CUNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
ESCOLA DE ENGENHARIA
DEPARTAMENTO DE ENGENHARIA QUÍMICA
PROGRAMA DE PÓS-GRADUAÇÃO EM ENGENHARIA QUÍMICA

SÍNTESE ENZIMÁTICA DE BIODIESEL EM REATORES CONTÍNUOS E EM BATELADA: ASPECTOS DO USO DE DIVERSAS FONTES DE ÓLEOS, DO CONCEITO DE *COMBI-LIPASES* E DO ULTRASSOM

TESE DE DOUTORADO

JAKELINE KATHIELE POPPE TODESCHINI

PORTO ALEGRE
2017

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Engenheira de Bioprocessos e Biotecnologia
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Tese de doutorado submetido ao Programa de Pós-Graduação em Engenharia Química da UFRGS como requisito para a obtenção do grau de Doutor em Engenharia Química

Orientador:
Prof. PhD. Marco Antônio Záchia Ayub
Co-orientador:
Prof. Dr. Rafael Costa Rodrigues

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A comissão examinadora, abaixo assinada, aprova a defesa de Tese de Doutorado, cujo projeto intitulado *síntese enzimática de biodiesel em reatores contínuos e em batelada: aspectos do uso de diversas fontes de óleos, do conceito de combi-lipases e do ultrassom* elaborada por Jakeline Kathiele Poppe Todeschini, como requisito para obtenção do grau de Doutor em Engenharia Química.

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NOMENCLATURA

Lista de Símbolos

g	grama
h	hora
L	litro
min	minuto
mL	mililitro
mg	miligramas
mm	milímetro
°C	graus Celcius
rpm	rotações por minuto
t	tempo
μL	microlitro
MHz	Mega Hertz

Lista de Símbolos Gregos

α – Conformação *α*-hélice enzimática

β – Conformação *β*-pregueada enzimática

Lista de Abreviaturas e Siglas

CG – Cromatógrafo gasoso

FID – Traduzido do inglês, “Detector de ionização de chama”

ANP **–** Agência Nacional do Petróleo, Gás Natural e Biocombustíveis

ASTM – Traduzido do inglês, “Sociedade Americana de Testes e Materiais”

DCCR – Delineamento Composto Central Rotacional

MSR – Metodologia de Superfície de Resposta

CBPBR – Traduzido do Inglês, “Reator de leito empacotado com recirculação”

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RESUMO

O processo de transesterificação de óleos vegetais para a síntese de biodiesel por catálise básica tem sido utilizado largamente em escala industrial e altas conversões são obtidas. Entretanto, uma grande quantidade de água é necessária para a purificação dos ésteres, gerando altos volumes de rejeitos aquosos inadequados para descarte, e dessa forma, a utilização de síntese enzimática catalisada por lipases imobilizadas destaca-se como uma alternativa ao método alcalino. Este trabalho teve como objetivo aperfeiçoar a produção de biodiesel a partir de diferentes fontes de óleos vegetais por diferentes lipases imobilizadas comercialmente. Na primeira fase do trabalho foi realizada uma análise dos principais fatores envolvidos na síntese enzimática de biodiesel, com foco nos parâmetros envolvidos na escolha e na configuração dos reatores. Uma extensa discussão foi apresentada sobre as vantagens e desvantagens de cada tipo de reator e seu modo de funcionamento. O cenário atual do mercado de síntese enzimática de biodiesel e algumas perspectivas futuras também foram apresentadas. Na segunda etapa desta pesquisa foi testado o conceito "*combi-lipase*", que se baseia no uso de misturas de lipases com diferentes especificidades para a cadeia molecular de um óleo em particular (o substrato). Neste caso, foram utilizadas as lipases comerciais imobilizadas Novozym 435 (CALB), Lipozyme TL-IM (TLL), e Lipozyme RM-IM (RML) como biocatalisadoras na síntese de biodiesel via transesterificação enzimática dos óleos de oliva e palma, com etanol como aceptor acila. Repetidas reações em batelada foram realizadas para testar a estabilidade operacional do sistema *combi-lipase*, em que elas puderam ser usadas em pelo menos sete ciclos, mantendo em torno de 80 % da sua atividade inicial. Dando sequencia ao conceito de *combi-lipase*, na terceira etapa dessa pesquisa, foi avaliada a utilização de substratos alternativos, como os provenientes de fritura doméstica e comercial, comparada ao óleo de soja. As reações foram conduzidas em banho de ultrassom, com a otimização dos parâmetros razão molar etanol:óleo, quantidade de água adicionada na reação e quantidade de biocatalisador (previamente à definição da composição do c*ombi-lipase*). O uso de tecnologia de ultrassom, concomitante com a aplicação de misturas de enzimas com diferentes preços de aquisição e uso de óleos residuais apresentou excelentes rendimentos, com 90 % (com óleo de soja) e 70 % (com óleo residual) de conversão de biodiesel. Na última etapa deste trabalho, foi conduzida a síntese contínua de biodiesel em um reator de leito empacotado (PBR) com o uso de etanol e dos substratos óleos de soja e óleo residual,

utilizando *combi-lipase* com biocatalisador. Após a otimização de alguns parâmetros de reação, foram definidas as seguintes condições: utilização de pérolas de vidro misturada ao *combi-lipase* para compor o leito enzimático; uso de *terc*-butanol como solvente de reação e velocidade de fluxo de 0,08 mL min^{-1}. O *combi-lipase* apresentou excelente estabilidade operacional, e o reator manteve-se operando continuamente por 30 dias em estado estacionário. Independente do tipo de substrato empregado, o rendimento de conversão manteve-se em torno de 50 %, com produtividade de 1,94 $g_{biodiesel}$ $g_{substrato}^{-1}$ h^{-1}.

Palavras-chaves: Biodiesel; *combi-lipase*; reatores enzimáticos em batelada; reatores enzimáticos contínuos, ultrassom; óleos residuais.

ABSTRACT

The process of transesterification of vegetable oils for biodiesel synthesis catalyzed by alkalis has been widely used on an industrial scale and high conversions are obtained. However, a large amount of water is required for the purification of esters, generating large amounts of aqueous wastes, unsuitable for disposal. Therefore, the enzymatic synthesis of biodiesel using immobilized lipases as biocatalysts stands as an alternative to the alkaline method. This work aimed at enhancing the production of biodiesel from different sources of vegetable oils using different immobilized commercially available lipases. In the first step of the work, an analysis of the main factors involved in enzymatic synthesis of biodiesel was carried out, focusing on choices of immobilization protocol and parameters involved in the selection and configuration of the reactors. An extensive discussion is presented on the advantages and disadvantages of each type of reactor and its operation. The current scenario of the enzymatic synthesis of biodiesel market and some future prospects are also presented. In the second stage of this study it was tested the concept "*combi-lipase*", which is based on the use of lipase mixtures with different specificities for a particular oil, the substrate. The immobilized commercial lipases Novozym 435 (CALB), Lipozyme TL-IM (TLL), and Lipozyme RM-IM (RML) were used as biocatalysts in enzymatic transesterification of biodiesel from olive and palm oils, with ethanol as acyl acceptor. Repeated batches of reaction were carried out in order to test the operational stability of the *combi-lipase* systems, with results showing that they could be used for at least seven cycles keeping higher than 80 % of their initial activities. Following the concept of *combi-lipase*, in the third stage of this research, it was evaluated the use of alternative substrates, such as waste frying oils, compared to soybean oil. The reactions were conducted in an ultrasonic bath, with the optimization of the molar ratio of ethanol: oil, amount of water added in the reaction and amount of biocatalyst (prior to the definition of the *combi-lipase* composition). The use of ultrasonic technology, concomitant with the application of mixtures of enzymes with different acquisition and use prices of residual oils presented excellent yields, with 90 % (with soybean oil) and 70 % (with residual oil) of biodiesel conversion. Finally, in the last stage of this work, the *combi-lipase* concept was applied in the continuous ethanolysis of biodiesel in a packed bed reactor (PBR) with the use of soybean and waste oils as substrates. After optimization of some reaction parameters, the following conditions were defined: use of glass beads mixed with lipases to compose the

enzymatic bed; Use of *tert*-butanol as reaction solvents and flow rate of 0.08 mL min^{-1}. The *combi-lipase* presented excellent operational stability, and the reactor was continuously operated for 30 days at steady state. Regardless of the type of substrate used, the conversion yield remained around 50 %, with productivity of 1.94 $g_{biodiesel}$ $g_{substrate}^{-1}$ h^{-1}.

Keywords: Biodiesel; *combi-lipase*; enzymatic batch reactors; enzymatic continuous reactors; ultrasound system; waste oils.

SUMÁRIO

INTRODUÇÃO

A possibilidade do emprego de combustíveis de origem agrícola em motores de ciclo diesel é bastante atrativa tendo em vista o aspecto ambiental, por ser uma fonte renovável de energia e na destinação de resíduos agrícolas e agroindustriais, além do fato do seu desenvolvimento permitir a redução da dependência de importação de petróleo para o Brasil. Nesse âmbito pesquisas científicas voltadas para a descoberta de novas fontes de energia ganham foco, entre elas, estudos com biodiesel. De acordo com a "National Biodiesel Board" (EUA), o biodiesel é definido como sendo um monoalquil éster de ácidos graxos de cadeia longa derivados de fontes renováveis, como óleos vegetais e gorduras animais (Knothe, 2006).

Diversas sementes oleaginosas podem ser utilizadas como matéria prima para produzir biodiesel, destacando-se a soja, o girassol, a canola e a palma, e a escolha entre elas é fortemente influenciada pela cultura de cada região. Por outro lado, dada a grande geração de resíduos oleosos advindos de atividades industriais, torna-se importante explorar fontes alternativas destes óleos, como óleos de cozinha e gorduras animais (Antczak *et al.*, 2009).

O biodiesel pode ser obtido através de diversas reações químicas, entre elas, o processo de transesterificação entre um triglicerídeo e um álcool de cadeia curta (metanol ou etanol) tem sido amplamente citado (Deng *et al.*, 2005; Chesterfield *et al.*, 2012; Aarthy *et al.*, 2014). Esta reação pode ser mediada por catálise básica ou ácida, entretanto, em razão da maior rapidez, simplicidade e eficiência, a catálise em meio alcalino ainda prevalece como a rota tecnológica mais frequentemente utilizada para a produção industrial de biodiesel. No entanto, um de seus maiores inconvenientes está relacionado à inevitável produção de sabões, tanto pela neutralização dos ácidos graxos livres quanto pela saponificação dos triglicerídeos. Reações secundárias como estas são indesejáveis, pois além de consumirem parte do catalisador, dificultam o processo de separação do glicerol e a purificação do biodiesel, diminuindo o rendimento da reação (Leung *et al.*, 2010).

O custo da purificação do glicerol é em torno de US$ 400,00/ton e o seu preço varia entre US$ 1,30 a US$ 2,00/kg. A glicerina bruta (50 % a 90 % em glicerol) é vendida por preços inferiores, que dependem do conteúdo de glicerol, do tipo e quantidade de contaminantes presentes (Mota *et al.*, 2009). Portanto, reduzir os custos envolvidos na purificação deste subproduto do biodiesel, e obter um biocombustível

com elevada pureza é um fator chave para a sua comercialização. Entre os processos testados para contornar alguns destes problemas, a utilização de enzimas é um dos mais promissores na produção do biodiesel por transesterificação (Zheng *et al.*, 2009). A síntese enzimática de biodiesel, mediada através do uso de lipases (EC 3.1.1.3, triacilglicerol hidrolases) apresenta vantagens importantes sobre os catalisadores químicos, tais como a especificidade, a regiosseletividade e a enantiosseletividade das reações, gerando dessa forma, menos produtos secundários e menor geração de efluentes. Além disso, as reações podem ser realizadas em condições moderadas de temperatura e pressão (Ghaly *et al.*, 2010).

O custo atual de aquisição de lipases ainda é a principal limitação que impede a sua utilização em processos de grande escala. Dessa forma, diversas pesquisas que visam à redução desses gastos têm sido desenvolvidas, como: produção de lipases utilizando substratos de baixo valor agregado para serem utilizadas em reações de síntese de biodiesel (Barbosa *et al.*, 2011); uso de solução aquosa de lipases insolúveis aos substratos da reação e ao biodiesel (Pedersen *et al.*, 2014); enzimas imobilizadas em ciclos repetidos de operação (Poppe *et al.*, 2013); o desenvolvimento e aperfeiçoamento de técnicas de imobilização (Forde *et al.*, 2010; Rodrigues *et al.*, 2012); a melhoria de parâmetros de reação em reatores em batelada e contínuos (Halim *et al.*, 2009).

A utilização de lipases imobilizadas facilita o desenvolvimento de processos em escala comercial, favorecendo numerosos processos biotecnológicos devido às vantagens que estes sistemas proporcionam, como por exemplo, o aumento da produtividade. Estas vantagens tornam-se ainda mais interessantes com estudos de aumento de escala de produção, onde as reações são desenvolvidas em reatores.

Com base nesses aspectos, o objetivo principal deste trabalho foi estudar a síntese de ésteres alquílicos (biodiesel), a partir de diferentes fontes lipídicas, via transesterificação enzimática, além de propor melhorias nos sistemas de reatores enzimáticos.

Os objetivos específicos foram:

1. Sintetizar ésteres etílicos via catálise enzimática;
2. Avaliar o uso combinado de lipases imobilizadas comercialmente na conversão em biodiesel;
3. Comparar os óleos vegetais de oliva, palma, soja e residual na síntese de biodiesel;

4. Verificar, através de um planejamento estatístico, a influência das variáveis de processo: quantidade de *combi-lipase*, temperatura de reação e razão molar álcool:óleo sobre a conversão de biodiesel;
5. Comparar sistema de agitação mecânico e em banho de ultrassom na conversão de biodiesel;
6. Testar o reuso dos biocatalisadores em vários ciclos de reação em batelada;
7. Verificar a influência de diferentes solventes orgânicos e diferentes taxas de fluxo sobre a conversão de biodiesel em reatores operados no modo contínuo;
8. Discutir aspectos importantes que envolvam a utilização de reatores aplicados no processo enzimático de síntese de biodiesel, mostrando o estado atual e as perspectivas futuras de suas aplicações.

Introdução aos capítulos I, II, III, IV e V.

Esta Tese encontra-se organizada em capítulos, os quais apresentarão os trabalhos resultantes do período de doutorado. O Capítulo I apresenta o embasamento teórico pertinente ao desenvolvimento deste estudo. Os Capítulos II e III estão na forma de artigo publicado, e os Capítulos IV e V estão na forma de artigo submetido para publicação. Os quatro trabalhos foram estruturados de acordo com a norma de cada revista. Neles são descritas as metodologias empregadas na condução dos experimentos, bem como a análise e discussão dos resultados.

No Capítulo II "Enzymatic reactors for biodiesel synthesis: Present status and future prospects", foi realizado um estudo de revisão bibliográfica onde foram analisados os principais fatores envolvidos na síntese enzimática de biodiesel, com foco na escolha do protocolo de imobilização e os parâmetros envolvidos na escolha e configuração dos reatores enzimáticos.

Este artigo encontra-se publicado no periódico Biotechnology Advances, 33(5):511-525, 2015. Doi:10.1016/j.biotecadv.2015.01.011.

No Capítulo III "Optimization of ethyl ester production from olive and palm oils using mixtures of immobilized lipases", foi realizada a otimização do processo de síntese de biodiesel em batelada através do emprego de planejamento experimental. Foram utilizadas as lipases imobilizadas comercialmente de *Candida antarctica*, *Thermomyces lanuginosus* e *Rhizomucor miehei*. Foram avaliados os parâmetros: variação na razão molar de etanol:óleo de palma e etanol:óleo de oliva; diferentes temperaturas de reação e quantidade de *combi-lipase*. Previamente a isso, foi realizado um planejamento de mistura para a definição da combinação ideal das lipases de acordo com a especificidade das enzimas pelos diferentes ácidos graxos presentes nos óleos, para a obtenção da máxima conversão de biodiesel.

Este artigo encontra-se publicado no periódico Applied Catalysis A: General, 490: 50-56, 2015. doi:10.1016/j.apcata.2014.10.050.

O Capítulo IV "Enzymatic synthesis of biodiesel from waste frying and soybean oils using the combi-lipase concept in ultrasound-assisted reactions", descreve o uso do sistema de ultrassom para a condução das reações de transesterificação enzimática de biodiesel. Óleo de soja e óleo residual foram utilizados como fonte lipídica, e com base

na composição de seus ácidos graxos foi novamente definido o *combi-lipase* entre as mesmas lipases: CALB, RML e TLL.

Esse trabalho foi submetido ao periódico Renewable Energy e ainda encontra-se em revisão.

No Capítulo V "Continuous enzymatic biodiesel synthesis in a plug-flow packed-bed reactor using combi-lipases and different oil sources", foram definidos as configurações de algumas variáveis envolvidas na utilização de reatores de leito empacotado, e por fim foi avaliada a estabilidade operacional do *combi-lipase* em processo contínuo.

Esse trabalho foi submetido para a revista Biochemical Engineering Journal e ainda encontra-se em revisão.

Para finalizar, são apresentadas as considerações finais deste trabalho e as principais conclusões, bem como as perspectivas futuras para dar continuidade a essa pesquisa. Ao final, estão incluídos anexos relevantes para a complementação do trabalho.

CAPÍTULO I – REVISÃO BIBLIOGRÁFICA

1.1. Aspectos gerais do biodiesel

A possibilidade do emprego de combustíveis de origem agrícola em motores de ciclo diesel é bastante atrativa tendo em vista o aspecto ambiental, por ser uma fonte renovável de energia e na destinação de resíduos agrícolas e agroindustriais. Com isso, destaca-se o biodiesel, que é tido como um substituto natural do diesel de petróleo.

Atualmente, a cadeia produtiva do biodiesel está em expansão devido às mudanças climáticas, segurança energética, necessidade crescente de combustíveis e ausência de outras soluções alternativas no curto prazo. Na comparação com o diesel de petróleo, o biodiesel tem significativas vantagens ambientais. Estudos do *National Biodiesel Board* (associação que representa a indústria de biodiesel nos Estados Unidos) demonstraram que a queima de biodiesel pode emitir em média 48 % menos monóxido de carbono; 47 % menos material particulado (que penetra nos pulmões); 67 % menos hidrocarbonetos. Esses percentuais variam de acordo com a quantidade de B100 adicionado ao diesel de petróleo (Parente Jr, 2016).

O biodiesel pode ser utilizado puro ou misturado com diesel, em diferentes proporções, pelo que é utilizada uma nomenclatura particular de modo a identificar mundialmente a concentração de biodiesel em mistura. É utilizada a classificação BXX de forma distintiva, onde XX é a percentagem de biodiesel na mistura, como, por exemplo, B20 que representa uma mistura de 20 % de biodiesel e 80 % de diesel (Issariyakul *et al.*, 2014).

No Brasil, o biodiesel foi introduzido na matriz energética pela Medida Provisória nº. 214, de 13/9/2004. Nesta norma, o biodiesel, combustível composto de monoalquil ésteres de ácidos graxos de cadeia longa, é conceituado como "um combustível para motores a combustão interna com ignição por compressão, renovável e biodegradável, derivado de óleos vegetais ou de gorduras animais, que possa substituir parcial ou totalmente o óleo diesel de origem fóssil" (ANP, 2008).

O Programa Nacional de Produção e Uso do Biodiesel (PNPB) foi lançado pelo Presidente da República em dezembro de 2004. De 2005 a 2007, a adição de dois por cento de biodiesel ao diesel fóssil era facultativa, evoluindo para ser obrigatória, no mesmo percentual (2 %), de 2008 a 2012. A antecipação do percentual de 5 % de

biodiesel ao diesel (antes prevista para 2013, mas efetivada em 2010) representou um consumo de 2,6 bilhões de litros em 2011, e acelerou a reivindicação de aumento da mistura (Ipea, 2012). Desde 1º de novembro de 2014, o óleo diesel comercializado em todo o Brasil contém 7 % de biodiesel (Anp, 2014). Com o Novo Marco Regulatório de 23 de março de 2016, esse percentual passará para 8 % até março de 2017, chegando a 10 % em até 36 meses (Ubrabio, 2016).

A indústria brasileira tem capacidade de produzir 7,4 bilhões de litros de biodiesel ao ano, mas, em 2016, foram produzidos apenas 3,8 bilhões de litros para atender o mercado obrigatório, limitado à demanda de diesel, que, por sua vez, reflete a atividade econômica reduzida nos últimos dois anos (Ubrabio, 2016). No entanto, essa retração econômica não freou o crescimento da indústria de biodiesel, que é a que mais cresce entre os combustíveis líquidos há 10 anos (Parente Jr, 2016).

O Brasil permanece como sendo o segundo maior produtor mundial de biodiesel, correspondendo a 23,8 % da produção total, estando atrás apenas dos EUA (53,6 %), ou seja, 3,4 e 4,8 bilhões de litros, respectivamente (Secretaria de Minas e Energia, 2012). No país, as regiões Sul e Centro-Oeste seguem como sendo as principais produtoras de biodiesel, correspondendo a 82,9 % da produção em 2015 (Parente Jr, 2016).

Várias políticas governamentais em todo o mundo estimulam a produção de biocombustíveis, definindo metas para as quotas de mistura, e impulsionando o desenvolvimento de tecnologias de biocombustíveis através da criação de mecanismos de apoio financeiro e político (Geraldes *et al.*, 2014). A produção e o uso do biodiesel no Brasil propiciam o desenvolvimento de uma fonte energética sustentável sob os aspectos ambiental, econômico e social. Além da diminuição da dependência do diesel importado, o biodiesel traz outros efeitos indiretos de sua produção e uso, como o incremento a economias locais e regionais, tanto na indústria de bens e serviços como na etapa agrícola de produção e processamento das matérias-primas utilizadas (Anp, 2014).

1.2. Matérias-primas para produção de biodiesel

O Biodiesel pode ser produzido a partir de óleo vegetal, gordura animal, óleos e gorduras residuais (Issariyakul *et al.*, 2014), óleos produzidos por algas (Nautiyal *et al.*, 2014) e cianobactérias (Karatay *et al.*, 2011), juntamente com um agente acilante. Considerando que a matéria-prima corresponde a 70-80 % do custo do biocombustível

(na rota alcalina), é importante uma cuidadosa seleção dos reagentes que irão compor o meio reacional (Hwang *et al.*, 2014)

Os óleos vegetais usados como matéria-prima para produção de biodiesel normalmente dependem da produção regional, por exemplo, como o óleo de canola nos países europeus e no Canadá, o óleo de soja nos Estados Unidos e Brasil, e óleo de palma em países tropicais como Indonésia e Malásia. O óleo de coco é outra matéria-prima lipídica utilizada em zonas costeiras (Issariyakul *et al.*, 2014). No Brasil, o óleo de soja representa cerca de 70 % de fonte lipídica, seguido de gordura bovina (16 %) (Anp, 2017).

Estas fontes lipídicas provenientes de oleaginosas são consideradas como a matéria-prima de biodiesel de primeira geração, sendo empregadas em mais de 95 % das indústrias devido à consolidada produção mundial. Entretanto, a competição com a cadeia alimentícia resulta na elevação dos preços do biocombustível e do óleo comestível, gerando limitações no emprego desses óleos na síntese (Atabani *et al.*, 2012). Matérias-primas de baixo custo tais como resíduos de óleo de fritura, óleos não comestíveis, banha, gorduras animais, entre outros, tidos como fontes para biodiesel de segunda geração, são utilizados no intuito de reduzir os custos de produção e de problemas ambientais, tornando a produção de biodiesel comercialmente mais competitiva com o diesel de petróleo (Christopher *et al.*, 2014).

No Brasil, em 2015 cerca de 700 mil toneladas de gordura animal foram utilizadas na indústria de biodiesel, representando cerca de 20 % da matéria-prima processada. Tal mercado gerou uma agregação de valor de mais de R$ 1 bilhão de um material que poderia ter sido descartado como resíduos por falta de destinação comercial (Parente Jr, 2016).

Óleos vegetais residuais são considerados uma fonte promissora para obtenção do biocombustível, em função do baixo custo e por envolver reciclagem de resíduos (Diya'uddeen *et al.*, 2012). Estes óleos representam riscos de poluição ambiental e a maior parte destes ainda prevalece sem qualquer proposta de destinação final adequada ou solução definitiva. Em estudo desenvolvido por Geris et al. a taxa de conversão de biodiesel a partir do óleo *in natura* e do usado em fritura foi muito similar, demonstrando ser uma alternativa viável e promissora o uso de óleos residuais. Dessa forma, ressalta-se a necessidade de que o óleo de fritura usado comece a ser aproveitado em larga escala para a fabricação de biodiesel, a fim de evitar os impactos ambientais advindos da incorreta destinação do mesmo (Geris *et al.*, 2007).

No que diz respeito a escolha do álcool utilizado na reação, sua seleção influencia na necessidade de uso de um solvente orgânico. Álcoois de cadeia menor, como metanol e etanol, apresentam uma solubilidade menor aos óleos normalmente utilizados para produção de biodiesel. Álcoois com cadeia maior, como propanol ou butanol, apresentam melhor solubilidade ao óleo e dispensam o uso de um solvente orgânico na reação. Em suma, os melhores resultados de rendimento de biodiesel são obtidos com a utilização de álcoois de cadeia linear, com baixo impedimento estérico, tal como metanol, etanol, propanol, butanol, pentanol e intermediários (Leung *et al.*, 2010). O metanol é mais amplamente aplicado em escala comercial e, por ser mais reativo, implica em menor temperatura e tempo de reação. O etanol, além de ter produção consolidada no Brasil, é consideravelmente menos tóxico, é renovável, produz biodiesel com maior número de cetano e lubricidade, com maior estabilidade oxidativa e índice de iodo inferior, entre outras propriedades (Banković-Ilić *et al.*, 2012).

1.3. Síntese de biodiesel

A aplicação direta dos óleos vegetais nos motores é limitada por algumas propriedades físicas e químicas dos mesmos, principalmente sua alta viscosidade, a acidez, a baixa volatilidade, os ácidos graxos livres contidos nos óleos, a presença de gomas, e o caráter poli-insaturado, que acarretam alguns problemas aos motores, bem como uma combustão incompleta (Knothe, 2006). Visando reduzir a viscosidade dos óleos vegetais, diferentes alternativas têm sido consideradas, e entre elas a reação de transesterificação tem se apresentado como a melhor opção, visto que o processo é relativamente simples promovendo a obtenção de um combustível cujas propriedades são similares as do óleo diesel (Geris *et al.*, 2007).

A transesterificação é composta por uma reação entre o óleo vegetal ou gordura animal e um álcool primário na presença de um catalisador, resultando em uma mistura de ésteres alquílicos de ácidos graxos (biodiesel) e glicerol (De Araújo *et al.*, 2013). O excesso de álcool na reação é necessário a fim de aumentar o rendimento de ésteres e de promover o deslocamento do equilíbrio da reação no sentido do produto (Suarez *et al.*, 2007). De forma geral, a reação de transesterificação pode ser descrita como uma reação reversível em que um éster é transformado em outro pela mudança na porção alcóxi. Esta transformação ocorre em três etapas sequenciais: inicialmente, as moléculas de triglicerídeos são convertidas em diglicerídeos, depois em monoglicerídeos e,

finalmente, em glicerol, produzindo um mol de éster a cada etapa reacional (Figura 1.1.).

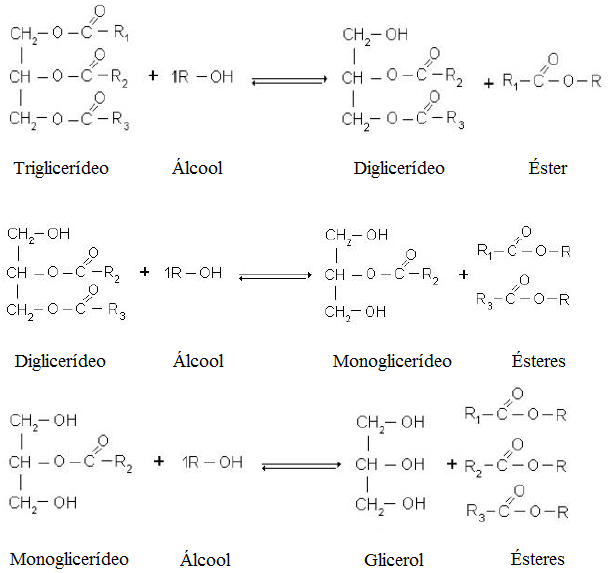

Figura 1.1. Etapas da transesterificação dos triglicerídeos.

Um catalisador é normalmente utilizado para acelerar a reação, podendo ser básico, ácido ou enzimático. O mais comum é a utilização de catalisadores básicos, tais como hidróxido de potássio (KOH) e hidróxido de sódio (NaOH) onde são observados maior rendimento e seletividade. Como catalisadores ácidos são geralmente usados ácido sulfúrico (H_2SO_4) e ácido clorídrico (HCl) (Tan *et al.*, 2010), entretanto, essa reação requer tempos mais longos e uma proporção molar mais elevada de álcool:óleo. Em razão da maior rapidez, simplicidade e eficiência, a catálise em meio alcalino ainda prevalece como a rota tecnológica mais frequentemente utilizada para a produção industrial de biodiesel, entretanto, apresenta o inconveniente da inevitável produção de sabões causada por saponificação dos ácidos graxos livres e pela hidrólise dos triglicerídeos em diglicerídeos e ácidos graxos livres causados pelo teor de água na reação. Estas reações secundárias são indesejáveis porque consomem parte do catalisador, dificultam o processo de separação e purificação de glicerol e do biodiesel, reduzindo o rendimento da reação (Leung *et al.*, 2010).

Embora a catálise química seja realizada industrialmente em muitos países para produção de biodiesel devido ao seu alto rendimento, a reação apresenta algumas desvantagens como o consumo elevado de energia, dificuldade na transesterificação de gorduras com alto teor de ácidos graxos livres, dificuldade de recuperação de glicerol e

remoção de sais inorgânicos e água do produto, e o tratamento de águas residuais alcalinas são processos complexos e requerem custos adicionais (Bajaj *et al.*, 2010).

O glicerol é um importante subproduto advindo da reação de síntese de biodiesel, representando cerca de 10 % do processo de transesterificação. É um produto nobre, que após purificação, pode ser utilizado principalmente na indústria farmacêutica, de cosméticos, alimentícia, de bebidas, tabaco e resinas alquilícas (Ayoub *et al.*, 2012). O glicerol derivado da catálise básica apresenta cerca de 30 % de impurezas, e são necessários complexos processos para que essa matéria-prima alcance tal grau de pureza visando aplicação industrial. Um dos procedimentos adotados é a cromatografia de troca iônica, o que tem um custo elevado e reduz a eficiência da reciclagem do álcool (Lopes *et al.*, 2014). Com isso, o surgimento de novas rotas de transesterificação de biodiesel, como as que utilizam catalisadores enzimáticos, emerge como alternativa para contornar algumas dessas dificuldades (Hwang *et al.*, 2014).

1.3.1. Transesterificação enzimática

Embora os processos de transesterificação enzimática para obtenção de biodiesel ainda não sejam amplamente desenvolvidos a nível industrial, novos e promissores resultados têm sido reportados, principalmente utilizando lipases imobilizadas (Huang *et al.*, 2010; Rodrigues, Pessela, *et al.*, 2010; Abdulla *et al.*, 2013; Bergamasco *et al.*, 2013; Arumugam *et al.*, 2014). Tais estudos são de extrema importância para o desenvolvimento de novas rotas de processo a custos mais competitivos.

A transesterificação catalisada por lipases é aplicável aos óleos vegetais refinados e brutos contendo ácidos graxos livres (AGL), resíduos de gorduras e de fritura. Como doadores acila, diversos álcoois podem ser empregados, entre os principais, destacam-se o metanol e o etanol, mas há relatos da utilização de butanol e propanol (Rodrigues *et al.*, 2008), todos utilizados em excesso para proporcionar alto rendimento na reação.

As lipases apresentam vantagens importantes sobre os catalisadores químicos, como a especificidade, a regiosseletividade e a enantiosseletividade, que permitem a catálise de reações com um número reduzido de coprodutos, menor geração de efluentes e sob condições mais brandas de temperatura e pressão (Ghaly *et al.*, 2010). Além disso, a atividade enzimática pode ser regulada com relativa facilidade, bastando modificar as condições do meio de reação, como, por exemplo, pela alteração do valor de pH ou pela

adição de algum inibidor. Os processos industriais que empregam enzimas são, em geral, relativamente simples, fáceis de controlar e energeticamente eficientes (Oliveira *et al.*, 2009).

