UNIVERSIDADE FEDERAL DE SÃO CARLOS
DEPARTAMENTO DE ENGENHARIA QUÍMICA
PROGRAMA DE PÓS-GRADUAÇÃO EM ENGENHARIA QUÍMICA

VINICIUS VESCOVI

LIPASES IMOBILIZADAS EM SUPORTES HÍBRIDOS COMO BIOCATALISADORES PARA A PRODUÇÃO DE ÉSTERES DE AÇÚCARES

SÃO CARLOS-SP
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Tese submetida ao Programa de Pós-Graduação em Engenharia Química da Universidade Federal de São Carlos como requisito para a obtenção do título de Doutor em Engenharia Química, área de concentração em Pesquisa e Desenvolvimento em Processos Químicos.

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RESUMO

O uso de lipases em larga escala é limitado devido ao seu alto custo. O reuso do biocatalisador contribuiria para tornar o processo custo-efetivo. Suportes hidrofóbicos são os mais utilizados na imobilização de lipases, devido ao mecanismo de ativação interfacial na presença de interfaces hidrofóbicas. Entretanto, o fato da enzima ligar-se fisicamente ao suporte não garante maior estabilidade operacional. Portanto, nesse trabalho foi avaliada a imobilização de lipases comerciais de *Candida antarctica* tipo B (CALB), *Thermomyces lanuginosus* (LTL) e *Pseudomonas fluorescens* (LPF) em suportes híbridos, os quais possibilitam adsorção hidrofóbica, seguida de ligação covalente enzima-suporte. Sílica foi funcionalizada com trietoxi(octil)silano (OCTES), (3-aminopropil)trietoxisilano (APTES) e 3-glicidiloxipropil)trimetoxisilano (GPTMS), para produzir suportes com diferentes funcionalidades: sílica contendo grupos octil (octil-silica, OS), sílica contendo grupos octil e aldeídos (octil-sílica-glioxil e octil-sílica-glutaraldeído, OSGlx e OSGlu, respectivamente) e sílica contendo grupos octil e epóxi (octil-sílica-epóxi, OSEpx). A modificação da sílica com OCTES aumentou significativamente a hidrofobicidade de todos os suportes, observado a partir de ensaios de adsorção do corante hidrofóbico Rosa de Bengala. Sílica modificada com grupos OCTES apresentou hidrofobicidade cerca de quatro vezes superior à apresentada pela sílica não modificada. O suporte OSGlu rendeu biocatalisadores mais ativos para CALB, enquanto OS rendeu biocatalisadores mais ativos para LPF and LTL. Todos os biocatalisadores apresentaram boa estabilidade em terc-butanol, especialmente CALB imobilizada em OSGlu (CALB-OSGlu), retendo em torno de 95 % de sua atividade inicial após 168 h a 60 ºC. CALB-OSGlu foi usada com sucesso na síntese de oleato de frutose a 55ºC, mantendo mais de 70% de conversão após nove ciclos de 6 horas, enquanto para o biocatalisador comercial Novozyme 435 a conversão foi de aproximadamente 53%. LTL e LPF foram aplicados na síntese de oleato de frutose a 35ºC na presença de diferentes percentuais de água. Todos os biocatalisadores mostram excelente desempenho na síntese do éster adicionando-se uma pequena quantidade de água (1%, v/v) na fase orgânica, exceto para as enzimas imobilizadas em OSEpx. A presença de água contribuiu para aumentar em até cinco vezes a produtividade do éster em comparação à reação na ausência de água. Uma conversão de aproximadamente 70% foi alcançada à baixa temperatura (35ºC) e curto período de tempo (30 min). Esses resultados representam um avanço nesta área do ponto de vista industrial, onde a produtividade é um parâmetro relevante para processos em larga escala. Por

fim, a lipase do pâncreas de porco (LPP) imobilizada em OS foi empregada na síntese de oleato de xilose e caprilato de xilose, devido ao seu menor custo dentre as lipases disponíveis comercialmente. Os resultados foram expressivos, obtendo-se uma conversão de aproximadamente 70% após 2 h de reação à 60ºC. De modo geral, esse trabalho mostrou que a modificação química da superfície da sílica com diferentes grupos ativos permitiu a preparação de biocatalisadores com diferentes microambientes, exercendo papel importante na atividade e estabilidade das lipases imobilizadas. Além disso, os biocatalisadores preparados neste trabalho apresentaram excelente desempenho e estabilidade operacional em reações de síntese de ésteres de açúcares, mostrando ter potencial para aplicação industrial.

Palavras-chave: Lipase; Sílica heterofuncionalizada; Imobilização; Ligação hidrofóbica/covalente; Ésteres de açúcares.

ABSTRACT

The use of lipases in large scale processes is limited due to their high cost. The reuse of the catalyst can contribute to make the enzymatic process more attractive. Hydrophobic supports are the mostly used for lipase immobilization, due to the mechanism of interfacial activation in the presence of hydrophobic interface. However, enzyme physically adsorbed to the support does not allow high operational stability. Therefore, in this work was evaluated the immobilization of commercial lipases from *Candida antarctica* type B (CALB), *Thermomyces lanuginosus* (LTL) e *Pseudomonas fluorescens* (LPF) on hybrid supports, that enable the hydrophobic adsorption, followed by covalent linkage between the adsorbed enzyme and the activated support. Silica was activated with trietoxy(octyl)silane (OCTES), (3-aminopropyl)trietoxysilane (APTES) e 3-glycidyloxypropyl)trimetoxysilane (GPTMS), aiming to produce supports with different functionality, as following: silica containing octyl groups (octyl-silica, OS), octyl and aldehyde groups (octyl-silica-glyoxyl and octyl-silica-aldehyde, OSGlx and OSGlu, respectively), and silica containing octyl and epoxy groups (octyl-silica-epoxy, OSEpx). From adsorption assays using the hydrophobic dye Rose of Bengal it was found that the modification of the silica with OCTES significantly increased the hydrophobicity of all the supports. Silica modified with OCTES groups showed to be 4 times more hydrophobic than non-modified silica. The support OSGlu yielded more active CALB biocatalyst, while OS yielded more active biocatalysts prepared with PFL and TLL. All the biocatalysts showed high stability in tert-butanol, specially CALB immobilized on OSGlu (OSGlu-CALB), maintaining 95% of its initial activity after 168 h at 60 ºC. CALB-OSGlu was successfully used in the synthesis of fructose oleate at 55ºC, yielding up to 70% conversion after 9 cycles of 6 hours, while the commercial biocatalyst Novozyme 435 retained around 53%. TLL and PFL were used in the synthesis of fructose oleate at 35ºC in presence of different amounts of water. All biocatalysts showed excellent performance in the ester synthesis when small amount of water (1%, v/v) was added to the organic phase, except for the lipases immobilized on silica modified with octyl and epoxy groups (OSEpx). Small amount of water increased around 5-times the ester productivity compared to reaction without water. Conversions around 70% were achieved at low temperature (35ºC) and short time of reaction (30 min). These results represent an advance in this field from of industrial point of view, where productivity is a relevant parameter for large-scale processes. Finally, porcine pancreatic lipase (PPL) immobilized on OS was used in the synthesis of xylose oleate and

xylose caprilate, because it is the most inexpensive lipase commercially available. The results showed to be promising, because conversions around of 70% were achieved after 2 h of reaction at 60 ^{o}C. Generally, this work showed that the chemical modification of the silica surface with different active groups allowed the preparation of biocatalysts with different microenvironment, which exhibits an important role in the activity and stability of the immobilized enzymes. Besides, the biocatalysts prepared in this work showed excellent performance and operational stability in syntheses of sugar esters, showing to have potential for industrial application.

Keywords: Lipase; Heterofunctionalized silica, Immobilization; Hydrophobic/covalent linkage, Sugar esters.

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LTL	Lipase de *Thermomyces lanuginosus*
TLL	*Thermomyces lanuginosus* Lipase
CALB	Lipase de *Candida antarctica* B
LPF	Lipase de *Pseudomonas fluorescens*
PFL	*Pseudomonas fluorescens* Lipase
LPP	Lipase do pâncreas de porco
PPL	Porcine Pancreatic Lipase
GPTMS	3-glicidoxipropiltrimetoxisilano (glycidoxypropyltrimethoxysilane)
APTES	3-aminopropiltrietoxisilano (3-aminopropyltrietoxysilane)
OCTES	Octiltrietoxisilano (Octyltrietoxysilane)
OS	Octil-Sílica (Octyl-Silica)
OSGlx	Octil-Sílica-Glioxil (Octyl-Silica-Glyoxyl)
OSEpx	Octil-Sílica-Epóxi (Octyl-Silica-Epoxy)
OSGlu	Octil-Sílica-Glutaraldeído (Octyl-Silica-Glutaraldehyde)
p-NPB	p-Nitrofenilbutirato (p-Nitrophenyl Butyrate)
Novozyme 435	CALB imobilizada em resina acrílica (CALB immobilized on macroporous acrylic resin)
FAEE	Fatty acid ethyl esters
HPLC	*High performance liquid chromatography*
GC	Gas Chromatography
DMSO	Dimetilsulfóxido
DMF	Dimetilformamida
U	Unidade enzimática
v/v	Volume/Volume
m/v	Massa/Volume
Å	Angstrom
Da	Dalton
Ala	Alanina
Arg	Arginina
Asn	Asparagina
Asp	Aspartato

Cys	Cisteína
Gln	Glutamina
Glu	Glutamato
Gly	Glicina
His	Histidina
Ileu	Isoleucina
Leu	Leucina
Lys	Lisina
Met	Metionina.
Phe	Fenilalanina
Pro	Prolina
Ser	Serina
Thr	Treonina
Trp	Triptofano
Tyr	Tirosina
Val	Valina

SUMÁRIO

1. INTRODUÇÃO

A busca por tecnologias "verdes" tem incentivado o desenvolvimento de novos processos. Segundo Malajovich (2011) não se trata de promessas ou de perspectivas futuras; os produtos e processos biotecnológicos fazem parte de nosso dia a dia, trazendo oportunidades de emprego e investimentos. Uma gama de produtos de interesse econômico (ácidos orgânicos, ésteres, biocombustíveis, antibióticos, vitaminas, aromas, proteínas, dentre muitos outros) pode ser obtida a partir de processos que utilizam microrganismos, células animais, vegetais ou enzimas.

A grande motivação pelo uso de enzimas deve-se, dentre outras vantagens, à redução do consumo energético do processo, pois enzimas atuam em condições brandas de temperatura e pressão, e à redução dos custos na etapa de purificação do produto final, pois, devido à alta seletividade e especificidade das enzimas, há formação é quase nula de subprodutos indesejáveis. O uso de enzimas industriais (lipases, amilases, celulases, proteases e outras) tem-se intensificado nos últimos anos. O mercado de enzimas industriais foi avaliado em US$ 4,2 bilhões em 2014 e está projetado para atingir US$ 6,2 bilhões em 2020, crescendo a uma taxa anual de 7,0% (2015-2020) (MARKETSANDMARKETS, 2015a).

A utilização de lipases nos setores de alimentos, bebidas, produtos de limpeza, e ainda, avanços biotecnológicos na área de biocombustíveis, alavancaram o crescimento do mercado mundial desta enzima. O mercado de lipases está projetado para atingir US$ 590,5 milhões em 2020, crescendo a uma taxa anual de 6,5% (2015-2020) (MARKETSANDMARKETS, 2015b).

No entanto lipases possuem estruturas frágeis, quando comparadas com catalisadores químicos, facilmente inativadas em condições extremas de pH e temperatura. Além disso, são solúveis e geralmente estão em baixa concentração no meio reacional, o que torna sua recuperação inviável sob o ponto de vista econômico. Tais inconvenientes podem ser amenizados pelo uso de enzimas imobilizadas. A imobilização de lipases em suportes sólidos produz um biocatalisador insolúvel e, geralmente, com maior estabilidade operacional, permitindo o desenvolvimento de processos de produção contínua, recuperação do biocatalisador e sua reutilização no processo. Essas características podem contribuir para a redução dos custos de produção do produto.

Inúmeros trabalhos são reportados na literatura sobre imobilização de lipases; particularmente, a imobilização destas enzimas por adsorção interfacial sobre suportes hidrofóbicos apresenta-se como um método vantajoso, a adsorção através de regiões

envolvidas na ativação interfacial de muitas lipases (aquelas que possuem uma tampa hidrofóbica) pode promover mudanças conformacionais relevantes em sua estrutura tridimensional, impactando sobre a atividade enzimática (BERNAL; ILLANES; WILSON, 2014; MATEO et al., 2010; REIS et al., 2006). No entanto, a adsorção interfacial representa uma ligação enzima-suporte mais frágil comparada à uma ligação covalente (LENINGHER.; NELSON e COX, 1995). Guisán (1988) reporta um sistema para imobilização covalente multipontual de enzimas sobre suportes ativados com alta densidade de grupos aldeídos. Sob condições alcalinas (pH 10), os grupos ε–aminos desprotonados de resíduos de lisinas interagem com os grupos aminos do suporte formando bases de Schiff, sendo transformadas posteriormente em ligações aminas estáveis através de redução com borohidreto de sódio. Essa técnica confere à molécula da enzima imobilizada alta rigidez, podendo restringir sua mobilidade que, muitas vezes é essencial à catálise. Particularmente para lipases que apresentam ativação interfacial, com modulação entre uma estrutura fechada (inativa) e uma estrutura aberta (ativa), a restrição da mobilidade da estrutura proteica pode levar à inativação da enzima imobilizada.

Uma estratégia muito promissora para a imobilização de lipase envolve a utilização de suportes heterofuncionais, que permitem obter em um único suporte as principais características apresentadas pelos métodos de imobilização via adsorção hidrofóbica (que proporciona a melhora no desempenho da enzima após a imobilização) e ligação covalente multipontual (alta estabilidade). O emprego de suportes heterofuncionais à base de sílica possibilita ainda a formação de diferentes microambientes, uma vez que a modificação da superfície da sílica pode ser realizada através de diferentes grupos reativos. Além disso, podem promover a orientação da enzima ao suporte através de diferentes regiões, afetando o desempenho catalítico da enzima, bem como sua estabilidade térmica e operacional (BERNAL; ILLANES; WILSON, 2014).

Diante deste contexto, este trabalho teve como objetivo principal a modificação da superfície da sílica com diferentes grupos funcionais, a saber: sílica monofuncionalizada com grupos octil e heterofuncionalizada com grupos octil/glutaraldeído, octil/epóxi e octil/glioxil, as quais foram empregadas na imobilização de lipases disponíveis comercialmente: lipase de *Candida antarctica* B, *Thermomyces lanuginosus, Pseudomonas fluorescens* e pâncreas de porco. A imobilização de lipases em sílica heterofuncionalizada, particularmente em sílica modificada com grupos octil/glutaraldeído e octil/epoxi, ainda não é reportada na literatura. Os derivados preparados foram avaliados quanto às atividades de

hidrólise, esterificação e transesterificação e estabilidade térmica em terc-butanol. Os melhores derivados foram selecionados para reações de síntese de ésteres de açúcares.

Ésteres de açúcares são moléculas anfifílicas com propriedades emulsificantes, muito utilizados na indústria farmacêutica e alimentícia. De acordo com Sandra Biben, gerente de negócios da BASF para a América Latina, em reportagem noticiada em 4 de setembro de 2011 pelo jornal ADCNEWS, a América Latina é o segundo maior mercado do mundo para o segmento de emulsificantes alimentares e está crescendo em torno de 5% a 6% ao ano, sendo o Brasil responsável por 80% desta demanda. No entanto, atualmente os emulsificantes são produzidos industrialmente na presença de um catalisador básico ou metálico, altas temperaturas (superiores à 100ºC) e pressões reduzidas que proporcionam a formação de subprodutos indesejados (FERRER et al., 2000). Além disso, a exigência de consumidores por aditivos naturais em substituição aos artificiais tem aumentado em todo mundo, na Europa até 80% dos consumidores preferem alimentos livres de aditivos artificiais (LEATHERHEAD, 2014). Esse crescente interesse por produtos mais saudáveis, aumentam as exigências de mercado, motivando a substituição da rota química pela rota enzimática.

1.1 Objetivo geral

O objetivo principal deste trabalho centrou-se na imobilização de lipases disponíveis comercialmente sobre sílica mono e heterofuncionalizada com grupos hidrofóbicos e grupos ativos que permitam a adsorção hidrofóbica e a formação de ligação covalente da enzima com o suporte.

1.2Objetivos específicos

Para o objetivo geral ser atingido, foram traçadas os seguintes objetivos específicos:

(i) Modificação química da superfície da sílica macroporosa com grupos octil (octil-silica), octil e glutaraldeído (octil-sílica-glutaraldeído), octil e glioxil (octil-sílica-glioxil) e octil e epóxi (octil-sílica-epóxi);

(ii) Imobilização das lipases de *Candida antarctica* B (CALB), *Thermomyces lanuginosus* (LTL) e *Pseudomonas fluorescens* (LPF) sobre os suportes preparados em (i), além da imobilização da lipase de pâncreas de porco (LPP) em octil-sílica;

(iii) Estudo da atividade de hidrólise, esterificação e transesterificação, assim como a análise da estabilidade térmica;

(iv) Avaliação dos melhores biocatalisadores na síntese de ésteres de açúcares;

(v) Extração, purificação e caracterização do éster de açúcar (oleato de frutose).

2. Revisão Bibliográfica

Este capítulo consiste de uma breve revisão bibliográfica acerca das informações fundamentais para a compreensão deste trabalho. Inicialmente, serão abordadas as estruturas, características e propriedades das enzimas utilizadas, bem como os métodos de imobilização de lipases por adsorção e ligação covalente. Em seguida, serão apresentados os aspectos gerais e desafios presentes na síntese enzimática de ésteres de açúcares.

2.1. Lipases

Lipases (triacilglicerol éster hidrolases, EC 3.1.1.3) compreendem um grupo de enzimas hidrolíticas que apresentam a capacidade única de agir na interface óleo/água, catalisando a hidrólise de óleos e gorduras com a liberação de ácidos graxos, diacilgliceróis, monoacilgliceróis e glicerol (CARVALHO et al., 2003). Em meios com baixa concentração de água, as lipases catalisam reações de esterificação, transesterificação e interesterificação (CASTRO et al., 2004). Essa versatilidade das lipases permite sua empregabilidade em diversos setores da indústria na formulação de detergentes, produção de fármacos, cosméticos, alimentos, perfumaria, produção de aromas e fragrâncias e biosurfactantes (PANDEY et al., 1999; PAULA; BARBOZA; CASTRO, 2005).

As lipases são encontradas em tecidos de vários animais e plantas, e podem ser produzidas por fermentação usando várias espécies de microrganismos (DALLA-VECCHIA; NASCIMENTO; SOLDI, 2004). Em eucariotos, as lipases estão envolvidas em vários estágios do metabolismo, incluindo digestão de gorduras, absorção, reconstituição e metabolismo de lipoproteínas (SHARMA; CHISTI; CHAND, 2001). Nas plantas, são encontradas em tecidos de reserva de energia, atuando durante os primeiros estágios da germinação, iniciando a metabolização de triglicerídeos estocados por meio de hidrólise dos ácidos graxos (QUETTIER; EASTMOND, 2009).

A estrutura tridimensional de muitas lipases tem sido elucidada por técnicas cristalográficas de difração de raios-X, mostrando que todas as lipases exibem um padrão de dobramento característico, conhecido como dobra α/β–hidrolase (GANJALIKHANY et al., 2012; JAEGER; REETZ, 1998; STAUCH; FISHER; CIANCI, 2015; UPPENBERG et al., 1994). O sítio ativo é formado por uma tríade catalítica que consiste dos resíduos de aminoácidos Ser-His-Asp/Glu, sendo essa triade frequentemente protegida por uma tampa

helicoidal hidrofóbica, chamada de “lid” (JAEGER; REETZ, 1998; UPPENBERG et al., 1994). A interação da lipase com a interface lipídeo/água promove uma mudança conformacional na estrutura tridimensional da enzima movendo a tampa, convertendo a enzima de uma forma "fechada" para uma forma "aberta", com o sítio ativo acessível ao substrato. Com a abertura da tampa, uma grande superfície hidrofóbica é exposta, facilitando a ligação da lipase ao substrato (DALLA-VECCHIA; NASCIMENTO; SOLDI, 2004; JAEGER; REETZ, 1998). Derewenda et al. (1992) reportaram que, durante o processo de ativação da lipase de *Rhizomucor miehei*, a tampa desloca-se cerca de 8 Å, expondo 12 aminoácidos hidrofóbicos, representando uma nova área hidrofóbica exposta de 732 $Å^2$ (cerca de 7% da superfície total de 10809 $Å^2$ da enzima). No entanto, de acordo com Dalla-Vecchia, Nascimento e Soldi (2004); Jaeger e Reetz (1998) a presença da tampa não está necessariamente correlacionada com a ativação interfacial, pois as lipases de *Pseudomonas aeruginosa*, *Burkholderia glumae* e *Candida antarctica* B apresentam tampa em suas estruturas, porém, não sofrem ativação interfacial.

A seguir, será feita uma breve revisão sobre as lipases utilizadas neste trabalho, ou seja, lipases de *Pseudomonas fluorescens* (LPF), *Thermomyces lanuginosus* (LTL), *Candida antarctica* (CALB) e lipase pancreática do pâncreas de porco (LPP).

2.1.1 Lipase de *Candida antarctica B*

Lipase de *Candida antartica* B (CALB) é a lipase mais amplamente estudada, com um grande número de patentes registadas e muitas aplicações industriais. Embora CALB não seja tão eficiente em hidrólise de triglicerídeos como as demais lipases, sua alta esterioespecificidade quanto a substratos quirais em hidrólises e sínteses orgânicas as tornam de particular interesse. Além disso, CALB exibe alta estabilidade térmica na presença de solventes orgânicos (GANJALIKHANY et al., 2012; STAUCH; FISHER; CIANCI, 2015; UPPENBERG et al., 1994).

CALB é uma enzima composta por 317 resíduos de aminoácidos (Tabela 2.1) com uma massa molecular de 33,3 kDa, pI de 6,0, pH ótimo para catálise igual a 7,0 e é estável em meio aquoso na faixa de pH de 3,5 a 9,5 (GANJALIKHANY et al., 2012; STAUCH; FISHER; STRZELCZYK et al., 2016; CIANCI, 2015; UPPENBERG et al., 1994). Sua estrutura é globular (30 Å × 40 Å × 50 Å) e apresenta dobramento clássico do tipo α/β-hidrolase, possuindo três pontes dissulfeto (Cys^{22}-Cys^{64}, Cys^{216}-Cys^{258}, Cys^{293}-Cys^{311}) e uma tríade catalítica clássica (Ser^{105}, Asp^{187} e His^{224}) (UPPENBERG et al., 1994).

Tabela 2.1 – Composição de aminoácidos da CALB.

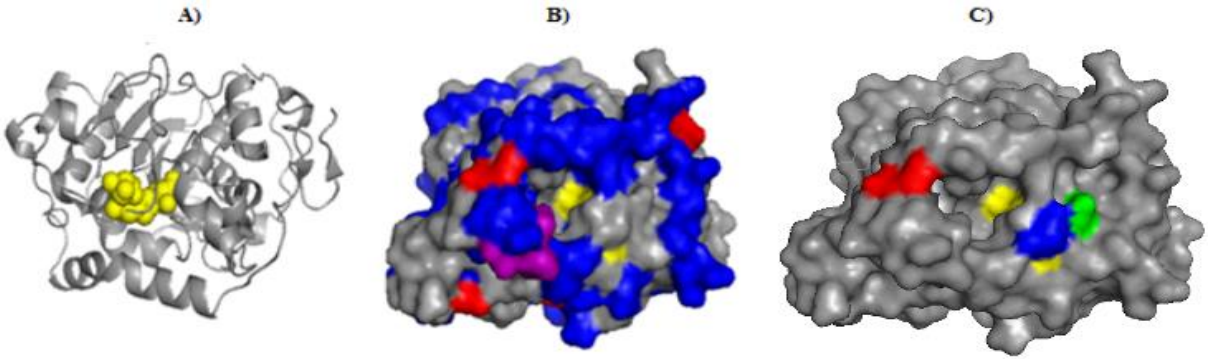
Aminoácidos	Unidades	Percentual (%, unidades)
Ala	36	11,36
Arg	8	2,52
Asn	14	4,42
Asp	14	4,42
Cys	6	1,89
Gln	18	5,68
Glu	4	1,26
Gly	26	8,20
His	1	0,32
Ile	11	3,47
Leu	31	9,78
Lys	9	2,84
Met	4	1,26
Phe	10	3,15
Pro	30	9,46
Ser	31	9,78
Thr	27	8,52
Trp	5	1,58
Tyr	9	2,84
Val	23	7,26

Fonte: Uppenberg et al., 1994.

Embora a CALB não tenha ativação interfacial significativa, seu sítio ativo é parcialmente coberto por dois segmentos de resíduos de aminoácidos em forma de α–hélices, α–hélice 5 (Leu^{140}-Ala^{141}-Gli^{142}-Pro^{143}-Leu^{144}-Asp^{145}-Ala^{146}-Leu^{147}) que funciona como uma tampa e α–hélice 10 (Pro^{280}-Ala^{281}-Ala^{282}-Ala^{283}-Ala^{284}-$Ileu^{285}$-Val^{286}-Ala^{287}-Gli^{288}) que funciona como um elemento de ativação (GANJALIKHANY et al., 2012; STAUCH; FISHER; CIANCI, 2015; UPPENBERG et al., 1994). Essas α–hélices são responsáveis pelas conformações aberta e fechada da CALB. Na conformação aberta, a α–hélice-5 expõe aminoácidos alifáticos responsáveis pelo revestimento de um canal hidrofóbico estreito (10 Å × 4 Å de largura e 12 Å de comprimento) até o sítio ativo, contendo a tríade catalítica

clássica. No fechamento do sítio ativo, a α–hélice-5 sofre uma grande mudança conformacional aproximando o grupo carboxílico da Asp^{145} ao resíduo Lys^{290} para a formação de uma ponte salina (ligação iônica). O rearranjo espacial da Lys^{290} aproxima a α–hélice-10 da região da tampa, completando o fechamento da cavidade catalítica, prevenindo o acesso de qualquer substrato ao sítio catalítico. O resíduo Asp^{145}, raramente conservado nas sequências de lipases, explicaria porque a CALB não apresenta ativação interfacial típica como as outras lipases, mas sim, ativação com o pH, pois a ponte salina é formada entre Asp^{145} e Lys^{290} em consequência do fechamento da tampa (STAUCH; FISHER; CIANCI, 2015). Estudos de simulação molecular, realizados por Ganjalikhany et al. (2012), mostraram que a abertura da tampa da CALB também é dependente da temperatura, mesmo na ausência de ligação enzima-substrato, devido ao natural aumento do movimento das moleculas com o aumento da temperatura. A Figura 2.1 mostra a representação da superfície da CALB construída a partir de uma estrutura cristalina de CALB disponível no Protein Data Bank (1TCA). A CALB tem uma grande superfície hidrofóbica (em torno de 450 $Å^2$) em torno da entrada do canal que dá acesso ao sítio ativo. Dois resíduos carboxílicos (Asp^{223} e Glu^{188}) próximos um do outro se situam perto da entrada do sítio ativo, e no lado oposto a essa entrada, situa-se o resíduo Lys-^{290} (GANJALIKHANY et al., 2012; UPPENBERG et al., 1994).

Figura 2.1. Estrutura 3D da CALB (Protein Data Bank, código PDB 1TCA) construída usando PyMOL Molecular Graphics System, v. 1.7.4. (A) Opção-Cartoon mostrando o sítio catalítico (amarelo); (B) Opção-Surface, mostrando resíduos hidrofóbicos (azul), resíduos Lys (vermelho), resíduos da tampa (roxo), resíduos do sítio ativo (amarelo) e (C) os resíduos carboxílicos Asp^{145} e Asp^{223} são apresentados na coloração verde, o resíduo Glu^{188} é na cor azul, o resíduo de Lys^{290} na cor vermelha enquanto a tríade catalítica é representada pela cor amarela.



Fonte: Acervo próprio.

2.1.2 Lipases de *Thermomyces lanuginosus*

Lipase de *Thermomyces lanuginosus* (LTL) é uma enzima termoestável disponível comercialmente na forma solúvel e imobilizada (Lipolase® e Lipozyme TL IM®, respectivamente). LTL é utilizada na modificação, hidrólise e transesterificação de óleos e gorduras, esterificação de ácidos graxos, acidólise ou interesterificação de óleos, resolução de misturas racêmicas, dentre outras (FERNANDEZ-LAFUENTE, 2010). A LTL é constituída por uma única cadeia de 269 resíduos de aminoácidos (Tabela 2.2), massa molecular de 31,7 kDa, ponto isoelétrico (pI) de 4,4, estável na faixa de 20 a 50ºC e com atividade máxima em pH em torno de 9,0 (DEREWENDA; SWENSON; WEI, 1994; FERNANDEZ-LAFUENTE, 2010).

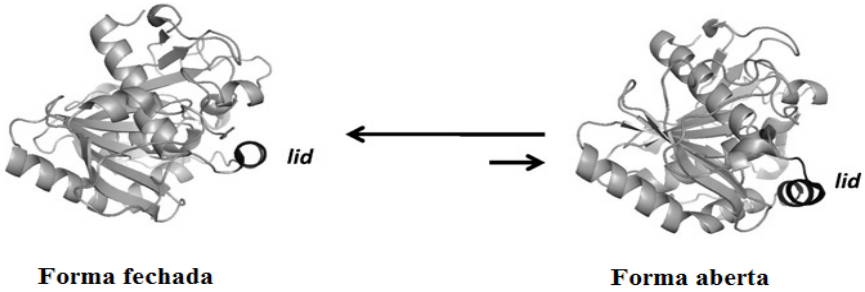
Tabela 2.2 – Composição de aminoácidos da LTL.

