Universidade Federal de Minas Gerais – UFMG

Departamento de Engenharia Química

Programa de Pós-Graduação em Engenharia Química

Preparação de biocatalisadores por ativação interfacial de lipase microbiana em suporte hidrofóbico e aplicação na síntese de biolubrificante por reação de esterificação

MICHELLE DIAS ALVES

Belo Horizonte – MG

2016

Preparação de biocatalisadores por ativação interfacial de lipase microbiana em suporte hidrofóbico e aplicação na síntese de biolubrificante por reação de esterificação

Universidade Federal de Minas Gerais – UFMG

Departamento de Engenharia Química

Programa de Pós-Graduação em Engenharia Química

Preparação de biocatalisadores por ativação interfacial de lipase microbiana em suporte hidrofóbico e aplicação na síntese de biolubrificante por reação de esterificação

Michelle Dias Alves

Dissertação de mestrado apresentada à Escola de Engenharia da Universidade Federal de Minas Gerais no Programa de Pós-Graduação em Engenharia Química na área de Concentração – Processos Bioquímicos como parte dos requisitos necessários à obtenção do grau de Mestre em Engenharia Química.

Orientador: Prof. Dr. Adriano Aguiar Mendes
Co-orientadora: Profa. Dra. Érika Cristina Cren

Belo Horizonte – MG

2016

Dedico esta dissertação aos meus pais, Geovani e Fernanda, à minha irmã Taís e ao meu noivo Marcus que desde o início me apoiaram, entenderam e incentivaram. Vocês fazem parte dessa conquista.

“Se pude enxergar mais longe, é porque me apoiei nos ombros de gigantes.”

Isaac Newton.

AGRADECIMENTOS

A Deus por tudo o que Ele sempre me concedeu, por me iluminar e guiar minhas escolhas.

Aos meus pais, Geovani e Fernanda, por todo carinho, apoio, incentivo, amor e por sempre acreditarem no meu potencial e viverem tudo isso comigo. Obrigada por serem minha base e por todo cuidado que vocês sempre tiveram comigo.

À minha irmã, Taís, pelo apoio e parceria de uma vida inteira, além de todo incentivo que você sempre me deu.

Ao meu noivo, Marcus, por me entender, apoiar, incentivar e me fazer acreditar quando eu começava a desanimar. Obrigada pela paciência, parceria e companheirismo em todos os momentos. Nossos sonhos me impulsionam a continuar.

À minha avó, Celma, pelo carinho e amor de 'vó', pelas palavras de conforto e proteção, e por sempre torcer por mim.

Ao meu orientador, Prof. Dr. Adriano Aguiar Mendes, pela confiança, carinho, atenção, cuidado e toda sua competência. Obrigada por embarcar comigo na proposta de concluir o mestrado em três semestres, por compartilhar seus conhecimentos, experiências e por todo suporte que você me deu desde que começamos esta jornada, se fazendo sempre presente apesar da distância. Tenho certeza de que este projeto me fez ganhar um grande amigo. À minha co-orientadora, Profa. Dra. Érika Cristina Cren, por todo apoio e suporte na UFMG. Agradeço ainda ao Prof. Dr. Éder Domingos de Oliveira pelo incentivo e por todo apoio que me deu durante o processo de seleção para o mestrado.

À Universidade Federal de Alfenas, pela excelente estrutura e por me permitir a realização de grande parte dos ensaios no Laboratório de Bioprocessos. Gostaria de agradecer a todas as meninas do laboratório, em especial a Fernanda e a Jaque, por me acolherem, me entenderem e, principalmente, por toda a ajuda que vocês me concederam. Este trabalho não teria saído sem vocês.

Ao pessoal do Laboratório de Corrosão da UFMG, em especial à Profa. Dra. Vanessa de Freitas Cunha Lins, por permitir que eu utilizasse os equipamentos e infraestrutura do

laboratório. Agradeço também ao Ricardo, por me socorrer e me ajudar todas as vezes que precisei, e ainda à Renata, ao Thalys e ao Túlio pelas companhias diárias e suporte no laboratório.

A todos os professores do Departamento de Engenharia Química da UFMG. Obrigada por me acolherem e ajudarem sempre que necessário, por compartilharem comigo os conhecimentos e as experiências de vocês e por todo o suporte durante a graduação e a pós-graduação.

À secretária da pós-graduação, Fernanda, por todo apoio, presteza e simpatia durante todo esse tempo.

Ao Ronald, por todo suporte e atenção durante os ensaios nos laboratórios do DEQ.

À FAPEMIG pelo apoio e à CAPES pelo suporte financeiro na concessão de bolsa durante todo o projeto.

RESUMO

O objetivo deste trabalho consistiu na preparação e caracterização de biocatalisadores por ativação interfacial de lipase de *Thermomyces lanuginosus* (TLL) em partículas hidrofóbicas de poli-(estireno-divinilbenzeno) (PSty-DVB) para a produção de ésteres de cera, compostos de interesse industrial com propriedades lubrificantes, por reação de esterificação. O efeito de certos fatores no processo de imobilização como pH, força iônica, temperatura, tempo de incubação e carregamento de proteína variando de 10 a 200 mg/g de suporte foi avaliado. Nas condições ótimas de adsorção da enzima (tampão acetato de sódio 5 mM, pH 5,0, 25 °C, 15 h de incubação e carregamento inicial de proteína de 150 mg/g de suporte), máxima concentração de proteína imobilizada da ordem de 134 mg/g foi observada. A imobilização da enzima no suporte empregado foi também confirmada por análise de espectroscopia de infravermelho e microscopia eletrônica de varredura. O biocatalisador obtido com a máxima concentração de proteína imobilizada foi testado na síntese de linoleato de cetila (biolubrificante) em um sistema isento de solventes, um importante éster de cera insaturado, com o intuito de estimar a sua atividade em reação de esterificação. Máxima conversão da ordem de 90% foi observada para 30 min e nas condições experimentais testadas, o biocatalisador foi altamente estável após cinco sucessivos ciclos de reação. Além disso, TLL imobilizada em PSty-DVB foi mais ativo na síntese de linoleato de cetila que Lipozyme TL-IM, um biocatalisador comercial. Em seguida, os biocatalisadores preparados foram também testados na síntese de decanoato de cetila, um éster de cera saturado e de grande aplicação industrial. Neste estudo, a influência de alguns parâmetros foi avaliada com o intuito de otimizar a síntese deste éster de cera (decanoato de cetila). Um modelo cinético de reação reversível de 2ª ordem foi proposto a fim de estimar constantes cinéticas e foi observada boa concordância entre os valores obtidos e os dados experimentais ($0{,}9430 \leq R^2 \leq 0{,}9938$). A reação de esterificação foi espontânea nas condições testadas e mostrou ser um processo endotérmico. O biocatalisador preparado com carregamento inicial de proteína de 115 mg/g de suporte (concentração de proteína imobilizada de 108,7 ± 3,1 mg/g) exibiu o maior valor de velocidade inicial de reação (113,5 mM/min de reação) e então selecionado para a síntese do éster. A reação em meio de heptano exigiu um pequeno excesso de álcool cetílico (razão molar ácido:álcool de 1:1,25) e 7,5% m/m de biocatalisador para atingir a conversão máxima de 92,5% em 30 min de reação. Por outro lado, após 50 min de reação foi observada conversão máxima de 85,4% em meio livre de solvente, conduzida com razão ácido:álcool 1:1 e 10% m/m de biocatalisador. A produtividade da reação realizada em meio de heptano foi 2,4 vezes maior que em meio livre de solvente (68,5 e 28,2 mM/min.g de biocatalisador, respectivamente). O biocatalisador preparado foi mais ativo que biocatalisadores comerciais como IMMTLL-T2-150 e Lipozyme TL-IM que exibiram máxima conversão de ≈92% após 45 e 75 min de reação, respectivamente. Os ensaios de estabilidade operacional foram realizados em meio de heptano e o biocatalisador não apresentou redução significativa de sua atividade após oito ciclos de reação.

Palavras-chave: Adsorção, Lipase imobilizada, Esterificação, Síntese de éster lubrificante, Otimização.

ABSTRACT

The aim of this study was the preparation and characterization of active biocatalysts prepared via interfacial activation of lipase from *Thermomyces lanuginosus* (TLL) on poly-(styrene-divinylbenzene) resin (PSty-DVB) to produce wax esters, an important class of compounds from the industrial point of view due to their lubricant properties, by esterification reaction. The influence of certain factors on the immobilization process such as pH, ionic strength, temperature, incubation time and initial protein loading varying from 10 to 200 mg/g of resin was evaluated. Under optimal experimental conditions (buffer sodium acetate 5 mM, pH 5.0, 25 °C, 15 h of incubation and initial protein loading of 150 mg/g of resin), maximum immobilized protein concentration around 134 mg/g was observed. The immobilization of TLL on the support was also confirmed by attenuated total reflection Fourier transform infrared (ATR–FTIR) and scanning electronic microscopy (SEM) analyses. The biocatalyst with the highest immobilized protein concentration was tested in the synthesis of cetyl linoleate, an important unsaturated wax ester, in a solvent-free system in order to estimate its catalytic activity in esterification reaction. Maximum ester conversion around 90% for 30 min of reaction was observed. Moreover, immobilized TLL on PSty-DVB resin was more active in cetyl linoleate synthesis than Lipozyme TL-IM, a commercial biocatalyst. This biocatalyst was highly stable after five consecutive cycles of reaction. In a subsequent study, these prepared biocatalysts were also used to catalyze the synthesis of cetyl decanoate, a saturated wax ester with large industrial application. Here, the influence of some factors on the ester synthesis was succesfully examined in order to optimize the esterification reaction. A second-order reversible reaction kinetic model was proposed to estimate apparent kinetic constants and good agreement with experimental data was observed ($0.9430 \leq R^2 \leq 0.9938$). The reaction was a spontaneous and endothermic process. The biocatalyst prepared with initial protein loading of 115 mg/g of support (immobilized protein concentration of 108.7 ± 3.1 mg/g) yielded the highest value of initial reaction rate (113.5 mM/min of reaction) and chosen for further tests. The reaction in heptane medium required a slight excess of cetyl alcohol (molar ratio acid:alcohol of 1:1.25) and 7.5% m/m of biocatalyst to attain a maximum conversion of 92.5% for 30 min of reaction. On the other hand, maximum conversion of 85.4% for the reaction performed in a solvent-free system was observed for 50 min of reaction conducted in an equimolar ratio acid:alcohol and 10% m/m of biocatalyst. The productivity for the reaction performed in heptane medium was 2.4 times higher than in a solvent-free system – 68.5 and 28.2 mM/min.g of biocatalyst, respectively. The prepared biocatalyst more active than commercial biocatalysts such as IMMTLL–T2–150 and Lipozyme TL–IM that exhibited maximum conversion ≈92% for 45 and 75 min of reaction, respectively. The biocatalyst could be reused at least eight times without significant decrease of its activity.

Keywords: Adsorption, Immobilized lipase, Esterification, Wax ester synthesis, Optimization.

APRESENTAÇÃO

Este projeto de dissertação de mestrado consiste na preparação de biocatalisadores por ativação interfacial de lipase para catalisar reações de esterificação para produção de ésteres de cera com propriedades lubrificantes.

O texto está dividido em três capítulos, além da introdução, da conclusão e dos anexos.

A Introdução trata da proposta do projeto, aplicação dos biocatalisadores na síntese de ésteres de cera, além dos objetivos gerais e específicos.

O capítulo 1 apresenta uma Revisão Bibliográfica que fundamenta a pesquisa e as discussões e considerações abordadas.

O capítulo 2 consiste em um artigo intitulado "*Isotherm, kinetic, mechanism and thermodynamic studies of adsorption of a microbial lipase on a mesoporous and hydrophobic resin"* publicado no periódico indexado *Chemical Engineering Journal.* O artigo aborda a preparação e caracterização de biocatalisadores preparados por adsorção física de lipase de *Thermomyces lanuginosus* em partículas de poli-(estireno-divinilbenzeno).

O capítulo 3 apresenta um segundo artigo, "*Kinetic, thermodynamic, optimization and reusability studies for the enzymatic synthesis of a saturated wax ester*", que reporta a otimização da síntese do decanoato de cetila utilizando o biocatalisador preparado. Aborda ainda os estudos cinéticos e termodinâmicos da reação e o reuso do biocatalisador após sucessivos ciclos de reação.

A conclusão apresenta as considerações finais do projeto. Além disso, recomendações para futuros trabalhos são também apresentados.

Por fim, parte dos resultados obtidos também foi também divulgado no XII Seminário Brasileiro de Tecnologia Enzimática (XII ENZITEC), realizado entre os dias 17 a 20/07/2016 na cidade de Caxias do Sul – RS, apresentado no Anexo I.

LISTA DE FIGURAS

Figura 1.1. Mecanismo de ativação interfacial de lipases em suportes hidrofóbicos..... 22

LISTA DE TABELAS

Tabela 1.1. Biodegradabilidade de lubrificantes de diferentes procedências. 7

Tabela 1.2. Revisão de literatura para a aplicação de lipases imobilizadas na síntese de ésteres lubrificantes. 15

Tabela 1.3. Levantamento bibliográfico para a imobilização de lipases via adsorção física em diferentes suportes..... 26

LISTA DE ABREVIATURAS

AGL	Ácidos graxos livres
API	Instituto de Petróleo Americano
ASTM	American Society for Testing and Materials
ATR-FTIR	Espectroscopia no infravermelho
EDMA	Etileno dimetacrilato
FFA	Ácidos graxos livres
GMA	Metacrilato de glicidila
HA	Atividade hidrolítica
IP	Proteína imobilizada
IY	Rendimento de imobilização
PHB	Poli-(hidroxibutirato)
PMA	Poli-metacrilato
PSty-DVB	Poli-(estireno-divinilbenzeno)
RM IM	Lipase imobilizada de *Rhizomucor miehei*
RSM	Metodologia de superfície de resposta
SA	Atividade específica
SEM	Microscopia eletrônica de varredura
TLL	Lipase de *Thermomyces lanuginosus*

SUMÁRIO

INTRODUÇÃO 1
CAPÍTULO 1 5
REVISÃO BIBLIOGRÁFICA 5
1.1. Biolubrificantes 5
1.2. Rotas de Produção de Ésteres Sintéticos 8
1.3. Produção de Biolubrificantes por Reação de Esterificação 10
1.4. Produção Enzimática de Ésteres por Esterificação 12
1.5. Imobilização de Lipases por Adsorção Física 20
1.6. Referências Bibliográficas 27
CAPÍTULO 2 34
CAPÍTULO 3 47
CONCLUSÃO 86
SUGESTÕES PARA TRABALHOS FUTUROS 87
ANEXO I 88

INTRODUÇÃO

Lipases (triacilglicerol acil hidrolases EC 3.1.1.3) são enzimas que catalisam a hidrólise de triacilgliceróis (óleos e gorduras) em glicerol e ácidos graxos livres (AGL) na interface água/óleo (Schmid e Verger, 1998). Estas enzimas também catalisam reações de esterificação, transesterificação e interesterificação em meios não aquosos (Schmid e Verger, 1998; Adlercreutz, 2013). Elas são obtidas a partir de fontes animais, vegetais e microbianas (Sharma et al., 2001). No entanto, lipases microbianas são as mais empregadas industrialmente devido à sua simplicidade na manipulação genética e em condições de cultivo (Adlercreutz, 2013).

A aplicação de lipases em processos industriais é, muitas vezes, limitada devido ao elevado custo e sensibilidade à alta temperatura e aos solventes orgânicos. Além disso, é difícil separá-las do meio de reação, o que limita a sua recuperação e reutilização. Neste sentido, a imobilização de lipases em um suporte apropriado é uma estratégia utilizada com o propósito de melhorar a sua atividade, especificidade e estabilidade e facilitar a sua reutilização (Adlercreutz, 2013; Fernandez-Lafuente, 2010). Dentre os diferentes protocolos de imobilização existentes, a adsorção física em suportes hidrofóbicos é um dos mais simples e mais baratos disponíveis. Este método permite a reutilização do suporte por dessorção de moléculas de enzima inativas empregando diferentes compostos químicos, como tensoativos e uréia (Cabrera et al., 2009; Bastida et al., 1998). Além disso, a adsorção física de lipases em suportes hidrofóbicos promove a hiperativação e estabilização de lipases na conformação aberta (mecanismo de ativação interfacial), tornando assim este protocolo especialmente adequado para a imobilização de lipases (Hernandez et al., 2011; Cabrera et al., 2009; Palomo et al., 2002).

A escolha de um suporte para a imobilização de enzimas, incluindo lipases, é um fator crucial na preparação de um biocatalisador com alta atividade catalítica e mínima limitação difusional de moléculas de reagentes e/ou produtos em seu microambiente. Uma variedade de suportes hidrofóbicos incluindo octil-Sepharose, octadecil-Sepabeads, poli-metacrilato, sílica funcionalizada, partículas magnéticas, poli-estireno, poli-hidroxibutirato, polipropileno, carvão ativado e poli-(estireno-divinilbenzeno) têm sido amplamente empregados na imobilização de lipases por adsorção (Jesionowski et al., 2014; Adlercreutz, 2013; Hernandez et al., 2011; Fernandez-Lafuente, 2010; Li et al., 2010; Cabrera et al., 2009; Palomo et al., 2002; Bastida et al., 1998). Suportes porosos são geralmente preferidos devido a sua elevada área superficial, o que permite uma maior carga de enzima e a enzima imobilizada possui maior efeito de proteção do meio reacional (Brena e Batista-Vieira, 2006). O tamanho de poros do suporte desempenha um papel importante nas propriedades catalíticas dos biocatalisadores como percentagem de imobilização, seletividade, especificidade e estabilidade térmica/operacional (Cunha et al., 2014; Li et al., 2010.). Nos últimos anos, a aplicação de suportes mesoporosos na preparação de biocatalisadores via adsorção de lipases para catalisar reações em meios aquosos e orgânicos tem sido amplamente reportada pela literatura especilizada (Lage et al., 2016; Cunha et al., 2014; Miranda et al., 2014; Hernandez et al., 2011). Embora a preparação de biocatalisadores ativos a partir da imobilização de TLL em poli-(estireno-divinilbenzeno) seja documentada, este é o primeiro estudo referente à determinação de modelos cinéticos e de isoterma de adsorção e de parâmetros termodinâmicos. Além disso, a aplicação deste biocatalisador na síntese de biolubrificante (decanoato de cetila) ainda não é reportada pela literatura.

Atualmente, ésteres lubrificantes são de origem sintética e produzidos por reação de esterificação de álcool com ácido carboxílico ou por transesterificação de óleos e

ácidos graxos com álcoois na presença de catalisadores químicos (homogêneos ou heterogêneos (Al-Arafi e Salimon, 2012). Contudo, diversas limitações como alto valor energético requerido, longos tempos de reação e geração de resíduos químicos têm sido reportadas para a rota química (Cirujano et al., 2015; Bouzidi et al., 2012; Al-Arafi e Salimon, 2011). Com o intuito de superar essas limitações, a aplicação de lipases imobilizadas em diversos suportes é proposta para a produção de ésteres lubrificantes sintéticos devido ao crescimento da demanda por produtos ambientalmente sustentáveis nos últimos anos (Alves et al., 2016; Khan et al., 2015; Ungcharoenwiwat e Kittikun, 2013; Kuo et al., 2012; Chen et al., 2011; Sellami et al., 2011). O biocatalisador preparado através da imobilização de lipase de *Thermomyces lanuginosus* é promissor na síntese de compostos de interesse industrial, como ésteres lubrificantes, devido a sua alta atividade catalítica na reação de esterificação (Alves et al., 2016) e a natureza hidrofóbica do suporte utilizado na preparação desse biocatalisador que pode permitir uma boa partição de reagentes não-polares como ácido decanoico e álcool cetílico no seu microambiente.

O objetivo geral deste trabalho consistiu na preparação e caracterização de biocatalisadores por adsorção física de lipase de *Thermomyces lanuginosus* (TLL) em partículas hidrofóbicas de poli-(estireno-divinilbenzeno) (PSty-DVB) para a produção de ésteres de cera (decanoato de cetila e linoleato de cetila) por reação de esterificação. Levando em consideração estes aspectos, o objetivo geral do projeto foi alcançado mediante a execução das seguintes etapas:

- Imobilização de lipase de *Thermomyces lanuginosus* em suporte de PSty-DVB e avaliação da influência do pH, força iônica, temperatura, tempo de incubação e

carregamento de proteína nas propriedades catalíticas dos biocatalisadores preparados;

- Estudos de isoterma, mecanismo, cinética e termodinâmica do processo de adsorção da lipase;

- Aplicação dos biocatalisadores preparados na síntese de ésteres de cera por reação de esterificação em meio de solvente e isento de solventes orgânicos;

- Avaliação da influência da concentração de proteína imobilizada, temperatura para determinação de parâmetros cinéticos e termodinâmicos da reação, concentração de biocatalisador, agitação, razão molar ácido:álcool e tempo de reação na síntese de decanoato de cetila;

- Estudos cinéticos e termodinâmicos da síntese de decanoato de cetila;

- Comparação da eficiência catalítica do biocatalisador preparado com aqueles disponíveis comercialmente;

- Estudos de estabilidade operacional dos biocatalisadores preparados em bateladas sucessivas de síntese de éster;

CAPÍTULO 1

REVISÃO BIBLIOGRÁFICA

1.1. Biolubrificantes

Lubrificantes são compostos orgânicos capazes de reduzir o atrito e o desgaste entre duas superfícies móveis pela formação de uma película de proteção. Eles estão disponíveis na forma sólida, líquida ou gasosa e, para ser considerado um bom lubrificante, espera-se que o composto apresente alto índice de viscosidade, alto ponto de ebulição, estabilidade térmica, baixo ponto de congelamento, capacidade de prevenir a corrosão e alta resistência à oxidação. Os óleos minerais vêm sendo amplamente utilizados como lubrificantes em motores de combustão interna, entretanto, por se tratar de um produto obtido da destilação do óleo bruto, essa aplicação somente poderá ocorrer enquanto houver petróleo disponível. Diante deste cenário, análises das perspectivas futuras da utilização de óleo mineral como lubrificante em motores automotivos foram realizadas e geraram conclusões pouco animadoras (Mobarak et al., 2014).

A síntese de lubrificantes iniciou-se antes da existência do motor de combustão interna. Em 1877, os primeiros hidrocarbonetos foram sintetizados por Friedel e Crafts utilizando tricloreto de alumínio como catalisador. Em 1937, o primeiro produto fabricado sinteticamente foi baseado na polimerização de olefinas. O Congresso de Aviação de Zurique também demonstrou interesse pela tecnologia de lubrificantes à base de ésteres, na qual foram desenvolvidos na Alemanha entre 1938 e 1944. Ésteres lubrificantes foram desenvolvidos pelo Laboratório de Pesquisa Naval dos Estados Unidos no início dos anos de 1940 e estes lubrificantes sintéticos foram estritamente

utilizados em aplicações militares e industriais. Antes de substâncias sintéticas serem desenvolvidas, eram utilizados óleos vegetais provenientes de mamona e colza. Na década de 1970, diversas formulações sintéticas foram também desenvolvidas. A corporação americana AMSOIL desenvolveu o primeiro óleo de motor de base 100% sintética a passar pela sequência de testes do Instituto de Petróleo Americano e alcançar a qualificação API em 1972. A Mobil Oil comercializou seu primeiro óleo de motor sintético nos Estados Unidos em 1975. As empresas Mobil, Quaker State, Castrol, Valvoline e Pennzoil também buscaram alternativas para seus óleos convencionais. Desde então, praticamente todas as grandes companhias têm introduzido um variante sintético ou semissintético em seus produtos (Nagendramma e Kaul, 2012).

A preocupação com a poluição e saúde ambiental é crescente e vem ganhando destaque perante as questões públicas. Na área de lubrificantes, o foco consiste em questões relacionadas à grande quantidade de lubrificantes descartada no meio ambiente, contaminando diretamente a água e o solo (Nagendramma e Kaul, 2012). Cerca de 50% de todo lubrificante comercializado no mundo tem como destino final o meio ambiente. Isso ocorre principalmente devido a perdas nas aplicações, derramamentos, volatilidade e acidentes. As perdas de fluidos hidráulicos, por exemplo, são estimadas em 70 a 80%, provocando contaminações do solo e lençóis freáticos (Akerman et al., 2011). Além disso, o ar também é afetado por substâncias voláteis presentes nestes compostos, além dos produtos de combustão, que emitem traços de metais como cálcio, fósforo, zinco, magnésio e nanopartículas de ferro, gerando danos ao meio ambiente (Mobarak et al., 2014). Portanto, a crescente consciência ambiental torna-se a principal força motriz para o desenvolvimento de novas tecnologias, de forma que os lubrificantes sintéticos biodegradáveis constituem uma área extensivamente explorada no âmbito de biotecnologias (Nagendramma e Kaul, 2012).

Biodegradabilidade é a capacidade de um material ser degradado por microrganismos. Um lubrificante é classificado como biodegradável se seu percentual de degradação em testes padrões excede determinado nível. A biodegradabilidade é diretamente influenciada pelo óleo base dos lubrificantes e depende da composição química do composto, que varia durante a sua aplicação. Isso significa que quando o lubrificante é submetido a fatores como ar, temperatura, metais, umidade e pressão, sua composição química pode ser alterada. Óleos vegetais e ésteres exibem melhor biodegradabilidade se comparados aos óleos minerais ou outros óleos, como pode ser observado na Tabela 1.1 (Mobarak et al., 2014).

Tabela 1.1. Biodegradabilidade de lubrificantes de diferentes procedências.

Tipo de Fluido	Biodegradabilidade (%)
Óleo mineral	20-40
Óleo vegetal	90-98
Ésteres	75-100
Polióis	70-100
Trimelitatos	0-70

Fonte: Mobarak et al. (2014).

A aplicação de lubrificantes é determinada por suas propriedades físico-químicas. Um requerimento básico é permanecer líquido em uma ampla faixa de temperatura de forma que os limites são determinados pelo ponto de fluidez e pelo ponto de *flash* do produto (Akerman et al., 2011). Os biolubrificantes são considerados uma alternativa aos óleos minerais devido ao fato de possuírem propriedades naturais e serem biodegradáveis. Se comparados aos óleos minerais, biolubrificantes à base de

óleo vegetal ou ésteres geralmente apresentam alta lubrificação, alto índice de viscosidade, alto ponto de *flash* e baixas perdas por evaporação, além de baixo ponto de fluidez, o que garante excelente lubrificação em arranques a frio (Bassi et al., 2016). De acordo com estudos reportados pela literatura especializada, diferentes óleos vegetais podem ser empregados na produção de biolubrificantes, incluindo linhaça, palma, girassol, coco, soja, oliva, colza, entre outros. Por outro lado, os óleos vegetais apresentam desvantagens como limitações de operação em baixas temperaturas, baixa compatibilidade com tintas e selantes e ainda a tendência de entupimento de filtros (Mobarak et al., 2014). Essas desvantagens podem ser superadas com o uso de aditivos, porém essa alternativa pode gerar impactos na biodegradabilidade, toxicidade e preço do produto. Entretanto, modificações estruturais por catálise química consistem em uma alternativa bastante interessante e mais econômica capaz de eliminar os aspectos desvantajosos dos biolubrificantes (Tao et al., 2014; Silva et al., 2013).

1.2. Rotas de Produção de Ésteres Sintéticos

Ésteres são importantes compostos químicos, atuando como intermediários na síntese de medicamentos, plastificantes, lubrificantes, cosméticos e fármacos (Wang et al., 2016; Chowdhury et al., 2014; Yadav e Dhoot, 2009). Os mais comumente utilizados para produzir óleos lubrificantes resultam da reação de ácidos carboxílicos e álcoois de cadeia longa, compostas por oito ou mais átomos de carbono (Chowdhury et al., 2014). O grupo éster apresenta efeito direto nas propriedades físicas dos lubrificantes, atuando principalmente na redução da volatilidade e no aumento do ponto de *flash* do composto (Dormo et al., 2004). Ésteres sintéticos são substâncias que apresentam uma combinação de excelente desempenho técnico com propriedades

ecológicas satisfatórias por se tratarem de compostos de fácil degradação, além de possuírem baixa toxicidade aquática. Além disso, conferem alta estabilidade química às moléculas quando comparados aos ésteres de glicerol, por exemplo, por apresentarem ao menos um átomo de carbono quaternário em sua cadeia. Esses ésteres são altamente recomendados para utilização como lubrificantes em diferentes aplicações industriais e automotivas como fluidos hidráulicos, óleos de perfuração, óleos de engrenagens ou lubrificantes para cadeias de serra elétrica (Bassi et al., 2016; Akerman et al., 2011).

Na indústria química, a esterificação normalmente é realizada na presença de ácido sulfúrico, composto tóxico e corrosivo, e das onerosas resinas de troca iônica. Além disso, as reações demandam longo tempo, altas temperaturas e apresentam baixas conversões e rendimentos, sendo, portanto, um procedimento pouco atrativo do ponto de vista industrial (Chowdhury et al., 2014; Yadav e Dhoot, 2009). Uma alternativa promissora consiste no uso de enzimas para a síntese de lubrificantes, principalmente devido à alta eficiência catalítica proporcionada, às brandas condições de reação e por se tratarem de processos ambientalmente sustentáveis (Wang et al., 2016; Tao et al., 2014). A síntese de ésteres de poliois é realizada por esterificação ou transesterificação com ácidos graxos, ésteres metílicos de ácidos graxos ou triglicerídeos, normalmente catalisadas por álcalis, enzimas ou ainda catalisadores ácidos homogêneos como o ácido *p*-toluenosulfônico em um sistema com solvente (Li et al., 2016; Akerman et al., 2011).

A transesterificação com triglicerídeos envolve dois passos: inicialmente o triglicerídeo é convertido em éster metílico normalmente em temperaturas entre 50 °C e 100 °C e em seguida o éster metílico de ácido graxo reage com o poliol a 110-160°C. Em outras palavras, esse processo consiste em consecutivas reações reversíveis nas quais o triglicerídeo é convertido passo a passo em diglicerídeo, monoglicerídeo e, finalmente, libera uma molécula de glicerol. Em cada passo desse processo, é obtido um

mol de éster metílico de ácido graxo. A reação de transesterificação pode ser catalisada por ácidos, bases e também por enzimas (Gryglewicz et al., 2013; Takisawa et al., 2013; Akerman et al., 2011).

Outra rota interessante utilizada na obtenção de ésteres consiste na hidroesterificação (Bressani et al., 2015; Silva et al., 2013; Mendes et al., 2012). Nesse processo, o óleo é inicialmente hidrolisado a fim de se obter ácidos graxos e esses ácidos graxos reagem com um álcool de cadeia ramificada ou poliois a temperaturas entre 135°C e 140°C na presença de solvente e catalisadores de forma a se obter os ésteres de interesse (Kamalakar et al., 2013). Porém, esse método pode ser considerado desvantajoso do ponto de vista econômico ou mesmo por questões de segurança (Takisawa et al., 2013).

A reação de epoxidação de lipídeos também tem sido empregada na síntese de ésteres. É uma das reações de funcionalização da ligação dupla carbono-carbono mais importante capaz de melhorar a estabilidade oxidativa de óleos. Um peroxi-ácido de cadeia curta (dois a quatro átomos de carbono) é preparado a partir de peróxido de hidrogênio e o ácido correspondente, em passos separados ou *in situ*. Outros métodos de epoxidação consistem no uso de dioxiranos, a geração de perácidos a partir de aldeídos e oxigênio molecular e a utilização de hidroperóxidos de alquilo com metais de transição como catalisadores. Epoxidação de óleos seguida por abertura do anel oxirano fornecem compostos polifuncionais interessantes, podendo ser utilizados como lubrificantes com melhoras nas propriedades em baixas temperaturas (Kulkarni et al., 2013; Salimon et al., 2011).