1.4. Lipases

1.4.1. Considerações importantes

As enzimas são catalisadores biológicos de natureza proteicos constituídos por longas cadeias de aminoácidos, unidos por ligações peptídicas, segundo um arranjo tridimensional. Apresentam uma elevada eficiência catalítica em condições operacionais suaves, como temperaturas brandas, pressão atmosférica e pH próximo do neutro, o que permite realizar transformações químicas complexas mesmo quando em pequenas quantidade (Betsy *et al.*, 2005). São, portanto, um tipo de proteína com atividade catalítica, sendo que a singularidade desses compostos decorre do elevado grau de especificidade ao substrato sob as quais atuam.

As lipases classificam-se como enzimas que pertencem à classe das hidrolases (E.C.3.1.1.3), ou seja, são glicerol éster hidrolases capazes de hidrolisar triacilgliceróis (principais componentes de óleos e gorduras) liberando ácidos graxos livres, glicerol, mono e diacilgliceróis. Essas enzimas também catalisam reações de esterificação e transesterificação quando presentes em meios aquo-restritos (Mendes *et al.*, 2011). Dependendo da fonte, as lipases variam sua massa molecular entre 20 e 75 KDa com cerca de 300 resíduos de aminoácidos, com atividade em pH na faixa de 4 a 9 e entre temperaturas desde ambiente até 70 ºC (Castro *et al.*, 2004).

A maioria das lipase utilizadas como catalisadoras em síntese orgânica é de origem microbiana, como as lipases de *Candida rugosa* (Moreno-Pirajàn *et al.*, 2011), *Pseudomonas fluorescens* (Devanesan *et al.*, 2007), *Rhizhopus oryzae* (Li *et al.*, 2007), *Burkholderia cepacia* (Kawakami *et al.*, 2011), *Aspergillus niger* (Xiao *et al.*, 2011), *Thermomyces lanuginosus* (Fernandez-Lafuente, 2010), *Rhizomucor miehei* (Al-Zuhair *et al.*, 2007; Rodrigues e Fernandez-Lafuente, 2010) e *Candida antarctica* (Liu *et al.*, 2010).

O sítio catalítico das lipases é bastante complexo e a estrutura da enzima ao redor do sítio ativo varia significativamente de uma lipase para outra. Entretanto, alguns elementos estruturais são comuns para todas as lipases: a tríade catalítica, formada por

resíduos dos aminoácidos serina, histidina e aspartato (ou glutamato) e um dobramento característico na conformação α/β hidrolase (Jaeger *et al.*, 1994).

A tríade de aminoácidos é frequentemente protegida na molécula por uma cadeia polipeptídica hidrofóbica em forma de α-hélice comumente chamada de tampa, ou *lid*, que, ao interagir com a interface lipídeo/água, sofre uma mudança conformacional expondo o sítio ativo. Em ambientes aquosos, sem o seu substrato natural, a "tampa" cobre o sítio catalítico, a lipase é inativa, e diz-se que ela está na sua forma "fechada" (Jaeger *et al.*, 1999). Na presença de substratos hidrofóbicos, as lipases são "adsorvidas" na interface hidrofóbica, o que promove mudanças drásticas na estrutura enzimática, levando à forma "aberta" da lipase, deixando a enzima ativa. Nesta conformação, o movimento do *lid*, não apenas abre o acesso ao sítio ativo como também expõe uma extensa zona hidrofóbica que interage favoravelmente com a interface lipídica (Derewenda *et al.*, 1992). Na Figura 1.2 é apresentado o mecanismo geral de ativação interfacial de lipases, mostrando o equilíbrio de ambas as formas fechadas e abertas.

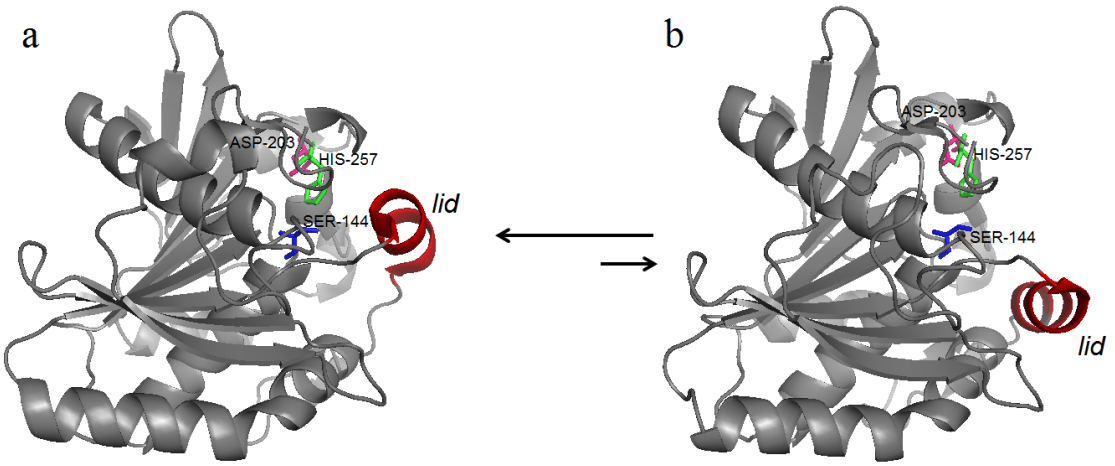

Figura 1.2. Distintas conformações da lipase de *Candida rugosa*. (a) Conformação fechada (PDB-3TGL); (b) Conformação aberta (PDB-4TGL). Estruturas 3D obtidas através do Protein Data Bank (PDB) utilizando o software Pymol v. 0.99.

As principais vantagens de utilizar lipases, em comparação com a reação química convencional, são a sua especificidade pelo substrato e a seletividade. Essas características são controladas pelas propriedades moleculares da enzima, a estrutura do substrato, e os fatores que afetam a ligação da enzima com o substrato. Com isso, pelo fato da alta especificidade da enzima pelo substrato, é necessário conhecer as

características da lipase para encontrar o substrato ideal para uso nas reações. Conforme a classificação das enzimas, as lipases podem ser divididas em: enantiosseletivas; tipo-seletivas; e regiosseletivas (Paques *et al.*, 2006).

Lipases ácido graxo específicas ou tipo seletiva são específicas em relação ao tamanho da cadeia carbônica e/ou ao número de insaturações do grupo acila, por outro lado, lipases enantiosseletivas têm capacidade de discriminar enantiômeros em uma mistura racêmica. Tal especificidade da lipase pode mudar com o substrato e esta mudança pode ser relacionada à natureza química do éster (De Carvalho, 2011). Por fim, as lipases regiosseletivas possuem especificidade em relação à posição do grupo funcional da molécula no substrato. Nesta última classificação, as enzimas ainda subdividem-se em (Valverde *et al.*, 2013):

- Lipases 1,3 específicas: catalisam a liberação de ácidos graxos especificamente das posições *sn*-1 e *sn*-3 dos acilgliceróis. As lipases de *Thermomyces lanuginosus, Rhizomucor miehei, Rhizopus delemar* e *Aspergillus niger* pertencem a este grupo.
- Lipases ácido graxo específicas: catalisam a hidrólise de tipos específicos de grupos acilas nas moléculas de triacilgliceróis. Um representante típico deste grupo é a lipase de *Geotrichum candidum* que hidrolisa preferencialmente grupos acila de cadeia longa, que contenham dupla ligação *cis* na posição 9;
- Lipases não específicas: catalisam a hidrólise de triacilgliceróis para ácidos graxos livres e glicerol, de modo aleatório. Não mostram especificidade com relação à natureza do grupo acil ou à posição em que este está esterificado no glicerol. São exemplos as lipases de *Candida antarctica, Penicilium cyclopium, Corynebacterium acnes, Pseudomonas fluorescens* e *Staphylococcus aureus*.

Como os óleos e gorduras utilizados nas reações são constituição heterogêneas, o uso combinado de lipases que atuam com diferentes especificidades para substratos é uma estratégia que tem sido amplamente relatado para aumentar a produtividade em reações de biocatálise. O trabalho de Alves et al. (2014) apresentou um novo conceito de biocatalisador denominado *combi-lipase* para substratos heterogêneos. Este conceito foi baseado no fato de que um biocatalisador composto por uma mistura de diferentes lipases deve ser mais eficaz sobre substratos heterogêneos do que uma lipase única e específica O uso do *combi-lipase* (*Candida antarctica+Thermomyces lanuginosus+Rhizomucor miehei*) proporcionou excelentes resultados quando

comparado com a utilização individual das enzimas na hidrólise do óleo de soja (Alves *et al.*, 2014).

Contudo, apesar das notáveis vantagens de utilizar a catálise enzimática, problemas como o alto custo de produção e purificação das enzimas e a cinética relativamente lenta do processo tem se tornado o maior obstáculo para a produção em grande escala de biodiesel. A recuperação com o reuso da enzima representa uma das opções e, para isso, a imobilização da enzima em suportes sólidos adequados tem sido utilizada para atingir esses objetivos.

1.4.2. Imobilização de lipases

A imobilização tem como finalidade aproveitar o potencial catalítico das enzimas e torná-las insolúveis ao meio reacional permitindo sua reutilização e facilitando a recuperação dos produtos, além de minimizar a produção de efluentes (Zanin *et al.*, 2004). Além disso, muitas vezes com o processo de imobilização, as propriedades enzimáticas são alteradas, produzindo biocatalisadores com atividade, especificidade e estabilidade aumentadas, dependendo do tipo de imobilização e da enzima (Narwal *et al.*, 2013). Dessa forma, ao se obter uma enzima imobilizada ativa, estável e com boa especificidade ao substrato, a maioria das desvantagens das enzimas durante o processo catalítico é eliminada.

Lipases imobilizadas são geralmente usadas para executar biotransformações das mais interessantes aplicações industriais, que muitas vezes ocorrem em meio não aquosos. Os suportes utilizados para imobilização de enzimas podem ser classificados pelo material como orgânicos e inorgânicos, ou também pela sua morfologia como não porosos ou porosos (Macario *et al.*, 2008; Mendes *et al.*, 2011).

Uma vez que a imobilização de enzimas envolve a interação da enzima e do suporte, as propriedades da superfície de ambos é de grande importância. Na superfície da enzima, os grupos polares (por exemplo, grupos amino, de lisina ou ácido glutâmico), as áreas de superfície não polares, ou porções de açúcar, podem influenciar as propriedades da sua superfície. Portanto, o suporte a ser utilizado tem de ser preparado de modo a coincidir com qualquer uma destas moléculas de superfície da enzima (Hanefeld *et al.*, 2009). Na escolha de um suporte, as suas propriedades físicas e químicas relevantes para o processo de imobilização, bem como as suas características para uma possível regeneração devem ser rigorosamente analisados.

As características físicas dos suportes serão da maior importância para o desempenho dos sistemas imobilizados quando as reações forem desenvolvidas em reatores, e será determinante na escolha do tipo de equipamento (ou seja, de operação contínua ou em bateladas) (Brena *et al.*, 2006). Parâmetros como porosidade, tamanho do poro, tamanho de partícula e área superficial disponível para imobilização afetam consideravelmente a capacidade de ligação das enzimas ao suporte.

Suportes porosos são preferencialmente escolhidos devido à sua grande área superficial interna que permite uma maior ligação de carga enzimática, e esta permanece protegida dos efeitos externos, como por exemplo, variações drásticas de pH (Hanefeld *et al.*, 2009). Entretanto, devem ter uma distribuição de poro controlada, a fim de otimizar a capacidade de carga enzimática disponível para ser imobilizada (Hernandez *et al.*, 2011; Liese *et al.*, 2013). Além disso, de acordo com Hernandez et al. suportes com diâmetro de poro muito pequeno podem ter os seus poros bloqueados pela enzima após a imobilização, deixando uma grande percentagem da superfície interna do suporte inacessível ao substrato. Isto ocasiona uma taxa de imobilização reduzida, restringindo a capacidade da ação catalítica da enzima.

As técnicas usualmente empregadas para imobilizar enzimas em suportes sólidos são baseadas em mecanismos físicos e químicos, e podem ser classificados em quatro tipos básicos: ligação ao suporte, confinamento, encapsulamento, e ligações intercruzadas (Figura 1.3.) (Dalla-Vecchia *et al.*, 2004). Nos métodos classificados como "ligação ao suporte", a ligação da enzima é mediada por ligações covalentes, iônicas ou de adsorção (através de interações iônicas, forças de van der Waals, ligações de hidrogênio, interações dipolo-dipolo ou interações hidrofóbicas) (Hanefeld *et al.*, 2009).

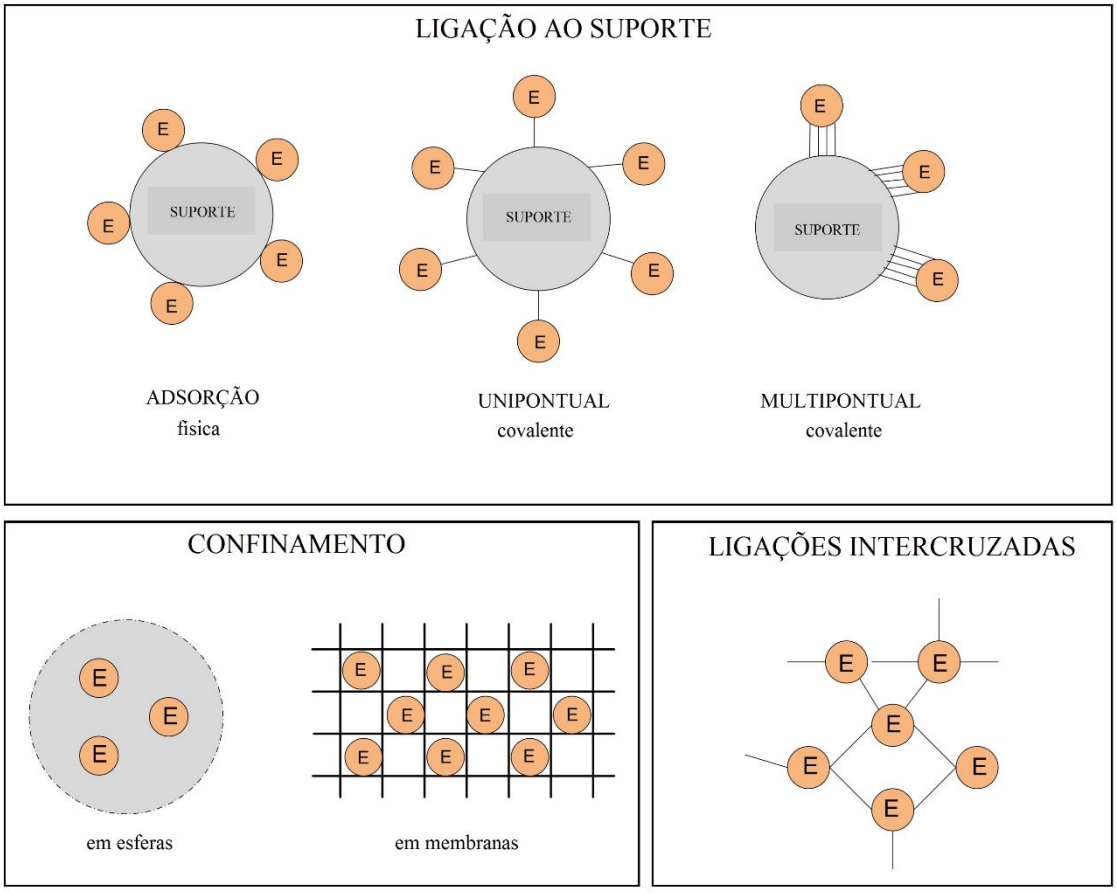

Figura 1.3. Representação das técnicas mais usualmente utilizadas para imobilizar enzimas.

A adsorção é o método mais simples e de menor custo, e consiste na incubação do suporte em uma solução enzimática e nesse processo ocorre a transferência de proteínas (adsorvato) para uma superfície sólida (adsorvente). As forças de ligação envolvidas nesse processo são estabilizadas por mudanças hidrofóbicas, onde enzimas que possuem uma extensa superfície hidrofóbica irão interagir melhor com suportes hidrofóbicos, enquanto que enzimas glicosiladas ou com extensa porção hidrofílica interagem favoravelmente com suportes hidrofílicos (Hanefeld *et al.*, 2009). Entretanto, este método de imobilização é limitado pela tendência de desorção da enzima do suporte e por ser sensível às condições do ambiente, como temperatura e concentração de íons (Grosova *et al.*, 2008).

Os métodos baseados no confinamento da enzima envolvem a polimerização de materiais orgânicos em torno da proteína, resultando em confinamento da molécula de enzima em uma matriz física. Embora seja um excelente método para manter a conformação da molécula de enzima, tem a desvantagem de dificultar a difusão do substrato através dos poros do suporte (Nunes *et al.*, 2006). O método de imobilização por encapsulamento consiste em aprisionar a enzima em um polímero insolúvel, isto é,

uma membrana porosa na qual pequenas moléculas como substratos e produtos são capazes de se difundir. Um exemplo desta técnica descrita na literatura é o encapsulamento de lipases em esferas de polissacarídeos, como agarose, quitosana e alginato (Nigam *et al.*, 2014).

Na imobilização por ligações covalentes, as lipases são covalentemente ligadas ao suporte por meio dos grupos funcionais não ativos sobre a superfície da enzima (grupos não essenciais para atividade catalítica) e os grupos reativos (hidroxila, carbonila, entre outros) presentes na superfície sólida do suporte. Por tratar-se de uma ligação forte, o derivado imobilizado obtido é estável, não permitindo que a enzima se desprenda do suporte em presença do substrato ou de soluções de alta concentração iônica (Mateo *et al.*, 2007; Brady *et al.*, 2009). Desta forma, evita-se o fenômeno de dessorção, fato comum no método de adsorção. As ligações covalentes podem também ser multipontuais, permitindo uma estabilidade adicional para a enzima imobilizada. Este procedimento é baseado na imobilização de enzimas em suportes via braços espaçadores curtos, isto é, os resíduos de aminoácidos na superfície da enzima se ligam ao suporte por um espaço muito pequeno, o que confere uma rigidez à estrutura de enzima (Mateo *et al.*, 2007).

Para permitir a imobilização covalente, a superfície do suporte deve ser ativada quimicamente por meio da adição de grupos químicos como o glicidol e epicloridrina que reagem preferencialmente com os grupos hidroxila do suporte. O tampão a ser empregado também é um fator importante no processo de imobilização, pois é necessário a utilização de uma solução tampão que facilite a interação dos grupos de interesse da enzima com os grupos disponíveis do suporte. Utilizando um pH alcalino, por exemplo, pode-se imobilizar enzimas através de seu grupo amino (cadeia lateral da lisina), grupos hidroxila (principalmente da cadeia fenol da tirosina) e os grupos tiol (a partir de resíduos de cisteína), ou utilizando um pH moderadamente ácido para imobilizar através dos seus grupos carboxílicos (cadeia lateral de aspartato e glutamato) (Barbosa *et al.*, 2013).

Outra possibilidade para utilização de enzimas imobilizadas para a síntese de biodiesel é através da produção de lipases por fermentação em estado sólido (FES). Nesse sistema, o microrganismo produtor de lipase cresce em um substrato orgânico sólido, com um mínimo de água livre nos espaços entre as partículas de substrato. Assim, o substrato fermentado pode atuar como suporte para a enzima, sem a

necessidade de uma etapa de extração prévia e imobilização do biocatalisador (Salum *et al.*, 2010; Zago *et al.*, 2014).

Devido às diferentes características químicas e composições de enzimas, diferentes propriedades do substrato, e a finalidade de aplicação do produto, não há um método universal de imobilização aplicável a todas as enzimas, nem um suporte ideal para imobilizá-las. Para cada caso, é necessário escolher o procedimento mais fácil e mais barato, e que resulte numa preparação com boa atividade e estabilidade para que assim, a enzima possa ser aplicada nos mais diversos equipamentos disponíveis na síntese de biocombustíveis.

1.5. Reatores enzimáticos

A seleção do reator mais apropriado para um determinado bioprocesso depende das características da bioconversão e das condições reacionais, tais como a cinética de conversão da enzima, a hidrodinâmica do sistema e os mecanismos de transferência de massa. Esses fatores serão determinantes na escolha do modo de operação do reator. No que diz respeito à síntese de biodiesel, a configuração do reator é muito importante e deve ser levada em conta tanto os problemas técnicos do processo quanto as propriedades do biocatalisador (Aires-Barros, 2002; Guisan, 2006). Nesse sentido, a Tabela 1.1. apresenta os principais fatores associados com a escolha do tipo de reator a ser utilizado em reações de síntese enzimática de biodiesel.

Tabela 1.1. Principais variáveis envolvidas na escolha do reator com enzimas imobilizadas.

Fatores	Características
Formato do suporte de imobilização	Partículas, membranas ou fibras
Natureza do substrato	Solução, sólidos suspensos, ou coloidais
Requisitos operacionais	Controle de temperatura e fluxo
Cinética de reação	Possível inibição pelo substrato, pelo produto, ou ambos
Superfície catalítica por unidade de volume de reator	Tamanho do suporte e presença de poros
Transferência de massa	Características de transferência interna e externa de massa
Substituição do catalisador e regeneração	Períodos de tempo morto
Construção do biorreator	Geometria, hidrodinâmica de fluido e condições operacionais
Custo operacional do biorreator	Energia e manutenção
Modo de operação	Batelada e contínuo

Os principais reatores enzimáticos são: reatores batelada de tanque agitado; reatores contínuos de tanque agitado; reatores em coluna de leito fixo, em que a enzima imobilizada encontra-se empacotada permanecendo imóvel enquanto a solução de substrato é bombeado através da coluna; e reator em coluna de leito fluidizado, em que a enzima imobilizada é mantida em suspensão através de recirculação da solução de substrato (Guisan, 2006; Kumar *et al.*, 2013). A escolha do modo de operação, seja a batelada quanto o contínuo, necessita ser avaliada de acordo com a necessidade do processo. Assim, é importante estudar esses dois modos de configuração, bem como as características dos diversos reatores enzimáticos que podem ser empregados nessas distintas formas de operação. A Figura 1.4. exibe uma representação esquemática dos principais tipos de reatores enzimáticos.

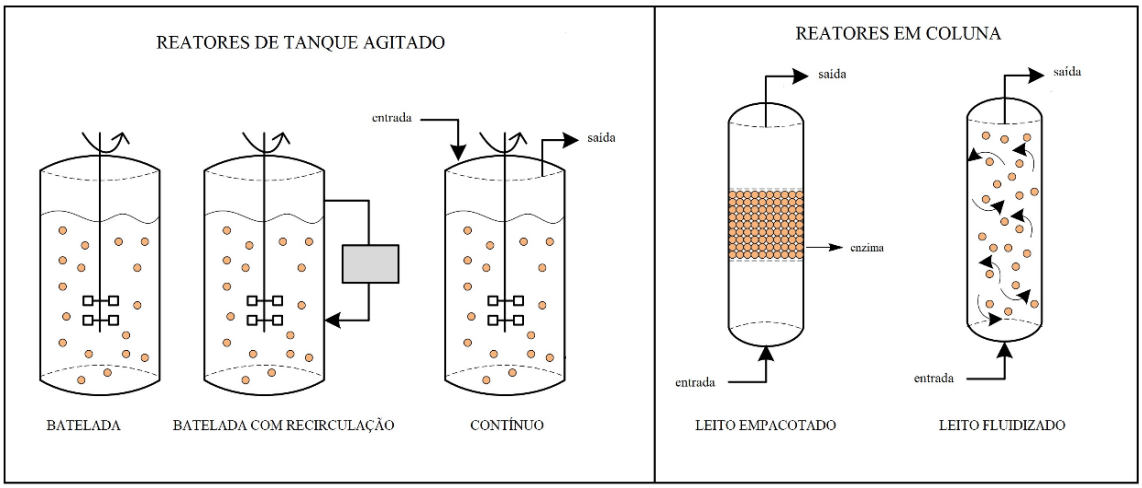

Figura 1.4. Representação dos principais reatores aplicados para síntese enzimática de biodiesel.

1.5.1. Processo em batelada

Os reatores em batelada são geralmente os mais comumente utilizados em processos com enzimas em solução, embora também sejam empregados com enzimas imobilizadas. Tipicamente, as partículas do biocatalisador são dispersos na solução de substrato e a agitação é proporcionada por agitadores mecânicos. O processo pode ser operado com a adição de todos os componentes logo no início, entretanto a adição de substrato em etapas, em alguns casos, é fortemente recomendada (Da Rós *et al.*, 2010). O reator mais rotineiramente utilizado para a síntese enzimática de biodiesel é o do tipo tanque agitado (Christopher *et al.*, 2014).

Reatores de tanque agitado são os mais simples, equipados com controle de temperatura, e um sistema de agitação (normalmente uma hélice). Depois de completada a reação, a separação das fases sólido-líquido é realizada por centrifugação, filtração ou decantação, com a finalidade de recuperar a enzima imobilizada e separar o produto. A realização de processos em batelada oferece certas vantagens operacionais, tais como o elevado grau de dispersão do substrato e um equipamento com estrutura simples e de controle simplificado (Tan *et al.*, 2010). Além disso, é fácil de coletar os dados sobre o processo, como por exemplo, a cinética da reação (Nielsen *et al.*, 2008). Contudo os reatores tem baixo volume de produção e elevado tempo de volume morto devido à necessidade de descarregar, limpar e recarregar o reator antes do início de um novo ciclo (Christopher *et al.*, 2014).

Processos em batelada geralmente ocasionam uma perda gradual da atividade enzimática devido as reutilizações e, desse modo, o tempo de reação tem de ser

aumentado para manter a taxa de conversão constante. Com o passar do tempo, há uma inevitável diminuição de produção e neste ponto a enzima deve ser substituída (Nielsen *et al.*, 2008).

Outro fator importante que deve ser considerado em operações em batelada é a velocidade de agitação e o tipo de agitador empregado, uma vez que as enzimas imobilizadas são susceptíveis à quebra devido às altas tensões de cisalhamento impostas pela agitação mecânica. Para avaliar esses inconvenientes, Basri et al. utilizaram a lipase de *Thermomyces lanuginosus* imobilizada em gel de sílica para síntese de ésteres de óleo de palma, através de alcoólise de óleo de palma e álcool oleílico em reator batelada. A velocidade de agitação do reator foi testada de 250 a 350 rpm. O rendimento da reação e a produtividade aumentaram ligeiramente com o aumento do impulsor até 325 rpm, o que provocou uma melhora substancial na área interfacial de contato entre o substrato e a enzima observada na fase não aquosa, como uma consequência da redução das dimensões de gotículas. No entanto, o rendimento e a produtividade diminuiu a 350 rpm (agitação máxima testada), provocada pela elevada tensão de cisalhamento sobre o suporte causando a desintegração do mesmo (Basri *et al.*, 2013). Nesse tipo de reator, para que haja um melhor transporte de massa, Liese e Hilterhaus (2013) sugerem a utilização de suportes com tamanho de partículas menores sob a agitação de fluidização constante.

1.5.2. Processo contínuo

A tecnologia dos processos contínuos pode ser caracterizada pelo uso de uma variedade de configurações de reatores tubulares, em que o biocatalisador e o meio reacional fluem através dos reatores. Neste caso, o tempo de reação é determinado pela taxa de vazão e o volume do reator, caracterizado pelo tempo de residência (De Souza *et al.*, 2014). O processo contínuo é um sistema que pode ser operado por longos períodos de tempo sob condições de estado estacionário na qual as variáveis de estado permanecem constantes ao longo do tempo. Processos que envolvem enzimas imobilizadas são preferencialmente operados no modo contínuo para evitar a perda de produtividade.

O sistema contínuo apresenta vantagens diretas e indiretas quando comparado com o sistema batelada: uma maior eficácia no controle dos parâmetros de reação e na mistura de reagentes (Zanin *et al.*, 2004); menor custo na otimização das condições de

reação; maior eficiência energética e menor número de operações unitárias durante a recuperação do produto (Itabaiana Jr *et al.*, 2013). Os reatores enzimáticos atualmente disponíveis e aplicados na indústria são totalmente automatizados, para que haja um controle rigoroso de temperatura, vazão e pressão. Devido a estas características, a transesterificação enzimática dos óleos vegetais pode ser realizada substancialmente mais rápida e sendo economicamente mais viável em reatores contínuos do que em reatores em batelada (Wang *et al.*, 2011).

Os reatores de leito empacotado (ou de leito fixo) são os reatores mais comuns para preparações enzimáticas em operação contínua devido à sua alta eficiência, baixo custo e facilidade de construção e operação (Chang *et al.*, 2007; Halim *et al.*, 2009; Séverac *et al.*, 2011; Itabaiana Jr *et al.*, 2013; De Souza *et al.*, 2014; Zhao *et al.*, 2014). Consistem basicamente de uma coluna cilíndrica com um conjunto de partículas de biocatalisador preso em um leito fixo, onde o meio de reação é bombeado através da coluna sob uma vazão específica. Os reagentes passam ao longo do leito compactado para várias vezes até que a conversão completa seja obtida, e o tempo de reação (tempo de residência) é calculado de acordo com o volume da coluna e da vazão aplicada (Fernandes, 2010). A opção por essa configuração de reator é a possibilidade de converter os reagentes em produtos em apenas um ciclo, evitando a necessidade de reciclagem do biocatalisador (Itabaiana Jr *et al.*, 2013). Além disso, devido as baixas tensões de cisalhamento existentes no reator, as partículas do biocatalisador permanecem protegidos de possíveis quebras.

Os suportes utilizados para imobilizar as enzimas não devem ter tamanhos menores que 0,05 milímetros, pois com o aumento do diâmetro do suporte, a queda de pressão diminui e há uma melhor transferência de massa, o que pode afetar positivamente a velocidade global de reação (Nigam *et al.*, 2014). As faixas de vazão utilizadas devem ser o suficiente para fornecer uma queda de pressão razoável, facilitar a difusão entre substratos e a enzima e fornecer alto rendimento de conversão (Fernandes, 2010). Esses reatores, no entanto, não são apropriados para a produção enzimática de biodiesel em reação isenta de solvente (Fjerbaek *et al.*, 2009) pois a alta viscosidade dos sistemas faz com que a queda de pressão torne-se significativa.

Mais algumas desvantagens do reator de leito empacotado devem ser citadas, como a facilidade de obstrução do leito, o aparecimento de caminhos preferenciais, e a ineficiência de transferência de calor e massa (Fernandes, 2010). Além disso, a operação por um longo período de tempo ainda é um desafio (Hermansyah *et al.*, 2011;

Zhao *et al.*, 2014). Em seus estudos, Hermansyah et al. utilizaram o reator de leito empacotado para a produção de biodiesel com a lipase imobilizada de *Candida rugosa* em esferas de zeólita, usando óleo de cozinha e acetato de metila como substratos. A enzima apresentou uma estabilidade operacional de apenas 40 h, com um rendimento máximo de 87 % (taxa de vazão de substrato de 2 mL h^{-1}). Depois desse tempo, o biocatalisador foi desativado devido ao enfraquecimento das ligações entre a enzima e o suporte, sendo a lipase incapaz de se ligar ao substrato.

O reator de leito fluidizado é basicamente uma variação do reator de leito empacotado, mas operado no modo de fluxo ascendente, onde a solução de substrato é alimentada a partir da base do reator a uma velocidade de fluxo suficientemente elevada para suspender o meio (Grosova *et al.*, 2008). O reator proporciona assim, livre circulação das partículas de biocatalisador por todo o leito, sendo a fluidização efetuada pelo próprio substrato ou por ar (Kosseva *et al.*, 2009).

A transferência de massa é facilitada nesse tipo de sistema devido a homogeneização do meio e a movimentação das partículas, o que evita a decantação de enzimas no fundo do reator. Entretanto, pode ocorrer a expansão do leito durante a fluidização, principalmente devido à natureza do suporte, a configuração do reator, a velocidade de fluidização, e a viscosidade dos substratos (Fernandes, 2010). Nesses reatores a queda de pressão não é afetada, o que possibilita a utilização de partículas pequenas de biocatalisador, no entanto, as partículas grandes geralmente são necessárias devido à diferença de densidade entre o fluido e a enzima imobilizada, e a elevada viscosidade dos líquidos (Feng *et al.*, 2013). Além disso, devido à fluidização, não há ocorrência de caminhos preferenciais (Ray, 2012).

A fim de operar de forma eficiente, os reatores de leito fluidizado requerem menores quantidades de enzima por unidade de volume do reator, reduzindo a eficiência global do reator. Além disso, a maior desvantagem do desenvolvimento desses reatores é a dificuldade de aumento de escala. Enquanto os reatores de leito empacotado permitem fatores de aumento de escala superiores a 50.000 ×, os reatores de leito fluidizado só podem ser escalonados de 10 a 100 × (Kosseva *et al.*, 2009).

1.5.3. Transferência de massa em reatores enzimáticos

A configuração apropriada de um reator requer um conhecimento adequado da cinética reacional, da hidrodinâmica do sistema e dos mecanismos de transferência de

massa. É a partir desta análise criteriosa que irá ser determinado o modo de operação e as características da vazão de alimentação do reator (Truppo *et al.*, 2008).

