UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE TECNOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM ENGENHARIA QUÍMICA

João Henrique Cabral Wancura

PRODUÇÃO DE BIODIESEL EM ESCALA PILOTO VIA HIDROESTERIFICAÇÃO ENZIMÁTICA

Santa Maria, RS
2021

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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Engenharia Química, Área de Concentração em Desenvolvimento de Processos Industriais e Ambientais, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de **Doutor em Engenharia Química.**

Orientador: Prof. Dr. Sergio Luiz Jahn
Co-orientador: Prof. Dr. Marcus Vinícius Tres

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2021

Wancura, João Henrique Cabral
PRODUÇÃO DE BIODIESEL EM ESCALA PILOTO VIA HIDROESTERIFICAÇÃO ENZIMÁTICA / João Henrique Cabral Wancura.- 2021.
159 p.; 30 cm

Orientador: Sergio Luiz Jahn
Coorientador: Marcus Vinícius Tres
Tese (doutorado) - Universidade Federal de Santa Maria, Centro de Tecnologia, Programa de Pós-Graduação em Engenharia Química, RS, 2021

1. Biodiesel 2. FAME 3. Lipase Solúvel 4. NS 40116 5. Hidroesterificação I. Jahn, Sergio Luiz II. Tres, Marcus Vinicius III. Titulo.

Sistema de geração automática de ficha catalográfica da UFSM. Dados fornecidos pelo autor(a). Sob supervisão da Direção da Divisão de Processos Técnicos da Biblioteca Central. Bibliotecária responsável Paula Schoenfeldt Patta CRB 10/1728.

Endereço: Rua Coronel Cabrita, n. 401, apartamento 405, Centro, Alegrete, RS, CEP: 97541-100

Fone: (55) 9 9697 8881; E-mail: jhwancura@hotmail.com

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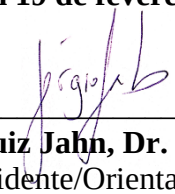
Aprovado em 19 de fevereiro de 2021:



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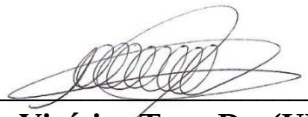

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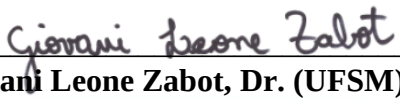

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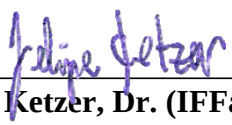

Felipe Ketzer, Dr. (IFFar)

José Vladimir de Oliveira, Dr. (UFSC)

Santa Maria, RS
2021

AGRADECIMENTOS

Gostaria de prestar minha sincera gratidão a todos que de alguma forma tornaram possível e viável a realização do meu doutorado. De forma especial, gostaria de agradecer:

À Deus;

À minha companheira Sara pela compreensão, atenção, carinho e apoio dado ao longos desses treze anos em que estamos juntos, em especial durante o período do doutorado;

À minha família, em especial meus pais Sergio e Bia, pelo suporte constante e de sempre me incentivarem a estudar e estudar cada vez mais;

Aos meus orientadores, Prof. Dr. Sergio Jahn e Prof. Dr. Marcus Tres pelos ensinamentos, confiança, disponibilidade e principalmente oportunidade em desenvolver este projeto, nesta parceria que já dura sete anos contando o tempo de mestrado e doutorado;

Um agradecimento especial ao Prof. Dr. José Vladimir de Oliveira por toda disponibilidade e suporte dado ao longo do meu mestrado e doutorado, seja na forma de apoio técnico ou fornecendo insumos essenciais ao desenvolvimento do projeto;

Ao Dr. Antonio Fantinel e ao Prof. Dr. Filipe Donato pela parceria firmada e ajuda durante a realização dos experimentos na Usina Piloto de Produção de Biodiesel do Colégio Politécnico da UFSM;

Ao Me. Gustavo Ugalde e à engenheira química Daniela Rosset pelas significativas contribuições prestadas durante a realização desta pesquisa, me auxiliando durante a a realização dos ensaios experimentais e análises de amostras;

Ao Programa de Pós-Graduação em Engenharia Química da Universidade Federal de Santa Maria, incluindo professores e funcionários, pela estrutura fornecida e serviços prestados durante a elaboração desta pesquisa;

Aos colegas do Programa de Pós-graduação em Engenharia Química pelo companheirismo e momentos de descontração ao longo dos últimos anos;

À CAPES pelo essencial suporte financeiro.

Sem a participação de todos vocês, dificilmente teria sido possível realizar este trabalho. Muito obrigado!

“[...] tenha fé em si mesmo, mas tenha fé na fé.
Não a fé como os outros a definem.
Fé como você a define.
A fé como ela se define no seu coração.”

Phil Knight

RESUMO

PRODUÇÃO DE BIODIESEL EM ESCALA PILOTO VIA HIDROESTERIFICAÇÃO ENZIMÁTICA

AUTOR: João Henrique Cabral Wancura
ORIENTADOR: Prof. Sergio Luiz Jahn
CO-ORIENTADOR: Prof. Marcus Vinícius Tres

O relatório *"State of the Climate"* divulgado pela Organização Meteorológica Mundial na 25ª Conferência Sobre Mudanças Climáticas destaca que 2019 encerra uma alarmante década em relação ao aumento histórico da temperatura média do planeta e recorde na elevação do nível do mar. Tais problemas ambientais foram potencializados pela emissão de gases de efeito estufa expelidos principalmente da queima de combustíveis de origem fóssil em motores à combustão interna. Neste contexto, o biodiesel (ésteres metílicos ou etílicos de ácidos graxos de cadeia longa) se consolidou na matriz energética mundial como uma alternativa ao seu similar derivado do petróleo, tornando-se um componente indispensável à ser misturado com o diesel para fins de comercialização. Dentre as distintas formas de sintetizar biodiesel, a aplicação de lipases em formulação líquida como catalisador da reação possui potencial para tornar o processo mais econômico, competitivo e sustentável em comparação ao uso da enzima imobilizada. No entanto, apesar dos benefícios, os longos tempos de reação necessários para atingir satisfatórios rendimentos bem como o efeito desnaturante do metanol (principal reagente da reação) sobre a enzima ainda são desvantagens do processo. Diante do exposto, esta tese teve por objetivo avaliar o processo de obtenção de biodiesel via hidroesterificação enzimática mediada por duas enzimas solúveis: Eversa® Transform e a recentemente lançada lipase termoestável Eversa® Transform 2.0 (também conhecida como NS 40116), ambas obtidas do microrganismo *Thermomyces lanuginosus* e fornecidas pela Novozymes A/K. Para isto, diferentes matérias-primas foram empregadas: óleo de soja degomado (principal matéria-prima utilizada industrialmente para produção de biodiesel), sebo de carne e óleo de cozinha usado de elevada acidez. Ensaios preliminares utilizando a lipase Eversa® Transform demonstraram que diferentes estratégias de alimentação dos insumos (enzima e álcool) à reação impactam consideravelmente no rendimento. A 35 °C, razão molar entre metanol e substrato (sebo) de 4,5:1, com alimentação do álcool à fluxo constante de 3,0 $g \cdot h^{-1}$, 1,0 m% de lipase, 6,0 m% de água e 8 h de reação, 85,08 % de rendimento de FAME foi obtido, um valor interessante mas aquém do exigido por normas regulamentadoras. Assim, melhorias no processo se fizeram necessárias. Para isto, uma proposta de configuração em dois estágios reacionais mostrou-se efetiva em alavancar a produtividade do processo: utilizando-se 0,70 m% da lipase NS 40116, 35 °C, relação molar total entre metanol e substrato de 6,3:1 e 8,0 m% de água, 97,1 % de rendimento de FAME foi alcançado em 8 h de reação. Tal rendimento é similar ao obtido por pesquisas similares que necessitaram de até 24 h de reação em um estágio reacional único, demonstrando que a configuração proposta é uma opção atraente para o processo. Assim, com os parâmetros reacionais para o processo em dois estágios otimizados via planejamento experimental, partiu-se para avaliação da rota biotecnológica em escala superior à laboratorial. Utilizando-se de uma unidade piloto com 60 L de capacidade de produção, óleo de cozinha usado como matéria-prima e com as condições reacionais encontradas previamente em laboratório, 96,2 % de rendimento de FAME foi alcançado. Ainda com estes resultados, foi proposto um modelo de pseudo-primeira ordem para ajuste dos dados experimentais, onde uma taxa reacional aparente (k_{App}) de $0{,}373 \cdot h^{-1}$ foi obtida. Além disso, uma análise econômica indicou a viabilidade do sistema por meio de um retorno líquido positivo e custo operacional de US$ $0{,}50 \cdot kg^{-1}$ de biocombustível. Estas informações serviram para concluir que a hidroesterificação enzimática catalisada por lipases líquidas possui as ferramentas necessárias para ser implementada na produção industrial de biodiesel.

