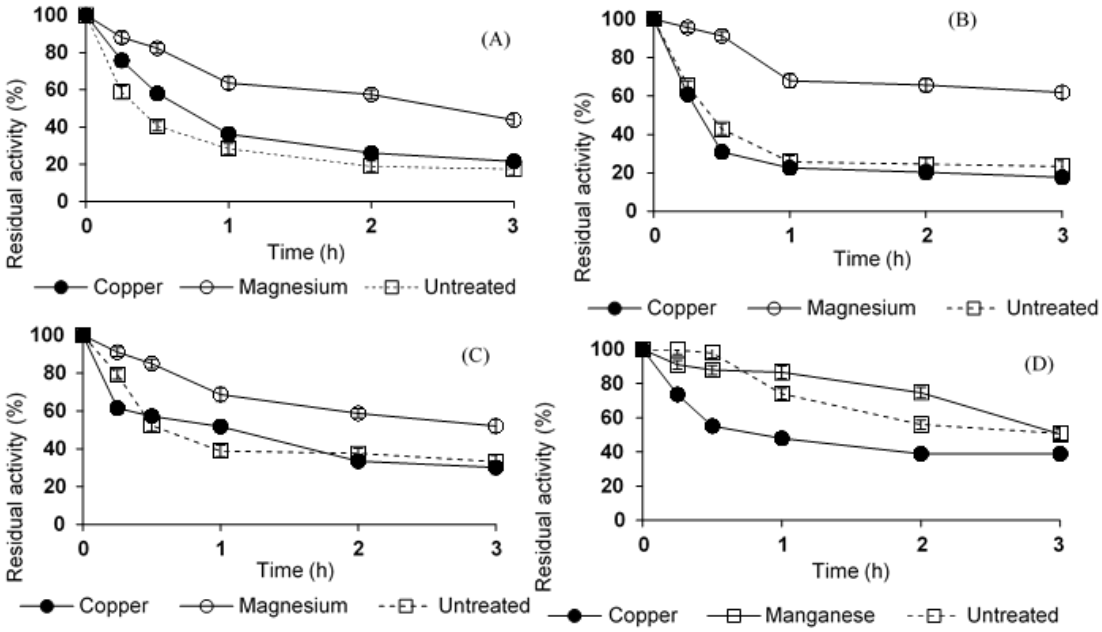

Figure A4. 9 - Inactivation courses of the most and least stable octyl-ETL biocatalyst modified with different metals in the presence of phosphate and unmodified biocatalysts. The biocatalysts were inactivated in 25 mM phosphate at pH 7 and 75 °C. Other specifications are described in Methods. (A): octyl-ETL immobilized at pH 5 and modified with different metallic salts; (B): octyl-ETL immobilized at pH 5 plus 100 mM NaCl and modified with different metallic salts (C): octyl-ETL immobilized in Tris at pH 7 plus 10% dioxane and modified with different metallic salts and (D): octyl-ETL immobilized in sodium bicarbonate at pH 9 and modified with different metallic salts.

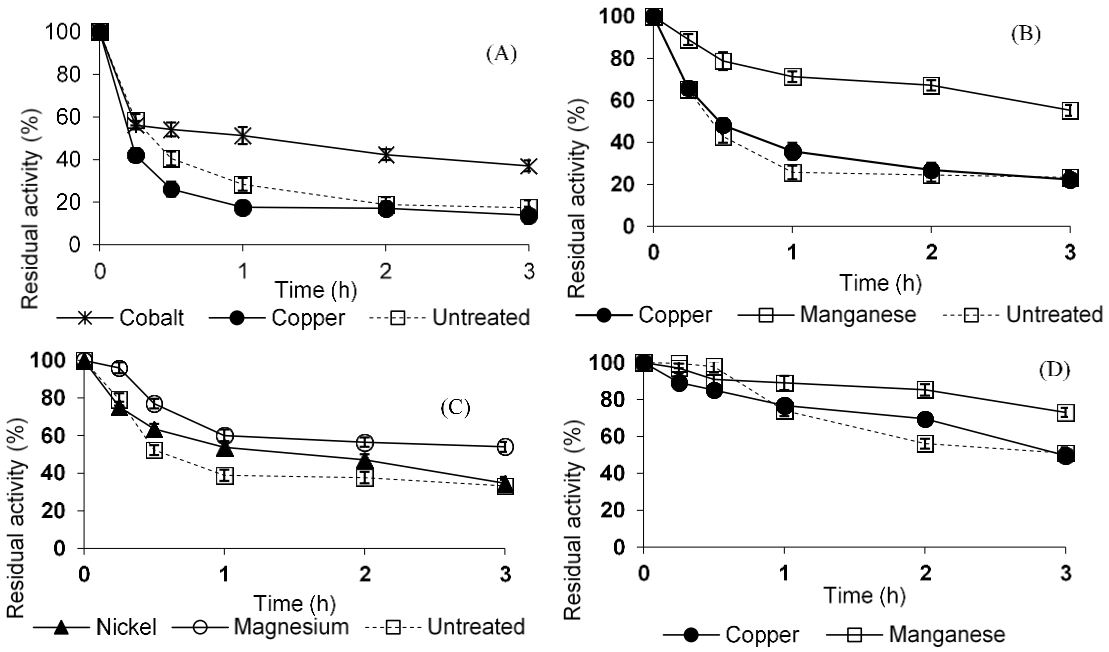

Figure A4. 10 - Inactivation courses of the most and least stable octyl-ETL biocatalyst modified with different metals in the presence of Tris and unmodified biocatalysts. The biocatalysts were inactivated in 25 mM phosphate at pH 7 and 75 °C. Other specifications are described in Methods. (A): octyl-ETL immobilized at pH 5 and modified with different metallic salts; (B): octyl-ETL immobilized at pH 5 plus 100 mM NaCl and modified with different metallic salts (C): octyl-ETL immobilized in Tris at pH 7 plus 10% dioxane and modified with different metallic salts and (D): octyl-ETL immobilized in sodium bicarbonate at pH 9 and modified with different metallic salts.

CHAPTER V

EFFECT OF pH VALUE ON THE IMMOBILIZATION OF *Candida antarctica* LIPASE B AND EVERSA® TRANSFORM 2.0 FOR THE DEVELOPMENT OF BIOCATALYSTS FOR XYLOSE ESTER SYNTHESIS

Sugar esters (SEs) are nonionic surfactants that have attracted considerable attention as sustainable alternatives to petroleum-derived surfactants due to their biodegradability, low toxicity, and compatibility with renewable feedstocks. Although enzymatic routes offer significant environmental and operational advantages over conventional chemical synthesis, their industrial implementation remains limited by issues related to enzyme stability, activity, and reuse. Enzyme immobilization is a well-established strategy to overcome these limitations and can also be exploited to modulate biocatalyst properties. In this study, the effect o pH during immobilization on the catalytic performance, stability, structure, and substrate specificity of lipase B from *Candida antarctica* (CALB) and Eversa® Transform 2.0 (ETL) was systematically investigated. Both enzymes were immobilized on Purolite® C18 via interfacial hydrophobic adsorption at pH 5, 7, and 9. The resulting biocatalysts were evaluated in hydrolysis, esterification, and transesterification reactions, as well as in xylose ester synthesis using fatty acids of different chain lengths. Thermal stability was assessed in aqueous and organic media, and immobilization-induced structural changes were analyzed by FTIR spectroscopy. High immobilization yields (>80%) were obtained under all conditions, with pH exerting no significant influence on adsorption efficiency but markedly affecting catalytic behavior. CALB immobilized at pH 5 exhibited enhanced esterification activity and higher selectivity in xylose ester synthesis, whereas pH 7 favored transesterification and pH 9 increased hydrolytic activity. In contrast, ETL showed optimal activity, stability, and substrate conversion when immobilized at pH 9, while acidic conditions were detrimental. FTIR analyses revealed pH-dependent alterations in secondary structure, particularly in α-helix and β-sheet contents, which correlated with variations in hydrolytic activity for both enzymes. Overall, this study demonstrates that pH during immobilization is a key parameter for tailoring the structure–function relationships of lipases, providing valuable insights for the design of robust biocatalysts for sugar ester synthesis and related biotechnological applications.

5.1. INTRODUCTION

Surfactants are amphiphilic molecules required in numerous industrial processes, ranging from cleaning applications to product formulation, due to their surfactant, emulsifying, lubricating, and stabilizing properties [1,2]. Sugar esters (SEs) are formed through the esterification of a sugar moiety (polar portion) with an acyl group (nonpolar portion) [1]. Both substrates can be obtained from renewable and low-cost sources [3].

In addition, SEs are widely recognized for their biodegradability, low toxicity, and low irritability [1–9], highlighting their strong potential for applications in the food, cosmetic, agricultural, and pharmaceutical industries [1,6]. SEs also stand out for their potential nutritional applications, as they can serve as a source of essential fatty acids such as omega-3 and omega-6 [10], in addition to exhibiting antimicrobial properties [1].

Although the first reports on the enzymatic synthesis of sugar esters date back to the late nineteenth century, most commercially available SEs are still produced predominantly via chemical synthesis [1] . Nevertheless, the enzymatic route offers several advantages, including the use of mild reaction conditions and a more environmentally friendly process [1,11]. Lipases (triacylglycerol acyl hydrolases, EC 3.1.1.3 – IUPAC) are the most widely applied enzymes as biocatalysts, since, in addition to triglyceride hydrolysis, they are capable of catalyzing a broad range of reactions, such as esterification, transesterification, alcoholysis, acidolysis, and aminolysis [12,13]. However, the industrial application of lipases, as with other enzymes, is often limited by their low stability under operational conditions and the challenges associated with enzyme recovery and reuse [12,13].

Heterogeneous biocatalysis, through the use of immobilized enzymes, offers effective strategies to overcome these limitations by enabling biocatalyst reuse and allowing the modulation of enzyme stability, activity, and specificity [13–15]. Changes in enzyme properties during immobilization are mainly associated with conformational alterations in the protein secondary structure, which may affect substrate accessibility and molecular rigidity [14,15].

Fourier transform infrared spectroscopy (FTIR) is a powerful technique for investigating protein secondary structures [16]. The amide I region (1700–1600 cm^{-1}) is commonly used to identify α-helix (1650–1658 cm^{-1}), β-sheet (1610–1640 cm^{-1}), β-turn (1660–1700 cm^{-1}), and random coil structures (1640–1650 cm^{-1}), as it is dominated by a limited number of molecular vibrations [16–19]. The resulting spectra can be analyzed using different approaches, such as deconvolution of characteristic peaks to estimate the relative

contribution of each structural element or correlation analysis based on the second derivative of spectra [16].

As previously shown **(Figure 4.1),** the immobilization pH of Eversa® Transform 2.0 (ETL) drastically affected its catalytic activity. Therefore, in this work, we focused on this variable and its influence on protein structural modifications, using ETL as well as *Candida antarctica* B lipase (CALB) as model enzymes. CALB has been widely used in the synthesis of SEs, both in its free and immobilized forms [11]. This enzyme exhibits high esterification activity, enantioselectivity, and high yields under mild reaction conditions [11]. In turn, ETL is a liquid formulation of *Thermomyces lanuginosus* lipase (TLL), genetically engineered and produced by submerged fermentation of the genetically modified microorganism *Aspergillus oryzae* [20], and is specially formulated for biodiesel synthesis [21]. Due to its low cost compared to the other commercial lipases [22], ETL may represent an attractive biocatalyst for the synthesis of SEs. Therefore, theses lipases were hydrophobically adsorbed onto an octadecyl inorganic resin, with the aim of applying them in the synthesis of xylose esters.