As limitações no transporte de massa podem ser diferenciadas em quatro passos de transporte distintos desde os reagentes até o sítio ativo da enzima (Liese *et al.*, 2013). Conforme é ilustrado na Figura 1.5. abaixo, a resistência à transferência de massa pode ser decorrida tanto pela difusão externa (etapas 1 e 4) quanto pela difusão interna (etapas 2 e 3). Ou seja, pode ocorrer resistência a transferência de substratos e produtos em direção a partícula devido a camada de filme estagnado que se forma ao redor do suporte (difusão externa), assim como ser decorrida pela difusão interna, devido à resistência a transferência de massa no interior da própria esfera.

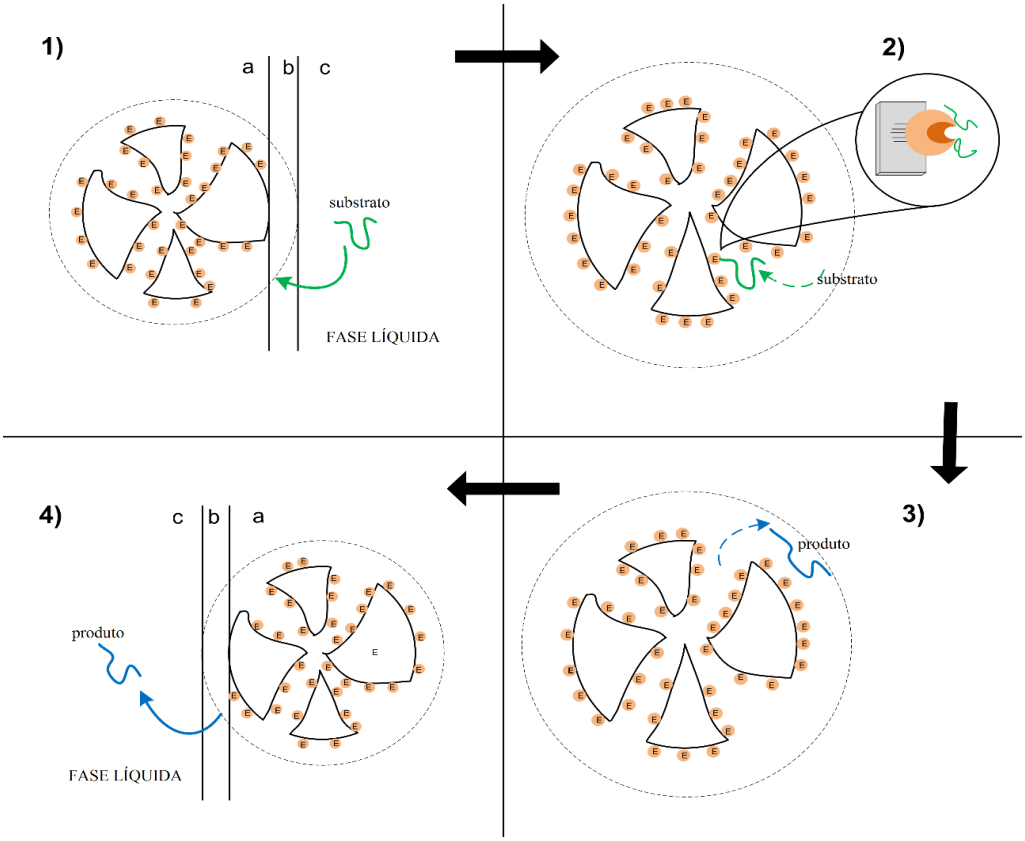

Figura 1.5. Quatro passos de limitações de transferência de massa: 1) difusão na camada de filme estagnado: o substrato que está na fase líquida atravessa a camada limite para as proximidades da superfície do suporte; 2) difusão através dos poros do suporte: o substrato penetra pela superfície do suporte poroso até o local do sítio ativo da enzima; 3) difusão através dos poros do suporte: produto afastar-se do sítio ativo para a superfície do suporte poroso; 4) difusão na camada de filme estagnado: o produto atravessa a camada de filme estagnado para a fase líquida do sistema. (a: suporte poroso; b: camada limite (filme estagnado); c: fase líquida).

As restrições na transferência de massa em reatores enzimáticos são estudadas por muitos autores, e em todos os trabalhos são abordados diversos parâmetros que

envolvem equações matemáticas para elucidar esses aspectos. Fatores como a altura do leito empacotado, tamanho de partícula de suporte, concentrações de substrato e vazão de alimentação ao longo do reator são cruciais para estimar a transferência de massa e identificar as regiões de limitação de difusão (Murty *et al.*, 2005).

Nesse sentido, Chesterfiel et al. avaliaram os efeitos da transferência de massa interna durante a etanólise de óleo alimentar usado (semente de algodão), em reatores em batelada catalisados por *Candida antarctica* imobilizada comercialmente em resina macroporosa. Foi avaliado a influência da difusão intra-partícula sobre a taxa da reação, através do uso de diferentes tamanhos de partícula de biocatalisador (100 a 800 µm). Além disso, a fim de negligenciar a resistência externa na camada interfacial óleo-etanol, a velocidade de agitação do reator foi mantido a 1.200 rpm.

Segundo os autores, o tamanho das partículas foi um fator de forte influência na taxa inicial de reação, sendo indicado um tamanho ótimo de suporte em torno de 400 µm. Além disso, é necessário verificar não apenas o tamanho da partícula, mas também o diâmetro de poro, pois durante a imersão na mistura de óleo e o etanol, as partículas sólidas do biocatalisador podem ser submetidas a um inchaço, que altera a estrutura dos poros, causando um efeito aparente de difusão (Chesterfield *et al.*, 2012). Portanto, o tamanho de partícula deve apresentar o tamanho certo para permitir uma fácil concepção do reator e para evitar as graves restrições de transferência de massa (Fernández *et al.*, 2013).

A avaliação dos efeitos de transferência de massa externa (difusão na camada de filme estagnado) também tem sido relatados. Na maioria dos casos, o primeiro parâmetro estudado é a velocidade de agitação em reatores em batelada, e vazões de alimentação em reatores contínuos (Fjerbaek *et al.*, 2009). Para avaliar esses parâmetros em reator PBR, Halin et al. (2009) utilizaram a lipase de *Candida antarctica* imobilizada comercialmente na catálise do óleo de palma usado com metanol. O efeitos de transferência de massa foram estudados através da variação da vazão de alimentação de substrato (de 0,18 a 1,02 mL min^{-1}) e a altura do leito empacotado (de 4,76 a 14 cm). Conforme relatado pelos autores, o rendimento máximo de biodiesel (80 %) foi alcançado com leito de 12 cm de altura e vazão de substrato de 0,55 mL min^{-1}. Com vazões muito baixas, baixos rendimentos de biodiesel foram obtidos causada pela resistência à transferência de massa na camada de filme estagnado. No entanto, com vazões muito elevadas (0,9 - 1,02 mL min^{-1}) o substrato apenas passou através da enzima, sem interagir com ela, não sendo convertido em produto (Halim *et al.*, 2009).

Em reatores contínuos, a vazão de entrada do substrato é de importância fundamental, pois se for demasiadamente elevada, o tempo de contato entre o substrato e a lipase será muito curto e a reação estará incompleta (Nie *et al.*, 2006).

O tempo de residência é uma importante informação sobre os reatores, que permite diagnosticar problemas de vazão, tais como a existência de zonas de fluido estagnado ou zonas mortas; existência de caminhos preferenciais; dispersão axial em reatores tubulares; segregação, resultante das condições de mistura no reator; e limitações na transferência de massa. Dessa forma, o tempo de residência fornece uma evidência do tipo de mistura que ocorre dentro do reator, e é uma das formas mais usuais para caracterizá-lo (Fogler, 2005).

O tempo de residência de um reator (*t*) pode ser calculado de acordo com a Eq. 1:

$$t = \frac{L}{v_S} \times \varepsilon \qquad (1)$$

em que: L é o comprimento do leito composto de biocatalisador; v_s é a velocidade superficial de fluido (razão entre a taxa de vazão volumétrica pela área da secção transversal do leito), e ε é a fração de leito vazio.

A relação entre o tempo de residência e a conversão foi avaliado em estudo desenvolvido por Hermansyah et al. (2011) para determinar a vazão de alimentação de substrato necessária para produzir uma conversão ótima de biodiesel. O reator utilizado foi do tipo PBR, em reação catalisada por lipase imobilizada de *Candida rugosa*. Os experimentos foram conduzidos com quatro níveis de vazão testadas: 1, 2, 4, e 5 mL h^{-1}, e o tempo de residência foi anotado para cada teste. A conversão mais elevada de biodiesel (71,47 %) foi obtida a uma vazão de 1 mL h^{-1} com tempo de residência de 5,5 h, e a conversão mais baixa (38,79 %) foi obtida com a maior vazão (5 mL h^{-1}) com tempo de residência de 1 h. De acordo com os autores, a concentração de biodiesel formada foi diretamente proporcional ao aumento do tempo de residência no reator, isto é, o contato prolongado entre o substrato e o biocatalisador criou uma forte reação catalítica que não sofreu limitação na transferência de massa, proporcionando o melhor rendimento (Hermansyah *et al.*, 2011).

1.5.4. Uso de solventes

A configuração do reator depende também de possíveis problemas técnicos que envolvem a homogeneidade da mistura de reação, a solubilidade do produto em álcool, o efeito do glicerol, entre outras (Itabaiana Jr *et al.*, 2013).

A utilização de solventes orgânicos tem várias finalidades, incluindo: assegurar mistura de reação homogênea; evitar problemas de formação de duas fases na reação; reduzir a viscosidade da mistura de reação para aumentar a velocidade de difusão, reduzindo assim os problemas de transferência de massa em torno da enzima; melhorar a estabilidade da enzima e acelerar a migração dos grupos acila (Fjerbaek *et al.*, 2009; Rodrigues, Pessela, *et al.*, 2010). Além disso, os solventes orgânicos, possivelmente, eliminam a necessidade de adição gradual de álcoois (Antczak *et al.*, 2009).

Uma importante discussão sobre a utilização de solventes ainda permanece em aberto, ou seja, qual o tipo de solventes orgânicos é mais adequado para as reações catalisadas por lipase. Até recentemente solventes não polares (log P > 4) foram relatados como sendo as melhores escolhas, pois a enzima apresenta atividade mais elevada em solventes orgânicos relativamente hidrofóbicos. O valor de log P é geralmente utilizado para correlacionar a polaridade do solvente com a atividade enzimática e a estabilidade em fases não aquosas, mas como essa correlação atua sob a desnaturação da enzima ainda é um dado difícil de prever (Laane *et al.*, 1987). Solventes orgânicos, tais como o hexano, éter de petróleo, e *terc*-butanol, têm sido amplamente utilizados (Christopher *et al.*, 2014).

Li et al. observaram os efeitos de diferentes solventes, estudando a metanólise com óleo de diferentes espécies de *Stillingia* catalisada por Novozym 435 e Lipozyme IM-TL em reatores em batelada. Os autores relataram melhores resultados com solventes cujos log P foram entre 1,4-1,52, tais como *terc*-butanol, acetonitrila, *terc*-pentanol, tetra-hidrofurano, e 1,4-dioxano. Rendimentos mais baixos foram obtidos com a utilização de solventes mais hidrofóbicos (log P > 2,0), tais como n-hexano e n-heptano. Estes resultados mostraram que os solventes polares são mais eficientes em reações com metanol, e evitam a desnaturação da lipase, além disso, *terc*-butanol produziu os melhores rendimentos com a maioria dos óleos vegetais. Devido a sua cadeia ramificada com três grupos metila, *terc*-butanol tem um impedimento estérico que impede a formação de ésteres *terc*-butílicos (Li *et al.*, 2010). Além disso, este solvente tem o atributo de preservar a atividade da lipase frente a desnaturação causada

por glicerol produzido na reação (Azócar *et al.*, 2014). A presença de glicerol afeta diretamente a eficiência da reação, pois ele se adere à superfície da enzima formando uma camada hidrofílica que impede a transferência de massa entre substratos hidrofóbicos, o que conduz a uma redução no rendimento da reação (Chattopadhyay *et al.*, 2011).

Contudo, a necessidade da utilização de solventes deve ser avaliada com cuidado pois esses reagentes, além de apresentam toxicidade e inflamabilidade, necessitam ser removidos no fim do processo, o que pode aumentar os custos de produção (Aires-Barros, 2002). Outros tipos de solventes, tais como o dióxido de carbono supercrítico (Lee *et al.*, 2011; Lozano *et al.*, 2011) e líquidos iônicos (LI) (Mohammad Fauzi *et al.*, 2012; Lin *et al.*, 2013) também têm sido relatados em processos de transesterificação enzimática. Esses últimos demonstram ser ótimos substitutos para os solventes orgânicos clássicos, com o diferencial de exibirem a possibilidade de moldagem de acordo com as necessidades da reação, e serem excelentes em promover a estabilização da enzima (Mohammad Fauzi *et al.*, 2012).

1.5.5. Reatores enzimáticos em ultrassom

O ultrassom é uma onda mecânica, como todo som, porém possui uma freqüência superior àquela que o ouvido do ser humano pode perceber, numa faixa de 20 kHz a um limite superior que não é precisamente definido, mas geralmente é definido para os gases em 5 MHz e de 500 MHz para líquido e sólidos (Sanderson, 2004).

As ondas ultrassônicas são produzidas por transdutores ultrassônicos, os quais são feitos de metais piezoelétricos, que apresentam um fenômeno conhecido como efeito piezoelétrico, ou seja, provocam o aparecimento de campos elétricos de forte vibração causando implosão de bolhas e cavidades na água. Essa vibração recebe a denominação de cavitação (Martines *et al.*, 2000).

O processo de produção de biodiesel utilizando banho ultrassônico surge como alternativa ao processo convencional de produção, uma vez que as cavitações geradas pelo ultrassom aumentam a miscibilidade entre os reagentes e são capazes de agitar o fluido mais eficientemente do que os reatores convencionais (Yu *et al.*, 2010). O uso de ultrassom em reações de catálise favorece a transferência de massa, bem como a taxa de

reação, proporcionando reações com menos tempo, menos reagentes e condições físicas mais leves (Mostafaei *et al.*, 2015).

Neste sentido, Yu et al. realizaram a catálise enzimática para a produção de biodiesel a partir de óleo de soja com metanol e Novozym 435. A reação foi realizada em banho de ultrassom com agitação de 50 rpm a 40 °C. Nas condições propostas foi obtido um rendimento de 96 % em apenas 4 h de reação, enquanto que para o procedimento de transesterificação sem ultrassom foi necessária 12 h para alcançar rendimentos comparáveis.

CAPÍTULO II – ENZYMATIC REACTORS FOR BIODIESEL SYNTHESIS: PRESENT STATUS AND FUTURE PROSPECTS.

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Enzymatic reactors for biodiesel synthesis: present status and future prospects

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Abstract

Lipases are being extensively researched for the production of biodiesel as a ―silver bullet in order to avoid the drawbacks of the traditional alkaline transesterification. In this review, we analyzed the main factors involved in the enzymatic synthesis of biodiesel, focusing in the choice of the immobilization protocol, and the parameters involved in the choice and configuration of the reactors. An extensive discussion is presented about the advantages and disadvantages of each type of reactor and their mode of operation. The current scenario of the market for enzymatic biodiesel and some future prospects and necessary developments are also briefly presented.

Keywords: Biodiesel; lipases; enzyme immobilization; batch reactor, continuous reactor.

1. Introduction

The possibility of using agricultural-based fuels in diesel cycle engines is very attractive in the view of environmental aspects because these are renewable energy sources and can use several agricultural and agro-industrial residues for their synthesis. Biodiesel is defined by the National Biodiesel Board (USA) as a mono ester of fatty acids derived from renewable sources of long chain, such as vegetable oils and animal fats (Knothe, 2006). Biodiesel is a renewable, carbon neutral biofuel, with neutral mass balance of CO_2 from emissions and absorption thereof by the plant (Yaakob et al., 2013).

Many oilseeds can be used to produce biodiesel, among them, the most important are soybean, sunflower, and palm oils, and the different alternatives depend upon the cultures in each region. On the other hand, given the large generation of waste oils by industrial and economical activities, it is becoming important to further explore alternative sources of oils, such as waste vegetable oils from cooking and various fats (Antczak et al., 2009).

Alkaline catalysis is still the most important technological route for the industrial production of biodiesel via transesterification reaction because of its shorter reaction time and high productivity. However, alkaline catalysis presents drawbacks related to the inevitable production of soaps caused by the saponification of free fatty acids, leading to losses of catalysts and difficult process of separation and purification of the formed glycerol, reducing the yields of the reaction (Leung et al., 2010).

The enzymatic synthesis of biodiesel, mediated through the use of lipases (EC 3.1.1.3, triacylglycerol hydrolases), presents important advantages over the chemical catalysts, such as the specificity, enantioselectivity, and regioselectivity of the reactions, therefore generating less side-products and wastes, and the reactions can be carried out at mild conditions of temperature and pressure (Ghaly et al., 2010). Enzymatic industrial processes are generally relatively simple, easy to control, and more efficient on energy input when compared to traditional chemical processes (Oliveira and Mantovani, 2009). Furthermore, in the case of lipase-based synthesis of biodiesel, the separation of glycerol generated as a by-product and the purification of the produced esters are easily performed (Fukuda et al., 2001).

The present market costs of lipases are still the main limitation that avoids their use in large-scale processes. Thus, many scientific groups are developing several studies

aiming at reducing these costs. Among strategies being investigated, we can highlight the use of aqueous solution of lipases that is non miscible with the oils and the biodiesel (Cesarini et al., 2013; Pedersen et al., 2014); immobilized enzymes in repeated cycles of operation (Calero et al., 2014; Chen et al., 2011; Noureddini et al., 2005; Poppe et al., 2013; Shimada et al., 1999; Wang et al., 2008); the development and improvement of immobilization techniques (Rodrigues et al., 2013; Silva et al., 2014); the use of new materials for immobilization to substitute expensive commercial supports (Miranda et al., 2014); and the improvement of reaction parameters in batch and continuous reactors (Hama et al., 2013; Kawakami et al., 2011; Lee et al., 2010).

The use of immobilized lipases may improve the development of commercial scale processes, favoring biotechnological processes based on their numerous advantages over the chemical process. In large-scale biodiesel production, selecting the most appropriate reactor depends on the characteristics of the reaction kinetics and reaction conditions based on the biocatalyst, which in turn will define the operation mode and the flow characteristics (Castro et al., 2008).

In this context, this review will discuss the some important aspects involving the use of reactors applied in the enzymatic process of biodiesel synthesis, showing the present status and the future prospects of their applications. Emphases will be given on the interactions between the lipase and the immobilization support and the efficient use of the biocatalyst in the reactors; the operation mode and settings of the enzymatic reactors; and analyzes of the main parameters involved in the transesterification reactions.

2. Technical aspects of biodiesel

The high demand for energy in the industrialized world and the recurrent environmental problems caused by the widespread use of fossil fuels push the need for developments on renewable energy sources. An alternative fuel must be technically feasible, economically competitive, environmentally acceptable, and readily available. One possible alternative to fossil fuel is the use of oils of plants or algae origin, and animal fats for the synthesis of biodiesel (Meher et al., 2006). Biodiesel accounted for approximately 5 % of the world biofuel production in 2000, but this value is continuously rising and, in 2011, biodiesel already represented about 20 % of the total biofuel production (Geraldes et al., 2014). Several governmental policies worldwide are

stimulating biofuels production by setting targets for blending quotas, and boosting the development of biofuels technologies by establishing financial and political support mechanisms (Geraldes et al., 2014).

Biodiesel is miscible with petroleum-based diesel in all proportions and can be used pure or blended with diesel. These blends are often coded such as B20, which indicates the blend of 20 % of volume biodiesel and 80 % of volume diesel (Issariyakul and Dalai, 2014). Biodiesel is predominantly produced by transesterification reaction, which process is relatively simple and generates a fuel whose properties are similar to diesel (Dorado et al., 2003; Geris et al., 2007). The transesterification consists of a reaction between vegetable oil or animal fat and a primary alcohol in the presence of a catalyst, resulting in a mixture of alkyl esters of fatty acids (biodiesel) and glycerol (de Araújo et al., 2013). Excess alcohol in the reaction is necessary in order to increase the yield of esters and to promote the shifting the reaction equilibrium towards the product (Suarez et al., 2007).

Another recent biodiesel synthesis technology has been widely reported, known as hydroesterification (Cavalcanti-Oliveira et al., 2011; de Sousa et al., 2010; Soares et al., 2013). This process occurs in two consecutive steps: The first is the hydrolysis of all glycerides (mono-, di- and triglycerides) that produces free fatty acids (FFAs) and glycerol; the second one is the esterification of the FFAs with a short chain alcohol to obtain esters (biodiesel) and water. Moreover, through this process, glycerol is separated in the first step, not by contacting the alcohol, being more pure than that obtained by transesterification (Aguieiras et al., 2014).

2.1 Raw materials for biodiesel production

The raw materials for biodiesel production are the lipid sources, mainly vegetable oils, animal fats, and, more recently, oils produced by algae (Nautiyal et al., 2014) and cyanobacteria (Karatay and Dönmez, 2011), and several types of alcohols.

The vegetable oils used as lipid feedstock for biodiesel production usually depend on regional production, for instance, as in rapeseed oil in the European countries and Canada, soybean oil in the United States and Brazil, and palm oil in tropical countries such as Indonesia and Malaysia. Coconut oil is another lipid feedstock used for synthesis of biodiesel in coastal areas (Issariyakul and Dalai, 2014).

Sources of oils such as soybean oil, palm oil, sunflower, rapeseed, coconut, and peanuts are considered as the first-generation biodiesel feedstock. However, their use leads to competition with the food industry, and may generate environmental problems such as serious destruction of vital soil resources, deforestation and the use of much of the available arable land (Atabani et al., 2012). Furthermore, the cost of raw materials accounts for 60-80 % of the total cost of biodiesel production (in the alkaline rout), indicating that selecting the appropriate feedstock is of considerable importance for ensuring the economic viability of the process (Aarthy et al., 2014). The use of low-cost feedstock such as wastes, such as frying oil, non-edible oils, such as colza, and oil extracted from other feedstock such as yellow grease, lard, animal fats, among others, are known as second generation biodiesel feedstock and are expected to reduce the production costs and environmental problems, making biodiesel production more commercially competitive with diesel (Christopher et al., 2014).

Many types of alcohols can be used to produce biodiesel, in some cases with the need to use organic solvents. Alcohols of higher chemical chains, such as propanol and butanol, posses better oil solubility and dispense the use of organic solvents in the reaction. Alcohols providing the best yields of biodiesel are linear, with low steric hindrance, such as methanol, ethanol, propanol, butanol, and amyl alcohol intermediates (Leung et al., 2010). These alcohols have lower activation energy, being methanol and ethanol the most frequently used, especially methanol, because of their low cost and distinct physical and chemical advantages, i.e. higher polarity and reactivity (Xiao et al., 2009). However, compared to ethanol, methanol is more toxic, presents higher risk of explosion and is produced mainly from petroleum gas, a non-renewable fossil fuel. Ethanol, besides being considerably less toxic than methanol, is a renewable raw materil and produces biodiesel of higher cetane number and oxidative stability, lower iodine value and better lubricity and other properties (Stamenković et al., 2011). One major drawback of ethanol is that it promotes a greater dispersion of glycerin in the formed biodiesel, which turns more difficult their purification (Lôbo et al., 2009).

In cases where the reaction occurs in organic alternative means and with the use of enzymes, the addition of alcohol in stages has been proposed as a way to improve the catalytic action of the enzyme. It is known that enzymes are often unstable in short chain alcohols, such as methanol and ethanol, so this would be a way to prevent denaturation or by inhibiting the enzyme alcohol and increase the reaction yield (Aguieiras et al., 2014; Rodrigues et al., 2010; Royon et al., 2007).

3. Enzymatic catalysis of biodiesel

Currently, the main synthetic approaches used for biodiesel production are alkaline-catalyzed and acid-catalyzed transesterification (with simultaneous esterification of free fatty acids) (Borugadda and Goud, 2012). The acid-catalyzed reaction requires longer times and a higher molar ratio of alcohol:oil. The alkaline-catalyzed reaction has the drawback of the inevitable production of soaps caused by saponification of free fatty acids and the hydrolysis of triglycerides into diglycerides and free fatty acids caused by the water content on reaction. These secondary reactions are undesirable because they consume part of the catalyst, and they make difficult the separation process and the purification of glycerol from biodiesel, reducing the yield of the reaction (Leung et al., 2010).

The technical problems associated with the chemical transesterification has led to a growing interest for the enzyme-catalyzed (lipase) transesterification reaction in the last few years (Borugadda and Goud, 2012; Rodrigues et al., 2008b; Séverac et al., 2011; Shieh et al., 2003; Yan et al., 2014). The glycerol derived from the basic catalysis needs to be purified to remove soaps and emulsions generated during the transesterification. This procedure is generally done by ion exchange, which has a high cost and reduces the efficiency of recycling alcohol (Quintella et al., 2009). According to Van Gerpen (2004), the glycerol purity content is around 15 %, which does not give a good commercial value. In enzyme catalysis, on the other hand, the glycerol produced has a high purity and higher market value, since the enzymes do not form soaps and may esterify the free fatty acids. In this way, this by-product can easily be recovered without complex processing, energy consumption in the process becomes lower, with a drastic reduction in the amount of waste generated (Nielsen et al., 2008).

3.1. Lipases

Lipases are named as glycerol ester hydrolases (EC 3.1.1.3), which catalyze the hydrolysis of long chain insoluble triglyceride in aqueous media and other insoluble esters of fatty acids. In addition, lipases can catalyze the transesterification, aminolysis and synthesis of a wide range of natural and synthetic esters, while retaining high enantio or regioselectivities (Mendes et al., 2011a).

Most of lipases utilized as catalysts in organic synthesis are of microbial origin such as lipases from *Candida rugosa* (Moreno-Pirajàn and Giraldo, 2011), *Pseudomonas fluorescens* (Devanesan et al., 2007), *Rhizhopus oryzae* (Li et al., 2007), *Burkholderia cepacia* (Kawakami et al., 2011), *Aspergillus niger* (Xiao et al., 2011), *Thermomyces lanuginosus* (Fernandez-Lafuente, 2010), *Rhizomucor miehei* (Al-Zuhair et al., 2007; Rodrigues and Fernandez-Lafuente, 2010) e *Candida antarctica* (Liu et al., 2010) Lipase active site consists of three amino acid residues: a nucleophilic residue (cysteine, serine, or aspartate), an acid catalyst residue (aspartate or glutamate), and one histidine residue (Jaeger et al., 1994). A structural feature common to most lipases is the presence of a peptide sequence in α-helix covering the active site, called lid. In the absence of a solvent or hydrophobic interface, the lid prevents access of substrate to the catalytic triad, maintaining the enzyme in the closed conformation (Jaeger and Reetz, 1998). The activation of lipases in the presence of hydrophobic surfaces, a phenomenon known as interfacial activation, is an important feature of these enzymes (Derewenda et al., 1992), and gives the enzyme functionality. In Fig.1 it is presented the general interfacial activation mechanism of lipases, showing the equilibrium of both closed and open forms.

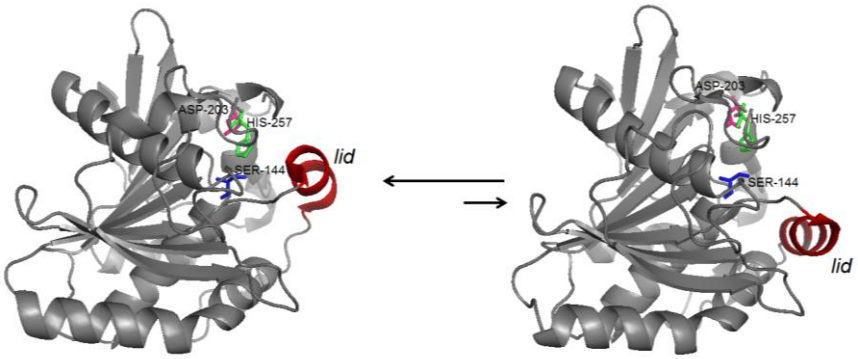

Fig. 1. Distinct forms of RML. The 3D structures were obtained from the Protein Data Bank (PDB) using Pymol vs. 0.99. A) Closed form (PDB-3TGL); B) Open form (PDB-4TGL).

As every other enzyme, lipases have properties that make them highly required as catalysts. They are versatile and perform a variety of transformations selectively and rapidly, under mild reaction conditions that are not feasible for the chemical synthesis of biodiesel. Furthermore, lipases activities can be modulated in a relatively easy way by simply adjusting reaction conditions, for example, by the addition of inhibitors (Oliveira and Mantovani, 2009).

The main advantages of using lipases, as compared to the conventional chemical reaction, are their substrate specificity and selectivity. These characteristics are controlled by the molecular properties of the enzyme, the structure of the substrate, and the factors that affect the binding of the enzyme with the substrate. Due to the specificity towards the substrate is too high, and sometimes absolute, it is necessary to know the characteristics of the lipase and find the ideal substrate for use in the reactions (Castro et al., 2004). As the oils and fats used in the reactions are heterogeneous constitution, the combined use of lipases that act with different specificities for substrates is a strategy that has been widely reported to increase productivity in biocatalysis reactions. The new concept of *combi-lipase* biocatalyst for heterogeneous substrates was first proposed in the work by Alves et al. (2014). The concept is based on the fact that a composed biocatalyst of a mixture of different lipases should be more effective on heterogeneous substrates than one specific lipase (Alves et al., 2014). The use of *combi-lipase* provided excellent results when compared to the individual use of enzymes, both in hydrolysis as transesterification reactions (Poppe et al., 2015).

Recently, it has been reported the use of new enzymes in liquid form (Callera^{TM}Trans L) developed by Novozymes (Denmark). Reactions using this lipase preparation need to be conducted with a high water content, which leads to the formation of a second liquid phase, creating an interface that is known to activate the catalytic action of many lipases. The proportion of alcohol should be higher so that the reaction equilibrium is shifted towards the synthesis of esters and does not promote hydrolysis, which to some extent increases the reaction rate. However, high alcohol content will increase the system's operating costs involved in the separation of excess alcohol, and possibly cause damage to lipase (Pedersen et al., 2014).

Since the main limitation of the lipases of use are still the cost and the difficulty to separate them from the reaction medium, the immobilization techniques continue to be focus of study in many works (Abdulla and Ravindra, 2013; Barbosa et al., 2011; Blanco et al., 2004; Brady and Jordaan, 2009; Fernandez-Lorente et al., 2008; Rodrigues et al., 2009).

3.2. Supports for lipase immobilization

The immobilization is intended to improve the catalytic potential of the enzyme and make them insoluble to the reaction medium allowing its reuse and facilitating the

recovery of products (Moreira et al., 2007). However, immobilization is not just a simple way to separate the enzyme from the reaction products. Often, the immobilization process alters enzymatic properties, producing biocatalyst with activity, specificity and enhanced stability (Hernandez et al., 2011; Rodrigues et al., 2013).

Because the immobilization of enzymes involves the interaction of the enzyme and the support, the surface properties of both are therefore important. In the enzyme molecule, polar groups (e.g. amino groups on lysine and acid groups on glutamic acid), non-polar surface areas, or sugar moieties can influence the properties of its surface. Therefore, the support to be used has to be prepared in order to match either of these surface properties of the enzyme (Hanefeld et al., 2009). In selecting a support, its physical and chemical properties relevant to the immobilization process, as well as its characteristics for possible regeneration, must be thorough analyzed. A wide variety of organic, synthetic and natural inorganic materials, with different characteristics such as size, shape, porosity, hydrophobicity, and density, has been tested for the immobilization of lipases (Mendes et al., 2011b).

These physical characteristics of the supports will be of major importance for the performance of the immobilized systems and will determine the type of reactor that can be used (i.e., stirred tank, fluidized or fixed beds) (Brena and Batista-Viera, 2006). In particular, parameters such as pore and particle sizes determine the total surface area and therefore critically affect the ability for binding enzymes. Non-porous substrates show some diffusion limitations and have a low bearing capacity. Therefore, porous supports are generally preferred because of their extensive surface area that allows for higher enzyme loading and the immobilized enzyme is better protected from the environment (Garcia-Galan et al., 2011; Hanefeld et al., 2009).

Porous supports should have a controlled pore distribution in order to optimize the load of enzymatic capacity available to be immobilized and to show optimum flow properties (Hernandez et al., 2011; Liese and Hilterhaus, 2013). Additionally, according to Hernandez et al. (2011), supports with very small pore diameter can have their pores blocked by the enzyme after immobilization, leaving a large percentage of the its internal surface inaccessible to substrate. This leads to a lower immobilization rate, reducing the ability of the catalytic action of the enzyme.