Palavras-chave: Biodiesel. FAME. Lipase Solúvel. NS 40116. Eversa® Transform. Hidroesterificação.

ABSTRACT

BIODIESEL PRODUCTION IN PILOT SCALE VIA ENZYMATIC HYDROSTERIFICATION

AUTHOR: João Henrique Cabral Wancura
ADVISOR: Prof. Sergio Luiz Jahn
CO-ADVISOR: Prof. Marcus Vinícius Tres

The report *"State of the Climate"* released by the World Meteorological Organization at 25th Climate Changes Conference highlights that 2019 ends an alarming decade regarding the historical increase in the average temperature of the planet and record rise in sea level. Such environmental problems were enhanced by the emission of greenhouse gases expelled mainly from the burning of fossil fuels in internal combustion engines. In this context, biodiesel (methyl or ethyl esters of long chain fatty acids) has consolidated itself in the global energy matrix as an alternative to its similar derivative from petroleum, becoming an indispensable component to be mixed with diesel for commercialization purposes. Among the distinct ways of synthesizing biodiesel, the application of lipases in liquid formulation as reaction catalyst has the potential to make the process more economical, competitive and sustainable compared to the use of immobilized enzymes. However, despite the benefits, the long reaction times required to achieve satisfactory yields as well as the denaturing effect of methanol (the main reaction reagent) on the enzyme are still drawbacks of the process. Before the exposed, this thesis aimed to evaluate the process of obtaining biodiesel via enzymatic hydroesterification mediated by two soluble enzymes: Eversa® Transform and the recently launched thermostable lipase Eversa® Transform 2.0 (also named as NS 40116), both obtained from the *Thermomyces lanuginosus* microorganism and supplied by Novozymes A/K. For this, different feedstocks were employed: degummed soybean oil (the main raw material used industrially for biodiesel production), beef tallow and waste cooking oil with high acidity. Preliminary tests using the lipase Eversa® Transform demonstrated that different feeding strategies of inputs (enzyme and alcohol) to the reaction significantly impact on the reaction yield. At 35 °C, a methanol to substrate (tallow) molar ratio of 4.5:1, with alcohol feeding at constant flow of 3.0 $g \cdot h^{-1}$, 1.0 wt% of lipase, 6.0 wt % of water and 8 h of reaction, 85.08% of FAME yield was obtained, an interesting value but below that required by regulatory standards. Thus, improvements in the process were necessary. For this, a proposal of reaction configuration in two stages showed to be effective in elevating the process productivity: using 0.70 wt% of lipase NS 40116, 35 °C, a total methanol to substrate molar ratio of 6.3:1 and 8 wt% of water, 97.1% FAME yield was achieved in 8 h of reaction. Such yield is similar to that obtained by similar researches that required up to 24 h of reaction in a single reaction stage, demonstrating that the proposed configuration is an attractive option for the process. Then, with the reaction parameters for the process in two stages optimized via experimental design, it was started to assess the biotechnological route on a superior scale than the lab. Using a pilot unit with 60 L of production capacity, waste oil used as raw material and with the reaction conditions found previously in the laboratory, 96.2 % of FAME yield was achieved. Still with these results, a pseudo-first order model was proposed to adjust the experimental data, where an apparent reaction rate (k_{App}) of $0.373 \cdot h^{-1}$ was obtained. Moreover, an economic analysis indicated the feasibility of the system through a positive net return and an operating cost of US$ $0.50 \cdot kg^{-1}$ of biofuel. This information served to conclude that enzymatic hydroesterification catalyzed by liquid lipases has the necessary tools to be implemented in the industrial production of biodiesel.

Keywords: Biodiesel. FAME. Soluble Lipase. NS 40116. Eversa® Transform. Hidroesterification.

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LISTA DE ABREVIATURAS E SIGLAS

ANOVA	Análise de Variância;
ANP	Agência Nacional do Petróleo, Gás Natural e Biocombustíveis;
AV	*Acid Value*, Valor de Acidez em inglês;
B15	Acrônimo para um diesel de petróleo contendo 15 vol% de biodiesel;
BT	*Beef Tallow*, Sebo de Carne em inglês;
CCRD	*Central Composite Rotational Design*, Planejamento Composto Central Rotacional em inglês;
CV	Coeficiente de Variação;
DAG	*Diacylglycerols*; Diacilgliceróis (diglicerídeos) em inglês;
FAAE	*Fatty Acid Acyl Esters*, Acil Ésteres de Ácidos Graxos em inglês;
FAEE	*Fatty Acid Ethyl Esters*, Etil Ésteres de Ácidos Graxos em inglês;
FAME	*Fatty Acid Methyl Esters*, sigla em inglês para Metil Ésteres de Ácidos Graxos (biodiesel);
FFA	*Free Fatty Acids*, Ácidos Graxos Livres em inglês;
GC	*Gas Chromatography*, sigla em inglês para Cromatografia Gasosa;
KOH	Hidróxido de potássio;
MAG	*Monoacylglycerols*, Monoacilgliceróis (monoglicerídeos) em inglês;
NaOH	Hidróxido de sódio;
NZ435	Enzima imobilizada Novozym 435;
MeOH	Metanol;
SWOT	*Strengths, Weaknesses, Opportunities and Threats*, termos em inglês para Pontos Fortes, Fraquezas, Oportunidades e Ameaças.
TAG	Triacilgliceróis (triglicerídeos);
TG	*Triglycerides*, sigla em inglês para triglicerídeos;
TSHR	*Two-step Hydroesterification Reaction*, Reação de Hidroesterificação em Duas Etapas em inglês;
UFSM	Universidade Federal de Santa Maria.

SUMÁRIO

1 INTRODUÇÃO

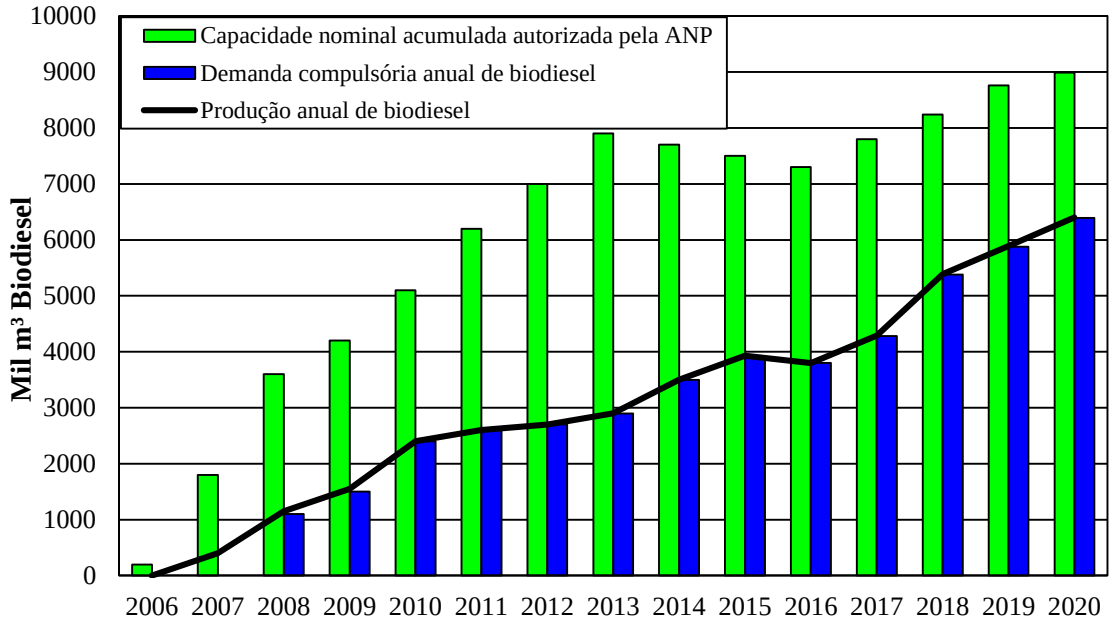
A constante alta na demanda por petróleo associada a quase exclusiva utilização do sistema rodoviário para escoamento e transporte de cargas em países em desenvolvimento, como o Brasil, destacam problemas de dependência deste setor por combustíveis de origem fóssil, principalmente o diesel. Esta questão somada as consequências do uso desenfreado deste tipo de combustível, tais como o seu iminente esgotamento em breve, além das pesadas cargas de emissões que ocasionam problemas de ordem ambiental e climática impulsionam pesquisas para o desenvolvimento de combustíveis alternativos aos usualmente consolidados na matriz energética mundial (WANCURA, 2017).