5.2. MATERIALS AND METHODS

5.3. Materials

Purolite® Lifetech™ ECR8806F beads (a macroporous methacrylate resin functionalized with octadecyl (C18) groups) were kindly donated by Purolite® Ltd. *Candida antarctica* lipase B (CALB) (total protein content determined by the Bradford method: 46.55 mg/mL) and Eversa® Transform 2.0 (total protein content determined by the Bradford method: 34.35 mg/mL), butyric acid, lauric acid, oleic acid, tributyrin, 3 Å molecular sieves, and ethyl heptadecanoate were purchased from Sigma Chemical Co. (St. Louis, USA). Methyl ethyl ketone (MEK) was obtained from Neon (São Carlos, Brazil). Xylose, butanol, and heptane were purchased from Synth (São Paulo, Brazil). All other reagents and solvents were of analytical grade and used without further purification.

5.4. Immobilization on Purolite C18 Support

Purolite C18 beads were used as supports for the immobilization of CALB and ETL lipases by interfacial hydrophobic adsorption at a support-to-solution ratio of 1:10 (m/v) and an enzyme loading of 25 mg of enzyme per gram of support. The commercial enzyme solutions were diluted to the appropriate volume in 50 mM acetate buffer (pH 5), 50 mM TRIS buffer (pH 7), or 50 mM bicarbonate buffer (pH 9). The support–enzyme suspensions were maintained under agitation at 25 °C, and the immobilization process was monitored by determining the hydrolytic activity of the reference suspension (enzyme solution without support) and the supernatant from the suspension containing both the enzyme and the support. [23].

5.5. Thermal Stability

The thermal stability of the produced biocatalysts was evaluated under two different conditions by incubating them at a concentration of 0.5% (w/v) in 50 mM TRIS–HCl buffer (pH 7) and methyl ethyl ketone for 6 h at 60 °C. Hydrolytic activity was measured before and after incubation. [24].

5.6. Enzymatic Synthesis of Xylose Esters

Xylose ester synthesis was carried out by enzymatic esterification of commercial xylose with oleic, lauric, and butyric acids at a 1:5 molar ratio (xylose:free fatty acid), using methyl

ethyl ketone to solubilize xylose. The reactions were performed in an orbital shaker (Marconi, MA 832/H) at 50 °C and 250 rpm, employing 10 g of biocatalyst per gram of xylose. Samples were collected at 0 and 24 h, and the free fatty acid concentration was determined by gas chromatography [25].

5.7. Analytical Methods

5.7.1. Hydrolytic Activity

The hydrolytic activity of the biocatalysts was determined by tributyrin hydrolysis in 100 mM phosphate buffer (pH 7.3) at 37 °C for 300 s. The amount of butyric acid released was titrated with 20 mM KOH using a pH-Stat titrator (Titrando 907, Metrohm, Herisau, Switzerland). Enzymatic activity was expressed as the amount of acid released per minute. One tributyrin hydrolytic unit (TBU) was defined as the rate of micromoles of butyric acid produced per minute under the conditions described. This method is an adaptation of the procedure described by Beisson *et al* [26].

5.7.2. Esterification Activity

The esterification activity of the immobilized biocatalysts was determined by butyl butyrate production at 40 °C, using 0.1 M butyric acid and 0.1 M tert-butanol in 15 mL of heptane, with the addition of 0.2 g of 3 Å molecular sieves. Gas chromatography was used to quantify butyl butyrate formation and butanol consumption. One esterification activity unit (U_{est}) was defined as the initial rate of butyl butyrate production (µmol/min) under the conditions described above [25].

5.7.3. Transesterification Activity

The transesterification activity of the biocatalysts was evaluated using the ethanolysis of olive oil (high oleic acid content), employing a Discover® microwave reactor (CEM Co., Matthews, USA) to achieve high reaction rates. Transesterification was carried out at 40 °C, using an olive oil/anhydrous ethanol molar ratio of 1:7 and 0.05 g of biocatalyst. All assays were performed at least in duplicate. Aliquots (400 µL) were withdrawn from the reaction medium at 30 min intervals for up to 3 h and analyzed by gas chromatography to quantify fatty acid ethyl esters (FAEE). Prior to analysis, samples were pretreated by phase separation, in which the oily phase (FAEE and unreacted acylglycerols) was recovered and dried at 60 °C for 24 h. One transesterification activity unit (U_{trans}) was defined as the initial rate of FAEE production (µmol FAEE per gram of sample per minute) under the conditions described above

[27].

5.7.4. Determination of Free Fatty Acids

The concentration of oleic acid in the reaction medium samples was quantified by gas chromatography using an Agilent 7890A system equipped with a flame ionization detector (FID), a split-splitless injector (split ratio 40:1) and an HP-INNOWAX capillary column (Agilent Technologies, Santa Clara, CA, USA). Compound separation was performed maintained at 200 °C for 1 min, 230 °C for 1 min (10 °C/min), and 250 °C for 5 min (5 °C/min). Helium was used as carrier gas at a flow rate of 1.8 mL/min, with a run time of 14 min, with helium as the carrier gas [28].

5.7.5. Determination of Fatty Acid Ethyl Ester Concentration

Samples (50 mg) were diluted in 10 mL of an ethyl heptadecanoate solution (10 mg/mL), used as an internal standard. Aliquots (1 µL) were injected into an Agilent 7890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a Restek 12423 capillary column (30 m × 0.25 mm × 0.25 µm), a split-splitless injector (split ratio 40:1) and a flame ionization detector (FID) operated at 250 °C. The analysis was carried out over 18 min using nitrogen as the carrier gas (25 mL/min), with the following temperature program: 150 °C for 2 min, ramp to 180 °C at 10 °C/min and held for 3 min, ramp to 230 °C at 10 °C/min, and held for 5 min [29].

5.7.6. Secondary Structure Analysis by FTIR

ATR–FTIR spectra were analyzed focusing on the amide I region, covering the wavenumber range of 1700–1600 cm^{-1}. Peak positions were identified using the second-derivative method, and peak deconvolution was performed using Origin 8.5 software [30]. The degree of similarity of the second-derivative spectra, obtained in transmittance mode within the amide I region for each biocatalyst, was evaluated using the QC similarity comparison (QCCS) function available in the OMNIC software (Thermo Scientific) [31].

5.7.7. Statistical Analysis

Data were subjected to analysis of variance (ANOVA), and statistical significance was determined using the F-test at a 5% significance level ($p \leq 0.05$). Mean comparisons were performed using Tukey's test at the same significance level. All analyses were conducted using SISVAR software, version 5.6 [32].

5.8. RESULTS AND DISCUSSION

5.8.1. Enzyme immobilization under different conditions

Figures 5.1 and **5.2** show the immobilization profiles of CALB and ETL on Purolite C18. The lipases were immobilized at an enzyme loading of 25 mg of enzyme per gram of support under three conditions: 50 mM sodium acetate buffer (pH 5), 50 mM TRIS–HCl buffer (pH 7), and 50 mM sodium bicarbonate buffer (pH 9). The hydrolytic activity of the reference solutions remained unchanged throughout the immobilization process, indicating that the observed variations in the enzyme activity were not associated with enzyme inactivation caused by the immobilization conditions.

The immobilization profile of ETL **(Figure 5.1)** showed similar behavior at all three evaluated pH values, reaching immobilization yields after 4 h of 92.3%, 92.6%, and 89.7% at pH 5, 7, and 9, respectively.

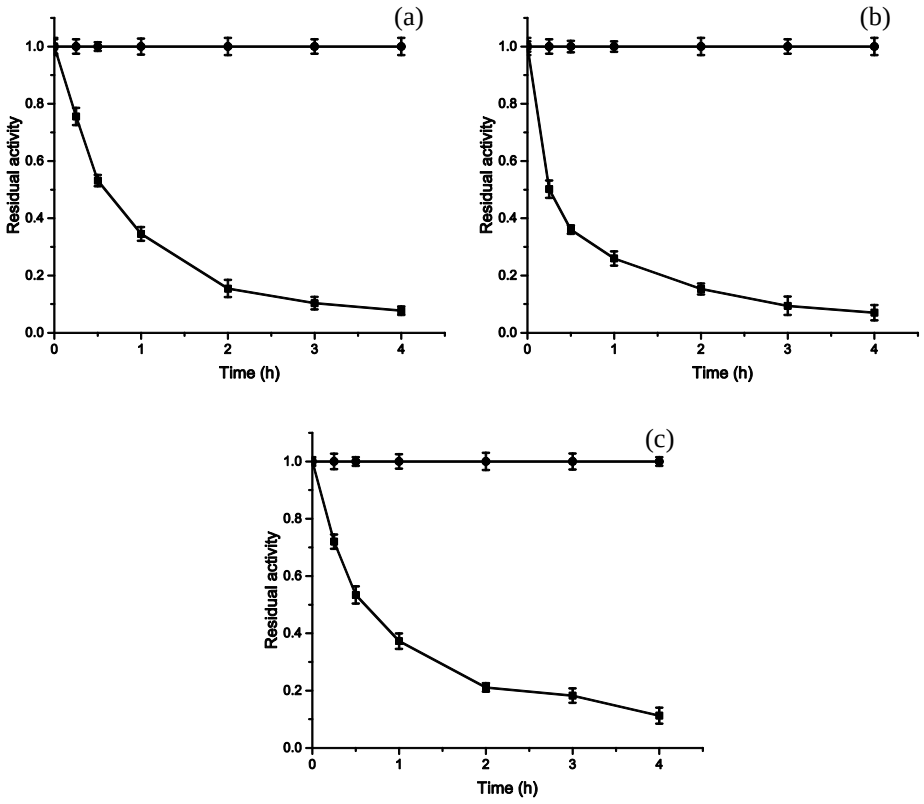

Figure 5.1 – Immobilization profiles of Eversa® Transform 2.0 (ETL) on Purolite C18 at an enzyme loading of 25 mg/g in (a) 50 mM acetate buffer, pH 5; (b) 50 mM TRIS buffer, pH 7; and (c) 50 mM bicarbonate buffer, pH 9. The circles represent the reference solution and the squares the supernatant.

The immobilization profiles of CALB **(Figure 5.2)** exhibited a higher immobilization

rate compared to ETL, with an approximately 80% reduction in supernatant activity within the first 30 min of immobilization. Immobilization yields at pH 5, 7, and 9 were 94.2%, 95.9%, and 95.2%, respectively. No significant differences in the immobilization profiles were observed as a function of pH variation.

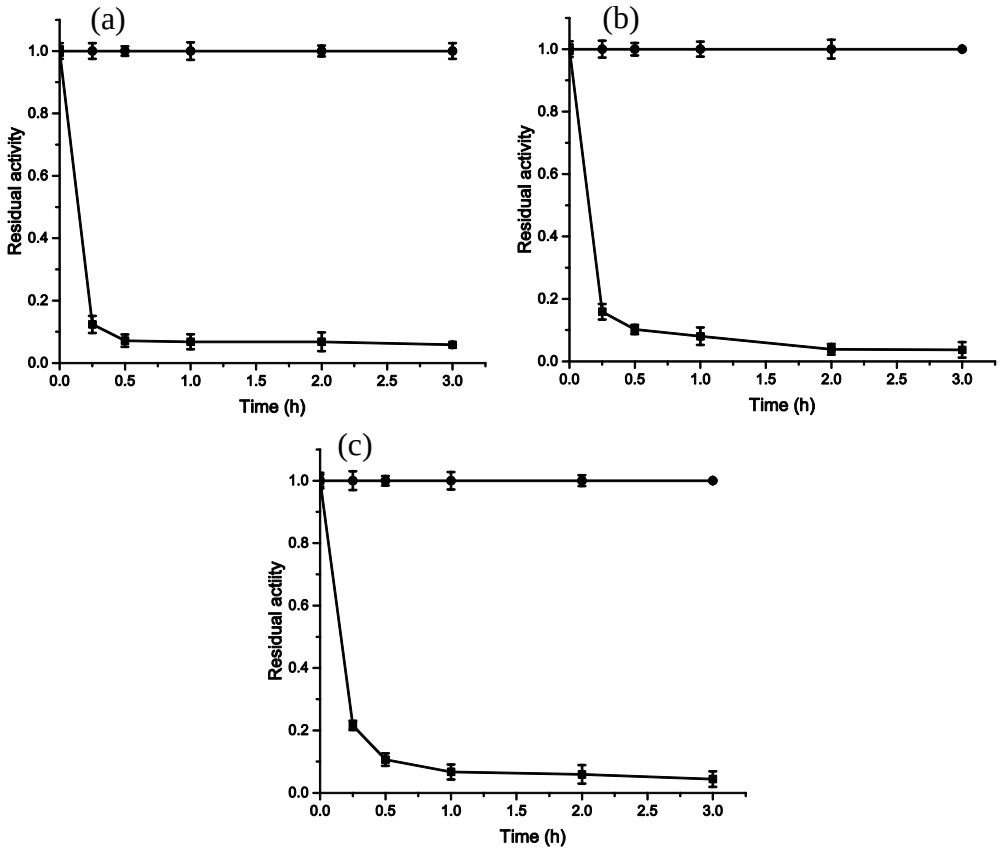

Figure 5.2 – Immobilization profile of lipase B from *Candida antarctica* (CALB) on Purolite C18 at an enzyme loading of 25 mg/g in (a) 50 mM acetate buffer, pH 5; (b) 50 mM TRIS buffer, pH 7; and (c) 50 mM bicarbonate buffer, pH 9. The circles represent the reference solution and the squares the supernatant.