Aminoácidos	Unidades	Percentual (%, unidades)
Ala	21	7,81
Arg	14	5,20
Asn	19	7,06
Asp	19	7,06
Cys	6	2,23
Gln	6	2,23
Glu	12	4,46
Gly	28	10,41
His	6	2,23
Ile	16	5,95
Leu	20	7,43
Lys	7	2,60
Met	0	0,00
Phe	15	5,58
Pro	12	4,46
Ser	17	6,32
Thr	19	7,06
Trp	4	1,49
Tyr	10	3,72
Val	18	6,69

Fonte: Derewenda; Swenson; Wei, 1994.

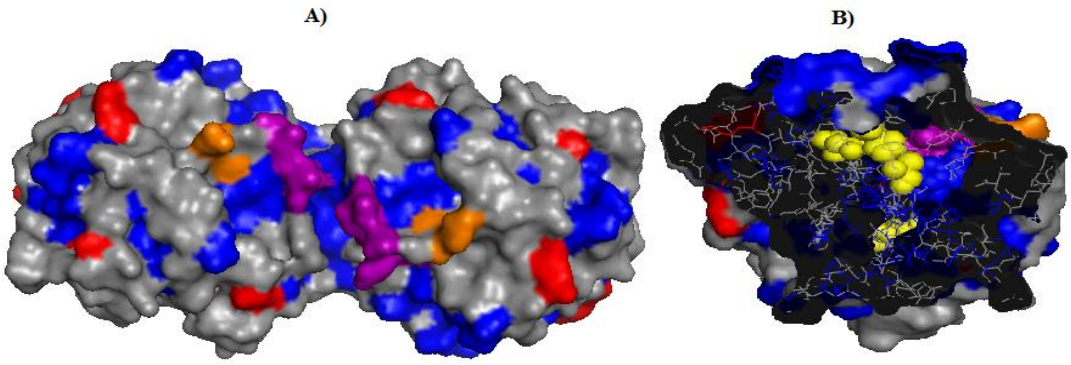
Sua estrutura é aproximadamente esférica com 35 Å × 45 Å × 50 Å. Seu sítio catalítico é composto pelos aminoácidos Ser^{146}-Asp^{201}-His^{258}, protegido do meio externo por uma tampa flexível composta pelos aminoácidos Ile^{86}-Glu^{87}-Asn^{88}-Trp^{89}-Ile^{90}-Gly^{91}-Asn^{92}-Leu^{93} (Figura 2.2) (DEREWENDA; SWENSON; WEI, 1994). De acordo com Derewenda, Swenson e Wei (1994) a alta mobilidade da tampa em meio aquoso implica que, mesmo na ausência de uma verdadeira interface água-óleo, há um equilíbrio sutil entre as duas conformações da enzima (sítios ativos, fechado e aberto) (Figura 2.2).

Figura 2.2: Equilíbrio estrutural da LTL.



Fonte: Adaptado de Fernandez-Lafuente, (2010).

Figura 2.3. (A) Visão externa da estrutura 3D do agregado bimolecular de LTL (Protein Data Bank, código PDB 1DT3), onde azul representa regiões hidrofóbicas, laranja representa o grupo amino terminal, roxo representa os resíduos da tampa da enzima e vermelho representa as lisinas. (**B)** Visão interna da estrutura (Figura 2.3A em perfil) 3D do agregado bimolecular de LTL (Protein Data Bank, código PDB 1DT3), onde azul representa regiões hidrofóbicas, laranja representa o grupo amino terminal, roxo representa os resíduas da tampa da enzima, vermelho representa as lisinas e amarelo representa a tríade catalítica da LTL. As estruturas foram construídas usando PyMOL Molecular Graphics System, v. 1.7.4 (Schrödinger, LLC).



Fonte: Acervo próprio

De acordo com Fernandez-Lafuente (2010) a LTL é uma das lipases com maior tendência para formar agregados bimoleculares, cujas as tampas estão em contato íntimo uma com a outra (Figura 2.3A). Portanto, este fato deve ser considerado ao se avaliar as propriedades catalíticas da enzima, pois as formas monomérica e dimérica apresentam atividade, estabilidade e seletividade diferentes (FERNANDEZ-LAFUENTE, 2010; PALOMO et al., 2003).

Uma estratégia bem sucedida para desfazer esses agregados bimoleculares menos ativos é a imobilização da LTL (e muitas outras lipases) em diferentes suportes hidrófóbicos por adsorção interfacial, favorecendo a imobilização da enzima na forma monomérica com a tampa aberta, cujas propriedades catalíticas irão depender da natureza do suporte (BASTIDA et al., 1998; FERNANDEZ-LAFUENTE et al., 1998; FERNANDEZ-LAFUENTE, 2010).

2.1.3 Lipase de *Pseudomonas fluorescens*

Lipase de *Pseudomonas fluorescens* (LPF) é uma das enzimas mais conhecidas lipases utilizadas em síntese orgânica, estando comercialmente disponível na sua forma livre e imobilizada. A LPF é constituída por uma cadeia de 320 resíduos de aminoácidos (Tabela 2.3), com massa molecular de aproximadamente 33 kDa (DEREWENDA; SWENSON; WEI, 1994). Sua estrutura é estavel na faixa de temperatura de 20 à 50ºC e pH 6-10, enquanto sua atividade ótima foi observada na temperatura de 55ºC e pH 8 à 10 (KOJIMA; YOKOE; MASE, 1994). Seu sítio catalítico é formado pela tríade Ser87-Asp264-His286 que se encontra no fundo de uma fenda profunda de 15 Å. As paredes internas dessa fenda, nas proximidades do sítio ativo, são revestidas por resíduos hidrofóbicos e sua entrada é protegida do meio externo por uma tampa flexível (hélice $\alpha-5$) composta pelos aminoácidos Thr^{137}-Val^{138}-Ile^{139}-Ala^{140}-Ala^{141}-Phe^{142}-Val^{143}-Asn^{144}-Val^{145}-Phe^{146}-Gly^{147}-Ileu-^{148}-Leu^{149}-Thr^{150} (Figura 2.4) (PALOMO et al., 2005; SCHRAG et al., 1997). Assim como a LTL, LPF pode formar agregados bimoleculares não covalentes. Fernández-Lorente et al (2003) mostraram que, mesmo em concentrações enzimáticas moderadas, PFL exibe uma forte tendência para formar estruturas bimoleculares, com grandes diferenças nas propriedades funcionais nas estruturas bimolecular e unimolecular. A estrutura bimolecular é mais estável e mais enantioseletiva que a unimolecular, porém menos ativa. Foi proposto que esses agregados moleculares são formados por mútua ativação interfacial, com as faces dos centros

ativos das duas moléculas em estreito contato entre si (Figura 2.4) (FERNÁNDEZ-LORENTE et al., 2003; LIMA et al., 2013; PALOMO et al., 2005).

Tabela 2.3 – Composição de aminoácidos da LPF.

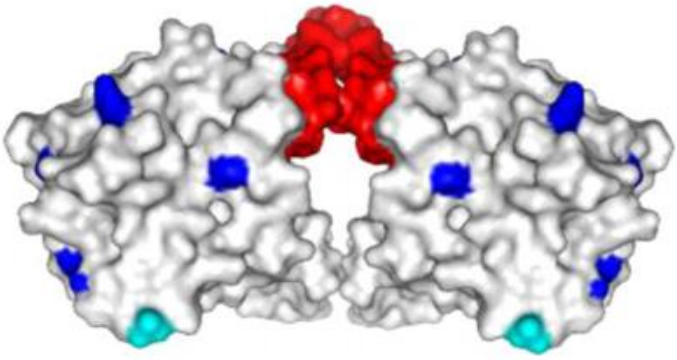
Aminoácidos	Unidades	Percentual (%, unidades)
Ala	39	12,19
Arg	9	2,81
Asn	18	5,63
Asp	15	4,69
Cys	2	0,63
Gln	15	4,69
Glu	7	2,19
Gly	36	11,25
His	6	1,88
Ile	11	3,44
Leu	31	9,69
Lys	7	2,19
Met	1	0,31
Phe	7	2,19
Pro	13	4,06
Ser	22	6,88
Thr	34	10,63
Trp	3	0,94
Tyr	14	4,38
Val	30	9,38

Fonte: Schrag et al. (1997)

O monômero da LPF contendo a distribuição dos resíduos de lisina (Lys) e resíduos hidrofóbicos é mostrado na Figura 2.5. É possível observar que esta lipase praticamente não apresenta áreas hidrofóbicas na superfície da enzima, exceto pela face interna da tampa e a área do sítio ativo (Figura 2.5A), o que significa que qualquer interacção hidrofóbica deve estar relacionada com esta área específica (PALOMO et al., 2005). Já os resíduos de Lys estão localizados no lado oposto do centro ativo (Figura 2.5B), e de acordo com Palomo et al. (2005) isto significa que um suporte de imobilização envolvendo esta

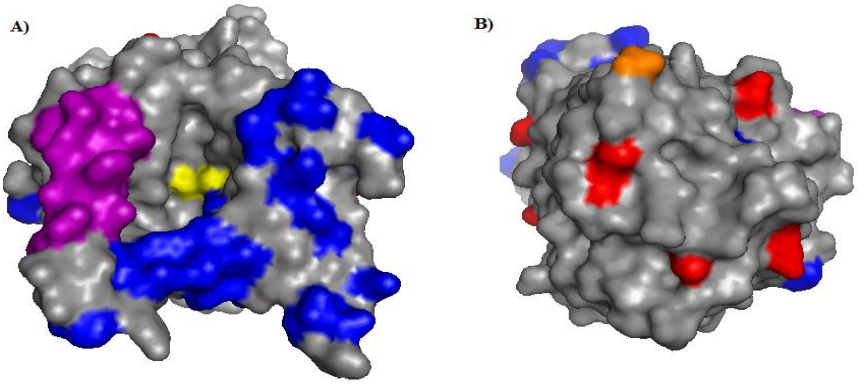
região rica em resíduos Lys deve permitir a imobilização da enzima com o seu sítio ativo exposto ao meio.

Figura 2.4: Representação esquemática de um agregado bimolecular de LPF (obtida a partir do PDB, código 3LIP). Azul claro: amino terminal; azul escuro: resíduos Lys; vermelho: tampas cobrindo os centros ativos da lipase.



Fonte: Lima et al. 2013.

Figura 2.5: Estrutura 3D da superfície da LPF (Protein Data Bank, código PDB 3LIP) construída usando PyMOL Molecular Graphics System, v. 1.7.4. (A) Face da tampa e (B) face oposta à tampa. Azul: resíduos hidrofóbicos; laranja: amino terminal; amarelo: tríade catalítica; vermelho: lisinas.



Fonte: Acervo próprio

2.1.4 Lipases de Pâncreas de Porco

Lipase de pâncreas de porco (LPP, EC 3.1.1.3) é amplamente aplicada em reações de biotransformação, que inclui hidrólise de óleos para a produção de ácidos graxos, hidrólise enantioseletiva, tratamento de águas residuais ricas em lipídeos, síntese de aromas, fragrâncias, biodiesel, emulsificantes e cosméticos (MENDES; OLIVEIRA; CASTRO, 2012). Dentre as inúmeras lipases comerciais disponíveis atualmente a lipase do pâncreas de porco se

destaca como a opção de menor custo (MENDES; OLIVEIRA; CASTRO, 2012). A LPP é estável na faixa de temperatura de 35 à 45ºC e pH 7,5-9,0 (MENDES; OLIVEIRA; CASTRO, 2012). Sua estrutura é composta por uma cadeia de 449 aminoácidos (Tabela 2.4), com peso molecular de 49,8 kDa (CARO et al., 1981).

Tabela 2.4. – Composição de aminoácidos da lipase do pâncreas de porco.

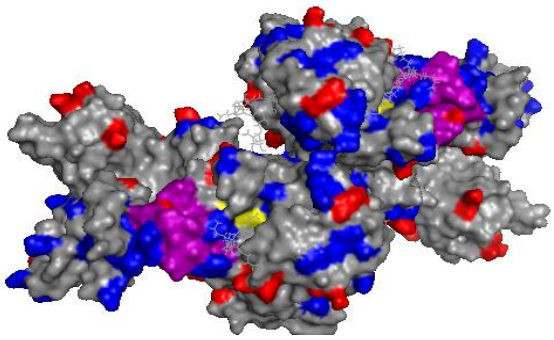
Aminoácidos	Unidades	Aminoácidos (%, unidades)
Ala	21	4,68
Arg	21	4,68
Asn	30	6,68
Asp	32	7,13
Cys	14	3,12
Gln	17	3,79
Glu	21	4,68
Gly	39	8,69
His	10	2,23
Ile	26	5,79
Leu	29	6,46
Lys	22	4,90
Met	4	0,89
Phe	25	5,57
Pro	25	5,57
Ser	31	6,90
Thr	25	5,57
Trp	6	1,34
Tyr	16	3,56
Val	35	7,80

Fonte - Caro et al. (1981)

Sua estrutura é divida em dois domínios, o domínio: (i) N-terminal que compreende os resíduos 1-336 e contém a tríade catalítica (Ser^{153}, Asp^{177}, His^{264}) e (ii) o domínio C-terminal composto pelos aminoácidos 337-449 (HERMOSO et al., 1996). A estrutura de cristal da LPP apresenta "loops" que cobrem a tríade catalítica, podendo dificultar

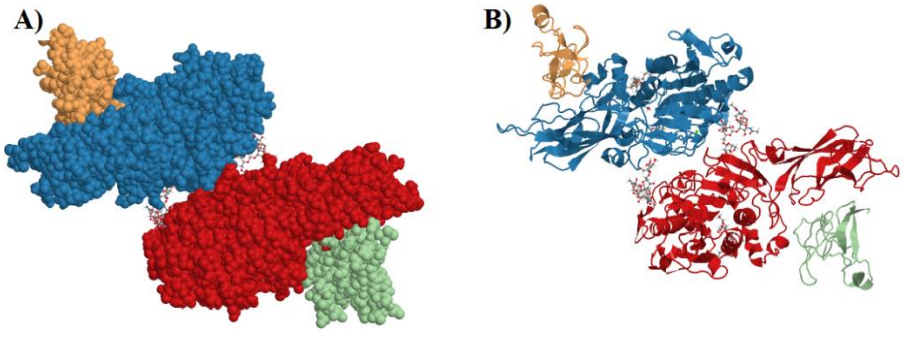
o acesso do substrato à tríade catalítica (HERMOSO et al., 1996; MENDES; OLIVEIRA; CASTRO, 2012; LOWE, 1997). Segundo Lowe (1997) e Mendes, Oliveira e Castro (2012) uma ligação dissulfeto entre Cys^{238} e Cys^{262} define o maior destes "loops", que representa o domínio da tampa (do inglês "lid", indicado pela coloração roxa na Figura 2.6). Outros "loop" que podem dificultar o acesso à tríade catalítica são formados pelos resíduos 76-86 (β5) e 213-217 (HERMOSO et al., 1996; LOWE, 1997). A estabilização da enzima na sua conformação aberta ocorre devido à ligação de hidrogênio entre a tampa da enzima e a colipase, que consiste numa proteína pequena de 10-11 kDa (Figura 2.7.A-B) secretada pelo pâncreas (MENDES; OLIVEIRA; CASTRO, 2012; LOWE, 1997). Segundo Hermoso et al. (1996) a interação entre a estrutura da LPP e a colipase ocorre através de várias ligações (8 de natureza polar, 17 por interações de van der Waals e 4 ligações mediada por água).

Figura 2.6 – Estrutura 3D do complexo LPP-Colipase-Monooctil (Protein Data Bank, código PDB 1ETH) construída usando PyMOL Molecular Graphics System, v. 1.7.4, onde azul representa os resíduos hidrofóbicos, amarelo o sítio ativo, vermelho os resíduos de lisinas e roxo os resíduos da tampa.



Fonte: Acervo próprio

Figura 2.7 - Estrutura 3D do complexo LPP-Colipase-Monooctil (Protein Data Bank, código PDB 1ETH), onde azul e vermelho representam as estruturas da LPP, enquanto laranja e cinza representam as proteínas da colipase



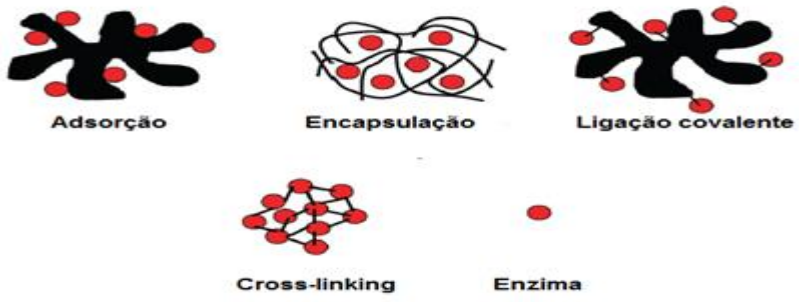
Fonte: RCSB, 2016.

2.2 Imobilização de Lipases

Desde a segunda metade do século passado, inúmeros esforços foram dedicados no desenvolvimento de catalisadores enzimáticos insolúveis para uma variedade de aplicações (CAO, 2005; SILMAN; KATCHALSKI, 1966). Catalisadores na forma insolúvel podem ser facilmente recuperados e reutilizados, frequentemente preservando sua atividade catalítica por longos períodos de tempo e permitindo o desenvolvimento de processos usando diferentes configurações de reatores (DICOSIMO et al., 2013).

As lipases podem ser imobilizadas usando a maior parte dos métodos desenvolvidos para a imobilização de enzimas em geral (ADLERCREUTZ, 2013). Alguns dos principais métodos são ilustrados na Figura 2.8. De acordo com (CAO, 2005) existem ainda métodos híbridos, ou seja, combinações de diferentes métodos de imobilização, as quais podem ser bastante úteis na preparação de um determinado biocatalisador imobilizado.

Figura 2.8 - Principais métodos de imobilização.



Fonte: Adaptado de Adlercreutz (2013)

Cada método apresenta suas vantagens e desvantagens, pois dependendo da enzima e do suporte, a imobilização pode causar a sua desativação (mudança da estrutura tridimensional da proteína) e apresentar problemas de limitações de transferência de massa, causando diminuição da atividade enzimática. Por outro lado, a imobilização facilita a reutilização da enzima, podendo assim, contribuir para a economicidade do processo (ADLERCREUTZ, 2013; DALLA-VECCHIA; NASCIMENTO; SOLDI, 2004; ZHANG; YUWEN; PENG, 2013). Além disso, a seleção do método de imobilização deve ser baseada em parâmetros como características de inativação, custo do procedimento e propriedades finais desejadas para a enzima imobilizada (MALCATA et al., 1990).

De acordo com Cao (2005) a estabilidade de uma enzima imobilizada é altamente dependente de muitos fatores, incluindo:

- As propriedades de sua interação com o suporte;
- A posição e o número de ligações;
- O microambiente no qual a enzima situa-se;
- Efeitos difusionais (mecanismos de difusão molecular e convectivo)
- Impedimento estérico (dificuldade do acesso de substratos volumosos ao sítio ativo)
- A estrutura química e física do suporte;
- As propriedades do espaçador (por exemplo, carregado ou neutro, hidrofílico ou hidrofóbico, tamanho, comprimento);
- As condições em que a enzima foi imobilizada.

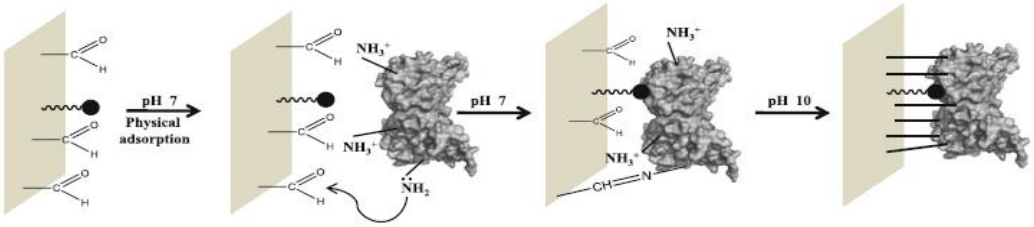
De acordo com Fernandez-Lafuente et al. (1998) e Secundo et al. (2008) o método de adsorção é o método de imobilização mais utilizado para lipases. A enzima é imobilizada em um suporte por ligações de baixa energia, tais como, interações de van der Waals ou hidrofóbicas, ligações de hidrogênio e iônicas (DALLA-VECCHIA; NASCIMENTO; SOLDI, 2004; FERNANDEZ-LAFUENTE et al., 1998; ZHANG; YUWEN; PENG, 2013). No caso de lipases, a interação hidrofóbica é mais comum, uma vez que lipases são adsorvidas espontaneamente a partir de soluções aquosas sobre superfícies hidrofóbicas, de forma seletiva preferencialmente em relação à maioria das outras proteínas, resultando ainda na maior parte dos casos e uma melhora da atividade catalítica (ADLERCREUTZ, 2013). Bastida et al. (1998) e Palomo et al. (2002) observaram que lipases imobilizadas por adsorção interfacial sobre suportes altamente hidrofóbicos, tais como, octadecil-sepabeads, phenil-sepharose e octil-agarose, exibiam atividade significativamente realçada (efeito este conhecido como hiperativação).

Outro método de imobilização de enzimas muito popular e difundido é o método da ligação covalente (KNEZEVIC; SILER-MARINKOVIC; MOJOVIC, 2004). Na imobilização por ligação covalente, a enzima é ligada ao suporte por ligações químicas covalentes, normalmente estabelecidas entre diferentes grupos das cadeias laterais dos resíduos de aminoácidos (amino, hidroxilas, tiol, dentre outros) e os grupos reativos do suporte. Entretanto, este método é pouco utilizado na imobilização de lipases (FERNANDEZ-LAFUENTE et al., 1998), uma vez que, a adsorção hidrofóbica é um método mais simples, barato e rende biocatalisadores melhores, devido sua hiperativação.

Suportes heterofuncionais (hidrofílico/hidrofóbico) têm despertando o interesse na imobilização de enzimas, particularmente de lipases, por permitirem aumento da atividade enzimática (hiperativação) e da estabilidade térmica/operacional (BERNAL; ILLANES;

WILSON, 2014; MATEO et al., 2010; MATEO et al., 2013). De acordo com Mateo et al. (2013) estes novos suportes contem grupos reativos capazes de promover a adsorção física de uma enzima em um pH neutro e, ainda, grupos capazes de reagir covalentemente com grupos funcionais da enzima, por exemplo, amino terminal e ε−amino de lisinas. Assim, o processo de imobilização nestes suportes ocorre em duas etapas: (i) na primeira etapa, a enzima é fisicamente adsorvida à superfície hidrofóbica do suporte; e (ii) na segunda etapa, a enzima adsorvida é incubada em condições alcalinas, favorecendo interações covalentes entre ε−amino desprotonados (reativos) de lisinas e grupos reativos do suporte (por exemplo, aldeídos) (Figura 2.9). De acordo com Bernal, Illanes, Wilson (2014) a imobilização de lipases em suportes heterofuncionais pode resultar em biocatalisadores mais ativos e estáveis, pois a primeira etapa (adsorção hidrofóbica) permitiria a modulação da lipase para a conformação aberta (mais ativa) e a segunda, a estabilização da proteína por ligações covalentes múltiplas.

Figura 2.9 - Imobilização de uma enzima em um suporte heterofuncional.



Fonte: Mateo et al. (2013).

A escolha do suporte é mais um importante fator responsável pelo bom desempenho do biocatalisador. Para ser efetivo na imobilização o suporte deve deixar a enzima acessível aos substratos, manter sua atividade por um longo período e permitir que o sistema (suporte/enzima) seja regenerado no final do processo, sem que ocorram perdas na atividade (MATEO et al., 2007). Dentre os diferentes suportes disponíveis, sílica modificada quimicamente se mostra como um suporte interessante para a imobilização de lipases. Além da sua natureza inorgânica, o que dispensa preocupação com degradação microbiana, permite a sua ativação com diferentes grupos reativos, os quais possibilitam a orientação da enzima ao suporte por diferentes regiões (BERNAL; ILLANES; WILSON, 2014).

Outro pré-requisito para o sucesso da imobilização consiste na escolha dos agentes modificadores da superfície do suporte. Sílica pode ser funcionalizada com diferentes grupos funcionais tais como: alquila, amino, carboxila, dentre outros, os quais possibilitam a

orientação da enzima ao suporte por diferentes regiões (CARVALHO; LIMA; SOARES, 2015). A ativação da sílica ocorre quando um alcoxissilano passa a ser quimicamente ligado covalentemente à sua superfície, cuja operação é denominada de funcionalização. De acordo com Airoldi; Farias (2000) a grande vantagem da utilização da sílica funcionalizada advém da possibilidade da reciclagem do substrato adsorvente (organosilano). Segundo Lazghab; Saleh; Guigon (2010) glicidoxipropiltrimetoxisilano (GPTMS) e (3-aminopropil)trietoxisilano (APTES) estão entre os organossilanos atualmente mais comumente utilizados como agentes de acoplamento para funcionalização da superfície da sílica. Outros agentes empregados na ativação da sílica são: 3-cloropropiltrimetoxissilano, epicloridrina, glutaraldeído, glioxal, formaldeído, glicidol, carbonildiimidazol, octiltrietoxisilano e outros (CARVALHO; LIMA; SOARES, 2015).

De modo geral, a ativação da sílica pode ocorrer em uma única reação, como por exemplo, a síntese realizada por Blanco et al. (2004) na ativação da sílica com octiltrietoxisilano, ou então com a ativação sendo realizada por duas ou mais reações, como por exemplo, a realizada por Sales, Prado e Airoldi (2002) que, inicialmente, ativaram a sílica com GPTMS e depois realizaram outra reação com etilenodiamina. Prado e Airoldi (2001) também realizaram procedimento similar, aminando inicialmente a sílica com APTES e, posteriormente, reagindo com 3-(3,4-diclorofenil)-1,1-dimetilurea.

2.3. Síntese de ésteres de açúcares

Ésteres graxos de açúcares, surfactantes e emulsificantes, são moléculas anfifílicas que possuem importantes propriedades, tais como, detergência, emulsificação, lubrificação, capacidade espumante, dentre outras (PAULA; BARBOZA; CASTRO, 2005). Essas propriedades são de extrema importância em vários campos, como biorremediação, biodegradação, recuperação de petróleo, alimentos, fármacos, e muitas outras aplicações em diferentes setores industriais (FERRER et al., 2005; NETA et al., 2011).

Atualmente, emulsificantes e outros aditivos alimentares (aromatizantes, corantes, antioxidantes e outros) se tornaram obrigatórios na alimentação moderna (HONORATO; BATISTA, NASCIMENTO, 2013). Embora apresente um mercado mundial altamente consolidado, o mercado global de aditivos alimentares continua a crescer. Entre 2009 e 2013, o mercado global de aditivos alimentares cresceu de USD 29,94 bilhões para USD 36,45 bilhões, representando um aumento médio de 5,4% ao ano (LEATHERHEAD, 2014). De acordo com Sandra Biben, gerente de negócios da BASF para a América Latina,

em reportagem noticiada em 4 de setembro de 2011 pelo jornal ADCNEWS, a América Latina é o segundo maior mercado do mundo para o segmento de emulsificantes alimentares e está crescendo em torno de 5% a 6% ao ano, sendo o Brasil responsável por 80% desta demanda.

Atualmente, os surfactantes são produzidos industrialmente a partir da reação de transesterificação entre carboidratos (glicose, frutose e sacarose) e ácidos graxos (ácidos esteárico, palmítico, oleico, linoleico, ou combinações) na presença de um catalisador básico ou metálico, altas temperaturas (superiores à 100ºC) e pressões reduzidas (FERRER et al., 2000). Contudo, a alta temperatura aplicada acaba se tornando um sério problema, pois propicia a formação de subprodutos indesejados, exigindo separações complexas e de múltiplas etapas, implicando em um alto custo para o processo (FERRER et al., 2000). Além disso, a exigência de consumidores por aditivos naturais em substituição aos artificiais tem aumentado em todo mundo. Por exemplo, na Europa até 80% dos consumidores preferem alimentos livres de aditivos artificiais (LEATHERHEAD, 2014). Esse crescente interesse por produtos mais saudáveis, aumentam as exigências de mercado, motivando a substituição da rota química pela rota enzimática.

A síntese de ésteres graxos de açúcares por via enzimática, utilizando lipases, apresenta muitas vantagens: condições mais suaves de temperatura, maior seletividade, menor número de etapas de purificação, além de ser uma rota ambientalmente "correta", tornando o produto mais atrativo ao consumidor, especialmente quando se trata de aditivos para a indústria biomédica, farmacêutica e alimentícia (BANAT et al., 2010; GUMEL et al., 2011). No entanto, a sua aplicação em larga escala é atualmente limitada pelo elevado custo de produção (BANAT et al., 2010).

Outro grande "gargalo" na derivatização de carboidratos em meio orgânico é a solubilização do sacarídeo. Somado a isso, a rota enzimática enfrenta ainda a tarefa desafiadora de preservar a atividade catalítica do biocatalisador nesse meio, uma vez que, solventes orgânicos usualmente empregados em processos químicos (DMF, PIRIDINA e DMSO) para dissolver sacarídeos, inativam enzimas e não são compatíveis com muitas aplicações dos derivados de carboidratos (EL SEOUD et al., 2007).

(DEGN; ZIMMERMANN, 2001) estudaram o efeito de diferentes solventes orgânicos na atividade de uma lipase (CALB imobilizada) e, ainda, na solubilidade de um carboidrato (glicose). Os solventes que menos afetaram a estrutura da enzima, mantendo alta a sua atividade residual percentual, foram: hexano (80%), *terc*-butanol (75%) e *terc*-pentanol

(71%), enquanto os melhores resultados obtidos na solubilização da glicose foram piridina (136 mM), *terc*-butanol (12 mM), dimetilformamida (12 mM) e *terc*-pentanol (10 mM). Piridina inativou completamente a enzima, inviabilizando o seu uso como solvente em qualquer processo com a CALB, além de ser um solvente altamente nocivo para a saúde humana. Os solventes mais adequados, de acordo com esse estudo, são *terc*-butanol e *terc*-pentanol.