1.3. Produção de Biolubrificantes por Reação de Esterificação

A esterificação direta tem-se mostrado ser mais efetiva que a transesterificação, embora demande longo tempo de reação em baixas temperaturas. Então, o tempo de reação pode ser reduzido com aumento da temperatura, porém, no caso da utilização de enzimas como catalisadores, deve-se atentar ao fato de muitas lipases não conseguirem manter a atividade em altas temperaturas (Bassi et al., 2016; Lage et al., 2016; Tao et al., 2014). A fim de melhorar as condições relacionadas ao tempo de reação, as reações de transesterificação e esterificação são, geralmente, realizadas com catalisadores ácidos ou básicos que podem ser tanto homogêneos quanto heterogêneos. Ácido fosfórico, ácido *p*-toluenosulfônico, ácido sulfúrico, hidróxido de sódio, etóxido e metóxido de sódio são tipicamente utilizados em catálises homogêneas (Silva et al., 2013).

Na produção de biodiesel, por exemplo, catalisadores homogêneos alcalinos como hidróxido de sódio e hidróxido de potássio são largamente utilizados devido à alta atividade catalítica proporcionada e ao curto tempo de reação demandado. Entretanto, existe grande dificuldade em separá-los da mistura reacional provocando o descarte de grandes quantidades de águas residuais (Li et al., 2016).

O crescente interesse na utilização de catalisadores heterogêneos é um importante passo na química verde visto que eles permitem simples processos de separação (sedimentação e/ou centrifugação) e de baixo custo, além de reduzirem a geração de resíduos e poderem ser reciclado por inúmeros ciclos de reações (Akerman et al., 2011). Os catalisadores heterogêneos oferecem um simples isolamento da mistura reacional de forma a reduzir consideravelmente a quantidade de água de lavagem necessária para o reciclo (Li et al., 2016). Sínteses de ésteres de poliois por transesterificação com triglicerídeos utilizando oxalato de zinco II como catalisador foram reportadas (Akerman et al., 2011). Em alguns casos, resinas de troca catiônica e oxalato de estanho também têm sido utilizados como catalisadores (Silva et al., 2013).

Entretanto, a catálise heterogênea gera um sistema com duas ou mais fases e a velocidade de reação pode ser lenta devido aos problemas de transferência de massa entre as fases (Li et al., 2016).

A catálise química ocorre geralmente em elevadas temperaturas (150 °C) e apresenta a vantagem de baixo custo industrial se comparados com outros processos, como, por exemplo, a catálise enzimática (Silva et al., 2013). Porém, a reação de esterificação realizada por meio de catalisadores químicos apresenta desvantagens relacionadas ao alto consumo de energia, geração de subprodutos indesejáveis e corrosão dos equipamentos (Chowdhury et al., 2014; Kleinaite et al., 2014). Além disso, os processos que evolvem esses catalisadores apresentam baixos rendimentos (cerca de 40%), formação de espuma, no caso de catalisadores básicos homogêneos e exigem condições de operação mais severas e alto consumo energético para executar a reação quando comparados a outros processos, como os que envolvem catálise enzimática (Silva et al., 2013).

1.4. Produção Enzimática de Ésteres por Esterificação

As reações de esterificação catalisadas por lipases têm sido extensivamente estudadas nos últimos anos devido às brandas condições de reação proporcionadas, alto grau de pureza dos produtos obtidos visto que as enzimas apresentam alta especificidade, o que reduz a geração de subprodutos e permite reduzir a geração de efluentes nas etapas de purificação dos ésteres, baixos custos energéticos exigidos e ainda por possibilitar a realização de processos ambientalmente favoráveis (Bassi et al., 2016; Lage et al., 2016; Chowdhury et al., 2014).

Lipases catalisam diferentes reações como hidrólise, esterificação e transesterificação, e constituem a base de muitos processos industriais (Kumar et al.,

2014). Estes catalisadores biológicos assumem importância em reações com precursores naturais, mas também na síntese de compostos sintéticos. Apesar de serem mais frequentemente utilizadas em reações em fase aquosa, a aplicação de enzimas em reações em meios não aquosos (aquorrestritos na ausência e/ou presença de solventes orgânicos) é tida como um potencial na química sintética. As reações de síntese enzimática de ésteres em meio aquorrestrito apresentam várias vantagens tais como alta solubilidade de reagentes em solventes orgânicos, capacidade de realizar novas reações impossíveis de serem realizadas em meio aquoso devido às restrições cinéticas ou termodinâmicas, maior estabilidade das enzimas e relativa facilidade de recuperação dos produtos e solventes orgânicos quando comparados com a água (Yadav e Dhoot, 2009).

A produção de óleos lubrificantes e aditivos biodegradáveis por meio de esterificação enzimática com lipase como catalisador é uma rota bastante promissora. Ao contrário de reações à base de alcalinos, nas reações enzimáticas os produtos podem ser facilmente coletados e separados (Tao et al., 2014). Dessa forma, a atividade das lipases é afetada tanto pela natureza do doador acila quanto do receptor acila e pela atividade de água do meio (Akerman et al., 2011).

A aplicação de lipases de diferentes fontes na síntese de ésteres com propriedades lubrificantes por esterificação de ácidos graxos com álcoois tem sido amplamente reportada pela literatura especializada, como mostrado na Tabela 1.2.

Oleato de oleíla foi sintetizado por esterificação catalisada pela lipase *Candida antarctica tipo B* imobilizada em Lewatit VPOC 1600 via adsorção física (Novozym 435). Nas condições ótimas de reação (razão molar álcool:ácido de 1:2, temperatura de 50°C e agitação de 400 rpm) foi obtido rendimento superior a 90% durante 30 min de reação em meio isento de solvente. Neste estudo foi analisada ainda a estabilidade do

biocatalisador que demonstrou alto percentual de rendimento, operando com atividade superior a 80% após quatro ciclos de reação (Radzi et al., 2005).

Lipases *Candida rugosa* e de pâncreas de porco foram imobilizadas por adsorção física em polipropileno (EP100) para catalisar a síntese de palmitato de octila. As reações foram realizadas na razão molar ácido:álcool de 1:1 a 50°C para a lipase *Candida rugosa* e a 40°C para a lipase de pâncreas de porco, com concentração de biocatalisador de 20% m/v em meio isento de solventes orgânicos e sem agitação. Após 10 horas de reação, foram obtidos rendimentos de 98% quando a lipase de *Candida rugosa* foi utilizada e de 60% para o caso da lipase de pâncreas de porco. Nos dois casos, foi analisado o reuso do biocatalisador e foi possível obter atividade de 83% após sete ciclos de operação (Guncheva e Zhiryakova, 2008).

Laurato de laurila foi sintetizado em meio de hexano por esterificação catalisada por lipase B de *Candida antarctica* imobilizada em poli(GMA-*co*-etileno dimetacrilato [EDMA]) via ligação covalente (Mugo e Ayton, 2013). As condições ótimas de reação foram obtidas na razão molar ácido:álcool de 1:1, temperatura de 50°C e em condições estáticas. Após 12 horas de reação, foi observada uma conversão de 97% e houve mínima redução de atividade do biocatalisador após execução de 15 ciclos de reação.

Lipase *Candida antarctica tipo B* foi imobilizada em Lewatit MonoPlus MP 64 via adsorção física para catalisar reação de esterificação para a produção de ricinoleato de cetila (Montiel et al., 2015). As condições ótimas foram atingidas na razão molar dos reagentes (ácido e álcool) de 1:1 a 60°C e concentração de biocatalisador de 9,9% m/v. A reação foi conduzida em meio isento de solventes orgânicos sob agitação de 350 rpm com remoção de moléculas de água produzidas na reação por um sistema de vácuo. Após 24 horas de procedimento, foi obtido um rendimento de 99,98% e não houve perdas consideráveis de atividade após três ciclos de reação.

Tabela 1.2. Revisão de literatura para a aplicação de lipases imobilizadas na síntese de ésteres lubrificantes.

Lipase	Suporte	Método de imobilização	Condições reacionais	Tempo de reação (h)	Conversão (%)	Estabilidade operacional (reuso)	Éster	Referência
Candida antarctica tipo B	Lewatit VPOC 1600	Adsorção física	Razão molar ácido:álcool de 1:2, 60°C, 0,5% m/v de biocatalisador, agitação de 150 rpm, concentração de água mantida em 0,5%	12	99,8	Não determinado	Laurato de propila	Dormo et al. (2004)
			Razão equimolar ácido:álcool, 80°C, 7% m/v de biocatalisador, ausência de solvente e agitação de 700 rpm.	2	7,34	Não determinado	Oleato de cetila	Garcia et al. (2000)
			Razão molar ácido:álcool de 1:2, 50°C, 90 g de enzima, meio livre de solvente e agitação de 400 rpm	0,5	Acima de 90	4 ciclos (atividade superior a 80%)	Oleato de oleíla	Radzi et al. (2005)

Tabela 1.2. Revisão de literatura para a aplicação de lipases imobilizadas na síntese de ésteres lubrificantes (continuação).

Lipase	Suporte	Método de imobilização	Condições reacionais	Tempo de reação (h)	Conversão (%)	Estabilidade operacional (reuso)	Éster	Referência
Candida antarctica tipo B	Lewatit MonoPlus MP 64	Adsorção física	Razão molar ácido:álcool de 1:1, 60°C, 9,9% de biocatalisador, ausência de solvente, 350 rpm, remoção de água por vácuo	24	99,98	3 ciclos sem perda aparente de atividade	Ricinoleato de cetila	Montiel et al. (2015)
	Poli(GMA-co-etileno dimetacrilato [EDMA])	Ligação covalente	Razão molar ácido:álcool de 1:1, 50°C em meio de hexano sem agitação	12	97	15 ciclos com mínima redução de atividade	Laurato de laurila	Mugo e Ayton (2013)
Candida rugosa	Polipropileno EP_{100}	Adsorção física	Razão equimolar de ácido:álcool, 50°C, 20% m/v de biocatalisador, meio isento de solvente e sem agitação	10	98	7 ciclos (atividade residual de 83%)	Palmitato de octila	Guncheva e Zhiryakova (2008)

Tabela 1.2. Revisão de literatura para a aplicação de lipases imobilizadas na síntese de ésteres lubrificantes (continuação).

Lipase	Suporte	Método de imobilização	Condições reacionais	Tempo de reação (h)	Conversão (%)	Estabilidade operacional (reuso)	Éster	Referência
Candida sp. 99-125	Fibra de seda modificada	Adsorção física	Razão molar álcool:ácido de 1:8,4, 45°C, 30% enzima em meio livre de solvente e agitação de 190 rpm	47	95,5	Não determinado	Octanoato de trimetilolpro-pano (TMP)	Tao et al. (2014)
Rhizomucor miehei	Q-Sepharose	Ligação covalente	Razão molar ácido:álcool de 1:1, 37°C, 0,5 g de enzima, em meio de acetona e agitação de 500 rpm	4	95	Não determinado	Fenilmalo-nato de monoglice-rila	Acosta et al. (2011)
	Resina de troca aniônica macroporosa	Adsorção física	Razão molar ácido:álcool de 1:1, 50°C, 4,33% de enzima, solvente – CO_2 supercrítico e sem agitação	1	80	Não determinado	Oleato de laurila	Knez et al. (2007)

Tabela 1.2. Revisão de literatura para a aplicação de lipases imobilizadas na síntese de ésteres lubrificantes (continuação).

Lipase	Suporte	Método de imobilização	Condições reacionais	Tempo de reação (h)	Conversão (%)	Estabilidade operacional (reuso)	Éster	Referência
Pâncreas de porco	Polipropileno EP_{100}	Adsorção física	Razão equimolar de ácido:álcool, 40°C, 20% m/v de biocatalisador, meio isento de solvente e sem agitação	10	60	7 ciclos (atividade de 83%)	Palmitato de octila	Guncheva e Zhiryakova (2008)
Rhizopus niveus	Partículas de celulose	Adsorção física	Razão molar ácido:álcool de 1:5, 50°C, 1,81% de enzima, em meio livre de solvente e 100 rpm	48	85 – oleato de laurila 67 – oleato de palmitila	Não determinado	Oleato de laurila e oleato de palmitila	Chen et al. (1995)
Rhizopus oryzae	Carbonato de cálcio ($CaCO_3$)	Adsorção física	Razão molar ácido:álcool de 1:3, 30°C, 300 IU/mL de enzima em meio de hexano e 200 rpm	2	98,52	Não determinado	Estearina de palma	Sellami et al. (2011)

Tabela 1.2. Revisão de literatura para a aplicação de lipases imobilizadas na síntese de ésteres lubrificantes (continuação).

Lipase	Suporte	Método de imobilização	Condições reacionais	Tempo de reação (h)	Conversão (%)	Estabilidade operacional (reuso)	Éster	Referência
Thermomyces lanuginosus	Poli-hidróxi butirato (PHB)	Adsorção física	Razão molar ácido:álcool de 1:1, 32,5°C, 15% m/v em meio isento de solvente e 200 rpm	0,25	90	5 ciclos (atividade residual de 73,3%)	Oleato de etila	Miranda et al. (2014)
	Poli-metacrilato (PMA)	Adsorção física	Razão molar ácido:álcool de 1:1, 45°C, 20% m/v em meio isento de solvente e 200 rpm	0,5	85	30 ciclos (atividade residual de 91,4%)	Oleato de isoamila	Lage et al. (2016)
	Poli-estireno-divinilbenzeno	Adsorção física	Razão molar ácido:álcool de 1:1,5, 45°C, 10% m/v em meio isento de solvente e 240 rpm	1	95,1 ± 1,3	12 ciclos de reação com perda desprezível de atividade	Oleato de *n*-octila	Bassi et al. (2016)

Lage et al. (2016) produziram o éster oleato de isoamila por esterificação catalisada pela lipase *Thermomyces lanuginosus* imobilizada por adsorção física em partículas mesoporosas de poli-metacrilato (PMA). Após 30 min de reação realizada na razão molar ácido:álcool de 1:1, temperatura de 45°C, enzima na concentração de 20% m/v em meio livre de solvente sob agitação de 200 rpm foi obtido rendimento de 85%. O reuso do biocatalisador foi analisado e após a realização de 30 ciclos houve perda de apenas 8,6% de atividade.

Em outro estudo recente, Bassi et al. (2016) sintetizaram oleato de *n*-octila por esterificação direta de ácido oleico e *n*-octanol. Neste estudo, cinco diferentes lipases foram imobilizadas por adsorção física em partículas de PMA e testadas na síntese do éster. Dentre os biocatalisadores preparados, lipase imobilizada de *Thermomyces lanuginosus* (TLL) foi o mais ativo. O efeito de certos fatores na reação de esterificação como concentração de biocatalisador, temperatura, razão molar ácido:álcool e concentração dos reagentes foi avaliado. Máxima produção de éster (conversão da ordem de 95,1 ± 1,3%) foi alcançada após 60 min de reação a 45 °C, 10% m/v de biocatalisador (TLL–PMA), razão molar ácido:álcool de 1:1,5 em meio isento de solvente. Após 12 ciclos de reação em um sistema batelada, o biocatalisador selecionado reteve toda a sua atividade inicial. O produto purificado obtido (oleato de *n*-octila) foi caracterizado por espectroscopia de infravermelho e suas propriedades físico-químicas foram determinadas de acordo com metodologias padrões estabelecidas pela *American Society for Testing and Materials* (ASTM).

1.5. Imobilização de Lipases por Adsorção Física

Embora a aplicação de lipases na síntese de ésteres seja altamente atrativa, a grande desvantagem na utilização destas enzimas na forma solúvel ou na forma de

preparados em pó (forma livre) é a sua separação para posterior aplicação, assim como a contaminação do produto desejado. Além disso, baixa atividade de esterificação para lipase na forma livre tem sido reportada devido à inativação da enzima por influência de moléculas de substratos como ácidos graxos e álcoois (Lage et al., 2016; Mendes et al., 2012). Com o intuito de superar estas desvantagens, a imobilização de lipases por diferentes protocolos tem sido amplamente empregada. Essa estratégia consiste no confinamento da enzima em um suporte sólido para posterior reutilização do biocatalisador, tornando o processo menos oneroso (Adlercreutz, 2013). As principais vantagens obtidas pelo processo de imobilização são: o aumento da estabilidade térmica do biocatalisador, aplicação em reatores com maior controle do processo, podendo ser usadas elevadas concentrações de enzimas, permitindo a sua reutilização sem perda significativa da sua atividade catalítica (Poppe et al., 2015; Zhao et al., 2015). As principais desvantagens deste processo são: alteração da conformação nativa da enzima, custo do suporte e perda de atividade durante o processo de imobilização (Adlercreutz, 2013). Diferentes protocolos têm sido empregados na imobilização de lipases tais como adsorção física em suportes hidrofóbicos e iônicos (catiônicos e aniônicos), encapsulação e ligação covalente em suportes hidrofílicos e hidrofóbicos de natureza orgânica e inorgânica (Poppe et al., 2015; Zhao et al., 2015; Adlercreutz, 2013).

Uma propriedade estrutural particular de muitas lipases é a presença de uma "tampa" ou "lid" que pode mudar sua conformação de maneira a controlar o acesso das moléculas de substrato ao sítio ativo da enzima. Essa tampa consiste em uma cadeia polipeptídica que possui duas conformações em meio aquoso: uma forma fechada em que o subdomínio cobre o sítio ativo de forma que a lipase se mantenha inativa e uma conformação aberta ou ativa em que o sítio ativo fica acessível às moléculas de substrato. Esses rearranjos de conformação geralmente induzem a adsorção da enzima

em uma interface hidrofóbica ou a um substrato de ligação (mecanismo de ativação interfacial de lipases), conforme ilustrado pela Figura 1.1. **Mecanismo de ativação interfacial de lipases em suportes hidrofóbicos** (Bassi et al., 2016). Normalmente, essas duas conformações da lipase estão em equilíbrio diretamente afetado pelas condições do meio (Tao et al., 2014).

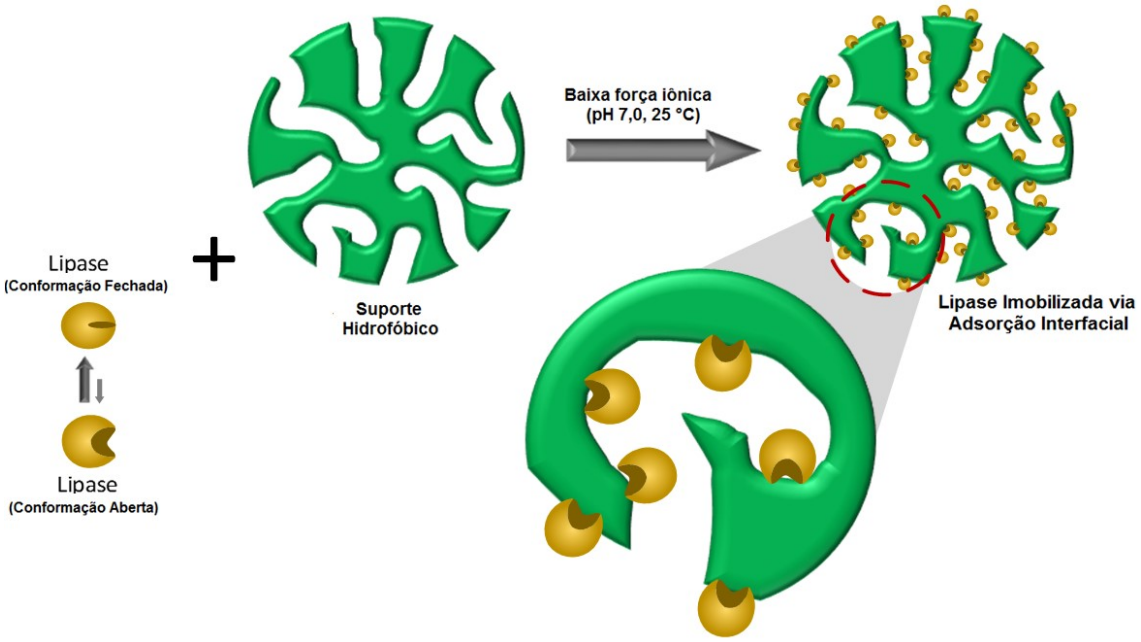

Figura 1.1. Mecanismo de ativação interfacial de lipases em suportes hidrofóbicos (Bassi et al., 2016).

A adsorção física é o método mais simples e mais empregado na imobilização de lipases. Esse método de imobilização é de baixo custo, pois não é necessária a ativação do suporte e permite fácil reuso do mesmo após vários ciclos por aplicação de agentes químicos que promovem a dessorção da enzima tais como ureia, guanidina e tensoativos (Cabrera et al., 2009; Secundo et al., 2008; Palomo et al., 2002). Na Tabela 1.3 estão sumarizados vários estudos reportados pela literatura especializada que abordam a imobilização de lipases via adsorção física em suportes iônicos e hidrofóbicos (ativação

interfacial). Além disso, lipases imobilizadas via adsorção física empregando diferentes suportes (orgânicos e inorgânicos) têm sido os principais biocatalisadores empregados na síntese de ésteres com propriedades lubrificantes por esterificação, como mostrado na Tabela 1.2. Esse procedimento de imobilização tem sido realizado pelo contato direto de uma solução enzimática com o suporte e forte interação enzima-suporte tem sido observada. No caso específico das lipases, a interação hidrofóbica é mais comum, embora interações iônicas também possam ocorrer. Devido ao fato dessas enzimas adsorverem espontaneamente em superfícies hidrofóbicas empregando baixa força iônica, esse método tem sido utilizado para a simultânea purificação/imobilização de lipases de extratos enzimáticos brutos (Adlercreutz, 2013; Cabrera et al., 2009; Palomo et al., 2002).

Lipases imobilizadas em suportes hidrofóbicos normalmente exibem maior atividade que enzimas livres ou outras formas de imobilização (Lage et al., 2016; Mendes et al., 2012). Lipases microbianas adsorvidas em suportes altamente hidrofóbicos são consideravelmente mais estáveis termicamente se comparadas com os biocatalisadores preparados via ligação covalente em suportes ativados (Palomo et al., 2002). Além disso, suportes diferentes com diferentes superfícies irão alterar consideravelmente as propriedades da enzima como estabilidade, atividade, seletividade ou especificidade (Tao et al., 2014). Portanto, a escolha do suporte para a imobilização é um fator crucial na preparação de biocatalisadores visto que o processo pode causar inativação da lipase e, nos casos de catálise heterogênea, limitações de transferência de massa podem causar diminuição da atividade específica da enzima (Adlercreutz, 2013).

O requerimento essencial para qualquer suporte consiste na necessidade de apresentar grande área superficial. Nesse sentido, materiais poliméricos porosos têm atraído muito a atenção devido à vantagem de possuírem elevada área superficial

interna. Em geral, os materiais porosos podem ser classificados de acordo com o tamanho dos poros. No caso de poros menores que 2 nm, são chamados microporosos, para poros entre 2 e 50 nm, são conhecidos como mesoporosos, de 50 a 200 nm são chamados macroporosos e para os poros maiores que 200 nm, são classificados como gigaporosos (Li et al., 2010).

A escolha do suporte a ser utilizado na imobilização é uma questão crítica (Li et al., 2010). De acordo com a Tabela 1.3, diversos materiais hidrofóbicos têm sido usados na imobilização de lipases como Accurel EP100 (partículas de polipropileno), sílica funcionalizada, poli-(hidróxibutirato), poli-metacrilato, partículas magnéticas, talo de milho, celulose e casca de ovo. Em alguns destes estudos, alguns parâmetros que influenciam o processo de adsorção como pH, força iônica, temperatura (determinação de parâmetros termodinâmicos), tempo de incubação e relação enzima/suporte têm sido criteriosamente avaliados com o intuito de maximizar o carregamento de enzima para a preparação de biocatalisadores ativos para aplicação em reações de interesse industrial. Conforme ainda pode ser observado, o modelo de isoterma de Langmuir é o mais aplicado para explicar o processo de adsorção de lipases nos diferentes suportes. Esse modelo assume que a adsorção da enzima na superfície do suporte ocorre na forma de monocamada com nível de energia uniforme em todos os sítios de adsorção (Lage et al., 2016; Foo e Hameed, 2010).

Lipase de pâncreas de porco foi imobilizada em matrizes de casca de ovo com carregamento máximo de proteína de 10,23 mg/g de suporte, obtendo retenção de atividade de enzima livre superior a 80%. A imobilização seguiu o modelo de isoterma de adsorção de Langmuir e foram observadas tanto interações iônicas quanto hidrofóbicas no processo (Chattopadhyay e Sen, 2012). A mesma lipase também foi imobilizada em partículas de poli-(hidroxibutirato) (PHB) obtendo atividade hidrolítica

máxima de 292,8 ± 8,60 UI/g. O modelo de isoterma de Langmuir foi aplicado e o carregamento máximo de proteína foi de 24,3 ± 1,70 mg/g (Silva et al., 2014).

A imobilização da lipase *Candida antarctica tipo B* em nanomateriais de carbono foi avaliada obtendo densidade de carregamento de enzima de 0,626 mg/g de suporte. Os dados ajustados geraram uma curva de isoterma característica do modelo de Langmuir, indicando que a cobertura ocorreu em monocamada em toda a superfície, não havendo agregação em multicamadas (Min et al., 2012).

A lipase *Thermomyces lanuginosus* foi imobilizada em partículas mesoporosas de poli-hidroxibutarato (PHB) via adsorção física a fim de catalisar a síntese de ésteres alquílicos por meio de esterificação direta de ácido oleico e álcoois de cadeia curta, com dois a quatro átomos de carbono, em meio orgânico. A adsorção seguiu o modelo de isoterma de Langmuir com máxima capacidade de adsorção de 26,5 ± 1,8 mg de proteína/g de suporte e atividade hidrolítica de 1300 UI/g de suporte. Sob condições ótimas, a conversão de éster foi próxima de 90% após 15 min de reação (Miranda et al., 2014).

Lewatit MonoPlus MP 64 foi empregado na imobilização de lipase de *Rhizopus oryzae* que foi imobiliza seguindo uma cinética de pseudo-segunda ordem, indicando que a imobilização ocorreu por quimissorção, enquanto o equilíbrio de adsorção seguiu a isoterma de Langmuir. Nesse caso, o carregamento máximo obtido foi de aproximadamente 84,7 mg/g de suporte (Ortega et al., 2013).

Lage et al. (2016) também imobilizaram a lipase de *Thermomyces lanuginosus* via adsorção física (ativação interfacial) em partículas hidrofóbicas de poli-metacrilato (PMA). O carregamento máximo de proteína foi de aproximadamente 100 mg/g e a atividade hidrolítica observada foi de 650 UI/g. O processo de adsorção também seguiu o modelo de isoterma de Langmuir, apresentando um valor de $R^2 = 0,9743$.

Tabela 1.3. Levantamento bibliográfico para a imobilização de lipases via adsorção física em diferentes suportes.

Lipase	Suporte	Isoterma	$q_{máx}$ (mg/g de suporte)	Referência
Candida antarctica tipo B	Nanomateriais de carbono	Langmuir	0,626	Min et al. (2012)
Rhizopus oryzae	Lewatit MonoPlus MP 64	Langmuir	≈84,7	Ortega et al. (2013)
Burkholderia sp.	Fe_3O_4-SiO_2 funcionalizado	Langmuir	29,45	Tran et al. (2012)
Pseudomonas aeruginosa	Talos de milho	Freundlich	≈8-9	Lv et al. (2013)
Pseudomonas (Lipase PS)	Accurel EP100	Freundlich	4500	Al-Duri e Yong (1997)
Mucor miehei	Sílica mesoporosa (SBA-15)	Langmuir	0,64	Jacoby et al. (2013)
Candida rugosa	n^+ - sílica porosa com alta oxidação (HO-Psi)	Langmuir	47	Salis et al. (2010)
	n^+ - sílica porosa com baixa oxidação (LO-Psi)	Langmuir	140	Salis et al. (2010)
	Nanofibras de celulose	Langmuir	41,02	Lu e Hsieh (2009)
	Aerogel de sílica magnética	Langmuir	81,9	Amirkhani et al. (2016)
Pâncreas de porco	Poli-hidroxibutirato (PHB)	Langmuir	24.3 ± 1.70	Silva et al. (2014)
	Casca de ovo	Langmuir	10,23	Chattopadhyay e Sen (2012)
	Na-Bentonita	Langmuir	80,43	Dong et al. (2013)
Thermomyces lanuginosus	Accurel EP_{100}	Langmuir	1200	Al-Duri e Yong (1997)
Thermomyces lanuginosus	Poli-hidroxibutirato (PHB)	Langmuir	26.5 ± 1.8	Miranda et al. (2014)
Thermomyces lanuginosus	Poli-metacrilato (PMA)	Langmuir	≈100	Lage et al. (2016)

1.6. Referências Bibliográficas

Acosta A., Filice M., Fernandez-Lorente G., Palomo J. M., Guisan J. M. Kinetically controlled synthesis of monoglyceryl esters from chiral and prochiral acids methyl esters catalyzed by immobilized *Rhizomucor miehei* lipase. *Bioresource Technology 102* (2011) 507-512.

Adlercreutz P. Immobilisation and application of lipases in organic media. *Chemical Society Reviews 42* (2013) 6406-6436.

Akerman C. O., Hagstrom A. E. V, Mollaahmad M. A., Karlsson S., Hatti-Kaul R. Biolubricant synthesis using immobilised lipase: Process optimisation of trimethylolpropane oleate production. *Process Biochemistry* 46 (2011) 2225-2231.

Akerman C. O., Gaber Y., Ghani N. A., Lamsa M., Hatti-Kaul R. Clean synthesis of biolubricants for low temperature applications using heterogeneous catalysts. *Journal of Molecular Catalysis B: Enzymatic* 72 (2011) 263-269.

Al-Arafi N., Salimon J. Production of oleic acid based wax ester using acidic homogeneous catalysts. *European Journal of Chemistry* 9 (2012) 99-106.

Al-Arafi N., Salimon J. Synthesis of long chain wax ester, suing the Bronsted acidic ionic liquid N-methyl-2-pyrrolidonium methyl sulfate ([NMP][CH3SO3]). *Research Journal of Applied Sciences* 6 (2011) 361-365.

Al-Duri B., Yong Y. P. Characterisation of the equilibrium behaviour of lipase PS (from *Pseudomonas*) and lipolase 100L (from *Humicola*) onto Accurel EP100. *Journal of Molecular Catalysis B: Enzymatic* 3 (1997) 177-188.

Alves M. D., Aracri F. M., Cren E. C., Mendes A. A. Isotherm, kinetic, mechanism and thermodynamic studies of adsorption of a microbial lipase on a mesoporous and hydrophobic resin. *Chemical Engineering Journal* (2016) doi: 10.1016/j.cej.2016.11.069.

Amirkhani L., Moghaddas J., Malmiri H. J. *Candida rugosa* lipase immobilization on magnetic silica aerogel nanodispersion. *Royal Society of Chemistry Advances* 6 (2016) 12676-12687.

Bassi J.J., Todero L.M., Lage F.A.P., Khedy G.I., Dell Ducas J., Custódio A.P., Pinto M.A., Mendes A.A. Interfacial activation of lipases on hydrophobic support andapplication in the synthesis of a lubricant ester. *International Journal of Biological Macromolecules* 92 (2016) 900–909.