Nesse cenário, o biodiesel consolidou-se nos últimos anos como um eficaz substituto ao diesel de petróleo. Tal fator se deve principalmente devido às suas propriedades ambientalmente favoráveis, não-toxicidade, capacidade de uso em motores à diesel com poucas ou até mesmo sem necessidade de modificações mecânicas, compatibilidade com a infraestrutura de distribuição de combustíveis existente e pelo fato de ser produzido a partir de fontes renováveis (SELVARAJ et al., 2016, AVHAD e MARCHETTI, 2015).

O Brasil, em termos de produção mundial, destaca-se como um dos principais produtores de biodiesel no mundo. O líder mundial segue sendo os Estados Unidos, que em 2019 alcançou a marca de 6,5 bilhões de litros, uma quantidade 7 % inferior à de 2018, observando-se pela primeira vez na história uma retração na produção americana (BIODIESEL BR, 2020). Por outro lado, o mercado de biodiesel da Indonésia segue em aguda ascenção uma vez que o governo do país asiático implantou uma mistura de 30 vol% de biodiesel ao diesel em 2017, o que fez o país ultrapassar o Brasil como o segundo maior produtor mundial de biodiesel, com 5,6 bilhões de litros produzidos em 2018. Projeta-se ainda que a Indonésia ultrapasse até mesmo os Estados Unidos nos próximos anos, uma vez que a intenção do governo em elevar a mistura para 40 vol%, resultaria em uma produção de 9,6 bilhões de litros ao ano (BIODIESEL BR, 2019a, b). No Brasil, o país produziu aproximadamente 6,4 bilhões de litros de biodiesel em 2020 (Figura 1), uma alta de 8,5 % em relação a 2019 mesmo em um período de crise mundial ocasionada pela pandemia do novo coronavírus (BRASIL, 2020). Perspectivas apresentadas pela Organização das Nações Unidas para a Alimentação e a Agricultura, estimam que até o ano de 2030 o Brasil ultrapassará os Estados Unidos como o maior produtor de soja no mundo, com uma estimativa de crescimento de produção de 2,6 % ao ano contra 1,0 % em relação ao país americano (FAO, 2017). Este crescimento pode ser considerado um fator que mantém otimista o desenvolvimento do setor de biodiesel no país, uma vez que o óleo de soja

é a principal matéria-prima utilizada na produção do biocombustível. Somado a isso, o Brasil apresenta políticas governamentais impostas no país prevendo um aumento gradual da mistura do biodiesel ao diesel. A proposta sugerida pelo governo visa aumentar a mistura do biocombustível ao diesel em 1 vol% ao ano, até atingir o B15 previsto pela Lei 13.263/2016 em março de 2023 (BRASIL, 2016). Isto tende à acarretar uma elevação da produção de biodiesel no Brasil – tendência que já pode ser observada na Figura 1 – para mais de 10 bilhões de litros anuais em 2023, um crescimento de 85 % da atual demanda doméstica (BRASIL, 2018). Cabe salientar que desde 2014, é regulamentado por lei realizar obrigatoriamente a mistura de 7 vol% de biodiesel ao diesel comercializado no país (BRASIL, 2014a). Ainda, em março de 2020 A ANP (Agência Nacional do Petróleo, Gás Natural e Biocombustíveis) regulamentou a mistura de biodiesel ao diesel aumentou para 12 vol%, uma estratégia adotada pelo governo visando diminuir custos com a importação de diesel e ao mesmo tempo impulsionar a indústria do setor.

Figura 1. Produção anual brasileira de biodiesel, demanda compulsória e capacidade nominal instalada no país. Fonte: BRASIL, 2020.



A principal rota utilizada para produção de biodiesel é através da transesterificação alcalina de óleos vegetais e gorduras animais. Tal tecnologia está bem estabelecida, sendo a principal rota utilizada para sintetizar o biocombustível em indústrias por todo o mundo devido a sua alta conversão em curtos tempos reacionais (KNOTHE e RAZON, 2017; ISSARIYAKUL e DALAI, 2014; ABBASZAADEH et al., 2012). Este processo baseia-se em uma reação entre

uma fonte oleaginosa (óleo vegetal ou gordura animal) e um álcool de cadeia curta (usualmente metanol) na presença de um catalisador básico, onde promove-se a clivagem das moléculas de triglicerídeos, gerando uma mistura de ésteres de ácidos graxos – *Fatty Acid Methyl Esters* ou *FAME* (KNOTHE et al., 2006). O uso de etanol no processo em escala industrial ao invés do metanol, o que tornaria o processo ainda mais sustentável, não acontece devido a importantes fatores: o etanol apresenta uma menor reatividade e maiores custos de produção em comparação ao metanol, além de apresentar uma maior imiscibilidade em fontes oleaginosas, ocasionando dificuldades para a separação entre as fases dos produtos obtidos (KNOTHE e RAZON, 2017).

No entanto, apesar da tecnologia alcalina ser bem difundida e ser a base da grande maioria dos projetos de plantas de produção de biodiesel, o processo apresenta diversos inconvenientes: necessidade de sucessivas etapas de lavagem do biodiesel para remoção de traços do catalisador, o que gera uma expressiva quantidade de água residual alcalina a ser posteriormente tratada; necessidade de uma matéria-prima com elevado grau de pureza a fim de se evitar reações secundárias indesejáveis de saponificação; e uma maior demanda energética em comparação a outras técnicas de síntese do biocombustível (BASKAR e AISWARYA, 2015; ARANSIOLA et al., 2014; HAMA e KONDO, 2013).

Dentre as diferentes formas de se produzir biodiesel, uma técnica que se mostrou interessante e eficaz frente à rota alcalina homogênea é o emprego de enzimas como catalisador da reação (WANCURA, 2017; NIELSEN et al., 2016; FJERBAEK et al., 2008; WATANABE, 2007). A tecnologia enzimática é capaz de produzir um biodiesel com elevado grau de pureza sob condições reacionais brandas, além de não necessitar de etapas de purificação do biocombustível para remoção de traços do catalisador, um dos principais pontos negativos da rota alcalina (NORJANNAH et al. 2016; CHRISTOPHER et al., 2014). Destaca-se também a possibilidade da utilização de matérias-primas com altos índices de acidez e umidade no processo enzimático (materiais ditos impuros e até mesmo residuais), uma vez que as enzimas conseguem converter simultaneamente os ácidos graxos livres e triglicerídeos contidos na fonte oleaginosa em biodiesel (MOUNGUENGUI et al., 2013).

No entanto, a rota enzimática para produção de biodiesel também possui seus inconvenientes que requerem atenção quando visa-se a ampliação da técnica para uma escala superior à laboratorial: baixas velocidades reacionais, o que acarreta longos tempos de reação; perda da atividade catalítica do biocatalisador quando em contato com metanol e custo elevado em comparação ao catalisador alcalino utilizado em processos industriais (HAMA et al, 2018; GULDHE et al., 2015). Frente ao exposto, esta de tese de doutorado apresenta resultados relativos a continuidade dos estudos realizados durante o período de mestrado do discente,

focados na síntese de biodiesel utilizando diferentes lipases comerciais em formulação líquida como biocatalisador da reação. Nesta oportunidade, sugeriu-se um eficaz sistema reacional em dois estágios para a reação de hidroesterificação, de forma que tal configuração quando aplicada em laboratório retornou resultados interessantes que possibilitou expandir o processo para aplicação em uma unidade piloto.

1.1 OBJETIVOS

1.1.1 Objetivo Geral

O objetivo principal desta tese foi apresentar resultados obtidos através do desenvolvimento de um processo de produção de biodiesel em escala piloto via hidroesterificação enzimática catalisada pela lipase solúvel NS 40116.

1.1.2 Objetivos Específicos e Metas

Para atender ao objetivo geral, fez-se necessário o cumprimento dos seguintes objetivos específicos:

Objetivo Específico 1:

- Avaliação das formas de alimentação de insumos ao processo (metanol e lipase solúvel Eversa® Transform) e sua influência sobre o rendimento de biodiesel.

Metas necessárias para atingir o objetivo:

Meta 1: Realização de ensaios alimentando a enzima e metanol de forma fracionada, manualmente (na forma de pulso) e empregando uma bomba dosadora;

Meta 2: Investigação do comportamento da capacidade catalítica da lipase após reutilização em subsequentes bateladas;

Meta 3: Determinação do rendimento de biodiesel (teor de metil ésteres) de cada processo através de análise cromatográfica;

Meta 4: Determinação do índice de acidez do produto obtido em cada ensaio por titulação;

Meta 5: Investigação da recuperação e reutilização da lipase solúvel em subsequentes ciclos.

Indicador: 34 ensaios realizados.