Another point to be considered is related to the dimensional stability of the material constituting the support, that is, if inorganic or organic (Dalla-Vecchia et al., 2004). In spite of the many advantages of inorganic supports (e.g., high stability against

physical, chemical, and microbial degradation), most of the industrial applications are performed with organic matrices (Brena and Batista-Viera, 2006). The rigid structure has the advantages of non-deformation of the matrix when compressed in column-type reactors, protection of the enzyme against shear forces and collaborate in maintaining the tertiary structure of the protein. Furthermore, a variety of reactive functional groups can be "introduced" in organic supports using chemical modifications improving the final characteristics of the enzyme (Rodrigues et al., 2008a).

Immobilization techniques can be classified into four basic types: binding to the support, confinement, encapsulation, and intercrossed links (Fig. 2) (Dalla-Vecchia et al., 2004). The methods classified as "binding support" assume the binding of the enzyme to the support by means of covalent linkages, ionic or adsorption (by ionic interactions, "van der Waals" forces, hydrogen bonding, dipole-dipole interactions or hydrophobic interactions) (Hanefeld et al., 2009).

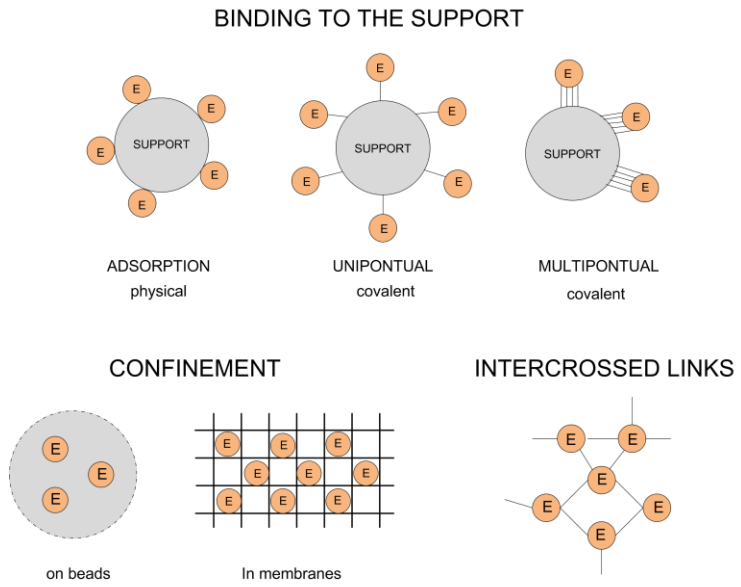

Fig. 2. Three basic types of immobilization techniques: binding to the support, confinement, and intercrossed links.

Adsorption is the simplest and oldest method of enzyme immobilization on supports. Immobilization by adsorption is based on weak physical interactions between the enzyme and support (Van der Waals, hydrogen bonds). Despite its simplicity, this immobilization method is limited by the tendency of desorption of the enzyme from the

support and for being sensitive to medium environmental conditions such as temperature and concentration of ions (Grosova et al., 2008).

Methods based on containment of the enzyme involve the polymerization of organic materials around the protein, resulting in confinement of the enzyme molecule in a physical matrix. Although a good method to maintain the conformation of the enzyme molecule, it has the drawback to difficult diffusion of the substrate through the pores of the support matrix (Nunes and Marty, 2006). The method of immobilization by encapsulation involves entrapping the enzyme in an insoluble gel polymer, i.e., one in which small molecules such as substrates and products are able to diffuse through the porous membrane. As an example, one of these gels involves the hydrolysis of siloxanes, which are polymerized and condensate to form a solid (Campàs and Marty, 2006). The enzyme is added to the reaction medium and is encapsulated when the gel is formed.

In the immobilization by covalent bonds, the enzymes are covalently bound to the support via the functional groups on the enzyme surface, if these groups are not essential for the catalytic activity. This method provides a high binding stability, easy handling of the derivative biocatalyst, and the enzyme load remains constant throughout use (Mateo et al., 2007). Covalent bonds may also be multipoint, allowing additional stability to the immobilized enzyme. This procedure is based on the immobilization of enzymes in supports via short spacer arms, where the amino acids residues in the surface of the enzyme will be connected very close to the support, conferring a rigidity of the enzyme structure (Mateo et al., 2006). To enable covalent immobilization, the chemical activation of the support should be performed by the addition of groups such as glycidol and epichlorohydrin that preferentially react with hydroxyl groups of the support. The glutaraldehyde is a bifunctional agent used as activation agent in immobilization techniques, which reacts with amino groups of the support (Barbosa et al., 2012). Another important feature of glutaraldehyde is its ability to adsorb hydrophobic dyes (such as rose bengal) showing the hydrophobic character of the support (Mendes et al., 2011a).

Among the immobilization supports used for lipases reported in several studies, many satisfy most requirements described, offering large areas for the enzyme-support interactions. Among them, the best appear to be: Decaoctyl Sepabeads, a polymetacrylate resin coated with decaoctyl groups (Palomo et al., 2002); chitosan beads (Palla et al., 2011; Ting et al., 2006); polysiloxane-polyvinyl alcohol (SiO_2-PVA)

hybrid matrix (Da Rós et al., 2010); glyoxyl activated agarose gels (Rodrigues et al., 2008a; Rodrigues et al., 2009); mesoporous carbon beads (MB) (Quirós et al., 2011); styrene–divinylbenzene beads (Hernandez et al., 2011); green coconut fiber (Brígida et al., 2008); silicate mesoporous materials (Khoobi et al., 2014); glass beads (Yilmaz et al., 2011); zeolites (de Vasconcellos et al., 2012); Fe_3O_4/ZnO core/shell magnetic nanoparticles (Ghasemi et al., 2014); aluminum oxide pellets (Kumar et al., 2013), and others.

Another possibility for use of immobilized enzyme for biodiesel synthesis is lipase production by solid state fermentation (SSF). In this system remains immobilized lipase produced naturally fermented on solid and can be used in reactions without the need for further extraction and immobilization (Liu et al., 2013; Salum et al., 2010). In this sense, Zago et al. (2014) used a fermented solid of *Rhizopus microsporus* on ethanolysis of corn oil to produce biodiesel. In this work, SSF was performed with a mixture of bagasse and sunflower seed flour (1:1, w/w), at 44 °C for 48 h (tricaprylin-hydrolyzing activity of 183 U g^{-1}). A 2^3 factorial design was used to optimize the reaction using n-heptane as solvent. The best conversion was 91 % at 48 h, obtained at 44 °C, with a molar ratio of ethanol:oil of 3:1 and the addition of 1:32 g of fermented solids/15 mL of reaction medium (Zago et al., 2014).

Because of the different characteristics and chemical compositions of enzymes, different properties of the substrate, and the purpose of application of the product, there is not one universal method of immobilization applicable to all enzymes, neither one ideal support to immobilize them. For each case is necessary to choose the easiest and cheapest procedure, which results in a preparation with good activity and stability.

Table 1 summarizes several studies regarding the enzymatic synthesis of biodiesel developed in the last years, showing the different lipidic sources, alcohols and lipases.

Table 1. Different raw material used in enzymatic biodiesel synthesis by different procedure.

Lipase	Support	Alcohol	Lipid sources	Reaction total time (h)	Process	Yield (%)	Reference
Candida rugosa lipase to hidrolyse and *Candida antarctica* lipase B (CALB) to esterification	Macroporous acrylic resin	Methanol	Acid oil	24 h	Hydroesterification	91.0 %	Watanabe et al., 2007
C. antarctica lipase B (CALB) - Novozym 435	Macroporous acrylic resin	Methanol	Sunflower oil	50 h	Transesterification	99.0 %	Orgajanovic 2009
C. antarctica lipase B (CALB) - Novozym 435	Macroporous acrylic resin	Methanol	Palm oil	0.5 h	Transesterification	92.0 %	Talukder et al., 2009
C. antarctica lipase B (CALB) - Novozym 435	Macroporous acrylic resin	Methanol	Soybean oil	15 h	Transesterification	97.0 %	Zeng et al., 2009
Burkholderia cepacia lipase	SiO2–PVA	Ethanol	Babassu oil	48 h	Transesterification	100.0 %	da Rós et al. (2010)
B. cepacia lipase	SiO2–PVA	Ethanol	Tallow beef	48 h	Transesterification	89.7 %	da Rós et al. (2010)
B. cepacia lipase	Nb2O5	Ethanol	Babassu oil	48 h	Transesterification	74.1 %	da Rós et al. (2010)
B. cepacia lipase	Nb2O5	Ethanol	Tallow beef	48 h	Transesterification	40.2 %	da Rós et al. (2010)
Lipase extract from germinated physic nut seeds	-	Methanol	Physic nut oil	4 h	Hydroesterification	97.1 %	de Souza et al., 2010
Combined Lipase AK from *Pseudomonas fluorescens* and Lipase AY from *Candida rugosa*	Accurel PE-100 - Microporous polypropylene powder	Ethanol	Palm oil	12 h	Transesterification	67.0 %	Tongboriboon et al. (2010)
Thermomyces lanuginosus lipase	-	Methanol	Soybean oil	49 h	Hydroesterification	92.0 %	Cavalcanti-Oliveira et al., 2011
C. antarctica lipase B (CALB) - Novozym 435	Macroporous acrylic resin	Ethanol	Soybean oil	24 h	Transesterification	85.0 %	Rosset et al. (2011)
B. cepacia	Silica monolith	Methanol	Crude jatropha oil	24 h	Transesterification	95.0 %	Kawakami et al. (2011)

Rhizopus oryzae	Macroporous resin (MI-ROL)	Methanol	*Pistacia chinensis bge* seed	60 h	Transesterification	92.0 %	Li et al. (2012)
R. oryzae	Anion exchange resin (AIROL)	Methanol	*Pistacia chinensis bge* seed	60 h	Transesterification	94.0 %	Li et al. (2012)
B. cepacia	Hydrophobic magnetic particles (HMPs)	Methanol	Olive oil	12 h	Transesterification	70.0 %	Liu et al., 2012
Burkholderia sp. C20 lipase	Fe3O4–SiO2	Methanol	Microalgal oil produced by *Chlorella vulgaris* ESP-31	288 h	Transesterification	97.3 %	Tran et al.(2012)
T. lanuginosus (Lipozyme TL-IM)	Macroporous ion-exchange resin	Oleyl alcohol	Palm oil	5 h	Transesterification	79.0 %	Basri et al. (2013)
C. antarctica lipase B (Novozym 435)		Methanol	Soybean oil	72 h	Transesterification	93.4 %	Poppe et al., 2013
C. antarctica lipase	Styrene-divinylbenzene beads	Methanol	Soybean oil	72 h	Transesterification	99.0 %	Poppe et al., 2013
Lipase from *B. cepacia* in the form of dry fermented solid	-	Ethanol	soybean soapstock acid oil	31 h	Hydroesterification	92.0 %	Soares et al., 2013
B. cepacia	Modified attapulgite	Methanol	Jatropha oil	30 h	Transesterification	94.0 %	You et al., 2013
T. lanuginosus	Mesoporous poly-hydroxybutyrate particles	Methanol and ethanol	Oleic acid	12 h	Transesterification	90.0 %	Miranda et al., 2014
R. oryzae	Polyvinyl alcohol–alginate matrix	Methanol	*Jatropha curcas*		Transesterification	87.1 %	Zarei et al. (2014)

C. antarctica lipase B (CALB) - Novozym 435	Macroporous acrylic resin	Blend of methanol and ethanol	Soybean oil	24 h	Transesterification	95.0 %	Zhao et al. (2014)
C. antarctica lipase B	Chitosan beads	Methanol	Waste frying canola oil	24 h	Transesterification	60.0 %	Aybastıer and Demir, 2014
Lipase from *Rhizomucor miehei* in the form of dry fermented solid	-	Ethanol	Acid oil from macauba pulp	32 h	Hydroesterification	91.0 %	Aguieiras et al., 2014

4. Enzymatic reactors

The use of immobilized lipase facilitates the development of commercial scale processes, favoring biotechnological processes upon their numerous advantages over the chemical synthesis that these systems provide, such as increased productivity. These advantages become even more interesting when scaling-up the process, where the reactions are carried out in reactors (or bioreactors) (Deng et al., 2010).

The main enzymatic reactors appearing in the literature are batch stirred tank reactors; continuous stirred tank reactors; fixed bed column reactors, in which the immobilized enzyme is packed, remaining stationary while the substrate solution is pumped through it; and fluidized bed column reactor, in which the immobilized enzyme is kept suspended by means of recycling of the substrate solution pumped through (Guisan, 2006; Kumar et al., 2013). The last two configurations can either be operated as batch or continuous mode, although their designs are best explored under the latter. In Fig. 3 is presented a schematic representation of the main types of reactors.

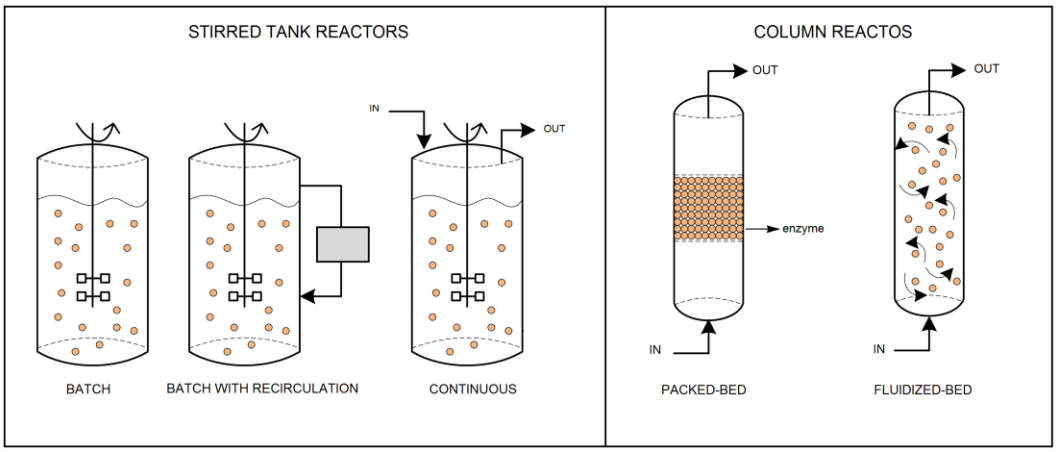

Fig. 3. Schematic representation of the main types of reactors.

The design of the enzymatic reactor requires adequate knowledge of the reaction kinetics, hydrodynamics of the system, and the mechanisms of mass transfer (Aires-Barros, 2002; Guisan, 2006). The main factors associated with the choice of the type of reactor to be used are summarized in Table 2.

Table 2. Main variables involved in choosing the reactor with immobilized enzymes.

Factors	Characteristics
Form of the immobilized enzyme	particles, membranes, or fibers
Nature of the substrate	solution, suspended solids, or colloidal
Operational requirements of reaction	temperature control and flow
Reaction kinetics	possible inhibition by substrate, product, or both
Catalytic surface per unit volume of reactor	support size and presence of pores
Mass transfer	transfer characteristics of external and internal mass
Replacement of the catalyst and regeneration	operational dead time
Construction of the bioreactor	geometry, the hydrodynamic fluid and operating conditions
Cost operacional of the bioreactor	energy and maintenance
Mode of operation	batch and continuous - designed for a specific process

(Castro et al., 2008; Christopher et al., 2014; Zanin and Moraes, 2004).

In the next topics it will be discussed the advantages and disadvantages of batch and continuous operation modes, and the reactor set-up used for enzymatic biodiesel synthesis.

4.1. Batch reactors

Batch reactors are usually the most commonly used in processes with enzymes in solution, though immobilized enzymes are also employed. Typically, the biocatalyst particles are dispersed in the solution of substrate and the agitation is provided by mechanical stirrers. The process can be operated with the addition of all components from the start, whereas stepwise addition of methanol, in special, is recommended (Da Rós et al., 2010). The reactor commonly used for biodiesel synthesis is the stirred tank reactor (STR) (Christopher et al., 2014). The batch STR is the simplest bioreactor consisting of a reactor equipped with temperature measurement devices and control, and

a stirring system, usually a propeller. After completion of reaction, the solid–liquid separation is carried out by centrifugation, filtration or decantation, in order to recover the immobilized enzyme. The STR operated in batch mode has a low throughput because of the need to unload, clean and reload the reactor before the start of new batch.

The low productivity disadvantage of the batch STR can be reduced by using a continuous STR where the enzyme is retained in the reactor by means of membrane technologies placed at the reactor outlet, with several configurations being possible (Christopher et al., 2014). Notwithstanding, conducting batch processes offers certain operational advantages, such as the high degree of substrate dispersion; simple equipment structure and easy reaction control; (Tan et al., 2010). In addition, it is simple to collect data about the process, for example, the reaction kinetics (Mendes et al., 2011a; Nielsen et al., 2008). In some reactions where there is deactivation of the biocatalyst by product, the batch system is highly indicated. In this case, the product concentration increases with time or length of the reactor (Liese and Hilterhaus, 2013).

Batch processes usually require long reaction times, and in order to be operated on a large scale, also required high-volume tanks. It is also important to consider the gradual loss of enzyme activity along reuses. When the enzymatic activity decreases, the reaction time needs to be increased to maintain a constant high degree of conversion. Along time, the production decreases and eventually is unacceptably low, and at this point the enzyme must be replaced (Nielsen et al., 2008). Batch systems have some other disadvantages for industrial scale use such as the biocatalysts are susceptible to disruption because of high shear stresses imposed by mechanical agitation (Balcão et al., 1996; Helwani et al., 2009).

In order to reduce these limitations, Fadnavis et al. (2007) proposed the use of STR fitted with a porous basket. This apparatus retains the immobilized enzyme, allowing the passage of reactants and products through their pores. This reactor has the advantage of allowing a more efficient contact among reactants and biocatalyst, which increases the reaction rate and efficiency. Another advantage is that the biocatalyst is separated from the reaction mixture simply by draining the circulating liquid (Baltaru et al., 2009; Fadnavis et al., 2007).

Another important factor that must be considered in batch operations is the agitation speed and the type of stirrer employed. Ognjanovic et al. (2009) explored the effect of the mode and intensity of stirring, reactor configuration, and the flow conditions on the activity and stability of the immobilized lipase. In their experiments,

the batch reactor was equipped with six-bladed turbine impeller (Rushton turbine). According to the authors, this stirring system was able to improve the contact between substrates and biocatalyst and provided a good dispersion of the biocatalyst in the reaction mixture. In addition, mass transfer was improved, whereas the reaction rate increased, and consequently the performance of the reactor. The mass transfer of triglycerides to the biocatalyst reaction sites could be a critical limiting step to the rate of the transesterification reaction because the reaction mixture is heterogeneous, consisting of two immiscible phases. Thus, the agitation intensity and the mode of agitation appear to be of particular importance for the transesterification process, because the agitation is the primary cause of enzyme deactivation in the batch STR systems (Ognjanovic et al., 2009).

Batch operations can also be carried out in packed-bed reactors (PBR). Veny et al. (2014) described the use of a packed-bed reactor with circulation (CBPBR) and down-flow to be applied in enzymatic methanolysis of *Jatropha curcas* oil. The CBPBR allowed the repeated use of enzymes while preventing enzyme inactivation and provided acceptable mixing conditions with solid retention times.

In CBPBR, the down-flow direction circulation of the reaction mixture can minimize the mass transfer limitation. Veny et al., 2014 studied the reaction kinetics, the external mass transfer, and internal diffusivity of immobilized enzyme of CBPBR, reporting low external mass transfer resistance (Damköhler number < 1), without any mass transfer limitation at a linear velocity of 6.1 cm / min in CBPBR. The internal diffusivity effect was studied with three different enzyme particle sizes with mean diameters of 398.16 µm, 558 µm, and 757.27 µm, showing no significant effects.

4.2. Continuous reactors

The technology of reactions in continuous flow can be characterized by the use of a range of various configurations tubular reactors, where the biocatalyst and the reaction medium flow through the reactors. In this case, the reaction time is determined by the flow rate and the reactor volume, characterizing the residence time (de Souza and Miranda, 2014).

The continuous process is a system that can be operated for long periods of time under steady state condition in which the state variables remain constant over time.

Processes involving immobilized enzymes would preferably be operated continuously to avoid loss of productivity.

The continuous system presents direct and indirect advantages when compared to batch system: greater efficiency in the control of reaction parameters and the mixing of reagents (Zanin and Moraes, 2004); lower cost in the optimization of reaction conditions; fewer steps to scale up; greater energy efficiency; fewer unit operations during the isolation of the product (Itabaiana Jr et al., 2013). Enzymatic reactors currently available are completely automated, so there is a strict control of temperature, flow and pressure. This monitoring is often not possible in batch conditions. Because of these characteristics, a large amount of biodiesel per volume unit can be obtained, enabling easy control of reaction conditions in terms of optimizing the quality of biodiesel (Halim et al., 2009; Wang et al., 2011).

The enzymatic transesterification of vegetable oils can be carried out substantially faster and more economically in continuous reactors than in batch reactors (Wang et al., 2011), and many works focus on performing comparisons between these two processes. In all reports, the continuous reactors were considered better than batch reactors (Kawakami et al., 2011; Lee et al., 2010; Ognjanovic et al., 2009).

A variety of continuous bioreactors has been proposed for lipase-catalyzed reactions. Packed-bed reactors (PBRs) (or fixed bed), which is the most common form of enzymatic reactors for continuous operation, is basically a cylindrical column holding a fixed bed of catalyst particles (support and enzyme), where the reaction medium is pumped throughout the column under a specific flow rate, which will determine the residence time (reaction time) according to the column volume (Fernandes, 2010). PBR is indicated to convert reagents to products in just one cycle, avoiding working on recycling conditions (Itabaiana Jr et al., 2013). Supports used to immobilize the enzymes should not have sizes smaller than 0.05 mm, in order to keep the pressure drop within reasonable limits. The range of flow rates used must be enough to provide a compromise between reasonable pressure drop, minimal diffusion layer and high conversion yield (Fernandes, 2010). These reactors have traditionally been used for most of large-scale enzymatic reactions because of their high efficiency, low cost, and ease of construction and operation (Chang et al., 2007; Halim et al., 2009; Hama et al., 2013; Séverac et al., 2011; Soares et al., 2013; Zhao et al., 2014). PBRs allow the reuse of the enzyme without a prior separation and protects enzyme particles from breaking down because of the low mechanical shear stress. Some disadvantages of this system,

however, may be cited, as the ease of obstruction of the bed, the appearance of preferential flow paths and heat and mass transfer inefficiencies (Fernandes, 2010). Additionally, long-term operation of a PBR is still a challenge (Zhao et al., 2014). An example of PBR was used by Hermansyah et al. (2011) for biodiesel production by *C. rugosa* lipase immobilized on zeolite beads, using cooking oil and methyl acetate as substrates. Maximal yield was about 87.09 % in 50 h (flow rate of substrate of 2 mL h^{-1}). The immobilized biocatalyst used in each experiment presented an operational stability of 40 h. After that, the biocatalyst was deactivated due to the weakening of bonds between the biocatalyst and support, and lipase was unable to bind to the substrate.

The Fluidized-bed reactor (FBR) is basically a variation of the packed-bed reactor, but operated only in up-flow mode, where the substrate solution is fed from the bottom of the bed at a flow rate high enough to lift the particles (Roy and Gupta, 2006). The pressure drop of the fluid flow effectively supports the weight of the bed. The reactor thus provides free movement of the catalyst particles throughout the bed. The fluidization may be carried out either by liquid or by gas (Kosseva et al., 2009).

The mass transfer in FBR is better when compared to PBR, but the residence time necessary to fluidize the bed, mediated by the flow rates, may result in lower yields. The bed expansion during fluidization is dependent on the nature of support, the reactor design, the velocity of fluidization, and the viscosity of the feeding substrates (Fernandes, 2010).

Advantages associated with FBRs are that small particles can be used because pressure drop is unaffected, and improved the mixing characteristics of the system, avoiding the formation of preferential paths, common in PBRs (Ray, 2012). However, large particles are usually required because of the low density difference between fluid and particles used in immobilized enzyme FBR systems, and the high viscosity of the fluids (Feng et al., 2013).

However, in order to operate efficiently, FBR reactors require lower amounts of enzyme per volume in the reactor, decreasing overall reactor efficiency. Furthermore, the major disadvantage of development of FBR is the difficulty in scaling-up these reactors, whereas PBRs allow scale-up factors of greater than 50,000. FBRs can only be scaled up by a factor of 10 to 100 times. In addition, changes in the flow rate of the substrate stream causes unexpected effects upon the conversion rate due to complex changes in the flow pattern within these reactors (Kosseva et al., 2009).

Stirred tank reactors (STR) described before for batch processes, have been used by some authors in continuous mode. In this case, it is necessary to keep the reactor being fed with a continuous supply of substrate and an equal volume of reaction mixture being withdraw, characterizing the dilution rate of the system. The project requires multiple tanks operating in series to ensure the same degree of conversion for the same reaction time. The main advantage of this design is the possibility of inserting steps of separation tanks for disposal product or by-products with the potential to inhibit the reaction (Fig. 4) (Nielsen et al., 2008). More details about continuous operation are discussed in the following topic.

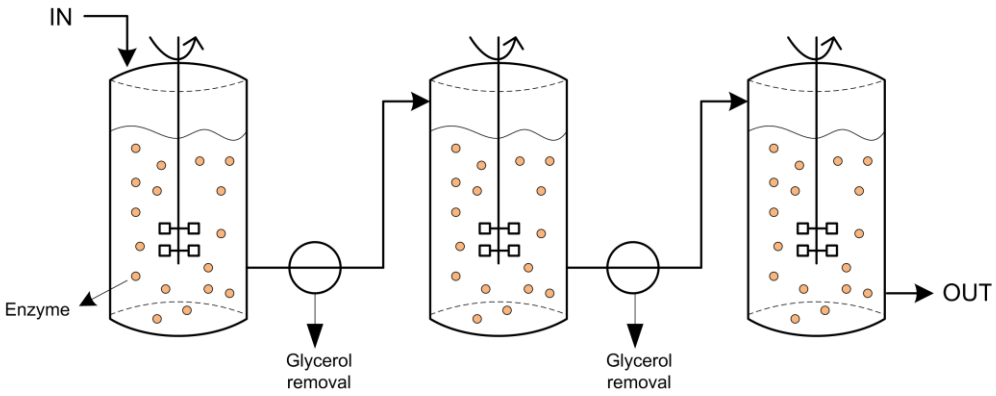

Fig. 4. Continuous stirred-tank reactor with three tanks in series.

4.3. General reactors settings

The performance of an enzymatic reactor depends directly and critically upon the properties of the biocatalyst employed. It is from this detailed analysis that will be determined the mode of operation and flow characteristics of the reactor. The reactor configuration also depends on possible foreseen technical problems, such as the homogeneity of the reaction mixture, product solubility in alcohol, type of alcohol, the enzyme stability, enzyme recovery, diffusion limitations, the effect of glycerol, among the most important (Castro et al., 2008; Itabaiana Jr et al., 2013).

The effect of the immobilized system is another factor for the reactor engineering, thus the choice of an optimal support for the immobilization is crucial for obtaining highly productive processes (Truppo et al., 2008). An ideal support must have high resistance to compression in operations at high flow rates PBR, frictional resistance in STR, and resistance towards settling velocity in FBR. Furthermore, for

continuous operation, rigid supports with spherical and uniform particle size are often more appropriate because these particles will produce little to no variations in operational pressure, and allow good flow characteristics of the interstitial fluid (Castro et al., 2008). The thermal, operational, and mechanical stability may limit the long-term application of the biocatalyst in the process. The mechanical strength of biocatalysts must allow for the use of filtration, centrifugation, and stirring, because continuous and repeated uses sometimes require these operations. Therefore, the mechanical stability of the support is crucial for many applications of immobilized enzymes in reactors. (Castro et al., 2008). Depending on the support material, the effect of mechanical stress can cause disintegration of the biocatalyst, further complicating the downstream processing (Liese and Hilterhaus, 2013).

The agitation speed in STR reactors influence yields of reaction, because it is closely related to the mechanical stability of the support (Basri et al., 2013). Immobilized *T. lanuginosus* lipase on silica gel was used in the synthesis of palm oil esters (POE) via alcoholysis of palm oil (PO) and oleyl alcohol (OA) in batch reactor. An important parameter tested was the stirring speed of the reactor, which ranged from 250 - 350 rpm. The conversion and system productivity were slightly increased with increasing impeller up to 325 rpm, which caused substantial improvement in the specific interfacial area between the substrate and enzyme observed in the non-aqueous phase, as a consequence of reduced droplet sizes. However, the yield and productivity decreased at 350 rpm (maximum tested agitation), caused by high shear and support disintegration (Basri et al., 2013). In this kind of reactor, some alternatives to enhance mass transport can also be achieved by reducing particle size to increase mass transport at a constant fluid stirring speed (Liese and Hilterhaus, 2013).

4.3.1. Mass transfer aspects in reactors

Mass transport limitations can be differentiated into four separate transport steps of the reactants to and from the active enzyme site (Liese and Hilterhaus, 2013). These are the (1) the film diffusion in, which is the passage of substrate through the boundary layer (stagnant film) around the particle surface; (2) pore diffusion in, which is the flow of substrate from the surface of the porous carrier to the active site; (3) pore diffusion out, which is the flow of product from the active site to the surface of the porous carrier; (4) film diffusion out, which is the flow of product from the surface through the

boundary layer (stagnant film) to the bulk medium. These steps are represented in Fig. 5.

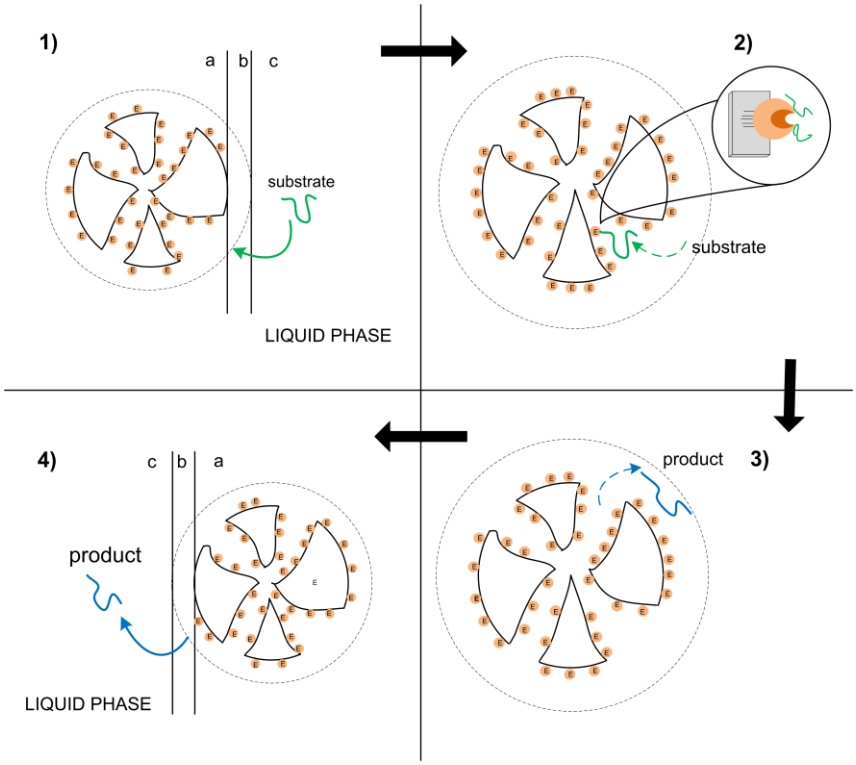

Fig. 5. Four steps ofmass transfer limitations: 1) Filmdiffusion in: substrate passing through the boundary layer close to the surface; 2) pore diffusion in: substrate passing fromthe surface of the porous support to the active site; 3) pore diffusion out: product passing from the active site to the surface of the porous support; 4) film diffusion out: product passing from the surface through the boundary layer to the bulk medium. (a: porous support; b: boundary layer (stagnant film); c: liquid phase).

The mass transfer aspects of PBR were studied by some authors, presenting general theoretical mathematical approaches to elucidate these aspects. Immobilized *C. rugosa* lipase in acid-washed activated glass beads were used in continuous-operated PBR to evaluate the effects of fluid flow rate and different feed substrate concentrations on the hydrolysis of rice bran oil (Murty et al., 2005). After a theoretical study of differential equations for the tubular reactor, based on the analysis of the height of the packed-bed, the superficial velocity and substrate concentrations throughout the reactor (Eq. (1)), it was possible to estimate the mass transfer coefficients and to identify the limiting diffusion and reaction regions:

$$h = \left(\frac{-v_S}{k_l a}\right) \times \int_{c_{S0}}^{c_{SF}} \frac{1}{(c_S - c_{S*})} dc_S \qquad (1)$$

Where h is the height of the packed-bed with immobilized lipase (m); v_S is the superficial velocity (m s^{-1}); $k_l a$ is the global mass transfer coefficient (1 s^{-1}); c_{SF} is the substrate concentration at the outlet of the reactor (kmol $(m^3)^{-1}$); c_{So} is the feed substrate concentration (kmol $(m^3)^{-1}$); c_{S*} is the interfacial substrate concentration (kmol $(m^3)^{-1}$) (Murty et al., 2005).