Bastida A., Sabuquillo P., Armisen P., Fernández-Lafuente R., Huguet J., Guisán J. M. A single step purification, immobilization, and hyperactivation of lipases via interfacial

adsorption on strongly hydrophobic supports. *Biotechnology and Bioengineering* 58 (1998) 486-493.

Bouzidi L., Li S., Di Biase S., Rizvi S. Q., Dawson P., Narine S. S. Lubricating and waxy esters II: Synthesis, crystallization, and melt behavior of branched monoesters. *Industrial and Engineering Chemistry Research* 51 (2012) 14892−14902.

Brena B. M., Batista-Vieira F. Immobilization of enzymes: A literature survey. In: *Immobilization of Enzymes and Cells*, edição: J. M. Guisan, 15-30. Totowa, NJ: Humana Press Inc., 2006.

Bressani A. P. P., Garcia K. C. A., Hirata D. B, Mendes A. A. Production of alkyl esters from macaw palm oil by a sequential hydrolysis/esterification process using heterogeneous biocatalysts: optimization by response surface methodology. *Bioprocess and Biosystems Engineering* 38 (2015) 287-297.

Cabrera Z., Fernandez-Lorente G., Fernandez-Lafuente R., Palomo J. M., Guisan J. M. Novozym 435 displays very different selectivity compared to lipase from *Candida antarctica* B adsorbed on other hydrophobic supports. *Journal of Molecular Catalysis B: Enzymatic* 57 (2009) 171-176.

Chattopadhyay S., Sen R. A comparative performance evaluation of jute and eggshell matrices to immobilize pancreatic lipase. *Process Biochemistry* 47 (2012) 749-757.

Chen H. C., Kuo C. H., Chen H. H., Liu Y. C, Shieh C. J. Optimization of enzymatic synthesis of cetyl 2-ethylhexanoate by Novozym 435. *Journal of the American Oil Chemists Society* 88 (2011) 1917-1923.

Chen J. P., Wang J. B., Liu H. S. Alcoholysis of olive oil for producing wax esters by intracellular lipase in immobilized fungus cells. *Biotechnology Letters* 17 (1995) 1177-1182.

Chowdhury A., Chakraborty R., Mitra D., Biswas D. Optimization of the production parameters of octyl ester biolubricant using Taguchi's design method and physico-chemical characterization of the product. *Industrial Crops and Products* 52 (2014) 783-789.

Cirujano F. G., Corma A., Llabrés i Xamena F. X. Zirconium-containing metal organic frameworks as solid acid catalysts for the esterification of free fatty acids: Synthesis of biodiesel and other compounds of interest. *Catalysis Today* 257 (2015) 213–220.

Cunha A. G., Besteti M. D., Manoel E. A., Silva A. A. T., Almeida R. V., Simas A. B. C., Fernandez-Lafuente R., Pinto J. C., Freire D. M. G. Preparation of core-shell polymer supports to immobilize lipase B from *Candida antarctica* - Effect of the support nature on catalytic properties. *Journal of Molecular Catalysis B: Enzymatic* 100 (2014) 59-67.

Dong H., Li Y., Li J., Sheng G., Chen H. Comparative study on lipases immobilized onto bentonite and modified bentonites and their catalytic properties. *Industrial and Engineering Chemistry Research* 52 (2013) 9030-9037.

Dormo N., Bélafi-Bakó K., Bartha L., Ehrenstein U., Gubicza L. Manufacture of an environmental-safe biolubricant from fusel oil by enzymatic esterification in solvent-free system. *Biochemical Engineering Journal* 21 (2004) 229-234.

Fernandez-Lafuente R. Lipase from *Thermomyces lanuginosus*: Uses and prospects as an industrial biocatalyst. *Journal of Molecular Catalysis B: Enzymatic* 62 (2010) 197-212.

Foo K. Y., Hameed B. H. Insights into the modeling of adsorption isotherm systems. *Chemical Engineering Journal* 156 (2010) 2-10.

Garcia, T., Coteron A, Martinez M., Aracil J. Kinetic model for the esterification of oleic acid and cetyl alcohol using an immobilized lipase as catalyst. *Chemical Engineering Science* 55 (2000) 1411-1423.

Gryglewicz S., Muszynski M., Nowicki J. Enzymatic synthesis of rapeseed oil-based lubricants. *Industrial Crops and Products* 45 (2013) 25-29.

Guncheva M. H., Zhiryakova D. High-yield synthesis of wax esters catalysed by modified Candida rugosa lipase. *Biotechnology Letters* 30 (2008) 509-512.

Hernandez K., Garcia-Galan C., Fernandez-Lafuente R. Simple and efficient immobilization of lipase B from *Candida antarctica* on porous styrene-divinylbenzene beads. *Enzyme and Microbial Technology* 49 (2011) 72-78.

Jacoby J., Pasc A., Carteret C., Dupire F., Stébé M. J., Coupard V., Blin J. L. Ordered mesoporous materials containing *Mucor miehei* lipase as biocatalyst for transesterification reaction. *Process Biochemistry* 48 (2013) 831-837.

Jesionowski T., Zdarta J., Krajewska B. Enzyme immobilization by adsorption: A review. *Adsorption* 20 (2014) 801-821.

Kamalakar K., Rajak A. K., Prasad R. B. N., Karuna M. S. L. Rubber seed oil-based biolubricant base stocks: A potential source for hydraulic oils. *Industrial Crops and Products* 51 (2013) 249-257.

Khan N. R., Jadhav S. V., Rathod V. K. Lipase catalysed synthesis of cetyl oleate using ultrasound: Optimisation and kinetic studies. *Ultrasonics Sonochemistry* 27 (2015) 522-529.

Kleinaite E., Jaska V., Tvaska B., Matijosyte I. A cleaner approach for biolubricant production using biodiesel as a starting material. *Journal of Cleaner Production* 75 (2014) 40-44.

Knez Z., Laudani C. G., Habulin M., Reverchon E. Exploiting the pressure effect on lipase-catalyzed wax ester synthesis in dense carbon dioxide. *Biotechnology and Bioengineering* 97 (2007) 1366-1375.

Kulkarni R. D., Deshpande P. S., Mahajan S. U., Mahulikar P. P. Epoxidation of mustard oil and ring opening with 2-ethylhexanol for biolubricants with enhanced thermo-oxidative and cold flow characteristics. *Industrial Crops and Products* 49 (2013) 586-592.

Kumar V., Jahan F., Kameswaran K., Mahajan R. V., Saxena R. K. Eco-friendly methodology for efficient synthesis and scale-up of 2-ethylhexyl-p-methoxycinnamate using *Rhizopus oryzae* lipase and its biological evaluation. *Journal of Industrial Microbiology and Biotechnology* 41 (2014) 907-912.

Kuo C. H., Chen H. H., Chen J. H., Liu Y. C., Shieh C. J., High yield of wax ester synthesized from cetyl alcohol and octanoic acid by Lipozyme RMIM and Novozym 435. *International Journal of Molecular Sciences* 13 (2012) 11694–11704.

Lage F. A. P., Bassi J. J., Corradini M. C. C., Todero L. M., Luiz J. H. H., Mendes A. A. Preparation of a biocatalyst via physical adsorption of lipase from *Thermomyces lanuginosus* on hydrophobic support to catalyze biolubricant synthesis by esterification reaction in a solvent-free. *Enzyme and Microbial Technology* 84 (2016) 56-67.

Li H., Niu S., Lu C., Li J. Calcium oxide functionalized with strontium as heterogeneous transesterification catalyst for biodiesel production. *Fuel* 176 (2016) 63-71.

Li Y., Gao F., Wei W., Qu J. B., Ma G. H., Zhou W. Q. Pore size of macroporous polystyrene microspheres affects lipase immobilization. *Journal of Molecular Catalysis B: Enzymatic* 66 (2010) 182-189.

Lu P., Hsieh Y. L. Lipase bound cellulose nanofibrous membrane via Cibacron Blue F3GA affinity ligand. *Journal of Membrane Science* 330 (2009) 288-296.

Lv J. S., Liu X. Y., Xu J. X., Deng Y. F., Wu Z., Wang Y. M., Fan M. Y., Xu H. Preparation and properties of adsorption material from corn stalks core when used for enzyme immobilization and the subsequent activities of the adsorbed enzymes. *Industrial Crops and Products* 50 (2013) 787-796.

Mendes A. A., Oliveira P. C., Vélez A. M., Giordano R. C., Giordano R. L. C., Castro H. F. Evaluation of immobilized lipases on poly-hydroxybutyrate beads to catalyze biodiesel synthesis. *International Journal of Biological Macromolecules* 50 (2012) 503-511.

Min K., Kim J., Park K., Yoo Y. J. Enzyme immobilization on carbon nanomaterials: Loading density investigation and zeta potential analysis. *Journal of Molecular Catalysis B: Emzymatic* 83 (2012) 87-93.

Miranda J. S., Silva N. C. A., Bassi J. J., Corradini M. C. C., Lage F. A. P., Hirata D. B., Mendes A. A. Immobilization of *Thermomyces lanuginosus* lipase on mesoporous poly-hydroxybutyrate particles and application in alkyl esters synthesis: Isotherm, thermodynamic and mass transfer studies. *Chemical Engineering Journal* 251 (2014) 392-403.

Mobarak H. M., Mohamad E. N., Masjuki H. H., Kalam M. A., Mahmud K. A. H. A., Habibullah M., Ashraful A. M. The prospects of biolubricants as alternatives in automotive applications. *Renewable and Sustainable Energy Reviews* 33 (2014) 34-43.

Montiel M.C., Serrano M., Máximo M. F., Gómez M., Ortega-Requena S., Bastida J. Synthesis of cetyl ricinoleate catalyzed by immobilized Lipozyme CalB lipase in a solvent-free system. *Catalysis Today* 255 (2015) 49-53.

Mugo S. M., Ayton K. Lipase immobilized methacrylate polymer monolith microreactor for lipid transformations and online analytics. *Journal of the American Oil Chemists' Society* 90 (2013) 65-72.

Nagendramma P., Kaul S. Development of ecofriendly/biodegradable lubricants: An overview. *Renewable and Sustainable Energy Reviews* 16 (2012) 764-774.

Ortega S., Máximo M. F., Montiel M. C., Murcia M. D., Arnold G., Bastida J. Esterification of polyglycerol with polycondesed ricinoleic acid catalysed by immobilised *Rhizopus oryzae* lipase. *Bioprocess and Biosystems Engineering* 36 (2013) 1291-1302.

Palomo J. M., Muñoz G., Fernández-Lorente G., Mateo C., Fernández-Lafuente R., Guisán J. M. 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* 19-20 (2002) 279-286.

Poppe J. K., Fernandes-Lafuente R., Rodrigues R. C., Ayub M. A. Z. Enzymatic reactors for biodiesel synthesis: Present status and future prospects. *Biotechnology Advances* 33 (2015) 511-525.

Radzi S. M., Basri M., Salleh A. B., Ariff A., Mohammad R., Rahman B. A., Rahman R. N. Z. R. A. Large scale production of liquid wax ester by immobilized lipase. *Journal of Oleo Science* 54 (2005) 203-209.

Salimon J., Salih N., Yousif E. Chemically modified biolubricant basestocks from epoxidized oleic acid: Improved low temperature properties and oxidative stability. *Journal of Saudi Chemical Society* 15 (2011) 195-201.

Salis A., Cugia F., Setzu S., Mula G., Monduzzi M. Effect of oxidation level of n+ -type mesoporous silicon surface on the adsorption and the catalytic activity of *Candida rugosa* lipase. *Journal of Colloid and Interface Science* 345 (2010) 448-453.

Schmid R. D., Verger R. Lipases: interfacial enzymes with attractive applications. *Angewandte Chemie International Edition* 37 (1998) 1608-1633.

Secundo F., Miehé-Brendlé J., Cheralu C., Ferrandi E.E., Dumitriu E. Adsorption and activities of lipases on synthetic beidellite clays with variable composition. *Microporous and Mesoporous Materials* 109 (2008) 350-361.

Sellami M., Aissa I., Frikha F., Gargouri Y., Miled N. Immobilized *Rhizopus oryzae* lipase catalyzed synthesis of palm stearin and cetyl alcohol wax esters: Optimization by Response Surface Methodology. *BMC Biotechnology* 68 (2011) 1-8.

Sharma R., Chisti Y., Banerjee U. C. Production, purification, characterization, and applications of lipases. *Biotechnology Advances* 19 (2001) 627-662.

Silva J. A. C., Habert A. C., Freire D. M. G. A potential biodegradable lubricant from castor biodiesel esters. *Lubrication Science* 25 (2013) 53-61.

Silva N. C. A., Miranda J. S., Bolina I. C. A., Silva W. C., Hirata D. B., Castro H. F., Mendes A. A. Immobilization of porcine pancreatic lipase on poly-hydroxybutyrate particles for the production of ethyl esters from macaw palm oils and pineapple flavor. *Biochemical Engineering Journal* 82 (2014) 139-139.

Takisawa K., Kanemoto K., Kartikawati M., Kitamura Y. Simultaneous hydrolysis-esterification of wet microalgal lipid using acid. *Bioresourse Technology* 149 (2013) 16-21.

Tao Y., Cui C., Shen H., Liu L., Chen B., Tan T. Enhancing trimethylolpropane esters synthesis through lipase immobilized on surface hydrophobic modified support and appropriate substrate feeding methods. *Enzyme and Microbial Technology* 58-59 (2014) 60-67.

Tran D. T., Chen C. L., Chang J. S. Immobilization of *Burkholderia sp*. lipase on a ferric silica nanocomposite for biodiesel production. *Journal of Biotechnology* 158 (2012) 112-119.

Ungcharoenwiwat P., Kittikun A. H. Synthesis of wax esters from crude fish fat by lipase of *Burkholderia* sp. EQ3 and commercial lipases. *Journal of the American Oil Chemists Society* 90 (2013) 359-367.

Wang Y., Zhang D. H., Zhang J. Y., Chen N., Zhi G. Y. High-yield synthesis of bioactive ethyl cinnamate by enzymatic esterification of cinnamic acid. *Food Chemistry* 190 (2016) 629-633.

Yadav G. D., Dhoot S. B. Immobilized lipase-catalysed synthesis of cinnamyl laurate in non-aqueous media. *Journal of Molecular Catalysis B: Enzymatic* 57 (2009) 34-39.

Zhao X., Qi F., Yuan C., Du W., Liu D. Lipase-catalyzed process for biodiesel production: Enzyme immobilization, process simulation and optimization. *Renewable and Sustainable Energy Reviews* 44 (2015) 182-197.

CAPÍTULO 2

"*Isotherm, kinetic, mechanism and thermodynamic studies of adsorption of a microbial lipase on a mesoporous and hydrophobic resin"* publicado no periódico indexado *Chemical Engineering Journal.*

Chemical Engineering Journal 311 (2017) 1–12

Contents lists available at ScienceDirect

Chemical Engineering Journal

journal homepage: www.elsevier.com/locate/cej

Isotherm, kinetic, mechanism and thermodynamic studies of adsorption of a microbial lipase on a mesoporous and hydrophobic resin

Michelle D. Alves [a,b], Fernanda M. Aracri [c], Érika C. Cren [a,b], Adriano A. Mendes [a,c,*]

[a] Graduate Program in Chemical Engineering, Federal University of Minas Gerais, 31270-901 Belo Horizonte, MG, Brazil
[b] Department of Chemical Engineering, School of Engineering, Federal University of Minas Gerais, 31270-901 Belo Horizonte, MG, Brazil
[c] Institute of Chemistry, Federal University of Alfenas, 37130-001, Alfenas, MG, Brazil

HIGHLIGHTS

- Poly-(styrene-divinylbenzene) resin was used in the adsorption of microbial lipase.
- The effect of certain factors on the adsorption process was evaluated.
- Maximum immobilized protein concentration was around 134 mg/g of support.
- Kinetic, isotherm, mechanism and thermodynamic studies were performed.
- The biocatalyst exhibited high activity and operational stability in ester synthesis.

ARTICLE INFO

Article history:
Received 6 July 2016
Received in revised form 2 November 2016
Accepted 8 November 2016
Available online 9 November 2016

Keywords:
Adsorption
Microbial lipase
Poly-(styrene-divinylbenzene) resin
Catalytic properties
Structural characterization

ABSTRACT

Lipase from *Thermomyces lanuginosus* (TLL) was immobilized via physical adsorption on mesoporous poly-(styrene-divinylbenzene) resin (PSty–DVB). The influence of pH, ionic strength, temperature, initial protein loading, and contact time on the adsorption process and catalytic properties of the biocatalysts was systematically investigated. The catalytic properties of the biocatalysts were determined in the hydrolysis of olive oil emulsion and immobilized protein concentration. Maximum adsorption capacity of 133.9 ± 1.3 mg of protein/g of support using initial protein loading of 150 mg/g was observed after 900 min of incubation at 5 mM buffer sodium acetate pH 5.0 and 25 °C. This biocatalyst presented hydrolytic activity of 443.0 ± 25.2 IU/g of support. Moreover, high esterification activity and operational stability in cetyl linoleate (wax ester) synthesis in a solvent-free system (conversion of 90.5 ± 0.6% after five cycles of reaction of 30 min each) was also observed. This biocatalyst showed better catalytic activity than commercial immobilized TLL (Lipozyme TL–IM) in wax ester synthesis (conversion ≈87% after 120 min of reaction). Thermodynamic analysis showed that the immobilization was a spontaneous and a physisorption process. The equilibrium adsorption data were fitted to the Sips isotherm model. The results of kinetic study showed that the adsorption process was described by a pseudo-first-order model. This process was influenced by intraparticle and film diffusion. Attenuated total reflection Fourier transform infrared (ATR–FTIR) and scanning electronic microscopy (SEM) analyses were also performed to confirm the adsorption of the enzyme on the support surface.

1. Introduction

Lipases (triacylglycerol ester acylhydrolases EC 3.1.1.3) are enzymes that catalyze the hydrolysis of triacylglycerols (oils and fats) to glycerol and free fatty acids (FFA) at the water/oil interface [1]. These enzymes can also catalyze esterification, transesterification, interesterification, thiotransesterification, oximolysis and aminolysis reactions in non-aqueous media [1,2]. They are produced by several plants, animal tissues and microorganisms [3]. However, microbial lipases are the most preferred source due to their great versatility to environmental conditions, simplicity in genetic manipulation and in cultivation conditions [4].

The application of lipases in large-scale processes is often limited due to their high cost, and sensitivity to high temperature and organic solvents. Moreover, it is difficult to separate them from the reaction system which limits its recovery and reuse. In this

* Corresponding author at: Institute of Chemistry, Federal University of Alfenas, 37130-001, Alfenas, MG, Brazil.
E-mail addresses: adriano.mendes@unifal-mg.edu.br, adriano_amendes@yahoo.com.br (A.A. Mendes).

http://dx.doi.org/10.1016/j.cej.2016.11.069
1385-8947/

field, immobilization of lipases on a suitable support is a commonly used strategy to improve their activity, specificity, and stability and to facilitate the reuse of the biocatalysts [4,5]. The immobilization procedure via physical adsorption on hydrophobic supports is one of the simplest and cheapest immobilization methods available. This method allows the reuse of the support by desorption of inactive enzyme molecules from the biocatalyst surface using several chemicals [6,7]. Moreover, the physical adsorption of lipases on hydrophobic supports promotes hyperactivation and stabilization of lipases in open conformation (mechanism of interfacial activation), thus making this protocol especially appropriate for lipase immobilization [6–9].

The choice of the support for enzyme immobilization is a crucial factor in the preparation of biocatalysts with high catalytic activity and minimal diffusional limitations of reactant and/or product molecules. A variety of hydrophobic supports, including octyl-Sepharose, octadecyl-Sepabeads, poly-methacrylate, functionalized silica, magnetic particles, poly-styrene, poly-hydroxybutyrate, polypropylene, activated carbon, and poly-(styrene-divinylbenzene) resin have been broadly investigated in the immobilization of lipases via physical adsorption [4–11]. Porous supports are generally preferred due to their high surface area which allows a higher enzyme loading and the immobilized enzyme receives greater protection effect from the reaction medium [12]. The pore size plays an important role on the catalytic properties of the biocatalysts such as immobilization yield, selectivity, specificity, and thermal/operational stabilities [11,13]. The porous supports may be classified as microporous (<2 nm), mesoporous (2–50 nm), macroporous (50–200 nm), and gigaporous (>200 nm) [11]. In the last years, the application of mesoporous supports in the preparation of biocatalysts via physical adsorption of lipases to catalyze reactions in aqueous and organic media has been well-documented [9,13–15].

The aim of this study was the immobilization of lipase from *Thermomyces lanuginosus* (TLL) via physical adsorption on mesoporous poly-(styrene-divinylbenzene) resin. The selection of this support was based on previous studies that report its promising application in the preparation of highly active and stable biocatalysts [16–19]. Dizge et al. [17] reported the immobilization of TLL via covalent attachment on activated support with polyglutaraldehyde and maximum protein loading of 11.4 mg/g of support was observed. Martins et al. [18] immobilized TLL on this support (MCI GEL CHP20P) via physical adsorption at fixed experimental conditions (10 mM sodium phosphate at pH 7.0 and 20 °C for 24 h of incubation using initial protein loading of 120 mg/g of support) for further application in butyl butyrate synthesis by esterification reaction. These authors observed an immobilized yield around 95% (immobilized protein loading around 114 mg/g of support). In a subsequent study, TLL was also immobilized via physical adsorption on hydrophobic supports, including MCI GEL CHP20P [19]. The catalytic properties of these biocatalysts were determined in the hydrolysis of several esters. According to the authors, maximum protein loading of 90 ± 8 mg/g of support was observed. In order to improve the adsorption capacity of TLL on this support, the influence of certain parameters (pH, ionic strength, temperature, initial protein loading and contact time) was investigated. Isotherm, kinetic, mechanism and thermodynamic studies were also performed to elucidate the adsorption process. The characterization of the support and prepared biocatalyst with the highest immobilized protein concentration was performed by ATR–FTIR and SEM analyses. This biocatalyst was also applied in the synthesis of cetyl linoleate (wax ester) and its operational stability was evaluated after five cycles of reaction. Moreover, its performance in wax ester synthesis was compared with commercial immobilized TLL (Lipozyme TL–IM) from Novozymes. This is the first study dealing with the determination of isotherm, kinetic, mechanism and thermodynamic parameters for the adsorption of TLL on PSty–DVB resin and its potential application in wax ester synthesis.

Lipase from *Thermomyces lanuginosus* (TLL), previously *Humicola lanuginosa*, is a microbial lipase produced on industrial scale by submerged fermentation of a genetically modified strain of *Aspergillus oryzae* [5]. This alkalophilic lipase preparation has been produced and commercially available as Lipolase® 100L by Novozymes S.A. since 1989 [20]. The lipase consists of a single chain protein of 269 amino acids with a molecular mass of 31700 Da [9]. The choice of this lipase as an enzyme model was due to its promising application in the synthesis of several esters by esterification and transesterification reactions [5,14,15,18,21].

2. Materials and methods

2.1. Materials

Lipase from *Thermomyces lanuginosus* (TLL) was purchased from Sigma-Aldrich (St. Louis, MO, USA). This is a liquid enzyme preparation with protein concentration of 18 mg/mL and specific activity of 1090.7 IU/mg of protein. Commercial immobilized lipase from *Thermomyces lanuginosus* (Lipozyme TL–IM) was acquired from Novozymes S.A. (Araucária, PR, Brazil). Poly-(styrene-divinylbenzene) resin (Diaion® HP 20) was purchased from Supelco (Bellefonte, PA, USA). This resin has surface area of 500 m^2/g, average particle size of 250–850 μm and average pore size of 260 Å (Supelco technical information). Olive oil from Carbonell was purchased at a local market (Alfenas, MG, Brazil). Arabic Gum was acquired from Synth® (São Paulo, SP, Brazil). Bovine serum albumin (BSA) was also purchased from Sigma-Aldrich. All other chemical reagents were of analytical grade acquired from Vetec Química Ltd. and Synth®.

2.2. Determination of protein concentration

The protein concentration was measured by the Bradford's method using BSA as a standard [22].

2.3. Determination of hydrolytic activity

The apparent hydrolytic activity of soluble and immobilized TLL was determined by titrimetric method [14]. In the standard condition, the reaction mixture was composed of 1.25 g of olive oil, 3.75 g of Arabic Gum solution at 3% m/v and 5 mL of 100 mM buffer sodium phosphate pH 8.0. The mixture was placed in a 125 mL conical flask and incubated in a conventional water bath shaker with controlled temperature (37 °C) under continuous agitation at 200 rpm for 5 min. The hydrolysis reaction was then started by adding 0.1 g of immobilized TLL or 20 μL of soluble lipase. After, 10 mL of ethanol solution at 95% m/m was added to the reaction mixture and titrated with a 25 mM NaOH solution using phenolphthalein as indicator. Control runs were performed by adding either soluble or immobilized TLL after ethanol solution. One international unit (IU) of activity was defined as being the mass of enzyme required to release 1 μmol of free fatty acid per minute of reaction. In this study, all assays were performed with three replications.

2.4. Adsorption procedure

The adsorption of TLL on PSty–DVB resin in a batch system was performed according previous studies [14,15,21]. The immobilization consisted of the preparation of a suspension containing the enzyme solution previously prepared in buffer solution and the support in a proportion of 1:19 (m/v) (support:enzyme solution). The suspension was kept under continuous agitation in an orbital

shaker at 200 rpm. The biocatalyst was then filtered using Whatman no. 41 filter paper and washed with distilled water. The immobilization was monitored by measuring the protein concentration and catalytic activity of the supernatant solution in the hydrolysis of emulsified olive oil, compared to an enzyme solution (control) incubated under the same experimental conditions. After, the prepared biocatalysts were then stored at 4 °C for 24 h prior to use.

2.4.1. Effect of pH

The effect of pH on the adsorption and catalytic properties of the prepared biocatalysts was evaluated. In this set of experiments, pH values varied from 4.0 to 9.0 (buffer sodium acetate – pH 4.0 and 5.0, buffer sodium phosphate – pH 6.0, 7.0, 8.0 and buffer sodium carbonate – pH 9.0) at 5 mM using initial protein loading of 10 mg/g of support in order to avoid diffusional delay. The suspensions were incubated at 200 rpm for 15 h at 25 °C.

2.4.2. Effect of ionic strength

The effect of ionic strength (5–200 mM) on the adsorption and catalytic properties of the prepared biocatalysts was studied. Adsorption procedure was performed at pH 5.0 (buffer sodium acetate) using initial protein loading of 10 mg/g of support. The suspensions were also incubated at 200 rpm for 15 h at 25 °C.

2.4.3. Effect of temperature

The effect of temperature on the adsorption and catalytic properties of the prepared biocatalysts was evaluated ranging from 10 to 25 °C, with increment of 5 °C. Adsorption procedure was performed at pH 5.0 (5 mM buffer sodium acetate) using initial protein loading of 10 mg/g of support under agitation at 200 rpm for 15 h.

2.4.4. Effect of initial protein loading

The effect of initial protein loading varying from 10 to 200 mg/g of support was studied. The adsorption procedure was performed at 5 mM buffer sodium acetate pH 5.0 under agitation at 200 rpm for 15 h at 25 °C.

2.4.5. Effect of contact time

The effect of the time of incubation on the adsorption was evaluated for four different initial protein loadings (10, 50, 115 and 150 mg/g of support). The adsorption procedure was also performed at pH 5.0 (5 mM buffer sodium acetate) under agitation (200 rpm) at 25 °C.

2.4.6. Determination of immobilization parameters

Immobilization yield percentage (IY) was determined as follows (Eq. (1)) [14]:

$$IY = \left(\frac{EA_0 - EA_f}{EA_0}\right) \times 100 \quad (1)$$

where EA_0 and EA_f are the enzymatic activity in solution before and after immobilization (IU/mL), respectively.

Immobilized protein loading (IP – mg/g of support) was calculated according to Eq. (2) [14]:

$$IP = \frac{V_{enz} \times (C_0 - C_f)}{m} \quad (2)$$

where V_{enz} is the volume of enzyme solution (mL), C_0 is the initial protein concentration (mg/mL), C_f is the protein concentration after immobilization (mg/mL) and m is the mass of support (g).

Specific activity (SA – IU/mg of IP) was calculated as follows (Eq. (3)) [14]:

$$SA = \frac{HA}{IP} \quad (3)$$

where HA is the apparent hydrolytic activity of the prepared biocatalysts (IU/g of support) and IP is the immobilized protein concentration (mg/g of support).

Gibbs free energy was determined according to Eq. (4) [23]:

$$\Delta G = -RT \ \ln \ K_c \therefore K_c = \frac{IP}{C_e} \quad (4)$$

where ΔG is the Gibbs free energy (kJ/mol), R is the gas universal constant (8.314×10^{-3} kJ/mol.K), T is the absolute temperature (298.15 K), and K_c is the equilibrium constant, determined as the ratio between immobilized protein concentration (IP) and residual protein concentration in solution (C_e) at equilibrium, respectively.

2.4.7. Adsorption isotherms

The experimental data obtained for adsorption equilibrium studies were fitted to non-linear isotherm models of Langmuir (Eq. (5)), Freundlich (Eq. (6)), Temkin (Eq. (7)), Sips (Eq. (8)) and Redlich–Peterson (Eq. (9)) [24]:

$$q_e = \frac{q_{max} \times C_e}{K_L + C_e} \quad (5)$$

$$q_e = K_F \times C_e^{\frac{1}{n}} \quad (6)$$

$$q_e = \frac{RT}{b_T} \times \ln(A_T \times C_e) \quad (7)$$

$$q_e = \frac{K_S \times C_e^{\beta_S}}{1 + a_S \times C_e^{\beta_S}} \quad (8)$$

$$q_e = \frac{K_R \times C_e}{1 + a_R \times C_e^{g}} \quad (9)$$

where q_e is the adsorption capacity at equilibrium (mg protein/g support), C_e is defined as the residual mass of protein in unit volume of lipase solution (mg protein/mL), q_{max} is the maximum adsorption capacity (mg protein/g support), K_L is the Langmuir constant related to the energy of adsorption (mL/mg protein), K_F is the Freundlich isotherm constant (mL/mg support), n is the Freundlich exponent (dimensionless), R is the gas universal constant (8.314×10^{-3} kJ/mol.K), T is the experimental temperature (298.15 K), A_T is the Temkin isotherm equilibrium binding constant (mL/g support), b_T is the Temkin isotherm constant (J/mol), K_S is the Sips isotherm model constant (mL/mg), β_S is the Sips exponent (dimensionless), a_S is the Sips model constant (mL/mg), K_R is the Redlich–Peterson isotherm constant (mL/mg), a_R is the Redlich–Peterson isotherm constant (1/mg) and g is the Redlich–Peterson exponent (dimensionless).

2.4.8. Adsorption kinetic studies

The kinetic studies of adsorption of TLL were performed and the experimental data were fitted to the pseudo-first-order (Eq. (10)), pseudo-second-order (Eq. (11)), Elovich (Eq. (12)) and Avrami (Eq. (13)) models as follows [25]:

$$q_t = q_e(1 - e^{-k_1 \times t}) \quad (10)$$

$$q_t = \frac{k_2 q_e^2 t}{1 + k_2 q_e t} \quad (11)$$

$$q_t = \frac{\ln(\alpha\beta)}{\beta} + \frac{\ln t}{\beta} \quad (12)$$

$$q_t = q_e \times \left(1 - e^{(-k_{AV} \times t)^{n_{AV}}}\right) \quad (13)$$

where: q_e and q_t are respectively the concentration of adsorbed lipase at equilibrium and at certain time t (mg/g), k_1 (1/min), k_2

(mg/g.min) and k_{AV} (1/min) are respectively the constants of first-, second-order and Avrami adsorption kinetic constants, n_{AV} is the constant relationed to the adsorption mechanism, α is the initial adsorption rate (mg/g.min), and β is the desorption constant (g/mg).