The immobilization profiles of ETL and CALB did not exhibit significant changes with pH variation, this behavior was also reported by Sabi *et al.* (2025) during the immobilization of ETL on octyl-agarose support [14]. CALB exhibited a high immobilization rate, reaching approximately 80% yield within the first 30 min, whereas ETL achieved only about 50% over the same period. This difference in immobilization behavior can be attributed to the structural differences between the lipases, mainly related to the size of the lid domain. ETL has a large lid, which leads to an equilibrium between open and closed conformations in solution. In contrast, the small and rigid lid of CALB restricts the closed conformation, thereby facilitating

faster adsorption onto hydrophobic supports [13,35,36].

5.8.2. Effect of pH during immobilization on hydrolysis, esterification, and transesterification activities.

To evaluate the effect of pH during immobilization on biocatalyst performance, hydrolytic activity toward tributyrin, esterification activity during butyl butyrate synthesis, and transesterification activity using olive oil and ethanol were assessed. The obtained activities are presented in **Figure 5.3**.

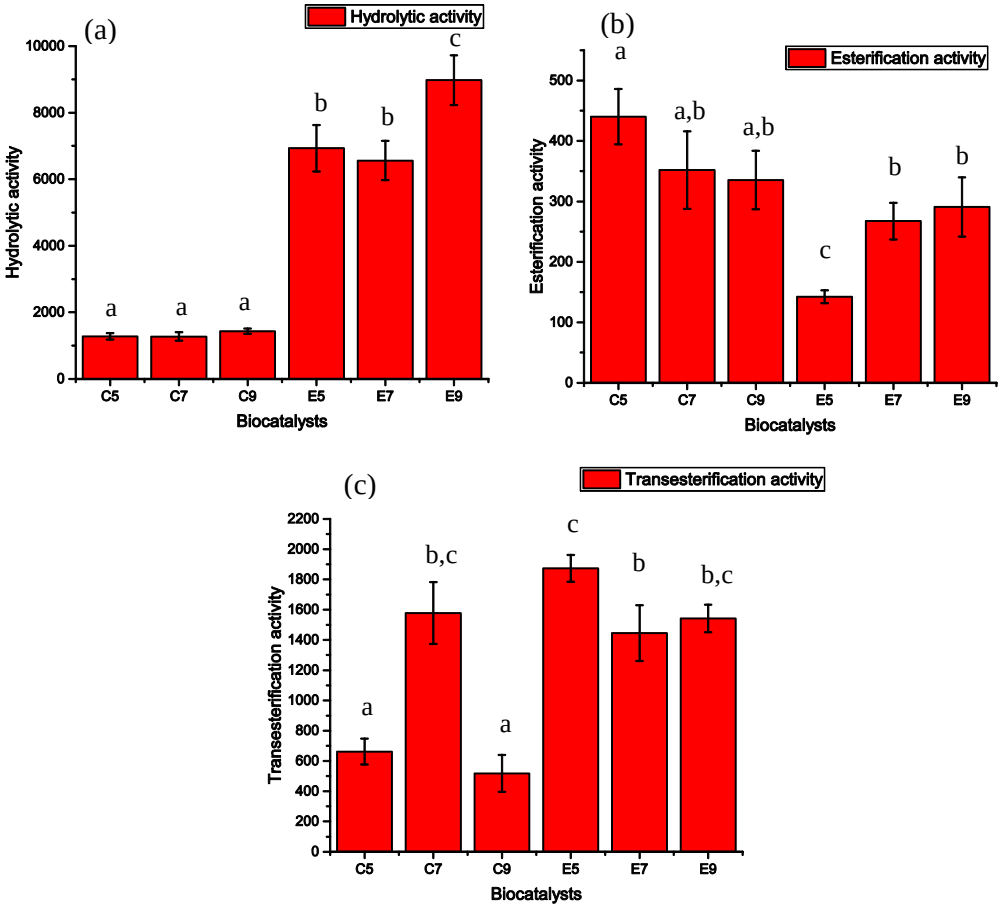

Figure 5.3 – Hydrolytic (a), esterification (b), and transesterification (c) activities of ETL and CALB immobilized on Purolite C18 in 50 mM acetate buffer (pH 5), 50 mM TRIS buffer (pH 7), and 50 mM bicarbonate buffer (pH 9). Different letters (a-c) indicate statistically significant differences according to Tukey's test (5% significance level).

The immobilization conditions significantly affected the catalytic activities of the resulting biocatalysts. CALB immobilized at pH 5 and 7 **[Figure 5.3 (a)]** showed no differences in hydrolytic activity; however, immobilization at pH 9 resulted in 13.15% increase

in activity. In terms of esterification **[Figure 5.3 (b)]**, CALB immobilized at pH 5 exhibited approximately 24% higher activity than enzymes immobilized at pH 7 and 9. In contrast, transesterification activity **[Figure 5.3 (c)]** was maximized when CALB was immobilized at pH 7, showing an increase of more than 130% compared to the other conditions.

The influence of immobilization pH on CALB activity can be largely attributed to changes in the protonation state of the catalytic triad residues (serine, histidine, and aspartate). The isoelectric point of CALB is approximately pH 6; therefore, under neutral or basic conditions, the enzyme is predominantly in its deprotonated form. Previous studies have shown that, for free CALB, the protonation state of amino acid residues within the catalytic site plays a key role in modulating hydrolytic activity [37,38]. Świderek *et al*.(2023) reported that free CALB exhibited higher regioselectivity toward bis(2-hydroxyethyl) terephthalate hydrolysis at pH 5 compared to pH 7 and pH 9, highlighting the sensitivity of catalytic behavior to pH [38].

Similarly, ETL immobilized at pH 5 and 7 showed comparable hydrolytic activities, whereas immobilization at at pH 9 led to an increase of more than 29%. In contrast, esterification activity was markedly reduced (approximately 46%) when ETL was immobilized at pH 5. The most pronounced effect was observed for transesterification activity, which decreased by around 80% under acidic immobilization conditions.

ETL is derived from the *Thermomyces lanuginosus* lipase (TLL), whose isoelectric point is approximately pH 4.4. Consequently, under neutral and basic immobilization conditions, the enzyme is expected to remain predominantly in a deprotonated state [39,40]. As shown in **Figure 5.3**, esterification and transesterification activities increased progressively with increasing immobilization pH, while the highest activities were observed at pH 9. These results suggest that the deprotonated state of catalytic residues favors the activity of ETL, particularly in synthesis reactions.

Overall, the observed changes in activity suggest that immobilization pH induces conformational changes in enzyme structure, which directly affect catalytic performance [41]. Sabi *et al*. (2025) investigated ETL immobilized on octyl agarose under different pH values, ionic strengths, and desorption conditions, reporting activity changes that depended on the both immobilization parameters and the substrate used. Notably, ETL immobilized at pH 5 exhibited higher hydrolytic activity toward pNPB, whereas immobilization at pH 9 favored triacetin hydrolysis [23]. These findings reinforce that immobilization conditions can be strategically exploited to modulate enzyme activity and substrate specificity.

5.8.3. Thermal Stability

The biocatalysts obtained under the different immobilization pH conditions were incubated for 6 h at 50 °C in 50 mM TRIS–HCl buffer (pH 7) and in methyl ethyl ketone. Hydrolytic activity was measured before and after incubation, and the resulting residual activities are presented in **Figure 5.4** and **Figure 5.5**.

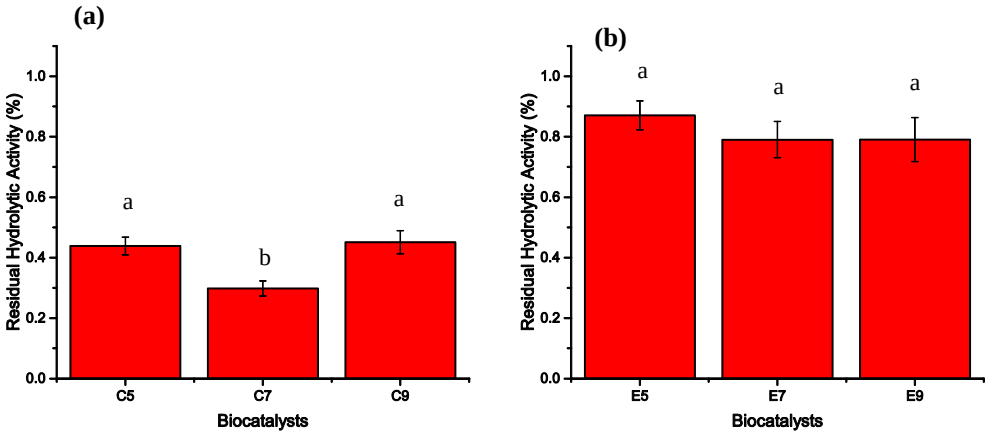

Figure 5. 4 – Residual hydrolytic activity of ETL and CALB immobilized on Purolite C18 in sodium acetate buffer (pH 5), TRIS–HCl buffer (pH 7), and sodium bicarbonate buffer (pH 9) after 6 h of incubation at 50 °C in 50 mM TRIS–HCl buffer (pH 7). Different letters (a-b) indicate statistically significant differences according to Tukey's test (5% significance level).

After incubation in 50 mM TRIS–HCl buffer (pH 7) for 6 h at 50 °C **[Figure 5.4 (a)],** the CALB derivatives immobilized at pH 5 and 9 exhibited approximately 55% reductions in hydrolytic activity, whereas CALB immobilized at pH 7 showed a slightly higher decrease of about 65%. In contrast, ETL immobilized under all three evaluated conditions **[Figure 5.4 (b)]** showed markedly higher stability, with activity reductions below 22%. No statistically significant differences in residual activity were observed among ETL biocatalysts as a functional of immobilization pH. The higher thermal stability of ETL in the aqueous medium may be related to the presence of a larger lid domain, which has an amphiphilic structure and plays a key role in lipase behavior in aqueous environments. In this closed conformation, the hydrophilic face of the lid is preferentially exposed to the medium, protecting the catalytic site and resulting in lower catalytic activity but enhanced structural stability [35].