Assim como o solvente, a presença de água pode desempenhar um papel importante no meio reacional de síntese de ésteres graxos de açúcares. Por um lado, sua concentração deve ser baixa para favorecer a reação de síntese, por outro lado, deve ser suficiente para preservar as propriedades catalíticas da enzima, atuando direta ou indiretamente em várias interações não covalentes, incluindo a solvatação de grupos iônicos e dipolos, ligação de hidrogênio e interacções hidrofóbicas (AFFLECK et al., 1992). Affleck et al. (1992) relataram um aumento de seis vezes na atividade de subtilisina Carlsberg na reação de transesterificação do éster etílico de N-acetil-L-fenilalanina-2-cloro com 1-propanol pela adição de 5 μL de água por mL de tetraidrofurano. No entanto, a eficiência catalítica da enzima reduziu sete vezes ao se aumentar a quantidade de água de 5 para 20 μL por mL de tetraidrofurano. Ferrer et al (2000) relataram um aumento significativo na síntese de 6-o-laurilsacarose, catalisada pela LTL imobilizada em celite, devido o aumento da quantidade inicial de água. Garlapati e Banerjee (2013) também observaram o mesmo comportamento na síntese de ésteres de aromas. Outra vantagem proveniente da utilização de um meio reacional com a presença de moléculas de água se deve ao fato da água proporcionar um aumento expressivo na solubilidade dos açúcares no meio orgânico, o que pode afetar diretamente a produtividade do processo.

Assim, a influência do solvente, a quantidade de água, temperatura e razão açúcar/ácido graxo são importantes parâmetros a serem considerados na otimização de qualquer processo envolvendo a síntese de biosurfactantes. Na Tabela 2.4 são apresentados alguns trabalhos envolvendo a síntese enzimática de ésteres graxos de açúcares.

2.3. Referências

ADCNEWS. Basf inaugura primeira fábrica de emulsificante alimentares em Jacareí. ADCNEWS, Jacareí, 4 de setembro de 2011, Indústria, p. 8. Disponível em: < http://www.adcnews.com.br/pdf_edicao/edicao313.pdf>, acessado 05/05/2016.

ADLERCREUTZ, P. Immobilisation and application of lipases in organic media. **Chemical Society Reviews**, v. 42, n. 15, p. 6406–36, 2013.

AFFLECK, R. et al. Enzymatic catalysis and dynamics in low-water environments. **Proceedings of the National Academy of Sciences**, v. 89, n. February, p. 1100–1104, 1992.

AIROLDI, C.; FARIAS, R. F. The use of organofuntionalized silica gel as sequestrating agent for metals. **Química Nova**, v. 23, n. 4, p. 496–503, 2000.

BANAT, I. M. et al. Microbial biosurfactants production, applications and future potential. **Applied Microbiology and Biotechnology**, v. 87, n. 2, p. 427–444, 2010.

BASTIDA, A. et al. A single step purification, immobilization, and hyperactivation of lipases via interfacial adsorption on strongly hydrophobic supports. **Biotechnology and Bioengineering**, v. 58, n. 5, p. 486–493, 1998.

BERNAL, C.; ILLANES, A.; WILSON, L. Heterofunctional hydrophilic-hydrophobic porous silica as support for multipoint covalent immobilization of lipases: Application to lactulose palmitate synthesis. **Langmuir**, v. 30, n. 12, p. 3557–3566, 2014.

BLANCO, R. M. et al. Functionalization of mesoporous silica for lipase immobilization: Characterization of the support and the catalysts. **Journal of Molecular Catalysis B: Enzymatic**, v. 30, n. 2, p. 83–93, 2004.

CAO, L. Introduction: Immobilized Enzymes: Past, Present and Prospects. In: **Carrier-bound Immobilized Enzymes.** Germany: WILEY-VCH, 2005. p. 1-52.

CARO, J. et al. Porcine pancreatic lipase. Completion of the primary structure. **Biochimica et Biophysica Acta (BBA) - Protein Structure**, v. 671, p. 129–138, 1981.

CARVALHO, P. et al. Aplicação de lipases microbianas na obtenção de concentrados de ácidos graxos poliinsaturados. **Quimica Nova**, v. 26, n. 1, p. 75–80, 2003.

CARVALHO, N. B.; LIMA, Á. S.; SOARES, C. M. F. USO DE SÍLICAS MODIFICADAS PARA IMOBILIZAÇÃO DE LIPASES. **Química Nova**, v. 38, n. 3, p. 399–409, 2015.

CASTRO, H. F. et al. Modificação de óleos e gorduras por biotransformação. **Quimica Nova**, v. 27, n. 1, p. 146–156, 2004.

COULON, D. et al. Investigation of keys parameters of fructose oleate enzymatic synthesis catalyzed by an immobilized lipase. **Industrial Crops and Products**, v. 6, n. 3-4, p. 375–381, 1997.

DALLA-VECCHIA, R.; NASCIMENTO, M. D. G.; SOLDI, V. Aplicações sintéticas de lipases imobilizadas em polímeros. **Quimica Nova**, v. 27, n. 4, p. 623–630, 2004.

DEGN, P.; ZIMMERMANN, W. Optimization of carbohydrate fatty acid ester synthesis in organic media by a lipase from Candida antarctica. **Biotechnology and Bioengineering**, v. 74, n. 6, p. 483–491, 2001.

DEREWENDA, U. et al. Catalysis at the interface: the anatomy of a conformational change in a triglyceride lipase. **Biochemistry**, v. 31, n. 5, p. 1532–1541, 1992.

DEREWENDA, U.; SWENSON, L.; WEI, Y. Conformational Lability Of Lipases Observed In The Absence Of An Oil-Water Interface: Crystallographic Studies Of Enzymes From The Fungi Humicola Lanuginosa, And Rhizopus Delemar. **Journal of Lipid Research**, v. 35, n. 1, p. 524–534, 1994.

DICOSIMO, R. et al. Industrial use of immobilized enzymes. **Chemical Society Reviews**, v. 42, n. 15, p. 6437–74, 2013.

DUCRET, A. et al. Enzymatic preparation of biosurfactants from sugars or sugar alcohols and fatty acids in organic media under reduced pressure. **Biotechnology and Bioengineering**, v. 48, n. 3, p. 214–221, 1995.

EL SEOUD, O. A. et al. Applications of ionic liquids in carbohydrate chemistry: A window of opportunities. **Biomacromolecules**, v. 8, n. 9, p. 2629–2647, 2007.

FERNANDEZ-LAFUENTE, R. et al. Immobilization of lipases by selective adsorption on hydrophobic supports. **Chemistry and Physics of Lipids**, v. 93, n. 1-2, p. 185–197, 1998.

FERNANDEZ-LAFUENTE, R. Lipase from Thermomyces lanuginosus: Uses and prospects as an industrial biocatalyst. **Journal of Molecular Catalysis B: Enzymatic**, v. 62, n. 3-4, p. 197–212, 2010.

FERNÁNDEZ-LORENTE, G. et al. Self-assembly of Pseudomonas fluorescens lipase into bimolecular aggregates dramatically affects functional properties. **Biotechnology and Bioengineering**, v. 82, n. 2, p. 232–237, 2003.

FERRER, M. et al. Synthesis of sugar esters in solvent mixtures by lipases from *Thermomyces lanuginosus* and *Candida antarctica* B, and their antimicrobial properties. **Enzyme and Microbial Technology**, v. 36, n. 4, p. 391–398, 2005.

FERRER, M.et al. Chemical versus enzymatic catalysis for the regioselective synthesis of sucrose esters of fatty acids. **Glass**, p. 509–514, 2000.

GANJALIKHANY, M. R. et al. Functional motions of candida antarctica lipase b: A survey through open-close conformations. **PLoS ONE**, v. 7, n. 7, 2012.

GARLAPATI, V. K.; BANERJEE, R. Solvent-free synthesis of flavour esters through immobilized lipase mediated transesterification. **Enzyme Research**, v. 2013, 2013.

GUISÁN, J. Aldehyde-agarose gels as activated supports for immobilization-stabilization of enzymes**. Enzyme and Microbial Technology**, 1988.

GUMEL, A. M. et al. Lipase mediated synthesis of sugar fatty acid esters. **Process Biochemistry**, v. 46, n. 11, p. 2079–2090, 2011.

HERMOSO, J. et al. Lipase Activation by Nonionic Detergents. **Journal of Biological Chemistry.** v. 271, n. 30, p. 18007–18016, 1996.

HONORATO, T. C.; BATISTA, E.; NASCIMENTO, K. O. Aditivos alimentares: aplicações e toxicologia. **Revista Verde de Agroecologia e Desenvolvimento Sustentável**, v. 8, n. 1981 - 8203, p. 1–11, 2013.

JAEGER, K. E.; REETZ, M. T. Microbial lipases form versatile tools for biotechnology. **Trends in Biotechnology**, v. 16, n. 9, p. 396–403, 1998.

KHALED, N. et al. Fructose oleate synthesis in a fixed catalyst bed reactor. **Biotechnology Letters**, v.13, p. 167-172, 1991.

KNEZEVIC, Z.; SILER-MARINKOVIC, S.; MOJOVIC, L. Immobilized lipases as practical catalysts. **Acta Periodica Technologica**, v. 280, n. 35, p. 151–164, 2004.

KOJIMA, Y.; YOKOE, M.; MASE, T. Purification and characterization of an alkaline lipase from Pseudomonas fluorescens AK102. **Bioscience, Biotechnology, and Biochemistry**, v. 58, n. 9, p. 1564–1568, 1994.

LAZGHAB, M.; SALEH, K.; GUIGON, P. Functionalisation of porous silica powders in a fluidised-bed reactor with glycidoxypropyltrimethoxysilane (GPTMS) and aminopropyltriethoxysilane (APTES). **Chemical Engineering Research and Design**, v. 88, n. 5-6, p. 686–692, 2010.

LEATHERHEAD. Food additives – A growing global Market. 2014.

LENINGHER, A. L.; NELSON, D. L.; COX, M. M. **Princípios de bioquímica**. 2.ed. São Paulo: Sarvier, 1995.

LIMA, L. N. et al. Immobilization and stabilization of a bimolecular aggregate of the lipase from Pseudomonas fluorescens by multipoint covalent attachment. **Process Biochemistry**, v. 48, n. 1, p. 118–123, 2013.

LOWE, M. E. Molecular Mechanisms of Rat and Human Pancreatic. **American Society for Nutritional Science**, p. 549–557, 1997.

MALAJOVICH, M. A. In: **Biotecnologia 2011**. Rio de Janeiro: BIBLIOTECA MAX FEFFER, 2011.

MALCATA, X, F. et al. Immobilized Lipase Reactors for Modification of Fats and Oils-A Review. **Journal of the American Oil Chemists' Society**, v. 67, n. 12, p. 890–910, 1990.

MARKETSANDMARKETS. **Industrial Enzymes Market worth 6.2 Billion USD by 2020.** 2015a**.** Disponível em: <http://www.marketsandmarkets.com/PressReleases/industrial-enzymes.asp>. Acessado dia 05/05/2016.

MARKETSANDMARKETS. **Lipase Market worth $590.5 Million by 2020,** 2015b. Disponível em: <http://www.marketsandmarkets.com/PressReleases/lipase.asp>. Acessado dia 05/05/2016

MATEO, C. et al. Advances in the design of new epoxy supports for enzyme immobilization-stabilization. **Biochemical Society Transactions**, v. 35, n. Pt 6, p. 1593–1601, 2007.

MATEO, C. et al. Improvement of enzyme properties with a two-step immobilizaton process on novel heterofunctional supports. **Biomacromolecules**, v. 11, n. 11, p. 3112–3117, 2010.

MATEO, C. et al. Oriented covalent immobilization of enzymes on heterofunctional-glyoxyl supports. **Methods in Molecular Biology**, v. 1051, p. 73–88, 2013.

MENDES, A. A.; OLIVEIRA, P. C.; CASTRO, H. F. Properties and biotechnological applications of porcine pancreatic lipase. **Journal of Molecular Catalysis B: Enzymatic**, v. 78, p. 119–134, 2012.

NETA, N. S. et al. Maximization of fructose esters synthesis by response surface methodology. **New Biotechnology**, v. 28, n. 4, p. 349–355, 2011.

PALOMO, J. M. et al. Interfacial adsorption of lipases on very hydrophobic support (octadecyl-Sepabeads): Immobilization, hyperactivation and stabilization of the open form of lipases. **Journal of Molecular Catalysis B: Enzymatic**. v.19, p. 279-286, 2002

PALOMO, J. M. et al. General trend of lipase to self-assemble giving bimolecular aggregates greatly modifies the enzyme functionality. **Biomacromolecules**, v. 4, n. 1, p. 1–6, 2003.

PALOMO, J. M. et al. Lipase-lipase interactions as a new tool to immobilize and modulate the lipase properties. **Enzyme and Microbial Technology**, v. 36, n. 4, p. 447–454, 2005.

PANDEY, A et al. The realm of microbial lipases in biotechnology. **Biotechnology and Applied Biochemistry**, v. 29, p. 119–131, 1999.

PRADO, A. G. S.; AIROLDI, C. The Pesticide 3-(3,4-Dichlorophenyl)-1,1-dimethylurea (Diuron) Immobilized on Silica Gel Surface. **Journal of Colloid and Interface Science**, v. 236, n. 1, p. 161–165, 2001.

PAULA, A. V.; BARBOZA, J. C. S.; CASTRO, H. F. Estudo da influência do solvente, carboidrato e ácido graxo na síntese enzimática de ésteres de açúcares. **Quimica Nova**, v. 28, n. 5, p. 792–796, 2005.

QUETTIER, A.-L.; EASTMOND, P. J. Storage oil hydrolysis during early seedling growth. **Plant Physiology and Biochemistry**, v. 47, n. 6, p. 485–490, 2009.

RCSB, 2016. Disponível em : <http://www.rcsb.org/pdb/explore/jmol.do?structureId=1ETH&opt=3>, acessado dia 03/05/2016.

REIS, P. et al. Lipase-catalyzed reactions at different surfaces. **Langmuir**, v. 22, n. 19, p. 8169–8177, 2006.

ŠABEDER, S.; HABULIN, M.; KNEZ, Ž. Lipase-catalyzed synthesis of fatty acid fructose esters. **Journal of Food Engineering**, v. 77, n. 4, p. 880–886, 2006.

SALES, J. A A; PRADO, A G. S.; AIROLDI, C. Thermodynamic Data for Divalent Cations Onto New Modified Glycidoxy Silica Surface At Solid / Liquid Interface. **Journal of Thermal Analysis and Calorimetry**. v. 70, p. 135–141, 2002.

SCHRAG, J. D. et al. The open conformation of a Pseudomonas lipase. **Structure**, v. 5, n. 2, p. 187–202, 1997.

SECUNDO, F. et al. Adsorption and activities of lipases on synthetic beidellite clays with variable composition. **Microporous and Mesoporous Materials**, v. 109, n. 1–3, p. 350–361, 2008.

SHARMA, R.; CHISTI, Y.; CHAND, U. Production, Purification, Characterization, and Applications of Lipases. **Biotechnology Advances**, v. 19, p. 627–662, 2001.

SILMAN, I. H. and KATCHALSKI, E. Water-insoluble derivatives of enzymes, antigens, and antibodies. **Annual Review of Biochemistry**, v. 35, p. 873–908, 1966.

STAUCH, B.; FISHER, S. J.; CIANCI, M. Open and closed states of *Candida antarctica* lipase B: protonation and the mechanism of interfacial activation. **Journal of Lipid Research**, v. 56, n. 12, p. 2348–2358, 2015.

STRZELCZYK, P.; BUJACZ, G. D.; KIELBASINSKI, P.; BLASZCYK, J. Crystal and molecular structure of hexagonal form of lipase B from *Candida antarctica*. **Acta Biochimica Polonica**, v. 63, p. 103-109, 2016.

UPPENBERG, J. et al. The sequence, crystal structure determination and refinement of two crystal forms of lipase B from Candida antarctica. **Structure (London, England : 1993)**, v. 2, n. 4, p. 293–308, 1994.

ZHANG, D.-H.; YUWEN, L.-X.; PENG, L.-J. Parameters Affecting the Performance of Immobilized Enzyme. **Journal of Chemistry**, v. 2013, p. 1–7, 2013.

3. Artigo 1 – Modulação das propriedades catalíticas de lipase de *Candida antarctica* B imobilizada sobre sílica heterofuncionalizada e aplicação na síntese de oleato de frutose

Neste capítulo, em forma de artigo científico, são apresentados os resultados da imobilização de lipase de *Candida antarctica* B sobre suportes heterofuncionais à base de sílica. Os derivados foram caracterizados quanto à sua atividade de esterificação, transesterificação e hidrólise, bem como quanto à sua estabilidade térmica em terc-butanol. O melhor derivado foi selecionado para avaliação de desempenho na produção de oleato de frutose comparado ao derivado comercial Novozyme 435.

A imobilização de uma enzima sobre superfícies sólidas contendo diferentes grupos funcionais pode modular as propriedades catalíticas de um biocatalisador, permitindo que esse seja mais adequado para uma biotransformação específica. A superfície da sílica foi quimicamente modificada com grupos octil, epóxi e aldeídos (glioxil e glutaraldeído) com o objetivo de se preparar suportes com diferentes microambientes para a imobilização de lipase de *Candida antarctica* B (CALB). CALB imobilizada sobre sílica modificada com grupos octil e glutaraldeído (OSGlu), de agora em diante denominado CALB-OSGlu, foi provavelmente orientada à superfície sólida pelas suas regiões mais hidrofóbicas, sendo uma delas localizada no lado oposto à tampa, contendo o amino terminal e resíduos de lisina que poderiam ligar-se covalentemente ao suporte. O derivado apresentou alta atividade recuperada (71,0 ± 2.3%) e alta estabilidade térmica em álcool *tert*-butílico (100% ativo após 100 h a 65ºC). CALB imobilizada sobre sílica monofuncionalizada rendeu biocatalisadores com menores atividades recuperadas, comparado ao biocatalisador CALB-OSGlu, com valores de 43,5% e 32% para CALB imobilizada sobre sílica modificada com grupos octil (OS) e grupos glutaraldeído (SGlu), respectivamente. CALB-OSGlu foi usado na síntese de oleato de frutose, atingindo conversões superiores a 70% (principalmente monooleato de frutose) em nove ciclos de 6 h em condições previamente estabelecidas, a saber: 55ºC, razão molar frutose/ácido oleico de 1:5, álcool *tert*-butílico como solvente e presença de peneira molecular (20% m/v). O biocatalisador CALB-OSGlu apresentou estabilidade operacional maior que do biocatalisador comercial Novozyme 435 nas mesmas condições reacionais, com a conversão diminuindo de 84% (primeiro ciclo) para 53% (nono ciclo).

Modulation of the catalytic properties of *Candida antarctica* type B lipase immobilized on heterofunctional silica-based supports and application in fructose oleate synthesis

ABSTRACT: The immobilization of an enzyme to solid surfaces containing different functional moieties can modulate the catalytic properties of the biocatalyst, thus allowing that it to be more suitable for a specific biotransformation. Silica surface was chemically modified with octyl, epoxy, and aldehyde (glyoxyl and glutaraldehyde) groups in order to prepare tailored supports with different microenvironments for the immobilization of *Candida antarctica* type B lipase (CALB). CALB immobilized on modified silica with octyl and glutaraldehyde groups (OSGlu), namely CALB-OSGlu, was probably orientated to the solid surface by its hydrophobic regions, one of them in the opposite side to the lid, which contains the *t*-NH2 and lysine residues capable to covalently link to the support. The derivative showed high recovered activity (71.0 ± 2.3%) and high thermal stability in *tert*-butyl alcohol (full activity was recovered after 100 h at 65^{o}C). CALB immobilized on monofunctionalized silica yielded biocatalysts with lower recovered activities, compared to CALB-OSGlu, with values of 43.5% and 32% for CALB immobilized on silica modified with octyl groups or glutaraldehyde groups, respectively. CALB-OSGlu was used in the synthesis of fructose oleate achieving conversions above 70% (mainly to fructose monooleate ester) in nine-6h cycles under optimized conditions (55^{o}C, fructose/oleic acid molar ratio of 1:5, *tert*-butyl alcohol as solvent, and molecular sieve at 20%, m/v). CALB-OSGlu biocatalyst showed better operational stability than the commercial biocatalyst Novozyme 435 in the same reaction conditions, with the conversion decreasing from 84% (first cycle) to 53% (ninth cycle).

Keywords: *Candida antarctica* type B lipase, CALB, silica-based supports, octyl-silica-glutaraldehyde, fructose monooleate ester.

3.1. Introduction

Sugar fatty acid esters are amphiphilic molecules with both hydrophilic and hydrophobic moieties (FRANZETTI et al., 2010). They have important emulsifying, stabilizing, and detergent properties, which make them widely used in the food, cosmetics, detergent, and pharmaceutical industries (ŠABEDER; HABULIN; KNEZ, 2006; YAN et al., 1999). The syntheses of the sugar esters can be performed either chemically or enzymatically. The chemical routes are energetically more costly than the enzymatic routes and have low selectivity, producing undesirable byproducts and toxic compounds (FERRER et al., 2000). Some of the byproducts are allergenic and possibly carcinogenic (CAUGLIA; CANEPA, 2008; GUMEL et al. 2011). These drawbacks can be overcome using lipases as potential biocatalysts. The enzymatic synthesis of sugar fatty acid esters offers advantages such as mild reaction conditions, simple operation, easy product separation, and excellent selectivity, when compared to the chemical processes (FERRER et al., 2000; GUMEL et al., 2011; MAI et al., 2014; SARNEY; VULFSON, 1995; YAN et al., 2001; YOO; PARK; YOON, 2007). These properties made the use of lipases an attractive biocatalyst for producing sugar fatty acid esters aiming use in foods, pharmaceuticals and cosmetics (products with high aggregate value), as the synthesis of vitamin-c fatty acid esters *via* lipase catalysis (KARMEE, 2008).

The advantages of the use of enzymes in the different industrial fields are notably known. Besides, it is well-documented that most enzymes are relatively unstable, the costs of its isolation and purification remain high, and it is technically difficult to recover active enzymes from the reaction medium when they are used in the soluble form (BRENA; GONZÁLEZ-POMBO; BATISTA-VIERA, 2006). These drawbacks can be overcome by the use of immobilized enzymes (ADLERCREUTZ, 2013). The immobilization technique is capable of producing an insoluble biocatalyst with high stability towards several denaturing agents (temperature, pH, and organic solvents), and an additional advantage is that the biocatalyst can be easily recovered from the reaction medium and reused (BRENA; GONZÁLEZ-POMBO; BATISTA-VIERA, 2006; DICOSIMO et al., 2013).

The most popular methods to immobilize lipases are based on the adsorption of the enzymes onto hydrophobic supports by means of hydrophobic, van der Waals, and electrostatic forces (FERNANDEZ-LAFUENTE et al., 1998). Lipases have a secondary structural element, known as a “lid”, which covers the active site, making it inaccessible to substrates. In the presence of a hydrophobic interface, conformational changes take place, opening the lid and allowing access of the substrate to the active site (DEREWENDA et al.,

1992; FERNANDEZ-LAFUENTE et al., 1998). According to Blanco et al. (2004), the activation of high surface area mesoporous silica with octyltriethoxysilane enabled the hydrophilic surface of the silica to be covered with a hydrophobic layer of octyl groups. However, due to the nature of the non-covalent immobilization forces, losses of the active enzyme can occur due desorption, mainly in aqueous media, changing the conditions that influence the enzyme-support interaction, such as pH, ionic strength, temperature, and polarity of the solvent (BRENA; GONZÁLEZ-POMBO; BATISTA-VIERA, 2006). On the other hand, covalent binding provides a stronger and more permanent linkage between the lipase and the support (NIGAM et al., 2014), which can result an increase on the thermal stability of the immobilized enzyme, stability against organic solvents, and avoidance of leakage during operation due to variations of pH and temperature (BERNAL; ILLANES; WILSON, 2014). Covalent attachment of the enzyme to the functional group from the support surface occurs through chemical binding between side-chains of amino acid residues of the enzyme and active groups of the support. However, in order to achieve high levels of bound activity, the amino acid residues essential for catalytic activity must not be involved in the covalent linkage to the support (BRENA; GONZÁLEZ-POMBO; BATISTA-VIERA, 2006). Besides, in the case of lipases it is advantageous to immobilize the enzyme molecule on the support by multipoint covalent attachment under conditions favoring its open form, because multipoint covalent attachments will avoid conformational change impairing the opening of the lid (ADLERCREUTZ, 2013).

Some studies have considered the influence of heterofunctional (hydrophilic-hydrophobic) supports on the activity and stability of enzymes (BERNAL; ILLANES; WILSON, 2014; MATEO et al., 2010; MATEO et al., 2013; REIS et al., 2006). Reis et al. (2006) reported that lipase covalently attached to a hydrophobic surface yielded a higher esterification:hydrolysis ratio than lipase adsorbed on a hydrophilic surface, and that the nature of the surface and the mode of immobilization were also important.

Bernal, Illanes e Wilson (2014) reported that heterofunctional supports possessing both hydrophilic and hydrophobic moieties were very suitable for the immobilization of lipases, thus enabling adsorption of the enzyme by interfacial activation and its stabilization by covalent attachment to the support. In their work, porous silica was chemically modified with octyl, glyoxyl, and octyl-glyoxyl groups, in order to immobilize lipases from *Pseudomonas stutzeri* and *Alcaligenes* sp. for use in the synthesis of lactulose

palmitate. Both lipases immobilized on hydrophobic-hydrophilic silica (octyl-glyoxyl-silica) showed the highest stabilities in acetone.

In this study, evaluation was made of chemical modification of the silica surface with octyl, octyl-epoxy, octyl-glyoxyl, octyl-glutaraldehyde, and glutaraldehyde for immobilization of lipase from *Candida antarctica* type B, to be used mainly in organic syntheses. Using these coatings, and changing the immobilization conditions, it was possible to immobilize the lipase to the silica surface by means of distinct regions where the coupling was favored, such as enzyme regions that were more hydrophobic or richer in lysine residues, or by the terminal amino group. On the other hand, the use of an excess of hydrophobic groups, relative to other reactive groups (such as aldehydes) enabled the hydrophobicity of the silica to be greatly increased, while providing a sufficient density of reactive groups to allow covalent linkages between enzyme and support. This strategy could be used to produce biocatalysts with improved properties.

The best immobilized lipase, chosen based on immobilization yield, recovered activity, and thermal stability in *tert*-butyl alcohol, as well as the highest activities in hydrolysis, esterification, and transesterification reactions, was used to investigate the synthesis of fructose oleate.

3.2. Experimental

3.2.1. Materials

(3-Glycidyloxypropyl)trimethoxysilane (GPTMS), triethoxy(octyl)silane (OCTES), lipase from *Candida antarctica* type B (CALB), Novozyme 435 (CALB immobilized on macroporous acrylic resin), (3-aminopropyl)triethoxysilane (APTES), Bengal Rose dye, bovine serum albumin, methyl heptadecanoate, butyric acid, n-butanol, and p-nitrophenyl butyrate (p-NPB) were purchased from Sigma-Aldrich Co. (St. Louis, USA). Anhydrous ethyl alcohol and molecular sieve (3 Å) were purchased from JT Baker (New Jersey, USA). Tert-butyl alcohol, tert-pentyl alcohol, sulfuric acid, and glutaraldehyde were purchased from Vetec (São Paulo, Brazil). Heptane, fructose, toluene, sodium periodate, sodium borohydride, and oleic acid were purchased from Synth (São Paulo, Brazil). Silica (Immobead S60S) was obtained from Chiral Vision (Leiden, The Netherlands). Olive oil (Carbonell®) was purchased in a local market.

3.2.2. Synthesis of the supports

Macroporous silica, average pore diameter 193 Å (LIMA et al., 2015), was chemically modified using OCTES, APTES, and GPTMS. The porous silica was previously treated with HCl (0.1 M) under reflux, followed by washing until neutral pH and drying at 50 ^{o}C for 12 h (TANI; SUZUKI, 1996).

Octyl-Silica (OS). Octyl groups were incorporated on the silica surface using the methodology described by Tani and Suzuki (1996), where a suspension containing 1 g of dry silica and 20 mL of a mixture of OCTES:toluene (1:10, v/v) was kept under reflux for 4 h at the boiling temperature of the solvent. The activated support was washed with toluene and distilled water, and dried at 40 °C for 24 h.

Octyl-Silica-Glutaraldehyde (OSGlu). 1 g of dry silica was chemically modified with octyl and amine groups using 1.5 mL of OCTES and 0.5 mL of APTES dissolved in 18 mL of toluene. The reaction was performed at boiling temperature under reflux for 4 h. The modified silica was washed with toluene and distilled water, and dried at 40 °C for 24 h.

The activation of the support with aldehyde groups was performed immediately before its use in the CALB immobilization, according to the methodology described by Tardioli, Zanin and Moraes (2000). Briefly, 1 g of dry silica modified with octyl and amine groups was suspended in 10 mL of 0.2% (v/v) glutaraldehyde solution prepared in sodium phosphate buffer (100 mM, pH 7.0). The suspension was stirred for 1 h at room temperature, next the activated support was recovered by filtration and washed with an excess of distilled water.

Octyl-Silica-Epoxy (OSEpx). 1 g of dry silica was chemically modified with octyl and epoxy groups using 1.5 mL of OCTES and 0.5 mL of GPTMS dissolved in 18 mL of toluene. The mixture was kept at boiling temperature under reflux for 4 h, as described by Pereira, Guisan and Giordano (1997). The support was washed with toluene and distilled water, then dried at 40 °C for 24 h.

Octyl-Silica-Glyoxyl (OSGlx). This support was prepared by acid hydrolysis of the epoxy groups of the OSEpx support obtained previously. 1 g of OSEpx was suspended in 30 mL of 0.1 M sulfuric acid and kept under reflux at 85 ^{o}C for 2 h (PEREIRA; GUISAN; GIORDANO, 1997). In this step, the opening of the epoxy rings generated glyceryl groups on the silica surface. The support was then washed with toluene and distilled water, and dried at 40 °C for 24 h.