The applicability of the isotherm and kinetic models to describe the adsorption process, apart from the correlation coefficient (R^2), was also validated by average relative error (ARE), as described in Eq. (14) [24]:

$$ARE = \frac{100}{N}\sum_{i=1}^{N}\left|\frac{q_t^{\exp} - q_t^{calc}}{q_t^{\exp}}\right|_i \quad (14)$$

where q_t^{exp} and q_t^{calc} are experimental and calculated immobilized protein loading on the support at certain time t, respectively, and N is the number of samples.

2.4.9. Adsorption mechanism studies

The adsorption mechanism was explained by the intraparticle diffusion model (Eq. (15)) [26]:

$$q_t = C + k_{id}t^{1/2} \quad (15)$$

where C is the intercept, k_{id} is the intraparticle diffusion rate constant (mg/g.min$^{1/2}$) and t is the contact time.

The intraparticle diffusion coefficients were determined as follows (Eq. (16)) [27]:

$$D_P = \frac{0.03 \times r_P^2}{t_e^{1/2}} \quad (16)$$

where D_P is the intraparticle diffusion (cm^2/s), r_P is the average radius of PSty–DVB resin (275 μm or 2.75×10^{-2} cm), and t_e is the contact time required to reach the equilibrium (s).

2.5. Characterization of the support and prepared biocatalyst by different techniques

2.5.1. ATR–FTIR analysis

Spectroscopy analysis was performed in an ATR–FTIR spectrophotometer (Nicolet iS50 FTIR – Thermo Scientific, Madison, WI, USA) with a diamond single bounce accessory (GladiATR, PIKE Technologies). The samples were directly applied on the crystal cell. ATR–FTIR spectra of the support and biocatalyst were acquired after 64 scans between 4000 and 400 cm^{-1} with spectral resolution of 4 cm^{-1}.

2.5.2. SEM analysis

Scanning electron microscopy (SEM) images with magnification of 30× and 20,000× were acquired on a JSM 6360 LV field-emission scanning electron microscope, at 10 kV accelerating voltage and spot size of 25 nm. All samples were sputter-coated with a thin layer (ca. 10 nm) of Au prior to SEM imaging.

2.6. Cetyl linoleate synthesis by esterification reaction

In this study, immobilized TLL on PSty–DVB and commercial immobilized TLL from Novozymes (Lipozyme TL–IM) were used as biocatalysts in the synthesis of cetyl linoleate by esterification reaction in a solvent-free system. The experimental conditions of reaction were established in a previous study [14], with slight modifications. The synthesis of the ester was performed in a screw-capped glass bottle with capacity of 100 mL containing 6 g of reactants at equimolar ratio cetyl alcohol to linoleic acid, 15% m/m of biocatalyst, 50 °C under continuous agitation at 240 rpm. The conversion percentage was determined by measuring the unreacted linoleic acid concentration by titration with 30 mM NaOH solution using phenolphthalein as an indicator. The operational stability of the biocatalyst was also evaluated after successive cycles of esterification reaction performed under the conditions described above. At the end of each cycle (five cycles of 30 min each), the biocatalyst was removed from the reaction medium and washed with *n*-hexane to remove adsorbed compounds on the biocatalyst surface.

3. Results and discussion

3.1. Effect of the pH on the adsorption of TLL on PSty–DVB resin

The pH is an important parameter in the preparation of biocatalysts via physical adsorption because it influences the surface charge of supports and degree of ionization of protein molecules [28]. In this context, the effect of this parameter on the adsorption of TLL on PSty–DVB was firstly evaluated. The results summarized in Table 1 show that the pH did not significantly influence the immobilization yield and immobilized protein concentration (immobilization yield higher than 95%), thus suggesting that the adsorption process was preferentially controlled by hydrophobic interactions [29]. These results are in agreement with those studies previously described in the literature for the adsorption of lipases from *Mucor javanicus* (MJL) on SBA-15 mesoporous silica particles [30], and PPL on poly(hydroxyethyl methacrylate-co-methacryloy lamidotryptophane) nanospheres [29] that were not also influenced by the pH of immobilization. On the other hand, the pH influenced the values of hydrolytic and specific activities of the biocatalyst and maximum apparent hydrolytic activity (235.0 ± 5.1 IU/g of support) and specific activity (23.5 ± 1.1 IU/mgIP) was observed at pH 5.0. The isoelectric point (*pI*) of TLL is around 4.4 [5]. Under this condition (pH 5.0), a maximum hydrophobic interaction between lipase and support surface (mechanism of interfacial activation of the enzyme on hydrophobic surfaces) could be expected. Thus, further tests were performed at pH 5.0.

3.2. Effect of the ionic strength on the adsorption of TLL on PSty–DVB resin

The ionic strength strongly influences the interaction of lipases on several supports [29,31]. The effect of ionic strength on the adsorption of TLL on the mesoporous support was also examined. As shown in Table 2, the parameters immobilization yield (above 99%) and immobilized protein concentration (above 9.7 mg/g of support) were not influenced for ionic strength ranging from 5 to 200 mM. Similar apparent hydrolytic and specific activities up to 100 mM were also observed. However, a slight decrease of activity at 200 mM was observed due to interaction of ions with ionic groups (carboxylate and protonated amino groups) from the enzyme surface that led to possible conformation changes in its three-dimensional structure that could result in charges in its isoelectric point and/or inactivation of some enzyme molecules. Moreover, the immobilization of TLL at low ionic strength is beneficial from the industrial point of view because minimizes the costs of preparation of the biocatalyst. Thus, the adsorption of TLL on the support performed at low ionic strength (5 mM) was chosen for subsequent studies. These results are also in agreement with previous studies that report the preparation of active biocatalysts via physical adsorption on hydrophobic supports performed at low ionic strength [6–9,14,15].

3.3. Effect of the temperature on the adsorption of TLL on PSty–DVB resin

Subsequently, the effect of the temperature varying from 5 to 25 °C, with increment of 5 °C, was investigated. In this set of exper-

Table 1
Influence of pH of immobilization on the catalytic properties of immobilized TLL on PSty–DVB resin.

pH	IY[a] (%)	IP[b] (mg/g of support)	HA[c] (IU/g of support)	SA[d] (IU/mgIP)
4.0	99.4 ± 0.1	9.9 ± 0.1	155.7 ± 16.3	15.6 ± 1.6
5.0	99.6 ± 0.1	9.9 ± 0.1	235.0 ± 5.1	23.5 ± 1.1
6.0	98.9 ± 0.2	9.9 ± 0.1	211.2 ± 12.8	21.2 ± 0.3
7.0	98.4 ± 1.1	9.8 ± 0.3	121.6 ± 3.2	12.2 ± 0.4
8.0	98.1 ± 1.0	9.7 ± 0.1	89.3 ± 11.0	8.9 ± 1.1
9.0	95.4 ± 1.2	9.5 ± 0.2	82.8 ± 1.8	8.7 ± 0.1

[a] Immobilization yield.
[b] Immobilized protein concentration.
[c] Apparent hydrolytic activity.
[d] Specific activity.

Table 2
Influence of the ionic strength on the catalytic properties of immobilized TLL on PSty–DVB resin.

Ionic strength (mM)	IY[a] (%)	IP[b] (mg/g of support)	HA[c] (IU/g of support)	SA[d] (IU/mgIP)
5	99.6 ± 0.1	9.9 ± 0.1	235.0 ± 5.1	23.7 ± 0.5
20	99.7 ± 0.2	9.8 ± 0.1	237.6 ± 8.8	24.2 ± 9.0
50	99.6 ± 0.2	9.8 ± 0.1	229.3 ± 5.0	23.6 ± 5.8
100	99.5 ± 0.4	9.7 ± 0.2	237.8 ± 1.3	24.5 ± 0.2
200	99.6 ± 0.2	9.8 ± 0.1	195.4 ± 5.4	19.9 ± 0.5

[a] Immobilization yield.
[b] Immobilized protein concentration.
[c] Apparent hydrolytic activity.
[d] Specific activity.

iments, the adsorption process was performed under optimal conditions (5 mM buffer sodium acetate pH 5.0). According to the results, all biocatalysts exhibited immobilized yield percentage above 98% and apparent hydrolytic activity around 235 IU/g of support (data not shown), as previously described in Table 2 for the adsorption process performed at 5 mM buffer sodium acetate pH 5.0. Although an increase of immobilized protein concentration by increasing the temperature is expected [29], the adsorption of TLL on the support was not significantly influenced in the interval from 5 to 25 °C. These results clearly show that this lipase has high affinity by hydrophobic surfaces as PSty–DVB resin same incubated at low temperature. Similar results were reported by Huang and coworkers [28] in the adsorption of lipase from *Candida rugosa* (CRL) on polyacrylic acid-coated magnetic nano-adsorbent and PPL on polyamine microspheres [32]. Further studies, including effect of initial protein concentration and contact time, were then conducted at 25 °C.

3.4. Effect of the initial protein loading: isotherm and thermodynamic studies

In this set of experiments, the effect of initial protein loading on the catalytic properties of the biocatalysts varying from 10 to 200 mg/g of support, which corresponds to 574.1 ± 31.7 and 11,482.0 ± 634.0 IU/mL of solution, was analyzed. The adsorption process of TLL on PSty–DVB resin was performed under optimal experimental conditions (5 mM buffer sodium acetate pH 5.0 at 25 °C under continuous agitation (200 rpm) for 18 h of incubation). According to Table 3, high immobilization yield percentage above 94% was observed for adsorption performed up to 125 mg protein/g of support, corresponding to immobilized enzyme activity around 130,000 IU/g of support. A decrease of immobilization yield percentage after 150 mg protein/g of support was verified due to support saturation and maximum immobilized enzyme activity was of 145,130.1 ± 2973.1 IU/g of support. The maximum capacity of PSty–DVB resin to adsorb TLL was around 134 mg/g of support. This high capacity to immobilize TLL is attributed to its high surface area and average pore diameter (260 Å) which is 5-fold higher than molecular diameter of this lipase – 53.2 Å [14]. Thus, high retention of lipase molecules on both internal and external microenvironments is expected.

Adsorption isotherm studies play an important role in the predictive modeling procedure for the analysis and design of an adsorption process. In this study, five adsorption isotherm models (Langmuir, Freundlich, Temkin, Sips and Redlich–Peterson) were applied to the experimental data from TLL immobilization. The Fig. 1 shows the non-linear plots of isotherm models fitted to these experimental data. The values of isotherm parameters, maximum adsorption capacity, correlation coefficients (R_2) and average relative error (ARE) are described in Table 4. As can be observed, Sips isotherm model had a better fit to the experimental data, considering its highest correlation coefficient (R^2 = 0.9873) and the lowest ARE value (6.59), followed by Redlich–Peterson model (R^2 = 0.9846 and ARE = 7.74). Moreover, the immobilized protein concentration at equilibrium state (q_e = 128.9 mg/g of support for initial protein loading of 150 mg/g) was very close to the experimental data, around 134 mg/g – see Table 3). Sips isotherm model consists of a combination between Langmuir and Freundlich models. At low sorbate concentration, it effectively fits to the Freundlich isotherm model and thus does not obey Henry's law [33]. On the other hand, at high adsorbate concentration it predicts a monolayer sorption capacity characteristic of Langmuir isotherm model [24,33]. These results were compared with those ones described in the literature dealing with the adsorption of lipases on several supports. According to Table 5, Langmuir isotherm model is the most applied to explain adsorption process of lipases. This model assumes that the monolayer adsorption takes place only at homogenous sites on the adsorbent surface with uniform energy level [24]. PSty–DVB resin exhibited higher adsorption capacity to immobilize lipase than several supports such as silica-based supports, carbon nanomaterials, bentonite, magnetic particles, eggshell, etc. (Table 5). However, this resin presented lower immobilized protein concentration than Accurel EP100, polypropylene particles with surface area of 70 m^2/g used to immobilize lipase PS and lipolase 100L (TLL) [38], and poly (hydroxyethyl methacrylate-co-methacryloylamidotryptophane) nanospheres which was applied in the adsorption of PPL [29].

Table 3
Influence of initial protein loading on the catalytic properties of immobilized TLL on PSty-DVB resin.

Protein loading (mg/g)	IA[a] (IU/mL)	IY[b] (%)	IP[c] (mg/g)	C_e[d] (mg/mL)	HA[e] (IU/g)	SA[f] (IU/mg_{IP})	Kc[g]	ΔG[h] (kJ/mol)
10	574.1 ± 31.7	99.7 ± 0.1	9.9 ± 0.1	0.005	235.0 ± 5.1	23.7 ± 0.5	1881.1	−18.7
20	1,148.2 ± 63.4	98.4 ± 0.8	19.2 ± 0.5	0.042	432.7 ± 10.1	23.8 ± 1.1	432.1	−15.0
30	1,722.3 ± 95.1	98.9 ± 0.4	29.0 ± 0.4	0.053	467.9 ± 22.9	16.1 ± 0.6	551.0	−15.0
55	3,157.6 ± 126.8	99.6 ± 0.2	53.6 ± 4.2	0.074	452.9 ± 56.2	8.5 ± 1.7	727.5	−16.3
70	4,018.7 ± 221.9	99.4 ± 0.1	68.3 ± 0.8	0.089	445.2 ± 49.4	6.5 ± 0.6	767.4	−16.4
115	6,602.3 ± 364.5	94.2 ± 0.7	108.7 ± 3.1	0.331	450.7 ± 30.7	4.2 ± 0.3	327.8	−14.4
125	7,176.3 ± 396.3	94.8 ± 0.8	117.0 ± 2.3	0.416	469.9 ± 60.2	4.0 ± 0.6	277.9	−14.0
150	8,611.5 ± 475.5	86.9 ± 1.1	133.9 ± 1.3	0.847	443.0 ± 25.2	3.3 ± 0.1	158.0	−12.5
175	10,046.8 ± 554.8	77.6 ± 0.2	133.9 ± 4.4	2.163	431.8 ± 50.6	3.2 ± 0.5	61.9	−10.2
200	11,482.0 ± 634.0	66.5 ± 0.1	133.5 ± 3.1	3.501	412.6 ± 19.3	3.1 ± 0.1	38.1	−9.0

[a] Initial activity.
[b] Immobilization yield.
[c] Immobilized protein loading.
[d] Residual protein concentration.
[e] Apparent hydrolytic activity.
[f] Specific activity.
[g] Equilibrium constant.
[h] Gibbs free energy.

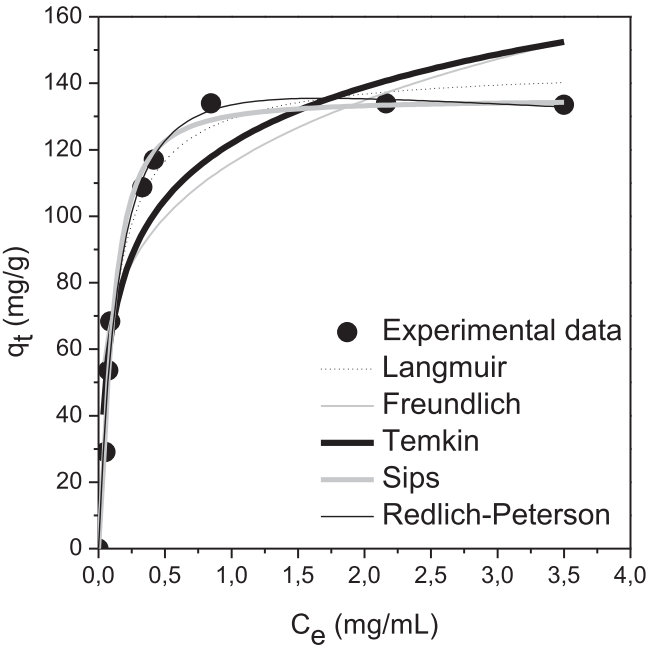

Fig. 1. Plots of non-linear isotherm models for the equilibrium data of TLL adsorption on PSty–DVB resin at 25 °C.

Table 4
Determination of isotherm parameters of the adsorption of TLL on PSty-DVB resin at 25 °C.

Isotherm model	Equation	Parameters	Values
Langmuir	$q_e = \frac{145.10 \times C_e}{0.12 + C_e}$	q_{max} (mg/g)	145.10
		K_L (mL/mg)	0.1218
		R^2	0.9768
		ARE	10.47
Freundlich	$q_e = 115.96 \times C_e^{0.22}$	K_F (mg/g)	115.96
		n	4.59
		R^2	0.7267
		ARE	24.99
Temkin	$q_e = 24.41 \times \ln(147.51 C_e)$	A_T (mL/mg)	147.51
		b_T (J/mol)	99.35
		R^2	0.9084
		ARE	20.28
Sips	$q_e = \frac{3604.48 \times C_e^{1.45}}{1 + 26.69 \times C_e^{1.45}}$	K_S (mL/mg)	3604.48
		β_S	1.45
		a_S	26.69
		R^2	0.9873
		ARE	6.59
Redlich-Peterson	$q_e = \frac{948.11 \times C_e}{1 + 6.10 \times C_e^{1.09}}$	K_R (mL/mg)	948.11
		a_R (1/mg)	6.10
		g	1.09
		R^2	0.9846
		ARE	7.74

The increase of protein loading from 10 to 20 mg/g of support increased the apparent hydrolytic activity (HA) by a 2-fold factor (Table 3). Although further increment of apparent hydrolytic activity by increasing the initial protein concentration is expected, similar values were observed. This could be attributed to possible blocking of pores of the support after immobilization procedure which could restrict the diffusion of droplets of oils to the internal microenvironment of the biocatalyst. Although similar apparent hydrolytic activity values were obtained for immobilization process performed between 20 and 200 mg protein/g of support, the specific activity of the biocatalysts was reduced by increasing the initial protein loading from 23.7 ± 0.5 to 3.1 ± 0.1 IU/mg of immobilized protein. These results confirm the possible reduction of the access of substrate molecules to the biocatalyst microenvironment with the increase of the initial protein concentration, as above described. Similar results were observed in previous studies performed in our lab for the adsorption of TLL on poly-methacrylate (PMA) and poly-hydroxybutyrate (PHB) particles [14,15].

Thermodynamic considerations of an adsorption process are necessary to conclude whether the process is spontaneous or not. Adsorption processes occur spontaneously at a given temperature whether ΔG is a negative value [23,46]. According to results summarized in Table 3, K_c values varied from 1881.1 (initial protein loading of 10 mg/g of support) to 38.1 (initial protein loading of 200 mg/g of support). This parameter was used to estimate ΔG values. The increase of initial protein loading reduced the spontaneity of the adsorption due to reduction of the surface area of the support available to adsorb enzyme molecules (support saturation). ΔG values varied from −18.7 to −9.0 kJ/mol which shows that the adsorption of TLL was a spontaneous process. Adsorption processes with ΔG values up to −20 kJ/mol correspond to interaction adsorbent/adsorbate mediated by physical adsorption (physisorption), while more negative ΔG values down to −40 kJ/mol correspond to interaction processes mediated by chemical bonds (chemisorption) [46]. These ΔG values show that the immobiliza-

Table 5
Literature survey for isotherm studies of physical adsorption of lipases on several supports.

Lipase	Support	Isotherm	IP[a](mg/g of support)	References
Candida antarctica type B	Carbon nanomaterials	Langmuir	0.625	[34]
Rhizopus oryzae	Lewatit MonoPlus MP 64	Langmuir	≈84.7	[35]
Burkholderia sp.	Alkyl-grafted Fe_3O_4–SiO_2	Langmuir	29.45	[36]
Pseudomonas aeruginosa	Corn stalks core (Core)	Freundlich	≈8–9	[37]
Pseudomonas (Lipase PS)	Accurel EP100	Freundlich	4500	[38]
Mucor miehei	Mesoporous sílica (SBA-15)	Langmuir	0.64	[39]
Candida rugosa	n^+-type porous silicon at high oxidation (HO-Psi)	Langmuir	47	[40]
	n^+-type porous silicon at low oxidation (LO-Psi)	Langmuir	140	[40]
	Cellulose nanofibrous membrane	Langmuir	41.02	[41]
	Magnetic silica aerogel	Langmuir	81.9	[42]
Porcine pancreas	Poly(hydroxyethyl methacrylate-co-methacryloylamidotryptophane) nanospheres	Langmuir	588.2	[29]
	Poly-hydroxybutyrate (PHB)	Langmuir	24.3 ± 1.70	[43]
	Eggshell	Langmuir	10.23	[44]
	Na-bentonite	Langmuir	80.43	[45]
Thermomyces lanuginosus	Accurel EP100	Langmuir	1200	[38]
	Poly-hydroxybutyrate (PHB)	Langmuir	26.5 ± 1.8	[15]
	Poly-methacrylate (PMA)	Langmuir	≈100	[14]
	Poly(styrene-divinylbenzene)	Sips	≈134	This study

[a] Immobilized protein concentration.

tion procedure of TLL on the support occurred via physical adsorption.

3.5. Effect of the contact time: kinetic and mechanism studies

The effect of contact time on the adsorption was performed at different initial protein loadings (10, 55, 115 and 150 mg/g of support). According to Fig. 2, the increase of protein loading required an increase of the contact time to reach equilibrium state and maximum adsorption capacity using initial protein loadings of 10, 55, 115 and 150 mg/g of support was observed after 50, 500, 600 and 900 min. This is attributed to decrease of pore size of the support by increasing the initial protein loading that drastically restricted the diffusion of enzyme molecules to its internal surface. Moreover, some lipases, including TLL, trend to form biomolecular aggregates in solution via interactions between hydrophobic areas surrounding their active sites [47]. Thus, an increase of these aggregates at high enzyme concentration is expected which could also reduce the diffusion rate to the support microenvironment. The intraparticle diffusion coefficient (D_P) for each adsorption system was determined as being 4.88×10^{-8} cm^2/s for 10 mg/g, 1.69×10^{-8} cm^2/s for 55 mg/g, 1.54×10^{-8} cm^2/s for 115 mg/g, and 1.26×10^{-8} cm^2/s for 150 mg/g.

In order to express the kinetic characteristics of the adsorption process of TLL on the support surface, non-linear pseudo-first-order, pseudo-second-order, Elovich and Avrami models were fitted to the experimental data (Fig. 2). The corresponding parameters, as well as correlation coefficients (R^2) and average relative error (ARE) values, are listed in Table 6. Elovich kinetic model presented high R^2 values (between 0.9478–0.9945), but this model was not suitable to describe the experimental data due to the highest ARE values varying from 81.66 to 99.32. Pseudo-seconder-order model exhibited also high R^2 and low ARE values, however the theoretical adsorption capacity at equilibrium (q_e) was not similar to the experimental data shown in Table 3. Although very similar theoretical q_e values to those experimentally obtained have been observed for Avrami kinetic model, ARE values were higher than pseudo-first-order model (Table 6). Thus, pseudo-first-order model was defined as being the best model to describe the kinetic data of the adsorption of TLL on PSty–DVB resin at different initial protein loadings. This model includes all the steps in the adsorption process such as external diffusion, pore diffusion and binding to active sites [48]. Moreover, this model assumes that the change in the adsorbed phase concentration with the time is directly proportional to the difference between the equilibrium concentration and adsorbed phase concentration at certain time t [49]. These results are in agreement with those one reported for the adsorption of lipase from *Aspergillus niger* on macroporous cross-linking polystyrene resin that followed also a pseudo-first-order kinetic model [50].

The kinetic adsorption models, above described, are not able to predict the adsorption mechanism and the rate-controlling step in a solid-liquid adsorption process. This can be explained by the mechanism adsorption models, including intraparticle diffusion model previously proposed by Weber and Morris [26]. This model has been applied to better identify the involved diffusion mechanism. The Fig. 3 shows the linear plots of q_t versus $t^{1/2}$ for different initial protein loadings and the linearized equations for the different steps of adsorption of TLL. Two steps for the adsorption performed at lowest initial protein loading (10 mg/g of support) were observed, while the adsorption process using high loading (55, 115 and 150 mg/g) was distributed in three steps. The first is attributed to rapid adsorption of lipase molecules on the external surface of the support or the boundary layer diffusion of adsorbate molecules. The high k_{id} values of the first step indicate that adsorption rate is high. The second step is due to diffusion of enzyme molecules from the solution to the internal support surface, resulting in lower k_{id} values (see slope values from the linearized equations shown in Fig. 3). The third step corresponds to the adsorption equilibrium which exhibits the lowest k_{id} values. According to Fig. 3, intercept values showed that the linear fitted curves did not completely pass through origin due to possible difference in the rate of mass transfer in the initial and final stages of adsorption [51]. This indicates that the intraparticle diffusion was not only the rate-limiting step of the adsorption process of TLL on the support, but also a possible involvement of film diffusion in the mechanism of adsorption [25,51]. Thus, the adsorption process of TLL on PSty–DVB could be controlled by both mechanisms (intraparticle and film diffusion).

3.6. Characterization of the support and adsorbed TLL by different techniques

The characterization of PSty–DVB resin and adsorbed TLL using initial protein loading of 150 mg/g of support (selected biocatalyst) was performed by scanning electron microscopy (SEM) and ATR–

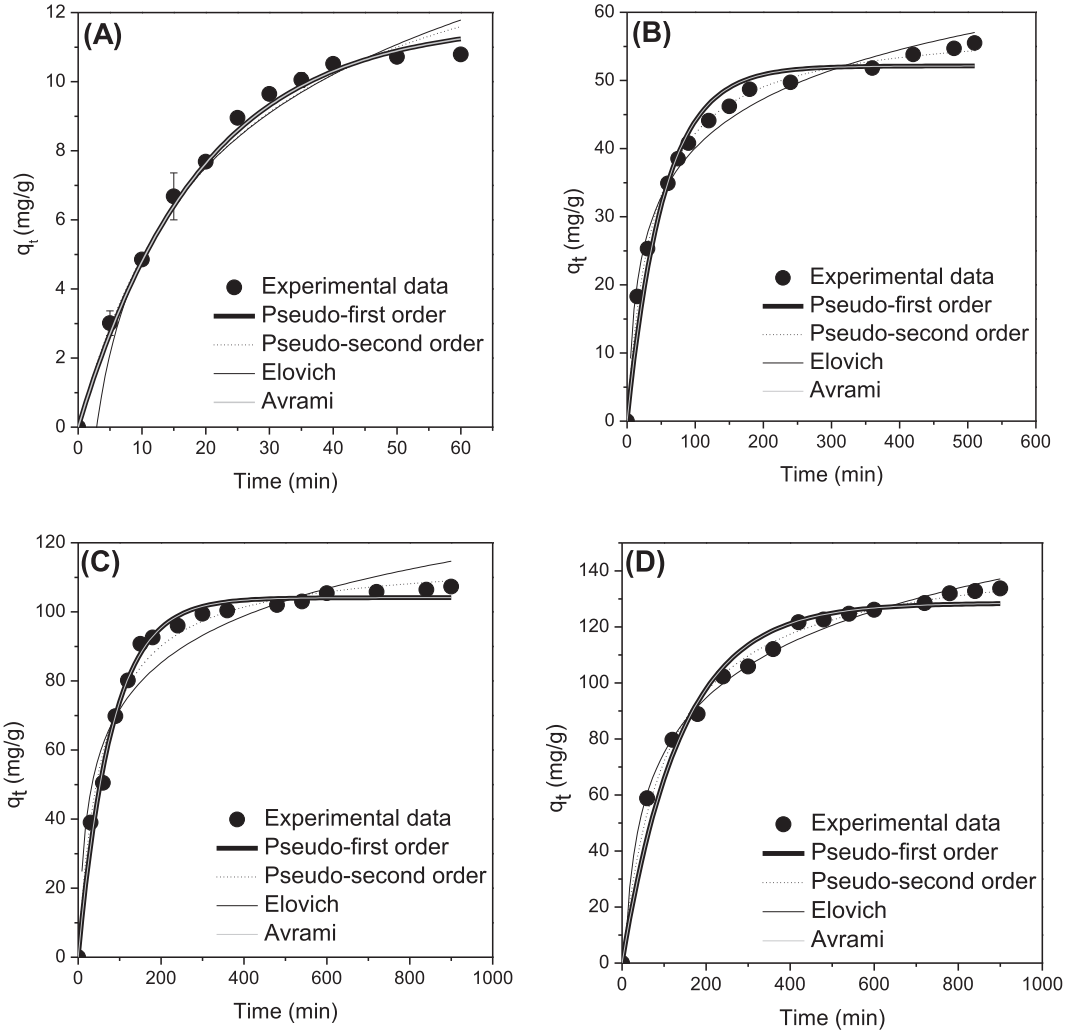

Fig. 2. Plots of non-linear kinetic models for the adsorption of TLL on PSty–DVB resin using initial protein loading of 10 mg/g (A), 55 mg/g (B), 115 mg/g (C) and 150 (D) mg/g of support.

Table 6
Determination of kinetic parameters of adsorption of TLL on PSty-DVB resin at different initial protein loadings.