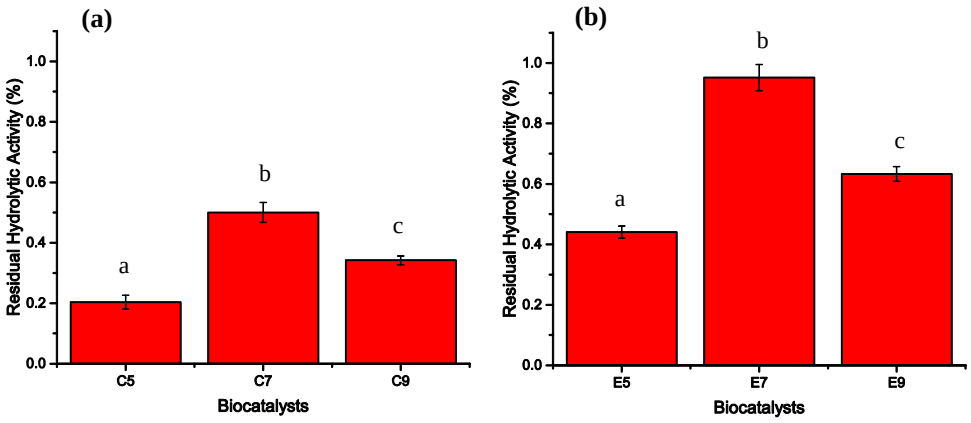

Figure 5. 5 – Residual hydrolytic activity of ETL and CALB immobilized on Purolite C18 in sodium acetate buffer (pH 5), TRIS–HCl buffer (pH 7), and sodium bicarbonate buffer (pH 9) after 6 h of incubation at 50 °C in methyl ethyl ketone. Different letters (a-c) indicate statistically significant differences according to Tukey's test (5% significance level).

When incubated in methyl ethyl ketone **(Figure 5.5)**, both CALB and ETL biocatalysts exhibited more pronounced activity losses. For CALB **[Figure 5.5 (a)]**, activity reductions of approximately 80%, 50%, and 70% were observed after 6 h for biocatalysts immobilized at pH 5, 7, and 9, respectively. ETL **[Figure 5.5 (b)]** showed reductions of about 66%, 5%, and 37% under the same conditions. The greater loss of activity in methyl ethyl ketone is mainly attributed to the ability of hydrophilic organic solvents to disrupt the enzyme solvation layer, leading to partial unfolding or decreased catalytic efficiency [38,41].

Notably, CALB and ETL immobilized at pH 7 exhibited the highest residual activity in methyl ethyl ketone. However, the extent of stability modulation was substantially greater for ETL, indicating that the immobilization pH has a stronger impact on ETL stability than on CALB. This behavior further suggests that the lid domain plays a central role in mediating conformational and stability changes induced by immobilization conditions. Consistent with these findings, Sabi *et al.*(2025) reported enhanced stability for ETL immobilized on octyl agarose at alkaline pH values (pH 9), reinforcing the idea that immobilization parameters can be strategically adjusted to tailor lipase stability [23]. Overall, these results demonstrate that immobilization conditions, particularly pH, can be effectively exploited to modulate the thermal stability of lipases in both aqueous and organic media.

5.8.4. Secondary Structure Analysis by FTIR

To investigate possible changes in the secondary structure of CALB and ETL

immobilized under different pH conditions, Fourier transform infrared (FTIR) spectroscopy was employed in both attenuated total reflectance (ATR) and transmission modes. The ATR-FTIR spectra recorded in the 1600–1700 cm^{-1} range (amide I region), which is primarily associated with C=O stretching vibrations of the polypeptide backbone and commonly used for secondary structure analysis, are shown in **Figures 5.6** and **5.7** for CALB and ETL, respectively. The percentage areas corresponding to α-helix (1650–1658 cm^{-1}), β-sheet (1610–1640 cm^{-1}), β-turn (1660–1700 cm^{-1}), and random coil (1640–1650 cm^{-1}) structures were calculated, and the results are summarized in **Tables 5.1** and **5.2,** respectively.

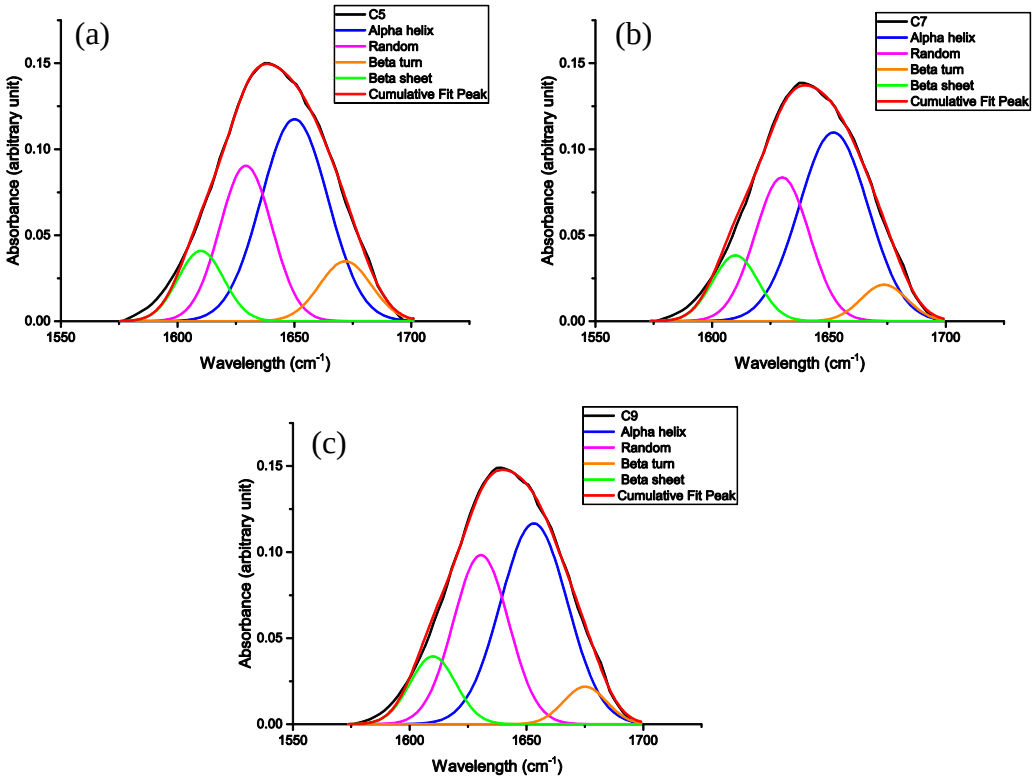

Figure 5. 6 – FTIR-ATR spectra of the amide I region for CALB immobilized at different pH values, after deconvolution.

Table 5. 1– Percentage area of deconvoluted peaks in the amide I region from FTIR-ATR spectra of biocatalysts obtained by immobilizing CALB on Purolite C18 at different pH values.

Biocatalyst	α-Helix (%)	β-Sheet (%)	Random (%)
C5	48.37	22.56	29.07
C7	51.15	18.29	30.56
C9	48.95	17.06	33.99

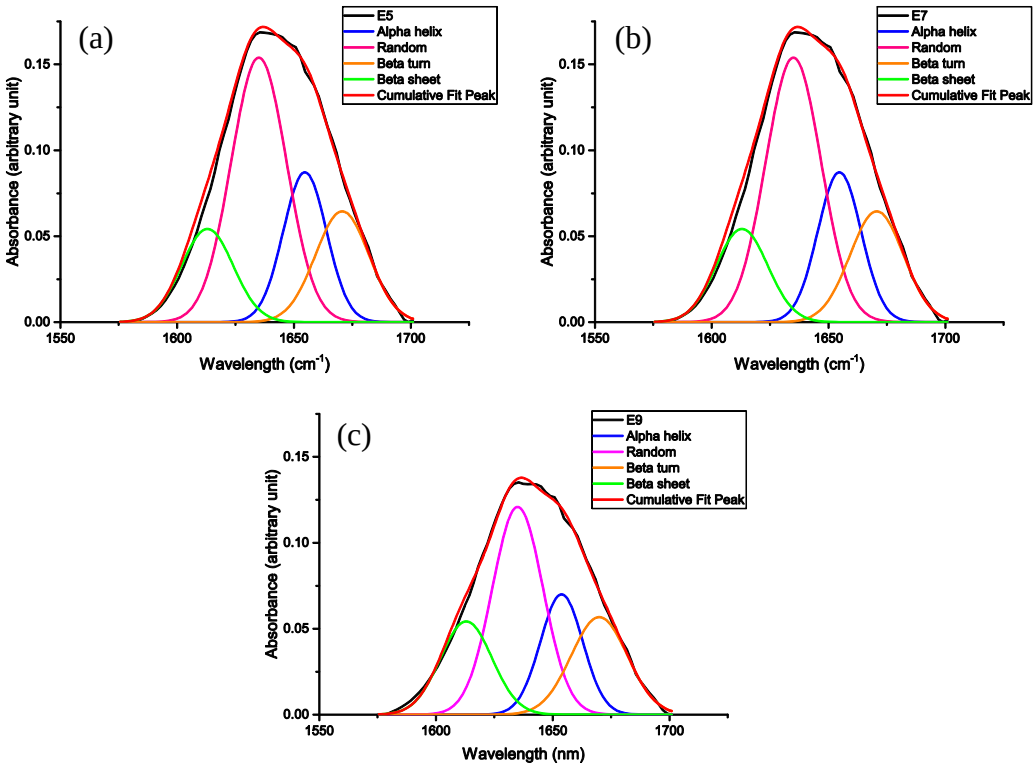

Figure 5. 7 – FTIR-ATR spectra of the amide I region for ETL immobilized at different pH values, after deconvolution.

Table 5. 2 – Percentage area of deconvoluted peaks in the amide I region from FTIR-ATR spectra of biocatalysts obtained by immobilizing ETL on Purolite C18 at different pH values.

Biocatalyst	**α-Helix (%)**	**β-Sheet (%)**	**Random (%)**
E5	18.76	38.33	42.91
E7	20.38	33.67	45.95
E9	19.62	39.61	40.77

CALB immobilized at pH 7 exhibited a higher α-helix content (51.15%), whereas the enzyme immobilized at pH 5 showed a higher percentage of β-sheet percentage (22.56%). A reduction in α-helix content under acidic conditions was also reported by Rabbani *et al*.(2015), who evaluated the α-helix percentage of free CALB by circular dichroism at neutral (pH 7.4) and acidic (pH 2.6) conditions, observing a decrease from 33% to 20% [37]. Moreover, literature reports indicate that a decrease in α-helix content can facilitate substrate access to the active site. Both the present study and previous reports suggest the possibility of modulating enzyme selectivity, whereby activity may increase for one substrate while decreasing for

another [15,23].

Despite the absence of interfacial activation and the presence of a small lid, CALB features two α-helices (α-5 and α-10) surrounding the active site, whose mobility varies with environmental conditions [42]. These α-helices do not participate directly in interfacial activation like the lids of most lipases but they influence catalytic properties such as activity and regioselectivity [42]. In addition to the variation in α-helix content observed in **Table 5.1**, changes in activity and stability can be attributed to the protonation state of amino acids in the catalytic site and to tertiary structure alterations occurring during immobilization [38].

ETL immobilized at pH 7 exhibited the highest α-helix content (20.38%), whereas the enzyme immobilized at pH 9 showed the highest β-sheet content (39.61%). Similar to CALB, ETL exhibited a reduction in α-helix content at lower pH, as reported previoysly [37]. These alterations in α-helix content likely influenced the observed changes in enzymatic activity.

Tables 5.1 and **5.2** indicate that variations in secondary structure percentages can affect substrate accessibility to the active site, leading to modifications in both the activity and specificity [38,43]. Notably, α-helix content is directly related to substrate access; an increase in α-helix content can hinder substrate entry to the lipase active site [38,44]. Therefore, observed changes in activity and stability likely result from a combination of α-helix content, protonation states of catalytic residues, and conformational adjustments of secondary structures in the presence of organic solvent [38].

In addition to FTIR-ATR analyses, transmission-mode FTIR measurements were performed. Second derivative spectra were obtained, and QC Compare function in OMNIC software was used to evaluate spectral similarity in the amide region. **Figure 5.8** shows the second derivative spectra, and **Table 5.3** presents the percentage similarity between spectra.