The glyceryl groups were oxidized to aldehyde groups using sodium periodate, employing the methodology described by Pereira, Guisan and Giordano (1997). Briefly, 1 g of dry silica modified with octyl and glyceryl groups was suspended in 10 mL of 0.1 M $NaIO_4$ and kept under stirring in a GyroTwister GX-1000 3-D Shaker (S1000-A, Labnet International, Inc., New Jersey, USA) at room temperature for 1 h. The support was then washed with distilled water and dried at 40 °C for 24 h.

3.2.3. Characterization of the supports

The supports were characterized in terms of their hydrophobicity by adsorption of Bengal Rose dye, using an adaptation of the methodology described by Williams et al. (1998). Typically, 0.1 g of the support was suspended in 15 mL of Bengal Rose solution (320 µg/mL) and kept under stirring in a GyroTwister GX-1000 3-D Shaker (S1000-A, Labnet International, Inc., New Jersey, USA) for 24 h at room temperature. The absorbance of the Bengal Rose solutions (using the supernatants obtained before and after the adsorption procedure) was measured spectrophotometrically at 549 nm, and the dye concentrations were calculated using a calibration curve. All assays were performed in triplicate.

3.2.4. CALB immobilization

Immobilization on OS and OSGlu. The immobilizations of CALB on OS and OSGlu were performed in a single step, where 1 g of OS or OSGlu was suspended in 15 mL of an enzyme solution prepared in 10 mM sodium phosphate buffer (pH 7.0). The suspension was kept under mild stirring in a GyroTwister GX-1000 3-D Shaker (S1000-A, Labnet International, Inc., New Jersey, USA) for 6 h, at room temperature. The biocatalysts were recovered by filtration, washed with distilled water, and dried/stored at 4 ^{o}C for further use. The immobilization yield was monitored by measuring the protein concentration using the Bradford method (BRADFORD, 1976) and by determining the *p*-NPB hydrolytic activity in the immobilization supernatants.

Immobilizations on OSEpx and OSGlx. The immobilizations on OSGlx and OSEpx required two steps. In the first step, 1 g of OSEpx or OSGlx was suspended in 15 mL of an enzyme solution prepared in 10 mM sodium phosphate buffer (pH 7.0), in order to promote the hydrophobic enzyme adsorption (similar to the immobilization on OS). The suspension was kept under mild stirring in a GyroTwister GX-1000 3-D Shaker (S1000-A,

Labnet International, Inc., New Jersey,USA) for 6 h, at room temperature. The biocatalysts were recovered by filtration, resuspended in 15 mL of 100 mM sodium phosphate buffer (pH 10.0), and gently stirred at room temperature for 3 h, as described by Bernal, Illanes and Wilson (2014), to allow the formation of Schiff bases between the amine groups of the enzyme and the aldehyde groups of the OSGlx support, and covalent linkages between the epoxy groups of the OSEpx support and the nucleophile groups on the enzyme surface, such as lysine, histidine, cysteine, tyrosine, etc (MATEO et al., 2007). Afterwards, the biocatalysts prepared on OSGlx were reduced with 1 mg/mL sodium borohydride for 1 h to convert the remaining active aldehydes on the support surface to inert hydroxyl groups, as well as to convert reversible Schiff bases to irreversible covalent linkages (BRENA; GONZÁLEZ-POMBO; BATISTA-VIERA, 2006). The remaining reactive epoxy groups on the OSEpx surface were blocked with glycine (3 mM, 1 h, room temperature) (MATEO et al., 2007). The immobilization yield was monitored by measuring the protein concentration using the Bradford method (BRADFORD, 1976), and by determining the *p*-NPB hydrolytic activity in the immobilization supernatants.

Protein loading assay. CALB was immobilized on the OSGlu support (the most suitable support to immobilize CALB) in the presence of different protein loadings (1, 10, and 40 mg of protein/g of support). Briefly, 1 g of support was suspended in 15 mL of an enzyme solution prepared in 10 mM sodium phosphate buffer (pH 7.0). The suspension was kept under mild stirring in a GyroTwister GX-1000 3-D Shaker (S1000-A, Labnet International, Inc., New Jersey, USA) for 6 h, at room temperature. The biocatalysts were recovered by filtration, washed with distilled water, and dried/stored at 4 ^{o}C for further use. The immobilization yield was monitored by measuring the protein concentration using the Bradford method (BRADFORD, 1976), and by determining the *p*-NPB hydrolytic activity in the immobilization supernatants.

3.2.5 Characterization of the biocatalysts

Hydrolysis activity. The hydrolysis activity of soluble CALB was assayed in a glass cuvette, measuring the absorbance increase at 405 nm caused by the release of *p*-nitrophenol (ε = 6080 M^{-1} cm^{-1}) during the hydrolysis of *p*-NPB solution (25 µL of *p*-NPB dissolved in 50 mM acetonitrile and 2 mL of 25 mM sodium phosphate buffer at pH 7.0) at 25 ^{o}C, based on the methodology of Martinelle, Holmquist and Hult (1995). One unit of

hydrolysis activity (U) was defined as the initial rate of hydrolysis of *p*-NPB (μmols/min) under the conditions described above.

The hydrolytic activity of the immobilized CALB was determined using a mechanically stirred 25 mL reactor, under the same conditions described for the soluble CALB, but on a larger scale, employing 0.25 mL of *p*-NPB solution and 20 mL of 25 mM sodium phosphate buffer at pH 7.0. Samples were withdrawn at 1 min intervals for quantification (at 405 nm) of the *p*-nitrophenyl released.

Esterification activity. The esterification activities of the immobilized CALB were assessed using the synthesis of butyl butyrate at 40 °C as a model reaction, employing 0.1 M of butyric acid and 0.1 M of *n*-butanol in 15 mL of heptane, and 0.2 g of molecular sieve, as described by Oliveira et al. (2000). The progress of the reaction was followed using gas chromatography to monitor the consumption of *n*-butanol. One unit of esterification activity (U) was defined as the initial rate of production of butyl butyrate (mM/min) under the conditions described above.

Transesterification activity. The transesterification activities of the immobilized CALB were assessed using the ethanolysis of olive oil (oil with high concentration of oleic acid) as model reaction, employing a Discover® microwave reactor (CEM Co., Matthews, USA) to achieve a high reaction rate in a short time period. The transesterification conditions were established previously: 40 °C, 1:7 olive oil/anhydrous ethanol ratio, and 0.5 g of immobilized biocatalyst (CALB immobilized on OS, OSGlu, OSGlx, or OSEpx). All assays were performed in duplicate. 1 mL aliquots were withdrawn from the reaction medium at intervals of 30 min, up to 2 h of reaction, for measurement of fatty acid ethyl esters (FAEE) by gas chromatography. The samples were previously pretreated using phase separation, where the oil phase (FAEE and unreacted acylglycerols) was decanted and dried at 100 °C for 2 h. One unit of transesterification activity (U) was defined as the initial rate of FAEE production (μg of FAEE/g of sample per minute) under the conditions described previously.

Thermal stability in tert-butyl alcohol. The stabilities of the biocatalysts (CALB immobilized on OS, OSGlu, OSGlx, or OSEpx) were evaluated by incubating them in pure *tert*-butyl alcohol at 45 °C. The stability of the selected biocatalyst (CALB immobilized on OSGlu) was then compared with the soluble CALB at 65 °C. Samples were periodically withdrawn for immediate measurement of the residual *p*-NPB hydrolysis activity.

Quantification of the percentage of lysine residues linked to the OSGlu support. Soluble and immobilized CALB (3.0-3.5 mg of total protein) were suspended in 1 mL of 6 M HCl, oxygen was withdrawn under nitrogen atmosphere, and kept at 105 °C for 24 h. The samples were cooled, neutralized with 1 mL of 10 M KOH, filtered, and fully dried using compressed air before measurement of soluble amino acids by liquid chromatography.

3.2.6 Enzymatic synthesis of fructose oleate

Solubility of fructose. Tert-butyl and *tert*-pentyl alcohols were evaluated for the solubilization of fructose, using 150 mM quantities of fructose added to the organic solvents in closed tubes. The solutions were stirred in a Marconi incubator (MA 430/1) equipped with 360°-stirring carrousel (Marconi®, Piracicaba, Brazil) at 60 °C for 24 h and then centrifuged at 10,000 rpm (12,857 × *g*). One milliliter of the supernatant was dried at 70 °C for 24 h. After evaporation of the tertiary alcohol, 1 mL of water was added prior to measurement of the fructose concentration by liquid chromatography, allowing calculation of the fructose solubility.

Optimization of fructose oleate synthesis. The synthesis of fructose oleate was evaluated considering the effects of the following parameters: fructose/oleic acid molar ratio, concentration of molecular sieve (%, m/v), and reaction temperature. The initial conditions were: 50 mM of oleic acid and fructose, 0.5 g of molecular sieve, 5 mL of *tert*-butyl alcohol, temperature of 45 °C, and 24 h under stirring in a Marconi incubator (MA 430/1) equipped with 360°-stirring carrousel (Marconi®, Piracicaba, Brazil). These initial conditions were chosen based on previously published studies (DEGN; ZIMMERMANN, 2001; DUCRET et al., 1996; FERRER et al., 2005; KHALED et al., 1991; ŠABEDER; HABULIN; KNEZ, 2006). Subsequently, the biocatalyst was separated by centrifugation at 10,000 rpm (12,857 × *g*) and the supernatant was dried at 70 °C for 24 h to evaporate the *tert*-butyl alcohol. Water (1 mL) was added to the dry extract prior to determination of the concentration of unconverted fructose by liquid chromatography.

Effects of reaction time and enzymatic loading on the fructose oleate synthesis. The kinetics of the fructose oleate synthesis was evaluated at a temperature of 55 °C, with a fructose/oleic acid molar ratio of 1:5 and 20% (m/v) molecular sieve (the best conditions determined). The reactions were performed in closed tubes maintained under stirring in a Marconi incubator (MA 430/1) equipped with 360°-stirring carrousel (Marconi®, Piracicaba, Brazil). The biocatalyst was CALB immobilized on OSGlu, loaded with 40 mg of protein/g of

support, and the kinetics was evaluated using enzymatic loadings of 1.05, 2.10, 4.20, and 8.40 U/mL of solvent. An assay was also performed using the maximum concentration of fructose (130 mM), and an enzymatic loading of 8.40 U/mL of solvent. Samples (1 mL) were withdrawn at regular intervals for quantification of unconverted fructose by liquid chromatography, as described previously.

Operational stability. The best biocatalyst prepared in this study (CALB immobilized on OSGlu) was evaluated for reuse in the synthesis of fructose oleate, using the optimal reaction conditions established previously. After each batch, the biocatalyst and the molecular sieve were recovered, washed with *tert*-butyl alcohol, and reused in a new synthesis. Just as a comparison, the operational stability of the most popular immobilized CALB (Novozyme 435) was also evaluated at the same conditions.

3.2.7 Chromatographic analyses

Fructose analysis. Fructose concentrations were determined using a Waters HPLC equipped with a refractive index detector and a Sugar Pak-I column (300 x 6.5 mm x 10 µm) kept at 80 °C. Ultrapure water was used as eluent, at 1 mL/min. The calibration curve was constructed using fructose standards at concentrations ranging from 0.1 to 8.0 g/L.

Fructose oleate purification. The purification of sugar esters by silica gel chromatography has been widely reported (DUCRET et al., 1995; DUCRET et al., 1996; SCHECKERMANN et al., 1995). Here, fructose oleate was purified by medium-pressure liquid chromatography using a Shimadzu HPLC equipped with a refractive index detector and a Nova-Pak silica column (150 × 3.9 mm, 5 µm, Waters) kept at 30 °C. A solution of hexane/ethanol (90:10, v/v) containing 0.1% acetic acid was used as eluent, at 1 mL/min.

Fatty acid ethyl esters. Samples (50 mg) were diluted in 10 mL of a solution of ethyl heptadecanoate (10 mg/mL), used as an internal standard. Aliquots (1 µL) were injected into an Agilent 7890 GC equipped with a Restek 12423 column (30 m × 0.25 mm × 0.25 µm) and a flame ionization detector operated at 250 °C as described by (LIMA et al., 2015). The analysis was performed for 18 min using nitrogen as carrier gas (25 mL/min), with the following heating program: 150 °C for 2 min, ramp to 180 °C at 10 °C/min, hold for 3 min, ramp to 230 °C at 10 °C/min, and hold for 5 min.

n-Butanol. The consumption of *n*-butanol during the synthesis of butyl butyrate was monitored by gas chromatography. Samples (1 µL) were injected into an Agilent 7890 GC equipped with a Restek 12423 column (30 m × 0.25 mm × 0.25 µm) and a flame

ionization detector operated at 250 °C. The analysis was performed for 6.33 min using nitrogen as carrier gas (25 mL/min), with the following heating program: 100 °C for 2 min, ramp to 200 °C at 30 °C/min, and hold for 1 min. The calibration curve was constructed using *n*-butanol standards at concentrations ranging from 0 to 200 mM.

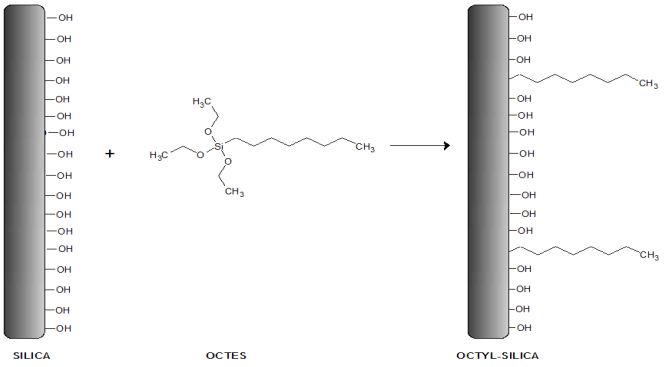
3.2.8. Extraction, purification, and characterization of fructose oleate

Under the optimized conditions, fructose oleate was recovered from the reaction medium (free from *tert*-butyl alcohol and biocatalyst) by liquid-liquid extraction using a mixture of dichloromethane/water (1:2, v/v). The aqueous and organic phases were separated by centrifugation at 10,000 rpm (12,857 × *g*) (4 ^{o}C) for 20 min. Each phase was again submitted to liquid-liquid extraction (3 × 20 mL) using dichloromethane and water, respectively. The organic phase was concentrated under reduced pressure, yielding an oily extract. The fructose oleate in this oily extract was purified by silica gel chromatography, as described previously, and was characterized by mass spectrometry using a Waters triple quadrupole Xevo TQ instrument equipped with an electrospray ionization source operated in positive mode. Data acquisition and analysis employed MassLynx software (Waters). A solution of purified fructose oleate (1 μg/mL) in methanol/water (50:50, v/v) was introduced into the system by infusion at a flow rate of 20 μL/min. The conditions established for the analysis were as follows: capillary voltage of 3.8 kV, cone voltage of 32 V, temperature of 200 °C, and collision energy of 20 eV. These ionization conditions were optimized with the MassLynx software, using the IntelliStart automatic adjustment tool. The mass spectrometer was operated in ion scanning mode, with evaluation of the 100-500 amu range and a scan time of 4 s.

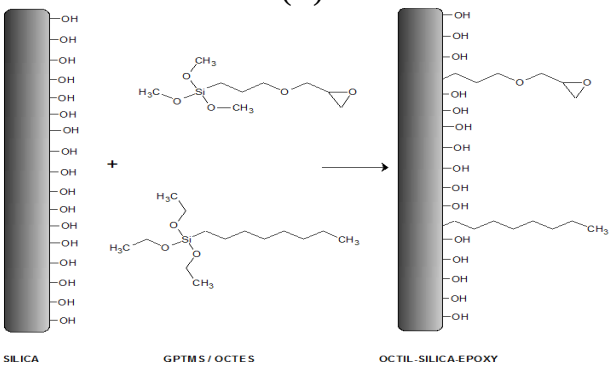
3.3. Results and Discussion

In this work was investigated the influence of coating the silica surface with different hydrophobic/hydrophilic moieties on its hydrophobicity, the immobilization yield, and the recovered activity of the immobilized lipase. In order to assist the readers in the discussion concerning the enzyme immobilization on the different silica surfaces, Figure 3.1 shows the chemical moieties introduced onto the supports surface. Only one group of each is shown as an illustration, although the surface was coated using different hydrophobic/hydrophilic moiety densities.

Figure 3.1 - Schematic representation of the chemical modification of the silica surface. (A) octyl groups (OS support), (B) epoxy and octyl groups (OSEpx support), (C) glutaraldehyde and octyl groups (OSGlu support), and (D) glyoxyl and octyl groups (OSGlx support).



(A)



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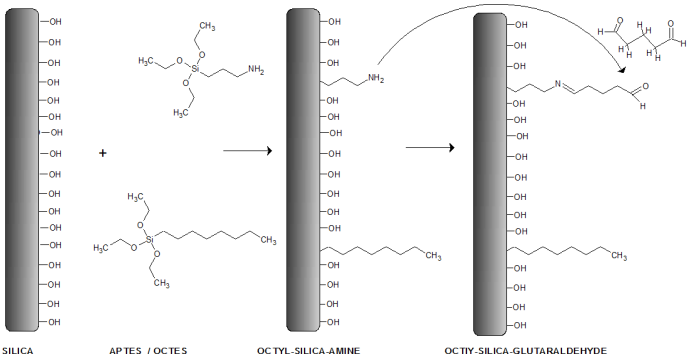
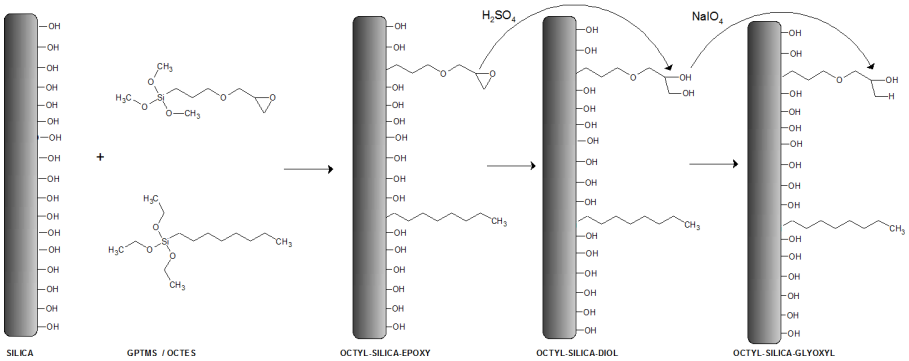


(C)



(D)

3.3.1. Hydrophobicity of the modified and unmodified silica

The hydrophobicity of the supports prepared in this study was evaluated by the amount of a hydrophobic compound (Bengal Rose dye) adsorbed per unit of surface area. The hydrophobicity of a support is an important property to be considered in the immobilization of lipases (LIMA et al., 2015). LIMA et al. (2015) showed that the chemical modification of the silica with OCTES no represented significant variation on area of the pore (74.80 m^2/g before and 73.20 m^2/g, after the modification with OCTES). The chemical modification of the silica with OCTES increased its capacity to adsorb the hydrophobic dye from 62.91 ± 5.83 $\mu g/m^2$ (unmodified silica) to 263.54 ± 14.83 $\mu g/m^2$ (octyl-silica, OS). On the other hand, the introduction of heterofunctional groups (octyl-epoxy and octyl-glyoxyl) decreased the adsorption capacity by approximately 30 to 50% (143.35 ± 8.50 $\mu g/m^2$ for OSEpx and 180.47 ± 8.62 $\mu g/m^2$ for OSGlx). The results of dye adsorption by the different supports are shown in table 3.1.

Table 3.1 – Adsorption of Bengal Rose dye by modified and non-modified silica

Supports	**μg_{dye} / $g_{support}$**	**μg_{dye} / $m^2_{support}$**
Silica	4705.44 ± 435.76	62.91 ± 5.83
OS	19713.00 ± 1109.01	263.54 ± 14.83
OSEpx	10722.67 ± 635.83	143.35 ± 8.50
OSGlx	13499.20 ± 644.83	180.47 ± 8.62
OSAmino	45718.33 ± 167.44	611.21 ± 2.24
OSGlu	36571.83 ± 1314.15	488.93 ± 17.57

The OSGlu surface showed the highest adsorption capacity for the hydrophobic dye (488.93 ± 17,57 $\mu g/m^2$). This could be explained by the presence of ionized groups in the chemical structure of the dye, which were able to interact ionically with the nitrogen atoms on the support surface (Figure 3.1-C), which had a partial positive charge. This could contribute to the immobilization of the lipase, due to differences between the hydrophobic and ionic forces in terms of the orientation of the enzyme molecule to the support surface.

3.3.2. CALB immobilization

The OS, OSGlu, OSEpx, and OSGlx supports were evaluated in the immobilization of *Candida antarctica* lipase (CALB). Table 3.2 shows the immobilization yield and recovered activity results for the biocatalysts with low loadings (1 mg of protein/g of support). All the heterofunctional supports showed high immobilization yields (92-98%). The highest recovered activity was observed for CALB immobilized on OSGlu (71%), while the lowest values (around 18%) were obtained for CALB immobilized on the OSEpx and OSGlx supports. CALB immobilized on monofunctionalized silica also yielded biocatalysts with lower recovered activities, compared to the use of OSGlu, with values of 43.5% and 32% for CALB immobilized on OS and SGlu (silica modified only with glutaraldehyde), respectively. As reported by Bernal, Illanes and Wilson (2014), the ratio of hydrophobic and hydrophilic moieties in heterofunctional supports has a crucial influence on the activities of immobilized biocatalysts. In addition, the choice of the functional groups depends on the unique characteristics of each enzyme. In the case of CALB, the combination of hydrophobic-hydrophilic moieties (octyl-glutaraldehyde) did not impair the conformational structure required for the catalysis.

Table 3.2 - Protein immobilization yields (in terms of protein) and recovered activities of CALB immobilized on chemically modified silica (protein loading of 1 mg/g of support).

Support	Protein immobilization yield, %	Recovered activity, %
OS	74.8 ± 4.3	43.5 ± 0.5
OSGlu	97.5 ± 0.4	71.0 ± 2.3
SGlu[a]	89.7 ± 1.4	32.6 ± 0.1
OSEpx	92.3 ± 1.5	17.8 ± 0.9
OSGlx	93.5 ± 0.5	18.8 ± 0.9

[a]The SGlu support (silica modified with glutaraldehyde) was used as a control to show that the heterofunctional support (octyl-silica-glutaraldehyde) was superior to the monofunctional supports.

Soluble CALB was stable under all the immobilization conditions (room temperature, pH 7.0 or 10.0). The lower recovered activities on the OS, OSEpx, and OSGlx supports could therefore be attributed to the higher hydrophobicity of these supports and/or excessive linkages between ε-amino groups of lysine residues in the lipase surface and epoxy (or aldehyde) groups of the support, which impaired the conformational structure of the enzyme.

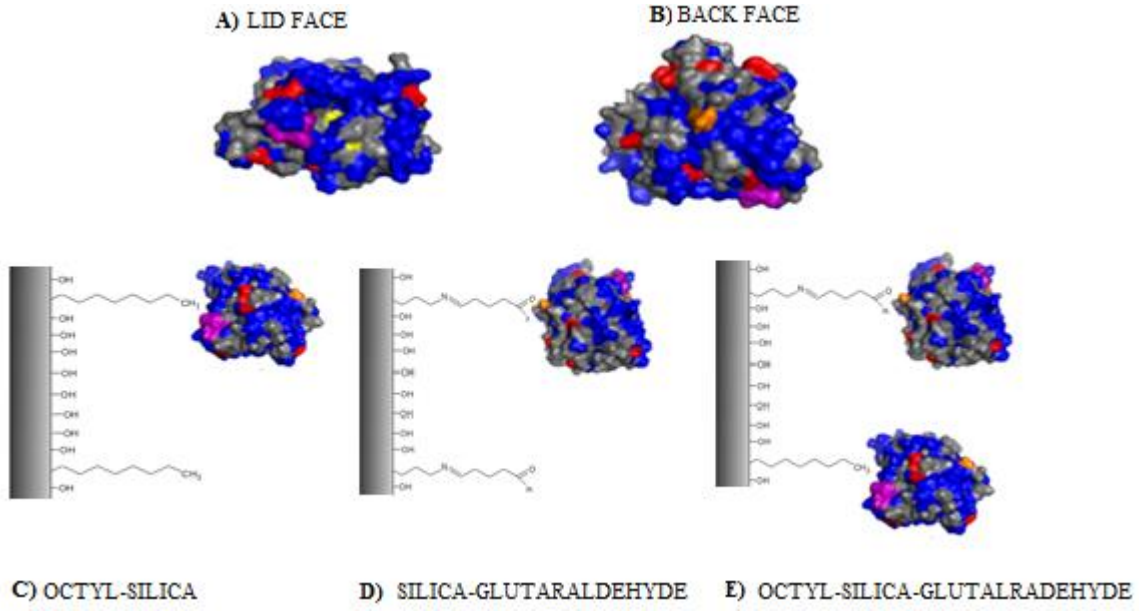
Lipases present high affinity not only for water-oil interfaces, but also for hydrophobic surfaces (such as water-immiscible organic solvents, glass and plastic surfaces, and air bubbles). These enzymes can therefore be irreversibly adsorbed on such interfaces and become deactivated (SCHMID; VERGER, 1998). According to Mateo et al. (2007), the presence of a hydrophobic surface close to the enzyme surface can have a large negative effect on its properties. Blanco et al. (2004) reported that the recovered activities for CALB immobilized on OS were 43% and 25%, using tributyrin and *p*-nitrophenyl propionate as substrates, respectively. The authors attributed the loss of activity to the small changes in the tertiary structure of the lipase promoted by hydrophobic interactions.

Another reasonable hypothesis for the loss of activity is the partitioning of water molecules in the microenvironment of the protein, affecting its catalytic mechanism. Lipases present essentially the same catalytic mechanism as proteases, with the catalytic triad composed of serine, histidine, and aspartate/glutamate residues, and the water molecules participating in the hydrolysis of the ester bonds (SCHMID; VERGER, 1998). Hence, a highly hydrophobic microenvironment could create partitioning delays for the water molecules, impairing the catalytic mechanism of the lipase. The OSGlu support had moieties containing nitrogen atoms (Figure 3.2-C) with a pair of free electrons, allowing the interaction with of H_3O^+ ions. This could create a hydrophilic microenvironment close to the enzyme, maintaining its catalytic activity. Mateo et al. (2007) reported that the negative effects on the properties of an enzyme due to its proximity to hydrophobic surfaces could be avoided using hydrophilic compounds in the blockage of the epoxy groups. Although the second step in the immobilization of CALB on the OSGlx support (reduction of remaining aldehyde groups to hydroxyl groups) and on the OSEpx support (blocking of remaining epoxy groups with glycine) created a more hydrophilic microenvironment, the hydrophobic adsorption in the first step of the immobilization could have already caused irreversible changes in the protein structure.

In addition to the hypotheses described above, the possibility of diffusional delays and steric hindrances caused by the immobilization procedure should not be discounted. The immobilization of the protein with the active site facing the support could lead to steric hindrance of access of the substrate to the active site of the enzyme. It has been reported that the active site of CALB is located in the center of the protein (as shown in Figure 3.2-A), and that its access is through a path in the form of a long and narrow funnel, with highly hydrophobic amino acid residues surrounding the internal cavity (UPPENBERG

et al., 1994). Consequently, compared to other lipases, CALB has a limited space in the active site, explaining its specificity for short and medium chain fatty acids (FERRARIO et al., 2013). For all the supports prepared in this work, the first step of the immobilization was performed at low ionic strength and pH 7.0, with the aim of immobilizing the enzyme to the support by means of its most hydrophobic face. It is likely that this face corresponded to the lid face, which contains five amino acid residues (Gly-Pro-Leu-Asp-Ala). In the case of the OS support, the immobilization of CALB to the support was probably by this face (Figure 3.2-C). The immobilization of CALB to the OSGlx and OSEpx supports was also expected to be by the lid face, because at pH 7.0 (in the first step of the immobilization) the ε-amino groups of lysine residues (which were more abundant in the back face of the molecule, as shown in Figure 3.2-B) are protonated, and therefore had low reactivity with the glyoxyl and epoxy groups of the support.

Figure 3.2. 3D surface structure model of CALB (Protein Data Bank, code PDB 1TCA) constructed using PyMOL Molecular Graphics System, v. 1.7.4 (Schrödinger, LLC). (A) Lid face and (B) back face. The color patterns represent amino acid residues, as follows: blue - hydrophobic residues; red - Lys; yellow - catalytic triad; orange - *t*-NH_2; purple - residues from the hydrophobic lid. Figures (C), (D) and (E) represent the probable orientations of CALB to the OS, SGlu and OSGlu supports, respectively.



On the other, hand the immobilization of CALB on SGlu at pH 7.0 is expected to be covalently immobilized on the support by the terminal amino (*t*-NH_2) face (Figure 3.2-

D), due to its high reactivity at pH 7.0 and low ionic strength (BARBOSA et al., 2012; BETANCOR et al., 2006). However, concerning the immobilization of CALB on heterofunctional hydrophobic-hydrophilic supports (OSGlu) it is expected that there is a balance between the two different methods (Figure 3.2-E). This hypothesis could explain the higher value of recovered activity presented by OSGlu.

The hypothesis that a fraction of CALB molecules was immobilized on OSGlu with the active site facing the medium was highlighted through the comparative study of the amino acid percentage of free and immobilized CALB (Table 3.3). The decrease of around 43% of the lysine residues after protein immobilization suggests that the aldehyde groups present on the support OSGlu reacted with the amino groups present on the surface of the enzyme (terminal amino and amino from lysine residues). It is also important observe that the highest density of lysines (around six residues) are located in the opposite face of the lid (Figure 3.2-B). In this way, it is very likely that many covalent linkages enzyme-support (OSGlu) occurred in the region located on the opposite face of the lid.