Kinetic model	Parameters	Initial protein loading (mg/g of support)			
		10	55	115	150
Pseudo-first-order	k_1 (1/min)	0.0525	0.0186	0.0124	0.0072
	q_e (mg/g)	11.7	52.1	104.1	128.5
	R^2	0.9867	0.9727	0.9913	0.9751
	ARE	3.51	6.25	3.20	4.73
Pseudo-second-order	k_2 (mg/g.min)	0.0028	0.0004	0.0002	0.00006
	q_e (mg/g)	15.9	58.5	115.9	148.5
	R^2	0.9748	0.9974	0.9864	0.9950
	ARE	4.06	3.32	6.95	2.48
Elovich	α (mg/g.min)	0.2572	0.0961	0.0512	0.0354
	β (g/mg)	1.3426	4.9000	7.6641	4.0282
	R^2	0.9794	0.9875	0.9478	0.9945
	ARE	81.66	98.03	99.32	99.12
Avrami	q_e (mg/g)	11.7	52.1	104.1	128.5
	k_{AV} (1/min)	0.3163	0.0141	0.0141	0.0143
	n_{AV}	0.1660	1.3179	0.8762	0.4999
	R^2	0.9851	0.9703	0.9906	0.9730
	ARE	32.35	13.19	3.19	8.30

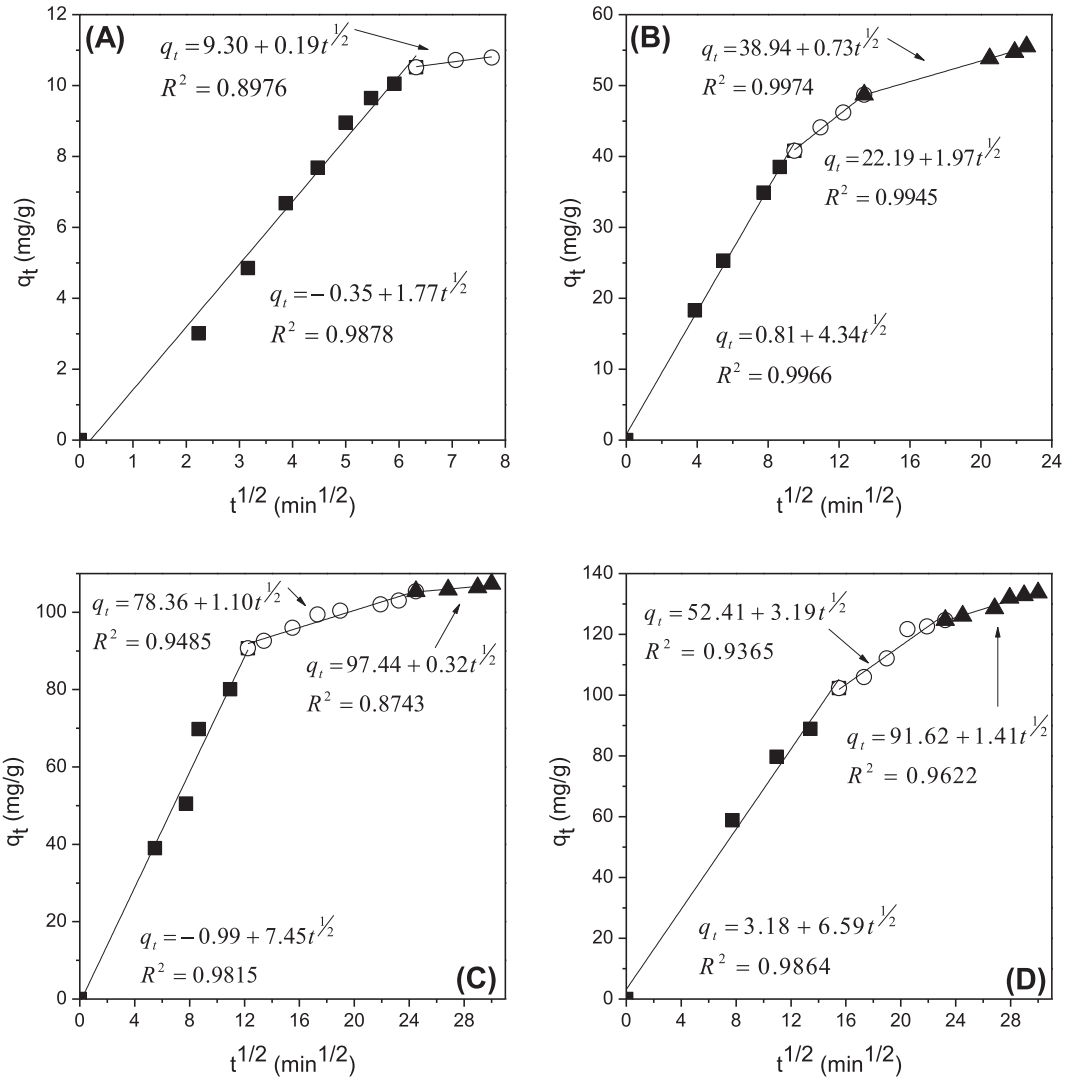

Fig. 3. Plots of intraparticle diffusion model for the adsorption of TLL on PSty–DVB resin using initial protein loading of 10 mg/g (A), 55 mg/g (B), 115 mg/g (C) and 150 (D) mg/g of support.

FTIR analyses. The differences in surface morphologies before and after adsorption process were investigated using scanning electron microscope (SEM).

SEM micrographs for PSty–DVB resin and adsorbed lipase are shown in Fig. 4. The support has a good spherical shape and different particles size ranging from 250 to 850 μm (magnification of 30×), as previously reported in Section 2.1, and porous structure – magnification of 20,000×(Fig. 4A). After adsorption process (Fig. 4B – magnification of 20,000×), a drastic reduction of pore size was observed. Moreover, an irregular structure may be also verified due to preferential adsorption of lipase molecules on the external support surface, as above described.

ATR–FTIR spectra of the resin before and after adsorption of TLL are shown in Fig. 5. The spectra for resin and adsorbed lipase show aromatic C–H stretching at 3022.9 cm^{-1}, aliphatic C–H stretching at 2923.7 cm^{-1}, and aromatic C=C stretching at 1602.6, 1487.4 and 1446.4 cm^{-1} which are characteristic bands of the resin [52]. The intense band at 1651.8 cm^{-1} observed after adsorption process refers to NH_2 deformation for amide I from the protein structure [52,53]. Moreover, a broad absorption band at 3300 cm^{-1} for adsorbed lipase is attributed to the asymmetric and symmetric NH_2 stretching [53], thus confirming the adsorption of the enzyme on the support surface.

3.7. Esterification reaction and operational stability study

The ability of adsorbed TLL to catalyze the synthesis of cetyl linoleate via esterification reaction in a solvent-free system was also evaluated. Cetyl esters are important compounds from the industrial point of view due to their potential applications in cosmetic and pharmaceuticals products and as additives in lubricant, polisher and plasticizer formulations [54,55]. The performance of the prepared biocatalyst was compared with Lipozyme TL–IM, an industrial biocatalyst broadly used in the modification of oils and fats [5]. Fig. 6 shows that the commercial biocatalyst was less active than immobilized TLL on PSty–DVB. Under the same experimental conditions, maximum conversion of 90.5 ± 0.6% catalyzed by immobilized TLL on PSty–DVB was observed after 30 min of reaction, while Lipozyme TL–IM required a higher reaction time to attain similar conversion percentage (around 87% after 120 min of reaction). Moreover, the prepared biocatalyst fully retained its original activity after successive cycles of reaction (Fig. 6 inset). These results show that this biocatalyst can be a promising alternative in the production of esters with lubricant properties due to its high catalytic activity in a solvent-free system and satisfactory operational stability (reuse).

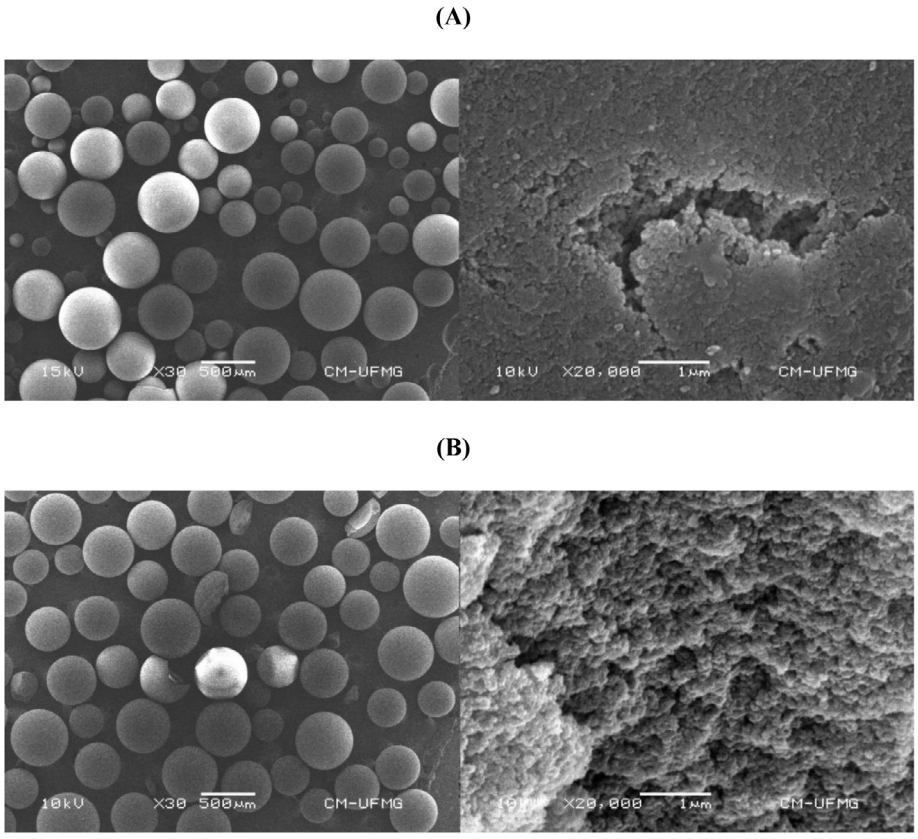

Fig. 4. SEM micrograph of PSty–DVB resin – magnification of 30× and 20,000×(A), and adsorbed TLL – magnification of 30× and 20,000×(B).

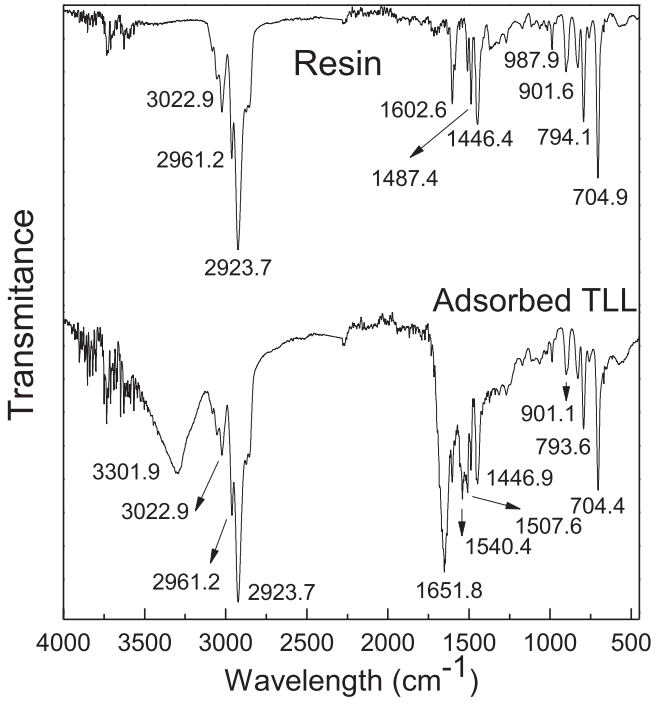

Fig. 5. ATR–FTIR spectra of PSty–DVB resin and adsorbed TLL.

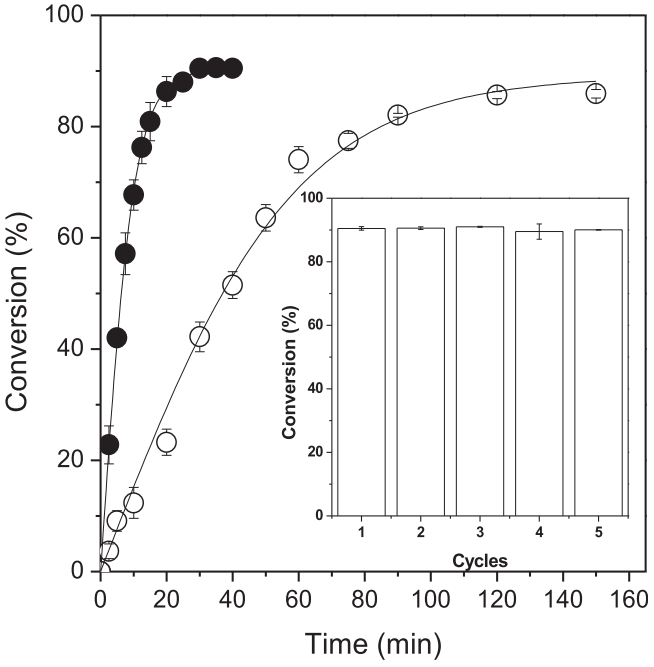

Fig. 6. Effect of reaction time on the synthesis of cetyl linoleate catalyzed by immobilized TLL on PSty–DVB resin (closed circle) and Lipozyme TL–IM (open circle) in a solvent-free system. Inset is the operational stability of immobilized TLL on PSty–DVB resin after five successive cycles of reaction of 30 min each.

4. Conclusion

The application of PSty–DVB resin as support in the preparation of biocatalysts was highly attractive due to its large surface area and porous size. According to the results, the adsorption process was independent of pH, ionic strength and temperature. However, maximum hydrolytic activity of the prepared biocatalyst was

reached at pH 5.0 (very close to the *pI* value of TLL) and low ionic strength (5 mM). The adsorption isotherm showed that Sips model had a better fit to the experimental data and maximum adsorption capacity was of 133.9 ± 1.3 mg protein/g of support. Thermodynamic studies revealed that the adsorption was a spontaneous and physorption process. The adsorption kinetic data were analyzed using four different kinetic models. Among them, the pseudo-first-order kinetic model provided the best fit to the adsorption kinetic data for TLL-resin system. The results indicated that the rate-limiting step of the adsorption process was influenced by intraparticle diffusion. However, the enzyme adsorption could also be controlled by film diffusion. The prepared biocatalyst showed better catalytic activity than Lipozyme TL–IM in the synthesis of an ester of industrial interest (cetyl linoleate) in a solvent-free system. Moreover, satisfactory operational stability after five cycles of esterification reaction was also observed. This confirms the promising application of PSty–DVB resin as support in the preparation of biocatalysts with high immobilized protein concentration and catalytic activity in ester synthesis.

Acknowledgements

This paper was financially supported by FAPEMIG (Process number APQ–02196–15), CNPq, CAPES and FINEP (Brazil). The authors are grateful to UFMG Microscopy Center (Belo Horizonte, Brazil) by the SEM analysis, MSc. Raphael A. B. Gomes (IQ–UNIFAL) by the ATR–FTIR spectroscopy analysis and Prof. Paulo W. Tardioli (DEQ–UFSCar) by the donation of Lipozyme TL–IM.

References

[1] R.D. Schmid, R. Verger, Lipases: interfacial enzymes with attractive applications, Angew. Chem. Int. Ed. 37 (1998) 1608–1633.
[2] A. Zaks, A.M. Klibanov, Enzyme-catalyzed processes in organic solvents, Proc. Natl. Acad. Sci. U.S.A. 82 (1985) 3192–3196.
[3] R. Sharma, Y. Chisti, U.C. Banerjee, Production, purification, characterization, and applications of lipases, Biotechnol. Adv. 19 (2001) 627–662.
[4] P. Adlercreutz, Immobilisation and application of lipases in organic media, Chem. Soc. Rev. 42 (2013) 6406–6436.
[5] R. Fernandez-Lafuente, Lipase from *Thermomyces lanuginosus*: uses and prospects as an industrial biocatalyst, J. Mol. Catal. B Enzym. 62 (2010) 197–212.
[6] A. Bastida, P. Sabuquillo, P. Armisen, R. Fernández-Lafuente, J. Huguet, J.M. Guisán, A single step purification, immobilization, and hyperactivation of lipases via interfacial adsorption on strongly hydrophobic supports, Biotechnol. Bioeng. 58 (1998) 486–493.
[7] Z. Cabrera, G. Fernandez-Lorente, R. Fernandez-Lafuente, J.M. Palomo, J.M. Guisan, Novozym 435 displays very different selectivity compared to lipase from *Candida antarctica* B adsorbed on other hydrophobic supports, J. Mol. Catal. B Enzym. 56 (2009) 171–176.
[8] J.M. Palomo, G. Muñoz, G. Fernández-Lorente, C. Mateo, R. Fernández-Lafuente, J.M. Guisán, Interfacial adsorption of lipases on very hydrophobic support (octadecyl–Sepabeads): immobilization, hyperactivation and stabilization of the open form of lipases, J. Mol. Catal. B Enzym. 19–20 (2002) 279–286.
[9] K. Hernández, C. Garcia-Galan, R. Fernández-Lafuente, Simple and efficient immobilization of lipase B from *Candida antarctica* on porous styrene–divinylbenzene beads, Enzyme Microb. Technol. 49 (2011) 72–78.
[10] T. Jesionowski, J. Zdarta, B. Krajewska, Enzyme immobilization by adsorption: a review, Adsorption 20 (2014) 801–821.
[11] Y. Li, F. Gao, W. Wei, J.B. Qu, G.H. Ma, W.Q. Zhou, Pore size of macroporous polystyrene microspheres affects lipase immobilization, J. Mol. Catal. B Enzym. 66 (2010) 182–189.
[12] B.M. Brena, F. Batista-Viera, Immobilization of enzymes: a literature survey, in: J.M. Guisán (Ed.), Immobilization of Enzymes and Cells, Humana Press Inc., Totowa, 2006, pp. 15–30.
[13] A.G. Cunha, M.D. Besteti, E.A. Manoel, A.A.T. Silva, R.V. Almeida, A.B.C. Simas, R. Fernández-Lafuente, J.C. Pinto, D.M.G. Freire, Preparation of core-shell polymer supports to immobilize lipase B from *Candida antarctica* – effect of the support nature on catalytic properties, J. Mol. Catal. B Enzym. 100 (2014) 59–67.
[14] F.A.P. Lage, J.J. Bassi, M.C.C. Corradini, L.M. Todero, J.H.H. Luiz, A.A. Mendes, Preparation of a biocatalyst via physical adsorption of lipase from *Thermomyces lanuginosus* on hydrophobic support to catalyze biolubricant synthesis by esterification reaction in a solvent-free system, Enzyme Microb. Technol. 84 (2016) 56–67.
[15] J.S. Miranda, N.C.A. Silva, J.J. Bassi, M.C.C. Corradini, F.A.P. Lage, D.B. Hirata, A.A. Mendes, Immobilization of *Thermomyces lanuginosus* lipase on mesoporous poly-hydroxybutyrate particles and application in alkyl esters synthesis: isotherm, thermodynamic and mass transfer studies, Chem. Eng. J. 251 (2014) 392–403.
[16] R.C. Rodrigues, K. Hernandez, O. Barbosa, N. Rueda, C. Garcia-Galan, J.C.S. dos Santos, A. Berenguer-Murcia, R. Fernandez-Lafuente, Immobilization of proteins in poly-styrene-divinylbenzene matrices: functional properties and applications, Curr. Org. Chem. 19 (2015) 1707–1718.
[17] N. Dizge, B. Keskinler, A. Tanriseven, Covalent attachment of microbial lipase onto microporous styrene-divinylbenzene copolymer by means of polyglutaraldehyde, Colloids Surf. B 66 (2008) 34–38.
[18] A.B. Martins, J.L.R. Friedrich, J.C. Cavalheiro, C. Garcia-Galan, O. Barbosa, M.A.Z. Ayub, R. Fernandez-Lafuente, R.C. Rodrigues, Improved production of butyl butyrate with lipase from *Thermomyces lanuginosus* immobilized on styrene-divinylbenzene beads, Bioresour. Technol. 134 (2013) 417–422.
[19] C. Garcia-Galan, O. Barbosa, K. Hernandez, J.C.S. dos Santos, R.C. Rodrigues, R. Fernandez-Lafuente, Evaluation of styrene-divinylbenzene beads as a support to immobilize lipases, Molecules 19 (2014) 7629–7645.
[20] D. Aaslyng, E. Gormsen, H. Malmos, Mechanistic studies of proteases and lipases for the detergent industry, J. Chem. Technol. Biotechnol. 50 (1991) 321–330.
[21] L.M. Todero, J.J. Bassi, F.A.P. Lage, M.C.C. Corradini, J.C.S. Barboza, D.B. Hirata, A. A. Mendes, Enzymatic synthesis of isoamyl butyrate catalyzed by immobilized lipase on poly-methacrylate particles: optimization, reusability and mass transfer studies, Bioprocess Biosyst. Eng. 38 (2015) 1601–1613.
[22] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[23] W. Song, Y. Liu, L. Qian, L. Niu, L. Xiao, Y. Hou, Y. Wang, X. Fan, Hyperbranched polymeric ionic liquid with imidazolium backbones for highly efficient removal of anionic dyes, Chem. Eng. J. 287 (2016) 482–491.
[24] K.Y. Foo, B.H. Hameed, Insights into the modeling of adsorption isotherm systems, Chem. Eng. J. 156 (2010) 2–10.
[25] A.C. Martins, O. Pezoti, A.L. Cazetta, K.C. Bedin, D.A.S. Yamazaki, G.F.G. Bandoch, T. Asefa, J.V. Visentainer, V.C. Almeida, Removal of tetracycline by NaOH-activated carbon produced from macadamia nut shells: kinetic and equilibrium studies, Chem. Eng. J. 260 (2015) 291–299.
[26] W.J. Weber, J.C. Morris, Kinetics of adsorption of carbon from solutions, J. Sanit. Eng. Div. Am. Soc. Civil Eng. 89 (1963) 31–63.
[27] J. Matusik, Arsenate, orthophosphate, sulfate, and nitrate sorption equilibria and kinetics for halloysite and kaolinites with an induced positive charge, Chem. Eng. J. 246 (2014) 244–253.
[28] S.H. Huang, M.H. Liao, D.H. Chen, Fast and efficient recovery of lipase by polyacrylic acid-coated magnetic nano-adsorbent with high activity retention, Sep. Purif. Technol. 51 (2006) 113–117.
[29] D.A. Uygun, M.E. Çorman, N. Öztürk, S. Akgöl, A. Denizli, Poly(hydroxyethyl methacrylate-co-methacryloylamidotryptophane) nanospheres and their utilization as affinity adsorbents for *porcine pancreas* lipase adsorption, Mater. Sci. Eng., C 30 (2010) 1285–1290.
[30] A. Salis, D. Meloni, S. Ligas, M.F. Casula, M. Monduzzi, V. Solinas, E. Dumitriu, Physical and chemical adsorption of *Mucor javanicus* lipase on SBA-15 mesoporous silica – synthesis, structural characterization, and activity performance, Langmuir 21 (2005) 5511–5516.
[31] Z. Ding, K. Zheng, X. Cao, Lipase purification by affinity precipitation with a thermo-responsive polymer immobilized Cibacron Blue F3GA ligand, Biotechnol. Bioprocess Eng. 19 (2014) 892–899.
[32] F. Wang, P. Liu, T. Nie, H. Wei, Z. Cui, Characterization of a polyamine microsphere and its adsorption for protein, Int. J. Mol. Sci. 14 (2013) 17–29.
[33] M.V. Subbaiah, D.S. Kim, Adsorption of methyl orange from aqueous solution by aminated pumpkin seed powder: kinetics, isotherms, and thermodynamic studies, Ecotoxicol. Environ. Saf. 128 (2016) 109–117.
[34] K. Min, J. Kim, K. Park, Y.J. Yoo, Enzyme immobilization on carbon nanomaterials: loading density investigation and zeta potential analysis, J. Mol. Catal. B Enzym. 83 (2012) 87–93.
[35] S. Ortega, M.F. Máximo, M.C. Montiel, M.D. Murcia, G. Arnold, J. Bastida, Esterification of polyglycerol with polycondensed ricinoleic acid catalysed by immobilised *Rhizopus oryzae* lipase, Bioprocess Biosyst. Eng. 36 (2013) 1291–1302.
[36] D.T. Tran, C.L. Chen, J.S. Chang, Immobilization of *Burkholderia* sp. lipase on a ferric silica nanocomposite for biodiesel production, J. Biotechnol. 158 (2012) 112–119.
[37] J.S. Lv, X.Y. Liu, J.X. Xu, Y.F. Deng, Z. Wu, Y.M. Wang, M.Y. Fan, H. Xu, Preparation and properties of adsorption material from corn stalks core when used for enzyme immobilization and the subsequent activities of the adsorbed enzymes, Ind. Crops Prod. 50 (2013) 787–796.
[38] B. Al-Duri, Y.P. Yong, Characterisation of the equilibrium behaviour of lipase PS (from *Pseudomonas*) and lipolase 100L (from *Humicola*) onto Accurel EP100, J. Mol. Catal. B Enzym. 3 (1997) 177–188.
[39] J. Jacoby, A. Pasc, C. Carteret, F. Dupire, M.J. Stébé, V. Coupard, J.L. Blin, Ordered mesoporous materials containing *Mucor miehei* lipase as biocatalyst for transesterification reaction, Process Biochem. 48 (2013) 831–837.
[40] A. Salis, F. Cugia, S. Setzu, G. Mula, M. Monduzzi, Effect of oxidation level of n^+-type mesoporous silicon surface on the adsorption and the catalytic activity of *Candida rugosa* lipase, J. Colloid Interface Sci. 345 (2010) 448–453.

[41] P. Lu, Y.L. Hsieh, Lipase bound cellulose nanofibrous membrane via Cibacron Blue F3GA affinity ligand, J. Membr. Sci. 330 (2009) 288–296.
[42] L. Amirkhani, J. Moghaddas, H. Jafarizadeh-Malmiri, *Candida rugosa* lipase immobilization on magnetic silica aerogel nanodispersion, RSC Adv. 6 (2016) 12676–12687.
[43] N.C.A. Silva, J.S. Miranda, I.C.A. Bolina, W.C. Silva, D.B. Hirata, H.F. Castro, A.A. Mendes, Immobilization of porcine pancreatic lipase on poly-hydroxybutyrate particles for the production of ethyl esters from macaw palm oils and pineapple flavor, Biochem. Eng. J. 82 (2014) 139–149.
[44] S. Chattopadhyay, R. Sen, A comparative performance evaluation of jute and eggshell matrices to immobilize pancreatic lipase, Process Biochem. 47 (2012) 749–757.
[45] H. Dong, Y. Li, J. Li, G. Sheng, H. Chen, Comparative study on lipases immobilized onto bentonite and modified bentonites and their catalytic properties, Ind. Eng. Chem. Res. 52 (2013) 9030–9037.
[46] M. Tu, X. Pan, J.N. Saddler, Adsorption of cellulase on cellulolytic enzyme lignin from Lodgepole Pine, J. Agric. Food Chem. 57 (2009) 7771–7778.
[47] J.M. Palomo, M. Fuentes, G. Fernandez-Lorente, C. Mateo, J.M. Guisan, R. Fernandez-Lafuente, General trend of lipase to auto-assemble giving bimolecular aggregates greatly modifies the enzyme functionality, Biomacromolecules 4 (2003) 1–6.
[48] Y. Chang, L. Chu, J. Tsai, S. Chiu, Kinetic study of immobilized lysozyme on the extrudate-shaped NaY zeolite, Process Biochem. 41 (2006) 1864–1874.
[49] D.D. Kavak, S. Ülkü, Kinetic and equilibrium studies of adsorption of β-glucuronidase by clinoptilolite-rich minerals, Process Biochem. 50 (2015) 221–1229.
[50] X. Zhao, M. Fan, J. Zeng, W. Du, C. Liu, D. Liu, Kinetics of lipase recovery from the aqueous phase of biodiesel production by macroporous resin adsorption and reuse of the adsorbed lipase for biodiesel preparation, Enzyme Microb. Technol. 52 (2013) 226–233.
[51] P.S. Kumar, S. Ramalingam, S.D. Kirupha, A. Murugesan, T. Vidhyadevi, S. Sivanesan, Adsorption behavior of nickel(II) onto cashew nut shell: equilibrium, thermodynamics, kinetics, mechanism and process design, Chem. Eng. J. 167 (2011) 122–131.
[52] O. Aybastier, C. Demir, Optimization of immobilization conditions of *Thermomyces lanuginosus* lipase on styrene–divinylbenzene copolymer using response surface methodology, J. Mol. Catal. B Enzym. 63 (2010) 170–178.
[53] L.C. Costa, A.S. Gomes, F.M.B. Coutinho, V.G. Teixeira, Chelating resins for mercury extraction based on grafting of polyacrylamide chains onto styrene–divinylbenzene copolymers by gamma irradiation, React. Funct. Polym. 70 (2010) 738–746.
[54] H.C. Chen, C.H. Kuo, H.H. Chen, Y.C. Liu, C.J. Shieh, Optimization of enzymatic synthesis of cetyl 2-ethylhexanoate by Novozym® 435, J. Am. Oil Chem. Soc. 88 (2011) 1917–1923.
[55] P. Ungcharoenwiwat, A. H-Kittikun, Synthesis of wax esters from crude fish fat by lipase of *Burkholderia* sp. EQ3 and commercial lipases, J. Am. Oil Chem. Soc. 90 (2013) 359–367.

CAPÍTULO 3

"*Kinetic, thermodynamic, optimization and reusability studies for the enzymatic synthesis of a saturated wax ester*".

Abstract

Cetyl decanoate was synthetized by esterification reaction catalyzed by immobilized lipase from *Thermomyces lanuginosus* (TLL) via physical adsorption on poly-(styrene-divinylbenzene) resin (PSty–DVB). The effect of some factors on the ester synthesis was evaluated. In this study, a second-order reversible reaction kinetic model was proposed in order to estimate apparent kinetic constants and good agreement with experimental data was observed ($0.9430 \leq R^2 \leq 0.9938$). The reaction was found to be a spontaneous and endothermic process. The biocatalyst prepared with initial protein loading of 115 mg/g of support (immobilized protein concentration of 108.7 ± 3.1 mg/g) yielded the highest initial reaction rate (113.5 mM/min of reaction), then selected for subsequent tests. The reaction in heptane medium required a slight excess of cetyl alcohol (molar ratio acid:alcohol of 1:1.25) and 7.5% m/m of biocatalyst to attain a maximum conversion of 92.5% for 30 min of reaction. In a solvent-free system, maximum conversion of 85.4% was observed for 50 min of reaction conducted in an equimolar ratio acid:alcohol and 10% m/m of biocatalyst. The productivity for the reaction performed in heptane medium was higher than in a solvent-free system – 68.5 and 56.4 mM/min.g of biocatalyst, respectively. The prepared biocatalyst was more active than commercial biocatalysts such as IMMTLL–T2–150 and Lipozyme TL–IM that exhibited maximum conversion around 92% for 45 and 75 min of reaction, respectively. The biocatalyst could be reused at least eight times without significant decrease of its activity.

Keywords: Wax ester synthesis, Esterification, Immobilized lipase, Optimization, Kinetic, Thermodynamic.

1. Introduction

Wax esters are important compounds derived from long chain alcohols and fatty acids with chain lengths of twelve carbon atoms or more. They have been extensively extracted from natural sources such as beeswax, spermaceti oil, jojoba oil, skin lipids, sheep wool and seafowl feathers for industrial application due to their excellent properties as additives or auxiliaries in lubricant polishes, plasticizers and coating materials [1–3]. In cosmetics and personal care products, they are used in a wide variety of products, including moisturizers, skin care and cleansing products, make-up, hair conditioners and hair dyes [4]. However, the application of natural wax esters is limited due to the high cost of extraction/purification and they are not easily available [1,5].

Currently, wax esters are of synthetic origin generally produced by esterification reaction of an alcohol with a carboxylic acid or transesterification of oils and fats with alcohols in the presence of chemical catalysts (homogeneous or heterogeneous) such as sulfuric acid [6], potassium hydroxide [7], sodium methoxide [8], acidic ionic liquids [9,10], zirconium-containing metal organic frameworks [11], etc. However, several limitations such as requirement for high energy and hazardous organic solvents as reaction medium (*e.g.* pyridine), removal of generated water molecules during the reaction, long reaction times, high generation of chemical wastes and formation of undesirable products have been reported for the chemical route [9–13]. In order to overcome these limitations, the application of immobilized lipases on several supports (heterogeneous biocatalysts) has been widely proposed in the production of synthetic wax esters because the demand for green products has significantly increased in recent years. The enzymatic route requires mild reaction conditions, exhibits high conversion in short reaction times and high catalytic activity towards several fatty acids and alcohols [1–3,5,14–16].

In the present study, immobilized TLL via physical adsorption on PSty–DVB was tested as the biocatalyst in the synthesis of wax esters by esterification reaction. This biocatalyst was prepared in a recent study performed in our lab aiming the evaluation of certain factors on the immobilization process [16]. The biocatalyst with the highest immobilized protein concentration was applied in the synthesis of an unsaturated wax ester (cetyl linoleate) under fixed experimental conditions. Here, immobilized TLL, previously prepared using different initial protein loadings varying from 10 to 200 mg/g of support, was applied in the synthesis of cetyl decanoate (so-called hexadecyl decanoate, cetyl caprate and palmityl decanoate) via esterification reaction. The most active prepared biocatalyst was then selected. The effect of important factors such as reaction temperature, agitation speed, organic solvent, molar ratio acid:alcohol and biocatalyst concentration on the ester synthesis was also examined.