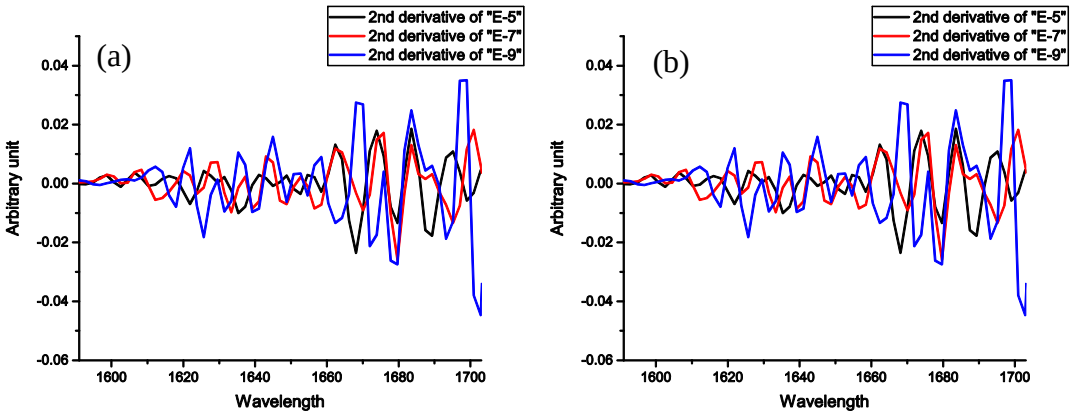

Figure 5. 8 – Second derivative of transmission-mode FTIR spectra in the amide I region for CALB immobilized at different pH values.

Table 5. 3 – Percentage correlation of the amide I region obtained from second derivative transmission-mode FTIR spectra of biocatalysts prepared by immobilizing CALB and ETL on Purolite C18 at different pH values.

Biocatalyst	Spectral similarity
E7-E5	31,30 %
E7-E9	14,49 %
E5-E9	23,71 %
C7-C5	64,68 %
C7-C9	32,33 %
C5-C9	2,64 %

For ETL, the highest degree of spectral similarity in the amide region was observed between biocatalysts immobilized at pH 5 and 7, which correspond to biocatalysts with statistically similar hydrolytic activities **(see Figure 5.3).** For CALB, a similar relationship between hydrolytic activity and spectral similarity was observed: biocatalysts immobilized at pH 5 and 7 exhibited the highest spectral similarity and showed no statistically significant difference in hydrolytic activity. These data suggest a correlation between spectral similarity in the amide I region (1600–1700 cm^{-1}) and hydrolytic activity for both CALB and ETL. In contrast, no clear pattern could be stablished between the degree of spectral similarly in the amide region and esterification or transesterification activities.

5.8.5. Effect of pH variation during the immobilization of *Candida antarctica* lipase B and Eversa® Transform 2.0 on their specificity toward different free fatty acids

To evaluate the effect of immobilization conditions on the selectivity of the obtained biocatalysts during xylose ester synthesis, three free fatty acids with short-, medium-, and long-chain lengths were used as acyl donors. The carboxylic acids chosen as models were oleic, lauric and butyric acids. Oleic acid is a monounsaturated fatty acid (C18:1, ω-9) widely found in vegetable oils and animal fats [45,46]. Lauric acid is a medium-chain saturated fatty acid (C12:0) with several industrial and biological applications, particularly due to its antimicrobial properties [47,48]. Butyric acid is a short-chain fatty acid (C4:0) widely used in lipase studies, ester synthesis, and industrial processes [49].

Figures 5.9 and 5.10 presents the conversion data for oleic, lauric, and butyric acids during xylose ester synthesis catalyzed by biocatalysts prepared by immobilizing ETL and CALB at different pH values.

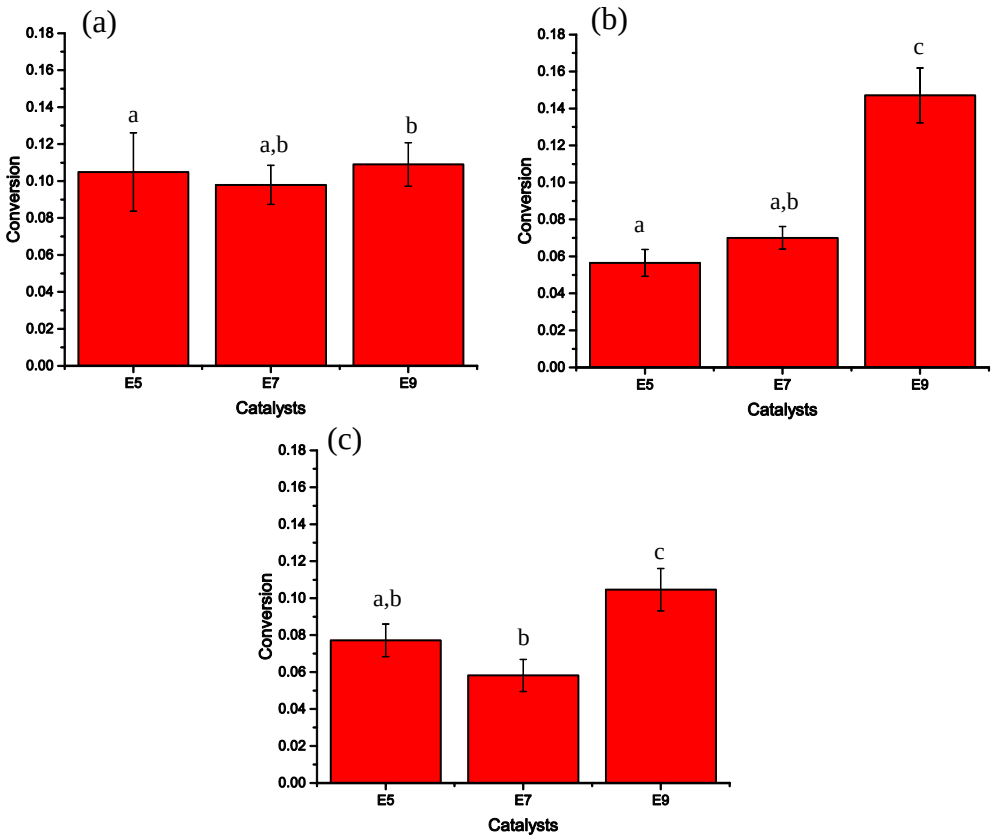

Figure 5. 9 – Conversion of (a) oleic acid, (b) lauric acid, and (c) butyric acid during xylose ester synthesis catalyzed by biocatalysts obtained by immobilizing ETL at pH 5, 7, and 9. Different letters (a-c) indicate statistically significant differences according to Tukey's test (5% significance level).

Figure 5.9 shows the conversion of oleic, lauric, and butyric acids during xylose ester synthesis catalyzed by immobilized Eversa Transform 2.0 at different pH values. Figure 5.9(a) shows the conversion of oleic acid by ETL immobilized at different pH values did not show statistically significant differences in oleic acid conversion during xylose ester synthesis. However, when lauric and butyric acids were used as acyl donors **[Figure 5.9 (b,c)]**, ETL immobilized at pH 9 exhibited higher conversion values compared to the other biocatalysts.

The conversion of oleic, lauric, and butyric acids during the synthesis of xylose esters catalyzed by *Candida antarctica* lipase B immobilized at different pH values is shown in Figure 5.10. CALB-catalyzed reactions **[Figure 5.10 (a)]** showed that the biocatalyst immobilized at pH 5 achieved the highest conversion of oleic acid. Similarly, when lauric and butyric acids were used as acyl donors **[Figure 5.10 (b,c)],** CALB immobilized at pH 5 also exhibited higher conversion values.

Overall, ETL immobilized at pH 9 and CALB immobilized at pH 5 showed the highest conversions in xylose ester synthesis, regardless of the acyl donor used. These findings are

consistent with the esterification activities shown in Figure 5.3, where CALB immobilized at pH 5 and ETL immobilized at pH 9 also demonstrated the highest esterification activities using butyric acid and butanol as acyl donor and acceptor, respectively.

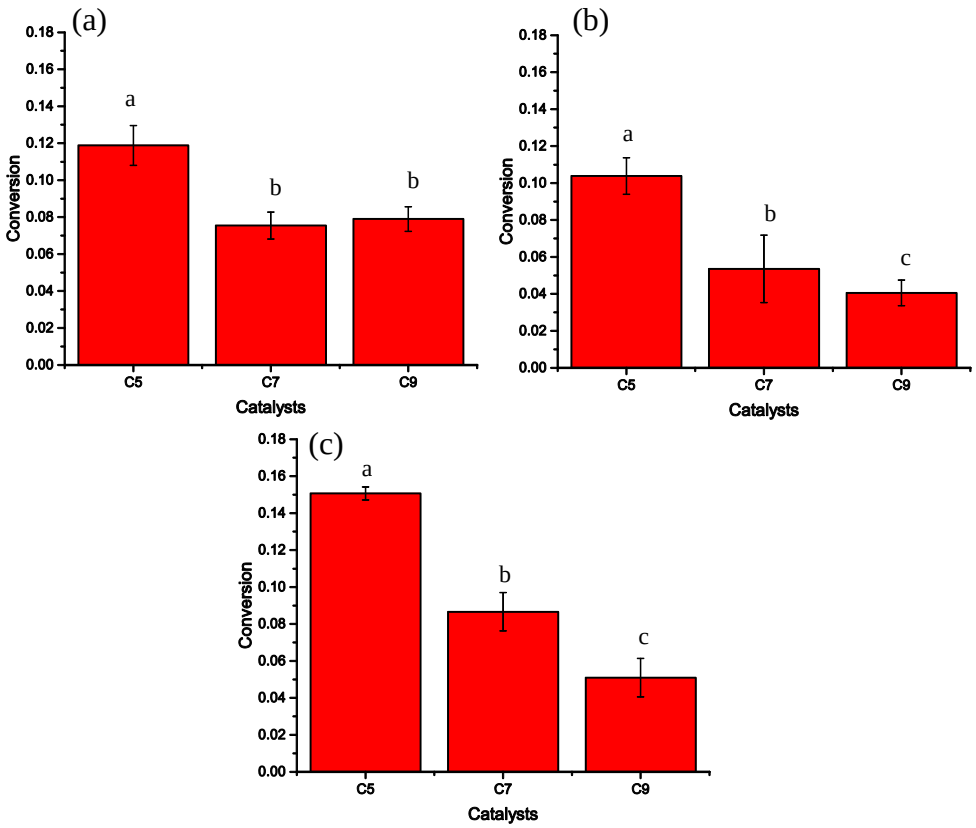

Figure 5. 10 – Conversion of (a) oleic acid, (b) lauric acid, and (c) butyric acid during xylose ester synthesis catalyzed by biocatalysts obtained by immobilizing ETL at pH 5, 7, and 9. Different letters (a-c) indicate statistically significant differences according to Tukey's test (5% significance level).

5.9. CONCLUSION

The pH applied during immobilization played a decisive role in modulating the catalytic activity performance, stability, and structural features of CALB and ETL, despite having no significant effect on overall immobilization yields. The distinct immobilization profiles observed for CALB and ETL were consistent with structural differences between the enzymes, particularly related to the size and flexibility of their lid domains. For CALB, immobilization pH strongly influenced reaction specificity: alkaline conditions favored hydrolytic activity, acidic conditions enhanced esterification, and neutral pH maximized transesterification performance. In contrast, ETL exhibited a clear preference for alkaline immobilization

conditions, with pH 9 resulting in the highest catalytic activities and improved stability, while acid conditions were detrimental, especially for synthesis reactions. These trends are consistent with the isoelectric points of the enzymes and highlight the role of protonation states of catalytic residues in governing enzyme performance. Thermal stability assays further demonstrated that immobilization pH affects enzyme robustness, particularly in organic media. Although both enzymes were more susceptible to deactivation in methyl ethyl ketone, ETL exhibited greater stability modulation as a function of immobilization pH, reinforcing the central role of the lid domain in mediating conformational and stability changes. FTIR analyses revealed pH-dependent alterations in secondary structure, particularly in α-helix and β-sheet contents, which correlated with variations in hydrolytic activity for both enzymes. The observed relationship between spectral similarity in the amide I region and hydrolytic activity performance supports the notion that subtle conformation changes induced during immobilization can significantly impact catalytic behavior. However, no direct correlation was observed between secondary structure similarity and esterification or transesterification activities, indicating that additional structural and mechanistic factors are involved in synthesis reactions. Finally, the selectivity of CALB and ETL toward different free fatty acids during xylose ester synthesis was strongly influenced by immobilization pH. CALB immobilized at pH 5 and ETL immobilized at pH 9 consistently exhibited the highest conversions, regardlees of acyl donor chain length, in agreement with their esterification activities. Overall, these results demonstrate that immobilization pH is a critical and versatile parameter for tailoring lipase activity, stability, and selectivity, providing a valuable strategy for the rational design of biocatalyst aimed of sugar ester synthesis and related applications.