Table 3.3 – Amino acid percentage of lysines for free and immobilized CALB after acid hydrolysis

Amino acid	Free-Theoretic	Free-Experimental	OSGlu
Lys	2.84	3.00 ± 0.12	1.61 ± 0.13

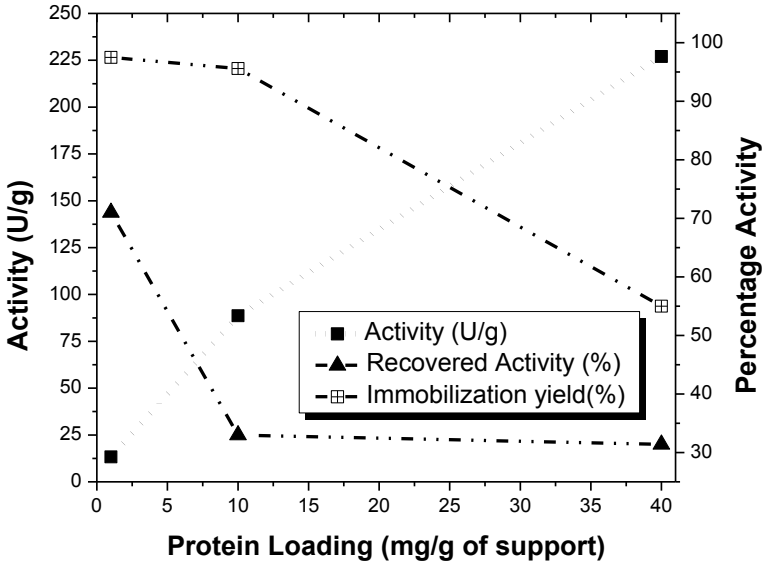
3.3.3 Protein loading assay

The amount of enzyme immobilized on carriers has a great influence on the performance of the immobilized enzyme. According to Singh et al. (2013), diffusional delays can occur in highly loaded biocatalysts, resulting in an unequal distribution of the enzyme within the porous support and causing a significant reduction of the pore volume available to the access substrate and product molecules. However, it is expected that the highly porous nature of the silica used here should enable high CALB loading. Blanco et al. (2004) reported the preparation of high loadings of CALB derivatives on octyl-silica, where CALB was immobilized in the monolayer form and the formation of enzyme aggregates was avoided up to loadings of 200 mg of protein per gram of support.

The effect of the loading of CALB on the OSGlu support (the best support evaluated in this work) on the immobilization yield, recovered activity, and expressed activity of the biocatalyst is shown in Figure 3.3. High loadings enabled the preparation of

biocatalysts with high apparent hydrolytic activities (approximately 230 U/g), although, as expected, the recovered activities decreased dramatically (from 71% for the lowly loaded biocatalyst to 31.4% for the highly loaded biocatalyst), because the existence of diffusional delays did not permit measurement of every active immobilized enzyme molecule. In the hydrolysis of *p*-NPB or other small substrates that are rapidly hydrolyzed, the reaction rate is expected to be faster than the diffusion rate. Diffusional delays could be decreased by using substrates that are hydrolyzed more slowly, such as tributyrin (BLANCO et al., 2004), fatty acid esters, and others. Changing the protein loading from 10 to 40 mg/g resulted in a significant reduction in the immobilization yield (from 95.6 to 55%), but almost no change in the recovered activity (around 32% for both biocatalysts). Blanco et al. (2004) also reported that the recovered activities of CALB immobilized on octyl-silica were the same for loadings of 50 and 200 mg/g support.

Figure 3.3 - Protein loading assay. Hydrolytic activity was determined at 25 ^{o}C with *p*-NPB as substrate.



3.3.4. Characterization of the biocatalysts

Activities. The OS, OSGlu, OSEpx, and OSGlx supports loaded with CALB immobilized using 10 mg of protein/g of support were evaluated in terms of their activities in hydrolysis (using *p*-NPB as substrate), esterification (synthesis of butyl butyrate), and transesterification (synthesis of FAEE using olive oil and ethanol). The results are shown in Table 3.4 as mean values of duplicates.

Table 3.4 - Enzymatic activities of CALB immobilized on hydrophobic and heterofunctional hydrophobic-hydrophilic supports.

CALB-IS[a]	Hydrolysis activity (Specific activity)[b]	Esterification activity (Specific activity)	Transesterification activity (Specific activity)
OSEpx	23.7 ± 0.9 (2.5)	4.8 ± 0.3 (0.5)	186.1 ± 19.7 (20.0)
OSGlx	8.9 ± 0.5 (1.0)	5.7 ± 0.5 (0.6)	130.8 ± 1.8 (14.2)
OSGlu	51.1 ± 1.3 (5.3)	18.9 ± 1.7 (1.9)	1807.2 ± 95.7 (186.3)
OS	11.3 ± 0.4 (1.5)	4.6 ± 0.1 (0.6)	524.8 ± 74.1 (70.0)

[a]Lipase from *Candida antarctica* B immobilized on derivatized silica (OSEpx, OSGlx, OSGlu, or OS). Protein loading: 10 mg protein/g of support. Immobilized protein (expressed as mg of protein/g support): 9.3 (OSEpx), 9.2 (OSGlx), 9.7 (OSGlu), and 7.5 (OS). Activities are expressed as U/g of support. [b]The values between parentheses correspond to specific activities (U/mg of immobilized protein).

The highest specific activities were found for CALB immobilized on OSGlu. The activities (in hydrolysis, esterification, and transesterification) of CALB immobilized on OSGlu were approximately 3-5 times higher than the activities of CALB immobilized on OS. These results showed that heterofunctional hydrophobic-hydrophilic supports had an important effect on the CALB activity. In addition, the higher activities of CALB immobilized on OSGlu towards a large substrate (oleic acid) corroborated the hypothesis of immobilization of large fraction of CALB with the active site facing the medium, which could have reduced the effects of diffusional delays and steric hindrances.

The hydrolysis, esterification, and transesterification activities of CALB immobilized on the OSGlu support were approximately 5.0, 3.5, and 11.0 times higher than for CALB immobilized on the OSGlx and OSEpx supports. These results suggested that higher hydrophobicity of the substrate resulted in decreased access to the active sites of the CALB immobilized on the OSGlx and OSEpx supports, compared to the OSGlu support. This behavior could be explained by the enzyme immobilization with the hydrophobic region that surrounded the active site facing the support (Figure 3.2-C). Hence, diffusional delays and steric hindrances could have been greater for the biocatalysts prepared on the OS, OSEpx, and OSGlx supports. The adsorption of CALB with the hydrophobic region surrounding the active

site facing the support surface was therefore not sufficient, because besides not exhibiting hyperactivation, steric hindrances were more noticeable. It was previously reported that the lid of CALB does not display modulation between the open and closed forms to regulate the access of the substrate to the active site; the lid seems to help the coupling of the lipase to the water/lipid interface (MARTINELLE; HOLMQUIST; HULT, 1995).

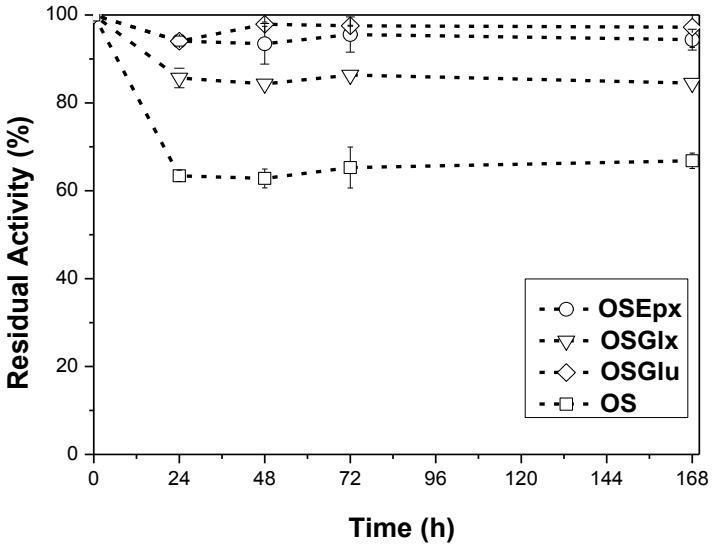
Stability. The thermal stabilities of CALB immobilized on OS, OSEpx, OSGlu, and OSGlx were evaluated at 45 ^{o}C in *tert*-butyl alcohol, because this solvent was more suitable for the solubilization of fructose, as shown below (Section 3.5). The residual activities during the incubation period were measured using *p*-NPB as substrate.

Figure 3.4-A shows the high stability in *tert*-butyl alcohol of CALB covalently immobilized on the heterofunctional supports. CALB immobilized on OSGlu and OSEpx retained around 95% of its initial activity after 168 h, while CALB immobilized on OSGlx retained around 85%. However, CALB immobilized by hydrophobic interactions proved to be the least stable, with around 65% of its initial activity retained after 168 h.

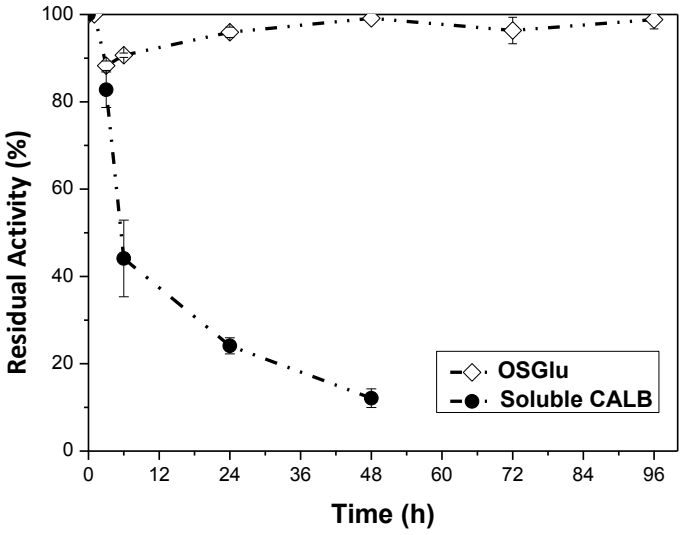
Based on immobilization yield, recovered activity, and thermal stability towards *tert*-butyl alcohol, as well as the highest hydrolysis, esterification, and transesterification activities, CALB immobilized on OSGlu was chosen as the biocatalyst to be used in the synthesis of fructose oleate. Its stability in *tert*-butyl alcohol at 65 °C was compared to that of the soluble enzyme (Figure 3.4-B). Very high stability was observed, with no significant loss of activity after 96 h, while the soluble enzyme lost 50% of its initial activity after only 6 h.

The high stability of CALB immobilized on OSGlu (here named CALB-OSGlu) makes it an excellent biocatalyst from the industrial point of view. In the synthesis of fructose oleate, where *tert*-butyl alcohol is required to solubilize the sugar, the high stability of the biocatalyst could enable its recovery and reuse, contributing to a reduction in manufacturing costs.

Figure 3.4 - Thermal stabilities of lightly loaded biocatalysts (1 mg of protein/g of support) in *tert*-butyl alcohol at (A) 45 °C and (B) 65 °C.



(A)



(B)

3.3.5 Synthesis of sugar fatty acid esters

Solubility of fructose in tertiary alcohols. Two tertiary alcohols were selected for experiments of solubilization of fructose, namely the *tert*-butyl and *tert*-pentyl alcohols. Fructose showed high solubility in *tert*-butyl alcohol, with solubilization of 23.64 ± 0.27 g/L (approximately 130 mM). The solubility of fructose in *tert*-pentyl alcohol was around 30% lower (16.98 ± 0.08 g/L). Based on these results, *tert*-butyl alcohol was selected for use in the synthesis of fructose fatty acid ester, because based on the reaction stoichiometry, a high fructose concentration contributed to an increase in the volumetric productivity of the ester.

Optimization of the fructose oleate synthesis (using OSGlu). In order to optimize the production of fructose oleate, an evaluation was performed of the main parameters affecting the reaction: fructose/oleic acid molar ratio, temperature, and need for molecular sieve to adsorb water released during the reaction. From the results shown in Figure 3.5, an excess of oleic acid (fructose/oleic acid molar ratio of 1:5) significantly favored the reaction, yielding the highest fructose-to-ester conversion. Coulon et al. (1996) reported the same behavior in the synthesis of fructose oleate by transesterification between fructose and oleic acid methyl ester, catalyzed by immobilized *Candida antarctica* lipase. While an excess of oleic acid significantly increased the conversion, an excess of sugar had the opposite effect, reducing the conversion. This effect was also reported by Tarahomjoo and Alemzadeh (2003) in the esterification of glucose and hexadecanoic acid, and by Yan et al. (2001) in the esterification of glucose and stearic acid. This behavior can be attributed to the increased hydrophilicity in the microenvironment of the enzyme caused by the interaction between the sugar and the hydrophilic groups of the enzyme molecules. Thus, a highly hydrophilic microenvironment close to the enzyme could create problems of partitioning for the fatty acid molecules, affecting the catalytic mechanism. Besides, excessive amount of fructose which exhibits water adsorption property may influence the essential water for the enzyme activity (TARAHOMJOO; ALEMZADEH, 2003).

It is well known that a higher temperature increases the reaction rate. However, within the time interval evaluated (24 h), the fructose-to-fructose oleate conversion was not greatly influenced by this parameter (Figure 3.6), with values of 45% at 35 ^{o}C and 58% at 75 ^{o}C. A temperature of 55 ^{o}C was chosen in subsequent assays, because at this temperature, the conversion was close to that at 75 ^{o}C, and from the industrial point of view, a lower temperature implies a lower energy cost of the process.

Figure 3.5 - Effect of the fructose/oleic acid molar ratio in the fructose-to-fructose oleate conversion. The reaction was performed at 45 °C for 24 h, using 0.5 g of molecular sieve and 0.05 g of OSGlu (protein loading of 10 mg/g of support and hydrolytic activity of 51.1 U/g of support, measured at 40 ^{o}C with *p*-NPB as substrate) in 5 mL of *tert*-butyl alcohol.

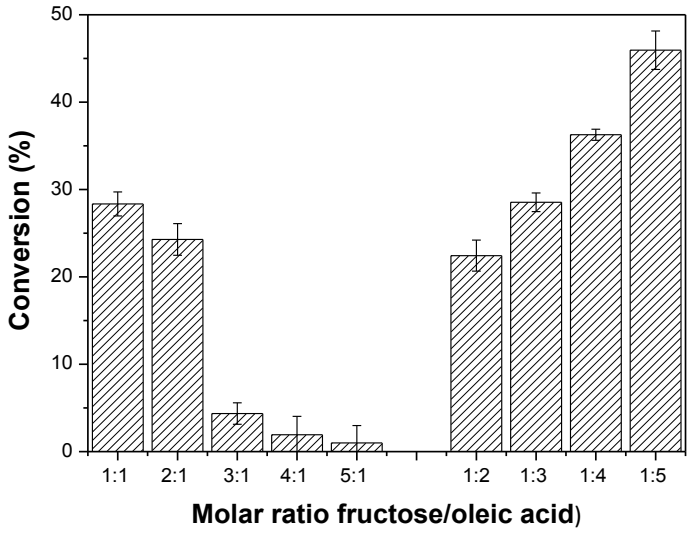
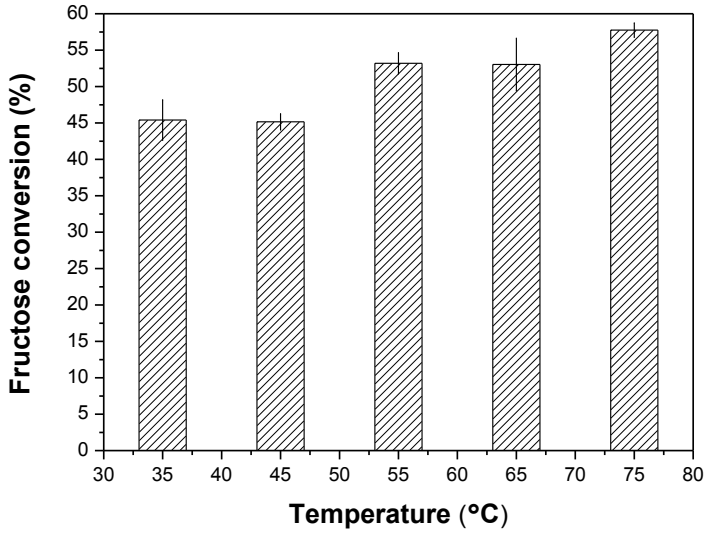


Figure 3.6 - Effect of temperature on fructose oleate conversion after 24 h. Conditions: fructose/oleic acid molar ratio of 1:5; 0.5 g of molecular sieve; 0.05 g of OSGlu (protein loading of 10 mg/g of support and hydrolytic activity of 51.1 U/g of support, measured at 40 ^{o}C with p-NPB as substrate) in 5 mL of tert-butyl alcohol.



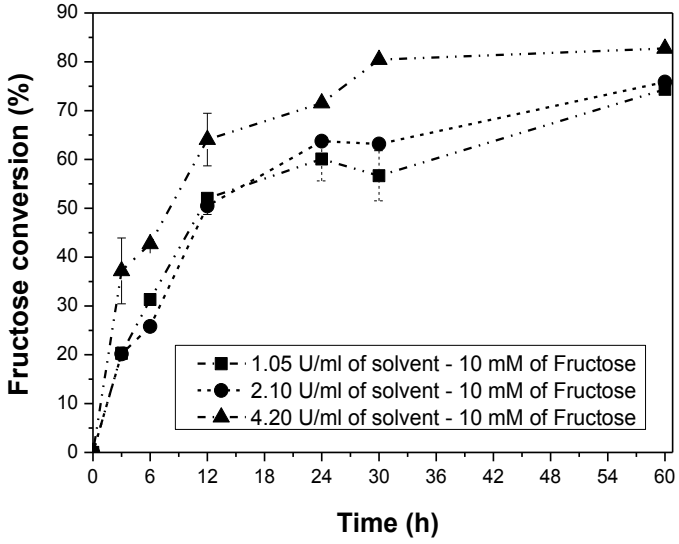
There was a strong effect of molecular sieve (type 3 Å) in the synthesis of fructose oleate (using a fructose/oleic acid molar ratio of 1:5 and 0.05 g of OSGlu with 51.1 U

of p-NPB hydrolysis/g of support in 5 mL of tert-butyl alcohol for 24 h). The percentage conversions as a function of the mass concentration of molecular sieve (%, m/v) were as follows: 16.5 ± 1.6% (without molecular sieve), 20.6 ± 2.2% (5% molecular sieve), 28.3 ± 2.1% (10%), and 35.6 ± 2.4% (20%). The conversion was increased 2-fold when the reaction medium without molecular sieve was changed for one containing 20% (m/v) molecular sieve. Chamouleau et al. (2001) reported that the presence of water strongly affected the esterification of fructose and palmitic acid catalyzed by immobilized *Candida antarctica* lipase (Novozyme 435). It was observed that water surrounded the particle of biocatalyst, forming a hydrophilic layer preventing the access of the lipophilic substrate to the enzyme. The problem of partitioning for the fatty acid molecules can severely affect the catalytic mechanism of the lipase. This drawback could be overcome using molecular sieves that adsorbed water molecules released during the reaction, shifting the reaction towards the products.

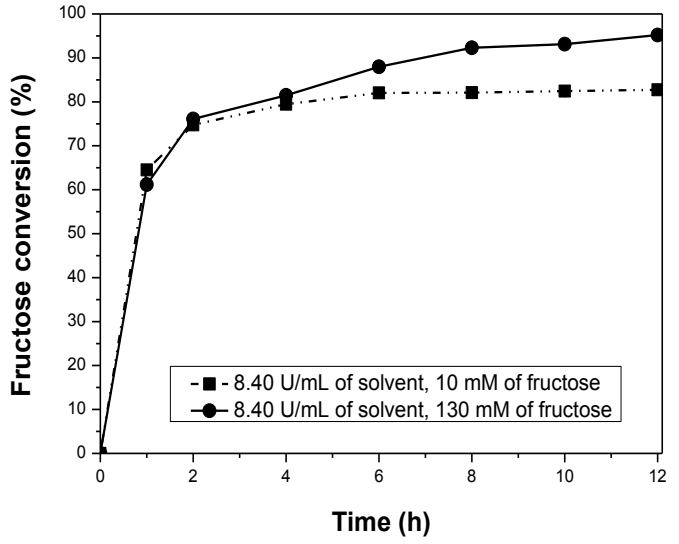
Effect of reaction time and enzymatic loading on the fructose oleate synthesis. Time is a crucial parameter in an industrial process, because it directly affects the volumetric productivity of a desired product, as well as the energy consumption of the process. Therefore, the reaction time and enzymatic loading used for the production of fructose oleate were evaluated (Figure 3.7) using the optimized parameters described above. Figures 3.7(a) and 3.7(b) show that the conversions increased from approximately 50% (for 1 U/mL) to 80% (for 8.4 U/mL), using a 12 h reaction time and 10 mM fructose.

Increasing the fructose concentration to 130 mM resulted in conversion of around 95% achieved using the same reaction time (12 h) and 8.4 U/mL. However, after only 6 h, the conversion of fructose to fructose oleate reached almost 90%. Hence, from an industrial point of view, a 6 h reaction time and a fructose concentration of 130 mM should be chosen, because the sugar ester productivity will be higher. Some studies concerning enzymatic syntheses of sugar fatty esters in organic media catalyzed by biocatalysts commercial are summarized in Table 3.5, which shows conversions on the same order of magnitude obtained in this work, although with reactional time higher. In Table 3.5, Novozyme 435 is lipase from *Candida antarctica* type B commercially available in the immobilized form; POS-PVA is polysiloxane-polyvinyl alcohol; CALB-OSGlu is lipase from *Candida antarctica* type B immobilized on octyl-silica-glutaraldehyde.

Figure 3.7 - Effects of reaction time, enzymatic loading, and fructose concentration on the fructose oleate synthesis. Conditions: temperature of 55 ^{o}C; fructose/oleic acid molar ratio of 1:5; 1 g of molecular sieve in 5 mL of *tert*-butyl alcohol.



A



B

Table 3.5 - Enzymatic syntheses of sugar fatty acid esters in organic media.

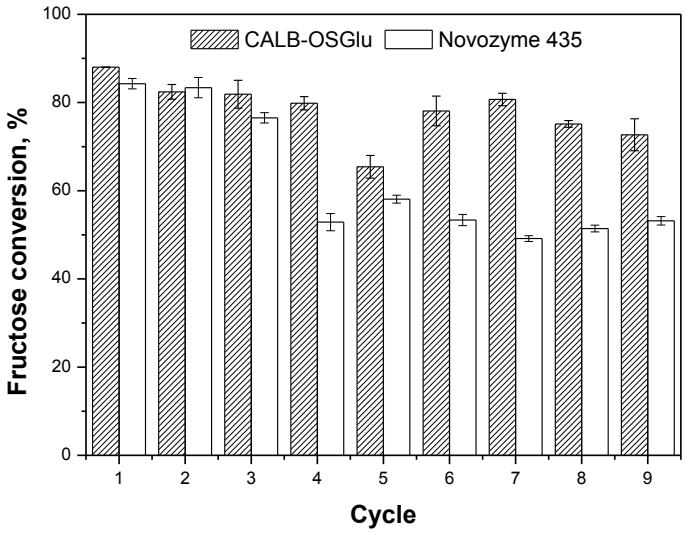
Sugar/fatty acid molar ratio	Solvent	Reaction conditions	Biocatalyst	Conversion (%)	Reference
Fructose/oleic acid 1:10	*Tert*-butyl alcohol	10 h in a fixed bed reactor at 0.06 mL/min flow rate	*Mucor miehei* lipase (Lipozyme)	83	(KHALED et al., 1991)
Sorbitol/oleic acid 10:1	*Tert*-pentyl alcohol	60 ˚C / 7 h	Novozyme 435	96.5	(DUCRET et al., 1995)
Xylitol/oleic acid 1:1		60 ˚C / 24 h		98.2	
Glucose/oleic acid 1:1		60 ˚C / 24 h		69.3	
Fructose/oleic acid 1:1		60 ˚C / 24 h		93.2	
Fructose/oleic acid 1:5	*Tert*-butyl alcohol	38 ^{o}C / 4 h	Porcine pancreas lipase immobilized on POS-PVA	52.8	(DE PAULA; DE SOUZA BARBOZA; DE CASTRO, 2005)
Fructose/ palmitic acid 1:1	*Tert*-pentyl alcohol	60 ˚C / 72 h	Novozyme 435	78.0	(ŠABEDER; HABULIN; KNEZ, 2006)
Fructose/oleic acid 1:5	*Tert*-butyl alcohol	55 ^{o}C / 6 h 55 ^{o}C / 12 h	CALB-OSGlu	90.0 95.0	This work

Novozyme 435 - lipase from Candida antarctica type B commercially available in the immobilized form; POS-PVA - polysiloxane-polyvinyl alcohol; CALB-OSGlu - lipase from Candida antarctica type B immobilized on octyl-silica-glutaraldehyde.

Operational stability. CALB-OSGlu showed high operational stability at 55 ^{o}C in the synthesis of fructose oleate (Figure 3.8). The high stability of this immobilized biocatalyst towards *tert*-butyl alcohol enabled its reuse in nine 6-h batches, with a small decrease in the fructose-to-fructose oleate conversion, from 88% for the first batch to 72% for the ninth batch. Novozyme 435 showed a lower operational stability in the same reaction conditions, with the conversion decreasing from 84% (first cycle) to 53% (ninth cycle). COULON et al. (1996) reported that the biocatalyst maintained a constant conversion up to the seventh cycle, while from the eighth cycle onwards there was a significant decrease in the conversion. Akoh and Mutua (1994) reported a drop in the conversion from 71.9% (first

cycle) to 38.3% (eighth cycle) in the reuse of immobilized lipase SP382 from *Candida antarctica* catalyzing the oleic acid incorporation into methyl glucoside in benzene/pyridine (molar ratio of 2:1). According to Akoh and Mutua (1994) this decrease could be attributed to the incomplete removal of residues of the biocatalyst between each new cycle, which may have hindered substrate contact with the enzyme. In the present work, a mass loss of the biocatalyst was observed in the pretreatment step between each batch, so the drop in the conversion could be overcome by addition of a very low fresh mass of biocatalyst at each new batch.

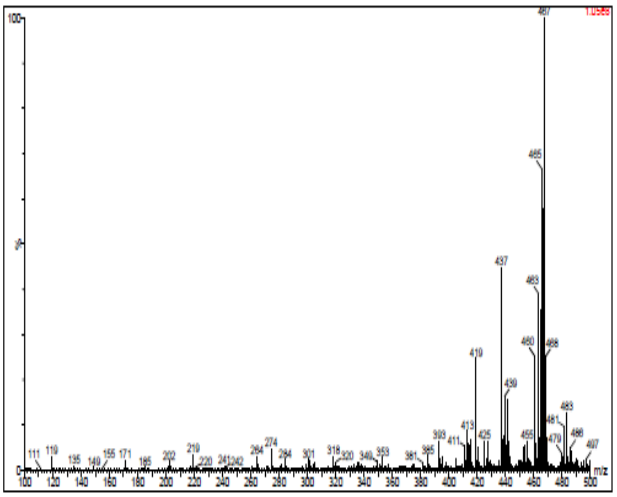
Figure 3.8 - Operational stability of the CALB-OSGlu and Novozyme 435 biocatalysts in the synthesis of fructose oleate at 55 ^{o}C in *tert*-butyl alcohol, using a fructose/oleic acid molar ratio of 1:5, 20% (m/v) molecular sieve, and 6 h reaction time.



3.3.6. Characterization of fructose oleate by mass spectrometry

The mass spectrum of the $[M+23]^+$ ion with m/z = 467 (Figure 3.9), corresponding to the sodium adduct of fructose monooleate, revealed that CALB immobilized on OSGlu mainly produced fructose monooleate. This specificity could be explained by the limited space of the active site of CALB, which took the form of a long and narrow funnel (FERRARIO et al., 2013; MARTINELLE; HOLMQUIST; HULT, 1995) capable of accommodating only single molecules of oleic acid and fructose.

Figure 3.9 - Mass spectrum of the fructose monooleate $[M+23]^+$ ion with m/z = 467.



3.4. Conclusions

Heterofunctional (hydrophobic-hydrophilic) silica-based supports were successfully used to immobilize *Candida antarctica* type B lipase. Due the orientation of enzyme and microenvironment, CALB immobilized on silica functionalized with octyl and glutaraldehyde groups (the OSGlu support) yielded the best biocatalyst in terms of immobilization yield and recovered activity, as well as hydrolysis, esterification, and transesterification activities, and thermal stability in *tert*-butyl alcohol. This biocatalyst (CALB-OSGlu) efficiently catalyzed the synthesis of fructose oleate. Conversions of around 90% and 95% were achieved after 6 h and 12 h of reaction, respectively, under the optimized conditions. It was performed with successful the extraction, purification and characterized of the ester of fructose oleate, obtaining in the end a product with high aggregate value. The presence of monooleate was confirmed by mass spectrometry. The CALB-OSGlu biocatalyst obtained in this study was more reactive and stable than commercial biocatalyst Novozyme 435. Thus CALB-OSGlu biocatalyst has considerable potential for use in industrial processes for the production of biosurfactants based on fructose and oleic acid.

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3.6 References

ADLERCREUTZ, P. Immobilisation and application of lipases in organic media. **Chemical Society Reviews**, v. 42, n. 15, p. 6406–36, 2013.

AKOH, C. C.; MUTUA, L. N. Synthesis of alkyl glycoside fatty acid esters: Effect of reaction parameters and the incorporation of n-3 polyunsaturated fatty acids. **Enzyme and Microbial Technology**, v.16, p.115–119, 1994.

BARBOSA, O. et al. Modulation of the properties of immobilized CALB by chemical modification with 2,3,4-trinitrobenzenesulfonate or ethylendiamine. Advantages of using adsorbed lipases on hydrophobic supports. **Process Biochemistry**, v. 47, n. 5, p. 867–876, 2012.