Objetivo Específico 2:

- Investigação das variáveis de processo “concentração de água adicionada a reação”, “carga de catalisador” e “razão molar entre metanol e óleo” utilizada em uma proposta de processo composto de uma reação de hidroesterificação desenvolvida em duas etapas. Neste ponto, a lipase solúvel NS 40116 (Eversa® Transform 2.0) foi utilizada nos ensaios. Os parâmetros foram analisados através de um planejamento de experimentos do tipo DCCR (delineamento composto central rotacional).

Metas necessárias para atingir o objetivo:

Meta 6: Avaliação de como a capacidade catalítica da lipase responde a variação dos parâmetros reacionais “concentração de água adicionada ao sistema”, “relação molar entre metanol e óleo” e “carga de lipase adicionada a reação”, para cada reação, via análise da variável resposta “rendimento de biodiesel;

Meta 7: Determinação o rendimento de biodiesel (teor de metil ésteres) de cada ensaio através de análise cromatográfica;

Meta 8: Determinação do valor de acidez do produto obtido em cada ensaio por titulação;

Indicador: 29 ensaios realizados.

Objetivo Específico 3:

Produção de biodiesel a partir de óleo de cozinha descartado, em uma unidade piloto com capacidade de produzir até 60 L de biocombustível, de forma a comparar o desempenho das lipases solúveis Eversa® Transform e Eversa® Transform 2.0.

Metas necessárias para atingir o objetivo:

Meta 9: Determinação do rendimento de biodiesel (teor de ésteres metílicos) de cada ensaio através de análise cromatográfica;

Meta 10: Determinação do valor de acidez do produto obtido em cada ensaio por titulação;

Meta 11: Avaliação da cinética reacional do sistema, através da aplicação de um sistema simplificado de pseudo-primeira ordem;

Meta 12: Realização de uma avaliação econômica da operação deste processo;

Indicador: 06 ensaios realizados.

1.2 ESTRUTURA DA TESE

Este documento está dividido em oito capítulos, onde os resultados obtidos estão dispostos na forma de artigos científicos.

O **Capítulo Um** apresenta a introdução do trabalho, onde é descrita a motivação para a realização dele, a estrutura do projeto da tese de doutorado e os objetivos do estudo.

No **Capítulo Dois** encontra-se o artigo *"Lipases in Liquid Formulation for Biodiesel Production: Current Status and Challenges"* publicado no periódico *Biotechnology and Applied Biochemistry*. O trabalho constitui-se de um *review* onde é apresentada uma revisão bibliográfica do tema, o qual se faz indispensável para dar embasamento a pesquisa desenvolvida, mostrando considerações importantes e necessárias à compreensão dos resultados obtidos.

O **Capítulo Três** apresenta o primeiro artigo escrito com os resultados obtidos com este trabalho, intitulado *"Feeding Strategies of Methanol and Lipase on Eversa® Transform-Mediated Hydroesterification for FAME Production"* o qual foi publicado no periódico *The Canadian Journal of Chemical Engineering*. Este artigo apresenta resultados referente a influência da adoção de diferentes formas de alimentar insumos à reação sobre o rendimento de biodiesel.

No **Capítulo Quatro** encontra-se o segundo artigo elaborado com resultados desta tese. O artigo intitulado *"Improving the Soluble Lipase-catalyzed Biodiesel Production through a Two-step Hydroesterification Reaction System"*, pubicado no periódico *Applied Microbiology and Biotechnology*, propõe um sistema de duas reações de hidroesterificação em série na produção de biodiesel utilizando a lipase solúvel NS 40116.

O **Capítulo Cinco** apresenta o terceiro artigo escrito com os resultados desta tese: *"Semi-continuous Production of Biodiesel on Pilot Scale via Enzymatic Hydroesterification of Waste Material: Process and Economics Considerations"*, publicado no periódico *Journal of Cleaner Production*, apresenta dados relativos a uma ampliação de escala de produção de biodiesel via hidroesterificação enzimática baseando-se em resultados alcançados em laboratório ao longo da pesquisa. No entanto, nesta oportunidade, utilizou-se de óleo de cozinha residual como matéria-prima do processo.

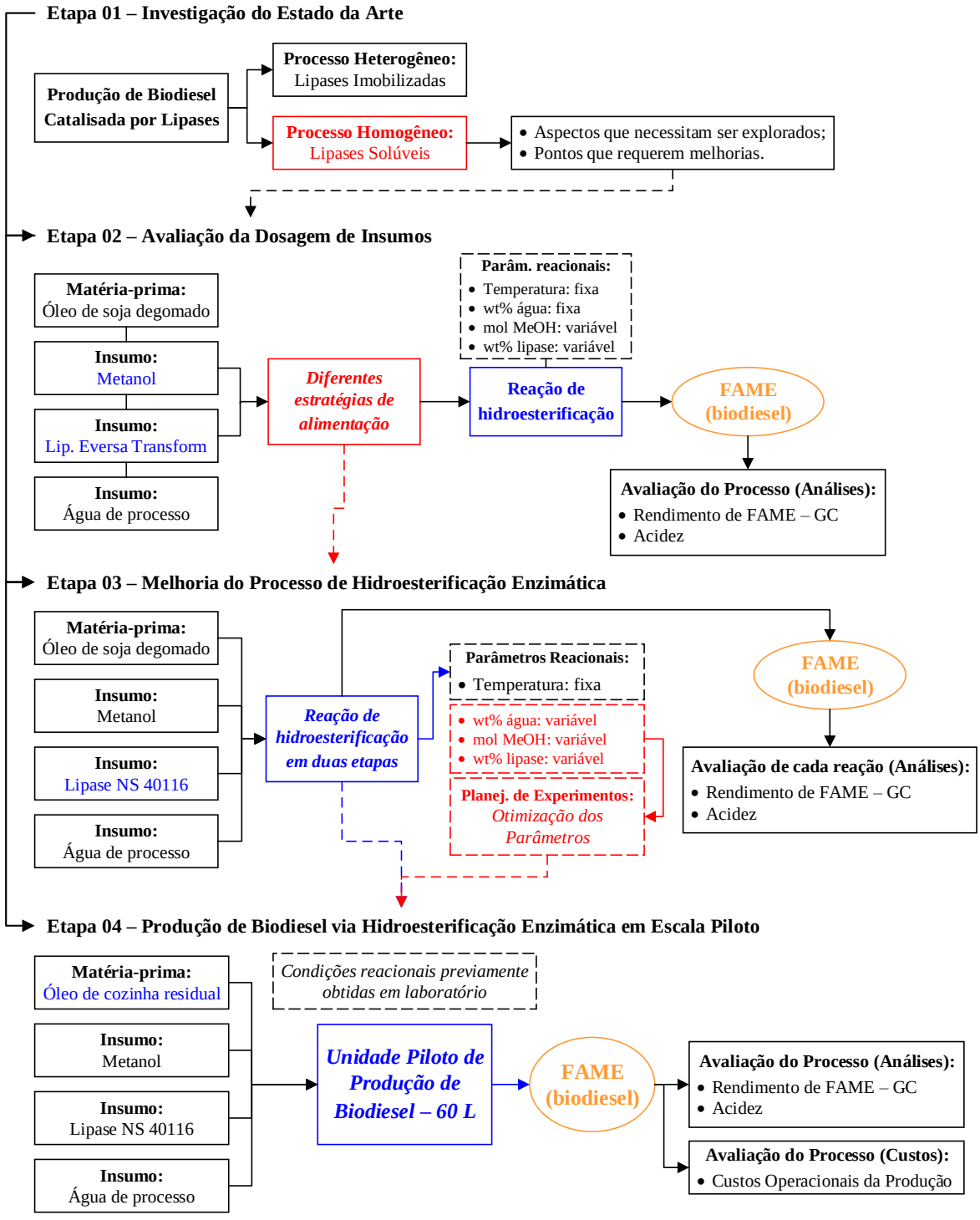
O **Capítulo Seis** apresenta uma discussão dos resultados dos artigos apresentados nos capítulos anteriores e a relação destes para fomentar os objetivos desta tese.

No **Capítulo Sete** são apresentadas conclusões a respeito da pesquisa, onde destacam-se sugestões para trabalhos futuros sobre o tema abordado.

E por fim, à título de informação e consulta, são apresentadas as referências que foram consultadas para a escrita da parte introdutória desta tese.

A Figura 2 apresenta um fluxograma representando todas as etapas do desenvolvimento desta tese.

Figura 2. Fluxograma das etapas de elaboração da presente tese.