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CHAPTER VI

EFFECT OF EVERSA TRANSFORM® 2.0 IMMOBILIZATION CONDITIONS AND POST-TREATMENT BY MAGNESIUM CHLORIDE METALLIZATION ON THE SYNTHESIS OF XYLOSE SUGAR ESTERS FROM SUGARCANE BAGASSE

The increasing demand for sustainable products and processes has intensified the use of agro-industrial biomass as a raw material across diverse industrial sectors. In this context, sugar esters have emerged as promising biosurfactants, particularly those derived from C5 sugars such as xylose, which can be obtained from abundant lignocellulosic residues, including sugarcane bagasse. Accordingly, this study evaluated biocatalysts obtained through the immobilization of Eversa® Transform 2.0 lipase, as well as post-treatment by metallization with magnesium chloride, for application in the acylation of xylose obtained from sugarcane bagasse. Pretreatment of the biomass with hydrogen peroxide and acetic acid effectively reduced recalcitrance, resulting in more than fourfold increase in the release of reducing sugars and a more than threefold increase in xylose concentration after enzymatic hydrolysis. Enzyme immobilization at pH 5 in the presence of 100 mM NaCl enhanced hydrolytic activity by approximately 45%, whereas post-treatment with magnesium chloride negatively affected enzymatic performance. In In the synthesis of xylose oleate, the biocatalyst immobilized at pH 5 with NaCl addition achieved the highest conversions of oleic acid (≈14–15%) and xylose (≈61–63%), using both commercial xylose and xylose derived from sugarcane bagasse. Overall, these results demonstrate the technical feasibility of valorizing the C5 fraction of lignocellulosic residues for the sustainable production of sugar esters.

6.1. INTRODUCTION

The increasing demand for sustainable products and processes have been driven by global challenges such as climate change, environmental pollution, and growing food insecurity [1,2]. In this context, the transition from products traditionally derived from finite fossil resources to those obtained from renewable feedstocks, particularly biomass, has becomes essential for the development of more sustainable industrial systems [2,3].

In this scenario, sugar esters (SEs) have emerged as a promising class of biosurfactants, as they can be produced via enzymatic catalysis, offering a sustainable alternative to petrochemical-derived surfactants [4,5]. SEs are synthesized through the esterification of a sugars with a free fatty acid and may exhibit superior stability under extreme operating conditions compared to conventional surfactants [6,7].

Among these compounds, esters derived from five-carbon sugars (C5 sugar esters) have attracted particular attention due to their excellent surfactant and lubricating properties, as well as their antimicrobial activity [5,8,9]. Xylose esters, in particular, stand out as environmentally friendly alternatives, since xylose can be obtained from abundant renewable lignocellulosic biomasses such as wood, sugarcane bagasse, straw, and other lignocellulosic residues [8,10–13].

Brazil is the world's largest producer of sugarcane, and most of this crop is processed for sugar and ethanol production, generating large amounts of agro-industrial residues, with sugarcane bagasse being the main by-product [14,15]. This lignocellulosic material exhibits high structural complexity, and is composed, on a dry basis, of approximately 50% cellulose, 25% hemicellulose, and 25% lignin [16,17]. Within the hemicellulosic fraction, xylan is the most abundant biopolymer and represents an important renewable source of xylose. [15,18].

Xylan consists of a backbone formed by D-xylose units linked by β-(1→4)-glycosidic bonds and may present varying degrees of branching, typically involving L-arabinose and 4-O-methyl-D-glucuronic acid residues [19,20]. Xylose can be obtained through the enzymatic hydrolysis of xylan by xylanases [21,22], in a synergistic process involving endoxylanases, which cleave the polymer backbone and generate xylooligosaccharides, and β-xylosidases, which subsequently hydrolyze these oligomers to release free xylose [22–24].

However, the direct hydrolysis of untreated sugarcane bagasse is inefficient due to the high recalcitrance of the biomass. The extensive presence of lignin within the cellulose-hemicellulose matrix increases structural rigidity and hydrophobicity, limiting enzyme accessibility to the polysaccharides. [15,25,26]. Consequently, pretreatment strategies are required to reduce biomass recalcitrance, primarily through partial or complete lignin removal.

Pretreatment methods for lignocellulosic biomass (LCB) are generally classified into four main categories: physical, chemical, physicochemical, and biological, each operating through distinct mechanisms to reduce structural recalcitrance [5,25,27,28]. Among chemical approaches, dilute acid pretreatment promotes hemicellulose hydrolysis, modifies lignin, and enhances cellulose accessibility while generating relatively low levels of inhibitory compounds [5,25,26]. In contrast, alkaline pretreatment primarily removes lignin and part of the hemicellulose by cleaving ester bonds, thereby minimizing excessive structural degradation [25,29,30]. Among these strategies, pretreatment with hydrogen peroxide and acetic acid has proven effective under relatively mild conditions, enabling significant lignin removal compared to more severe chemical processes [15,26,30].

In parallel, previous studies have demonstrated that the immobilization conditions and post-treatment of Eversa® Transform 2.0 lipase (ETL) can significantly modulate its catalytic performance [31]. Additionally, post-treatment of the immobilized enzyme, at pH 5 with the addition of 100 mM NaCl, with magnesium chloride in Tris-HCl or sodium phosphate buffers was shown to enhance activity and thermal stability across different pH values [32].

Therefore, the present study aims to evaluate the performance of biocatalysts obtained through the immobilization of ETL on octyl agarose and subsequent post-treatment with magnesium chloride in the synthesis of SEs from the C5 fraction of sugarcane bagasse, contributing to the development of more sustainable and integrated biorefinery processes.

6.2. MATERIALS AND METHODS

6.2.1. Materials

Eversa® Transform 2.0 lipase (ETL), octyl agarose, xylan, p-nitrophenyl butyrate (p-NPB), and dinitrosalicylic acid (DNS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Multifect CX XL A03139 was kindly provided by DuPont™ Genencor® (Palo Alto, CA, USA). Glacial acetic acid, hydrogen peroxide, sodium acetate, sodium phosphate, sodium chloride, magnesium chloride, xylose, and oleic acid were obtained from Synth (São Paulo, Brazil). All other reagents and solvents were of analytical grade and used without further purification.

6.2.2. Bagasse Pretreatment

The fibrous fraction of sugarcane bagasse was initially ground using a knife mill (HM100 POWTEC® multiprocessor). Subsequently, the biomass was incubated at 60 °C under agitation at 100 rpm in a 1:1 (v/v) mixture of 8.74 M glacial acetic acid and 21.6 M hydrogen peroxide, using a solid-to-liquid ratio of 1:20 (w/v). After pretreatment, the bagasse was separated by filtration and extensively washed with distilled water until the filtrate reached neutral pH. Finally, the bagasse was dried at 60 °C for 24 h [15].

6.2.3. Enzymatic Hydrolysis

Enzymatic hydrolysis of untreated and pretreated sugarcane bagasse was performed using 3% (w/v) bagasse in 50 mM sodium citrate buffer (pH 5.5), employing the enzymatic preparation Multifect CX XL A03139, which contains xylanases and β-xylosidases. An enzyme loading of 10 enzymatic units per gram of bagasse was applied. The reactions were conducted at 50 °C under constant agitation at 100 rpm. Aliquots were withdrawn after 30, 60, 120, 180, and 240 min and centrifuged at 3000 rpm for 3 min [23]. The concentrations of reducing sugars and xylose in the hydrolysate supernatants were determined using the DNS method [33] and high-performance liquid chromatography (HPLC), respectively.

6.2.4. Immobilization of ETL on Octyl-Agarose Beads

ETL was immobilized on octyl agarose beads via interfacial hydrophobic activation using an enzyme loading of 25 mg of enzyme per gram of support. The commercial enzyme solution was diluted to the appropriate volume in 50 mM sodium acetate buffer (pH 5.0), either in the absence or presence of 100 mM NaCl. Subsequently, 10 g of support were added to 100

mL of enzyme solution, and the suspension was agitated at 25 °C and 120 rpm in a shaker. The immobilization process was monitored by measuring the activity of the reference solution (enzyme solution without support), the immobilization suspension, and the supernatant via p-NPB hydrolysis. After 1 h, the suspensions were filtered, and the immobilized biocatalysts were washed five times with 10 volumes of distilled water and stored at 4 °C until future use [31].

6.2.5. Modification of Immobilized ETL with Metal Salts

The immobilized ETL biocatalyst was subjected to post-treatment with magnesium chloride. For this process, 1 g of immobilized ETL was suspended in 10 mL of 10 mM sodium phosphate buffer containing 125 mM NaCl at pH 7.4, followed by the addition of 400 µL of a 230 mM magnesium chloride solution. Alternatively, the treatment was performed using 10 mM Tris buffer containing 125 mM NaCl at pH 7.4. In both cases, the suspensions were incubated at room temperature under agitation of 100 rpm for 5 hAfter mineralization, the biocatalysts were filtered, washed five times with 10 volumes of distilled water, and stored at 4 °C [32].

6.2.6. Determination of Xylanase Activity

The enzymatic activity of the Multifect CX XL A03139 preparation was determined according to the IUPAC methodology [34], based on the initial rate of xylan hydrolysis catalyzed by a known amount of enzyme. The standard substrate consisted of 1% (w/v) birchwood xylan in 50 mM citrate buffer (pH 5.5). Reactions were conducted at 50 °C under agitation at 100 rpm for 10 min. Aliquots were withdrawn every 2 min, and the released reducing sugars were quantified using the DNS method [33]. One unit of activity (IU) was defined as the amount of enzyme required to release 1 µmol of xylose per minute under the assay conditions [23].

6.2.7. Enzymatic Activity by *p*-NPB Hydrolysis

The hydrolytic activity of free and immobilized enzymes was measured using p-NPB as substrate. The reaction was initiated by adding 50 µL of a 20 mM *p*-NPB solution in acetonitrile to 2.5 mL of 25 mM sodium phosphate buffer (pH 7.0) at 25 °C, followed by the addition of 50 µL of enzyme solution or suspension. The reaction was carried out under magnetic stirring for 90 s, and the increase in absorbance at 348 nm due to the *p*-nitrophenol release was monitored using a Jasco V-730 spectrophotometer. Enzymatic activity was

expressed as micromoles of *p*-nitrophenol produced per minute [32].

6.2.8. Enzymatic Synthesis of Xylose Esters

Xylose ester synthesis was carried out by enzymatic esterification of commercial xylose with oleic, lauric, and butyric acids at a 1:5 molar ratio (xylose:free fatty acid), using methyl ethyl ketone to solubilize xylose. The reactions were performed in an orbital shaker (Marconi, MA 832/H) at 50 °C and 250 rpm, employing 10 g of biocatalyst per gram of xylose. Samples were collected at 0 and 24 h, and the free fatty acid concentration was determined by gas chromatography [35].

6.2.9. Determination of Free Fatty Acids

Oleic acid concentration was determined by gas chromatography. The reaction medium was centrifuged, and 1 mL of the supernatant was collected for analysis. Measurements were performed using an Agilent 7890A gas chromatograph (Agilent Techologies, Santa Clara, CA, EUA) equipped with a flame ionization detector (FID), a split-splitless injector (split ratio 40:1) and a Restek Rtx-wax column (30 m × 0.25 mm × 0.25 μm; Restek Corporation, Bellefonte, PA, USA). The oven temperature program consisted of an initial hold at 200 °C for 1 min, followed by heating to 230 °C at 10 °C/min (1 min hold) and then to 250 °C at 5 °C/min, with a final hold of 5 min. Helium was used as the carrier gas at a flow rate of 1.8 mL/min, with a total run time of 14 min [35].