The synthesis of wax esters has been preferentially performed by using oleic acid or oils/fats containing in their composition high concentration of unsaturated fatty acids as potential feedstocks [1,6,7,14,15,17–19]. However, some previous studies related the application of saturated oils/fats and their derived (free fatty acids) as acyl donors. These studies have been preferentially performed by applying commercial biocatalysts from the Novozymes such as Lipozyme RM IM (immobilized lipase from *Rhizomucor miehei* via physical adsorption on macroporous anion exchange resin – Duolite ES 562) and Novozym 435 (immobilized lipase B from *Candida antarctica* via physical adsorption on macroporous acrylic resin – Lewatit VP OC 1600). The heavy triglyceride fraction from the fractionation of sheep milk fat, a rich-fraction into saturated fatty acids (mainly decanoic, myristic, palmitic and stearic), was used in the enzymatic production of wax esters in hexane medium via transesterification reaction

[20]. Maximum ester synthesis around 80% after 100 min of reaction was observed using Lipozyme RM IM as biocatalyst. Cetyl esters were produced by transesterification of palm stearin, a vegetable oil rich in palmitic and oleic acids, catalyzed by immobilized lipase from *Rhizopus oryzae* on $CaCO_3$ [2]. Response surface methodology (RSM) was used in order to evaluate the effect of certain parameters on the ester synthesis. Under optimal conditions, a high conversion yield of 98.52% was reached for 2 h of reaction. RSM was also used to optimize the enzymatic synthesis of cetyl octanoate by esterification reaction in hexane medium [3]. Lipozyme RM IM and Novozym 435 were tested as biocatalysts. Among them, the latter was the most active and maximum ester conversion of 98.24 ± 0.11% was observed after 3.65 h of reaction performed at 57.84 °C, molar ratio acid:alcohol of 2.35:1 and biocatalyst concentration of 34.38% m/m. In a recent study, several saturated fatty acids such as lauric, myristic, palmitic and stearic were esterified with cetyl alcohol to produce esters analogue to spermaceti catalyzed by Novozym 435 [21]. The authors observed maximum conversion around 98.5% for esterification reactions performed between 70 and 80 °C after 50–90 min of reaction in solvent-free systems. Although the application of immobilized lipases in the synthesis of cetyl esters has been well-documented, the enzymatic production of wax esters, including cetyl decanoate, catalyzed by immobilized TLL on PSty–DVB resin has not been reported in the literature yet. This biocatalyst is promising in the synthesis of compounds of industrial interest as wax esters due to its high catalytic activity in esterification reaction [16], and hydrophobic nature of the support used in the preparation of this biocatalyst that could allow good partition of non-polar reactants as decanoic acid and cetyl alcohol to its microenvironment.

2. Materials and methods

2.1. Materials

Lipase from *Thermomyces lanuginosus* was purchased from Sigma-Aldrich (St. Louis, MO, USA). PSty–DVB (Diaion® HP 20) was purchased from Supelco (Bellefonte, PA, USA). Commercial immobilized TLL as Lipozyme TL–IM and IMMTLL–T2–150 (immobilized TLL via covalent attachment on Immobead IB–150A) were acquired from Novozymes S.A. (Araucária, PR, Brazil) and Chiral Vision (Leiden, The Netherlands), respectively. Cetyl alcohol and decanoic acid were purchased from Sigma-Aldrich. All other chemical reagents and organic solvents were of analytical grade acquired from Vetec Química Ltd. and Synth® (São Paulo, SP, Brazil).

2.2. Preparation of the biocatalysts via physical adsorption

The adsorption of TLL on PSty–DVB in a batch system was performed at 5 mM buffer sodium acetate pH 5.0, 25 °C by a maximum period of 15 h under continuous agitation in an orbital shaker (200 rpm) using initial protein loading from 10 to 200 mg/g of support, according to previous study performed in our lab [16].

2.3. General procedure of ester synthesis

The enzymatic synthesis of cetyl decanoate was performed in screw-capped glass bottles with capacity of 100 mL containing 6 g of reaction mixture. Cetyl alcohol and decanoic acid were dissolved by preheating at 50 °C by 15 min under agitation (200 rpm). The reaction was initiated by adding the biocatalysts previously prepared. The suspensions (reaction mixture + biocatalyst) were incubated in a conventional water bath shaker with a temperature control system. The samples were withdrawn from the reaction mixture at various time intervals (0.1 mL) for each set of reactions, diluted in

10 mL of ethanol solution (95% m/m) and titrated against a standard 30 mM NaOH solution using phenolphthalein as indicator in order to determine the residual decanoic acid concentration and, thus, the conversion percentage [21–23,25,26]. Previous studies related good agreement between the titration method and chromatography analyses for the determination of ester conversion via enzymatic esterification [23,24]. Control assays (reaction mixture incubated with PSty–DVB) were performed and no conversion was observed for 2 h of incubation under different reaction conditions. The results were the mean of three replications and were graphically represented including error bars (± standard deviation). These data were analyzed using software OriginPro version 8.0 (OriginLab Corporation, Northampton, USA).

2.4. Optimization of the enzymatic synthesis of cetyl decanoate

2.4.1. Effect of initial protein loading

The effect of initial protein loading on the initial reaction rate (determined from the slope of the curve of consumption of decanoic acid *versus* reaction time) was firstly studied. The reactions were conducted in heptane medium at 45 °C, 10% m/m of biocatalyst, agitation speed of 250 rpm and equimolar ratio acid:alcohol (1:1).

2.4.2. Effect of reaction temperature

In order to determine the effect of reaction temperature on the esterification reaction, the reaction mixtures (equimolar ratio acid:alcohol in heptane medium) were incubated in an orbital shaker at 250 rpm in the interval of 30 to 50 °C, with increment of 5 °C, using 10% m/m of biocatalyst previously prepared with initial protein loading of 115 mg/g of support.

2.4.3. Effect of agitation speed

Esterification reactions were performed at equimolar ratio acid:alcohol in heptane medium, 45 °C, 10% m/m of biocatalyst previously prepared with initial protein loading of 115 mg/g of support under different agitation speeds (0, 60, 125, 190 and 250 rpm).

2.4.4. Effect of organic solvents

In this set of experiments, wax ester synthesis was conducted using either heptane or hexane as reaction media. Ester synthesis in a solvent-free system was also performed under similar experimental conditions (equimolar ratio acid:alcohol of 1:1, 45 °C, 10% m/m of biocatalyst previously prepared with initial protein loading of 115 mg/g of support and 250 rpm).

2.4.5. Effect of molar ratio acid:alcohol

The effect of molar ratio acid:alcohol on the esterification reaction in heptane or solvent-free system was studied under fixed experimental conditions (45 °C, 10% m/m of biocatalyst previously prepared with initial protein loading of 115 mg/g of support and 250 rpm).

2.4.6. Effect of biocatalyst concentration

The concentration of biocatalyst varied from 5% m/m to 12.5% m/m for heptane medium and from 7.5% m/m to 12.5% m/m for solvent-free system. The reactions were conducted at 45 °C and agitation speed of 250 rpm using equimolar ratio acid:alcohol (1:1) for solvent-free system and molar ratio acid:alcohol of 1:1.25 for heptane medium.

2.4.7. Comparing with commercial biocatalytsts

Under optimal experimental conditions (45 °C, 250 rpm, molar ratio acid:alcohol of 1:1.25 and 7.5% m/m of biocatalyst), cetyl decanoate was also produced by using commercial biocatalysts (Lipozyme TL–IM and IMMTLL–T2–150) and their performance were compared with the selected biocatalyst prepared in this study (immobilized TLL on PSty-DVB).

2.5. Reusability tests

The synthesis of cetyl ester was conducted under optimal experimental conditions for heptane medium (45 °C, 250 rpm, molar ratio acid:alcohol of 1:1.25 and 7.5% m/m of biocatalyst). At the end of each esterification reaction (eight consecutive cycles of reaction of 30 min each), the biocatalyst was withdraw from the reaction mixture, washed with chilled hexane (100 mL) to remove reactant and/or product molecules retained in its microenvironment, followed by filtration in a Buchner funnel under vacuum for 1.5 h in order to remove water molecules. After, the biocatalyst was then introduced into a fresh reaction mixture.

2.6. Determination of kinetic constants

A second order reversible kinetic model was proposed to determine apparent kinetic constants [25,26]. The mechanism of reaction is shown in Eq. 1:

$$C_A + C_B \underset{k_{-1}}{\overset{k_1}{\Leftrightarrow}} C_C + C_D \quad (1)$$

where C_A, C_B, C_C and C_D are respectively the concentrations of decanoic acid, cetyl alcohol, ester and water (mM), and k_1 and k_{-1} are the apparent rate constants for forward and backward reactions ($mM^{-1}.h^{-1}$), respectively.

The reaction rate according to this model is described as follows (Eq. 2):

$$r_A = -\frac{dC_A}{dt} = k_1 C_A C_B - k_{-1} C_C C_D \quad (2)$$

where r_A or $-dC_A/dt$ is the consumption rate of decanoic acid in the reaction (mM/h).

This equation can be also described according to Eq. 3:

$$\frac{dX}{dt} = k_1 (1 - X)(C_{B0} - X C_{A0}) - k_{-1} C_{A0} X^2 \quad (3)$$

where X is the conversion of acid at certain time t, and C_{A0} and C_{B0} are the initial concentration of decanoic acid and cetyl alcohol (mM), respectively.

The integrated equation is expressed as follows (Eq. 4):

$$X = \frac{X_E (1 - e^{\varepsilon t})}{e^{\varepsilon t}\left[1 - \left(1 + \frac{C_{A0}}{C_{B0}}\right) X_E\right] + 1} \quad (4)$$

where X and X_E are the theoretical conversion values at certain time t and equilibrium, respectively.

The parameter ε was determined according to Eq. 5:

$$\varepsilon = \frac{k_1 [X_E (C_{A0} + C_{B0}) - 2 C_{B0}]}{X_E} \quad (5)$$

The apparent constants k_1 and k_{-1} were calculated according to Eq. 6 and 7, respectively.

$$k_1 = \frac{\varepsilon}{\left(C_{A0} + C_{B0} - \frac{2 C_{B0}}{X_E}\right)} \quad (6)$$

$$k_{-1} = \frac{k_1 (1 - X_E)(C_{B0} - X_E C_{A0})}{C_{A0} {X_E}^2} \quad (7)$$

At equilibrium ($\frac{dX}{dt} = 0$) and $X=X_E$, (from Eq. 3), apparent equilibrium constant (K_e) was determined as follows (Eq. 8) [27]:

$$K_e = \frac{k_1}{k_{-1}} \tag{8}$$

2.7. Determination of thermodynamic parameters

The experimental data from the effect of reaction temperature on the ester synthesis were used to determine thermodynamic parameters. The apparent activation energy for forward reaction was determined using linearized Arrhenius equation (Eq. 9) [28]:

$$\ln k_1 = \ln A - \frac{E_a}{R} \times \frac{1}{T} \tag{9}$$

where A is the Arrhenius collision factor, E_a is the apparent energy activation (kJ/mol), R is the gas universal constant (8.314×10^{-3} kJ/mol.K) and T is the temperature (K).

Apparent Gibbs free energy (ΔG – kJ/mol) values were calculated as follows (Eq. 10) [27]:

$$\Delta G = -RT \ln K_e \tag{10}$$

The values of apparent entropy (ΔS – J/mol.K) and enthalpy (ΔH – kJ/mol) were determined by Van't Hoff's equation (Eq. 11) [27,29]:

$$\ln K_e = \frac{\Delta S}{R} - \frac{\Delta H}{R} \times \frac{1}{T} \tag{11}$$

3. Results and discussion

The synthesis of cetyl decanoate via esterification of cetyl alcohol and decanoic acid was catalyzed by adsorbed TLL on PSty–DVB resin. These biocatalysts were prepared in a recent study performed in our lab by varying the initial protein loading from 10 to 200 mg/g of support [16]. The immobilized protein concentration for the different prepared biocatalysts is summarized in Table 1.

Table 1. Influence of immobilized protein concentration on the initial reaction rate of cetyl decanoate synthesis by esterification reaction.

Initial protein loading (mg/g)	Immobilized protein concentration (mg/g)[a]	Equations	R^2	Initial reaction rate (mM/min)
10	19.2 ± 0.5	$y = 7.53x + 0.69$	0.9957	7.53
20	29.0 ± 0.4	$y = 11.15x + 5.65$	0.9966	11.15
30	53.6 ± 4.2	$y = 22.84x + 38.32$	0.9754	22.84
55	19.2 ± 0.5	$y = 36.71x + 26.69$	0.9938	36.71
70	68.3 ± 0.8	$y = 68.74x + 31.36$	0.9871	68.74
115	108.7 ± 3.1	$y = 113.51x + 17.37$	0.9913	113.51
125	117.0 ± 2.3	$y = 116.47x + 14.58$	0.9878	116.47
150	133.9 ± 1.3	$y = 114.32x + 27.97$	0.9891	114.32
175	133.9 ± 4.4	$y = 119.88x + 27.98$	0.9829	119.88
200	133.5 ± 3.1	$y = 113.51x + 17.37$	0.9913	113.51

a – Experimental data reported in a previous study [16].

The experimental data of conversion percentage *versus* reaction time were fitted to a second-order reversible reaction kinetic model in order to estimate apparent kinetic constants and, thus, thermodynamic parameters. The applicability of this model for the enzymatic synthesis of esters of industrial interest, including wax esters, has been previously proposed [26,30]. According to results summarized in Table 2, good agreement with theoretical ($X_E^{theor.}$) and experimental ($X_E^{exp.}$) data was observed which shows that this model may be successfully used to describe the enzymatic synthesis of cetyl decanoate. In this study, the apparent kinetic constants for forward – k_1 (Eq. 6) and backward – k_{-1} (Eq. 7) reactions, as well as apparent equilibrium constant – K_e (Eq. 8), were determined using the values of experimental conversion at equilibrium ($X_E^{exp.}$).

3.1. Effect of initial protein loading

The effect of initial protein loading on the initial esterification reaction rate (plot of consumption of acid decanoic *versus* reaction time) was firstly evaluated in order to select the most active biocatalyst. High correlation coefficients (R^2) for the determination of initial reaction rate values may be observed in Table 1. According to results in Fig. 1 and Table 1, the consumption of decanoic acid increased by increasing the initial protein loading from 10 mg/g (7.53 mM/min) to 115 mg/g (113.51 mM/min). After, similar initial reaction rate values were observed due to restrict diffusion of reactant molecules to the biocatalyst microenvironment. Thus, further studies regarding with the effect of certain parameters on the reaction were performed using initial protein loading of 115 mg/g of support (immobilized protein concentration of 108.7 ± 3.1 mg/g).

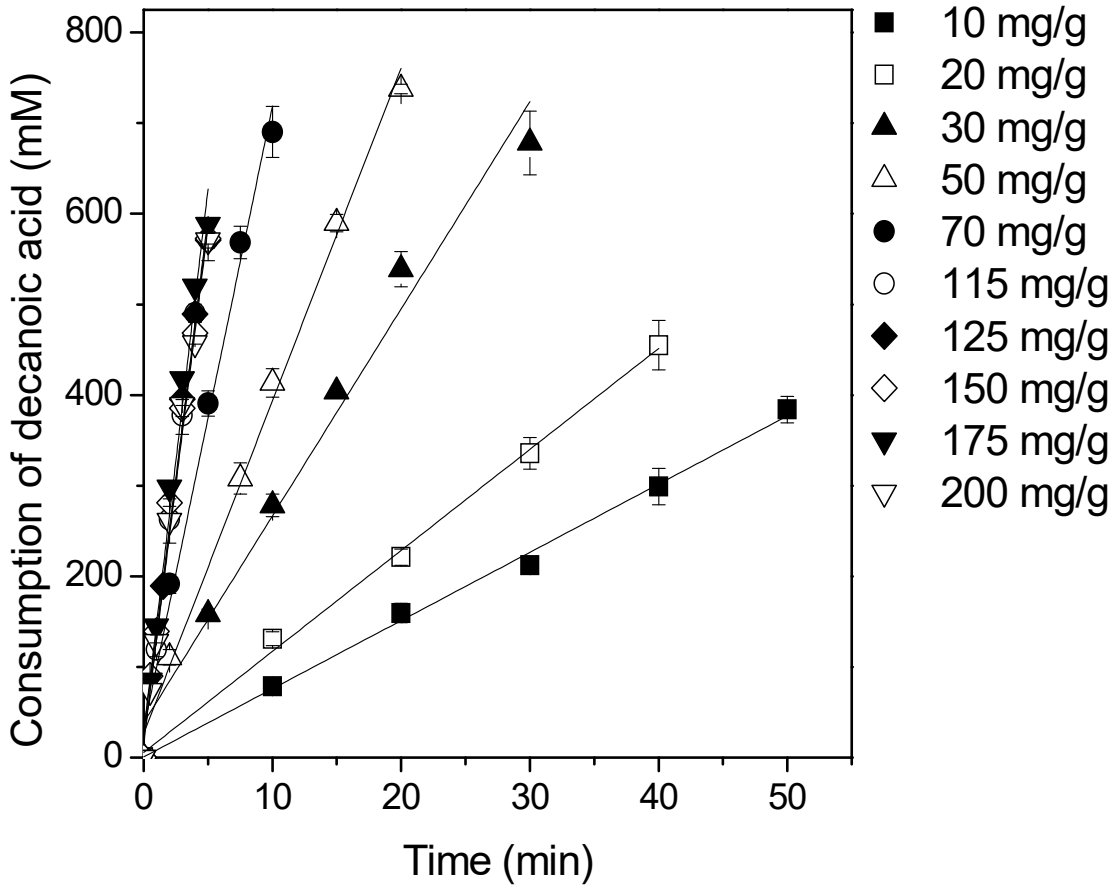

Figure 1. Effect of immobilized protein concentration on the initial reaction rate. The reactions were performed using equimolar ratio acid:alcohol, 45 °C, biocatalyst concentration of 10% m/m and 250 rpm.

3.2. Effect of reaction temperature: Determination of thermodynamic parameters

The increment of temperature allows more energy delivery to overcome the minimum activation energy required in a reaction in order to result in more successful particles collisions, thus improving the initial reaction rate and conversion percentage [31]. Moreover, the increment of temperature decreases the viscosity of the reaction mixture and increases the solubility of reactants [32]. In this set of experiments, the

effect of reaction temperature on the apparent kinetic constants and conversion percentage was investigated.

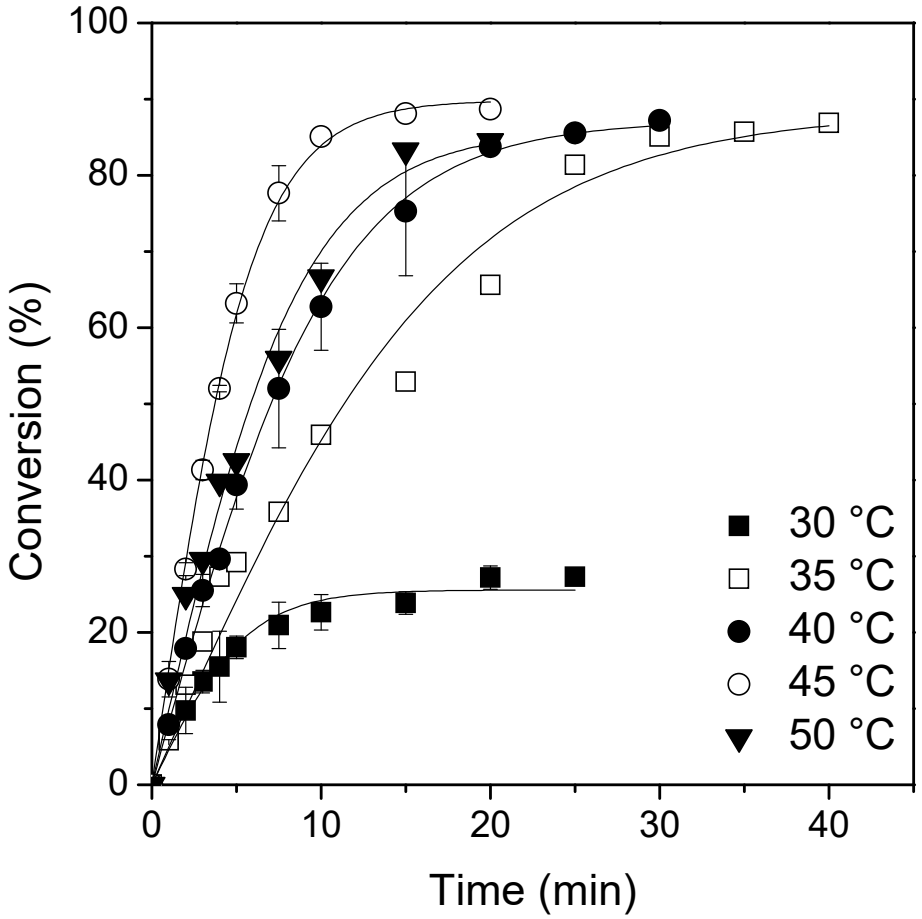

Figure 2. Effect of reaction temperature on the ester conversion percentage. The reactions were performed using equimolar ratio acid:alcohol, biocatalyst concentration of 10% m/m and 250 rpm.

According to Fig. 2 and Table 2 (entries 1–5), the conversion ($X_E^{exp.}$) and kinetic constant for forward reaction (k_1) were functions of temperature. On the other hand, the values of apparent kinetic constant for backward reaction (k_{-1}) decreased with the increment of temperature. Thus, an increase of apparent equilibrium constant values (K_e) was observed. At lowest reaction temperature (30 °C), equilibrium conversion

percentage of only 27.3% for 20 min of reaction was observed due to high viscosity of the reaction mixture that decreased the diffusion of reactants to the external and internal surface of the biocatalyst (Table 2 – entry 1). On the other hand, the reactions performed at 35, 40 and 45 °C exhibited high conversion percentage (between 87% and 88.7%), as shown in Fig. 2. Although similar conversion percentage values have been observed, the equilibrium was attained for 40, 30 and 15 min of reaction, respectively. These results may be credited to the increase of kinetic energy in the reaction mixture that increases the number of collisions per unit time and improves the homogeneity between reactant molecules that facilitates the formation of the ester. However, further increase in temperature reduced the ester formation and k_1 value (Table 2 – entry 5) due to possible inactivation of lipase molecules and maximum conversion percentage at 50 °C was around 84% for 20 min of reaction. Thus, subsequent tests were conducted at 45 °C.

Table 2. Estimation of apparent kinetic constants and Gibbs free energy for the synthesis of cetyl decanoate under different reaction conditions.

Parameter	Entry	Experimental conditions	R^2	X_E^{exp}	X_E^{theor}	ε	k_1 ($mM^{-1}.h^{-1}$)	k_{-1} ($mM^{-1}.h^{-1}$)	K_e	ΔG (kJ/mol)
Temperature	1	30	0.9736	0.273	0.256	–0.360	6.759×10^{-5}	1.800×10^{-4}	0.37	2.5
(°C)	2	35	0.9910	0.869	0.865	–0.122	4.046×10^{-4}	9.195×10^{-6}	44.0	–9.7
	3	40	0.9984	0.876	0.876	–0.165	5.814×10^{-4}	1.165×10^{-5}	54.4	–10.4
	4	45	0.9989	0.884	0.898	–0.338	1.487×10^{-3}	1.918×10^{-5}	77.5	–11.5
	5	50	0.9853	0.846	0.859	–0.228	6.945×10^{-4}	1.871×10^{-5}	37.1	–9.7
Agitation speed	6	0	0.9912	0.858	0.855	–0.267	8.066×10^{-4}	2.209×10^{-5}	36.5	–9.5
(rpm)	7	60	0.9798	0.858	0.870	–0.319	9.637×10^{-4}	2.640×10^{-5}	36.5	–9.5
	8	125	0.9622	0.855	0.874	–0.360	1.061×10^{-3}	3.052×10^{-5}	34.8	–9.4
	9	190	0.9852	0.866	0.880	–0.402	1.299×10^{-3}	3.110×10^{-5}	41.8	–9.9
	10	250	0.9989	0.884	0.898	–0.338	1.487×10^{-3}	1.918×10^{-5}	77.5	–11.5
Solvent	11	Hexane	0.9926	0.844	0.850	–0.382	1.033×10^{-3}	3.529×10^{-5}	29.3	–8.9
	12	Heptane	0.9846	0.866	0.876	–0.327	1.057×10^{-3}	2.531×10^{-5}	41.8	–9.9
	13	Solvent-free	0.9938	0.854	0.862	–0.125	1.828×10^{-4}	5.343×10^{-6}	34.2	–9.4
Molar ratio	14	1:1	0.9938	0.854	0.862	–0.125	1.828×10^{-4}	5.343×10^{-6}	34.2	–9.4
acid:alcohol	15	1:1.25	0.9894	0.886	0.867	–0.156	1.569×10^{-4}	1.399×10^{-5}	11.2	–6.4
(solvent-free)	16	1:1.5	0.9885	0.876	0.868	–0.139	9.960×10^{-5}	1.809×10^{-5}	5.51	–4.5
	17	1.5:1	0.9785	0.599	0.586	–0.165	1.967×10^{-4}	1.487×10^{-5}	13.2	–6.8
	18	2:1	0.9751	0.532	0.520	–0.206	2.713×10^{-4}	1.435×10^{-5}	18.9	–7.8
Molar ratio	19	1:1	0.9846	0.866	0.876	–0.327	1.220×10^{-3}	2.921×10^{-5}	41.8	–9.9
acid:alcohol	20	1:1.25	0.9960	0.925	0.923	–0.279	6.163×10^{-4}	1.756×10^{-5}	35.1	–9.4
(heptane)	21	1:1.5	0.9737	0.902	0.933	–0.184	2.228×10^{-4}	1.642×10^{-5}	13.9	–7.0
Biocatalyst	22	5	0.9430	0.856	0.824	–0.179	2.283×10^{-4}	2.521×10^{-5}	9.05	–5.8
(% m/m)	23	7.5	0.9738	0.924	0.914	–0.206	4.521×10^{-4}	1.312×10^{-5}	34.4	–9.4
(heptane)	24	10	0.9960	0.925	0.923	–0.279	6.163×10^{-4}	1.756×10^{-5}	35.1	–9.4
	25	12.5	0.9938	0.922	0.922	–0.314	6.804×10^{-4}	2.048×10^{-5}	33.2	–9.3
Biocatalyst	26	7.5	0.9669	0.744	0.752	–0.078	1.133×10^{-4}	1.341×10^{-5}	8.45	–5.6
(% m/m)	27	10	0.9938	0.854	0.862	–0.125	1.828×10^{-4}	5.343×10^{-6}	34.2	–9.4
(solvent-free)	28	12.5	0.9927	0.766	0.802	–0.087	1.424×10^{-4}	1.329×10^{-5}	10.7	–6.3
Biocatalyst	29	TLL–PSty–DVB	0.9738	0.924	0.914	–0.206	4.521×10^{-4}	1.312×10^{-5}	34.4	–9.4
	30	Lipozyme TL–IM	0.9932	0.929	0.970	–0.048	1.088×10^{-4}	2.873×10^{-6}	37.9	–9.6
	31	IMMTLL–T2–150	0.9825	0.923	0.969	–0.063	1.374×10^{-4}	4.061×10^{-6}	33.8	–9.3

The apparent kinetic constants for forward reaction reported in Table 2 (entries 2– 4) were used to determine apparent activation energy (E_a) by Arrhenius plot (natural logarithm of k values *versus* reciprocal temperature) and the results are presented in Fig. 3A. The value of apparent E_a for the synthesis of the ester was calculated as 105.7 kJ/mol (25.3 kcal/mol). This result is in agreement with the synthesis of oleyl oleate, an unsaturated wax ester, by esterification reaction catalyzed by Novozym 435 (E_a=21.15 kcal/mol) [30].

The values of apparent K_e in Table 2 were used to estimate ΔG, ΔH and ΔS. With increasing temperature, the value of ΔG decreased which indicates that the reaction is more favorable at higher temperatures. At 30 °C, apparent ΔG value was 2.5 kJ/mol and for the reaction performed between 35 and 45 °C these values varied from –9.5 to –11.3 kJ/mol. Van't Hoff plot was then applied to estimate apparent ΔH value from the slope of the curve and apparent ΔS from the intercept (Fig. 3B). In this set of experiments, K_e values for the reaction performed at lowest (30 °C) and highest (50 °C) temperatures were excluded. High correlation coefficient was observed (R^2=0.9636). The reaction was found to be endothermic in nature (ΔH=47.1 kJ/mol). Positive ΔH value was consistent with the observed increase of k_1 values by increasing reaction temperature from 35 to 45 °C (Table 2, entries 2–4). ΔS was estimated as 183.8 J/mol.K, thus indicating that the reaction was entropically favorable. This positive value of apparent ΔS could be credited to formation of water molecules that led to the increase of the disorder in the reaction system [33]. Similar behavior was reported for the synthesis of a flavonoid ester by esterification of rutin and lauric acid catalyzed by Novozym 435 [33].

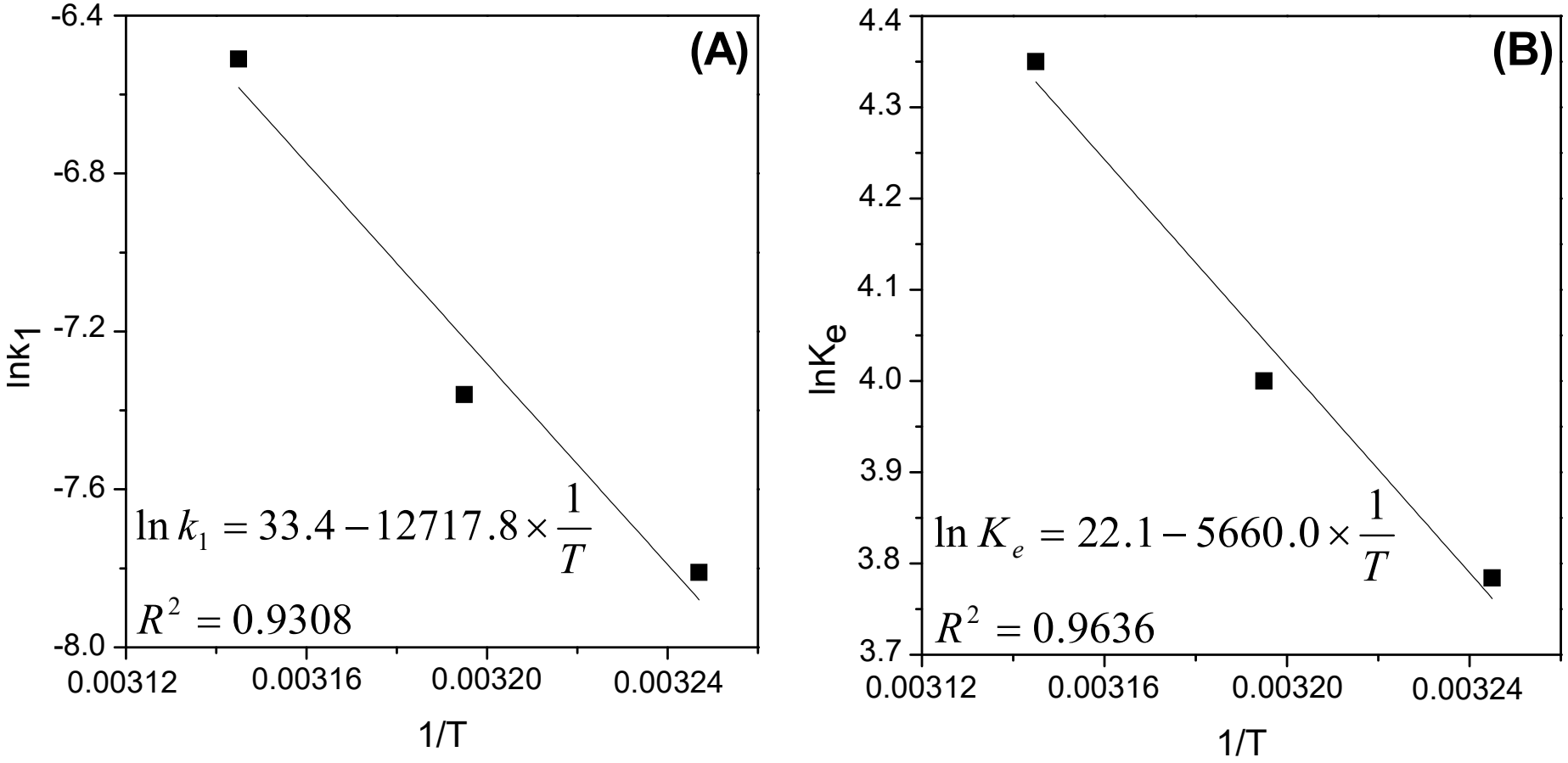

Figure 3. Determination of thermodynamic parameters for the ester synthesis by Arrhenius (A) and Van't Hoff (B) plots.