1.3 CONSIDERAÇÕES A RESPEITO DO ESTADO DA ARTE

É importante destacar que os trabalhos publicados até o momento focando na catálise enzimática como ferramenta para produção de biodiesel utilizaram, em sua vasta maioria, lipases imobilizadas a suportes poliméricos como catalisador da reação. O emprego de lipases em formulação líquida/solúvel nesse tipo de tecnologia é recente, sendo que, até a presente data, poucos trabalhos sobre o tema foram publicados. Resultados para uma produção em escala superior à laboratorial são ainda mais raros.

Considerando o potencial catalítico demonstrado por lipases solúveis em catalisar reações de produção de biodiesel, se faz necessário encontrar uma configuração reacional que permita reduzir o tempo reacional do processo necessário para atingir um satisfatório rendimento de biodiesel e assim alavancar tal tecnologia para ser empregada em projetos de síntese de biodiesel em larga escala. Um processo inovador realizado em duas etapas, utilizando-se da lipase líquida, termoestável e de menor custo de produção NS 40116 possui potencial para aplicação em escala industrial, sendo uma interessante alternativa ao sistema.

Outro ponto fundamental ao utilizar-se da catálise enzimática para produção de biodiesel elaborado em pesquisas sobre o tema é o emprego de uma matéria-prima residual de baixo custo/elevado teor de impurezas no processo, aproveitando-se da não-sensibilidade das lipases em processar este tipo de material. No presente trabalho, ao realizar-se um aumento na escala de produção do sistema reacional proposto, utilizou-se como matéria-prima óleo de cozinha residual recolhido dos restaurantes universitários da UFSM.

Sendo assim, considerando as variáveis que afetam a reação, a correta identificação da combinação dos fatores que direcionam a um rendimento satisfatório do processo é um desafio que este trabalho se propôs a investigar. A utilização de lipases em sua formulação líquida para catalisar a reação de produção de biodiesel é uma técnica promissora, onde apenas seus benefícios ambientais já servem como incentivo. Os resultados obtidos através deste estudo estão dispostos na forma de artigos que estão publicados em periódicos da área.

1.4 PUBLICAÇÕES GERADAS NA EXECUÇÃO DO TRABALHO

Ao longo da pesquisa, interessantes resultados foram alcançados, o que possibilitou a publicação de trabalhos, os quais estão listados a seguir:

i. Trabalho *"Produção de Biodiesel em Escala Piloto via Hidroesterificação Enzimática"* aceito para apresentação no *XXIV Congresso Brasileiro de Engenharia Química* que ocorrerá em maio de 2021;

ii. Artigo *"Semi-continuos Production of Biodiesel on Pilot Scale via Enzymatic Hydroesterification of Waste Material: Process and Economics Considerations"* publicado no periódico *Journal of Cleaner Production* (ISSN: 0959-6526) em outubro de 2020 e disponível em https://doi.org/10.1016/j.jclepro.2020.124838;

iii. Artigo *"Lipases in Liquid Formulation for Biodiesel Production: Current Status and Challenges"* publicado no periódico *Biotechnology and Applied Biochemistry* (ISSN: 1470-8744) em outubro de 2019 e disponível em https://doi.org/10.1002/bab.1835;

iv. Trabalho *"Produção de Biodiesel via Hidroesterificação de Óleo de Soja Catalisada pela Lipase Solúvel NS 40116"* publicado na *34ª Jornada Acadêmica Integrada da Universidade Federal de Santa Maria* em outubro de 2019;

v. Artigo *"Improving the Soluble Lipase-catalyzed Biodiesel Production through a Two-step Hydroesterification Reaction System"* pubicado no periódico *Applied Microbiology and Biotechnology* (ISSN: 0175-7598) em agosto de 2019 e disponível em https://doi.org/10.1007/s00253-019-10075-y;

vi. Artigo *"Enzyme-Catalyzed Production of FAME by Hydroesterification of Soybean Oil Using the Novel Soluble Lipase NS 40116"* publicado no periódico *Applied Biochemistry and Biotechnology* (ISSN 1559-0291) em fevereiro de 2019 e disponível em https://doi.org/10.1007/s12010-019-02966-7;

vii. Artigo *"Feeding Strategies of Methanol and Lipase on Eversa® Transform-Mediated Hydroesterification for FAME Production"* publicado no periódico *The Canadian Journal of Chemical Engineering* (ISSN: 1939-019X) em novembro de 2018 e disponível em https://doi.org/10.1002/cjce.23404;

viii. Trabalho *"Produção de Biodiesel Utilizando Lipases Livres como Catalisador da Reação e Sebo Animal como Matéria-Prima do Processo"* apresentado na *33ª Jornada Acadêmica Integrada da Universidade Federal de Santa Maria* em outubro de 2018;

ix. Trabalho *"Síntese de Ésteres Metílicos via Hidroesterificação Enzimática de Sebo Animal Catalisada pela Lipase Solúvel Eversa® Transform"* apresentado na *33ª Jornada Acadêmica Integrada da Universidade Federal de Santa Maria* em outubro de 2018;

x. Trabalho "Avaliação do Efeito Inibitório do Metanol sobre a Lipase Solúvel Eversa® Transform na Produção de Biodiesel via Hidroesterificação" apresentado no *XXII*

Congresso Brasileiro de Engenharia Química em setembro de 2018 http://dx.doi.org/10.5151/cobeq2018-co.024;

xi. Trabalho *"Influência da Temperatura Reacional e Carga de Catalisador na Produção de Biodiesel Catalisada por Lipases em Formulação Líquida"* apresentado na *32ª Jornada Acadêmica Integrada da Universidade Federal de Santa Maria* em outubro de 2017;

xii. Trabalho *"Efeito do Excesso de Metanol e Concentração de Água na Produção de Biodiesel Catalisada por Lipase em Formulação Líquida"* apresentado na *32ª Jornada Acadêmica Integrada da Universidade Federal de Santa Maria* em outubro de 2017;

xiii. Trabalho *"Produção de Biodiesel Catalisada por Lipases Solúveis: Influência do Excesso de Metanol e da Concentração de Água na Reação"* apresentado no *XII Congresso Brasileiro de Engenharia Química em Iniciação Científica* em julho de 2017 e disponível em http://dx.doi.org/10.5151/chemeng-cobeqic2017-350.

2 LIPASES EM FORMULAÇÃO LÍQUIDA PARA PRODUÇÃO DE BIODIESEL: STATUS ATUAL E DESAFIOS

Neste capítulo está apresentado um artigo *review* abordando uma revisão bibliográfica a respeito do tema da tese. Tal trabalho se faz necessário para embasar a pesquisa desenvolvida, discutindo considerações importantes e fundamentais à compreensão dos resultados alcançados.

Lipases in Liquid Formulation for Biodiesel Production: Current Status and Challenges

DOI: https://doi.org/10.1002/bab.1835

João H. C. Wancura[a], Marcus V. Tres[b], Sérgio L. Jahn[a], J. Vladimir Oliveira[c]

[a] Department of Chemical Engineering, Federal University of Santa Maria, 1000, Roraima Avenue, Santa Maria, 97105-900, Brazil;

[b] Laboratory of Agroindustrial Processes Engineering (LAPE), Federal University of Santa Maria, 1345, Ernesto Barros Street, Cachoeira do Sul, 96506-322, Brazil;

[c] Department of Chemical and Food Engineering, Federal University of Santa Catarina, Florianópolis, SC, 88040-900, Brazil.

ABSTRACT

Enzymatic synthesis of biodiesel showed advantageous characteristics in relation to other technologies once it works under bland conditions, no generation of wastewater, no occurrence of saponifications reactions and production of a biodiesel with high quality. Although many researches still apply immobilized lipases, the high costs associated with this biocatalyst hamper the economic viability of the process. Lipases in free/soluble/liquid formulation employed to biodiesel production via hydroesterification reaction have attracted interest from researchers because they are more cost effective than the immobilized form, making the enzymatic route more competitive. In addition, soluble lipases present higher reaction rates, reducing the time required to obtain a satisfactory biodiesel yield. Despite the fact that already exist industrial plants producing biodiesel with the assistance of lipases in liquid formulation, results of researches show that the process still needs to overcome some drawbacks. This paper is a comprehensive and critical discussion on the publications where soluble lipases were applied on biodiesel synthesis, as well as the challenges that the technology faces and its current status in pilot and industrial applications.

Keywords: biodiesel, enzymatic hydroesterification, liquid lipase, soluble lipase, transesterification.

Abbreviations: AV, acid value; DAG, diacylglycerols; FAAE, fatty acid of alkyl esters; FAEE, fatty acid ethyl esters; FAME, fatty acid methyl esters; FFA, free fatty acids; MAG, monoacylglycerols; MeOH, methanol; NZ435, immobilized enzyme Novozym 435; TAG, triacylglycerols.