BERNAL, C.; ILLANES, A.; WILSON, L. Heterofunctional hydrophilic-hydrophobic porous silica as support for multipoint covalent immobilization of lipases: Application to lactulose palmitate synthesis. **Langmuir**, v. 30, n. 12, p. 3557–3566, 2014.

BETANCOR, L. et al. Glutaraldehyde in Protein Immobilization, In: GUISAN; J.; M. **Immobilization of Enzymes and Cells.** United States of America: 2006. p. 57-64.

BLANCO, R. M. et al. Functionalization of mesoporous silica for lipase immobilization: Characterization of the support and the catalysts. **Journal of Molecular Catalysis B: Enzymatic**, v. 30, n. 2, p. 83–93, 2004.

BRADFORD, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. **Analytical Biochemistry**, v. 72, p. 248–254, 1976.

BRENA, B.; GONZÁLEZ-POMBO, P.; BATISTA-VIERA, F. Immobilization of Enzymes: A Literature Survey, In: GUISAN; J.; M. **Immobilization of Enzymes and Cells.** United States of America: 2006. p. 15-30.

CAUGLIA, F.; CANEPA, P. The enzymatic synthesis of glucosylmyristate as a reaction model for general considerations on "sugar esters" production. **Bioresource Technology**, v.99, p. 4065-4072, 2008.

CHAMOULEAU, F. et al. Influence of water activity and water content on sugar esters lipase-catalyzed synthesis in organic media. **Journal of Molecular Catalysis A: Chemical**, v. 11, p. 949–954, 2001.

COULON, D. et al. Effect of different biochemical parameters on the enzymatic synthesis of fructose oleate. **Journal of Biotechnology**, v. 51, n. 2, p. 115–121, 1996.

DEGN, P.; ZIMMERMANN, W. Optimization of carbohydrate fatty acid ester synthesis in organic media by a lipase from *Candida antarctica*. **Biotechnology and Bioengineering**, v. 74, n. 6, p. 483–491, 2001.

DEREWENDA, U. et al. Catalysis at the interface: the anatomy of a conformational change in a triglyceride lipase. **Biochemistry**, v. 31, n. 5, p. 1532–1541, 1992.

DICOSIMO, R. et al. Industrial use of immobilized enzymes. **Chemical Society reviews**, v. 42, n. 15, p. 6437–74, 2013.

DUCRET, A. et al. Enzymatic preparation of biosurfactants from sugars or sugar alcohols and fatty acids in organic media under reduced pressure. **Biotechnology and Bioengineering**, v. 48, n. 3, p. 214–221, 1995.

DUCRET, A. et al. Characterization of enzymatically prepared biosurfactants. **Journal of the American Oil Chemists' Society**, v. 73, n. 1, p. 109–113, 1996.

FERNANDEZ-LAFUENTE, R. et al. Immobilization of lipases by selective adsorption on hydrophobic supports. **Chemistry and Physics of Lipids**, v. 93, n. 1-2, p. 185–197, 1998.

FERRARIO, V. et al. Lipases Immobilization for Effective Synthesis of Biodiesel Starting from Coffee Waste Oils. **Biomolecules**, v. 3, n. 3, p. 514–534, 2013.

FERRER, M. et al. Synthesis of sugar esters in solvent mixtures by lipases from *Thermomyces lanuginosus* and *Candida antarctica* B, and their antimicrobial properties. **Enzyme and Microbial Technology**, v. 36, n. 4, p. 391–398, 2005.

FERRER, M. et al. Chemical versus enzymatic catalysis for the regioselective synthesis of sucrose esters of fatty acids. **Glass**, p. 509–514, 2000.

FRANZETTI, A. et al. (Bio)surfactant and Bioremediation, Successes and Failures. **Trends in Bioremediation and Phytoremediation**, v. 661, n. 2, p. 145–156, 2010.

GUMEL, A. M. et al. Lipase mediated synthesis of sugar fatty acid esters. **Process Biochemistry**, v. 46, n. 11, p. 2079–2090, 2011.

KARMEE, S. K. Lipase catalyzed synthesis of ester-based surfactants from biomass derivatives. **Biofuels, Bioproducts and Biorefining**, v.2, p. 144-154, 2008.

KHALED, N. et al. Fructose oleate synthesis in a fixed catalyst bed reactor. **Biotechnology Letters**, v. 13, n. 3, p. 167-172, 1991.

LIMA, L. N. et al. Immobilization of *Pseudomonas fluorescens* lipase on hydrophobic supports and application in biodiesel synthesis by transesterification of vegetable oils in solvent-free systems. **Journal of Industrial Microbiology & Biotechnology**, 2015.

MAI, N. L. et al. Ionic liquids as novel solvents for the synthesis of sugar fatty acid ester. **Biotechnology Journal**, p. 1–8, 2014.

MARTINELLE, M.; HOLMQUIST, M.; HULT, K. On the interfacial activation of *Candida antarctica* lipase A and B as compared with *Humicola lanuginos*a lipase. **Biochimica et Biophysica Acta - Lipids and Lipid Metabolism**, v. 1258, n. 3, p. 272–276, 1995.

MATEO, C. et al. Advances in the design of new epoxy supports for enzyme immobilization-stabilization. **Biochemical Society Transactions**, v. 35, n. Pt 6, p. 1593–1601, 2007.

MATEO, C. et al. Improvement of enzyme properties with a two-step immobilizaton process on novel heterofunctional supports. **Biomacromolecules**, v. 11, n. 11, p. 3112–3117, 2010.

MATEO, C. et al. Oriented covalent immobilization of enzymes on heterofunctional-glyoxyl supports. **Methods in Molecular Biology**, v. 1051, p. 73–88, 2013.

NIGAM, S. et al. Lipase Immobilization Techniques for Biodiesel Production: An Overview. **International Journal of Renewable Energy and Biofuels**, v. 2014, p. 1–16, 2014.

OLIVEIRA, P. C. et al. Síntese do butirato de N-butila empregando lipase microbiana imobilizada em copolímero de estireno-divinilbenzeno. **Quimica Nova**, v. 23, n. 5, p. 632–636, 2000.

PEREIRA, G. H. A.; GUISAN, J. M.; GIORDANO, R. L. C. Multi-point immobilization of penicillin G acylase on silica-glyoxyl: influence of the degree of activation. **Brazilian Journal of Chemical Engineering**, v. 14, n. 4, p. 327–331, 1997.

REIS, P. et al. Lipase-catalyzed reactions at different surfaces. **Langmuir**, v. 22, n. 19, p. 8169–8177, 2006.

ŠABEDER, S.; HABULIN, M.; KNEZ, Ž. Lipase-catalyzed synthesis of fatty acid fructose esters. **Journal of Food Engineering**, v. 77, n. 4, p. 880–886, 2006.

SARNEY, D. B.; VULFSON, E. N. Application of enzymes to the synthesis of surfactants. **Trends in Biotechnology**, v. 13, n. 5, p. 164–172, 1995.

SCHECKERMANN, C. et al. Enzymatic monoacylation of fructose by two procedures. **Enzyme and Microbial Technology**, v. 17, n. 2, p. 157–162, 1995.

SCHMID, R. D.; VERGER, R. the open form . As with all lipases whose Lipases : Interfacial Enzymes with Attractive Applications. **Analysis**, v. 37, n. 12, p. 1608–1633, 1998.

SINGH, R. K. et al. From protein engineering to immobilization: Promising strategies for the upgrade of industrial enzymes. **International Journal of Molecular Sciences**, v. 14, n. 1, p. 1232–1277, 2013.

TANI, K.; SUZUKI, Y. Influence of titania matrix on retention behaviour in reversed-phase liquid chromatography. **Journal of Chromatography A**, v. 722, n. 1-2, p. 129–134, 1996.

TARAHOMJOO, S.; ALEMZADEH, I. Surfactant production by an enzymatic method. **Enzyme and Microbial Technology**, v. 33, n. 1, p. 33–37, 2003.

TARDIOLI, P. W.; ZANIN, G. M.; DE MORAES, F. F. Production of cyclodextrins in a fluidized-bed reactor using cyclodextrin-glycosyl-transferase. **Applied Biochemistry and Biotechnology**, v. 84-86, p. 1003–1019, 2000.

UPPENBERG, J. et al. The sequence, crystal structure determination and refinement of two crystal forms of lipase B from Candida antarctica. **Structure (London, England : 1993)**, v. 2, n. 4, p. 293–308, 1994.

WILLIAMS, R. O. et al. Investigation of a pMDI system containing chitosan microspheres and P134a. **International Journal of Pharmaceutics**, v. 174, n. 1-2, p. 209–222, 1998.

YAN, Y. et al. Lipase-catalyzed solid-phase synthesis of sugar fatty acid esters - Removal of byproducts by azeotropic distillation. **Enzyme and Microbial Technology**, v. 25, n. 8-9, p. 725–728, 1999.

YAN, Y. et al. Production of sugar fatty acid estrs by enzymatic esterification in a stirred-tank membrane reactor: Optimization of parameters by response surface methodology. **Journal of the American Oil Chemists' Society**, v. 78, n. 2, p. 147–153, 2001.

YOO, I. S.; PARK, S. J.; YOON, H. H. Enzymatic Synthesis of Sugar Fatty Acid Esters. **Journal of Industrial and Engineering Chemistry**. v. 13, n. 1, p. 1–6, 2007.

4. ARTIGO 2 - Efeito da água na síntese de oleato de frutose por lipases imobilizadas em suportes heterofuncional à base de sílica

Neste capítulo, em forma de artigo científico, são apresentados os resultados da imobilização de lipase de *Thermomyces lanuginosus* e *Pseudomonas fluorescens em* suportes híbridos à base de sílica visando seu uso na síntese de oleato de frutose em meio aquoso/orgânico (terc-butanol/água). Sílica foi modificada quimicamente com octil, octil mais glutaraldeído, octil mais glioxil e octil mais grupos epóxi. Todos os suportes renderam biocatalisadores hiperativados (mais de 100% de atividade recuperada), fenômeno atribuído à ativação interfacial desta classe de enzima. Além disso, os suportes heterofuncionais contendo porções hidrofóbicas/hidrofílicas provavelmente permitiram a dissociação de agregados bimoleculares (mais estáveis, porém menos ativos) das lipases em solução, permitindo a imobilização das formas monoméricas (menos estável, porém mais ativas).

Os derivados foram caracterizados quanto à sua estabilidade térmica em terc-butanol e aplicados na síntese de oleato de frutose. Todos os derivados exibiram uma elevada estabilidade em terc-butanol (meia-vida de cerca de 50 h a 65 ºC). Exceto para as lipases imobilizadas em sílica modificada com grupos octil e epóxi, todos os derivados mostraram excelente desempenho na síntese de oleato de frutose quando pequena quantidade de água (1%, v / v) foi adicionada à fase orgânica, permitindo uma produtividade do éster até cinco vezes mais elevada comparada àquela obtida na ausência de água. Conversões de cerca de 70% foram obtidas á baixa temperatura (35 ºC) e tempo de reação curto (30 min). Estes resultados representam um avanço neste campo a partir de um ponto de vista industrial, cuja produtividade é um parâmetro relevante para processos de grande escala.

Effect of water in the synthesis of fructose oleate using immobilized lipases on heterofunctional silica-based support

Abstract: Lipases from *Thermomyces lanuginosus* (TLL) and *Pseudomonas fluorescens* (PFL) were immobilized on heterofunctional silica-based supports aiming their use in the synthesis of fructose oleate in *tert*-butyl alcohol/water medium. Silica was chemically modified with octyl, octyl plus glutaraldehyde, octyl plus glyoxyl, and octyl plus epoxy groups. All functionalized supports yielded hyperactivated biocatalysts (more than 100% recovered activity), phenomenon that could be attributed to the atypical interfacial activation of this class of enzymes. Besides, the heterofunctional supports containing hydrophobic/hydrophilic moieties probably allowed the dissociation of bimolecular aggregates (more stable but less active) of theses lipases in solution, allowing the immobilization of the monomeric forms (less stable but more active). All derivatives exhibited high stability against *tert*-butyl alcohol (half-lives around 50 h at 65 ºC). Except the lipases immobilized on silica modified with octyl and epoxy groups, all derivatives showed excellent performance in the synthesis of fructose oleate when small amount of water (1%, v/v) was added to the organic phase, allowing an ester productivity until 5 times higher than that in the absence of water. Conversions around 70% were achieved at low temperature (35 ºC) and short time reaction (30 min). These results represent an advance in this field from an industrial point of view, where productivity is a relevant parameter for large-scale processes.

Keywords: Microbial lipases, silica, hetero-functionalization, bio-surfactant, organic/water system.

4.1. Introduction

Lipases are enzymes extremely versatile, widely used in hydrolysis, esterification, and transesterification reactions. However, the use these enzymes in their soluble form, as well as any other enzyme, is currently no economically attractive, because the costs of these biocatalysts is still very high, and their operational stability is usually very low, particularly when they are expected to be used in an unusual medium, such as, organic medium. Several drawbacks of the soluble enzymes can be overcome by immobilization, either on solid supports, membranes, crosslinking, etc. (BASTIDA et al., 1998; DALLA-VECCHIA; NASCIMENTO; SOLDI, 2004; LIMA et al., 2013; PALOMO et al., 2005); VELASCO-LOZANO et al., 2014; ZHOU et al., 2015).

Despite on wide number of immobilization techniques, lipases have been widely immobilized on hydrophobic supports by adsorption attachments (LIMA et al., 2015). The physical adsorption is a simple, gentle, and cheap method to insolubilize enzymes (ZHANG; YUWEN; PENG, 2013). However, the biocatalysts prepared by this technique are usually as fragile as the soluble enzyme. Lipases may behave atypically, yielding biocatalysts immobilized on hydrophobic supports widely stable (particularly in organic solvents where leaching is prevent) and hyperactivated (recovered activity) due to their atypical catalytic mechanism (interfacial activation), where a lid that covers the active site in most lipases are modulated from a closed form (inactive enzyme) to an open form (active enzyme) in presence of hydrophobic interfaces (ADLERCREUTZ, 2013; ZHANG; YUWEN; PENG, 2013). The hyperactivation phenomenon for lipases adsorbed on hydrophobic supports is a well-documented strategy (BASTIDA et al., 1998; KOPP et al., 2015; LIMA et al., 2015; LIMA et al., 2013; MARTINELLE; HOLMQUIST; HULT, 1995; PALOMO et al., 2002). However, due to the labile forces involved in physical adsorption of proteins on solid surfaces, such as, van der Walls and electrostatic forces, hydrophobic and hydrogen linkages, lipases can be easily leached from the support, depending on reaction conditions (NIGAM et al., 2014). Hybrid supports, i.e., supports chemically modified with different moieties to allow the coupling of the lipase by ionic or hydrophobic adsorption, followed by strong and irreversible covalent links between the adsorbed lipase and the activated support can be an interesting alternative to the supports conventionally used (BERNAL; ILLANES; WILSON, 2014; BRENA; GONZÁLEZ-POMBO; BATISTA-VIERA, 2006; MATEO et al., 2013; MATEO et al., 2010). On the other hand, very strong linkages enzyme-support can dramatically reduce the catalytic activity of the immobilized enzyme due to protein 3D-distorcion, as well as,

steric hindrances in a new microenvironment surrounding the active center of the immobilized enzyme (ADLERCREUTZ, 2013; TARDIOLI et al., 2003; ZHANG; YUWEN; PENG, 2013). This work investigated this new strategy to immobilize two microbial lipases (*Thermomyces lanuginosus* and *Pseudomonas fluorescens* lipases) on silica chemically modified with different moieties to allow in a first step the hydrophobic adsorption of the open-lid lipase, followed by covalent linkages between reactive groups on the support surface (epoxy and aldehyde groups) and functional groups on the enzyme surface, such NH_2-terminal and ε-NH_2 from lysine residues (GUISÁN, 1988; MATEO et al., 2000). The catalytic properties of the biocatalysts were evaluated as recovered activity and thermal stability in *tert*-butyl alcohol, aiming their application in the synthesis of an industrial important class of bio-surfactant, i.e., sugar fatty esters. In this work, it was evaluated the synthesis of fructose oleate in organic medium (*tert*-butyl alcohol) containing small amounts of water (~ 1%, v/v) that contributed to dissolve high amounts of fructose in *tert*-butyl alcohol, besides to reach high esterification conversions at low temperature (35°C) and short reaction time (30 min). Under an industrial point of view, an important parameter for large scale processing was significantly improved by addition of small amount of water in the esterification medium.

4.2. Experimental

4.2.1. Material

(3-Glycidyloxypropyl)trimethoxysilane (GPTMS), triethoxy(octyl)silane (OCTES), (3-aminopropyl)triethoxysilane (APTES), lipases from *Thermomyces lanuginosus* (TLL) and *Pseudomonas fluorescens* (PFL), bovine serum albumin, and *p*-nitrophenyl butyrate (*p*-NPB) were purchased from Sigma-Aldrich Co. (St. Louis, USA). *Tert*-Butyl alcohol and glutaraldehyde were purchased from Vetec Química Fina Ltda (São Paulo, Brazil). Fructose, sodium periodate, sodium borohydride and oleic acid were purchased from Synth (São Paulo, Brazil). Silica (Immobead S60S) was obtained from Chiral Vision (Leiden, The Netherlands). All other chemicals were of analytical purity.

4.2.2. Support activation

Macroporous silica (average pore diameter 193 Å, LIMA et al., 2015) was chemically modified using OCTES, APTES, and GTPMS. The porous silica was previously

treated with HCl (0.1 M) under reflux, followed by washing until neutral pH and drying at 50 ºC for 12 h (TANI; SUZUKI, 1996).

Octyl-Silica (OS). Octyl groups were incorporated on the silica surface using the methodology described by Tani and Suzuki (1996), where a suspension containing 1 g of dry silica and 20 mL of a mixture of OCTES:toluene (1:10, v/v) was kept under reflux for 4 h at the boiling temperature of the solvent. The activated support was washed with toluene and distilled water, and dried at 40 ºC for 24 h.

Octyl-Silica-Glutaraldehyde (OSGlu). 1 g of dry silica was chemically modified with octyl and amine groups using 1.5 mL of OCTES and 0.5 mL of APTES dissolved in 18 mL of toluene. The reaction was performed at boiling temperature under reflux for 4 h. The modified silica was washed with toluene and distilled water, and dried at 40 ºC for 24 h.

The activation of the support with aldehyde groups was performed immediately before its use in the CALB immobilization, according to the methodology described by Tardioli, Zanin and Moraes (2000). Briefly, 1 g of dry silica modified with octyl and amine groups was suspended in 10 mL of 0.2% (v/v) glutaraldehyde solution prepared in sodium phosphate buffer (100 mM, pH 7.0). The suspension was stirred for 1 h at room temperature, after which the activated support was recovered by filtration and washed with an excess of distilled water.

Octyl-Silica-Epoxy (OSEpx). 1 g of dry silica was chemically modified with octyl and epoxy groups using 1.5 mL of OCTES and 0.5 mL of GPTMS dissolved in 18 mL of toluene. The mixture was kept at boiling temperature under reflux for 4 h, as described by Pereira, Guisan and Giordano (1997). The support was washed with toluene and distilled water, then dried at 40 ºC for 24 h.

Octyl-Silica-Glyoxyl (OSGlx). This support was prepared by acid hydrolysis of the epoxy groups of the OSEpx support obtained previously. 1 g of OSEpx was suspended in 30 mL of 0.1 M sulfuric acid solution and kept under reflux at 85 $^{\circ}$C for 2 h (PEREIRA; GUISAN; GIORDANO, 1997). In this step, the opening of the epoxy rings generated glyceryl groups on the silica surface. The support was then washed with toluene and distilled water, and dried at 40 ºC for 24 h.

The glyceryl groups were oxidized to aldehyde groups using sodium periodate, employing the methodology described by Pereira, Guisan and Giordano (1997). Briefly, 1 g of dry silica modified with octyl and glyceryl groups was suspended in 10 mL of 0.1 M $NaIO_4$

solution and kept under stirring in a GyroTwister GX-1000 3-D Shaker (S1000-A, Labnet International, Inc., New Jersey, USA) at room temperature for 1 h. The support was then washed with distilled water and dried at 40 ºC for 24 h.

4.2.3. Immobilization of TLL and PFL

Immobilization on OS and OSGlu. The immobilizations of TLL and PFL on OS and OSGlu were performed in a single step, where 1 g of OS or OSGlu was suspended in 15 mL of an enzyme solution prepared in 10 mM sodium phosphate buffer (pH 7.0). The suspension was kept under mild stirring in in a GyroTwister GX-1000 3-D Shaker (S1000-A, Labnet International, Inc., New Jersey, USA) for 6 h, at room temperature. The biocatalysts were recovered by filtration, washed with distilled water, and dried/stored at 4 ºC for further use. The immobilization yield was monitored by measuring the protein concentration using the Bradford method (BRADFORD, 1976) and by determining the hydrolytic activity in the immobilization supernatants.

Immobilizations on OSEpx and OSGlx. The immobilizations on OSGlx and OSEpx required two steps. In the first step, 1 g of OSEpx or OSGlx was suspended in 15 mL of an enzyme solution prepared in 10 mM sodium phosphate buffer (pH 7.0), in order to promote the hydrophobic enzyme adsorption (similar to the immobilization on OS). The suspension was kept under mild stirring in in a GyroTwister GX-1000 3-D Shaker (S1000-A, Labnet International, Inc., New Jersey, USA) for 6 h, at room temperature. The biocatalysts were recovered by filtration, resuspended in 15 mL of 100 mM sodium phosphate buffer (pH 10.0), and gently stirred at room temperature for 3 h, as described by Bernal, Illanes and Wilson (2014), to allow the formation of Schiff bases between the amine groups of the enzyme and the aldehyde groups of the OSGlx support, and covalent linkages between the epoxy groups of the OSEpx support and the nucleophile groups placed on the enzyme surface, such as lysine, histidine, cysteine, tyrosine (MATEO et al., 2007). Afterwards, the biocatalysts prepared on OSGlx were reduced with 1 mg/mL sodium borohydride for 1 h to convert the remaining active aldehydes on the support surface to inert hydroxyl groups, as well as to convert reversible Schiff bases to irreversible covalent linkages (BRENA; GONZÁLEZ-POMBO; BATISTA-VIERA, 2006). The remaining reactive epoxy groups on the OSEpx surface were blocked with glycine (3 mM, 1 h, room temperature (MATEO et al., 2007). The immobilization yield was monitored by measuring the protein concentration using

the Bradford method (BRADFORD, 1976), and by determining the *p*-NPB hydrolytic activity in the immobilization supernatants.

4.2.4. Hydrolysis activity

The hydrolysis activity of soluble TLL and PFL was assayed in a glass cuvette, measuring the absorbance increase at 405 nm caused by the release of *p*-nitrophenol (ε = 6080 M^{-1} cm^{-1}) during the hydrolysis of *p*-NPB (25 µL of 50 mM *p*-NPB solution in acetonitrile and 2 mL of 10 mM sodium phosphate buffer at pH 7.0) at 25 ºC, based on the methodology of Martinelle, Holmquist and Hult (1995). One unit of hydrolysis activity (U) was defined as the initial rate of hydrolysis of *p*-NPB (µmols/min) under the conditions described above.

The hydrolytic activity of the immobilized TLL and PFL was determined using a mechanically stirred 25 mL reactor, under the same conditions described for the soluble lipases, but on a larger scale, employing 0.25 mL of 50 mM *p*-NPB solution in acetonitrile and 20 mL of 10 mM sodium phosphate buffer at pH 7.0 at 25 ºC. Samples were withdrawn at 1 min intervals for quantification (at 405 nm) of the *p*-nitrophenyl released.

4.2.5. Thermal stability in *tert*-butyl alcohol

The stabilities of the biocatalysts (TLL and PFL immobilized on OS, OSGlu, OSGlx, or OSEpx) were evaluated by incubating them in pure *tert*-butyl alcohol at 65 ºC. Samples were periodically withdrawn for immediate measurement of the residual *p*-NPB hydrolysis activity.

4.2.6. Solubility of fructose

The fructose solubility was evaluated in aqueous-organic systems containing different water amounts. In closed tubes were added 2500 mM fructose in *tert*-butyl and different amounts of water (0, 1, 5, and 10%, v/v). After 24 h stirring in a Marconi incubator (MA 430/1) equipped with 360º-stirring carrousel (Marconi®, Piracicaba, Brazil) at 35 ºC the solutions were centrifuged at 10,000 rpm (12,857 × g) for 2 min. One milliliter of the supernatant was dried at 70 ºC for 24 h. After evaporation of the tertiary alcohol, 1 mL of water was added prior to measurement of the fructose concentration using a Water HPLC equipped with a refractive index detector and a Sugar Pak-I column (300 x 6.5 mm x 10 µm) kept at 80 ºC. Ultrapure water was used as eluent, at 0.5 mL/min.

4.2.7. Synthesis of fructose oleate

The reaction conditions, chosen based on previously work (data not shown), were: oleic acid:fructose molar ratio of 5:1, enzyme concentration of 0.5 U/mL of solvent (p-NPB activity), temperature of 35 ºC, and 12 h under stirring in a Marconi incubator (MA 430/1) equipped with 360º-stirring carrousel (Marconi®, Piracicaba, Brazil). The solvent was *tert*-butyl alcohol containing different amounts of water (0, 1, 5, and 10%, v/v). The biocatalysts were separated by centrifugation at 10,000 rpm (12,857 × *g*) and the supernatant was dried at 70 ºC for 24 h to evaporate the *tert*-butyl alcohol. Water was added to the dry extract prior to determination of the concentration of unconverted fructose by liquid chromatography, as described previously.

4.2.8. Effects of reaction time in the fructose oleate synthesis

To increase the ester productivity was increased the amount of enzyme. The reaction time, another important factor to productivity, was evaluated. At intervals predefined (30 min during 8 h) the samples were withdrawn for quantification of unconverted fructose by liquid chromatography, as described previously. The reaction conditions were oleic acid:fructose molar ratio of 5:1, enzyme concentration of 8.4 U/mL of *tert*-butyl alcohol containing 1% of water (p-NPB activity) and temperature of 35 ºC.

4.2.9. Operational stability on the fructose oleate synthesis

The reaction conditions were oleic acid:fructose molar ratio of 5:1, enzyme concentration of 8.4 U/mL of *tert*-butyl alcohol containing 1% of water (p-NPB activity), temperature of 35 ºC and 30 min under stirring in a Marconi incubator (MA 430/1) equipped with 360º-stirring carrousel (Marconi®, Piracicaba, Brazil). Samples were withdrawn for quantification of unconverted fructose by liquid chromatography, as described previously.

4.3. Results and Discussion

4.3.1. Immobilization of lipases

Heterofunctional silica-based supports were evaluated in the immobilization, in triplicate, of PFL and TLL. High immobilization yields were obtained for both lipases (Table

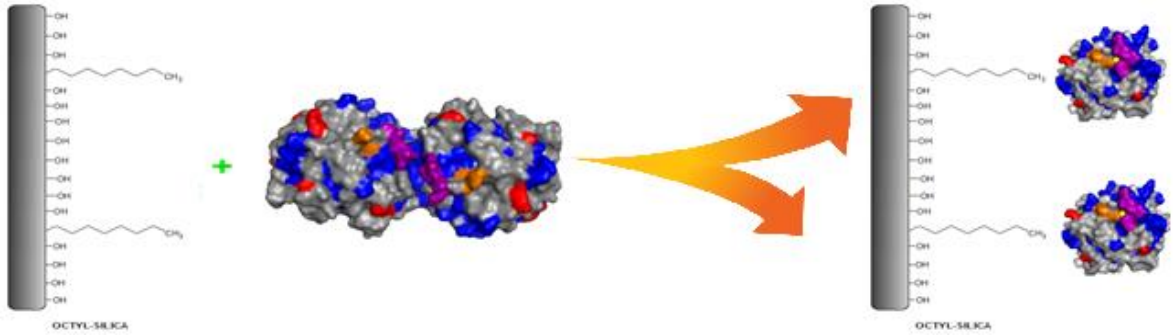
4.1): 80.4 – 92.0% for PFL and 84.3 – 89.2 for TLL. Almost all supports allowed hyperactivation of the lipases (recovered activities above 100%), a typical behavior reported for PFL and TLL immobilized on hydrophobic supports (BASTIDA et al., 1998; FERNANDEZ-LAFUENTE et al., 1998; KOPP et al., 2015; LIMA et al., 2015; LIMA et al., 2013; MARTINS et al., 2013; MATTE et al., 2014).

The hyperactivation of PFL and TLL (only for OS) immobilized on heterofunctional hydrophobic supports was probably due to dissociation of the bimolecular aggregates (Figure 4.1). Soluble PFL and TLL exhibit high tendency to form bimolecular aggregates (less active and more stable) in solution and in these aggregates the hydrophobic active sites of the monomers (more active and less stable) are in close contact with each other (FERNÁNDEZ-LORENTE et al., 2003; FERNANDEZ-LAFUENTE, 2010; LIMA et al., 2013). Thus, the immobilization of PFL and TLL on hydrophobic supports should have favored the dissociacion of these bimolecular aggregates. Particularly, PFL has practically no hydrophobic areas on its surface, except for the inner face of the lid and the area surrounding the active site. Therefore, any hydrophobic interaction between PFL and support should be related to these hydrophobic areas (PALOMO et al., 2005). Indeed, Lima et al. (2013) reported hyperactivation of PFL immobilized on highly hydrophobic octyl-Sepabeads (300% recovered activity) and octyl-Sepharose (150% recovered activity). Kopp et al. (2015) also reported hyperactivation of PFL immobilized on silica magnetic microparticles functionalized with octyl groups (163% recovered activity). Hyperactivation of TLL immobilized on hydrophobic supports was also previously reported by Martinelle, Holmquist and Hult (1995).

Table 4.1 – Immobilization yields (YI,%) and recovered activities (RA,%) of PFL and TLL immobilized on chemically modified silica (protein loading of 1 mg/g of support).