The global mass transfer coefficient ($k_l a$) increased with flow rates and is more sensitive at higher velocities, above 2.8 cm min^{-1}. Furthermore, feed substrate concentration seems to affect $k_l a$ less significantly compared to the flow rates. The data were represented in dimensionless Eadie-Hofstee plot (Fig. 6) in which R (dimensionless rate) was plotted against R / c_{Bb} with Damköhler number as the parameter (Eq. (2) and (3), respectively):

$$R = \frac{\gamma}{\gamma^{1}_{\max}} = \frac{c_{B*}}{(1 + c_{B*})} \tag{2}$$

$$c_{B*} = \frac{-(1 + Da - c_{Bb}) + \sqrt{(1 + Da - c_{Bb})^2 + 4 \times c_{Bb}}}{2} \tag{3}$$

Where γ^{-1}_{max} is the apparent maximum reaction rate (kmol $(m^3 \times s)^{-1}$); c_{B*} is the dimensionless interfacial concentration; Da is the Damköhler number.

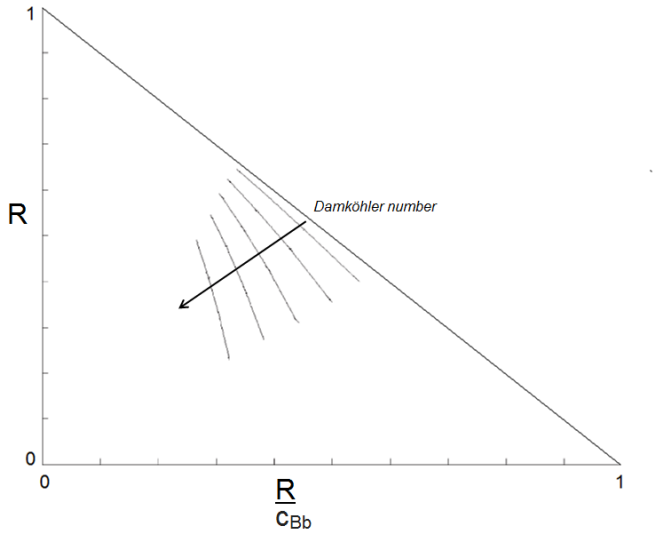

Fig. 6. Dimensionless Eadie–Hofstee plot with Damköhler number as the parameter. Adapted from Murty et al. (2005).

The dimensionless rate R is evaluated using eq. (2), in which c_{B*} is the dimensionless interfacial concentration, which varies from bottom to top of the packed bed reactor. The interfacial concentration in dimensionless form can be obtained as eq. (3) and used for calculation of R at particular velocity or Damköhler number.

Da can be used to assess the controlling region and the extent of mass transfer difficulty in the reactions. When mass transfer coefficient (k_l) is very large or Da is very small, the diffusional rate is very fast compared to the surface reaction and the system is governed by the kinetics. When the situation inverts, the concentration difference in the film is very steep and the system is governed by diffusion control (Murty et al., 2005). Summarizing, a linear plot at low Damköhler number indicates kinetic control and plots at higher values indicate various degree of diffusion control.

The effects of many parameters of reaction including the mass transfer influences, were evaluated during the ethanolysis of waste cooking oil (cottonseed) in batch reactors catalyzed by *C. antarctica* immobilized-lipase on macroporous acrylic resin (Novozym 435) (Chesterfield et al., 2012). In order to neglect the external resistance in the interfacial layer oil-ethanol, the reactor agitation speed was kept at 1,200 rpm. It was observed that the limiting step that controls the reaction rate is dependent on both intra-particle diffusion and surface reaction. Thus, to delineate the influence of the intra-particle diffusion on the reaction rate, different particle sizes of the biocatalyst (100-800 μm) were evaluated. Results showed that the size of the particles was a strong factor influencing the initial reaction rate, being indicated as optimal size of support around 400 μm. During the immersion in the mixture of oil and ethanol, the solid particles of immobilized lipase undergone swelling, which changes the pore structure, causing an apparent effect of pore diffusion (Chesterfield et al., 2012).

Therefore, particle size must be the right size to enable an easy design of the reactor and to prevent serious mass transfer restrictions (Fernández et al., 2013). In view of applicability, the particle diameter of biocatalysts to be used in PBRs, should ideally be between 200 and 400 μm to ensure low backpressures. For applications in STRs, the diameter can be reduced to ≥ 10 μm, enabling separation by filtration or sedimentation (Liese and Hilterhaus, 2013).

Research evaluating the effects of external mass transfer (film diffusion) has also been extensively reported. In most cases, the primary evaluation parameter is the speed of agitation (batch reactors), and flow rates (continuous reactors) (Fjerbaek et al., 2009).

In continuous reactors, the substrate flow rate is of fundamental importance: if it is too high, the contact time between substrate and lipase will be too short and the reaction will be incomplete; if it is too low, the throughput of the reactor will be small (Nie et al., 2006). The continuous methanolsis of olive oil was carried out using *Candida* sp. lipase immobilized on cotton membranes in a PBR with three columns. Flow rates varying from 12 to 40 L h^{-1} were tested, data showing that conversion to methyl esters decreased with increasing flow rates. The operational stability of the immobilized lipase was over 500 h and yields varied from 25 % to 35 % (Nie et al., 2006).

Reaction parameters affecting enzymatic transesterification catalyzed by Novozym 435 of waste cooking palm oil with methanol using tert-butanol as solvent in a continuously PBR was evaluated by Halin et al. (2009). The mass transfer effects have been studied by varying the flow rate and the packed-bed height, and a model of mass transfer has been developed. The height of the packed-bed ranged from 4.76 cm to 14 cm, and the substrate flow rate from 0.18 to 1.02 mL min^{-1}. The methyl esters of fatty acid (FAME) yield increased initially when there was an increase of substrate flow rate and packed-bed height, the maximum FAME yield of 80 % being achieved at 12 cm packed-bed height and substrate flow rate of 0.55 mL min^{-1}. At lower flow rates, low yields of FAME were obtained caused by mass transfer resistance at liquid film layer. However, at very high flow rates (0.9 to 1.02 mL min^{-1}) the substrate only passed through the enzyme without interacting with it (Halim et al., 2009).

The residence time is an important information on operating reactors, which allows to diagnose flow problems, such as the existence of zones of stagnant fluid or dead zones; extreme short circuitry and fluid sub-passage; existence of channeling; axial dispersion in tubular reactors; and segregation, resulting from the mixing conditions in the reactor. STR batch reactors, being thoroughly mixed, have a residence time very different from reactors operated continuously. Thus, the residence time displayed for a given reactor provides clear evidence of the kind of mixture that occurs within, and is one of the most informative ways to characterize the reactor (Fogler, 2005). The residence time (t) can be calculated according to Eq. (4):

$$t = \frac{L}{v_S} \times \varepsilon \qquad (4)$$

Where L is the length of the catalyst bed, v_s is the superficial velocity of the fluid (volumetric flow rate divided by the cross-sectional area of the bed), and ε is the void fraction of the bed.

Several studies focused on further examining the effect of residence time in enzymatic reactors. Hermansyah et al. (2011) investigated the biodiesel synthesis from waste cooking oil using immobilized *C. rugosa* lipase as a biocatalyst in a PBR system and methyl acetate. The relationship between residence time and the conversion was evaluated to determine the flow rate of substrate needed to produce an optimal biodiesel conversion. The experiments were conducted with four levels of flow rate: 1, 2, 4, and 5 mL h^{-1}, and the residence time was noted for each flow rate. Highest biodiesel conversion (71.47 %) was obtained at flow rate of 1 mL h^{-1} or residence time of 5.5 h, and the lowest conversion (38.79 %) was obtained at highest flow rate (5 mL h^{-1}) and residence time of 1 h. According to these authors, the concentration of biodiesel formed is directly proportional to the increased residence time in the reactor, i.e., prolonged contact between the substrate and the biocatalyst creates a strong catalytic reaction (Hermansyah et al., 2011).

Xu et al. (2012) developed a two-stage enzymatic process for the production of ethyl esters of fatty acids (FAEE) on a PBR, passing repeatedly the reaction mixture through a single column to simulate the effects of continuous production in a series of columns. Two immobilized lipases, those from *T. lanuginosus* (NS 88001) and Novozym 435, were used as catalysts for transesterification (first stage) and esterification (second stage), respectively. For transesterification, a mixture of rapeseed oil and ethanol was pumped into the reactor in a down-flow by a peristaltic pump at a flow rate of 0.14-6 mL min^{-1}. After all the reaction mixture passed through the column, glycerol was separated and additional oil was pumped through the column. This procedure was repeated until equilibrium conversion was reached. For esterification, either Novozym 435 or NS 88001 were packed into a column of about 22 cm long. The esterification reaction was carried out in multiple steps of a single pass through the column. The substrate was supplied by a peristaltic pump in down-flow through the column in a single pass at a fixed flow rate of 1.0 mL min^{-1}. In this way, the effects of flow and reaction rate (defined as mass-% of FAEE formed per residence time) were evaluated and an optimal superficial velocity (the corresponding Reynolds numbers based on the starting reaction condition) for conversion in a single pass was found to be around 1 cm min^{-1} for transesterification reaction. However, the reaction rate increased

almost linearly with increasing velocity, and this can be explained by an improved mass transfer at high flow velocities even though the Reynolds number, calculated by the authors, indicated laminar flow (Re < 10) at all tested velocities. Therefore, high velocities were used in the subsequent experiments because the limited residence time could be overcome by increasing the number of passes of the reaction mixture through the column. At flow velocity of 7.6 cm min^{-1}, 92.8 % of FAEE was obtained after the reaction mixture had passed 20 times through the reactor. The pressure drop was 1.1 bar at 7.6 cm min^{-1} and therefore higher flow rates were not tested due to limitations in the experimental set up. The calculation of the pressure drop was established according to Darcy's law, which is based on empirical observations. For a laminar flow through a column packed with porous support, the term is given by Eq. (5).

$$\Delta P = \frac{\mu \times L \times v_S}{\beta} \tag{5}$$

Where ΔP is the pressure drop, L is the length of the column; β is the permeability of the porous support, related to the porosity of the bed and the size of the packing particles.

The Reynolds number (Re) for flow through a packed-bed of identical and spherical porous particles is defined by Eq. (6):

$$\mathrm{Re} = \frac{\rho \times v_S \times d_S}{\mu(1-\varepsilon)} \tag{6}$$

Where, ρ is the fluid density, μ is the fluid viscosity, and d_s is the diameter of the spherical particles.

The effect of loading of biocatalyst in a PBR for transesterification has also been studied concerning the length of biocatalyst bed. As expected, reaction rates in longer bed lengths are generally found to be higher than those obtained in shorter length columns. This occurs due to to decreased viscosity of the reaction mixture, which is explained by the production of FAEEs and partial glycerides in the first part of the bed. Thus, a better mass transfer is obtained, which considerably increases the reaction yields (Xu et al., 2012). Furthermore, more active sites are available to promote the reaction between oil and alcohol at a given flow rate (Feng et al., 2011).

The use of reactors in series, in order to optimize conversion yields, is another aspect of research concern. Wang et al. (2011) evaluated continuous methanolysis using a battery of four columns packed with immobilized *P. cepacia* lipase on magnetized Fe_3O_4 nanoparticles, and this process was compared to using a single reactor. The maximum conversion rate of 75 % of biodiesel was obtained in the single PBR at 12 h, followed by reaction equilibrium phase lasting 132 h approximately. After 240 h, the conversion rate decreased to only 45 %. In contrast, the conversion rate and stability achieved using the four PBRs were kept over 88 % for 192 h, and after 240 h of operation, the conversion rate slightly dropped to approximately 75 %. The authors attributed the differences occurred because the four-packed-bed reactor provided a longer residence time and reduced the inhibition of the lipase-nanoparticle biocomposite by products, which may have further improved the reaction efficiency (Wang et al., 2011).

Owing to mass transfer limitations, PBRs do not seem to be appropriated for solvent-free enzymatic FAME production (Fjerbaek et al., 2009). The high viscosity of solvent-free systems causes the pressure drop becomes significant. In order to minimize the pressure drop, PBRs need to operate at low flow rates, and the size of carrier must be increased or solvent must be added. With increasing support particle diameter, the pressure drop decreases improving the mass transfer rate, which can affect the overall reaction rate (Nigam et al., 2014). With all such limitations, the use of solvents becomes strongly indicated.

4.4. Use of solvents in reactors

The use of organic solvents has several purposes, including: ensuring homogeneous reaction mixture; avoid problems of formation of two phases in the reaction; reducing the viscosity of the reaction mixture to increase the rate of diffusion, thus reducing the problems of mass transfer around the enzyme; improve the stability of the enzyme and accelerate the acyl migration (Fjerbaek et al., 2009; Rodrigues et al., 2010). Furthermore, organic solvents possibly eliminate the need for stepwise addition of alcohols (Antczak et al., 2009).

One open discussion concerning the use of solvents remains, that is the most adequate organic solvents for lipase-catalyzed reactions. Until recently non-polar solvents ($\log P > 4$), were reported to be the best choices for these reactions because of

enzyme higher activities in relatively hydrophobic organic solvents. The log P value is generally used to correlate solvent polarity with enzyme activity and stability in non-aqueous phases, but correlations between a simple parameter, such as solvent log P, and a complex factor as enzyme denaturation might be difficult to predict (Laane et al., 1987). Organic solvents such as hexane, petroleum ether, and *tert*-butanol have been widely used (Christopher et al., 2014).

Li et al. (2010) observed the effects of solvents by studying the methanolysis of oil of stillingia species catalyzed by Novozym 435 and Lipozyme TL-IM in the presence of organic solvents with different polarities on a batch reactor. The authors reported best results with solvents whose log P were in the range of 1.4 to 1.52, such as *tert*-butanol, acetonitrile, *tert*-pentanol, tetrahydrofuran, and 1,4-dioxane. Lower yields were obtained with the use of more hydrophobic solvents (log P > 2.0), such as n-hexane and n-heptane. These results showed that polar solvents are more efficient to dissolve methanol, avoiding denaturation of the lipase. Among the mentioned solvents, *tert*-butanol has been reported as producing the best results with most vegetable oils and a wide range of lipases (Aybastıer and Demir, 2014; Chen et al., 2011; Yan et al., 2014). Because of its branched chain of three methyl groups, *tert*-butanol has a steric hindrance, preventing the formation of *tert*-butyl esters. Furthermore, this solvent has a characteristic, not entirely understood, of preserving lipase activity from denaturation caused by glycerol (Azócar et al., 2014). The presence of glycerol directly affects the reaction efficiency because it adheres to the surface of the enzyme forming a hydrophilic layer and hindering the mass transfer of hydrophobic substrates, leading to a decrease in yield (Chattopadhyay et al., 2011). The negative effects of glycerol were studied by Li et al. (2006), who investigated the methanolysis of rapeseed oil in the presence of *tert*-butanol using Lipozyme TL IM and Novozym 435. According to the authors, glycerol was well dissolved on the solvent that prevented the formation of film layer on the biocatalyst. The solvent increased the operational stability of both lipases, eliminating the negative effects caused by excessive methanol and glycerol (Li et al., 2006).

On the other hand, the use of solvents should be avoided because of their toxicity and flammability, damaging effects on the environment and consequent requirement for solvent removal and reuse, increasing production costs (Aires-Barros, 2002).

More recently, other types of solvents such as supercritical carbon dioxide (Lee et al., 2011; Lozano et al., 2011) and ionic liquids (IL) (Lin et al., 2013; Mohammad et al., 2012) have also been used for enzymatic transesterification processes. Ionic liquids proved to be excellent substitutes to traditional organic solvents, having the possibility of shaping the IL according to the needs of the reaction. Gamba et al. (2008) conducted the methanolysis of soybean oil with immobilized *P. cepacia* lipase in the presence of IL 1-n-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide (6 g of enzyme was supported on 24 mL of IL). The reaction could be performed at room temperature, at 24 h, in the presence of water and without the use of others organic solvents, with up to 96 % yield under best conditions. The biodiesel was separated by a simple decantation and the recovered ionic liquid/enzyme catalytic system could be reused for at least four times without any loss of catalytic activity and selectivity.

4.5. Current context in the industrial enzymatic production of biodiesel

Although lipases as biocatalysts provide advantages in the synthesis of biodiesel, industrial application of the technology remains low. Two Chinese companies have established biodiesel production with enzymatic catalysis: Hunan Rivers Bioengineering Co. Ltd. (Hunan, China), which uses technology developed by Tsinghua University using Novozym 435 lipase in STRs with a designed capacity of 20,000 t per year; and Lvming and Environmental Protection Technology Co. Ltd. (Shanghai, China), which uses spent frying oil as substrates in STRs, with technology developed by the University of Chemical Technology, Beijing. In this second plant, the lipase of *Candida* sp. 99-125 is used as biocatalyst, with a cost of 32.6 US$/t biodiesel. Yields of FAMEs of up to 90 % under optimal conditions are reported, with production line capable of producing 10,000 t per year of biodiesel (Tan et al., 2010).

In 2008, biotechnology company Novozymes (Denmark) began collaboration with Piedmont Biofuels in Pittsboro, North Carolina, USA. The objectives of the project were to find a lipase with a selling price low enough to compete in the chemical biodiesel market and try to develop the enzymatic biodiesel process in pilot or production scale (Nielsen, 2014). The results led to the a patent filing in 2012, which describes the basis for the BioFAME® process utilizing liquid-formulated lipases as a catalyst and includes the reuse of the enzyme (Patent WO2012/098114, 2012) (Nielsen, 2012). In the first quarter of 2014, both Blue Sun Biodiesel in St. Joseph, Missouri,

USA, and Vieselfuel LLC in Stuart, Florida, USA, have announced the full-scale production of biodiesel based on lipase as catalyst. Novozymes has been the enzyme supplier and partner to accomplish full-scale production. According to Steve Bond, the company's business manager, Blue Sun produces 100 % enzymatic synthesis biodiesel on a large scale (30 million gallons per year), with production costs of US$ 75 to 80 cents per gallon (Scherer, 2014).

Process simulation and economical evaluation of enzymatic biodiesel production plant was conducted by Sotoft et al. (2010), where several scenarios have been investigated with different production scales (8 to 200 millions kg biodiesel/year) and enzyme prices (current price US$ 968.60/kg enzyme; future prices projected to US$ 9.69/kg enzyme). The process was modeled based on the use of methanol, high quality rapeseed oil, and with or without the use of solvents. Simulations with methanol and solvent-free/co-solvent operations were carried out to investigate how this affect the enzyme performance and process design and to elucidate what effect it has on the process economy. On the simulations, the solvent-free process is viable for scale productions of 200,000 kg biodiesel/year under current enzyme prices (50 % of the total process costs) For co-solvent operation, oil represents 73 % of costs, whereas enzymes costs drops to 22 % of process. The continuous operation was the only realistic option with stepwise addition of methanol. Biodiesel could be produced with enzymes and co-solvent to a final market price of US$ 1.90 to 3.02/kg biodiesel.

5. Concluding remarks

In order to reduce generation of waste, environmental impacts, and improve the processes of biodiesel production, the enzymatic catalysis has been extensively studied and developed by various authors and companies. As reported in this review, several microbial lipases have been used as biocatalysts, immobilized in a variety of supports in numerous configurations of reactors. Reported studies involved reactions with a wide range of fats and oils as substrates, and ethanol and methanol as acyl acceptors. In some cases, it was reported the gradual addition of alcohols, and the use of solvents in the reaction, which is often strongly indicated.

The analysis of the reaction parameters aimed at assisting the choice of the most suitable process for a given reactor. It is important to note that this choice is strongly

influenced by the type of support used in the immobilization of lipase, the substrate and the presence or absence of solvent in the reaction.

In the case of reactions involving continuous flow reactors, many authors have reported a high stability of the immobilized enzyme, and some reactors were able to operate continuously for many days. In addition, the shear stress over the immobilized system is smaller in this type of operation due to the absence of mechanical agitation. For packed-bed reactors, it is strongly suggested that the immobilized preparation presents sizes over than 0.05 mm, in order to keep the pressure drop within reasonable limits. For fluidized-bed reactor, smaller particles could be used, although bigger particles are usually required to support differences in the density of solid and liquid phases.

For processes involving batch system in STR, the immobilized preparations are susceptible to breakage due to high shear stresses imposed by the mechanical agitation (especially when inorganic matrices are used). Therefore, for the applications of these types of reactors, it is recommended the use of supports with particle diameters > 10 μm to reduce this inconvenience, and be easily filtered at the end of the process.

When studying new methods to improve a process already widely established, or even to develop a completely new protocol, the general aim is to reach industrial scales. Specifically addressing the biodiesel industry, the enzymatic production of this biofuel is already a reality in some refineries. However, still it is necessary improve existing techniques and develop new methodologies to increase its synthesis. Numerous strategies to reduce the economic impacts are studied, such as immobilization of enzymes, reuse of biocatalyst, the correct reactor configuration, the use of alternative substrates, among others. Thus, it is expected that very soon the enzymatic technology can be expanded to a larger number of research centers and refineries.

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CAPÍTULO III – OPTIMIZATION OF ETHYL ESTER PRODUCTION FROM OLIVE AND PALM OILS USING MIXTURES OF IMMOBILIZED LIPASES.

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A model enzymatic system for the synthesis of ethyl esters (biodiesel) using mixtures of immobilized lipases

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Abstract

Although reactions of transesterification are generally catalyzed by one specific lipase preparation, the concept of "*combi*-lipase" could be better explored for the production of biodiesel, since oils are heterogeneous substrates. In this research we tested this concept by evaluating the enzymatic transesterification of olive and palm oil, two diverse fatty acid compositions, using stand alone or mixtures of three immobilized lipases as biocatalysts: Novozym 435 (CALB), Lipozyme TL-IM (TLL), and Lipozyme RM-IM (RML). For olive oil, the combination of 29.0 % of TLL, 12.5 % of RML, and 58.5 % of CALB was the best combination, allowing for 95 % conversion efficiency in 18 h of reaction, up from 50 % for the best individual lipase (CALB). For palm oil, the best enzyme combination was 52.5 % of TLL and 47.5 % of RML, resulting in 80 % of conversion of ethyl esters in 18 h, compared to only 44 % when stand alone TLL was used. Repeated batches of reaction were carried out in order to test the operational stability of the *combi*-lipase systems, with results showing that they could be used for at least seven cycles keeping higher than 80 % of their initial activities.

Keywords: Biodiesel; *Thermomyces lanuginosus* lipase; *Rhizomucor miehei* lipase; *Candida antarctica* lipase B; *combi*-lipases; enzyme reuse.

1. Introduction

The transesterification of vegetable oils and animal fats using short-chain alcohols (methanol and ethanol) to produce alkyl esters to be used as biodiesel fuel has been extensively researched [1-3]. In industrial scale, biodiesel is mainly produced following the conventional chemical process where basic catalysts are used, which is efficient in terms of reaction time and yields. However, the chemical synthesis presents some drawbacks in terms of glycerol recovery, removal of salt residues, large amounts of waste water, and high energy costs [4]. In addition, the basic catalysis cannot be carried out using acid oils. Acid catalysts, which could be used with these acid substrates, are generally less efficient [5]. In this context, the research on enzyme catalysis for biodiesel synthesis, in special using lipases, is in continuous development to overcome setbacks of the chemical catalysis [6].

Lipases (EC 3.1.1.3) are largely used enzymes to catalyze alcoholysis, hydrolysis, esterification, and transesterification of fat acids. Lipases have excellent catalytic activities and stabilities in non-aqueous media and their specificity, regioselectivity, and enantioselectivity can be successfully used for many applications in organic synthesis, including the production of biodiesel [7]. Most of lipases are classified into two groups: sn-1,3-specific, which hydrolyzes ester bonds at sn-1 and sn-3 positions; and non-regiospecific (or random), which act on all three positions [8].

The main advantages of using lipases, as compared to the conventional chemical reaction, are their substrate specificity and selectivity, besides their ability to react at low temperatures, avoiding high costs of energy input [5]. The reaction is controlled by the molecular properties of the enzyme in use, the structure of the substrate, and factors affecting binding of the enzyme to the substrate [9]. These factors avoid the production of undesired by-products, allowing the production of purer biodiesel and glycerol [10].

On the other hand, enzyme regiospecificity to one type of fatty acid is a reason for decreased conversions, which also depends on the type of oil or fat in use as substrate [11]. This fact reflects the extensive research for finding best sources of lipases for optimal reaction rates and conversions for one specific substrate [8, 12, 13]. Vegetable oils, which are the main raw materials for biodiesel synthesis, are heterogeneous substrates, containing triglycerides formed by very different fatty acids, glycerides with lower substitutions, and even free fatty acids [14]. Therefore, it is difficult to find a lipase that optimally works for all available substrates [15].

In a previous work, we proposed the concept of *combi*-lipase biocatalyst for heterogeneous substrates. The concept is based on the fact that a composed biocatalyst of a mixture of different lipases should be more effective on heterogeneous substrates than one specific lipase [16]. It was applied for the hydrolysis of soybean oil, with results showing that the mixture of lipases from *Rhizomucor miehie* (RML) and *Candida antarctica* (CALB) was more efficient than the lipase from *Thermomyces lanuginosus* (TLL), which was the most active enzyme, when used individually [16].

In this context, the objective of this work was to evaluate the concept of *combi*-lipase biocatalyst for the transesterification reaction in the synthesis of biodiesel. Two vegetable oils, with very different compositions of fatty acids, olive oil and palm oil were used. Ethanol was used as acyl acceptor because this alcohol poses lower environmental impacts than methanol. The commercial immobilized lipases from *Candida antarctica* (CALB, Novozym 435), *Thermomyces lanuginosus* (TLL, Lipozyme TL-IM), and *Rhizomucor miehei* (RML, Lipozyme RM-IM) were used to compose the *combi*-lipase biocatalyst. The enzymes were also tested individually in order to verify their independent activities. The transesterification reaction parameters were optimized for the best *combi*-lipase composition. In addition, the recycling of the *combi*-lipase biocatalyst was tested by multiple batches of reuse in order to test its stability and activity.

2. Experimental

2.1. Materials

TLL immobilized on acrylic resin (Lipozyme TL-IM), RML immobilized on anion-exchange resin (Lipozyme RM-IM), and CALB immobilized on macroporous resin (Novozym 435) were kindly donated by Novozymes (Novozymes, Spain). Olive and palm oils were purchased at a local market and were used without any treatment. The composition of fatty acids of these oils is presented in Table 1 [17, 18]. All other chemicals were of analytical or chromatographic grade.

Table 1.

Fatty acid composition of palm and olive oils used in this research.

Fatty acid	Structure	Average composition (%)	
		Palm	Olive
Palmitic	16:0	52.71	10.2
Palmitoleic	16:1	-	-
Stearic	18:0	3.80	2.50
Oleic	18:1	36.71	78.10
Linoleic	18:2	6.70	7.10
Linolenic	18:3	-	0.76

2.2. Reaction of transesterification and its analysis

The transesterification reactions were carried out in 50 mL Erlenmeyer flasks containing 1 g of oil and appropriated amounts of ethanol, temperature, and enzyme content according to the experimental design. The reactions were carried out in an orbital shaker at 180 rpm.

After reaction completion, 5 mL of distilled water was added, followed by centrifugation (2 500 × *g*, 7 min, 4 °C). The upper phase, containing esters, was analyzed by gas chromatography (Shimadzu, model GC-17A) equipped with a flame ionization detector (FID) and DB5 capillary column (30 m × 0.25 mm id × 0.25 mM; J&W Scientific). The injector temperature was 300 °C, split ratio = 1:30 and the FID detector temperature was 310 °C. The carrier gas used was nitrogen at a flow of 1.0 mL min^{-1}. The chromatographic conditions were: initial column temperature of 50 °C, heating rate of 10 °C min^{-1} reaching a final temperature of 310 °C. The amount of sample injected was 1 µL, and total time of the analysis was 30 min.

Methyl heptadecanoate, which was used as an internal standard, was mixed with heptane to prepare a stock solution. After a sample was accurately weighted, an internal standard stock solution was added to the sample. A standard FAEE (Fatty Acid Ethyl Esters) mix (C4–C24) from Supelco was used to identify the peaks at different retention times and to correct the peak area using the response factors of the compound. The FAEE content was calculated using the compensated normalization method with internal standardization, based on the European standard DIN EN 14103 [19].

2.3. Reactions using the combination of lipase mixtures

A 3-factor mixture design and triangular surface analyses were performed to evaluate the best combination of lipases. The simplex-centroid design with interior points composed of 7 experiments with two replications at the center point is shown in Table 2.

Table 2.

Experiments performed in the mixture design.

Experiment	TLL	RML	CALB	Conversion (%)	
				Olive	Palm
1	1.00	0.00	0.00	48.57	23.29
2	0.00	1.00	0.00	32.19	20.12
3	0.00	0.00	1.00	56.04	16.48
4	0.50	0.50	0.00	49.77	38.18
5	0.50	0.00	0.50	57.19	26.00
6	0.00	0.50	0.50	44.17	20.98
7	0.33	0.33	0.33	54.91	30.46
8	0.33	0.33	0.33	55.34	33.11
9	0.33	0.33	0.33	54.30	35.86

The reactions were carried out in 50 mL Erlenmeyer flasks at 40 °C in an orbital shaker (180 rpm) for 18 h. The conditions were: substrate molar ratio, 6:1 (ethanol:oil); temperature, 40 °C; biocatalyst content 10 % as oil mass. All reaction conditions, including time, were defined based on previous studies [20-22]. The biocatalyst content corresponds to individual or mixtures of lipases according to Table 2.

2.4. Central composite design

A central composite design (CCD) of three variables was carried out in order to obtain the optimal conditions for the transesterification reaction (the optimum mixture of enzymes was selected as in section 2.3 and used as the biocatalyst). The variables and

their coded and uncoded values are presented in Table 3, whereas in Table 4 are shown the 17 treatments obtained for the three variables, each at five levels.

Table 3.

Processes variables and their levels used in the CCD.

Variables	Name	Coded Levels				
		-1.68	-1	0	1	1.68
X_1	Substrate molar ratio (ethanol:oil)	3	4.21	6	7.78	9
X_2	Temperature (°C)	30	32.59	37	41.16	44
X_3	Amount of biocatalyst (% based on oil mass)	5	7.03	10	12.97	15

Table 4.

Experimental design and results of the CCD.

Treatment	X_1	X_2	X_3	Olive (%)	Palm (%)
1	-1	-1	-1	68.32	25.36
2	-1	-1	1	72.77	34.85
3	-1	1	-1	51.23	7.20
4	-1	1	1	56.01	10.67
5	1	-1	-1	69.61	76.21
6	1	-1	1	77.18	79.05
7	1	1	-1	64.73	12.64
8	1	1	1	76.72	13.09
9	-1.68	0	0	71.06	24.53
10	1.68	0	0	79.26	75.51
11	0	-1.68	0	81.90	41.16
12	0	1.68	0	56.61	25.64
13	0	0	-1.68	58.85	17.29
14	0	0	1.68	98.30	64.67
15	0	0	0	80.15	24.45
16	0	0	0	83.24	24.35
17	0	0	0	83.31	25.28

The design was constructed of eight factorial points, six axial points (two axial points on the axis of design variable), and three replications at the central point. In each case, the percentage of conversion for transesterification was determined after 18 h of reaction. The second-order polynomial equation for the variables is as follows:

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ij} X_i X_j + \Sigma\beta_{ii} X_i^2 \quad (1)$$

Where Y is the response variable, β_0 the constant, β_i, β_{ii}, β_{ij} were the coefficients for the linear, quadratic, and for the interaction effects, respectively, and X_i and X_j the coded level of variables x_i and x_j. The above quadratic equation was used to plot surfaces for all variables.

2.5. Statistical analysis

The experimental design and analysis of results were carried out using Statistica 7.0 (Statsoft, USA). The significance of the regression coefficients and the associated probabilities, *p(t)*, was determined by Student's t-test; the second order model equation significance was determined using the Fisher's F-test. Statistical analysis of the model was performed as analysis of variance (ANOVA). The variance explained by model was given by the multiple determination coefficients, R^2. For each variable, the quadratic models were represented as contour plots (2D).