2.1 INTRODUCTION

Research divulged in April 2019 by *Potsdam Institute for Climate Impact Research*, conducted by Willeit et al. [1], reports alarming data concerning climate changes that are occurring on our planet: carbon dioxide, one of the combustion gases responsible for global warming, is at the highest concentration level ever recorded in 3 million years, which makes it inevitable to raise of the Earth's temperature and the level of the oceans. The use of fossil fuels, such as petroleum diesel, has a considerable share of these emissions responsible for promoting the pollution of our ecosystem [2, 3]. In addition, the current reserves of nonrenewable fossil fuels are depleting each day. Currently, we live in a society where the demand for energy is constantly growing, intensifying the exploration of nonrenewable fuels and consequently leading to energy crises and environmental issues [4–7]. Thus, the search for a fuel from renewable sources that at the same time being safe, "greener," and socially benign, has influenced several studies in recent years [8–12]. In this scenario, the biodiesel has become a promising alternative to petrodiesel. This circumstance is mainly due to its environmentally friendly properties, nontoxicity, capacity of use in diesel engines with few or no mechanical alterations, compatibility with the existing fuel distribution infrastructure, and by the fact that it is produced from renewable sources [13, 14].

Among the distinct techniques suggested for biodiesel synthesis, the main route used is the alkaline transesterification (alcoholysis) of vegetable oils and animal fats [15, 16]. Such technology is well established, being the main pathway employed to produce the biofuel industrially worldwide due to its high conversion in brief reaction times [17, 18]. However, despite the benefits in terms of efficiency, this method has drawbacks of process (requirement of a high purity feedstock in order to avoid saponification) and under the environmental point of view (generation of alkaline wastewater from the washing stages of the crude biodiesel) [19], going against the proposal of utilization of the biodiesel as a substitute for the diesel.

Thus, a technique that has showed promising and effective results in circumventing the problems of the industrial-alkaline process is the use of enzymes as catalyst of the transesterification reaction [20, 21]. The enzymatic methodology has the ability to originate a high purity biodiesel under bland reaction conditions, no requiring biofuel purification steps, as well as having the capacity to process raw materials with high content of impurities (moisture and acidity) without the occurrence of undesired side reactions in the system [22–24].

In terms of "enzymatic biodiesel," most of researches consider the application of lipases immobilized at different supports as catalysts of the reaction justified by a higher operational stability of the enzyme and possibility of the biocatalyst to be recovered and reutilized in the process [25–27]. However, recently published researches has demonstrated the great potential that lipases in liquid (or soluble or also free) formulation have in catalyzing the biodiesel synthesis [28–32]. Soluble lipases have a lower cost of production in relation to the immobilized form, which considerably reduces process expenses, making all the biodiesel production more competitive and sustainable [33].

In this context, this paper presents a comprehensive review about the biodiesel production catalyzed by lipases in liquid formulation, disclosing the recent literature published to date. For this, the following aspects will be addressed:

1. General information on biodiesel and the main techniques of oleaginous sources transformation in this biofuel;

2. Aspects about the enzymatic production of biodiesel, focusing mainly on the utilization of soluble lipase in the reaction, but also bringing recent researches about the application of lipases immobilized in the process;

3. The challenges that the liquid lipase-mediated synthesis of biodiesel confront to consolidate as an alternative pathway for biodiesel production on a scale superior to laboratory or pilot one;

4. The current status of the enzymatic biodiesel productioncatalyzed by soluble lipases

2.2 BIODIESEL: GENERAL INFORMATION

Long before of the energy crisis of the 1970s and early 1980s, researches already focused their attentions on the seek for renewable fuels, being one of the main themes of these works the use of vegetable oils and animal fats as an energy source for combustion engines [34]. Rudolf Diesel (1858–1913) was interested in this research and was the inventor of the

motors that took his name. Knothe and Razon [34] report that R. Diesel devoted part of his research to the utilization of vegetable oils as fuel for internal combustion engines. However, the high kinematic viscosity of these materials led to operational problems in the equipment, mainly the creation of deposits, impeding a reasonable thermodynamic efficiency by the engine.

Thus, it was necessary to search for a fuel that had a lower viscosity to be employed in these engines and at the same time had characteristics similar to petrodiesel in terms of burning efficiency (acceptable thermodynamic efficiency). Within this scenario and through developed research, the synthesis of the alternative and renewable fuel that we know today as biodiesel became possible.

Biodiesel (fatty acid of alkyl esters – FAAE or usually fatty acid methyl esters – FAME when methanol is used as alkyl acceptor) is defined as a fuel composed of alkyl esters derived from vegetable oils or animal fats [35]. Due to its chemical and physical properties very similar to petroleum diesel, biodiesel has consolidated as a promising substitute to the diesel and therefore it is able to be used in diesel engines without the need for mechanical modifications in the equipment [36]. Biodegradability, nontoxicity, lubricity, compatibility with the current fuel distribution infrastructure, miscibility with petroleum diesel in any proportion, high flash point, low emissions profile (including potential carcinogens), besides the possibility of being synthesized from renewable or residual feedstock are some of the characteristics that evidenced biodiesel as a fuel able to fit within of the currently existing energy matrix [17, 37, 38].

In terms of world production, United States and Brazil stand out as two of the world's leading biodiesel producers (Fig. 1), followed by Germany and Argentina. In 2017, the sum of the amount produced by the two main producers countries exceeded 10 billion of liters [39]. It is estimated that by 2030, biodiesel can replace up to 7 vol% of the total amount consumed of fossil fuels in the world [40].

The benefits of the biodiesel go beyond of environmental and energetic aspects. The biodiesel industry has the objective to assist the improvement of a balanced energy policy and a self-sufficient society through a production planning based on the capacities of a determined community [41, 42]. Živković et al. [41] cite that the local economies including the rural farmers, biodiesel producers, and consumers will benefit economically and socially as the capital generated will remain in the community and the environmental security will be improved.

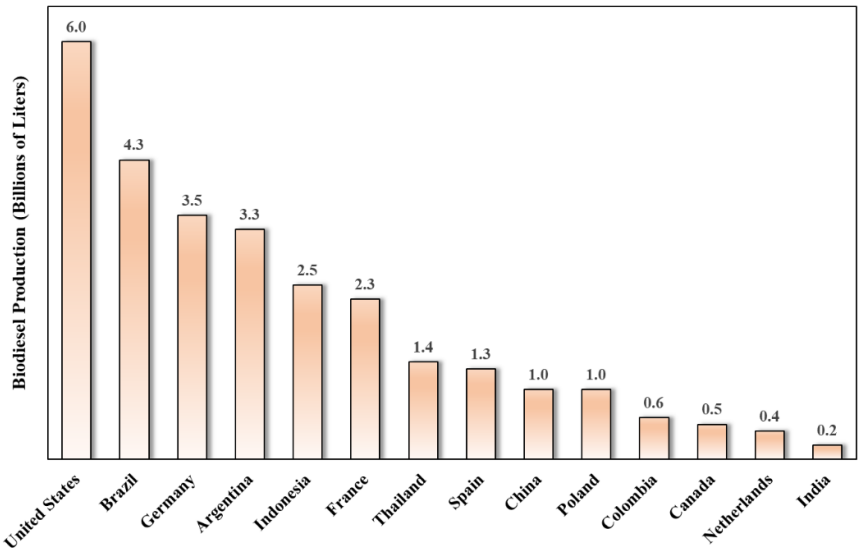


Figure 1. Leading biodiesel producers worldwide in 2017 [39].

Several processes and technologies have been developed in recent years focusing on biodiesel synthesis or as an attempt to reduce the kinematic viscosity of vegetable oils to enable their use in diesel engines. In general, the routes can be divided into "catalytic processes," where mainly acid, alkaline, and enzymatic catalysts are employed; and "noncatalytic processes," such as microemulsification, pyrolysis, and reactions under supercritical conditions. Figure 2 illustrates this classification.