3.3. Effect of agitation speed

In the present study, the reaction system was composed by a liquid phase, containing reactants in heptane medium, and solid phase (immobilized TLL). Thus, the reaction may be influenced by diffusion limitations of reactant molecules from the reaction mixture to the biocatalyst microenvironment. The increase of the agitation frequency positively improves the synthesis of esters due to possible reduction of the stagnant film thickness surrounding the biocatalyst surface that may minimize the influence of the external diffusion resistance on the reaction [34,35].

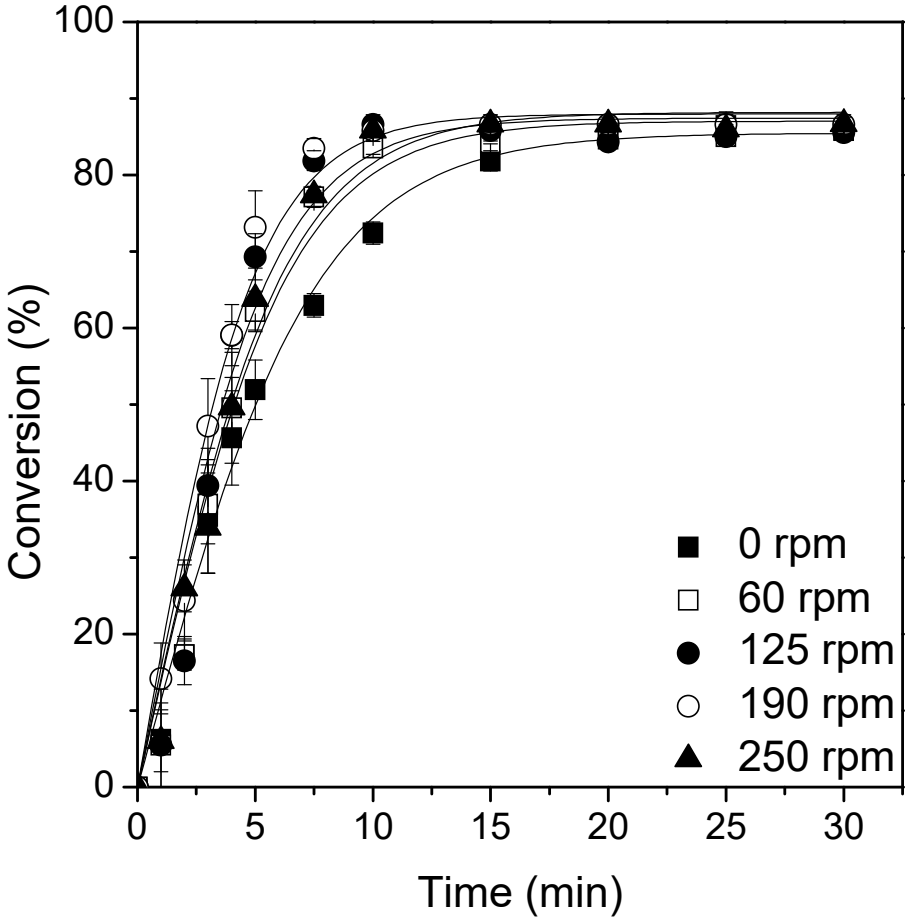

Figure 4. Effect of agitation speed on the ester conversion percentage. The reactions were performed using equimolar ratio acid:alcohol, 45 °C and biocatalyst concentration of 10% m/m.

Here, the esterification reactions were performed using different agitation speeds varying from 0 (static condition) to 250 rpm. According to Fig. 4, conversion percentage between 85 and 88% ($0.855 \leq X_E^{exp.} \leq 0.884$) was observed which indicates that the reaction was not controlled by external diffusion same under static condition. These results suggest good dispersion of lipase molecules on the external support surface and excellent external diffusion of reactants from the liquid phase to the microenvironment of the biocatalyst. Although similar conversion percentage has been observed for both reaction systems, the highest values of k_1 and K_e were observed for the reaction performed at 250 rpm (Table 2, entry 6–10). Moreover, the reaction was more favorable under this condition ($\Delta G = -11.5$ kJ/mol, see Table 2 – entry 10). Thus, agitation speed of 250 rpm was chosen as the optimal condition.

3.4. Effect of organic solvent

Most of the synthetic reactions on industrial scale are performed in organic solvents [36]. This is attributed to the improvement of the solubility of some reactants and reduction of the viscosity of the reaction system which increases the accessibility of reactant molecules from the reaction mixture to the biocatalyst microenvironment [37,38]. It has been reported that a minimum quantity of water is essential surrounding the lipase structures for maintaining its catalytic activity. Thus, hydrophobic solvents are more preferred as compared to the hydrophilic solvents due to the removal of the essential water layer around the enzyme which is necessary for its catalytic activity [34]. In this study, the synthesis of cetyl decanoate was performed using hexane and heptane as organic solvents (see Table 2, entries 11 and 12). The choice of these hydrophobic solvents was based on previous studies reported in the literature for the enzymatic synthesis of several biolubricants, including wax esters [3,14,18,22,26,28]. The reaction

in a solvent-free system was also performed in the same experimental conditions (Table 2, entry 13). From the data presented in Fig. 5, it is possible to observe that the conversion in heptane medium ($X_E^{exp.}$=0.866) was slightly higher than in hexane ($X_E^{exp.}$=0.844) for 15 min of reaction. Moreover, the reaction performed in heptane medium was thermodynamically more favorable (lowest apparent ΔG value: –9.9 kJ/mol – see Table 2, entry 12). Thus, heptane provided the highest conversion percentage and it was chosen as the organic solvent for further tests.

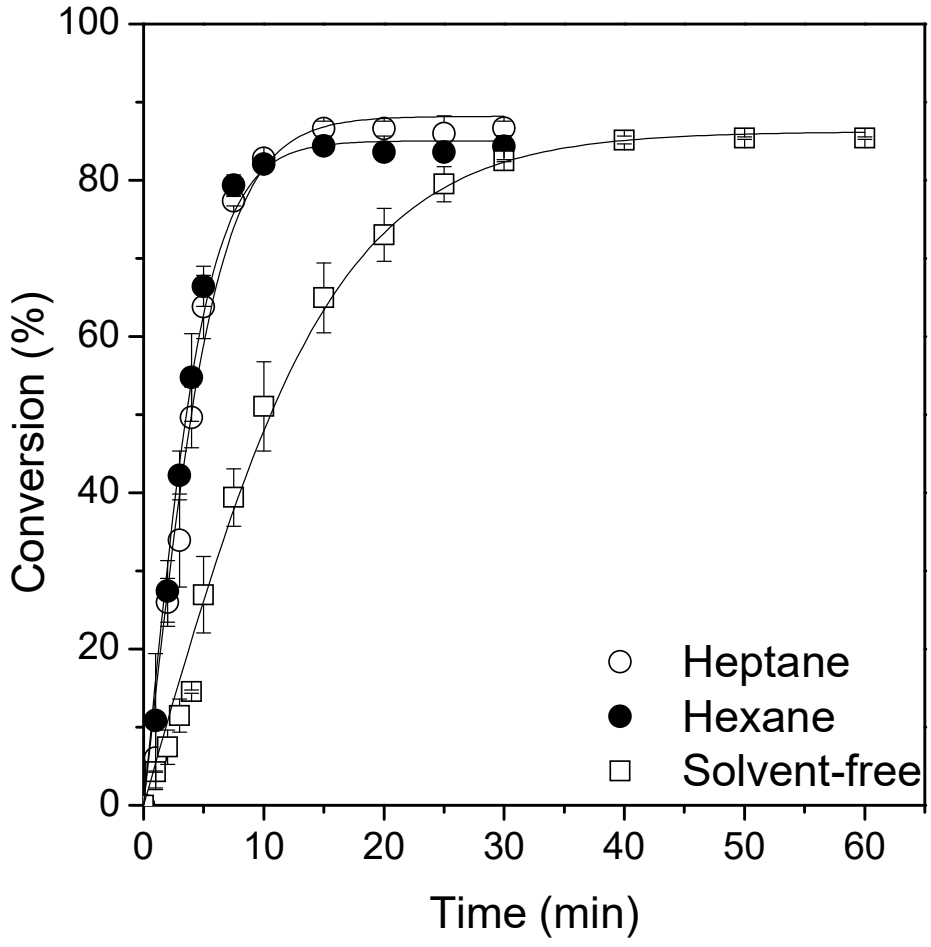

Figure 5. Effect of organic solvent on the ester conversion percentage. The reactions were performed using equimolar ratio acid:alcohol, 45 °C, biocatalyst concentration of 10% m/m and 250 rpm.

As can be observed in Fig. 5, the esterification reaction conducted in a solvent-free system exhibited similar conversion percentage, however the reaction time required for attaining maximum conversion was of 50 min. These results indicate that the reaction performed in solvent systems allowed better accessibility of reactant molecules to the biocatalyst microenvironment due to possible reduction of the viscosity of the reaction mixture. On the other hand, the production of esters in solvent-free systems is more advantageous from the industrial point of view because reduces steps and costs of their recovery and recycling [28]. Due to the high ester conversion observed in a solvent-free system, this reaction system was also selected and its performance was compared with the solvent system using heptane as the reaction medium.

3.5. Effect of molar ratio acid:alcohol

The molar ratio acid:alcohol is an important parameter in the synthesis of several esters, including wax esters [1,3,17]. Since the reaction is a reversible process, the increase of reactants (cetyl alcohol or decanoic acid) may shift the chemical equilibrium towards the ester production [22]. On the other hand, this increase of reactants concentration may also result in a reduction of the initial reaction rate and conversion percentage due to possible inhibition of the biocatalyst [22,31,34]. The ester synthesis in solvent-free system was firstly studied. In this system, the reactions were performed at equimolar ratio acid to alcohol (1:1) and using excess of decanoic acid (1.5:1 and 2:1) and cetyl alcohol (1:1.25 and 1:1.5), as shown in Table 2 (entries 14–18). As can be noted in Fig. 6A, similar ester conversion percentage (between 85–88.6%) by increasing the cetyl alcohol concentration was observed. This indicates that the biocatalyst was not inhibited by increasing the alcohol concentration under these experimental conditions.

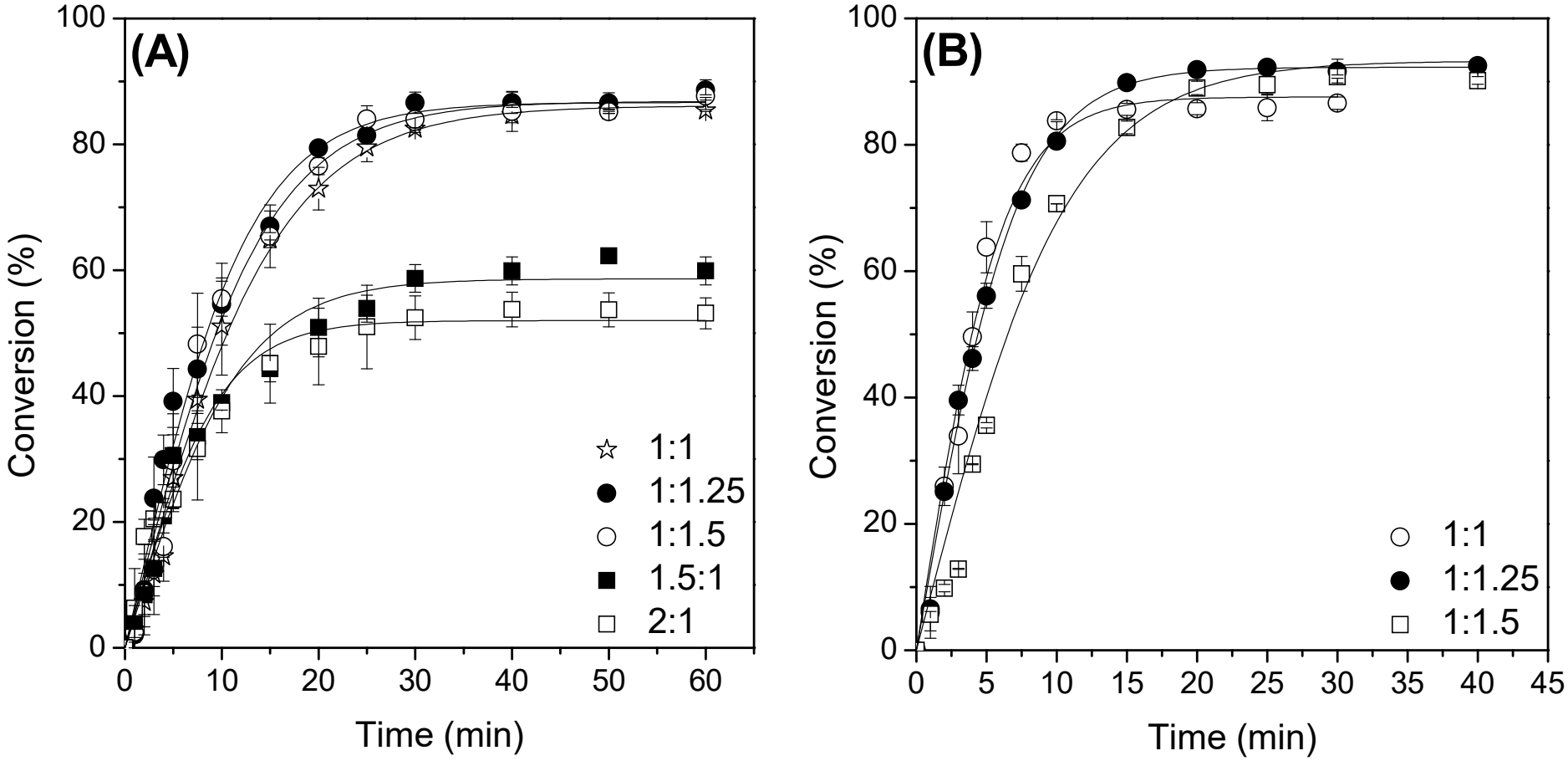

Figure 6. Effect of molar ratio acid:alcohol on the ester conversion percentage in a solvent-free system (A) and in heptane medium (B). The reactions were performed at 45 °C, biocatalyst concentration of 10% m/m and 250 rpm.

However, the increase of acid concentration drastically decreased the conversion percentage from ≈85% at equimolar ratio acid:alcohol to 59% and 53% using molar ratio acid:alcohol of 1.5:1 and 2:1, respectively. These results demonstrated that an increase of acid concentration had a higher inhibitory effect on the biocatalyst than cetyl alcohol due to strong acidification of its microenvironment [3,38], which could lead to conformational changes in the three-dimensional structure of the enzyme.

The effect of molar ratio acid:alcohol varying from 1:1 to 1:1.5 on the ester synthesis in heptane medium was also performed. In this set of experiments, the excess of decanoic acid was not evaluated due to its inhibitory effect on the catalytic activity of immobilized TLL, as described above. According to Fig. 6B, the increase of cetyl alcohol concentration led to an improvement in ester synthesis from ≈85% (equimolar ratio acid:alcohol) to 92.5% ($X_E^{exp.}$=0.925) by using molar ratio acid:alcohol of 1:1.25. However, the reaction time required to attain maximum conversion increased from 15 min to 20-25 min due to possible increase of viscosity in the reaction system. Under these conditions, similar apparent ΔG value was also observed (Table 2, entries 19–20). Thus, subsequent study concerning the effect of biocatalyst concentration was conducted at equimolar ratio acid:alcohol for solvent-free system and molar ratio acid:alcohol of 1:1.25 for the reaction in heptane medium.

3.6. Effect of biocatalyst concentration

Ester synthesis catalyzed by immobilized lipases may be strongly improved by using suitable biocatalyst concentration [21,31]. However, high biocatalyst concentration could drastically reduce the mass transfer due to its strong aggregation in the reaction media that, thus, reduces the process efficiency and increases the production costs [39]. In this set of experiments, the effect of biocatalyst concentration

on the ester synthesis in heptane (from 5% m/m to 12.5% m/m) and solvent-free system (from 7.5% m/m to 12.5% m/m) was examined in order to find optimum biocatalyst concentration (Fig. 7A,B). The increase of biocatalyst concentration from 5% m/m to 10% m/m for the reaction in a solvent system increased the values of apparent kinetic constant for forward reaction (k_1) and equilibrium constant (K_e), as expected. Thus, the reaction was more spontaneous by increasing the biocatalyst concentration (Table 2, entries 22–24). However, maximum ester conversion around 92.5% by using 10 and 12.5% m/m was observed for 20 min of reaction and the same conversion for the reaction performed at 7.5% m/m was attained at 30 min of reaction (Fig. 7A). Moreover, similar apparent ΔG values were obtained above 7.5% m/m of biocatalyst (Table 2, entries 23–25). Then, 7.5% m/m of biocatalyst was selected for reusability tests as an optimum concentration for the ester synthesis.

For the reaction performed in a solvent-free system, the increase of biocatalyst concentration from 7.5% m/m (0.45 g) to 10% m/m (0.6 g) improved also the conversion percentage from 73% ($X_E^{exp.}$=0.730) to ≈85% ($X_E^{exp.}$=0.845), as shown in Fig. 7B. According to Table 2, the lowest apparent Gibbs free energy value (–9.4 kJ/mol) was observed at 10% m/m (entry 27). However, drastic reduction of ester conversion was observed at 12.5% m/m of biocatalyst which was very similar those one performed at lowest biocatalyst concentration – 7.5% m/m. This could be attributed to possible aggregation of the biocatalyst in the reaction medium that reduced the diffusion of reactants from the reaction mixture to the biocatalyst microenvironment.

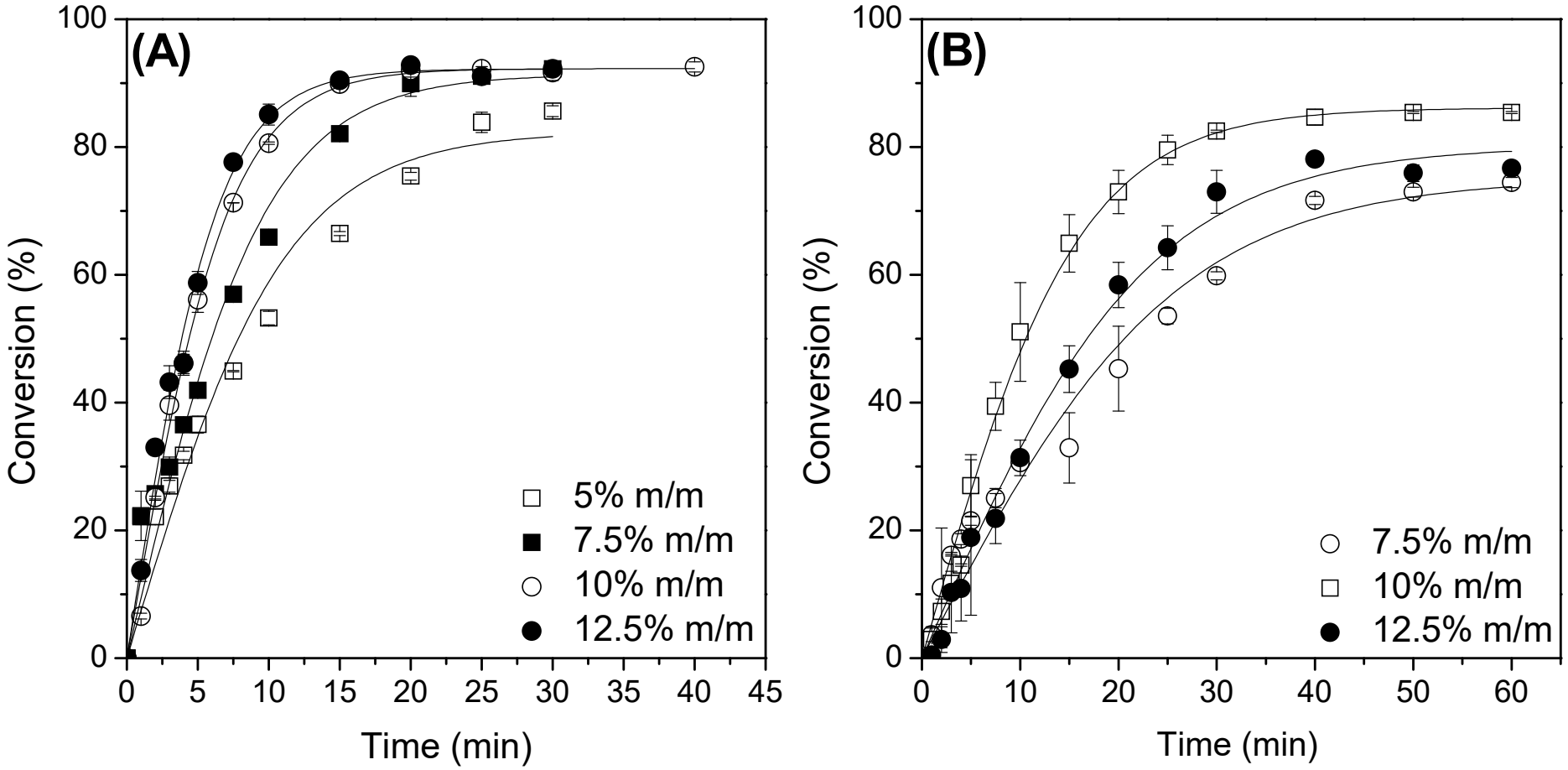

Figure 7. Effect of biocatalyst concentration on the ester conversion performed in solvent-free system (A) and in heptane medium (B). The reactions were conducted at 45 °C and 250 rpm using molar ratio acid:alcohol of 1:1 and 1:1.25 for solvent-free and heptane medium.

The productivity for both reaction systems was determined according to Eq. 12:

$$P = \frac{C}{t \times m} \quad (12)$$

where: P is the productivity (mM/min.g of biocatalyst), C is the total ester concentration produced at equilibrium (mM), t is the reaction at equilibrium and m is the mass of biocatalyst (g).

Under optimal conditions, the productivity (equivalent to the consumption of decanoic acid in the reaction – see Section 2.3) for the ester synthesis performed in a solvent-free and heptane medium was 68.5 mM/min.g of biocatalyst and 56.4 mM/min.g of biocatalyst, respectively. These results clearly indicate that the enzymatic synthesis of cetyl decanoate required the application of heptane as reaction medium to increase the diffusion of reactants to the biocatalyst microenvironment and to avoid possible aggregation of the biocatalyst, thus requiring the lowest biocatalyst concentration and reaction time. In fact, reusability tests were conducted in a solvent system only due to its highest productivity and conversion.

3.7. Comparing with commercial immobilized TLL

The performance of immobilized TLL on PSty–DVB in the synthesis of cetyl decanoate was compared with two commercial immobilized TLL supplied by Novozymes (Lipozyme TL–IM) and Chiral Vision (IMMTLL–T2–150). The prepared biocatalyst was more active than commercial available and maximum conversion percentage of 92.5% for 30 min of reaction was observed (Fig. 8). The reaction catalyzed by IMMTLL–T2–150 and Lipozyme TL–IM exhibited similar conversion percentage for 45 and 75 min of reaction, respectively. Similar ΔG values for both reaction systems were also observed (Table 2, entries 29–31).

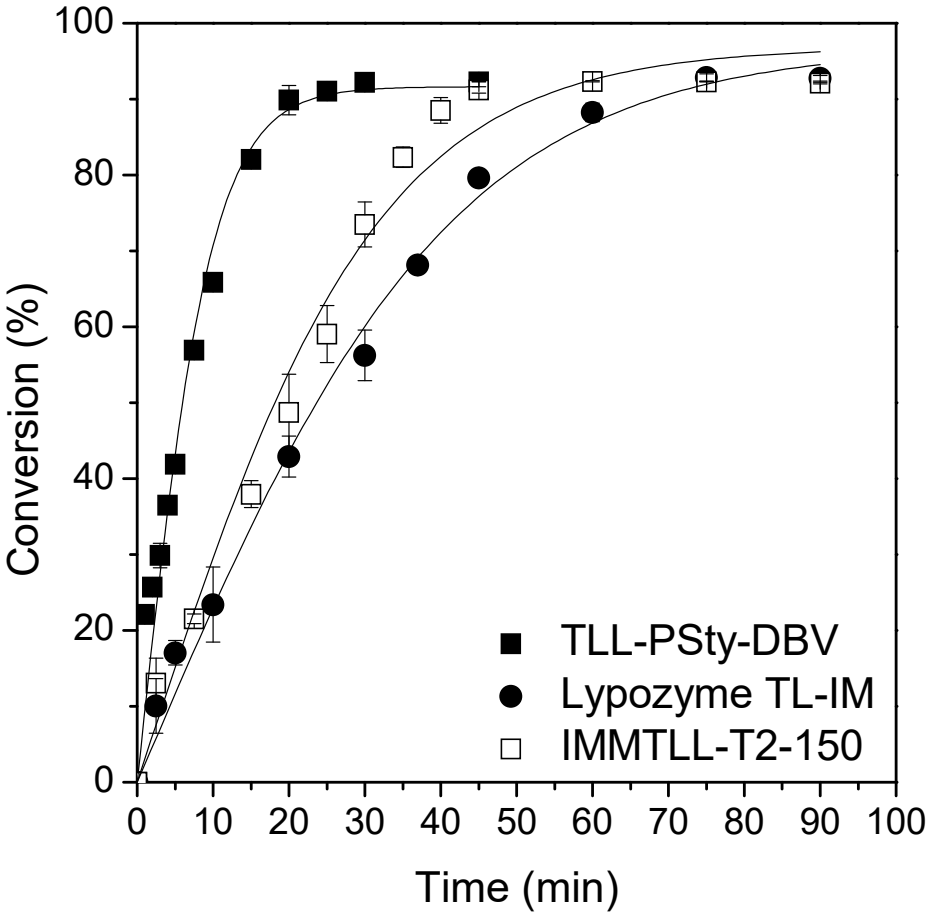

Figure 8. Cetyl decanoate synthesis catalyzed by immobilized TLL on PSty–DVB resin and commercial immobilized TLL (IMMTLL–T2–150 and Lipozyme TL–IM). The reactions were performed at 45 °C and 250 rpm using molar ratio acid:alcohol of 1:1.25 in heptane medium and 7.5% m/m of biocatalyst.

Table 3. Literature survey for the enzymatic synthesis of several wax esters.

Lipase source	Support	Immobilization protocol	Ester	Reaction	Solvent	Reaction time (h)	Conversion (%)	Reusability tests (Residual activity–%)	Reference
Candida sp. 99-125	Textile membrane	Physical adsorption	Cetyl oleate	Esterification	Solvent-free	24	95	7 (≈50)	[1]
Candida rugosa	Amino macroporous resin (LX-1000HA)	Physical adsorption	Oleyl oleate	Esterification	Solvent-free	12	92.6	8 (60)	[17]
	Amino macroporous resin (LX-1000HA) crosslinked with aldehyde dextran (6,000 Da)	Covalent attachment						8 (86.7)	
	Polypropylene (Accurel EP100)	Physical adsorption (native enzyme)	Dodecyl myristate	Esterification	Solvent-free	10	≈100	10 (≈30)	[40]
		Physical adsorption (PEG_{2000}-modified lipase)						10 (≈70)	
Bacillus stearothermophilus MC7	Nanosized tin dioxide ($nanoSnO_2$)	Physical adsorption	Stearyl stearate	Esterification	Solvent-free	5	75	8 (47)	[41]
					Ionic liquid [OMIM][Cl]		95	8 (90)	

Table 3. Literature survey for the enzymatic synthesis of several wax esters (continuation).

Lipase source	Support	Immobilization protocol	Ester	Reaction	Solvent	Reaction time (h)	Conversion (%)	Reusability tests (Residual activity–%)	Reference
Burkholderia sp. EQ3	Polypropylene (Accurel MP–100)	Physical adsorption	Oleyl esters from jatropha oil	Transesterification	Isooctane	12	89	5 (≈100)	[19]
Rhizomucor miehei (Lipozyme RM–IM)	Anionic resin (Duolite A568)				Hexane	12	86	5 (≈27)	
Lipase B from *Candida antarctica* (Fermase CALB™10000)	Polyacrylate beads	Covalent attachment	Cetyl oleate	Esterification	Solvent-free	0.5	95.96	7 (≈70)	[15]
Lipase B from *Candida antarctica* (Novozym 435)	Lewatit VP OC 1600	Physical adsorption	Oleyl oleate	Esterification	Hexane	0.083 (5 min)	≈95	9 (≈96.7)	[42]
Thermomyces lanuginosus	Poly-(styrene-divinylbenzene) resin	Physical adsorption	Cetyl linoleate	Esterification	Solvent-free	0.5	90.5 ± 0.6	5 (100)	[16]
			Cetyl decanoate		Heptane		92.5	8 (95.6)	This study

These results were also compared with previous studies reported in the literature concerning the enzymatic synthesis of wax esters. According to Table 3, immobilized TLL on PSty–DVB required a lower reaction time to attain maximum ester synthesis, with exception of oleyl oleate synthesis catalyzed by Novozym 435 which maximum conversion was observed in only 5 min of reaction [42]. In another study, Khan et al. observed also maximum cetyl oleate synthesis for 30 min of reaction under ultrasound irradiation using Novozym 435 as biocatalyst while the reaction performed under conventional system (very similar to our experimental conditions) presented the highest conversion above 2 h of reaction [15]. These results confirm the promising application of the prepared biocatalyst in the synthesis of cetyl decanoate.

3.8. Reusability tests

The reusability of heterogeneous biocatalysts is very important for their application, especially from the industrial point of view [28,43]. Here, the reusability of immobilized TLL on PSty–DVB was investigated in order to determine its stability after eight successive cycles of reaction of 30 min each under optimal experimental conditions. The results illustrated in Fig. 9 show that after eight cycles of reaction, the biocatalyst retained around 96% of its original activity. These results could be attributed to strong adsorption of TLL on the support surface which avoid its possible desorption and efficient removal of residual reactants or product molecules, including water molecules formed during the reaction which could accelerate the desorption of TLL molecules [16,22,28].