2.6. Enzyme reuse

After the transesterification reaction, the immobilized enzymes were separated from the reaction medium by vacuum filtration using a sintered glass funnel. The biocatalyst was washed 3 times with 5 volumes of hexane and the solvent was eliminated by incubation for 24 h at 30 °C, following the methodology described in a previous work [22]. The biocatalyst was then reused following the reaction conditions described above.

3. Results and discussion

3.1. Selection of the best *combi*-lipase for transesterification

Two vegetable oils (olive and palm oils), were used as substrates for the enzyme transesterifications. The choice of oils was made upon their very different compositions: palm oil has a significant content of C16, whereas olive oils is rich in C18:1. These differences of fatty acids compositions may affect some important properties of biodiesel, such as oxidation, which is a very common problem depending on the source vegetable oil used. Therefore, the three immobilized lipases were tested in transesterification reactions, alone and in combinations, according to a 3-factor mixture design. The results obtained for the mixture designs are shown in Table 2, and are graphically represented in Fig. 1.

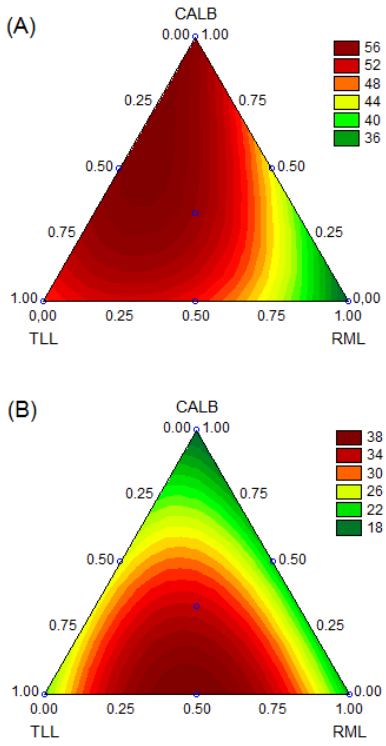

Fig. 1. Triangular surface for the mixture design. (A) Olive oil and (B) palm oil. Reac-tion conditions: substrate molar ratio, 6:1 ethanol:oil; amount of biocatalysts 10%;water 4% (in relation to oil mass); 37◦C; 180 rpm; 18 h.

As can be inferred from Fig. 1, the mixture of the lipases was always more effective than the best individual lipase. In addition, the most efficient combi-lipase biocatalyst changed for each of the oils. For olive oil, the presence of CALB and TLL in the mixture was very significant, whereas for palm oil, the best results were obtained with the combination of TLL and RML.

Some studies suggested that CALB and TLL exhibit different activities on transesterification of triglycerides, in which CALB is considered a nonspecific lipase

[23] whereas TLL could only act upon the acyl group positioned in sn-1,3, thus requiring the acyl migration from sn-2 to sn-1,3 in order to complete the reaction [24]. Therefore, it could be suggested that the combined use of these two lipases showing different specificities would increase the rate of the reaction as compared with other combinations. Furthermore, the combination of these enzymes could be an economically viable alternative when compared to their individual applications because the TLL is cheaper than the CALB [25].

For palm oil, the *combi-lipase* that presented the best reaction rate was the mixture of 50 % TLL and RML, each. Ibrahim et al. [26], studied the synergistic effect of mixtures of lipases TLL, RML, and CALB for the esterification of palm stearin (coconut oil), and reported an important and positive result for the combination of RML and TLL (50:50). When CALB was mixed with one of the other two lipases, this effect was reduced. This effect was also observed by Rodrigues and Ayub [15]. Individually, TLL was more active than RML over most of the tri, di, and mono-glycerides present in soybean oil. However, the mixture of both lipases was much more efficient than the individual enzymes.

The statistical analysis indicates that the best combination of lipase for transesterification of palm oil is a mixture of 52.5 % of TLL and 47.5 % of RML. When olive oil is used, the best transesterification performance was obtained for the *combi*-lipase composed of 29 % of TLL, 12.5 % of RML, and 58.5 % of CALB. Therefore, these two mixtures of biocatalysts were subsequently used in the next experiments.

3.2. Optimization of reaction parameters

3.2.1. Model fitting and ANOVA

After the selection of the best *combi*-lipase biocatalyst for both palm and olive oils, the reaction parameters were optimized in order to achieve the highest transesterification productivity. A CCD was carried out to evaluate the reaction temperature, amount of biocatalyst, and substrate molar ratio (ethanol:oil), and the results are presented in Table 4.

Using olive oil, the highest conversion obtained was 98.30 % at conditions in treatment 14 (6:1, ethanol:olive oil molar ratio; 37 °C; 15 % of enzyme relative to oil mass), whereas for palm oil, the highest conversion of 79.05 % was obtained at

treatment 6 (7.78:1, ethanol:palm oil; 32.6 °C and 12.97 % of enzyme relative to oil mass).

The experimental data have been adjusted to the proposed model in Eq. (1) and the second-order polynomial model to transesterification reaction are presented in Eq. (2) and (3) for olive and palm oils respectively.

$$Y = 83.36 + 3.93X_1 - 4.24X_1^2 - 5.98X_2 - 6.33X_2^2 + 6.96X_3 - 3.03X_3^2 + 3.56X_1X_2 + 1.29X_1X_3 \quad (2)$$

$$Y = 25.37 + 13.81X_1 + 6.71X_1^2 - 14.50X_2 - 0.82X_2^2 + 7.02X_3 + 3.51X_3^2 - 10.90X_1X_2 - 1.20X_1X_3 - 1.05X_2X_3 \quad (3)$$

Where Y is the percentage of conversion for transesterification reaction, and X_1, X_2, and X_3, are the coded values of substrate molar ratio, temperature and biocatalyst content, respectively.

Fisher's statistical test for analysis of variance (ANOVA) showed computed *F*-values of 3.805 (p = 0.045) for olive oil, and 3.37 (p = 0.05) for palm oil, both statistically significant. The goodness of a model was checked by the correlation coefficient (R). For olive oil, R = 0.91 and for palm oil, R = 0.90. These results showed a satisfactory representation of the process model and a good correlation between the experimental results and the theoretical values predicted by the model equation.

3.2.2. Effect of parameters on the transesterification rates

Table 5 presents the linear, quadratic, and the interaction effects of the variables substrate molar ratio (X_1), temperature (X_2), and amount of biocatalyst (X_3) on the enzymatic reaction. Linear effects are the most important, and they represent the average change in the response changing each variable from level -1 to 1. If the effect is positive, the change represents an increase in the response, and the contrary when the effect is negative.

Table 5.

Statistical analysis of CCD.

Variable	Olive			Palm
	Effect	Standard error	p-value	Effect
Mean	83.36*	0.36	<0.005	25.37*
Linear				
X_1	7.87*	0.33	0.002	27.63*
X_2	-11.97*	0.33	<0.005	-29.00*
X_3	13.93*	0.33	<0.005	14.04*
Quadratic				
X_1X_1	-8.48*	0.37	0.002	13.42*
X_2X_2	-12.67*	0.37	<0.005	1.64*
X_3X_3	-6.07*	0.37	0.004	7.02*
Interactions				
X_1X_2	7.13*	0.44	0.00	-21.80*
X_2X_3	2.58*	0.44	0.03	-2.41*
X_2X_3	1.18*	0.44	0.11	-2.10*

* Statistically significant at 95 % confidence level

All linear effects were statistically significant for the reactions with both oils, as shown in Fig. 2 (A) and (B). For olive oil, the most impacting effects were temperature (negative) and the amount of biocatalyst (positive, Fig. 2 (A)). For palm oil, substrate molar ratio (positive) and temperature (negative) had the most significant effects (Fig. 2 (B)).

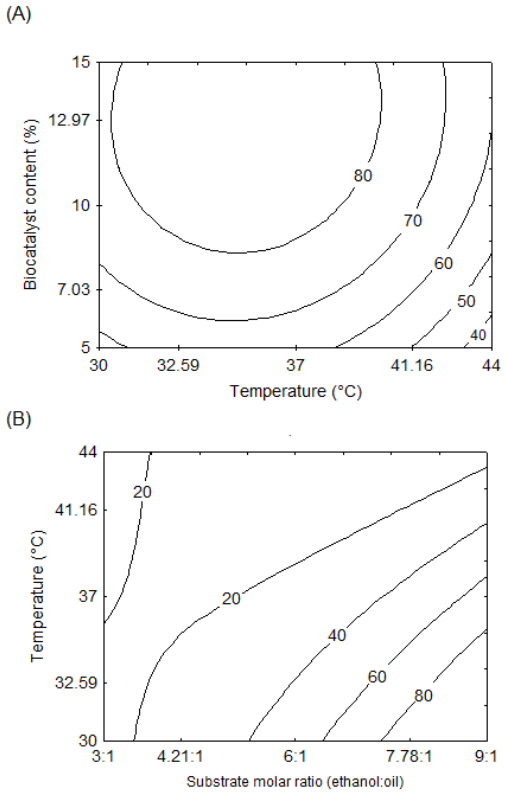

Fig. 2. Contour plots of transesterification reaction. (A) Olive oil, amount of biocat-alyst versus temperature; (B) palm oil, temperature versus substrate molar ratio.The numbers inside the contour plots indicate the values of yields of conversion (%)under the tested reaction conditions. The missing variable was fixed at the centralpoint.

The negative effect of temperature might be associated with loss of activity. Jeong and Park [27], reported loss of activity CALB when temperatures exceed 40 °C in the range 25-55 °C, producing decreased yields of conversion of transesterification of rapeseed oil. Some authors reported optima operating temperatures of TLL, RML, and CALB in the range of 30 to 35 °C [13], similar to results in this work. More specifically for TLL, Xu and Liu [28] reported a significant decrease in the yields of the transesterification of soybean oil when the temperature increases from 40 to 50 °C, which causes a decay in enzyme activity. Thus, milder reaction temperatures, as obtained in this optimization, are considered advantageous because of reduction of energy input and enzyme activity maintenance.

The highest yields of conversion promoted by increasing the amount of the *combi*-biocatalyst observed for olive oil is somehow expected as there are more active sites in the reaction medium [29]. However, according to Krishna et al. [30] there is a limit for the amount of added enzyme, above which further additions will not result in faster reaction rates. The authors tested the Lipozyme IM-20 in the transesterification

reactions, varying the enzyme amounts from 0.5 g to 10 g. They verified that with 3 g of enzyme was possible to achieve a 95 % conversion, the highest possible.

Concerning the positive effects of substrate molar ratio observed in the reaction using palm oil, an excess of alcohol will shift the thermodynamic equilibrium of the reaction towards the formation of ester, minimizing diffusion limitations and keeping the glycerol formed during the reaction in solution [20]. This mechanism can reduce the glycerol-mediated inhibition of the lipase, which takes place when glycerol liberated in the reaction blocks the entrance of catalyst pores [13, 31].

3.2.3. Optimal conditions for biodiesel synthesis and model validation

The optimal conditions for lipase-catalyzed biodiesel synthesis, using the *combi*-lipase biocatalysts of CALB:TLL:RML (58.5:29:12.5) for olive oil, and TLL:RML (52.5:47.5) for palm oil were determined by the response desirability profile calculated using the software Statistica 7.0. The optimal values of each variable were obtained for the desired response that, in this work, was the maximal yield of conversion after 18 h of reaction. For transesterification of olive oil the results were: substrate molar ratio of 7.04:1; temperature of 35.9 °C; 13.7 % of *combi*-lipase biocatalyst, relative to oil mass. For transesterification of palm oil the results were: substrate molar ratio of 9:1; temperature of 37.7 °C; and 15 % of *combi*-lipase biocatalyst, relative to oil mass.

Under these conditions, the theoretical value for the yields of conversion using olive oil predicted by the model was 98.3 %, whereas for palm oil it was 79.05 %. Experimental validation of the proposed model was conducted under optimized conditions with three repetitions and the average yields of conversion with the standard deviation obtained was 95.12 ± 1.56 % for olive oil and 81.04 ± 1.97 % for palm oil, showing excellent correlations between experimental results and the model prediction.

3.3. Time course of enzymatic reactions

In order to compare the transesterification reactions catalyzed by the *combi*-lipase biocatalysts and the lipases individually, reactions were carried out following the optimized conditions (CCD). The results of the performances of lipases in the transesterification reactions are shown in Fig. 3 (A) and (B) for olive and palm oils, respectively. In both cases, the conversion rates and the yields using *combi*-lipases were

much higher than those of stand-alone enzymes. Conversions peaked at 18 h of reaction, reaching 95 % using olive oil with the optimal *combi*-lipase biocatalyst (RML-TLL-CALB), and over 81 % using palm oil (TLL-RML), whereas none of the stand alone enzymes converted more than 50 % of their substrates.

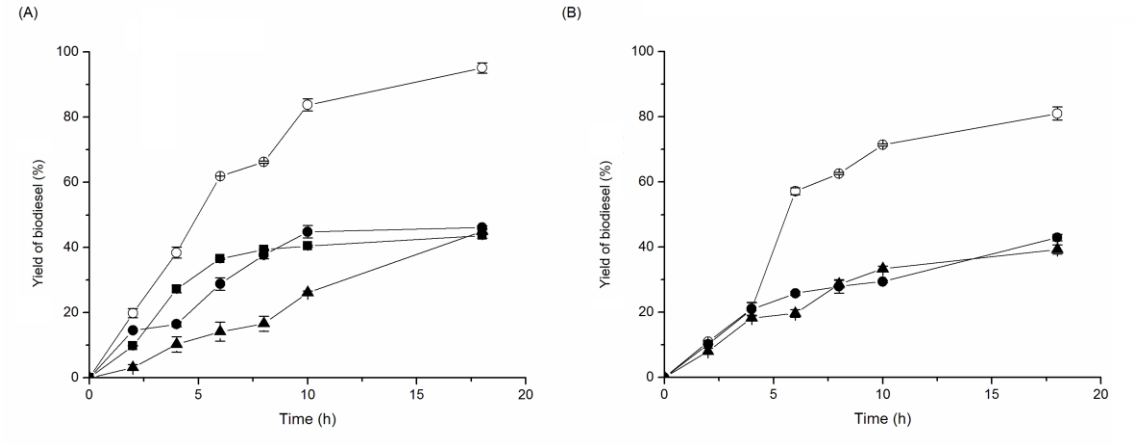

Fig. 3. Time course of transesterification of olive (A) and palm (B) oil catalyzed by *combi*-lipases (–○–), or stand-alone enzymes (–●–) TLL, (–▲–) RML, and (–■–) CALB. Reaction conditions for olive oil: substrate molar ratio 7.04:1 ethanol:oil; temperature of 35.9 °C; amount of enzyme 13.7 % as oil mass; and amount of water 4 % as oil mass. Reaction conditions for palm oil: substrate molar ratio, 9:1 ethanol:oil; temperature of 37.7 °C; amount of enzyme 15 % as oil mass; and amount of water 4 % as oil mass.

Considerations for these results emerge from enzymes differences in specificities related to type of fatty acids, besides their regio-specificities. Non-specific lipases, such as the one from *Candida antarctica*, act in all positions in the substrate molecule. On the other hand, sn-1,3-specific lipases (*Thermomyces lanuginosus* and *Rhizomucor miehei*) will preferably act at positions sn-1 and sn-3 of acylglycerol molecules [32]. Therefore, both the fatty acid composition of oils and the position of each fatty acid in the triglyceride will affect the best combination of the *combi*-lipase biocatalyst. Pleiss et al. [33], reported that CALB presents higher activities for short and medium chain length fatty acids, decreasing for long-chain fatty acids. Oppositely, RML has relatively low activities towards short chain fatty acids, but not for long fatty acids.

The results obtained in our work compared well with reports on transesterification for other similar systems. Lee et al. [34] used sn-1,3-specific immobilized *R. oryzae* lipase in combination with non-specific immobilized *Candida rugosa* lipase to catalyze transesterification of soybean oil with methanol, and obtained conversions up to 99 % within 21 h, whereas using the two lipases separately the

conversion was down to 70 % and 20 %, respectively. The enzymatic synthesis of biodiesel using palm oil and ethanol with immobilized lipase AY and lipase AK, both non-specific in a solvent-free continuous packed-bed reactor carried out by Tongboriboon et al. [8] was able to produce 67 % of biodiesel. The combination of lipases has also been demonstrated to synergistically act to reduce the total amount of enzyme required for efficient transesterification. For instance, Li et al. [25] reported that the combination of 3 % of TLL and 1 % of CALB (sn-1,3-specific and non-specific lipases, respectively) produced 95 % of conversion in the methanolysis of rapeseed oil with tert-butanol in 12 h. In contrast, the yield of methyl esters was 85 % in a reaction using 20 % of stand alone TLL, and 90 % using 2 % of CALB. Novozym 435, the lipase in CALB, is an excellent lipase capable of efficient biodiesel synthesis at high conversion rates. However, it is much more expensive than other commercial lipases [24]. The use of TLL reduced the required amount of CALB, thus representing lower costs of biodiesel synthesis [13, 21]. Finally, the use of the mixture of TLL and RML (both sn-1,3-specific), was reported by Rodrigues and Ayub [15], in the optimized ethanolysis of soybean oil. The authors also carried out the reaction using the enzymes individually under the same conditions. The yields obtained with the combined lipases were 90 %, around 10 % higher than that for TLL alone and 50 % than for RML.

3.4. Repeated batches of transesterification for biodiesel synthesis using the same biocatalyst preparation

Lipases are high-cost biochemicals and it would be very important if these enzymes could be reused in several batches of reaction cycles in order to allow their industrial application in the synthesis of biodiesel. Therefore, we design a repeated batch operation using the *combi*-lipase biocatalysts (TLL:RML:CALB for olive oil; TLL:RML for palm oil). The reactions were run for seven repeated batches under the optimal conditions, performing a hexane washing of the biocatalysts between batches. The washings with this non-polar solvent were necessary because it removes the oil layer/biodiesel formed around the enzyme, which could cause loss of activity and limits substrate and product diffusion [6]. The results of these experiments are presented in Fig. 4.

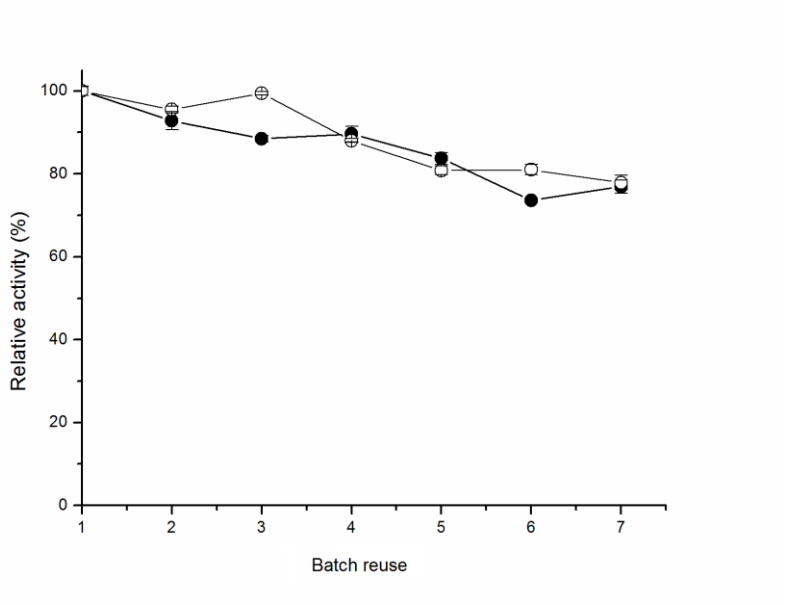

Fig. 4. Enzyme stability over repeated batches catalyzed by the mixture of lipases. (–○–) palm oil; (–●–) olive oil. The reactions were carried out at the optimal conditions.

At the end of the runs, the *combi*-lipase biocatalysts activities were around 80 % compared to the first batch, suggesting that both enzymes retained their activities.

4. Conclusions

The concept of *combi*-lipase biocatalysts for transesterification reactions using the heterogeneous substrates olive and palm oils was successfully employed in the synthesis of biodiesel, with high conversion yields in relatively shorter times. The enzyme combinations resulted in much higher conversions than when they were used individually, for both oils tested. Over repeated batches of reuse, both *combi*-biocatalysts showed to be stable, with over 80 % of their initial activity kept after seven batches, demonstrating that the enzymatic transesterification using mixtures of lipases is a very promising technology for large-scale production of biodiesel. Further studies are granted to test these preparations in continuous flow reactors.

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CAPÍTULO IV – ENZYMATIC SYNTHESIS OF BIODIESEL FROM WASTE FRYING AND SOYBEAN OILS USING THE *COMBI-LIPASE* CONCEPT IN ULTRASOUND-ASSISTED REACTIONS

Este artigo foi submetido para publicação no periódico Renewable Energy e ainda está sob avaliação.

Enzymatic synthesis of biodiesel from waste frying and soybean oils using the *combi-lipase* concept in ultrasound-assisted reactions

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Abstract

This work describes the use of an ultrasound system for the enzymatic transesterification of biodiesel using *combi-lipases* as biocatalyst. The reactions were carried out evaluating the use of both waste oil and fresh soybean oil, and the immobilized lipases CALB, TLL, and RML were used as biocatalysts. It was performed a mixture design of 3-factors to obtain the ideal mixture of lipases according to the composition of fatty acids present in each oil, and it was performed an optimization of the main reaction variables. The waste oil came out as an excellent substrate, producing biodiesel yields of 82 %. The use of the ultrasound technology in combination with the application of enzyme mixtures best suited to each reaction and the use of waste oil is a promising route to reduce the overall process costs of enzymatic biodiesel production.

Keywords: Biodiesel; waste oil; soybean oil; *combi-lipase*; ultrasound system.

1. Introduction

Lipases (EC 3.1.1.3) are enzymes that exhibit high catalytic activity and have great biotechnological potential based on their unique characteristics. In aqueous medium, lipases catalyze the hydrolysis of triglycerides, whereas under conditions of low amounts of water, they promote esterification, transesterification, and interesterification [1]. Lipases are thus applied in a variety of industrial sectors, especially in the bioenergy industry for the production of biodiesel, a biofuel that is a mixture of fatty acid alkyl esters (FAAE) [2].

Most of the lipases utilized as catalysts in organic synthesis are of microbial origin, such as from *Candida antarctica* (CALB) [3] *Thermomyces lanuginosus* (TLL) [4] and *Rhizomucor miehei* (RML) [5, 6] and their application requires the knowledge about their substrate specificity. Lipases, in their reactions with triacylglycerols, present different selectivities, such as typoselectivity (specificity for a particular fatty acid or group of fatty acids), regioselectivity (specificity for primary carboxylic ester groups at the sn-1 and sn-3 positions, or secondary carboxylic ester group at the sn-2 position), and enantioselectivity (ability to distinguish between two enantiomers in a racemic substrate) [1, 7].

In this sense, the combined use of lipases with different specificities for total modification of heterogeneous substrates such as oils is a newly reported strategy that allows increasing the conversion rates and process productivity, even when one specific lipase alone may be much better than each of the other individual lipases. These combinations of lipases are known as *combi-lipase*s [8]. In a previous work of our group [9], we have shown that the combination of commercially immobilized TLL (1,3-specific), RML (1,3-specific), and CALB (non-specific) in specific percentages could improve the conversion yields of olive oil to ethyl ester up to 95 % in 18 h, compared to maximal yields of 50 % obtained using the best individual lipase (CALB).

Lipid sources from oilseeds are considered as first generation raw materials for biodiesel synthesis, being employed in more than 95 % of industries due to consolidated production technologies worldwide. However, the competition with the food chain raises social and cost concerns, especially in relation to biofuel and edible oil prices, creating limitations on the use of these oils in the synthesis of biodiesel [10]. Low-cost raw materials such as spent frying oil deriving from industrial and commercial activities, are known as second generation sources for biodiesel production, and they are

used to reduce costs and environmental problems. Geris et al.[11] evaluated the biodiesel conversion rates of refined soybean oil and of spent frying oil, and reported similar results for both types of oils, showing that spent frying oil is a viable and promising alternative of use for this material.

In the transesterification process, reaction occurs at the interface between the two very low miscible phases formed between the substrates (oil and alcohol). Thus, the interfacial surface area between these two phases must be maximized to improve the product conversion rate. Despite the conventional mechanical agitation system to improve the mass transfer process being well established for transesterification [12], new technologies have been tested such as the application of ultrasound system [13].

The biodiesel production process using ultrasound is an alternative to the conventional process where the waves created at ultrasonic frequencies are able to shake the fluid on microscopic scale, producing cavitations bubbles that mix the phases more efficiently than in conventional reactors [14]. The use of ultrasound in catalysis reactions favors the mass transfer, as well as the reaction rate, allowing shorter-time reactions using smaller amounts of reagents, and under milder physical conditions [15]. Furthermore, the main advantage offered by this process is the need for less energy input, which consequently can optimize production costs, while providing less environmental impacts [16]. The ultrasound technology can be classified as fulfilling the sixth principle of green chemistry, which proposes the reduction of energy consumption in chemical processes [17]. Ultrasound system, in opposition to mechanical stirring, helps even inside porous catalyst, improving the substrate mixture, reducing diffusion problems and avoiding the formation of glycerin or water (side products in transesterification and esterification reactions, respectively) layers on the biocatalyst surface, that have been reported one main problem in this reactions [18, 19].

In the lights of these considerations, the objective of this work was to use waste (or spent) frying oil as a substrate for the enzymatic synthesis of biodiesel using the combination of ultrasound system technology and the concept of *combi-lipases*. We also run all tests using refined soybean oil under similar conditions to allow the comparison of yields using these two raw materials. The commercial immobilized lipases from *Candida antarctica* (Novozym 435), *Thermomyces lanuginosus* (Lipozyme TL-IM), and *Rhizomucor miehei* (Lipozyme RM-IM) were used to compound the *combi-lipase* biocatalyst, that has been optimized for this new product using statistical

tools, as this used cooking oil may have significant differences in composition with the previously used oils.

2. Material and Methods

2.1. Material

Three commercial immobilized lipases were used in this work and were kindly donated by Novozymes (Novozymes, Spain): Lipozyme TL-IM, immobilized on acrylic resin; Lipozyme RM-IM, immobilized on anion-exchange resin; and Novozym 435, immobilized on macroporous resin. Refined soybean oil was purchased at a local market, waste (spent) frying oil was obtained from a local commercial restaurant; both oils were used without any further treatment. Ethanol, hexane, and other chemicals were of analytical or chromatographic grade. Ultrasonic bath with temperature control model USC 2880A with 40 kHz and 220 W (Unique, Indaiatuba, Brazil) was used in all experiments. The equipment presents the capacity volume of 9.5 L with the following dimensions: 300 x 240 x 150 mm (length x width x height). Two disc transducers were placed at the bottom of the ultrasound vessel.

2.2. Physicochemical Characterization of Substrates

The substrates (soybean and waste oils) were characterized according to AOCS (American Oil Chemists Society) standards: Iodine index (AOCS Cd 1d-92); Saponification index (AOCS Cd 3-25); Acidity (AOCS – Ca 5a-40); Peroxide Index (AOCS Cd8b-90), and fatty acid composition (by gas chromatography AOCS Ce1b-89) [20]. Table 1 shows the results of the physicochemical analysis of the waste oil and commercial soybean oil.

Table 1.

Physicochemical properties of soybean and waste oils

Parameter		Soybean	Waste
Iodine index	g I_2 100 g^{-1}	120-141	98.5
Saponification index	mg KOH g^{-1}	180-200	214
Acidity	g oleic acid 100 g^{-1}	< 0.3	< 0.5
Peroxide Index	m_{eq} kg^{-1}	< 10	23.3
Molecular weight	g mol^{-1}	884	840

2.3. Transesterification Reactions

The transesterification reactions were carried out in 50 mL Erlenmeyer flasks containing 1 g of oil and appropriated amounts of ethanol (molar ratio to oil) and *combi-lipase* according to the experimental design (described in item 2.5). Oils molecular weights were based on their fatty acid composition (Table 2). The reactions were carried out at 40 °C.

Table 2.

Fatty acid of different oils and *combi-lipase* composition

Fatty acid	Chemical Structure	Average composition (%)			
		Palm	Olive	Soybean	Waste frying oil
Palmitic	C16:0	52.7	10.2	11.4	7.4
Palmitoleic	C16:1	0.1	-	0.2	0.4
Stearic	C18:0	3.80	2.5	4.1	2.4
Oleic	C18:1	36.7	78.1	23.0	0.1
Elaidic	(C18:1*n*9*t*)	-	-	-	56.2
Linoleic	C18:2	6.7	7.1	53.4	31.1
Linolenic	C18:3		0.7	6.6	0.3

***Combi-lipase* (%)**		Palm[a*]	Olive[a*]	Soybean[b*]	Waste[b*] frying oil
	TLL	52.5	29	22.5	40
	RML	47.5	12.5	27.5	25
	CALB	-	58.5	50	35

[a] Poppe et al., 2015b; [b]This work.
*The reactions were carried out in batch reactors under mechanical stirring

2.4. Gas Chromatography Analysis

Methyl heptadecanoate was used as an internal standard and mixed with heptane to prepare a stock solution. The sample was accurately weighted (50 mg) and 1 mL of internal standard stock solution was added. A standard FAEE (Fatty Acid Ethyl Esters) mix (C_4-C_{24}) from Supelco was used to identify the peaks at different retention times and to correct the peak area using the response factors of the compound. The FAEE content was calculated using the compensated normalization method with internal standardization, based on the European Standard DIN EN 14103 [21].

2.5. Statistical Design

A 3-factor mixture design and triangular surface analysis were performed to evaluate the best lipase combination for the transesterification reaction for each oil. The simplex-centroid design with interior points composed of 7 experiments, with two replications at the center point, is shown in Table 2.

The reactions were carried out in 50 mL Erlenmeyer flasks at 40 °C for 18 h, using 6:1 ethanol:oil molar ratio, and 15 % (by oil mass) of biocatalyst. All reaction conditions were based on a previous study [9]. The biocatalyst content corresponds to individual or mixtures of lipases as shown in Table 3.

Table 3. Experiments performed in the mixture designs

Experiments	TLL	CALB	RML	Conversion (%)	
				Waste oil	Soybean oil
1	1.00	0.00	0.00	64.48	66.81
2	0.00	1.00	0.00	66.44	73.41
3	0.00	0.00	1.00	58.29	33.05
4	0.50	0.50	0.00	65.19	55.75
5	0.50	0.00	0.50	41.67	44.23
6	0.00	0.50	0.50	65.89	75.20
7	0.33	0.33	0.33	61.13	49.00
8	0.33	0.33	0.33	56.93	49.39
9	0.33	0.33	0.33	60.40	55.99

After selection of the best lipase mixture, it was performed the optimization of transesterification reaction conditions by a central composite design (CCD). Three variables were evaluated: substrate molar ratio; *combi-lipase* content; and water content. The variables and their coded and uncoded values are presented in Table 4, whereas in Table 5 are shown the 17 treatments for the three variables, each at five levels.

Table 4. Processes variables and their levels used in the CCD

Variable	Name	Coded Levels				
		-1.68	-1	0	1	1.68
X_1	Substrate molar ratio (ethanol:oil)	3:1	4.21:1	6:1	7.78:1	9:1
X_2	Amount of *combi-lipase**	5	9.09	15	20.9	25
X_3	Added water*	0	2.02	5	7.97	10

* % based on oil mass

Table 5. Experimental design and results of the CCD

Treatment	X_1	X_2	X_3	Conversion (%)	
				Waste oil	Soybean oil
1	-1	-1	-1	17.50	54.55
2	-1	-1	1	9.88	3.27
3	-1	1	-1	38.70	79.88
4	-1	1	1	20.55	18.79
5	1	-1	-1	37.15	42.58
6	1	-1	1	11.31	2.17
7	1	1	-1	49.65	54.43
8	1	1	1	26.20	15.42
9	-1.68	0	0	51.26	29.50
10	1.68	0	0	23.69	13.20
11	0	-1.68	0	12.14	26.98
12	0	1.68	0	26.51	39.20
13	0	0	-1.68	63.79	74.34
14	0	0	1.68	11.70	66.57
15	0	0	0	18.48	34.08
16	0	0	0	22.76	37.85
17	0	0	0	23.52	27.79

In each case, the percentage of conversion for transesterification was determined after 18 h of reaction. The second-order polynomial equation for the variables is as follows:

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ij} X_i X_j + \Sigma\beta_{ii} X_i^2 \quad (1)$$

Where Y is the response variable, β_0 is the constant, β_i, β_{ii}, β_{ij} were the coefficients for the linear, quadratic, and for the interaction effects, respectively, and X_i and X_j are the coded level of variables x_i and x_j. The above quadratic equation was used to plot surfaces for all variables.

The experimental design and analysis of results were carried out using Statistica 12.0 (Statsoft, USA). Statistical analysis of the model was performed as analysis of variance (ANOVA). The variance explained by model was given by the multiple determination coefficients, R^2.