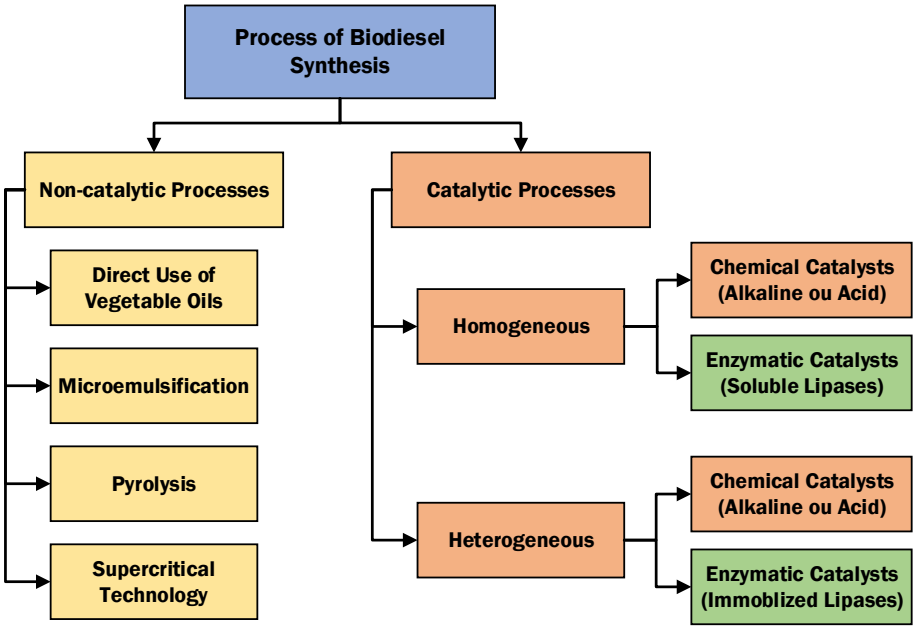


Figure 2. General methods of biodiesel (FAAE) production.

2.3 NON-CATALYTIC PROCESSES FOR BIODIESEL PRODUCTION

2.3.1 Dilution of Vegetable Oils, Microemulsification and Pyrolysis

It is known that the use of vegetable oils as an alternative fuel already would have been tested around the 1900s when the inventor of the diesel engines, Rudolph Diesel, used peanut oil in his engine of internal compression [34]. As consequence, mechanical problems were observed. Thus, alternatives such as the dilution of vegetable oils in blends with diesel oil or with solvents of low viscosity were tested. Nevertheless, unsatisfactory results persisted due to the formation of gums originated from the oxidation and polymerization reactions that occur during the fuel combustion, as well as to the presence of deposits in the engine [36].

A second noncatalytic technique also evaluated aiming to reduce the viscosity of vegetable oils is the microemulsification of oleaginous sources. According Ali and Hanna [43], a microemulsion is a system consisting of a dispersed liquid, with or without an emulsifier, in a liquid immiscible on droplets larger than a colloidal size, which can be a solution to the question of high viscosity of vegetable oils. The main purpose of this method is to blend vegetable oils with low-molecularweight alcohols, in order to formulate a hybrid diesel with low viscosity. However, the procedure results in an irregular injection of material into the engine, creating deposits of compounds and incomplete combustion [38]. Do et al. [44] investigated the use of surfactants and cosurfactants in a formulating reverse micelle microemulsion of oleaginous resources (canola, palm, and algae oil)/diesel fuel blended with ethanol, where the microemulsioned fuel was tested for temperature stability, viscosity, water tolerance, and their combustion performance. The authors formulated a satisfactory canola and algae/diesel microemulsion with cloud and pour points that satisfied the ASTM standards.

On the other hand, the pyrolysis procedure (or thermal cracking) involves the cleavage of chemical molecules via decarboxylation reaction to form a mixture of hydrocarbons with properties similar to those of petrofuels, by the action of heat (temperatures above 350 °C) in the absence of air or oxygen [36]. This technique is more suitable for biodiesel production in regions where there is a shortage of oil sources, where animal fats and residual cooking oils can be used as feedstock [45]. However, the product formed has characteristics more similar to gasoline than diesel. Moreover, the pyrolysis process follows different reaction mechanisms, and maintaining a process control in order to direct the reaction selectivity to the product of interest is a challenge [16]. Ito et al. [46] investigated the synthesis of biodiesel from waste cooking oil via pyrolysis. The authors verified that the raw material utilized decompose at 360

– 390 °C, generating fatty acids by cleavage of the ester bond of the triacylglycerols (TAG), shortchain hydrocarbons, and short-chain fatty acids, generated by cleavage of the unsaturated bonds of the hydrocarbon chain. Although the process of pyrolysis operates conventionally in a noncatalytic pathway, there are several studies investigating the employment of catalysts as a form of minimizing the temperature required to achieve the activation energy of the reaction, reducing the energetic demand of the system [46, 47].

2.3.2 Supercritical Technology

In a conventional process for biodiesel production (homogeneous alkali-mediated transesterification), steps for purification and removal of catalyst traces from the biofuel are inevitably involved. Considering the drawbacks of the main route of biodiesel synthesis in an industrial scale, the use of alcohols under supercritical conditions has aroused the interest of researchers by the fact that the process does not require stages for catalyst traces removal, since the reaction is conducted without catalyst, as also enables the processing of raw materials with high moisture content [48]. In the biodiesel production under supercritical reaction conditions, the medium becomes homogeneous, occurring simultaneously the esterification of free fatty acids (FFA) and the transesterification of TAG, enabling this technology to process a large variety of substrates [13]. Abbaszaadeh et al. [15] cite that the reactions are fast and have high conversions (50 % – 95 % in only 10 min), however occurring at temperatures of 250 – 400 °C. Such high reaction temperatures and pressures tend to improve the solubility of the system, reducing limitations in the mass transfer, resulting in higher reaction rates [49].

Several acyl acceptors have been considered for use on the process: methanol (MeOH), ethanol, methyl acetate, and dimethyl carbonate [50]. Saka and Isayama [51] investigated the transesterification of rapeseed oil using supercritical methyl acetate in a range of temperature from 270 to 380 °C for a reaction time from 10 to 120 min, a pressure of 20 MPa and a molar ratio of methyl acetate and oil of 42:1, obtaining a biodiesel yield of 105 wt%. Farobie and Matsumura [52] developed a spiral reactor to produce biodiesel from canola oil using supercritical MeOH: in 10 min at 350 °C, 20 MPa, and a methanol to oil molar ratio of 1:40; 100 % of yield was obtained. As can be seen, the main disadvantage of using supercritical conditions for biodiesel synthesis is the high demand of energy required to achieve the reaction temperature/pressure needed by the process, challenging researchers to develop the method at scales superior to laboratory ones.

2.4 CATALYTIC PROCESSES FOR BIODIESEL PRODUCTION

2.4.1 Homogeneous Catalysis

The main mode for production of biodiesel is through the use of homogeneous catalysts, mainly due to the simplicity of handling and shorter time required to complete the reaction [4]. The reaction using homogeneous catalysts can be mediated by acidic or alkaline compounds disposed in liquid formulations.

Alkaline transesterification in homogeneous medium is the technique most employed at industrial level for the biodiesel production, once the process offers high reaction yields in short intervals of time [7, 9, 17, 38, 49]. Baskar and Aiswarya [35] cite that homogeneous alkaline catalysis can be up to 4,000 times faster than acid catalysis to achieve an equivalent biodiesel yield. The foremost basic catalysts utilized are sodium hydroxide, potassium hydroxide, and their corresponding alkoxides such as sodium methoxide or ethoxide and potassium methoxide or ethoxide [45]. The most advantageous reaction condition established for biodiesel production is for an alcohol to oil molar ratio of 6:1, 60 °C, 1 h of reaction applying 0.5 wt% of sodium methoxide (CH3ONa) or 1.0 wt% of sodium hydroxide as catalyst when methanol is applied as the acyl acceptor [34]. Usually, a short-chain alcohol, like methanol or ethanol, is used in the process [53].

Figure 3 present a block flow diagram of the biodiesel production process usually applied in an industrial scale, where alkaline transesterification using sodium methoxide and methanol predominates among the routes selected for developing plant designs. The process develops in successive stages of reaction and separation of the heavy (glycerol) and light (biodiesel) phases formed during the process. The biodiesel formed is conducted to a washing step to remove traces of the catalyst and then to polishing steps (operations of filtration) to finally be led to storage tanks. Before the stages of separation of the light and heavy phases, the glycerol containing traces of catalyst is neutralized by the addition of acid in the process stream (usually hydrochloric, sulfuric, sulfonic, or phosphoric acid). The alcohol used in the synthesis is recovered from the glycerol and biodiesel by evaporation followed by distillation, to thereby be reused in the process.

Despite being an industrially established process, homogeneous alkaline transesterification has drawbacks that must be highlighted, which is the requirement of a raw material with high purity (low moisture and FFA content) due to the risk of saponification reactions occurring during the process and difficulty in removing the catalyst from the biofuel,

where the biodiesel stream must go through successive washing steps to remove the material, generating a large amount of alkaline water that needs to be treated later [54, 55].