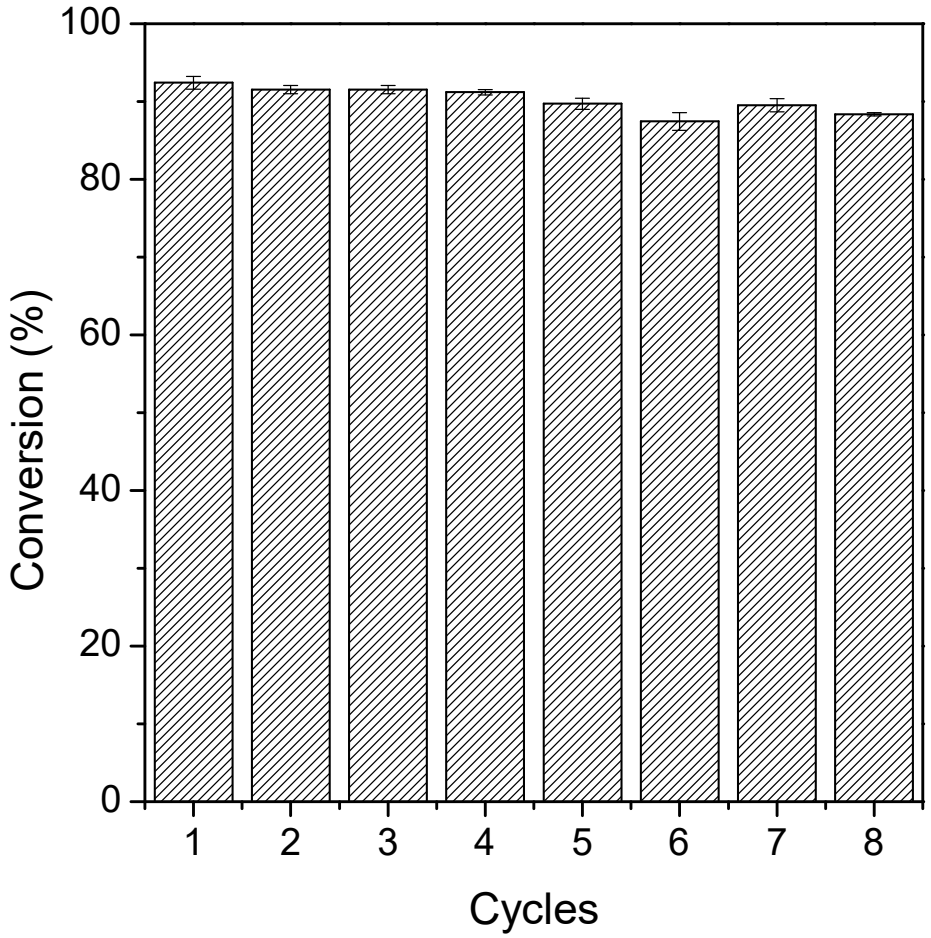

Figure 9. Reusability tests for the synthesis of cetyl decanoate in heptane medium. The reactions were conducted at 45 °C and 250 rpm using molar ratio acid:alcohol of 1:1.25, 7.5% m/m of biocatalyst and 30 min of reaction for each cycle.

These results were compared with those ones performed in batch systems previously reported in Table 3. Immobilized TLL on PSty–DVB was found to be more stable after eight successive cycles of reaction than several biocatalysts prepared by physical adsorption and covalent attachment of lipases from *Candida* sp. 99-125 [1], *Candida rugosa* [17,40], *Bacillus stearothermophilus* MC7 [41], *Rhizomucor miehei* (Lipozyme RM–IM) [19], and *Candida antarctica* B (Fermase CALB™10000) [15].

Similar results may be observed for the synthesis of oleyl oleate catalyzed by Novozym 435, the most used biocatalyst in ester syntheses by either esterification or transesterification reactions, that presented also negligible decrease of activity after 9th cycle of reaction [42]. These results clearly show that the immobilization of TLL on PSty–DVB is an important strategy to prepare a robust biocatalyst to be applied in successive cycles of esterification reaction in a batch system.

Conclusion

Immobilized TLL on PSty–DVB resin was applied as a biocatalyst in the synthesis of a saturated wax ester by esterification reaction. This biocatalyst exhibited high esterification activity due to some features of the support such as high surface area and porous size that allowed immobilize high protein concentration. The biocatalyst prepared by offering initial protein loading of 115 mg/g was selected as the potential biocatalyst in the production of cetyl decanoate (immobilized protein concentration of 108.7 ± 3.1 mg/g) due to its highest initial reaction rate. The synthesis of this ester was improved by adding heptane as the solvent in order to improve the mass transfer of reactant molecules from the reaction mixture to the biocatalyst microenvironment. A second-order reversible kinetic model could be successfully applied to the experimental data to determine apparent kinetic constants and thermodynamic parameters which showed that the reaction was a spontaneous and endothermic process in nature. The biocatalyst prepared was also more active than heterogeneous biocatalysts commercially available such as Lipozyme TL–IM and IMMTLL–T2–150. Moreover, immobilized TLL on PSty–DVB was also highly stable after successive cycles of reaction. These results showed that the biocatalyst previously prepared by using a simple protocol

(interfacial activation of lipase on hydrophobic support) and the process used (esterification reaction in a batch system using classical equipment) could be an interesting process to produce an important compound from the industrial point of view as cetyl decanoate.

Acknowledgements

This study was financially supported by FAPEMIG (Process APQ–02196–15) and CNPq (Process 404929/2016-8). Michelle D. Alves thanks the CAPES Foundation for the student fellowship. The authors are also grateful to Prof. Paulo W. Tardioli (DEQ–UFSCar/Brazil) by the donation of commercial immobilized TLL (Lipozyme TL–IM and IMMTLL–T2–150).

References

[1] D. Li, W. Xiaojing, N. Kaili, W. Fang, L. Junfeng, W. Pu, T. Tianwei, Synthesis of wax esters by lipase-catalyzed esterification with immobilized lipase from *Candida* sp. 99-125, Chin. J. Chem. Eng. 19 (2011) 978–982.

[2] M. Sellami, I. Aissa, F. Frikha, Y. Gargouri, N. Miled, Immobilized *Rhizopus oryzae* lipase catalyzed synthesis of palm stearin and cetyl alcohol wax esters: Optimization by response surface methodology, BMC Biotechnol. 11 (2011) 1–8.

[3] C.H. Kuo, H.H. Chen, J.H. Chen, Y.C. Liu, C.J. Shieh, High yield of wax ester synthesized from cetyl alcohol and octanoic acid by Lipozyme RMIM and Novozym 435, Int. J. Mol. Sci. 13 (2012) 11694–11704.

[4] Cosmetics Info – The Science & Safety Behind Your Favorite Products (2016). http://www.cosmeticsinfo.org/ingredient/cetyl-esters-0 (Accessed 25 November 2016).

[5] H.C. Chen, C.H. Kuo, H.H. Chen, Y.C. Liu, C.J. Shieh, Optimization of enzymatic synthesis of cetyl 2-ethylhexanoate by Novozym® 435, J. Am. Oil Chem. Soc. 88 (2011) 1917–1923.

[6] N. Al-Arafi, J. Salimon, Production of oleic acid based wax ester using acidic homogeneous catalysts, E-J. Chem. 9 (2012) 99–106.

[7] G. Steinke, S. Schönwiese, K.D. Mukherjee, Alkali-catalyzed alcoholysis of crambe oil and camelina oil for the preparation of long-chain esters, J. Am. Oil Chem. Soc. 77 (2000) 367–371.

[8] L. Yao, J.Y. Lio, T. Wang, D.H. Jarboe, Synthesis and characterization of acetylated and stearylyzed soy wax, J. Am. Oil Chem. Soc. 90 (2013) 1063–1071.

[9] S. Sunitha, S. Kanjilal, P.S. Reddy, R.B.N. Prasad, Liquid–liquid biphasic synthesis of long chain wax esters using the Lewis acidic ionic liquid choline chloride.2$ZnCl_2$, Tetrahedron Lett. 48 (2007) 6962–6965.

[10] N. Al-Arafi, J. Salimon, Synthesis of long chain wax ester, suing the Bronsted acidic ionic liquid N-methyl-2-pyrrolidonium methyl sulfate ([NMP][CH_3SO_3]), Res. J. Appl. Sci. 6 (2011) 361–365.

[11] F.G. Cirujano, A. Corma, F.X. Llabrés i Xamena, Zirconium-containing metal organic frameworks as solid acid catalysts for the esterification of free fatty acids: Synthesis of biodiesel and other compounds of interest, Catal. Today 257 (2015) 213–220.

[12] L. Bouzidi, S. Li, S. Di Biase, S.Q. Rizvi, P. Dawson, S.S. Narine, Lubricating and waxy esters II: Synthesis, crystallization, and melt behavior of branched monoesters, Ind. Eng. Chem. Res. 51 (2012) 14892−14902.

[13] L. Bouzidi, S. Li, S. Di Biase, S.Q. Rizvi, S.S. Narine, Lubricating and waxy esters. 4. Synthesis, crystallization behavior, melt behavior, and flow behavior of linear monoesters incorporating 9-decenol and 9-decenoic acid, Ind. Eng. Chem. Res. 52 (2013) 2740−2749.

[14] P. Ungcharoenwiwat, A. H-Kittikun, Synthesis of wax esters from crude fish fat by lipase of *Burkholderia* sp. EQ3 and commercial lipases, J. Am. Oil Chem. Soc. 90 (2013) 359–367.

[15] N.R. Khan, S.V. Jadhav, V.K. Rathod, Lipase catalysed synthesis of cetyl oleate using ultrasound: Optimisation and kinetic studies, Ultrason. Sonochem. 27 (2015) 522–529.

[16] M.D. Alves, F.M. Aracri, E.C. Cren, A.A. Mendes, Isotherm, kinetic, mechanism and thermodynamic studies of adsorption of a microbial lipase on a mesoporous and hydrophobic resin, Chem. Eng. J. (2016) doi: 10.1016/j.cej.2016.11.069

[17] Y. Bi, M. Yu, H. Zhou, H. Zhou, P. Wei, Biosynthesis of oleyl oleate in solvent-free system by *Candida rugosa* Lipase (CRL) immobilized in macroporous resin with cross-linking of aldehyde-dextran, J. Mol. Catal. B: Enzym. 133 (2016) 1–5.

[18] P. Ungcharoenwiwat, A. H-Kittikun, Purification and characterization of lipase from *Burkholderia* sp. EQ3 isolated from wastewater from a canned fish factory and its application for the synthesis of wax esters, J. Mol. Catal. B: Enzym. 115 (2015) 96–104.

[19] P. Ungcharoenwiwat, B. Canyuk, A. H-Kittikun, Synthesis of jatropha oil based wax esters using an immobilized lipase from *Burkholderia* sp. EQ3 and Lipozyme RM IM, Process Biochem. 51 (2016) 392–398.

[20] A. Salis, V. Solinas, M. Monduzzi, Wax esters synthesis from heavy fraction of sheep milk fat and cetyl alcohol by immobilised lipases, J. Mol. Catal. B: Enzym. 21 (2003) 167–174.

[21] M. Serrano-Arnaldos, M.F. Máximo-Martín, M.C. Montiel-Morte, S. Ortega-Requena, E. Gómez-Gómez, J. Bastida-Rodríguez, Solvent-free enzymatic production of high quality cetyl esters, Bioprocess Biosyst. Eng. 39 (2016) 641–649.

[22] J.J. Bassi, L.M. Todero, F.A.P. Lage, G.I. Khedy, J. Dell Ducas, A.P. Custódio, M.A. Pinto, A.A. Mendes, Interfacial activation of lipases on hydrophobic support and application in the synthesis of a lubricant ester, Int. J. Biol. Macromol. 92 (2016) 900–909.

[23] M. Ladero, M. de Gracia, J.J. Tamayo, I.L. Ahumada, F. Trujillo, F. Garcia-Ochoa, Kinetic modelling of the esterification of rosin and glycerol: Application to industrial operation, Chem. Eng. J. 169 (2011) 319–328.

[24] I.B.B. Romdhane, Z.B. Romdhane, A. Gargouri, H. Belghith, Esterification activity and stability of *Talaromyces thermophiles* lipase immobilized onto chitosan, J. Mol. Catal. B: Enzym. 68 (2011) 230–239.

[25] F. Peter, G. Preda, Characterisation of pancreatic lipase substrate specificity in organic reaction media by a kinetic method, J. Mol. Catal. B: Enzym. 19–20 (2002) 467–472.

[26] K.N.P. Rani, T.S.V.R. Neeharika, T.P. Kumar, B. Satyavathi, C. Sailu, R.B.N.Prasad, Kinetics of enzymatic esterification of oleic acid and decanol for wax ester and evaluation of its physico-chemical properties, J. Taiwan Inst. Chem. Eng. 55 (2015) 12–16.

[27] P.S. Kumar, S. Ramalingam, S.D. Kirupha, A. Murugesan, T. Vidhyadevi, S. Sivanesan, Adsorption behavior of nickel(II) onto cashew nut shell: Equilibrium, thermodynamics, kinetics, mechanism and process design, Chem. Eng. J. 167 (2011) 122–131.

[28] F.A.P. Lage, J.J. Bassi, M.C.C. Corradini, L.M. Todero, J.H.H. Luiz, A.A. Mendes, Preparation of a biocatalyst via physical adsorption of lipase from *Thermomyces lanuginosus* on hydrophobic support to catalyze biolubricant synthesis by esterification reaction in a solvent-free system, Enzyme Microb. Technol. 84 (2016) 56–67.

[29] J. Liu, H.T. Wu, J.F. Lu, X.Y. Wen, J. Kan, C.H. Jin, Preparation and characterization of novel phenolic acid (hydroxybenzoic and hydroxycinnamic acid derivatives) grafted chitosan microspheres with enhanced adsorption properties for Fe(II), Chem. Eng. J. 262 (2015) 803–812.

[30] S.M. Radzi, R. Mohamad, M. Basri, A.B. Salleh, A. Ariff, M.B.A. Rahman, R.N.Z.R.A. Rahman, Kinetics of enzymatic synthesis of liquid wax ester from oleic acid and oleyl alcohol, J. Oleo Sci. 59 (2010) 127–134.

[31] K.C. Badgujar, B.M. Bhanage, Application of lipase immobilized on the biocompatible ternary blend polymer matrix for synthesis of citonellyl acetate in non-aqueous media: kinetic modelling study, Enzyme Microb. Technol. 57 (2014) 16–25.

[32] Z. Guo, X. Xu, Lipase-catalyzed glycerolysis of fats and oils in ionic liquids: A further study on the reaction system, Green Chem. 8 (2006) 54–62.

[33] N.N.A. Razak, M.S.M. Annuar, Enzymatic synthesis of flavonoid ester: Elucidation of its kinetic mechanism and equilibrium thermodynamic behavior, *Ind. Eng. Chem. Res.* 54 (2015) 5604–5612.

[34] K.C. Badgujar, B.M. Bhanage, The combine use of ultrasound and lipase immobilized on co-polymer matrix for efficient biocatalytic application studies, J. Mol. Catal. B: Enzym. 122 (2015) 255–264.

[35] L.M. Todero, J.J. Bassi, F.A.P. Lage, M.C.C. Corradini, J.C.S. Barboza, D.B. Hirata, A.A. Mendes, Enzymatic synthesis of isoamyl butyrate catalyzed by immobilized lipase on poly-methacrylate particles: optimization, reusability and mass transfer studies, Bioprocess Biosyst. Eng. 38 (2015) 1601–1613.

[36] A. Kumar, K. Dhar, S.S. Kanwar, P.K. Arora, Lipase catalysis in organic solvents: Advantages and applications, Biol. Proced. Online 18 (2016) 1–11.

[37] N. Chaibakhsh, M.B.A. Rahman, S. Abd-Aziz, M. Basri, A.B. Salleh, R.N.Z.R.A. Rahman, Optimized lipase-catalyzed synthesis of adipate ester in a solvent-free system, J. Ind. Microbiol. Biotechnol. 36 (2009) 1149–1155.

[38] J.S. Miranda, N.C.A. Silva, J.J. Bassi, M.C.C. Corradini, F.A.P. Lage, D.B. Hirata, A.A. Mendes, Immobilization of *Thermomyces lanuginosus* lipase on mesoporous poly-hydroxybutyrate particles and application in alkyl esters synthesis: Isotherm, thermodynamic and mass transfer studies, Chem. Eng. J. 251 (2014) 392–403.

[39] N.C.A. Silva, J.S. Miranda, I.C.A. Bolina, W.C. Silva, D.B. Hirata, H.F. Castro, A.A. Mendes, Immobilization of porcine pancreatic lipase on poly-hydroxybutyrate particles for the production of ethyl esters from macaw palm oils and pineapple flavor, Biochem. Eng. J. 82 (2014) 139–149.

[40] M.H. Guncheva, D. Zhiryakova, High-yield synthesis of wax esters catalysed by modified *Candida rugosa* lipase, Biotechnol. Lett. 30 (2008) 509–512.

[41] M. Guncheva, M. Dimitrov, M. Kambourova, Excellent stability and synthetic activity of lipase from *B. stearothermophilus* MC7 immobilized on tin dioxide in environmentally friendly medium, Biotechnol. Biotechnol. Eq. 27 (2013) 4317–4322.

[42] S.M. Radzi, M. Basri, A.B. Salleh, A. Ariff, R. Mohammad, M.B.A. Rahman, R.N.Z.R.A. Rahman, High performance enzymatic synthesis of oleyl oleate using immobilized lipase from *Candida antartica*, Elec. J. Biotechnol. 8 (2005) 291–298.

[43] H. Dong, J. Li, Y. Li, L. Hu, D. Luo, Improvement of catalytic activity and stability of lipase by immobilization on organobentonite, Chem. Eng. J. 181–182 (2012) 590–596.

CONCLUSÃO

Lipase de *T. lanuginosus* (TLL) imobilizada em partículas de poli-estireno-divinilbenzeno possibilitou a preparação de um biocatalisador altamente ativo e estável na síntese de ésteres de cera. A elevada concentração de lipase imobilizada neste suporte (da ordem de 134 mg/g de suporte) foi atribuída à elevada área superficial do suporte e diâmetro de poros que permitiu imobilizar moléculas de enzima em seu microambiente interno e externo. Entretanto, estudos cinéticos e análise de microscopia eletrônica de varredura revelaram preferencial imobilização da enzima na sua parte externa. A alta atividade catalítica em reações de síntese de ésteres de cera insaturado (linoleato de cetila) e saturado (decanoato de cetila) por reação de esterificação foi também atribuída à alta hidrofobicidade do suporte que permitiu boa partição dos materiais de partida ao seu microambiente. Além disso, o biocatalisador preparado se mostrou altamente estável após sucessivos ciclos de reação em meio de solvente (síntese de decanoato de cetila) e em meio isento de solvente (síntese de linoleato de cetila). Isto foi atribuído à alta afinidade da enzima com o suporte, mecanismo de ativação interfacial da lipase na presença de suporte hidrofóbico, que reduziu a sua dessorção e a metodologia empregada na etapa de recuperação do suporte que eficientemente removeu materiais de partida não convertidos e produtos da reação que, consequentemente, permitiu excelente difusão de reagentes e elevada conversão em éster. De acordo com os resultados obtidos, este biocatalisador foi mais ativo que aqueles disponíveis comercialmente previamente preparados por imobilização de TLL por diferentes protocolos. Estes resultados mostraram a promissora aplicação do biocatalisador obtido na síntese de compostos de grande interesse industrial como ésteres de cera por esterificação em meios de solvente e isento de solventes orgânicos.

SUGESTÕES PARA TRABALHOS FUTUROS

- Caracterização das propriedades físico-químicas dos ésteres produzidos como ponto de fusão, ponto de ebulição, viscosidade cinemática, índice de viscosidade, densidade, índice de acidez.

- Produção de ésteres de cera empregando óleos vegetais ricos em ácidos linoleico (soja e algodão) e decanoico (coco, palmiste e macaúba) por reações de hidroesterificação (um processo sequencial de hidrólise de óleos vegetais, seguido de purificação dos ácidos graxos e esterificação dos ácidos graxos obtidos com álcoois de cadeia longa) e transesterificação (reação direta de óleos vegetais e álcool d cadeia longa).

- Produção de ésteres de cera em reatores de leito fixo. Influência da vazão de alimentação, tempo de residência e relação diâmetro/altura na síntese do éster.

- Preparação de biocatalisadores ativos empregando suportes não comerciais e de baixo custo como hidrogéis de quitosana hidrofobizados e resíduos agroindustriais como casca de café e macaúba.

ANEXO I

XII Seminário Brasileiro de Tecnologia Enzimática
ENZITEC 2016

Influence of protein loading on the catalytic properties of immobilized lipase from *Thermomyces lanuginosus* on poly-(styrene-divinylbenzene): Determination of thermodynamic and isotherm parameters

Michelle D. Alves[1], Fernanda M. Aracri[2], Jaquelinne J. Bassi[2], Érika C. Cren[1] and Adriano A. Mendes[2]

[1]Universidade Federal de Minas Gerais – Depto. de Engenharia Química
31270-901 Belo Horizonte – MG
[2]Universidade Federal de Alfenas – Instituto de Química
37130-000 Alfenas – MG - E-mail: adriano.mendes@unifal-mg.edu.br

ABSTRACT

In the present study, Thermomyces lanuginosus lipase (TLL) was physically adsorbed on poly-(styrene-divinylbenzene) (PSty-DVB) resin in order to prepare highly active biocatalysts. The effect of initial protein loading on the catalytic properties of the biocatalysts was evaluated. The adsorption capacity at 25 °C and pH 5.0 at low ionic strength (5 mM buffer sodium acetate) was around 134 mg of protein/g of support using initial protein loading of 150 mg/g of support. Maximum hydrolytic activity varied from 235 to 470 IU/g of support. The equilibrium adsorption data fitted to the Langmuir isotherm model (R^2=0.9776). Thermodynamic analysis showed that the adsorption was a spontaneous process ($-18.7 \leq \Delta G \leq -9.0$ kJ/mol).

Keywords: Physical adsorption, Lipase, Poly-(styrene-divinylbenzene) resin, Catalytic properties.

INTRODUCTION

Lipases (triacylglycerol ester acylhydrolases, EC 3.1.1.3) are hydrolases that cleavage carboxylic ester bonds in tri-, di-, and monoacylglycerols to glycerol and free fatty acids at the water-lipid interface. In environments with low water content, these enzymes also catalyze other biotransformation reactions such as esterification, interesterification and transesterification (Adlercreutz, 2013). The use of free lipases in industrial processes has some limitations such as difficult reusability and poor solvent tolerance capability (Lage et al., 2016). These problems can be overcome by immobilizing lipases using different techniques. Among them, physical adsorption has attracted significant commercial attention in the recent years because it is simpler and less expensive than other techniques and high catalytic activity and stability may be retained (Fernández-Lafuente, 2010; Adlercreutz, 2013). This method allows the reusability of supports after inactivation of immobilized enzyme using several chemicals, including surfactants, urea and guanidine (Fernández-Lafuente, 2010). In this study, TLL was immobilized on PSty-DVB resin via physical adsorption at low ionic strength. The effect of initial protein loading on the catalytic properties was evaluated in hydrolysis reaction and immobilized protein concentration. Thermodynamic and isotherm studies were also performed.

MATERIALS AND METHODS

Materials

Thermomyces lanuginosus lipase (TLL) was purchased from Sigma-Aldrich (St. Louis, MO, USA). PSty-DVB resin (average particle diameter of 250–850 µm, surface area of ≈500 m^2/g and average porous size of 260 Å) was purchased from Supelco (Bellefonte, PA, USA). Olive oil from Carbonell (Córdoba, Spain) was purchased at a local market (Alfenas, MG, Brazil). Arabic Gum was acquired from Synth® (São Paulo, SP, Brazil). All other chemical reagents were of analytical grade acquired from Vetec Química Ltd. and Synth®.

Determination of the hydrolytic activity and immobilized protein concentration

The hydrolytic activity (HA) of the biocatalysts was determined in the hydrolysis of olive oil emulsion at pH 8.0 (buffer sodium phosphate 100 mM), 37 °C and 5 min under agitation in an orbital shaker (200 rpm) (Lage et al., 2016). One international unit (IU) of hydrolytic activity was defined as the mass of enzyme required to release 1 µmol of free fatty acids per minute of reaction under the conditions above described. Protein was determined by the Bradford's method (Bradford, 1976), using bovine serum albumin as standard protein.

Physical adsorption of TLL on PSTy-DVB resin – Isotherm and thermodynamic studies

10 g of wet PSty-DVB resin was incubated in 190 mL of 5 mM sodium acetate pH 5.0 containing different protein loadings to vary the support loading from 10 to 200 mg protein/g of support (Lage et al., 2016). The suspensions were kept under agitation (200 rpm) in an orbital shaker at room temperature for 15 h. The biocatalysts were filtered under vacuum, washed with distilled water (volume ratio 1:5) and stored at 4 °C for 24 h prior to use. Immobilized protein (IP) was calculated after determining the amount of protein disappeared in the supernatant and comparing to the initial protein concentration offered (mg/g support). Specific activity (SA) was calculated as the hydrolytic activity of the biocatalyst per milligram of immobilized protein (IU/mg_{IP}). Equilibrium constant (K_c) was determined as the ratio between immobilized protein concentration (mg/g) and residual protein in solution (mg/mL), respectively. Free energy Gibbs (ΔG) was determined according to Eq. 1:

$$\Delta G\left(kJ/mol\right) = -RT\ln K_c \quad (1)$$

where R is the gas universal constant (8.314×10^{-3} kJ/mol.K), T is the experimental temperature (298.15 K), and K_c is the equilibrium constant.

In this study, Langmuir isotherm model (Eq. 2) was used to fit the experimental data from TLL adsorption on PSty-DVB (Lage et al., 2016).

$$q_e = \frac{q_{\max} \times C_e}{K_L + C_e} \quad (2)$$

where q_e is the adsorption capacity at equilibrium (mg protein/g of support), C_e is the residual protein concentration after immobilization (mg protein/mL), q_{max} is the maximum adsorption capacity (mg protein/g support), K_L is the Langmuir constant (mL/mg protein).

RESULTS AND DISCUSSION

The influence of protein loading on the catalytic properties of the prepared biocatalysts by immobilizing TLL on PSty-DVB resin via physical adsorption is shown in Table 1.

Table 1. Influence of initial protein loading on the catalytic properties of immobilized TLL on STY-DVB particles

Protein loading (mg/g of support)	IP (mg/g)	HA (IU/g)	SA (IU/mg_{IP})	Kc	ΔG (kJ/mol)
10	9.9 ± 0.1	235.0 ± 5.1	23.7 ± 0.5	1881.1	–18.7
20	19.2 ± 0.5	432.7 ± 10.1	23.8 ± 1.1	432.1	–15.0
30	29.0 ± 0.4	467.9 ± 22.9	16.1 ± 0.6	551.0	–15.0
55	53.6 ± 4.2	452.9 ± 56.2	8.5 ± 1.7	727.5	–16.3
70	69.3 ± 0.6	445.2 ± 49.4	6.5 ± 0.6	1454.5	–18.1
115	108.7 ± 3.1	450.7 ± 30.7	4.2 ± 0.3	327.8	–14.4
125	117.0 ± 2.3	469.9 ± 60.2	4.0 ± 0.6	277.9	–14.0
150	133.9 ± 1.3	443.0 ± 25.2	3.3 ± 0.1	158.0	–12.5
175	133.9 ± 4.4	431.8 ± 50.6	3.2 ± 0.5	61.9	–10.2
200	133.5 ± 3.1	412.6 ± 19.3	3.1 ± 0.1	38.1	–9.0

The immobilized lipase concentration (IP) increased greatly with the offered initial protein concentration up to 150 mg/g of support as expected. Maximum immobilized protein concentration was around 134 mg/g of PMA particles. After, no significant effect was verified due to possible support saturation. Hydrolytic activity of the prepared biocatalysts varied from 235 to 470 IU/g of support. The biocatalysts previously prepared using initial protein loading from 20 to 200 mg/g presented similar hydrolytic activity (HA). These results indicate strong diffusional limitation of olive oil molecules to the internal microenvironment of the biocatalysts. On the other hand, specific activity (SA) values were influenced by the protein loading, varying from 23.7 ± 0.5 to 3.1 ± 0.1 IU/mgIP. The reduction of SA values may be attributed to strong mass transfer effects because the substrate molecules (droplets of oil) are large, as above described, thus reducing their accessibility to the internal biocatalyst surface (possible reduction of porous diameter after immobilization) or steric hindrances because the active center of the enzyme is oriented towards the support surface.

Thermodynamic considerations of an adsorption process are necessary to conclude whether the process is spontaneous or not. Adsorption processes occur spontaneously at a given temperature whether ΔG is a negative value. K_c values varied from 1881.1 to 38.1 for lowest and highest protein loading, as shown in Table 1. This parameter was then used to determine ΔG values by using Eq. (1). ΔG values varied from −18.7 to –9.0 kJ/mol, thus indicating that the adsorption of TLL on PSty-DVB was a spontaneous process.

The physical adsorption results were analyzed by applying the Langmuir isotherm model (Fig. 1). The experimental data from the adsorption of TLL on PMA particles were adequately explained by the Langmuir isotherm model (R^2=0.9776). From these observations, it is possible to verify that the immobilization of TLL on PMA particles occurred via monolayer adsorption (Lage et al., 2016). The theoretical value of maximum adsorbed protein

amount on PMA particles (q_{max}) was 148.1 mg/g of support. The difference between maximum observed loading (experimental – 133.9 mg/g of PMA particles) and theoretical q_{max} value could be attributed to compounds (e.g. salts, polyols and sugars) present in crude TLL extract that could also adsorb on the support surface (Fernández-Lafuente, 2010).

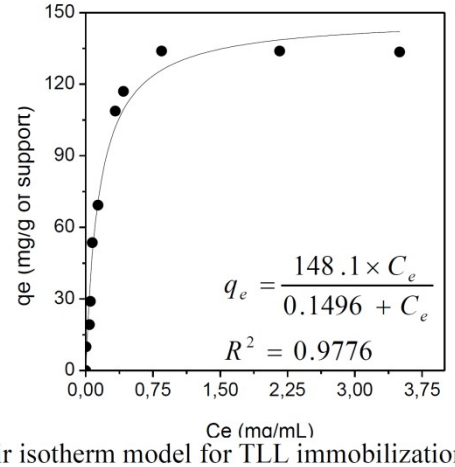

Figure 1. Langmuir isotherm model for TLL immobilization on PSty-DVB resin.

CONCLUSION

The present study showed that TLL was successfully immobilized on PSty-DVB resin. The adsorption of the enzyme was a spontaneous process ($\Delta G<0$). Hydrolytic activity and specific activity values indicated preferential immobilization of lipases molecules in the internal support surface. Maximum immobilized protein amount was around 134 mg protein/g. The selected support was found to be promising to prepare highly robust biocatalysts due to its large surface area and porous size.

ACKNOWLEDGEMENTS

This paper was financially supported by FAPEMIG (Process number APQ–02196–15), CNPq and CAPES (Brazil).

REFERENCES

Adlercreutz P. 2013. Immobilisation and application of lipases in organic media. Chem Soc Rev 42:6406-6436.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254.

Fernandez-Lafuente R. 2010. Lipase from *Thermomyces lanuginosus*: Uses and prospects as an industrial biocatalyst. J Mol Catal B Enzym 62:197-212.

Lage FAP, Bassi JJ, Corradini MCC, Todero LM, Luiz JHH, Mendes AA. 2016. Preparation of a biocatalyst via physical adsorption of lipase from *Thermomyces lanuginosus* on hydrophobic support to catalyze biolubricant synthesis by esterification reaction in a solvent-free system. Enzyme Microb Technol 84:56-67.