2.6. Test of Operational Stability

After the transesterification reaction, the immobilized enzymes were separated from the reaction medium and reused in subsequent reactions. At the end of each reaction, the biocatalysts were separated from the reaction medium, washed with hexane, dried at room temperature and used in a new fresh reaction [9].

3. Results and Discussion

3.1. Optimization of *Combi-lipase* Biocatalysts Composition for Used oil Transesterification

A 3-factor simplex-centroid design was performed in order to define the best enzyme combination to catalyze the transesterification of waste oil and soybean oil. The results obtained for the mixture designs are shown in Table 3, and are graphically represented in Fig. 1.

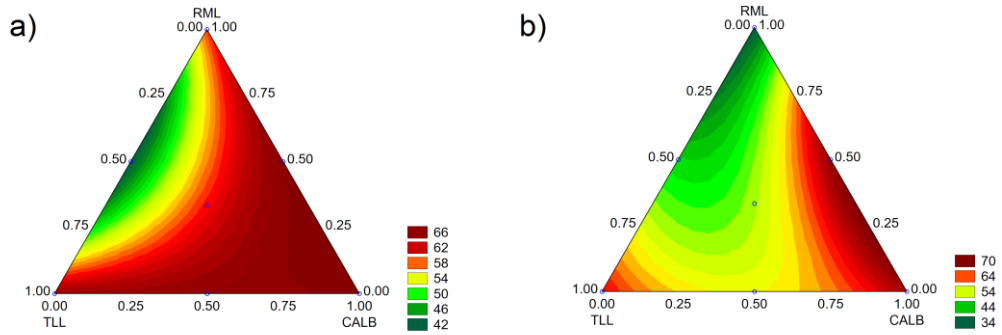

Fig.1. Triangular surface for the mixture design. a) waste oil; b) soybean oil. Reaction conditions: substrate molar ratio, 6:1 ethanol:oil; amount of biocatalysts 15 % (in relation to oil mass); 40 °C; 18 h.

According to the set of experiments represented in Table 3, the highest yields using waste oil as substrate were obtained using only CALB as biocatalyst, whereas for soybean oil, the highest yields were obtained using the mixture composed by 50 % CALB and 50% RML. This again exemplify that one *combi-lipase* is valid only for a specific oil.

However, although the best experimental results were obtained using only CALB, the surface response analysis predicted that they could be improved using a *combi-lipase* composed by 20 % of TLL, 60 % of CALB and 20 % of RML.

Also, statistical method predict that for soybean oil the best *combi-lipase* is composed by 10 % of TLL, 75 % of CALB and 15 % of RML. Experimental validation of the *combi-lipase* composition was performed under the same conditions of the mixing experiment and the mean conversion yield with the standard deviation obtained were 73.11 ± 0.81%. In all the following experiments, these lipase combinations were used as biocatalyst.

The test of these lipase combinations is important because they can be optimized according to the specific composition of oil sources, at the same time representing cost reductions depending on the enzyme of choice. Tiosso et al. [7] suggested that to produce biodiesel from triglycerides, sn-1,3-specific lipases (regioselective) should be combined in a way that all di- and monoglycerides could be converted into fatty acid alkyl esters. On this suggestion, Su et al [22] evaluated the synergistic effect of lipases with different classifications for the synthesis of biodiesel using soybean oil. The authors evaluated the effect of the combination of *Rhizopus oryzae* lipase (sn-1,3-specific), which has low catalytic efficiency for the conversion of ethyl esters, with the lipases of *Pseudomonas fluorescens*, *Candida rugosa*, *Candida antarctica* (all non-

specific enzymes), plus *Thermomyces lanuginosus* and *Mucor miehei* lipases (both sn-1,3-specific). The authors reported positive synergistic effects only for the combination of lipases of different regio-specificity, i.e. between sn-1,3-specific and non-specific enzymes, with best results obtained when using the combination of *R. oryzae* (ROL) and *C. antarctica* (Novozym 435) lipases [23]. This combination allowed to reach reaction equilibrium at 21 h, with yields of 98.3 %, whereas for the use of individual lipases, reaction equilibrium was reached at around 60 h, with yields of 93.2 % for ROL and 90.3 % for Novozym 435, respectively, showing that the mixture of lipases acting at different positions of the substrate molecule could increase the catalytic efficiency of the reaction.

However, besides regio-specific lipases also show specificity as to the type and length of the fatty acid chain of the substrates. This selective characteristic of the enzyme is due to the conformation of the active site and to the residues of amino acids that surround it, which may have different charge and reaction groups and are therefore susceptible to substrate molecules [24].

Analyzing the composition of each oil presented in Table 2, it is possible to make some considerations about the specificities of each lipase. The results in this work were compared to a previous report [9], when we used palm and olive oils. For soybean, used frying oil, and olive oil, the main fatty acids present in their composition are unsaturated fatty acids, comprising around 85 % of their composition. In the case of these 3 oils, the best results were obtained when CALB was the main enzyme in the *combi-lipase* mixture. One possible explanation for this result is because the small lid in the surface of CALB [25] makes easier the access of the unsaturated chains to the active site [26]. The main fatty acid in palm oil is the palmitic acid, and in this case, RML and TLL were in the best *combi-lipase* mixture [26], suggesting that linear chain fatty acids work better with these two lipases [27]. Based on the comparison of all results in Table 2, it seems to be plausible that TLL has some specificity for oleic acid, the second main fatty acid in palm oil, also present in olive oil. Taking into account the results obtained in this study and reports on the same subject in the literature, it would be necessary to study reactions using pure triglycerides of each fatty acid, as well as investigating the reactions in a heterogeneous mixture, where the mechanisms of each lipase could be affected.

3.2. Optimization of Transesterification Reaction Parameters

A central composite design was employed to optimize the biodiesel synthesis conditions using each respective optimal *combi-lipase* and oil, and the results are presented in Table 5. The highest conversion for waste oil was obtained in experiment 13 (63.79 %), whereas for soybean oil, the highest conversion was obtained in experiment 3 (79.88 %).

The experimental data were adjusted to the proposed model in Eq. (1) and the second-order polynomial model to transesterification reaction are presented in Eq. (2) and (3) for waste and soybean oils, respectively:

$$Y = 21.97 + 4.34X_1^2 + 6.11X_2 - 11.91X_3 + 4.43\ X_3^2 \quad (2)$$

$$Y = 33.79 + 6.33X_2 - 15.01X_3 + 11.33X_3^2 \quad (3)$$

In Table 6 are represented the linear, quadratic, and the interaction effects of the variables on the transesterification reaction using waste oil and soybean oil. The added water (X_3) showed significant negative effect (linear), while the *combi-lipase* variable (X_2) showed a significant positive effect (linear), for both oils.

Table 6. Statistical analysis of CCD to waste and soybean oil

Variable	Waste oil			Soybean oil		
	Effect	Standard error	*p*-value	Effect	Standard error	*p*-value
Mean	21.97*	1.56	<0.00	33.79*	2.29	0.00
Linear						
X_1	-1.26	0.73	0.48	-10.15	1.37	0.06
X_2	12.22*	0.73	0.01	12.67*	1.37	0.04
X_3	-23.82*	0.73	0.00	-30.02*	1.37	0.00
Quadratic						
X_1X_1	8.68*	0.81	0.03	-12.11	1.51	0.05
X_2X_2	-4.17	0.81	0.12	-3.79	1.51	0.33
X_3X_3	8.87*	0.81	0.03	22.67*	1.51	0.01
Interactions						
X_1X_2	-1.11	0.96	0.62	-3.94	1.79	0.38
X_2X_3	-5.87	0.96	0.09	8.23	1.79	0.15
X_2X_3	-2.03	0.96	0.40	-2.10	1.79	0.62

The effect of these variables can be better visualized in Fig. 2, in which the lowest the amount of added water in the reaction and highest the amount of *combi-lipase*, provided the maximum conversion yield of biodiesel.

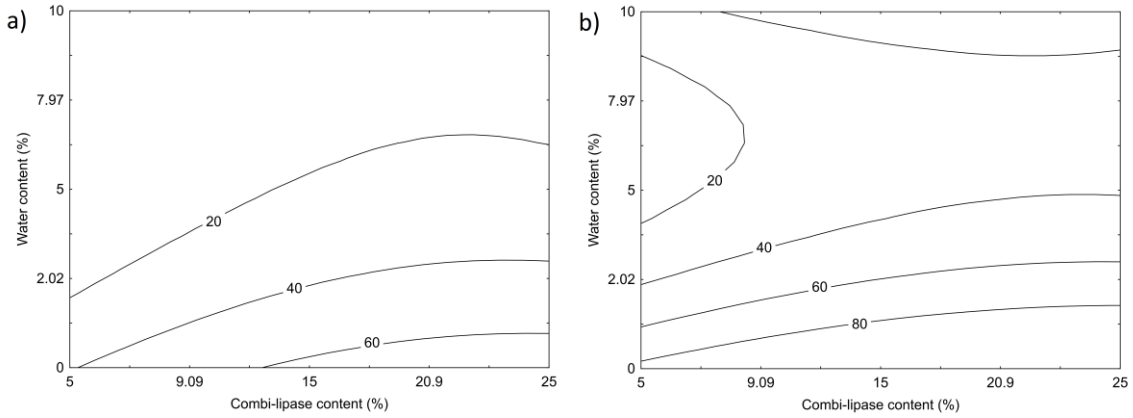

Fig. 2. Contour plots of conversion yields on biodiesel synthesis. a) waste oil, and b) soybean oil. The numbers inside the contour plots indicate the percentage of conversion under the tested reaction conditions. The substrate molar ratio variable was fixed at the central point.

In the transesterification process, exceeding amounts of water might lead to hydrolysis reactions or the formation of water layers on the enzyme support that could drive to the enzyme inactivation due to the very high concentration of acids. Therefore, low water activities, in transesterification reaction positively affects synthesis of fatty acid ethyl esters [28]. Regarding the amount of lipases added to reaction, Tretin et al. [13] reported that in the ethanolysis of soybean oil in a solvent-free system using Novozym 435, the concentration of enzyme positively affected the yields of reaction, which was expected, promoting shorter reaction times.

3.3. Comparisons of transesterification of Waste Oil and Refined Soybean Oil

Experimental validation of the proposed model was conducted under optimized conditions with three repetitions. Fisher's statistical test for analysis of variance (ANOVA) showed the following computed *F*-values: 2.82 for waste oil and 2.77 for soybean oil, both values were statistically significant ($p < 0.05$). The goodness of the model was checked by the determination coefficient ($R^2 = 0.78$) and the correlation coefficient (R = 0.88), the same values for waste and soybean oils.

Optimal conditions for biodiesel synthesis catalyzed by *combi-lipase* were determined by the response desirability profile calculated using Statistica 12.0 software. The optimal values of each variable were obtained for the desired response that, in this work, was the maximum yield of conversion after 18 h of reaction. For waste oil, the optimal conditions were: substrate molar ratio 9:1 ethanol:oil, and 25 % of *combi-lipase* content as oil mass, whereas for soybean oil, best conditions were: substrate molar ratio 4.95:1 ethanol:oil, and 25 % of *combi-lipase* content as oil mass.

Under these conditions, the theoretical values using waste oil predicted by the model were 76.97 %, whereas for soybean oil it was 100 %. Experimental validation reached 71.10 ± 2.15 % for waste oil and 94.98 ± 0.95 % for soybean oil. Compared to soybean oil, the waste oil presented excellent yields of biodiesel synthesis, proving to be a good source of raw material for this productive chain. These results showed a satisfactory representation of the process model and a good correlation between the experimental results and the theoretical values predicted by the model equation.

Besides the cost of the enzymes, another drawback in biodiesel production is the high value of refined oils, which are frequently used with high quality, also competing for food applications. A considerable number of works suggested the use of waste oil

for the enzymatic synthesis of biodiesel in order to reduce costs and turn the process more environmentally friendly [11, 29-31].

Spent frying oils are cheap, but may present some problems of oxidation, high free fatty acid composition, high polymerization products, and high viscosities. As a result, the pretreatment of such oils are necessary in order to reduce these problems [32]. In the alkaline (chemical) biodiesel synthesis, the use of NaOH or KOH requires low-acid feedstocks to avoid the formation of emulsions, whereas in the enzymatic route, this problem is averted by the high selectivity of lipases [33]. In the present study, the waste oil showed a higher content of free fatty acids compared to soybean oil. However, despite this characteristic, the waste oil could be used without pretreatments, suggesting that the oil composition showed no negative influence on the catalytic efficiency of the biocatalyst. The time-course of the transesterification reaction was followed under the optimal conditions (Fig. 3).

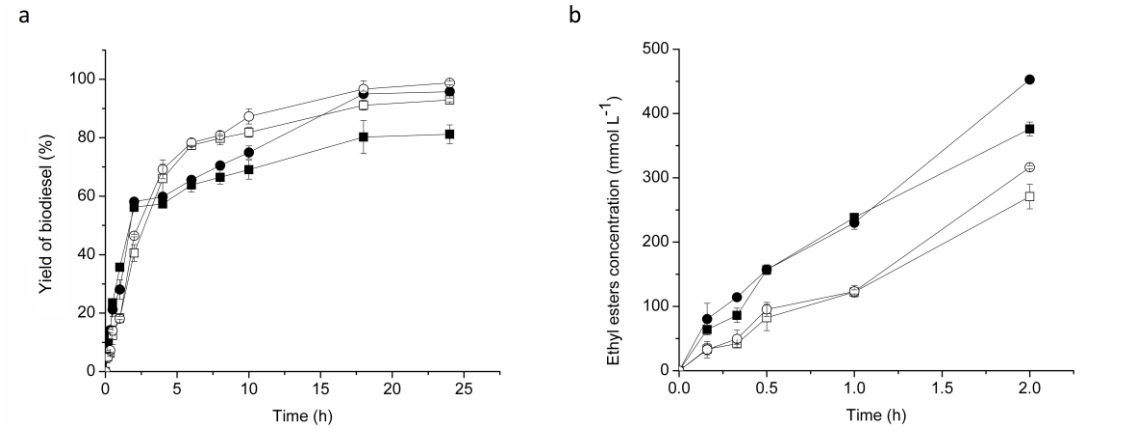

Fig. 3. Time course of transesterification of waste oil (square) and soybean oil (circle) by *combi-lipase.* The open symbols are the reactions conducted under mechanical stirring (MS), whereas the closed symbols are the reactions conducted under ultrasound system (US). Conversion yields at 24 h (a); Ethyl esters concentration at 2 h (b). Reaction conditions for soybean oil under US: substrate molar ratio 4.95:1 ethanol:oil, and 25 % of *combi-lipase* content as oil mass; under MS: substrate molar ratio 8.09:1 ethanol:oil, and 25 % of *combi-lipase* content as oil mass. For waste oil under US and MS, conditions were: substrate molar ratio 9:1 ethanol:oil and 25 % of *combi-lipase* content as oil mass.

The results of ultrasonic energy were compared to the conventional stirring. Usually, the enzymatic transesterification reactions using soybean oil under mechanical agitation exhibit high conversion yields. However, the shear stress may disrupt the enzyme molecule or the biocatalyst (physical particles when immobilized) and reduce the operating efficiency [12]. Thus, the use of alternative methods such as ultrasound system may avert these problems and reduce the energy input needed in the process [34]. It has been reported that ultrasound provides conversion efficiencies similar to

mechanical stirring and, in some cases, being even higher [15, 35]. In this study, after 24 h of reaction, biodiesel yields under ultrasound were very similar to those obtained using mechanical stirring (around 90 %, Fig. 3a) confirming the applicability of ultrasound.

Comparing results plotted in Fig. 3b, it is possible to verify that ultrasound system presented higher initial reaction rate, almost twice as high than mechanical stirring (at 2 h of reaction). Similar results were reported by Liu et al. (2008), who studied the effect of ultrasound in soybean oil hydrolysis under free-solvent system using immobilized *Candida lipolytica* lipase. Compared to mechanical agitation, the reaction rate under the ultrasound-assisted was 2.3-times higher in the first 12 h, probably because better homogenization and reduction of enzyme agglomeration promoted by the ultrasound. As suggested by Kojima et al. [36], the cavitation effect promoted by the formation of bubbles, generates intense turbulence and fluid circulation in a micro-scale, contributing to an effective mixing of the solution, disrupting immiscible liquid layers, and promoting mass transfer at the liquid–solid interface. The authors also suggested these effects may be due to the removal of impurities from the solid surface or at liquid–liquid interface region, as a consequence of increased liquid–liquid interfacial area caused by the emulsification of the mixture. Under ultrasound, the reaction time is shortened, making the ultrasound system more efficient and economically feasible [35, 36].

3.4. Reuse of *Combi-lipase*

The operational stability of *combi-lipase* tested in this work was evaluated in 5 cycles of reaction under the optimal conditions defined in section 3.2 for both oils. The biocatalyst was washed with hexane solvent between each batch. The results plotted in Fig. 4 show that the *combi-lipases* lost approximately 50 % of their initial activities after the second cycle, decreasing to around 10 % remaining activities after the fourth cycle. The reasons for this behavior are not clear. One possibility would be the removal of enzyme molecules from their immobilization supports. Another fact might be the enzyme inactivation by the ultrasound, although we did not check this possibility.

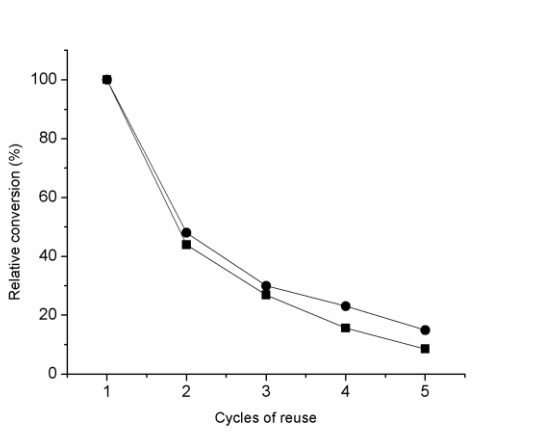

Fig. 4. *Combi-lipase* **s**tability over repeated batches of transtererification using (–■–) waste oil and (–●–) soybean oil. The reactions were carried out at the optimal conditions. Reaction conditions for soybean oil: substrate molar ratio 4.95:1 ethanol:oil, and 25 % of *combi-lipase* content as oil mass; for waste oil: substrate molar ratio 9:1 ethanol:oil, and 25 % of *combi-lipase* content as oil mass.

Similar results were reported by Tretin et al. [13] on the enzymatic (Novozyme 435) production of biodiesel using soybean oil and ethanol as substrates under ultrasound-assisted homogenization in a solvent-free system. The authors reported reductions of activity around 33 % after the first cycle, and practically no activity was detected after the second cycle. Batistella et al. [37] also reported low operational stability of Lipozyme RM IM and Novozym 435 during the transesterification of soybean oil in presence of organic solvent, ultrasound mediated. The authors showed that ultrasound-assisted method provided high product conversion (90 %). However, repeated batches of catalysts resulted in decreased activities for both enzyme and reduced product conversion after two cycles. It remains unclear the reasons for this inactivations, more research being required to optimize these reactions.

4. Conclusions

Biodiesel was synthesized using soybean and waste frying oil and *combi-lipase* biocatalysts, conducted under ultrasound-assisted reaction. The *combi-lipase* was fine-tune optimized according to the fatty acid composition of substrates, which is an important strategy to be applied on an industrial scale of biodiesel production. The ultrasonic technique provided excellent results on the initial rate of reaction compared to the use of conventional mechanical stirring. Results shown here suggest that the combination of optimized *combi-lipases*, waste frying oil and ultrasound can be an interesting alternative to reduce the costs of enzyme use, raw materials, and energy

inputs, making feasible the enzymatic synthesis of biodiesel. However, further research is required to optimize the use of ultrasound-assisted reaction in order to avoid enzyme loss of activity.

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CAPÍTULO V – CONTINUOUS ENZYMATIC BIODIESEL SYNTHESIS IN A PLUG-FLOW PACKED-BED REACTOR USING COMBI-LIPASES AND DIFFERENT OIL SOURCES

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Continuous enzymatic biodiesel synthesis in a plug-flow packed-bed reactor using *combi-lipases* and different oil sources

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Abstract

This work describes the continuous synthesis of biodiesel by enzymatic catalysis on a packed-bed reactor, using the *combi-lipase* of immobilized lipases of *Candida antarctica* (CALB), *Thermomyces lanuginosus* (TLL), and *Rhizomucor miehei* (RML). Reaction conditions were tested considering the addition of glass beads to the reactor bed, the best organic solvent, flow rate, the substrates molar ratio (ethanol:oil), and *combi-lipase* composition, for soybean oil and for waste oil (spent commercial and domestic frying oil). Best reaction conditions using soybean oil was found to be the use of glass beads in the reactor bed, *tert*-butanol as solvent, flow rate of 0.08 mL min^{-1}, and the *combi-lipase* of 22.5% of TLL, 50% of CALB, and 27.5% of RML. Using waste oil, the best *combi-lipase* was found to be 40 % of TLL, 35 % of CALB, and 25 % of RML, and a substrate molar ratio of 9:1. The enzymatic stabilities of the systems were high, showing that the *combi-lipase* reactors operating at steady state for over 30 days, keeping conversion yields of approximately 50 %, with average productivity of 1.94 $g_{biodiesel}\ g_{substrate}^{-1}\ h^{-1}$, regardless of the type of oil in use. These results show that the use of packed-bed reactors of specific *combi-lipases* and waste oil as substrate is a promising route to reduce the overall costs of the enzymatic biodiesel synthesis.

Keywords: Packed-bed reactor; Biodiesel; waste oil; soybean oil; *combi-lipase*.

1. Introduction

Lipases (EC 3.1.1.3) [1] are hydrolases that efficiently catalyze hydrolysis, esterification, and transesterification reactions involving water insoluble esters [2]. Lipases are considered as the most important group of biocatalyst in biotechnology, mainly because of their versatility [3, 4], which consist of their substrate specificity, regio-, chemo-, and stereo-selectivity, allowing these enzymes to perform well with a variety of substrates, including low-quality sources of oils to obtain one of the most important biofuels, the biodiesel.

However, one of the main drawbacks for the enzymatic synthesis of biodiesel, is the high cost of the enzymes used in the process [1]. The use of a mixture of different lipases having different fatty acids specificities and region- selectivities, named *combi-lipase*, has already been reported and proved to be an excellent alternative to reduce these costs [5]. Another important technical result when using *combi-lipases* is the reduced reaction time and increased conversion rates of biodiesel obtained [5].

The choices of oil sources for biodiesel production is another important point to be considered for the enzymatic route. The oil used in the reaction will be dictated by the geographic region, the type of plant and its cultivation conditions, and its availability. Presently, edible oil represents 95 % of the raw material used for biodiesel production, which generates price competition with the food chain, and may compromise the supply balance in the food market, which is undesirable [1, 6-8]. Some reports in the literature propose to mitigate this conflict, and at the same time reducing the cost of raw material for biodiesel production, proposing the use of waste edible oil coming from spent commercial and domestic frying oil as a feedstock in the biodiesel production, with projected costs reductions varying from 60 and 90 % [9]. However, waste oils usually present high amounts of free fatty acid (FFA), which leads to saponification in the presence of alkali catalysts, impairing the conversion to fatty acid methyl ester (FAME) [10]. This technical drawback is avoided when enzymatic catalysis is used because the esterification of FFA and transesterification of acid glycerides are simultaneously achieved by the enzyme [11].

Although most of the commercial preparation of biodiesel is still obtained in batch reactions, the development of a continuous flow reactor would possibly increase the efficiency of this process, with reactors operating at steady state for long periods of time [12]. The continuous process has many advantages over the batch, such as higher

reaction performance due to continuous removal of product, and fewer steps to scale up, with increased energy efficiency [11]. The processes involving immobilized enzymes are preferably operated in continuous mode to improve productivity and promote lower shear stress on immobilized enzymes, generally leading to long-term enzyme stability [13].

Among continuous reactors, packed-bed reactor (PBR) is one of the simplest configurations, where the immobilized biocatalyst is trapped in the reactor while the substrate solution is pumped through at a specific flow rate that will determine the residence time [14-17]. This configuration reduces the process cost, besides having faster and higher catalytic activity compared to free enzymes [1, 18].

The use of solvents in lipase-catalyzed transesterifications depends on the necessity of each process. In PBR, the use of solvents is recommended because during the transesterification reaction, the produced glycerol can be accumulated and cause column clogging, increasing reactor pressure. Furthermore, the glycerol produced is adsorbed on the surface of the support of immobilization, turning it inaccessible to hydrophobic substrates [13, 14]. The use of solvent avoids this problems, protecting the enzyme and reducing the substrate viscosity, further improving mass transfer rates through the bed [19].

Considering all these aspects, in this work, we applied the concept of *combi-lipases* for biodiesel synthesis in a continuous packed-bed-reactor, combination which so far has not been reported before. Two different oil sources, namely waste frying oil and soybean oil were compared, and two different *combi-lipases* optimized for each substrate were tested (Poppe et al., submitted). Reaction conditions were investigated for the use of different solvents, the flow rate of substrates in the PBR, and for the necessity of using glass beads in the bed composition.

2. Material and methods

2.1. Material

The enzymes used were kindly donated by Novozymes (Novozymes, Spain). These were Lipozyme TL-IM, immobilized on acrylic resin (TLL); Lipozyme RM-IM, immobilized on anion-exchange resin (RML); and Novozym 435, immobilized on macroporous resin (CALB). Refined soybean oil (molecular weight of 884 g mol^{-1}) was

purchased at a local market, waste frying oil (molecular weight of 840 g mol^{-1}) was obtained from domestic frying, both oils used without any treatments. Ethanol and organic solvents were of analytical or chromatographic grade. Methyl heptadecanoate and polyethylene sorbitol ester (Tween-80) were purchased from Sigma Aldrich Co. (St. Louis, USA).

2.2. Reactor configuration and set-up

The enzymatic reactor used in this work was designed and constructed in our group, and Fig. 1 shows a schematic representation of the apparatus set up. The unit consists of a cylindrical plug-flow, packed-bed reactor (PBR), dimensions of 65 mm of high, 10 mm of inner diameter, and working volume of 1.4 mL, equipped with water-jacket for temperature control. The column reactor was fed from bottom to top and a sintered glass disk retained the enzyme particles within the column. The flow rate was controlled with adjustable peristaltic pumps.

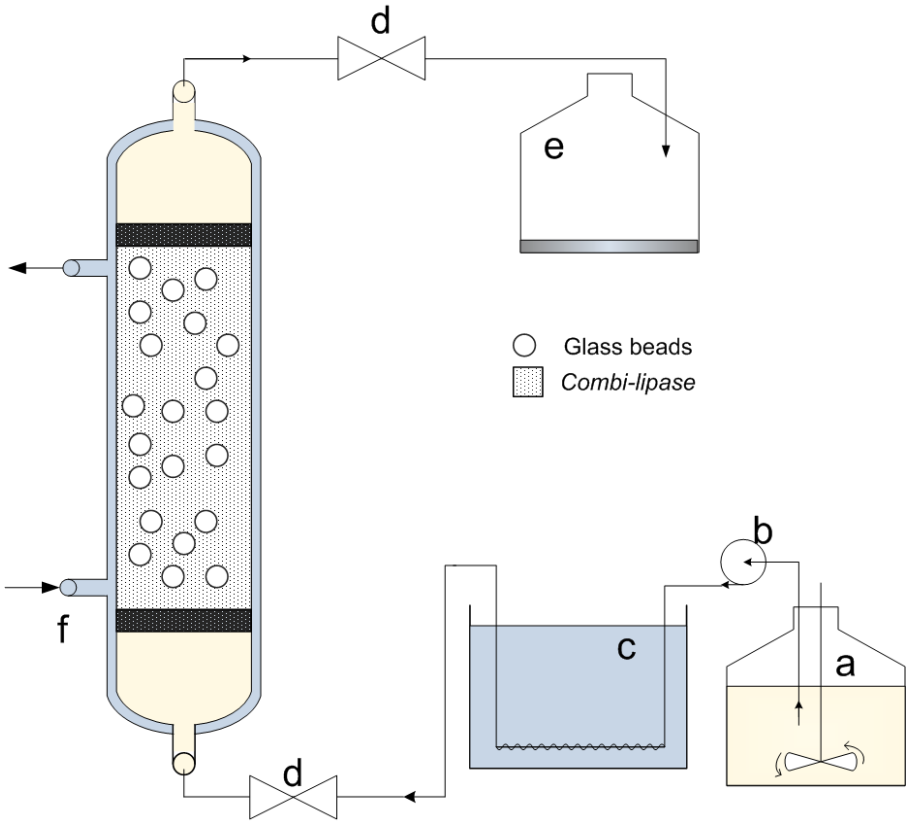

Fig 1. Schematic representation of the packed-bed reactor, loaded with 1.5 g of *combi-lipase* and glass beads. a) substrate reservoir; b) peristaltic pump; c) reservoir for temperature control equipped with glass serpentine; d) sampling valve; e) product reservoir; f) water jacket with cooling water.

2.3. Transesterification reaction conditions

The composition of *combi-lipases* used as biocatalysts and the optimized conditions of molar ratio of substrate and temperature were based on previous experiments (Poppe et al., submitted). In the experiments using soybean oil as substrate, the conditions were: substrate molar ratio of 8.09:1, and *combi-lipase* 22.5 % of TLL, 50 % of CALB, and 27.5 % of RML. The reaction conditions for the reactor using waste oil were: substrate molar ratio of 9:1; and *combi-lipase* 40 % of TLL, 35 % of CALB, and 25 % of RML. The reaction temperature was set at 40 °C in both systems.

2.4. Effect of the addition of inert particles

We predicted possible mass transfer problems in the column because of the reduced particle size of the support of immobilized lipases, thus we tested the addition of inert particles (glass beads) in the enzyme bed to reduce the compaction of the enzymes. Two sets of reactors were tested: in the first reactor, the bed was packed with 1.5 g of *combi-lipase* mixed with 5 g glass beads (21 particles of mean diameter of 3 mm); in the second reactor, the bed was packed using only 1.85 g of *combi-lipase*, without glass beads. In the test of both reactors, soybean oil was used without solvent. The substrate, kept in a circulating water bath at 40 °C, was pumped at a fixed flow rate of 0.15 mL min^{-1}, with bed height of approximately 6.5 cm.

2.5. Effect of solvents

Tween-80 and four organic solvents (*tert*-butanol, log P 0.58; hexane, log P 3.5; heptane, log P 4.0; isooctane, log P 4.78) were evaluated to increase the solubility and emulsification of oils and ethanol in the transesterification reaction. Tested conditions were: Tween-80, 5 % (volume of tween per total volume of substrate); organic solvents, 20 % (volume of solvent per total of substrate). Soybean oil and 21 glass beads were used in all reactors. The substrate, kept in a circulating water bath, was pumped at a fixed flow rate of 0.15 mL min^{-1}. The amounts of tween-80 and the organic solvents were chosen based on previous works [16, 20, 21].

2.6. Effect of flow rate

To determine the effect of the flow rate, the substrate solution was pumped into the column at 8 flow rates of 0.04, 0.08, 0.12, 0.21, 0.35, 0.45, 0.9, and 1.4 mL min^{-1}.

2.7. *Combi-lipase* stability

Refined soybean oil and waste oil were evaluated in a long-term stability test for 30 days. The reactors conditions (*combi-lipase* mixture and substrate molar ratio) were prepared according to each oil.

2.8. Gas chromatography analysis

Concentrations of biodiesel produced were analyzed using gas chromatography. Methyl heptadecanoate was used as an internal standard and mixed with heptane to prepare a stock solution. The sample was accurately weighted (50 mg) and 1 mL of internal standard stock solution was added. A standard FAEE (Fatty Acid Ethyl Esters) mix (C_4-C_{24}) from Supelco was used to identify the peaks at different retention times and to correct the peak area using the response factors of the compound. The FAEE content was calculated using the compensated normalization method with internal standardization, based on the European Standard DIN EN 14103 [22].

3. Theory and calculations

3.1. Space time (τ)

The space time was calculated according to Simões et al. [23], as described in Eq. 1:

$$\tau = \frac{V}{v_0} \quad (1)$$

Where τ is the space time (h), V is the working volume of the reactor (mL) and v_0 is the flow rate (mL h^{-1}).

3.2. The combi-lipase productivity

The enzymatic productivity (P), was defined as the concentration of biodiesel produced per h of reaction ($g_{biodiesel}\ g_{substrate}^{-1}\ h^{-1}$), calculated using Eq. 2:

$$P = \frac{[biodiesel]}{\tau} \quad (2)$$

Were [biodiesel] is the total concentration of biodiesel (g biodiesel per g of substrate) and τ is the space time (h).