6.2.10. Determination of Xylose

Xylose concentration was determined by HPLC. The final reaction medium was centrifuged, and 1 mL of the supernatant was collected. After solvent evaporation, the residue was reconstituted with 1 mL of distilled water, homogenized, and filtered through a 0.22 μm membrane filter. Analyses were carried out using a Breeze HPLC system (Waters Corporation, Milford, MA, EUA) equipped with a refractive index detector (RID) and a Sugar-Pak I column (300 × 6.5 mm × 10 μm) maintained at 80 °C. An EDTA–Ca solution (50 mg/L) was used as the mobile phase at a flow rate of 0.5 mL/min. The injection volume was 20 μL, and the total run time was 20 min. As an alternative procedure to confirm xylose quantification, the final reaction medium was washed and homogenized with distilled water to promote xylose transfer to the aqueous phase, which was then separated, filtered (0,22 mm), and analyzed under the same chromatographic conditions. Similar xylose conversions were obtained using both procedures, confirming the reliability of the analytical protocol [35].

6.3. RESULTS

6.3.1. Pretreatment

Pretreatment of sugarcane bagasse is an important step to maximize sugar recovery during enzymatic hydrolysis of cellulose and hemicellulose, as it aims to remove lignin and increase the surface area, thereby enhancing enzyme accessibility to polysaccharide chains [36]. To evaluate the effect of pretreatment on hemicellulose hydrolysis and xylose production, the release of reducing sugars from untreated sugarcane bagasse and from bagasse pretreated with hydrogen peroxide and acetic acid was assessed [15]. **Figures 6.1** shows the concentration of reducing sugars obtained during enzymatic hydrolysis, while **Figure 6.2** illustrates the physical appearance of the bagasse before and after pretreatment.

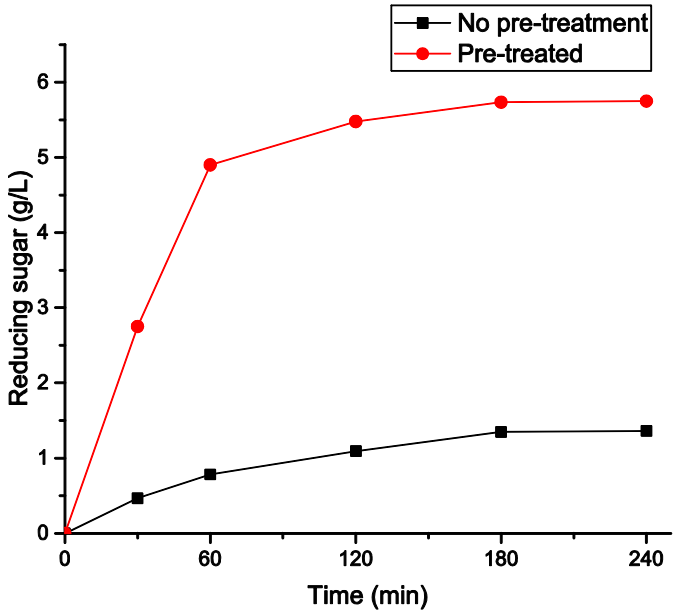

Figure 6. 1 - Concentration of reducing sugars released during the enzymatic hydrolysis of untreated and pretreated sugarcane bagasse using the Multifect CX XL A03139 enzymatic preparation at 50 °C, pH 5.5 and 100 rpm.

As shown in **Figure 6.1**, pretreatment with hydrogen peroxide and acetic acid resulted in more than fourfold increase in the concentration of reducing sugars after the enzymatic hydrolysis. This enhancement is primarily attributed to lignin removal, as lignin increases biomass rigidity and hydrophobicity, limiting enzyme access to cellulose and hemicellulose chains [29,37,38]. Visual inspection of the biomass **(Figure 6.2)** further supports lignin removal, as the pretreated exhibited a lighter color and thinner fibers compared to the untreated material. Similar morphological changes were reported by Bragatto *et al*. (2013), who observed

a substantial reduction in fiber thickness associated with an approximately tenfold decrease in lignin content after pretreated with hydrogen peroxide and acetic acid [15]. Increased glucose and xylose yields following pretreatment have also been widely reported in the literature as a consequence of improved enzyme accessibility [29,37,38].

Figure 6. 2 - Physical appearance of sugarcane bagasse (1g): (left) untreated and (right) pretreated with hydrogen peroxide and acetic acid for 4 h at 60 °C and 100 rpm.

Although reducing sugar concentrations provide an overall indication of hydrolysis efficiency, they may not accurately reflect xylose content due to the possible presence of xylooligosaccharides [15,39]. Therefore, xylose concentration in the hydrolysates was determined by HPLC. **Figure 6.3** presents the concentrations of both reducing sugars and xylose obtained from the hydrolysis of untreated and pretreated bagasse. The reducing sugars released during enzymatic hydrolysis were not composed exclusively of xylose. Nevertheless, pretreatment led to an approximately threefold increase in xylose concentration compared to untreated bagasse. The difference between reducing sugar and xylose concentrations has been previously reported and is mainly associated with the DNS method, which tends to overestimate xylose due to its nonspecific response toward reducing ends [15,40].

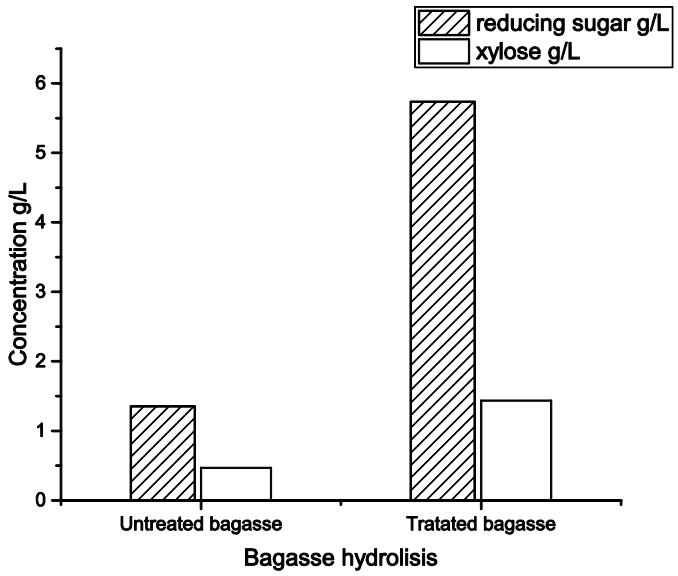

Figure 6. 3 – Concentrations of reducing sugars and xylose obtained during the enzymatic hydrolysis of untreated and pretreated sugarcane bagasse.

6.3.2. Biocatalyst Preparation

The development of a robust biocatalyst is of paramount importance, as increased stability and activity directly enable the implementation of more efficient and cost-effective processes [41]. In this context, immobilization conditions and post-treatment strategies represent key tools in biocatalyst design, as they allow modulation of properties such as catalytic activity, stability, and selectivity [31,32,41]. Previous studies have demonstrated that immobilizing ETL on octyl agarose at pH 5 in the presence of 100 mM NaCl enhances enzymatic activity, while post-treatment with magnesium chloride has been shown to improve biocatalyst stability [31,32].

ETL was immobilized on octyl-agarose support under the conditions described in Section 6.2.4.

Figure 6.4 shows the immobilization profiles at pH 5 and at pH 5 with the addition of 100 mM NaCl. Both immobilization conditions exhibited similar kinetic profiles; however, increasing the ionic strength led to a decrease in immobilization yield from 74% to 64% after 1 h. The hydrolytic activity of the reference enzyme solutions remained unchanged throughout the immobilization process, indicating that the observed differences in activity were not associated with enzyme inactivation induced by the immobilization conditions.

To evaluate the effect of immobilization conditions and post-treatment on biocatalyst activity performance, hydrolytic activity was determined by p-NPB hydrolysis. The resulting

activities are summarized in **Table 6.1**.

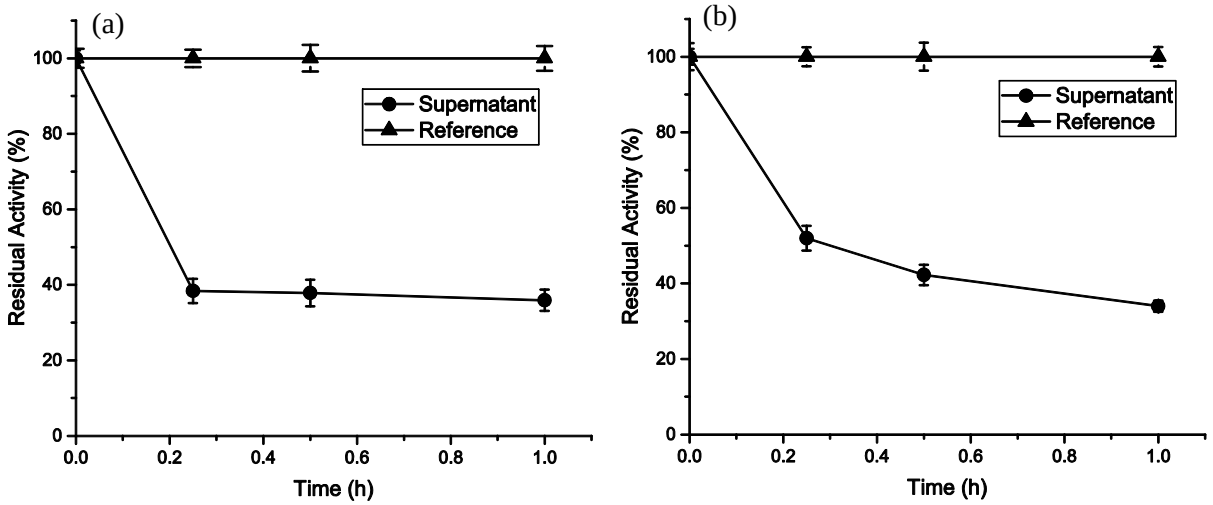

Figure 6. 4 – Immobilization profile of Eversa® Transform 2.0 on octyl agarose at (a) pH 5 and (b) pH 5 with the addition of 100 mM sodium chloride.

Table 6. 1 - Hydrolytic activity of biocatalysts immobilized at pH 5 (E5), at pH 5 with 100 mM sodium chloride (E5-100), and of biocatalysts immobilized at pH 5 with 100 mM sodium chloride and post-treated with magnesium chloride in Tris-HCl buffer (E5-100 MG TRIS) or iphosphate buffer (E5-100 MG FOS), as measured by p-NPB hydrolysis.

Biocatalyst	**Hydrolytic activity (UI/g)**
E5	535 ± 23.7
E5-100	776 ± 37.2
E5-100 MG TRIS	419 ± 50.2
E5-100 MG FOS	397 ± 34.9

As shown in **Table 6.1**, the addition of 100 mM sodium chloride during immobilization increased the hydrolytic activity of ETL immobilized on octyl-agarose by approximately 45% compared to the biocatalyst immobilized in the absence of salt. A similar effect was reported by Sabi *et al.* (2025), who observed enhanced activity at moderate ionic strength (100 mM NaCl), whereas higher salt concentrations (250 mM NaCl) led to activity losses [31].

Post-treatment with magnesium chloride resulted in an activity decrease of approximately 46% relative to the biocatalyst immobilized in the presence of NaCl, regardless of whether Tris-HCl or sodium phosphate buffer was used. Although this post-treatment negatively affected hydrolytic activity, Souza *et al*. (2025) reported that magnesium chloride

mineralization, particularly in the presence of phosphate ions, significantly improved biocatalyst stability under different inactivation conditions [32], suggesting a trade-off between activity and stability depending on the application.