	PFL		**TLL**	
Support	**IY,%**	**RA,%**	**IY,%**	**RA,%**
OS	92.0 ± 1.8	208.1 ± 4.1	88.6 ± 1.0	110.4 ± 0.4
OSGlu	87.5 ± 0.6	110.3 ± 5.7	89.2 ± 1.6	36.9 ± 1.3
OSEpx	80.4 ± 3.6	157.1 ± 7.0	84.3 ± 0.1	58.6 ± 1.3
OSGlx	90.0 ± 1.5	139.7 ± 2.4	86.9 ± 0.4	50.45 ± 3.5

Figure 4.1 – TLL 3D-surface as bimolecular aggregates (Protein Data Bank, code PDB 1DT3) and monomers (Protein Data Bank, code PDB 1TIB) dissociated in presence of hydrophobic ocyl-silica support (only an illustration). PyMOL Molecular Graphics System, v. 1.7.4 (Schrödinger, LLC) was used to generate the surfaces. Different colors represent the lid (purple), active site (yellow), lysine residues (red), amino-terminal (orange), and hydrophobic areas (blue).



TLL immobilized on OSGlu, OSEpx and OSGlx did not exhibit hyperactivation, except for OS. These results suggest that the enzyme-enzyme interaction is very strong. Therefore, dissociation of the bimolecular aggregates requires high hydrophobicity of the support. Indeed, Lima et al. (2013) and Lima et al. (2015) also have reported as more active PFL derivatives as more hydrophobic the supports. In this way, the lower hydrophobicity of the supports OSGlu, OSEpx and OSGlx than the OS probably allowed the immobilization of the lipase in its closed form (inactive form) and as bimolecular aggregates with active sites facing each other.

It should also be taken into account that at the end of immobilization reactions, the supports, and therefore the microenvironments of the immobilized lipases, exhibits different moieties, as following: hydrophobic (OS), hydrophobic/hydrophilic (OSGlx), and hydrophobic/negatively charged (OSEpx). The hydrophilic moieties of the OSGlx are due to reduction of the remnant aldehyde groups to hydroxyl groups (GUISÁN, 1988), and the negatively charged moieties of the OSEpx are due to carboxylic groups inserted in the blocking of the epoxy groups with glycine (MATEO et al., 2007). The hydrolytic activities using *p*-NPB (a molecule containing a charged moiety) as substrate are performed at pH 7, and at this pH the carboxylic groups are predominantly deprotonated. These different moieties could affect the partition of the *p*-NPB from the reaction bulk to closer of the immobilized

enzyme, yielding lower or higher activities depending to the substrate concentration closer to the enzyme. This effect was confirmed immobilizing TLL on OSEpx and blocking or not the remaining epoxy groups with glycine. The use of glycine represented an increase of 21% in the recovery activity.

4.3.2. Stability of immobilized lipases against *tert*-butyl alcohol

Figure 4.2 (A, B) shows the inactivation profiles of PFL and TLL immobilized on heterofunctional silica-based supports. They present high stability in *tert*-butyl alcohol at high temperature. Almost all derivatives exhibited half-lives above 50 h at 65 ºC. The high stabilities of the derivatives make them excellent biocatalysts for industrial purposes, like in the process of synthesis of fructose oleate (a biosurfactant), where *tert*-butyl alcohol is required to solubilize the fructose.

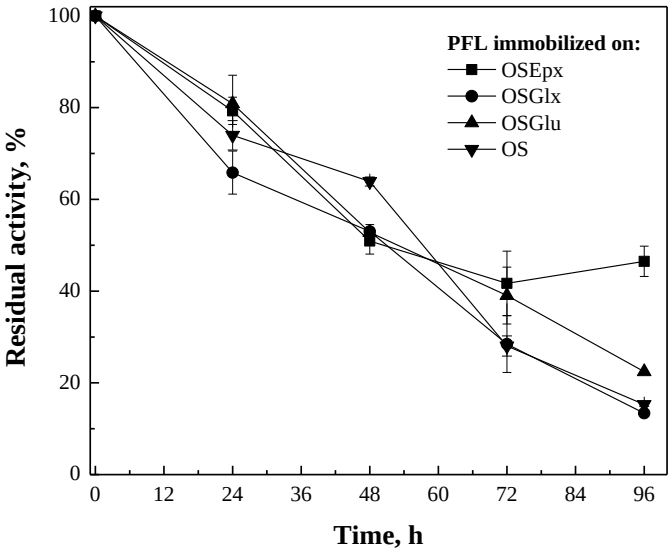
4.3.3. Solubility of fructose in *tert*-butyl alcohol/water system

Sugars are hardly solubilized in organic solvents, such as *tert*-butyl alcohol (DEGN; ZIMMERMANN, 2001). The addition of small amounts of water could have two positive effects: to increase the fructose solubility and to prevent enzyme inactivation. Indeed, the solubility of fructose in water/*tert* butyl alcohol system was greatly increased (more than 4 times), as shown in Table 4.2. The high solubility of fructose is attractive under an industrial point of view because high concentration of solubilized fructose allows increasing the productivity of the sugar fatty ester.

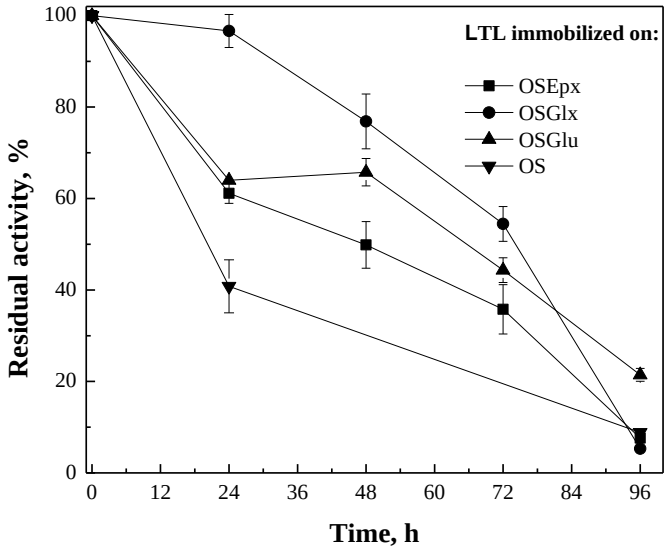
Table 4.2 – Solubility of fructose in *tert*-butyl alcohol/water system at 35 ºC.

Water content (v/v, %)	Soluble fructose (g/L)
0	9.5 ± 0.1
1	12.7 ± 0.3
5	37.2 ± 0.8
10	42.2 ± 2.1

Figure 4.2 – Residual activities of immobilized lipases at 65 ºC incubated in *tert*-butyl-alcohol



(A)



(B)

4.3.4. Synthesis of fructose oleate in *tert*-butyl alcohol/water system

Figure 4.3 shows the conversions of the esterification reactions catalyzed by PFL and TLL immobilized on heterofunctional silica-based supports in water-free medium. Low conversions (below 50%) were obtained with all biocatalysts. However, the lowest conversions were obtained with PFL and TLL immobilized on OSEpx. Probably, the microenvironment negatively charged of these biocatalysts may have hindered the mass transfer of the oleic acid to the active site of the lipase. These biocatalysts were discarded in further studies. Indeed, Yan et al. (1999) have reported that hydrophobic supports facilitate the partition of the lipophilic fatty acids into the pores, increasing synthetic activity.

Figure 4.3. – Synthesis of fructose oleate at 35 ºC for 12 h using a oleic acid:fructose molar ratio of 5:1 in *tert*-butyl alcohol medium (0.51 U/mL of solvent).

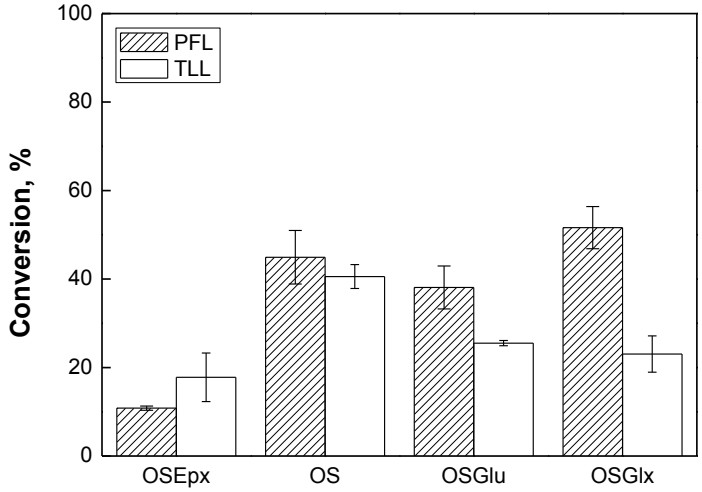
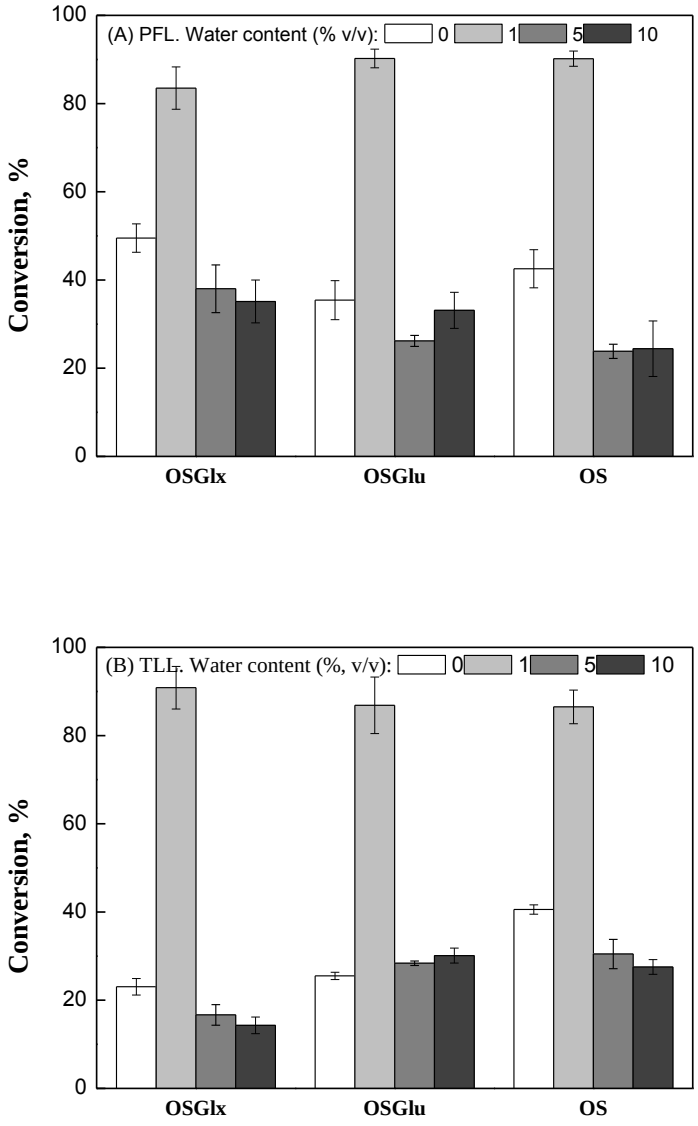


Figure 4.4 (A and B) shows that a small amount of water (1%, v/v) in the reaction medium greatly increased the conversions of oleic acid-to-fructose oleate. All biocatalysts allowed around 90% conversions, while in the absence of water all conversions were below 50%. Although the solubility of fructose was favored by high water concentration, the formation of ester was hindered, probably due to the shifting the thermodynamic equilibrium towards the hydrolysis reaction. Indeed, Leitgeb and Knez (1990) also report a significant increase in the conversion of the synthesis of butyl oleate catalyzed by *Mucor miehei* lipase in presence of small amounts of water. The improvement in the conversions was attributed to the better performance of the enzyme due to hydration of its 3D

structure. On the other hand, the presence of large amount of water negatively did displace the equilibrium towards to hydrolysis reaction.

Figure 4.4. – Synthesis of fructose oleate at 35 ºC for 12 h using a oleic acid:fructose molar ratio of 5:1 in tert-butyl alcohol/water systems (0.51 U/mL of solvent, catalyzed by immobilized (A) PFL and (B) TLL.



Other important parameter to be considered in this study is the ester productivity. The productivity of fructose oleate in presence of water was around 2-5 times higher than in the water absence, as shown in Table 4.3 and Table 4.4.

Table 4.3 – Productivities of fructose oleate at 35 ºC by enzymatic syntheses in *tert*-butyl alcohol/water systems. The reactions were catalyzed by PFL for 12 h using a oleic acid:fructose molar ratio of 5:1 and 0.51 U/mL of solvent.

PFL immobilized on:	Productivity (g L^{-1} h^{-1}) as function of water amount in the reaction medium			
	0%	**1%**	**5%**	**10%**
OSEpx	0.06 ± 0.01			
OSGlx	0.39 ± 0.03	0.88 ± 0.05	1.18 ± 0.17	1.24 ± 0.17
OSGlu	0.28 ± 0.04	0.95 ± 0.02	0.81 ± 0.04	1.17 ± 0.14
OS	0.34 ± 0.03	0.95 ± 0.02	0.74 ± 0.05	0.86 ± 0.22

Table 4.4 – Productivities of fructose oleate at 35 ºC by enzymatic syntheses in *tert*-butyl alcohol/water systems. The reactions were catalyzed by TLL for 12 h using a oleic acid:fructose molar ratio of 5:1 and 0.51 U/ml of solvent.

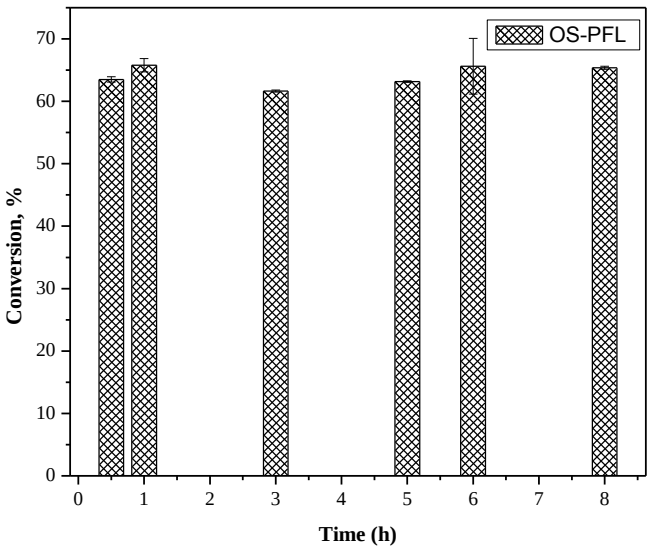
TLL immobilized on:	Productivity (g L^{-1} h^{-1}) as function of water amount in the reaction medium			
	0%	**1%**	**5%**	**10%**
OSEpx	0.14 ± 0.01			
OSGlx	0.18 ± 0.01	0.96 ± 0.05	0.52 ± 0.07	0.50 ± 0.07
OSGlu	0.20 ± 0.01	0.92 ± 0.07	0.88 ± 0.02	1.06 ± 0.06
OS	0.32 ± 0.01	0.92 ± 0.04	0.94 ± 0.10	0.97 ± 0.06

The system containing 1% of water was chosen for further studies because in this system the productivities are similar to those obtained in the other systems, besides the conversion be higher (around 90%) than in the others (20-40%).

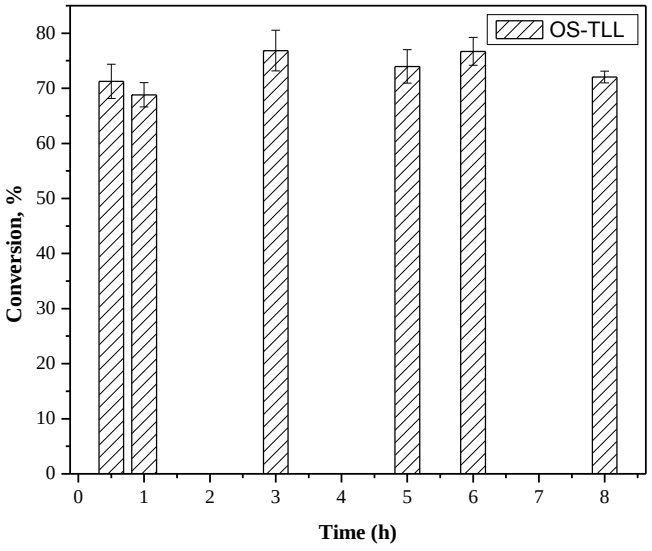
Aiming to increase the ester productivity, fructose oleate was produced using PFL and TLL immobilized on OS at the following conditions: oleic acid:fructose molar ratio of 5:1, temperature of 35 ºC, amount of water (1%, v/v) and 8.4 U/mL of solvent. Lipases immobilized on OS were chosen because its high recovery activity, stability in organic solvent and easy support activation compared to the other evaluated in this work. The increase in the enzyme load allowed ester productivities of 18.10 and 16.13 g $L^{-1}h^{-1}$, considering ester conversions of around 70% and 63% after 30 min of reaction for TLL and PFL (Figure 4.5),

respectively. These results are higher than those previously reported by Ferrer et al. (1999), who reported a productivity of 1.25 g/L^{-1} h^{-1} in the optimized conditions of the synthesis of the 6-o-lauroylsucrose in *tert*-butanol/DMSO (1/4, v/v) catalyzed by TLL immobilized on celite.

Figure 4.5. – Synthesis of fructose oleate at 35 ºC, oleic acid:fructose molar ratio of 5:1, (1% water, v/v) and 8.4 U/mL of solvent, catalyzed by (A) PFL and (B) TLL immobilized on octyl-silica



(A)

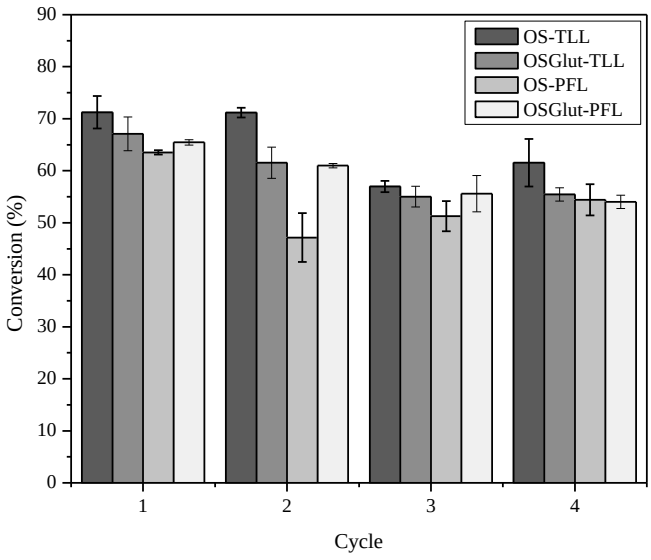


(B)

4.3.5. Operational stability

The operational stabilities of four biocatalysts were also evaluated and the results are showed in the Figure 4.6. The biocatalyst PFL-OSGlu, PFL-OS, TLL-OSGlu and TLL-OS showed high operational stability at 35 ºC in the synthesis of fructose oleate (Figure 4.5). The high stability of these immobilized biocatalysts towards *tert*-butyl alcohol enabled their reuse in four 30 min-batches. However, is important to stress that the biocatalysts PFL-OS and TLL-OS were previously used by 8 h at 35 ºC in the assay to estimate the maximum ester productivities describe above. So, it could be considered that these biocatalysts were previously used in sixteen 30 min-cycles.

Figure 4.6. – Operational stability of the OS-TLL, OS-PFL, OSGlu-TLL and OSGlu-PFL in the synthesis of fructose oleate at 35 ºC in *tert*-butyl alcohol, using a fructose/oleic acid molar ratio of 1:5 and 30 min reaction time.



4.4. Conclusion

The chemical modification of the silica surface with hydrophobic/hydrophilic moieties did contribute to prepare immobilized biocatalysts of *Thermomyces lanuginosus* and *Pseudomonas fluorescens* lipases hyperactivated and highly stable in *tert*-butyl alcohol. These biocatalysts were successfully used in the synthesis of fructose oleate in organic/aqueous medium (*tert*-butyl alcohol/water) yielding high ester conversions and increased productivities.

The addition of small amount of water to the tert-butanol, instead the use of other toxic polar solvents, such as DMSO, pyridine, hexane and so on, showed to be a promising process, because it allows high enzyme stability, high sugar solubilization, high conversion and high productivity. Besides, the process could be considered more environmental friendly than those using toxic polar solvents.

4.5. Acknowledgements

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4.6. References

ADLERCREUTZ, P. Immobilisation and application of lipases in organic media. **Chemical Society Reviews**, v. 42, n. 15, p. 6406–36, 2013.

BASTIDA, A. et al. A single step purification, immobilization, and hyperactivation of lipases via interfacial adsorption on strongly hydrophobic supports. **Biotechnology and Bioengineering**, v. 58, n. 5, p. 486–493, 1998.

BERNAL, C.; ILLANES, A.; WILSON, L. Heterofunctional hydrophilic-hydrophobic porous silica as support for multipoint covalent immobilization of lipases: Application to lactulose palmitate synthesis. **Langmuir**, v. 30, n. 12, p. 3557–3566, 2014.

BRADFORD, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. **Analytical Biochemistry**, v. 72, p. 248–254, 1976.

BRENA, B.; GONZÁLEZ-POMBO, P.; BATISTA-VIERA, F. Immobilization of Enzymes: A Literature Survey, In: GUISAN; J.; M. **Immobilization of Enzymes and Cells.** United States of America: 2006. p. 15-30.

DALLA-VECCHIA, R.; NASCIMENTO, M. D. G.; SOLDI, V. APLICAÇÕES SINTÉTICAS DE LIPASES IMOBILIZADAS EM POLÍMEROS. **Quimica Nova**, v. 27, n. 4, p. 623–630, 2004.

DEGN, P.; ZIMMERMANN, W. Optimization of carbohydrate fatty acid ester synthesis in organic media by a lipase from Candida antarctica. **Biotechnology and Bioengineering**, v. 74, n. 6, p. 483–491, 2001.

FERNANDEZ-LAFUENTE, R. et al. Immobilization of lipases by selective adsorption on hydrophobic supports. **Chemistry and Physics of Lipids**, v. 93, n. 1-2, p. 185–197, 1998.

FERNANDEZ-LAFUENTE, R. Lipase from *Thermomyces lanuginosus*: Uses and prospects as an industrial biocatalyst. **Journal of Molecular Catalysis B: Enzymatic**, v. 62, n. 3-4, p. 197–212, 2010.

FERNÁNDEZ-LORENTE, G. et al. Self-assembly of *Pseudomonas fluorescens* lipase into bimolecular aggregates dramatically affects functional properties. **Biotechnology and Bioengineering**, v. 82, n. 2, p. 232–237, 2003.

FERRER, M. et al. Lipase-catalyzed regioselective acylation of sucrose in two-solvent mixtures. **Biotechnology and Bioengineering**, v. 65, n. 1, p. 10–16, 1999.

GUISÁN, J. Aldehyde-agarose gels as activated supports for immobilization-stabilization of enzymes. **Enzyme and Microbial Technology**, v.10, p. 375-382, 1988.

KOPP, W. et al. Synthesis and characterization of robust magnetic carriers for bioprocess applications. **Materials Science and Engineering: B**, v. 193, p. 217–228, 2015.

LEITGEB, M.; KNEZ, Z. The influence of water on the synthesis of n-butyl oleate by immobilized *Mucor miehei* lipase. **Journal of the American Oil Chemists' Society**, v. 67, n. 11, p. 775–778, 1990.

LIMA, L. N. et al. Immobilization and stabilization of a bimolecular aggregate of the lipase from *Pseudomonas fluorescens* by multipoint covalent attachment. **Process Biochemistry**, v. 48, n. 1, p. 118–123, 2013.

LIMA, L. N. et al. Immobilization of *Pseudomonas fluorescens* lipase on hydrophobic supports and application in biodiesel synthesis by transesterification of vegetable oils in solvent-free systems. **Journal of Industrial Microbiology & Biotechnology**, 2015.

MARTINELLE, M.; HOLMQUIST, M.; HULT, K. On the interfacial activation of *Candida antarctica* lipase A and B as compared with *Humicola lanuginosa* lipase. **Biochimica et Biophysica Acta - Lipids and Lipid Metabolism**, v. 1258, n. 3, p. 272–276, 1995.

MARTINS, A. B. et al. Improved production of butyl butyrate with lipase from *Thermomyces lanuginosus* immobilized on styrene-divinylbenzene beads. **Bioresource Technology**, v. 134, p. 417–422, 2013.

MATEO, C. et al. Increase in conformational stability of enzymes immobilized on epoxy-activated supports by favoring additional multipoint covalent attachment. **Enzyme and Microbial Technology**, v. 26, n. 7, p. 509–515, 2000.

MATEO, C. et al. Advances in the design of new epoxy supports for enzyme immobilization-stabilization. **Biochemical Society transactions**, v. 35, n. Pt 6, p. 1593–1601, 2007.

MATEO, C. et al. Improvement of enzyme properties with a two-step immobilizaton process on novel heterofunctional supports. **Biomacromolecules**, v. 11, n. 11, p. 3112–3117, 2010.

MATEO, C. et al. Oriented covalent immobilization of enzymes on heterofunctional-glyoxyl supports. **Methods in Molecular Biology**, v. 1051, p. 73–88, 2013.

MATTE, C. R. et al. Immobilization of Thermomyces lanuginosus lipase by different techniques on Immobead 150 support: Characterization and applications. **Applied Biochemistry and Biotechnology**, v. 172, n. 5, p. 2507–2520, 2014.

NIGAM, S. et al. Lipase Immobilization Techniques for Biodiesel Production: An Overview. **International Journal of Renewable Energy and Biofuels**, v. 2014, p. 1–16, 2014.

PALOMO, J. M. et al. Interfacial adsorption of lipases on very hydrophobic support (octadecyl-Sepabeads): Immobilization, hyperactivation and stabilization of the open form of lipases. **Journal of Molecular Catalysis B: Enzymatic**. v.19, p. 279-286, 2002

PALOMO, J. M. et al. Lipase-lipase interactions as a new tool to immobilize and modulate the lipase properties. **Enzyme and Microbial Technology**, v. 36, n. 4, p. 447–454, 2005.

PEREIRA, G. H. A.; GUISAN, J. M.; GIORDANO, R. L. C. Multi-point immobilization of penicillin G acylase on silica-glyoxyl: influence of the degree of activation. **Brazilian Journal of Chemical Engineering**, v. 14, n. 4, p. 327–331, 1997.

TANI, K.; SUZUKI, Y. Influence of titania matrix on retention behaviour in reversed-phase liquid chromatography. **Journal of Chromatography A**, v. 722, n. 1-2, p. 129–134, 1996.

TARDIOLI, P. W.; ZANIN, G. M.; DE MORAES, F. F. Production of cyclodextrins in a fluidized-bed reactor using cyclodextrin-glycosyl-transferase. **Applied biochemistry and biotechnology**, v. 84-86, p. 1003–1019, 2000.

TARDIOLI, P. W. et al. Design of new immobilized-stabilized carboxypeptidase a derivative for production of aromatic free hydrolysates of proteins. **Biotechnology Progress**, v. 19, n. 2, p. 565–574, 2003.

VELASCO-LOZANO, S. et al. Carrier-free immobilization of lipase from *Candida rugosa* with polyethyleneimines by carboxyl-activated cross-linking. **Biomacromolecules**, v. 15, n. 5, p. 1896–1903, 2014.

YAN, Y. et al. Lipase-catalyzed solid-phase synthesis of sugar fatty acid esters - Removal of byproducts by azeotropic distillation. **Enzyme and Microbial Technology**, v. 25, n. 8-9, p. 725–728, 1999.

ZHANG, D.-H.; YUWEN, L.-X.; PENG, L.-J. Parameters Affecting the Performance of Immobilized Enzyme. **Journal of Chemistry**, v. 2013, p. 1–7, 2013.

ZHOU, Z. et al. Immobilization of lipase in cage-type mesoporous organosilicas via covalent bonding and crosslinking. **Catalysis Today**, v. 243, n. C, p. 173–183, 2015.

5. ARTIGO 3 – Esterificação de ácidos graxos e xilose catalisada por lipase de pâncreas de porco adsorvida hidrofobicamente em silica-octil

Neste trabalho, em forma de artigo científico, lipase de pâncreas de porco (LPP) foi imobilizada em sílica modificada com grupos octil (octil-silica). LPP foi escolhida devido ao seu baixo custo comparado aos das lipases microbianas comerciais. Além disso, a técnica de imobilização utilizada (adsorção hidrofóbica) é uma técnica simples e rápida, contribuindo para a obtenção de um biocatalisador de baixo custo para aplicação em reações de síntese de ésteres de açúcares, que são biosurfactantes com alto valor agregado.

LPP foi imobilizada com sucesso em sílica-octil, rendendo um biocatalisador hiperativado (144% de atividade recuperada), fenômeno atribuído à ativação interfacial desta enzima. Os valores de atividade enzimática e concentração de proteínas adsorvidos no suporte (98% e 75%, respectivamente), sugerem que a LPP presente no extrato bruto foi seletivamente adsorvida pelo suporte octil-silica (OS).

As sínteses dos ésteres oleato de xilose e caprilato de xilose renderam conversões de 70% e 63%, respectivamente, após 2 h de reação a 60 ºC. O derivado LPP-OS (LPP imobilizada em octil-silica) foi reutilizado em quatro ciclos reacionais de 2 h (60 ºC, razão molar frutose/ácido oleico de 1:5, terc-butanol como solvente), mantendo sua atividade residual em aproximadamente 100%.