4. Results and discussion

4.1. Glass beads effect

Packed-bed reactors (PBR) working under continuous mode can minimize the damage to the biocatalyst because it generates lower shear stress, [12], but they can cause enzyme bed compaction, forming preferential flow paths that limit mass transfer [24]. To avoid this problem, a first experiment was carried out to evaluate the effect of addition of inert particles to the bed, and for this glass beads were mixed with the *combi-lipase*. The comparison of results of reactors with and without glass beads can be seen in Fig. 2.

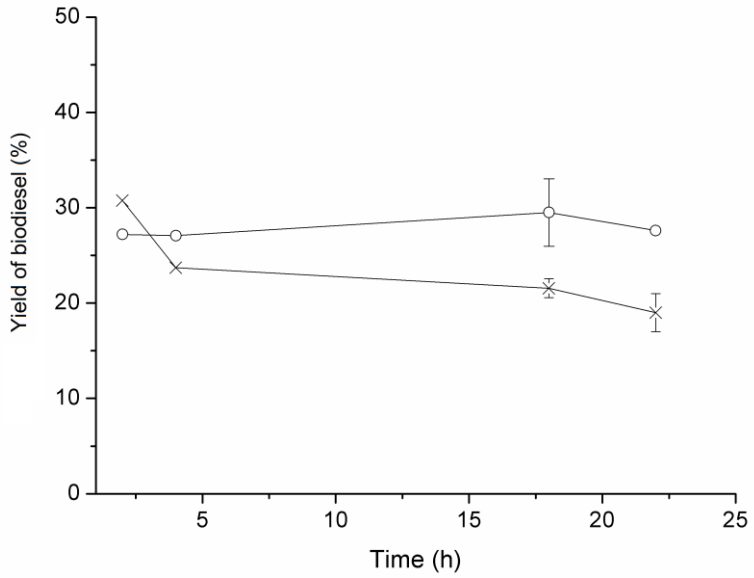

Fig 2. Evaluation of the use of glass beads in the enzyme bed. (–×–) without glass beads and 1.85 of *combi-lipase*; (–○–) with 21 glass beads and 1.5 g of *combi-lipase*. Reaction conditions: substrate molar ratio 8.09:1 ethanol: soybean oil; 40 °C; 0.15 mL min^{-1} of flow rate; without solvent.

As can be seen there, the reactor containing the inert support produced a higher conversion compared to the reactor without this element, probably because the glass beads avoided compaction and formation of preferential channels, thus increasing substrate dispersion and reducing the diffusion limitation [25]. In this way, the mass transfer in the reaction system was improved. Another aspect concerning the use of the glass beads, is the fact that less immobilized enzyme was loaded into the reactor, further contributing to operational cost reduction. In this work, the use of the glass beads showed to be of importance to the process, doubling the yields of biodiesel, from 0.097 $g_{biodiesel}\ g_{substrate}^{-1}\ g_{enzyme}^{-1}$ in the reactor without spheres, to 0.18 $g_{biodiesel}\ g_{substrate}^{-1}\ g_{enzyme}^{-1}$ in the reactor containing these inert elements.

Considering these results, the next experiments were performed using glass beads mixed with the bed of *combi-lipase*.

4.2. Effect of the solvent in the transesterification reaction

In some case, enzymatic reactions in column reactors require the use of organic solvents that exert multiple effects on both reactants and products. These effects include increased solubility of alcohol, which protects lipase from denaturation and creates a single-phase reaction mixture, leading to an improvement in mass transfer and reaction rates, reduction in the viscosity, and stabilization of immobilized enzymes. In this work, four organic solvents with different log P values and tween-80 were tested for their applicability in continuous biodiesel synthesis. The Fig. 3 shows the results of biodiesel yields over time.

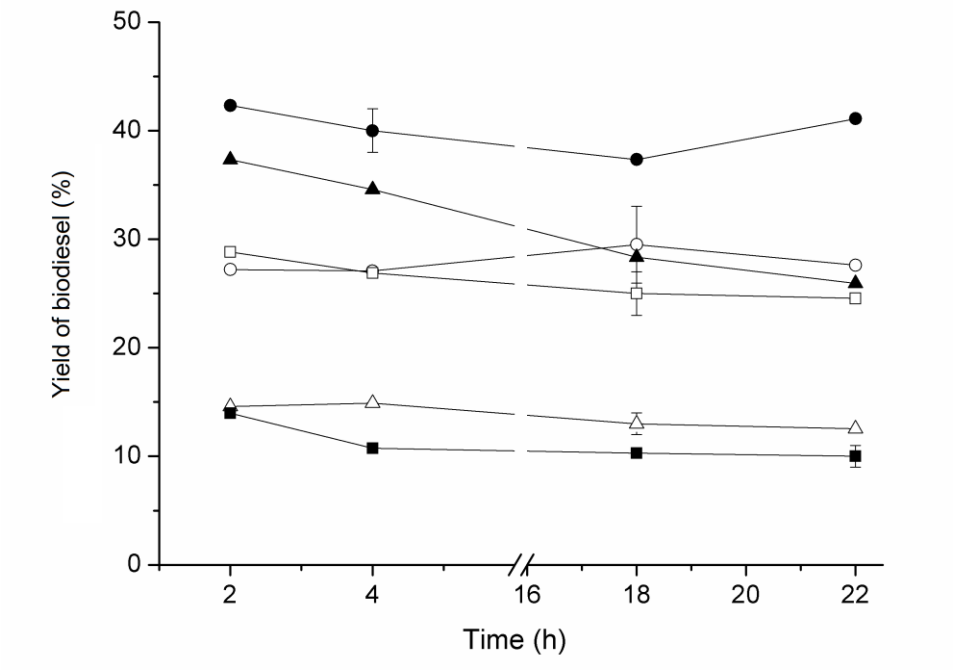

Fig 3. Evaluation of organic solvents and tween-80 in the biodiesel transesterification in the continuous packed-bed enzymatic reactor. Reaction conditions: substrate molar ratio 8.09:1 ethanol:soybean oil; 40 °C; 0.15 mL min^{-1} of flow rate, 21 glass beads, 1.5 g of *combi-lipase*. (–○–) without solvent; (–Δ–) tween-80; (–●–) *tert*-butanol; (–□–) hexane; (–■–) heptanes; and (–▲–) isooctane.

Usually solvents with log P > 4.0 are reported to be best choices for biodiesel synthesis, because lipases show higher activities in hydrophobic organic solvents [26]. The log P value is generally used to correlate solvent polarity with enzyme activity and stability in non-aqueous phases, but correlations between a simple parameter, such as solvent log P, and a complex factor as enzyme denaturation might be difficult to predict [12]. In this work, the results showed that hydrophobic solvents with a high log P value, such as heptane (log P 4.0), performed poorly in terms of yields. In contrast, *tert*-butanol, a solvent showing a moderate polarity, with low a log P value (0.58), provided the highest yields of biodiesel (42 %).

This is possibly because *tert*-butanol eliminates the negative effects of alcohol and glycerol on lipases, since glycerol creates a hydrophilic layer around the enzyme, limiting diffusion of the hydrophobic substrate to the active site. Since ethanol and glycerol are both soluble in *tert*-butanol, it is expected that glycerol molecules were not adsorbed onto the surface of the lipases [21].

Furthermore, *tert*-butanol is a branched alcohol chain, with three methyl groups, and there is a steric hindrance that prevents the formation of *tert*-butyl esters, thus favoring the effect on the conversion of ethyl esters, since this alcohol does not compete as a substrate for lipase [27]. Royon et al. [28] reported high stabilization of

immobilized lipase Novozyme 435 - longer than 500 h of operation in a packed-bed reactor - without appreciable loss in substrate conversion, for the transesterification of cotton seed oil using methanol in presence of *tert*-butanol.

The main disadvantage of using organic solvents for transesterification would be the need of their recovery. However, the amount of *tert*-butanol used for optimized conversion is relatively small and consequently the energy required for its recovery would be acceptable, especially because solvent recovery is a common unit operation in the chemical catalyzed production of biodiesel, being necessary in all cases to remove the excess alcohol. The low boiling point of *tert*-butanol makes it easy to separate this solvent along with the alcohol [28].

In addition to the organic solvents, we also tested the effect of a surfactant, Tween-80, in order to prevent phase separation of the substrate in the reactor feeding. However, despite avoiding phase separation, it was not possible to observe a satisfactory response regarding to biodiesel production (12 % of yield at 22 h, Figure 3).

Considering these results, and the improved yields using *tert*-butanol, this solvent was chosen for the next steps.

4.3. Effect of the flow rate

The evaluation of the flow rate of the substrate is critical in continuous reactors because it will affect the residence time in the reactor, and thus the yields of reaction and volumes of the equipment. If the flow rate is too high, the contact time between the substrates and lipase will be very short and the reaction will be incomplete. However, at low flow rates, low yields of biodiesel can be achieved due to the mass transfer resistance at liquid film layer [29].

In this work, eight levels of flow rates were studied in the PBR to verify the relationship between the substrate flow rate and the space time required to produce an optimum biodiesel conversion (productivity). These results are detailed in Table 1, showing that the flow rate strongly influenced biodiesel production by *combi-lipase* in PBR. The highest productivity was achieved at the flow rate 1.4 mL min^{-1} reaching 3.2 $g_{biodiesel}\ g_{substrate}^{-1}\ h^{-1}$. At high flow rates, reduction of the space time was observed and the reaction was incomplete, producing conversions as low as 5 %.

Table 1

Relationship between space time, flow rate, productivity and biodiesel yields.

Flow rate (mL min^{-1})	Space time (min)	Productivity ($g_{biodiesel}$ $g_{substrate}^{-1}$ h^{-1})	Biodiesel yield (%)
1.4	1	3.2	5
0.8	1.5	3.2	9
0.45	3	3.2	15
0.35	4	3.1	21
0.21	6.5	2	42
0.12	11.3	2	40
0.08	17	1.84	50
0.04	39	0.95	62

Similar findings were reported by Hallin et al. [30], who carried out the continuous production of biodiesel using waste cooking palm oil in a packed-bed reactor using Novozym 435 as a catalyst. According to the authors, increased flow rates (from 0.9 to 1.02 mL min^{-1}) caused the substrate to only pass through the enzyme bed without interacting with it, consequently failing to bind at the enzyme active site, lowering the reaction yields.

In this work, it was possible to verify that at the lowest flow rate (0.04 mL min^{-1}), the maximum conversion of biodiesel was obtained, but a very low productivity value was reached. On the other hand, by increasing the flow rate to 0.08 mL min^{-1}, the productivity doubled. The best reaction condition is a compromise between the yields and productivities. Thus, to conduct the next experiments, we decided for a substrate flow rate of 0.08 mL min^{-1}, which provided 50 % of yields, in a space time of 17 min, and a productivity of 1.94 $g_{biodiesel}$ $g_{substrate}^{-1}$ h^{-1}.

4.4. Stability of *combi-lipase* in a continuous packed-bed reactor

Operational stability of the immobilized lipase is an important parameter to be considered in an industrial process, because it directly affects the total process cost. Development of a continuous reaction would be required to guarantee the application of

this kind of enzymatic catalysis. In Fig. 4 it is shown the yields of biodiesel synthesis during the continuous operation of the PBR at the optimum reaction conditions previously described for each oil source, both at feed flow rate of 0.08 mL min^{-1}.

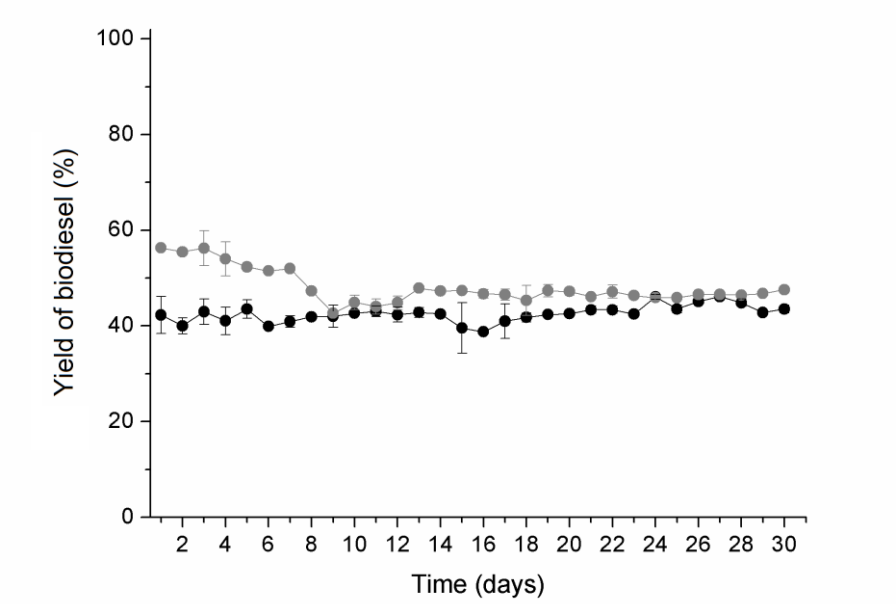

Fig 4. Biodiesel synthesis in the continuous enzymatic packed-bed reactor. Black circles, reaction with soybean oil, conditions: *combi-lipase* of 22.5 % TLL, 50 % CALB, and 27.5 % RML; substrate molar ratio of 8.09:1 ethanol:soybean oil. Grey circles, reaction with waste oil, conditions: *combi-lipase* of 40 % TLL, 35 % CALB, and 25 % RML; substrate molar ratio of 9:1 ethanol:waste oil. All reactions with 21 glass beads, 20 % of *tert*-butanol, 40 °C and 0.08 mL min^{-1} of flow rate.

After 30 days (720 h) of continuous operation, a high operational stability was observed, regardless of the type of substrate used. There was no difference in stability between the different *combi-lipases* and the biodiesel synthesis yields were kept at steady state, with approximately 50 % conversion for both oils. In addition, the process productivity also remained stable, in the range of 1.62 $g_{biodiesel}$ $g_{substrate}^{-1}$ h^{-1}.

Lee et al. [15] also evaluated the production of biodiesel in a continuous packed-bed reactor, using a 1:1 mixture of commercial immobilized lipases of *Candida rugosa* and *Rhizopus oryzae*, and soybean oil as substrate. The authors reported yields of 90 % at optimum flow rate of 0.8 mL min^{-1}, but as glycerol accumulated in the system, yields steadily decreased to 61 % after in 108 h of operation. This negative effect of glycerol accumulation in the system was also observed by other authors [23]. Using *Burkholderia cepacia* lipase immobilized on SiO2-PVA composite, Tran et al. [14] studied the transesterification of babassu oil with ethanol in a continuous packed-bed reactor. A high transesterification yield of 96.0 % was obtained, using an oil to ethanol

molar ratio of 1:12 and for space times equal or higher than 11 h. However, the productivity was only 0.041 g_{ester} $g_{catalyst}^{-1}$ h^{-1}, negatively affected by the high reaction yield. This effect was attributed to fast rate of glycerol production and its consequent effects on the biocatalyst, forming a hydrophilic layer that, in turn, makes the lipases inaccessible to hydrophobic substrates [23].

In our work, the negative influence of the presence of glycerol in the enzyme bed was not observed. Although the yields were relatively smaller compared to the above-mentioned values, the flow rate used showed to be adequate to remove a possible excess of glycerol. Furthermore, as previously mentioned, *tert*-butanol probably provided the stability of the *combi-lipase*, which helped to keep the system operating at steady-state for several days.

There are few studies using mixtures of lipases in continuous process [15] and this is a promising alternative that could be applied in order to reduce the costs of the reaction. Because lipases have varying commercial prices, the mixture of lipases including low-priced ones, but reaching similar yields, is an excellent choice to reduce the final process cost [5]. Furthermore, because of the different rate-limiting steps of the different lipases in the transesterification reaction, the combined use of 1,3-specific and nonspecific (or mono- and di-acylglycerol) lipases instead of one lipase can potentially eliminate rate-limiting steps and provide a way to significantly increase biodiesel yields [31].

5. Conclusions

In this work, we evaluated the use of *combi-lipases* as biocatalysts for continuous biodiesel synthesis in a packed-bed reactor, using both soybean oil and waste frying oil as substrates. The addition of inert supports as components of the bed improved the reaction yield, reducing column pressure and preventing the formation of preferential paths. The use of *tert*-butanol was found to be positive in the continuous process, providing to the combi-lipases an excellent stability, for at least 30 days, without losses in activity. It can be emphasized that the use of combi-lipase is a viable and promising technology for the large-scale production of biodiesel. However, some design studies in the reactors configuration may be necessary to enhance their performance and ensure that the reaction yields increase, such as to evaluate different *tert*-butanol concentrations in the reaction medium, to test varying amounts of glass

spheres in the reactor, and to determine best operational possible temperatures for the reaction according to the materials in use.

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CAPÍTULO VI – CONSIDERAÇÕES FINAIS

Esta pesquisa foi desenvolvida visando o aprimoramento do processo da produção de biodiesel a partir de catálise enzimática com diferentes fontes lipídicas, contribuindo para a geração de uma fonte de energia renovável e menos poluente que a convencional catálise química. Para isso, muitos processos foram desenvolvidos, abrangendo desde experimentos com diferentes fontes de lipases imobilizadas comercialmente, variação na temperatura de reação e diferentes fontes de óleos vegetais, realizados na tentativa de melhorar a eficiência do processo e parâmetros da reação de transesterificação.

Em um primeiro momento, foi desenvolvido um estudo teórico aprofundado das variáveis envolvidas no desenvolvimento das reações de transesterificação enzimática para síntese de biodiesel, desde a escolha do suporte de imobilização, dos processos de imobilização das enzimas, da reutilização do biocatalisador, da configuração correta do reator e do uso de substratos alternativos. Como relatado na pesquisa, a análise dos parâmetros de reação é destinada a favorecer a escolha do processo mais adequado para um dado reator e assim contribuir para elevar ao máximo o rendimento da reação. No caso de reações que envolvem reatores de fluxo contínuo, diversos autores relataram uma alta estabilidade da enzima imobilizada, e alguns reatores foram capazes de operar de forma contínua durante muitos dias. Além disso, a tensão de cisalhamento sobre o sistema imobilizado é menor neste tipo de operação, devido à ausência de agitação mecânica.

Ao estudar novos métodos para melhorar processos já amplamente estabelecidos, ou mesmo para desenvolver um protocolo completamente novo, o objetivo geral é alcançar escalas industriais. Especificamente voltada para a indústria de biodiesel, a produção enzimática deste biocombustível já está sendo desenvolvida em algumas refinarias. No entanto, ainda é necessário melhorar as técnicas existentes, e desenvolver novas metodologias para aumentar a sua síntese, para que dessa forma a tecnologia enzimática pode ser expandida para um maior número de centros de pesquisa.

Em uma segunda etapa, buscou-se aprimorar a produção de biodiesel através da utilização de lipases imobilizados operadas em processo de batelada. Foi avaliada a capacidade de síntese enzimática das lipases de *Candida antarctica*, *Thermomyces lanuginosus* e *Rhizomuchor miehei*, todas imobilizadas comercialmente, testadas

individualmente e em mistura como forma de maximizar o rendimento da reação. Estes testes foram conduzidos a partir de um planejamento fatorial de mistura de 3 fatores com superfície triangular. Após a definição do *combi-lipase*, foram conduzidos os experimentos de otimização das condições de reação de síntese de biodiesel a partir de um delineamento composto central rotacional (DCCR) e metodologia de superfície de resposta (MSR). Os parâmentros avaliados foram a proporção molar entre o álcool etílico e os óleos de oliva e palma, a temperatura de reação e a quantidade de biocatalisador (*combi-lipase*), sendo relatados os seguintes resultados para óleo de palma: proporção molar entre etanol e óleo de 9:1; 37,7 °C e 15 % de adição de *combi-lipase* (constituído por 52,5 % de TLL e 47,5 % de RML); para óleo de oliva, os resultados foram: proporção molar entre etanol e óleo de 7,04:1; 36 °C e 13,7 % de adição de *combi-lipase* (constituído por 29 % de TLL , 12,5 % de RML e 58,5 % de CALB). Após a otimização, as combinações de enzimas resultaram em conversões de biodiesel muito mais elevadas do que quando foram utilizadas individualmente, em torno de 95 % para o óleo de oliva e 80 % para o óleo de palma. Esses resultados demonstram o potencial de aplicação de reações catalisadas por combinação de lipases, uma vez que evidenciam o efeito sinergístico entre as enzimas.

Na sequência do trabalho, o terceiro artigo teve por finalidade conduzir as reações de transesterificação em banho de ultrassom e dar continuidade ao estudo do conceito *combi-lipase* aplicado à síntese de biodiesel. Devido a vasta disponibilidade de rejeitos oleosos e a necessidade de redução do custo da matéria-prima utilizada na produção de biodiesel, óleo residual proveniente de fritura doméstica ou comercial foi comparado ao óleo de soja refinado. Foi definido a composição do *combi-lipase* para os dois diferentes substratos e a otimização dos parâmetros: proporção molar etanol:óleo; quantidade de água adicionada na reação e quantidade de *combi-lipase*. Para o óleo de soja, as condições ótimas de síntese de biodiesel obtidas em ultrassom foram: razão molar de substrato 4,95:1 e 25 % de adição de *combi-lipase*, constituída por 10 % TLL + 15 % RML + 75 % CALB; para o óleo residual, a razão molar de substrato foi de 9:1 e 25 % de adição de *combi-lipase*, composto de 20 % TLL + 20 % RML + 60 % CALB. Em todas as reações utilizando o *combi-lipase* os valores de rendimento de biodiesel foram superiores às reações conduzidas com as lipases individuais. As transesterificações em ultrassom apresentaram maiores taxa inicial de reação (2 vezes mais rápido comparado a agitação mecânica em shaker), proporcionando benefícios significativos que podem ajudar a reduzir os custos com energia no processo. Após os

experimentos de otimização, não foram descritas diferenças significativas na síntese de biodiesel entre a utilização do óleo de soja e do óleo residual, com os valores superiores a 90 % de conversão para ambos os substratos.

Apesar do óleo de soja ser a matéria-prima de melhor qualidade, seu consumo para obtenção do biodiesel gera vários conflitos sociais e econômicos, pois além de combustível, ele também é fonte de alimento para a humanidade. A partir deste entendimento, e diante do potencial de aplicação de um *combi-lipase*, a última fase desse trabalho de doutorado visou conduzir os experimentos em reatores enzimáticos de leito empacotado com utilização tanto de óleo de soja quanto de óleos residuais como substratos. Para definir os parâmetros de configuração do reator contínuo, foi necessário definir primeiramente as condições ideais de reação em um sistema de batelada. Os dados obtidos para o óleo de soja foram os seguintes: razão molar etanol:óleo 8.09:1 e *combi-lipase* constituído de 22,5 % TLL + 27,5 % RML + 50 % CALB; para o óleo residual foram relatados os seguintes dados: razão molar etanol:óleo 9:1 e *combi-lipase* constiuído de 40 % TLL + 25 % RML + 35 % CALB. A temperatura de reação foi fixada em 40 °C. A partir dessas condições otimizadas foram configurados os parâmetros de fluxo de alimentação de substrato e avaliação da necessidade do uso de solventes orgânicos. O leito de *combi-lipase* foi preenchido com a adição de 21 esferas de vidro, pois assim houve um favorecimento na transferência de massa do sistema. *Terc*-butanol foi relatado como sendo o melhor solvente de reação e o fluxo de alimentação de substrato foi definido como 0,08 mL min^{-1}, pois foi o fluxo que forneceu a melhor relação entre o rendimento de conversão de biodiesel e a produtividade do processo.

Na sequencia, avaliou-se a estabilidade operacional do *combi-lipase* em processo contínuo com leito empacotado. A conversão de biodiesel manteve-se em estado estacionário durante 30 dias ininterruptos, com 50 % de conversão de biodiesel e produtividade média de 1,94 $g_{biodiesel}\ g_{substrate}^{-1} h^{-1}$. É importante ressaltar que este resultado foi obtido com os dois óleos utilizados como matéria-prima, demonstrando mais uma vez o potencial de aplicação dos óleos residuais na indústria de biodiesel.

Dentro dos objetivos gerais propostos nesse trabalho, foi possível produzir biodiesel aplicando a biocatálise em reações de transesterificação. A parir do que foi exposto, pode-se perceber que é possível a utilização dos óleos residuais para a produção de biodiesel, e que os resultados se equipararam aos tradicionais óleos refinados. Torna-se evidente as vantagens que se obtêm com essa produção, uma vez

que a reciclagem do óleo de fritura como biocombustível não somente retira um composto indesejado do meio ambiente, mas também permite a geração de uma fonte de energia alternativa, renovável e menos poluente, constituindo-se, assim, em um forte apelo ambiental.

Após ter sido relatada todas as vantagens da utilização de um *combi-lipase*, definido com base na composição em ácidos graxos da matéria-prima oleosa, os dados expostos neste trabalho contribuem para melhorar o desenvolvimento de pesquisas de síntese enzimática de biodiesel. Inúmeras estratégias para reduzir os impactos econômicos foram estudadas, e assim, espera-se que muito em breve a tecnologia enzimática possa ser expandida para um maior número de centros de pesquisa e refinarias.

PERSPECTIVAS

O presente trabalho demonstrou o potencial de aplicação de misturas de lipases como biocatalisadoras na síntese de biodiesel e o emprego de diferentes fontes de matéria-prima. No entanto, melhorias no processo ainda podem ser feitas visando aprimorar esta bioconversão, como as seguintes sugestões:

1. Estudo e aplicação de outras lipases para compor o *combi-lipase*;
2. Aumentar a escala do reator de leito empacotado;
3. Analisar um sistema constituído de vários leitos fixos funcionando em série;
4. Estudar a viabilidade econômica do processo de produção de biodiesel catalisado pelo *combi-lipase* em reatores contínuos*;*
5. Testar a utilização de reatores de leito fluidizado;
6. Desenvolver um reator contínuo configurado com sistema de ultrassom.

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ANEXOS

Anexo A – Figuras

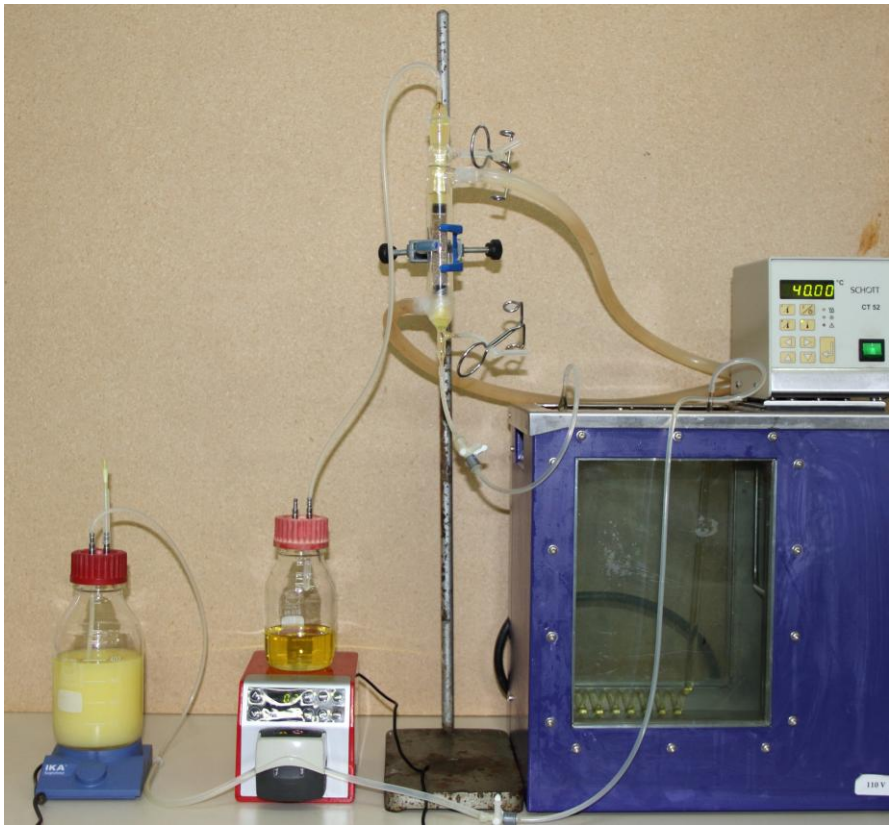

Figura A. Visão geral do aparato experimental para a síntese contínua de biodiesel em reator de leito empacotado.

Figura B. Serpentina utilizada para aumentar a área de contato do substrato ao banho termostatizado.

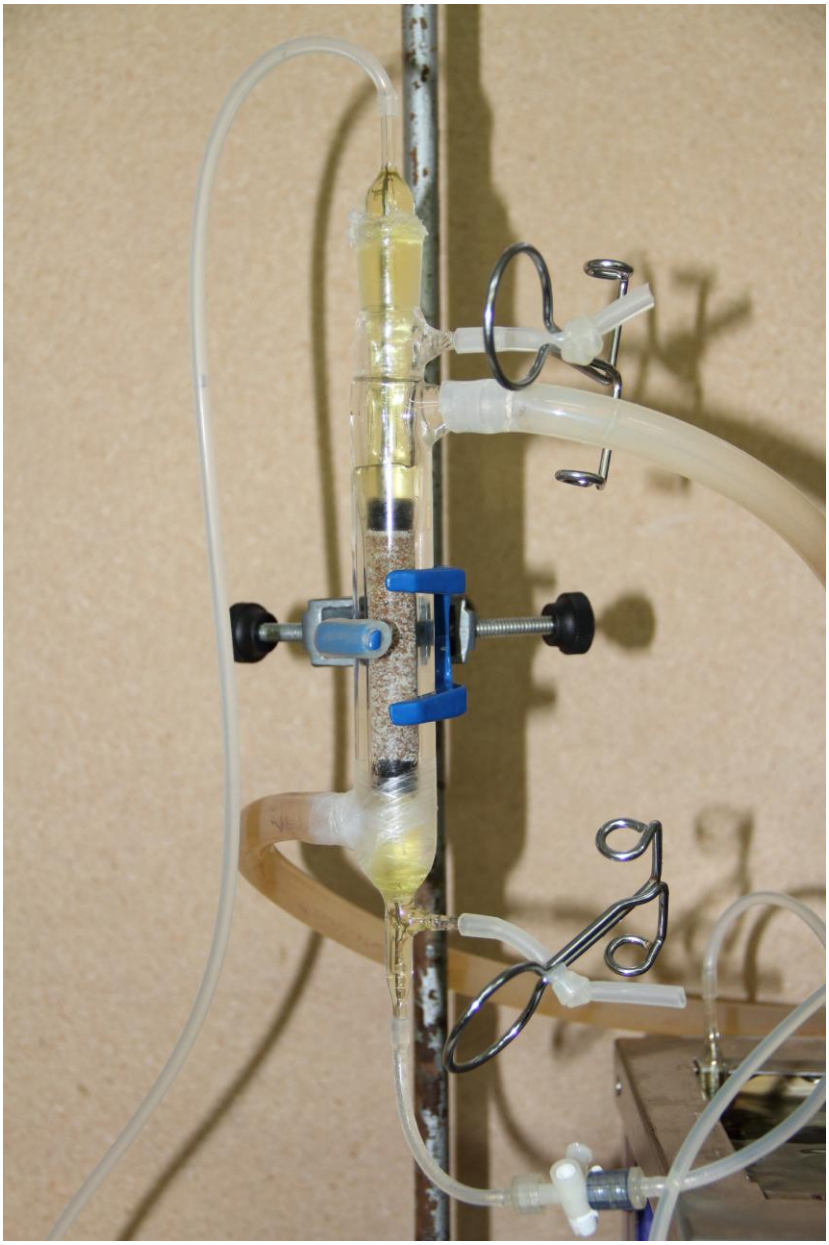

Figura C. Reator de leito empacotado preenchido com *combi-lipase* (CALB + RML + TLL).

Anexo B

O Anexo B apresenta dois trabalhos que foram publicados em Periódicos, e constituam-se como uma complementação ao trabalho de doutorado. Não constam como requisitos para a obtenção do Título de doutorado.

Physical-Chemical Properties of the Support Immobead 150 Before and After the Immobilization Process of Lipase.

Journal of the Brazilian Chemichal Society, v.0, n.0, p. 1-10, 2017.

Carla R. Matte, Carolina Bordinhão, Jakeline K. Poppe, Edilson V. Benvenutti, Tania M. H. Costa, Rafael C. Rodrigues, Plinho F. Hertz and Marco A. Z. Ayub

http://dx.doi.org/10.21577/0103-5053.20160319

Synthesis of butyl butyrate in batch and continuous enzymatic reactors using *Thermomyces lanuginosus* lipase immobilized in Immobead 150

Journal of Molecular Catalysis B: Enzymatic v.127, p. 67–75, 2016.

Carla R. Matte, Carolina Bordinhão, Jakeline K. Poppe, Rafael C. Rodrigues, Plinho F. Hertz, Marco A.Z. Ayub

http://dx.doi.org/10.1016/j.molcatb.2016.02.016