6.3.3. Sugar Ester Synthesis

The biocatalysts obtained by ETL immobilization and post-treatment with magnesium chloride were applied to the synthesis of xylose oleate using both commercial xylose and xylose obtained from sugarcane bagasse hydrolysates. **Figure 6.5** shows the conversions of xylose and oleic acid achieved during the esterification reactions catalyzed by the different ETL-derived biocatalysts.

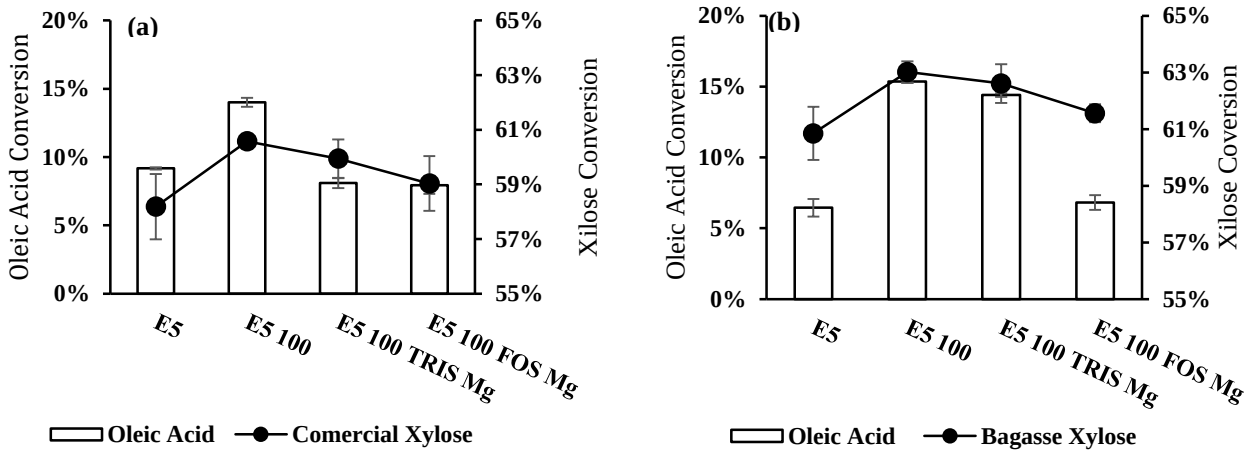

Figure 6. 5 - Conversions of xylose and oleic acid during xylose oleate synthesis at 50 °C and 250 rpm, using a 1:5 xylose-to-oleic acid molar ratio and 10 g of biocatalyst per g of xylose, with (a) commercial xylose and (b) xylose obtained from sugarcane bagasse.

As shown in **Figure 6.5**, the conversions of oleic acid (bars) and xylose (symbols) were strongly influenced by the immobilization conditions and post-treatments applied to the biocatalysts, regardless of the xylose source. When commercial xylose was used **(Figure 6.5a)**, the E5-100 biocatalyst exhibited the highest oleic acid conversion, reaching approximately 14%, while xylose conversion remained around 61%. Considering the 1:5 xylose-to-oleic acid molar ratio, the theoretical oleic acid conversion expected for exclusive monoester formation, based on the observed xylose conversion, would be approximately 12.2%. The higher experimental oleic acid consumption therefore suggests the simultaneous formation of esters with different degrees of acylation, including diesters.

After post-treatment of the biocatalysts with magnesium chloride resulted in a reduction

in substrate conversions, indicating a negative effect on catalytic performance. This behavior is consistent with the decrease in hydrolytic activity observed after mineralization **(Table 6.1)**, reinforcing the trade-off between activity and stability associated with this post-treatment.

When xylose derived from sugarcane bagasse was employed **(Figure 6.5b)**, the E5-100 biocatalyst again showed superior performance, achieving oleic acid conversions of approximately 15% and xylose conversions close to 63%, slightly higher than those obtained with commercial xylose. As observed previously, the discrepancy between experimental oleic acid conversion and the theoretical value for manometer formation supports the formation of a mixture of xylose esters with multiple acylations.

Unlike the results obtained with commercial xylose, post-treatment in Tris/HCl buffer did not lead to a significant reduction in conversion when lignocellulosic xylose was used. In contrast, the marked decrease in conversion observed for the E5-100 MG FOS biocatalyst confirms the detrimental effect of phosphate ions on catalytic performance, irrespective of the xylose source.

The oleic acid conversions obtained with the E5-100 biocatalyst using both commercial and lignocellulosic xylose differed from the theoretical values expected for exclusive monoester synthesis, further corroborating the formation of multiply acylated esters. Gonçalves *et al*. [28] reported higher conversions for xylose oleate synthesis catalyzed by Novozym® 435 when lignocellulosic xylose was used instead of commercial xylose. However, differences in biocatalyst type, immobilization method, and reactions conditions may account for the distinct performances observed. Overall, the results of the present study demonstrate the feasibility of producing sugar esters from the C5 fraction of lignocellulosic residues, such as sugarcane bagasse, reinforcing their potential within integrated biorefinery platforms.

6.4. CONCLUSION

The results demonstrate that the integrating of an efficient pretreatment strategy for sugarcane bagasse with tailored enzyme immobilization approaches enables the valorization of the C5 fraction of the lignocellulosic biomass for enzymatic sugar ester synthesis. Pretreatment with hydrogen peroxide and acetic acid proved essential for reducing biomass recalcitrance and increasing xylose availability for subsequent biocatalyst conversion. Immobilization of Eversa® Transform 2.0 lipase on octyl-agarose, particularly in the presence of 100 mM NaCl, resulted in biocatalysts with improved catalytic performance in xylose oleate synthesis. Although post-treatment with magnesium chloride led to a decrease in catalytic activity, these findings highlight the possibility of modulating biocatalyst properties through immobilization

conditions and post-treatment strategies, depending on the targeted process requirements. Furthermore, the use of xylose obtained from sugarcane bagasse hydrolysates resulted in conversions comparable to or higher than those obtained with commercial xylose, confirming technical feasibility of using lignocellulosic streams as renewable and low-cost substrate sources. Overall, this study reinforces the potential of integrating biomass pretreatment and enzyme engineering strategies to develop sustainable and efficient biocatalyst routes for sugar ester production within the biorefinery framework.

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CHAPTER 7
CONCLUSIONS, SUGGESTIONS FOR FUTURE WORK, AND ACADEMIC OUTPUT

7.1. CONCLUSIONS

This thesis demonstrated that the enzymatic synthesis of xylose fatty acid esters represents a sustainable and technically viable route for the production of high value-added nonionic biosurfactants. Throughout this work, the importance of valorizing the C5 fraction of lignocellulosic biomass was highlighted, particularly xylose derived from sugarcane bagasse, which is an abundant and renewable feedstock with potential for industrial applications.

A critical analysis of the state of the art enabled the identification of major advances in the enzymatic synthesis of xylose esters, as well as technological gaps related to sugar solubility, enzyme stability in highly polar media, and the lack of efficient purification and scale-up strategies. These challenges provided the scientific rationale for the experimental approaches adopted in this thesis, which focused on biocatalyst improvement, solvent evaluation, and the use of xylose obtained from sugarcane bagasse.

Studies involving the post-treatment of the commercial biocatalyst Novozym® 435 demonstrated that coating with polyethylenimine followed by chemical crosslinking with glutaraldehyde led to more robust catalytic systems, exhibiting enhanced operational stability, reduced enzyme leaching, and significantly higher conversions in xylose oleate synthesis. In addition, the use of unconventional solvents, such as xylose-based natural deep eutectic solvents, proved to be an environmentally friendly alternative that improved xylose solubility. The esters produced under these conditions exhibited emulsifying properties superior to those of commercial surfactants, reinforcing their potential for industrial applications.

The investigation of the different immobilization protocols and post-treatment strategies based on mineralization with phosphate salts revealed that catalytic properties such as activity, stability, and specificity can be effectively modulated through structural modifications of the biocatalyst. The results showed that even subtle conformational changes induced during immobilization or post-treatment can lead to significant variations in catalytic performance, highlighting the importance of system-specific optimization. The use of spectroscopic techniques, particularly FTIR, enable correlations between conformational changes and catalytic behavior, contributing to a deeper understanding of the structure-function relationships governing immobilized lipases.

Finally, the integration of sugarcane bagasse pretreatment and enzymatic hydrolysis with the enzymatic esterification of the resulting xylose confirmed the feasibility of converting the C5 fraction of the biomass into xylose esters. Pretreatment with hydrogen peroxide and acetic acid was essential to reduce biomass recalcitrance and increase xylose availability, while immobilization of the Eversa® Transform 2.0 lipase on octyl-agarose, particularly in the presence of NaCl, resulted in biocatalysts with superior performance in xylose oleate synthesis. Xylose obtained from sugarcane bagasse showed comparable or superior performance to commercial xylose, reinforcing the potential of lignocellulosic streams as renewable and low-cost substrate sources.

Overall, this thesis makes a significant contribution to the scientific and technological advancement of enzymatic sugar ester synthesis by demonstrating an integrated approach that combines biomass pretreatment, biocatalyst engineering, and the use of sustainable reaction media. The results strengthen the applicability of this process within the biorefinery concept and open perspectives for the development of C5 sugar-based biosurfactants with potential for scale-up and application across different industrial sectors.

7.2. SUGGESTIONS FOR FUTURE WORK

- Conduct technical, economic, and environmental assessments of xylose ester production from sugarcane bagasse to evaluate the industrial feasibility of the proposed process.
- Investigate the effects of enzyme immobilization conditions on the three-dimensional protein structure using circular dichroism spectroscopy in combination with FTIR analysis.
- Develop and evaluate separation and purification strategies for xylose esters produced in reaction media based on deep eutectic solvents.
- Assess the synthesis, physicochemical properties, and potential applications of xylose esters obtained using aromatic acids as acyl group donors.

7.3. ACADEMIC OUTPUT

The results of this thesis were published in indexed scientific journals, as listed below:

L. de Souza, J.R. Guimarães, J.C. Amaral, R. Fernandez-Lafuente, P.W. Tardioli, Polyethylenimine-glutaraldehyde modification of Novozym® 435 enhances stability and sugar ester synthesis in a xylose-based natural deep eutectic solvent, Biomass and Bioenergy 205 (2026) 108523.

L. de Souza, G.J. Sabi, P. Abellanas-Perez, A.A. Mendes, P.W. Tardioli, J. Rocha-Martin, R. Fernandez-Lafuente, The structure of the immobilized Eversa Transform determines the activity/stability effects of the biocatalyst metallization, Int. J. Biol. Macromol. 333 (2025) 148803. https://doi.org/https://doi.org/10.1016/j.ijbiomac.2025.148803.

G.J. Sabi, **L. de Souza**, P. Abellanas-Perez, P.W. Tardioli, A.A. Mendes, J. Rocha-Martin, R. Fernandez-Lafuente, Enzyme loading in the support and medium composition during immobilization alter activity, specificity and stability of octyl agarose-immobilized Eversa Transform, Int. J. Biol. Macromol. 295 (2025) 139667. https://doi.org/10.1016/j.ijbiomac.2025.139667.

The results of this thesis were also presented at national scientific conferences, as listed below:

25th Brazilian Congress of Chemical Engineering and 20th Brazilian Meeting on Chemical Engineering Education.

- Treatment of the commercial biocatalyst N435® with polyethyleneimine and glutaraldehyde to prevent enzymatic desorption during sugar ester synthesis.
- Effect of pH variation on the immobilization of *Candida antarctica* lipase B and Eversa Transform 2.0 for the development of biocatalysts for xylose laurate synthesis.

23rd Brazilian Congress on Catalysis: Catalysis for a Sustainable Energy Transition.

- Use of eutectic solvents as an alternative to conventional organic solvents in the enzymatic synthesis of xylose oleate.
- Influence of immobilization conditions on the activity and stability of Eversa Transform® 2.0 lipase and the effects of post-treatment with metal salts.