Esterification of fatty acids and xylose catalyzed by porcine pancreatic lipase hydrophobically adsorbed on octyl-silica

Abstract: In this work, porcine pancreatic lipase (LPP) was immobilized on silica modified with octyl groups (octyl-silica). LPP was chosen due to its low cost compared with the microbial lipases commercially available. Besides, the immobilization technique used (hydrophobic adsorption) is a simple and rapid immobilization protocol, contributing to the preparation of a low-cost biocatalyst to be used in reaction of syntheses of sugar esters, which high-value compounds. LPP was successfully immobilized on octyl-silica, yielding a hyperactivated biocatalyst (144% of recovered activity), a phenomenon attributed to interfacial activation of the enzyme. The values of enzyme activity and protein content adsorbed on the support (98% and 75%, respectively), suggest that PPL, present in the crude extract, was selectively immobilized by the support octyl-silica (OS). The synthesis of xylose oleate and xylose caprylate esters resulted in conversion of 70% and 63%, respectively, after 2 h reaction at 60 °C. The biocatalyst PPL-OS was reused in 4 cycles of 2 h reaction (60 °C, molar ratio sugar/acid 1: 2, *tert*-butanol as solvent), maintaining its residual activity around 100%.

Keywords: Porcine pancreatic lipase, octyl-silica, hydrophobic adsorption, xylose, bio-surfactant.

5.1. Introduction

Sugar esters are non-ionic surfactants widely used in the food, cosmetics, oral-care, detergent and pharmaceutical industries. These compounds can be synthesized by chemical or enzymatic catalysts. Due to several drawbacks showed by the chemical route, such as poor selectivity towards the various hydroxyl groups in carbohydrates and formation of coloured side-products, enzymatic route using lipases have been preferred (HAJOUR, KLAI, 2013).

Lipases (EC 3.1.1.3) are hydrolases, which catalyze the hydrolysis of triacylglycerides to free fatty acids and glycerol in aqueous media, and esterification, interesterification and transesterification in the presence of organic solvents. Moreover, lipases display enantioselective properties. Due to the ability to catalyze several types of biotransformations, lipases has been increasingly applied in pharmaceuticals, detergent, organic synthesis, oil processing, cosmetics, and food processing industries (THAKUR, 2012).

Lipases can be obtained from different sources, such as animals, plants or microbial cells. An example of lipase derived from animal tissue is the porcine pancreatic lipase (PPL), the cheapest lipase preparation among the commercially available (MENDES; OLIVEIRA; CASTRO, 2012). Although microbial lipases has been more extensively used due to their features (stability, selectivity, and broad specificity to the substrate) (GRIEBELER et al., 2011), lipase from porcine pancreas has also demonstrated stability and activity in esterification and transesterification reactions (MENDES; OLIVEIRA; CASTRO, 2012).

The successful use of porcine pancreatic lipase in fat digestibility (LIU et al., 2010), detergents formulations (SHARMA et al., 2008), enantioselective synthesis (ANGOLI et al., 2003), and synthesis of fatty acid sugar esters (HAIOUR; KLAI, 2013) have been reported. The PPL appears as an interesting alternative to overcome the limitation of the use of enzymes in industrial processes, which often lies in the high cost of commercial enzymes. Immobilization of the enzyme may also be a strategy in an attempt to facilitate its application, since this technique enhances its catalytic activity, storage, operational and thermal stability (MENDES et al., 2012).

In order to explore the potential application of the low-cost PPL, this work evaluated the immobilization of the enzyme on silica covered by octyl groups, a highly

hydrophobic support which does not require high cost of preparation, and application of the immobilized PPL in the synthesis of xylose esters.

D-xylose fatty acid esters are compounds of great relevance in cosmetics because of skin and eye tolerance (HAJOUR, KLAI, 2013). Besides, xylose can be obtained from renewable resources, as well as lignocellulosic biomasses. Particularly in Brazil, sugarcane bagasse largely generated in ethanol's facilities could be an important source of xylose.

5.2. Experimental

5.2.1. Material

Crude porcine pancreatic lipase type II (PPL) and octyl triethoxysilane (OCTES) were purchased from Sigma Aldrich (St. Louis, EUA); macroporous silica (Immobead S60S) from Chiral Vision (Leiden, Holanda). Olive oil (Carbonell®) was acquired at local market. Gum arabic was obtained from Synth (São Paulo, Brazil), and phenolphthalein from Qhemis (Brazil). *Tert*-butyl alcohol and *tert*-pentyl alcohol were purchased from Vetec Química Fina Ltda (São Paulo, Brazil). Sucrose, glucose, xylose, oleic and caprylic acids were acquired from Synth (São Paulo, Brazil). All other chemicals were of analytical purity.

5.2.2. Methods

5.2.3. Chemical modification of the silica surface with octyl groups

Macroporous silica (average pore diameter 193 Å, according to Lima et al 2015) was chemically modified using OCTES. The porous silica was previously treated with HCl (0.1 M) under reflux, followed by washing until neutral pH and drying at 50 ºC for 12 h (TANI; SUZUKI, 1996). Octyl groups were incorporated on the silica surface using the methodology described by Tani and Suzuki (1996), where a suspension containing 1 g of dry silica and 20 mL of a mixture of OCTES:toluene (1:10, v/v) was kept under reflux for 4 h at the boiling temperature of the solvent. The activated support was washed with toluene and distilled water, and dried at 40 °C for 24 h.

5.2.4. Determination of the PPL hydrolytic activity

The hydrolysis of olive oil was performed according to the modified methodology, previously described by Soares et al., 1999. The substrate was prepared by mixing 50 mL of the olive oil and 50 mL of emulsification reagent (Gum Arabic solution at 7%, m/v). The reaction mixture consisted of 5 mL of emulsion, 2 mL of 100 mM sodium phosphate buffer at pH 7.0 and an amount of free or immobilized enzyme, conveniently diluted. The reaction system was incubated for 5 min at 37 ºC under continuous stirring (300 rpm) in a orbital shaker (Marconi, Piracicaba, Brazil) The reaction was stopped by adding 10 mL acetone-ethanol solution 1:1 (v/v). The released free fatty acids were tritated with standard 0.025 M potassium hydroxide solution, using phenolphthalein as indicator. Lipase activity was calculated by Equation (1) using the rate of alkali addition, expressing the activity as units per millilitre or gram of sample. One unit (U) of lipase activity was defined as the enzyme amount that releases 1µmol of fatty acids per minute under the assay conditions.

$$\text{Activity}\left(\frac{\mu\text{mol}}{\text{min}\,\text{min}}\right) = \frac{(V_A - V_B)\times M\times 1000}{t\times E} \qquad (1)$$

Where: E is the amount of enzyme (g), M is molarity of KOH solution, t is the reaction time (min), V_A is the KOH volume used to titrate the sample (mL) e V_B is the KOH volume used to titrate the blank (mL).

5.2.5. Determination of the PPL esterification activity

The esterification activity of the immobilized lipase was measured according to the method described by Paula et al. (2008), which consists of butyl butyrate formation in the reaction of 1-butanol (0.10 M) and butyric acid (0.10 M) in heptane at 37 °C.

5.2.6. PPL immobilization on octyl-silica

A suspension containing 1 g of octyl-silica support (OS) and 10 mL of PPL solution (0.534 mg protein total.mL^{-1}, in sodium phosphate buffer 5 mM, pH 7.0) was prepared for the immobilization assay. The suspension was kept under mild stirring in a GyroTwister GX-1000 3-D Shaker (S1000-A, Labnet International, Inc., New Jersey, USA)

for 18 h at room temperature. The supernatant samples were withdrawn over the immobilization period for enzyme hydrolytic activity and protein concentration assays. The protein content of the enzyme preparation was determined by colorimetric method (BRADFORD, 1976). After 18 h, the immobilized biocatalyst was recovered by filtration and washed with distilled water.

5.2.7. Sugar solubility in organic solvents

A 150 mM solution of each sugar (sucrose, glucose and xylose) was prepared in the presence of organic solvent (*tert*-butyl alcohol and *tert*-pentyl alcohol) and placed in closed flasks. After 24 h under constant stirring in a Marconi incubator (MA 430/1) equipped with 360°-stirring carrousel (Marconi®, Piracicaba, Brazil) at 60 ºC, the preparation was centrifuged at 10,000 rpm (750 × *g*) and 35 ºC for 2 min. One milliliter of the supernatant was dried at 70 °C for 24 h. After evaporation of the tertiary alcohol, 1 mL of water was added prior to measurement of the sugar concentration using a Water HPLC equipped with a refractive index detector and a Sugar Pak-I column (300 x 6.5 mm x 10 µm) kept at 80°C. Ultrapure water was used as eluent, at 0.5 mL/min.

5.2.8. Synthesis of sugar esters

The synthesis of esters was carried out at 60 °C in closed flasks under predefined conditions (60 mM of xylose in *tert*-butyl alcohol, 30 mM caprylic acid and oleic acid, 6 mL of *tert*-butyl alcohol and 51 esterification Unit). Samples of 1 mL were withdrawn and dried in oven at 70 °C for 24 h. After evaporation of the tertiary alcohol, 1 mL of water was added prior to measurement of the xylose concentration using a Water HPLC equipped with a refractive index detector and a Sugar Pak-I column (300 x 6.5 mm x 10 µm) kept at 80 ºC. Ultrapure water was used as eluent, at 0.5 mL/min.

5.3. Results

Silica covered by hydrophobic groups OCTES was used as support to immobilized the crude extract containing porcine pancreatic lipase. Table 1 presents the immobilization results.

Table 5.1 – Immobilization parameters of porcine pancreatic lipase (PPL) on octyl-silica (OS) support at pH 7.0, room temperature for 18 h

Immobilization parameters	
Protein loading ($mg.g_{support}^{-1}$)	5.34 ± 0.02
Immobilization yield (% protein)	75.60 ± 0.01
Immobilization yield (% hydrolytic activity)	98.13 ± 1.54
Recovered Activity (%)	**144.27 ± 3.4**

It can be observed from the data in Table 5.1 that there was a partially selective immobilization of PPL, since the yield of immobilization regarding activity was around 98%, while the yield regarding protein was approximately 75%. Thus, it is probable that the percentage of non-immobilized proteins correspond to other proteins in the crude extract (Table 5.1). According to the information provided by the manufacturer (Sigma-Aldrich), the commercial crude lipase from porcine pancreas also contains amylase and protease. The selective immobilization of lipases on hydrophobic support was previously reported by Bastida et al. (1997) and Fernandez-Lafuente et al. (1998). According to the authors, the immobilization of lipases in the presence of hydrophobic supports, such as OS, in medium with low ionic strength results in a rapid and selective adsorption of the enzyme on the support, due to the high affinity displayed by lipases, which has as its natural substrate highly hydrophobic molecules (fats and oils).

The hyperactivation of PPL immobilized on OS support (PPL-OS) may be associated with conformational changes of the enzyme, such as lid opening, thus exposing its active site. Pregnolato et al. (2001) reported that the immobilization of PPL on octyl-agarose resulted in improvement of the catalytic properties of the enzyme compared to the free enzyme. Indeed, it has been reported in the literature the phenomenon of hyperactivation for different lipases immobilized on hydrophobic surfaces (BASTIDA et al., 1997; FERNANDEZ-LAFUENTE et al., 1998; LIMA et al., 2013). However, as shown in Table 5.2, despite the immobilization of PPL on different supports be well studied, the hyperactivation phenomenon is rarely reported (BAI; ZHOU, 2004; BAI et al., 2006; BAGI; SIMON, 1999; CASTRO et al., 1999; LI et al, 2009; OZCAN; SAGIROGLU, 2009; SCHERER et al, 2011; XU et al, 2011).

Therefore, the choice of octyl-silica as support may be an interesting alternative to the immobilization of PPL, because besides enabling the enzyme

hyperactivation and allowing rapid and selective immobilization, the OS support has a simple activation process.

In order to apply the PPL immobilized on OS support in synthesis reactions of biosurfactants, a preliminary study of solubility of different sugars was performed (xylose, sucrose and glucose) in *tert*-butyl and *tert*-pentyl alcohols, both previously evaluated by Degn and Zimmermann (2001).

As shown in Table 5.3, xylose was the most soluble sugar in both organic solvents studied, about 4 and 22 times more soluble than glucose and sucrose in *tert*-butyl alcohol. Based on these results, xylose and *tert*-butyl alcohol were chosen for synthesis reactions of sugar fatty ester.

Table 5.2 - Recovered activity of PPL immobilized on different supports

Support	Recovered Activity	Source
Octyl-agarose	300.0%	FERNANDEZ-LAFUENTE et al. 1998
Akrilex C-100	12.0%	BAGI e SIMON 1999
Celite	25.0%	BAGI e SIMON 1999
Celite[a]	6.7%	CASTRO et al. 1999
Celite[b]	26.7%	CASTRO et al. 1999
Silica	94.0%	BAI e ZHOU 2004
Silica-(APTES)[c]	79.0%	BAI et al. 2006[a]
Silica-(VTES)[d]	118.0%	BAI et al. 2006[a]
Celite	25.0%	OZCAN and SAGIROGLU 2009
Silica (pH 7.0)	41.4%	LI et al. 2009
Silica (pH 8.0)	47.0%	LI et al. 2009

[a] Immobilized in sodium phosphate buffer 0.1 M pH 7.0.

[b] Immobilized in acetone medium.

[c] 3-Aminopropyltriethoxysilane (APTES)

[d] Vinyltriethoxysilane (VTES)

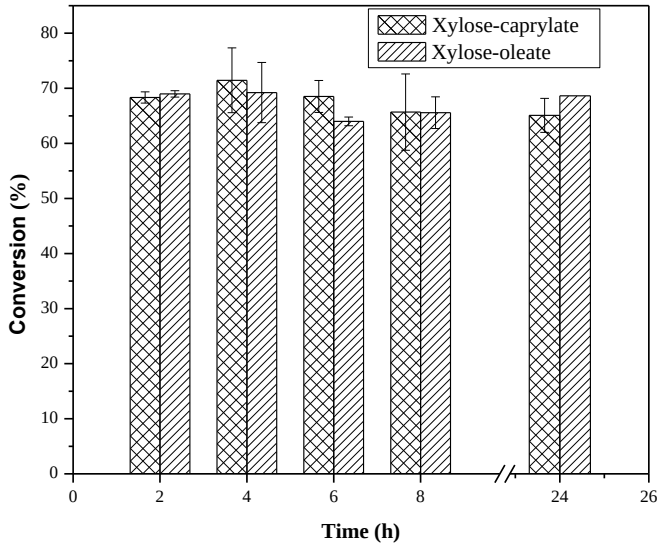
Table 5.3 – Sugar solubility in organic solvents at 60 ºC

Sugar	*tert*-Butyl alcohol ($g.L^{-1}$)	*terc*-Pentyl alcohol ($g.L^{-1}$)
Sucrose	0.47 ± 0.03	0.35 ± 0.01
Xylose	10.35 ± 0.19	7.92 ± 0.10
Glucose	2.37 ± 0.03	0.86 ± 0.03
	2.16[a]	
	2.30[b]	

[a](DEGN; ZIMMERMANN, 2001) at 45ºC
[b](YOO; PARK; YOON, 2007) at 60ºC

LPP immobilized on octyl-silica (85 U/g, where U is esterification unit in the synthesis of butyl butyrate) was used in the batch synthesis of xylose fatty ester under the following conditions: molar ratio xylose/fatty acid of 2:1, 60 °C and absence of molecular sieve. Figure 5.1 shows that the maximum conversion of esters (around 70%) was achieved after a short period of time (2 h), representing a productivity of 3.62 $g.L^{-1}.h^{-1}$.

Figure 5.1 – Conversions of synthesis reactions of xylose-caprylate and xylose-oleate at 60 ºC for 24 h, molar ratio xylose/fatty acid of 2:1 in *tert*-butyl alcohol, 51 esterification unit of PPL immobilized on octyl-silica (PPL-OS).

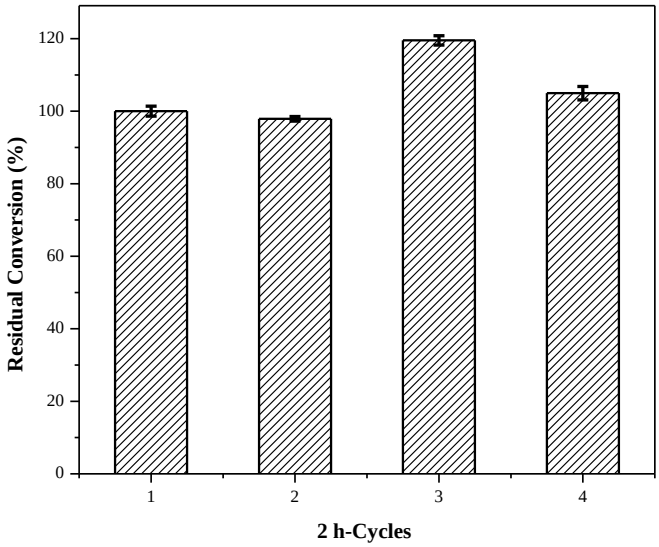


These results are more relevant when compared to xylose-laurate synthesis in *tert*-butyl alcohol at 60 ºC using PPL immobilized on Celite previously reported by Bidjou-Haiour and Klai (2013), although it is another ester. The authors reported a conversion of 34%

in 72 h reaction. Paula et al. (2007) reported that in the syntheses of glucose and fructose oleate in *tert*-butyl alcohol using PPL immobilized on POS-PVA (polysiloxane-polyvinyl alcohol), the ester conversions were 49.8% (6 h) and 52.8% (4 h) at 45 ºC, respectively.

The operational stability of PPL immobilized on OS in the synthesis reaction of xylose oleate was evaluated. Figure 5.2 shows that the biocatalyst could be used in four 2 h-cycles. It is worth noting that the biocatalyst was stable under the synthesis conditions; even working at high temperature, there was not loss in the performance of the biocatalyst.

Figure 5.2 – Operational stability of PPL-OS in xylose oleate syntheses at 60 ^{o}C for 2 h, using molar ratio xylose/oleic acid of 2:1 in *tert*-butyl alcohol, 51 esterification unit of PPL-OS



5.4. Conclusion

Lipase from porcine pancreas was successfully immobilized on silica activated with octyl groups. Immobilization yield regarding activity and protein suggested that lipase was immobilized selectively. The high-recovered activity presented by the biocatalyst PPL-OS suggested conformational changes in the three-dimensional structure of the enzyme. The biocatalyst had an excellent performance in the synthesis of xylose fatty ester, yielding high conversions in short period of time (about 70% in 2 h reaction). Thus, it can be concluded that the use of PPL immobilized on OS may represent a significant cost savings to the production process of bioproducts, especially those for pharmaceutical and food industries, which have a high-added value.

5.5. Acknowledgements

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5.6. References

ANGOLI, M. et al. Remote Stereocenter Discrimination in the Enzymatic Resolution of Piperidine-2-ethanol. Short Enantioselective Synthesis of Sedamine and Allosedamine. **Journal of Organic Chemistry**, v. 68, n. 24, p. 9525–9527, 2003.

BAGI, K.; SIMON, L. M. Comparison of esterificafion and transesterification of fructose by porcine pancreas lipase immobilized on different supports. **Biotechnology Techniques**, v. 13, n. 5, p. 309–312, 1999.

BAI, Y. X. et al. Covalent immobilization of triacylglycerol lipase onto functionalized nanoscale SiO2 spheres. **Process Biochemistry**, v. 41, n. 4, p. 770–777, 2006.

BAI, Z. W.; ZHOU, Y. K. A novel enzyme support derived from aminated silica gel and polysuccinimide: Preparation and application for the immobilization of porcine pancreatic lipase. **Reactive and Functional Polymers**, v. 59, n. 1, p. 93–98, 2004.

BASTIDA, A. et al. A single step purification, immobilization, and hyperactivation of lipases via interfacial adsorption on strongly hydrophobic supports. **Biotechnology and Bioengineering**, v. 58, n. 5, p. 486–493, 1997.

BRADFORD, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. **Analytical biochemistry**, v. 72, p. 248–254, 1976.

CASTRO, H. F. et al. Immobilization of porcine pancreatic lipase on Celite for application in the synthesis of butyl butyrate in a nonaqueous system. **J. Am. Oil Chem. Soc.**, v. 76, n. 1, p. 147–152, 1999.

DEGN, P.; ZIMMERMANN, W. Optimization of carbohydrate fatty acid ester synthesis in organic media by a lipase from *Candida antarctica*. **Biotechnology and bioengineering**, v. 74, n. 6, p. 483–491, 2001.

FERNANDEZ-LAFUENTE, R. et al. Immobilization of lipases by selective adsorption on hydrophobic supports. **Chemistry and Physics of Lipids**, v. 93, n. 1-2, p. 185–197, 1998.

GRIEBELER, N.; POLLONI, A. E.; REMONATTO, D.; ARBTER, F.; VARDANEGA, R.; CECHET, J. L.; et al. Isolation and screening of lipase producing fungi with hydrolytic activity. **Food Bioprocess Tech.**, vol.4, n. 4, p. 578-586, 2011.

HAIOUR, C.; KLAI, N. Lipase catalyzed synthesis of fatty acid xylose esters and their surfactant properties. **Asian Journal of Chemistry**, v. 25, n. 8, p. 4347–4350, 2013.

LI, Y. et al. Adsorption and catalytic activity of Porcine pancreatic lipase on rod-like SBA-15 mesoporous material. **Colloids and Surfaces A: Physicochemical and Engineering Aspects**, v. 341, n. 1-3, p. 79–85, 2009.

LIMA, L. N. et al. Immobilization and stabilization of a bimolecular aggregate of the lipase from Pseudomonas fluorescens by multipoint covalent attachment. **Process Biochemistry**, v. 48, n. 1, p. 118–123, 2013.

LIMA, L. N. et al. Immobilization of *Pseudomonas fluorescens* lipase on hydrophobic supports and application in biodiesel synthesis by transesterification of vegetable oils in solvent-free systems. **Journal of Industrial Microbiology & Biotechnology**, 2015.

LIU, F. C. et al. Application of porcine lipase secreted by pichia pastoris to improve fat digestion and growth performance of postweaning piglets. **Journal of Agricultural and Food Chemistry**, v. 58, n. 6, p. 3322–3329, 2010.

MENDES, A. A.; OLIVEIRA, P. C.; DE CASTRO, H. F. Properties and biotechnological applications of porcine pancreatic lipase. **Journal of Molecular Catalysis B: Enzymatic**, v. 78, p. 119–134, 2012.

OZCAN, H. M.; SAGIROGLU, A. Production of ricinoleic acid from castor oil by immobilised lipases. **Preparative Biochemistry & Biotechnology**, v. 39, n. 2, p. 170–182, 2009.

PAULA, A. V. et al. Porcine pancreatic lipase immobilized on polysiloxane-polyvinyl alcohol hybrid matrix: Catalytic properties and feasibility to mediate synthesis of surfactants and biodiesel. **Journal of Chemical Technology and Biotechnology**, v. 82, n. 3, p. 281–288, 2007.

PAULA, A. V.; MOREIRA, A. B. R.; BRAGA, L. P.; CASTRO, H. F. Comparação do desempenho da lipase de *Candida rugosa* imobilizada em suporte híbrido de polissiloxanopolivinilálcool empregando diferentes metodologias. **Química Nova**, v. 31, p. 35-40, 2008

PREGNOLATO, M. et al. Enantioselective enzymatic hydrolysis of racemic glycidyl esters by using immobilized porcine pancreas lipase with improved catalytic properties. **Journal of Molecular Catalysis - B Enzymatic**, v. 11, n. 4-6, p. 757–763, 2001.

SCHERER, R. et al. Screening of supports for immobilization of commercial porcine pancreatic lipase. **Materials Research**, v. 14, n. 4, p. 483–492, 2011.

SHARMA, M.; KUMAR, V.; PUNDIR, C. S. Immobilization of porcine pancreas lipase onto free and affixed arylamine glass beads and its application in removal of oil stains. **Indian Journal of Biotechnology**, v. 7, n. 3, p. 328–332, 2008.

SOARES, C. M. et al. Characterization and utilization of *Candida rugosa* lipase immobilized on controlled pore silica. **Applied Biochemistry and Biotechnology**, v. 77-79, p. 745–757, 1999.

TANI, K.; SUZUKI, Y. Influence of titania matrix on retention behaviour in reversed-phase liquid chromatography. **Journal of Chromatography A**, v. 722, n. 1-2, p. 129–134, 1996.

THAKUR, S. Lipases, its sources, properties and applications: a review. Int. J. Sci. Eng. Res., vol. 3, n. 7, p. 1-29, 2012.

XU, Y. Q. et al. Improving adsorption and activation of the lipase immobilized in amino-functionalized ordered mesoporous SBA-15. **Solid State Sciences**, v. 13, n. 5, p. 867–874, 2011.

YOO, I. S.; PARK, S. J.; YOON, H. H. Enzymatic Synthesis of Sugar Fatty Acid Esters. **Journal of Industrial and Engineering Chemistry.** v. 13, n. 1, p. 1–6, 2007.

6. CONCLUSÃO

- Sílica modificada quimicamente com diferentes grupos funcionais (octil, aldeído e epóxi) rendeu suportes contendo microambientes complexos. Os suportes híbridos contendo diferentes porções hidrofóbicas/hidrofílicas foram utilizados com sucesso na imobilização das lipases comerciais: lipases de *Candida antarctica* B, *Thermomyces lanuginosus, Pseudomonas fluorescens* e ainda lipase de pâncreas de porco, imobilizada apenas em OS.
- Para a CALB o suporte funcionalizado com grupos octil e glutaraldeído (OSGlu) rendeu o melhor biocatalisador em termos de recuperação de atividade (71,0 ± 2,3%), assim como em termos das mais altas atividades de hidrólise, esterificação, transesterificação e estabilidade em terc-butanol (atividade total retida após 100 h a 65ºC).
- Para a LTL e PFL o suporte funcionalizado com grupos octil (OS) rendeu os melhores biocatalisadores em termos de recuperação de atividade (110,4 ± 0. e 208,1% ± 4,1%, respectivamente). Enquanto para a LPP imobilizada em OS a recuperação de atividade foi 144,3 ± 3,4. O alto valor de atividade recuperada observado para estas enzimas se deve à dissociação dos agregados bimoleculares ou a mudanças conformacionais da enzima (por exemplo, abertura da tampa) durante a imobilização sobre suportes altamente hidrofóbicos.
- O biocatalisador (CALB-OSGlu) catalisou eficientemente a síntese de mono-oleato de frutose (confirmado por espectroscopia de massa) sob condições otimizadas: 55ºC, razão molar frutose/ácido oleico de 1:5 (130 mM de frutose); 20% (m/v) de peneira molecular e terc-butanol como solvente orgânico para solubilizar a frutose. Usando essas condições, a conversão foi de aproximadamente 90% após 6 h de reação.
- O derivado CALB-OSGlu pode ser reutilizado em nove ciclos reacionais de 6 h (55ºC, razão molar frutose/ácido oleico de 1:5, terc-butanol como solvente e peneira molecular, 20%, m/v), atingindo conversão final acima de 70%, enquanto o derivado comercial Novozyme 435 mostrou uma estabilidade operacional mais baixa nas mesmas condições de reação, com a conversão reduzindo de 84% (primeira batelada) para 53% (nona batelada). Portanto, CALB-OSGlu apresenta

considerável potencial para uso industrial na produção de biosurfactantes do tipo ésteres graxos de açúcares.

- Os biocatalisadores da LTL e LPF exibiram uma elevada estabilidade em terc-butanol (meia-vida de cerca de 50 h a 65ºC). Todos os derivados apresentaram excelente desempenho na síntese de oleato de frutose, aproximadamente 90% em 12 h, na presença de 1% (v/v) de água na fase orgânica, exceto para os biocatalisadores imobilizados em sílica modificada com grupos octil e epóxi (OSEpx). Este resultado é possivelmente proporcionado pela presença de grupos carboxílicos na superfície do suporte, formados na reação de redução dos grupos epóxidos com a glicina, criando um microambiente mais hidrofílico.
- O aumento na carga enzimática na síntese de oleato de frutose catalisada por LTL e LPF imobilizadas em OS proporcionou uma alta conversão do éster (70% e 63%, respectivamente) em um curto intervalo de tempo (30 min), resultando em alta produtividade (18 e 16 $g_{éster} l^{-1} h^{-1}$).
- A estratégia de utilização da mistura água/terc-butanol ao invés de solventes tóxicos (DMSO, piridina, hexano e outros) se mostrou como uma alternativa interessante, uma vez que a utilização de pequenas frações de água possibilitou, além do aumento significativo da solubilidade da frutose em terc-butanol, um aumento de até cinco vezes na produtividade do éster comparada àquela obtida na ausência de água.
- LTL e LPF LTL imobilizadas em OSGlu e OS foram reutilizadas em quatro ciclos reacionais de 30 min a 35ºC (razão molar frutose/ácido oleico de 1:5, terc-butanol como solvente), mantendo a conversão do último ciclo acima de 60%.
- As sínteses dos ésteres oleato de xilose e caprilato de xilose foram eficientemente catalisadas pela LPP imobilizada em OS, rendendo conversão de aproximadamente 70% após 2 h de reação à 60ºC. O derivado LPP-OS foi reutilizado em quatro ciclos reacionais de 2 h a 60ºC (razão molar frutose/ácido oleico de 1:5, terc-butanol como solvente) sem redução do seu desempenho catalítico.

7. Sugestões para trabalhos futuro

- Estudar a orientação das lipases aos suportes heterofuncionais a partir de técnicas físico-químicas;
- Caracterizar os suportes quanto à concentração das espécies químicas inseridas na superfície da sílica;
- Avaliar o efeito da concentração das espécies químicas na superfície da sílica sobre as atividades catalíticas das lipases imobilizadas;
- Avaliar outras configurações de reatores (leito fixo, fluidizado, aquecimento por micro-ondas, etc.) na produção de ésteres de açúcares, visando obter máxima produtividade no processo;
- Estudar a purificação dos bioprodutos e sua caracterização química;
- Avaliar a imobilização de lipases vegetais (menor custo comparada às microbianas) nos suportes heterofuncionais à base de sílica;
- Estudar a transferência de massa nas reações heterogêneas de síntese de ésteres de açúcares, avaliando restrições difusionais externas e internas;
- Estudar a modelagem do sistema reacional envolvendo as enzimas imobilizadas para a produção de ésteres de açúcares.