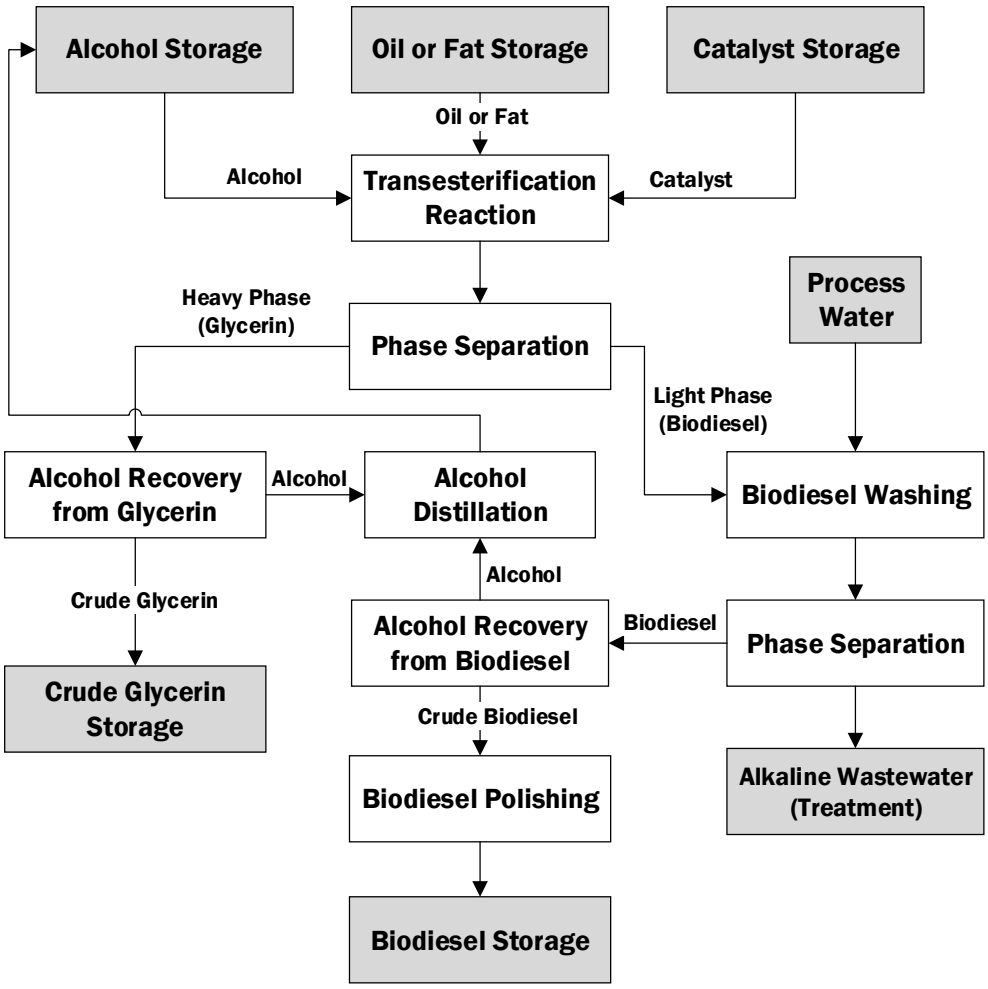


Figure 3. Conventional pathway to produce biodiesel industrially.

In terms of homogeneous acid catalysis, sulfuric, sulfonic, phosphoric, and hydrochloric acid are the foremost catalysts utilized [45]. The main advantage of the homogeneous acid catalysis compared with other techniques is that this route is not sensitive to the presence of FFA and moisture in the raw material [38]. Nevertheless, homogeneous acid catalysis did not develop industrially as it requires high reaction temperatures (usually 50 – 150 °C [56]), equipment made of materials that support inevitable problems with corrosion, as well as reactors with high volumes once the reaction rates of this route are low [17]. Mohadesi et al. [57] investigated the transesterification of waste cooking oil in the presence of potassium hydroxide in a pilot microreactor to produce 5 $L \cdot h^{-1}$ of biodiesel. First, the acidity of the raw material had to be reduced to less than 1 mg $KOH \cdot g^{-1}$ of oil employing an acid esterification in the presence of sulfuric acid (1 wt%). With the FFA content reduced, the authors applied a

homogeneous alkaline transesterification under 62 °C, a methanol to oil molar ratio of 9.4:1, and 1.16 wt% of potassium hydroxide to achieve 98.3 % of biodiesel yield. In a similar work, Banani et al. [58] suggested a two-step catalyzed process to convert waste frying oil with an acid value (AV) of 32.8 mg $KOH \cdot g^{-1}$ of oil in biodiesel. In the first step, sulfuric acid was applied as catalyst to esterify the FFA present in the substrate for then, in a second step, to use an alkaline-mediated transesterification to convert the pretreated frying oil into biodiesel: under the best conditions found, 98 % of biodiesel yield and 0.2 wt% of FFA was obtained.

2.4.2 Heterogeneous Catalysis

Although homogeneous catalysts have been extensively investigated in the biodiesel production, washing steps required to remove traces of material from the biofuel generate a considerable volume of wastewater that needs to be treated [36]. In this sense, heterogeneous catalysts appear as an interesting alternative for biodiesel synthesis in economic and ecological terms, being easier to be separated from the reaction system, simplifying the stages of FAAE purification [59, 60]. Moreover, heterogeneous catalysts are tolerant to fatty acids and moisture present in the raw material, besides having a larger surface area with pores for the active interaction between reagent and oleaginous source [35]. Notwithstanding, according to Baskar and Aiswarya [35], the main disadvantage related to heterogeneous catalysis is the tendency of formation of three phases with the substrate and alcohol, leading to limitations on reactive diffusion with decreasing in the reaction rate. Knothe and Razon [34] cite that several works related to heterogeneous catalysts applied to the biodiesel production do not address the issue about the leaching of these catalysts and how this can affect the properties of the biofuel (and the meeting of regulatory specifications), especially to heteroatoms, such as sodium, potassium, magnesium, phosphor, and sulfur.

Heterogeneous acid catalysts have the capacity to simultaneously catalyze reactions of esterification and transesterification, which become interesting when low-quality raw materials are employed [16]. Guldhe et al. [61] evaluated the biodiesel production from microalgae lipids using WO_3/ZrO_2, comparing the efficiency of this catalyst with a homogeneous acid and enzymatic ones. Results showed that the process using the heterogeneous catalyst reached a biodiesel yield of 94.6 % at 100 °C, using a molar ratio between methanol and oil of 12:1 and 15 wt% of catalyst load after 3 h of reaction, a conversion comparable to the homogeneous and higher than the enzymatic process. Nonetheless, the authors reported that the system presented a higher energy consumption than the homogenous and enzymatic-mediated processes. Yu et

al. [62] reported the carbonization of powder coal under nitrogen atmosphere followed by treatment with sulfuric acid under microwave radiation assistance for preparation of an heterogeneous acid catalyst to be employed for biodiesel production: 98.1 % of esterification yield was achieved applying 10 wt% of catalyst loading, molar ratio of alcohol to feedstock of 12:1, 65 °C, and 180 min of reaction.

On the other hand, heterogeneous alkaline transesterification for biodiesel synthesis has been an interesting alternative in terms of "process simplification," mainly in the purification stages, minimizing the generation of alkaline wastewater [35]. The majority of the studies published involving heterogeneous alkaline catalysis are focused in the use of metal oxides, with calcium oxide (CaO) being one of the most studied due its high basicity, low solubility and cost, nontoxic, and high stability [59]. In one of these researches, Devaraj et al. [63] reports results of the application of activated CaO on the production of biodiesel from waste cooking oil in a pilot plant of 15 L: 96 % of biodiesel was achieved in 2 h under 80 °C, 3 wt% of catalyst and methanol to oil molar ratio of 6:1. In a similar work, Navas et al. [64] reported 97 % of biodiesel yield obtained from castor oil and using butanol as acyl acceptor, using as catalyst the compound $MgO/\gamma\text{-}Al_2O_3$ under 80 °C, 5 wt% of catalyst, alcohol to oil molar ratio of 6:1, and 6 h of reaction.

2.5 ENZYMATIC BIODIESEL PRODUCTION

2.5.1 Lipases

Enzymes are alternative tools that have the possibility of introducing environmental and sustainable processes at an industrial level [65]. Lipases (*triacylglycerol ester hydrolases*, EC.3.1.1.3) are enzymes that have demonstrated to be useful in different hydrolytic and synthetic reactions, being the class of enzymes most widely employed in organic synthesis due to a considerable number of commercially available preparations and better stability compared with other enzymes in media containing organic solvents [66, 67]. The potential of application for lipases is noticeable in the production of biofuels, leather, foods, detergents, cosmetics, fragrances, and pharmaceuticals [68, 69]. Moreover, they are easily obtained from a diversity of biological sources by extraction from animal and plant tissues or cultivation of microorganisms, providing enzymes with different substrate specificities and catalytic properties [66, 70]. Most lipases utilized as catalysts in organic synthesis are of microbial and fungal origin, such as *Candida rugosa, Candida antarctica, Pseudomonas fluorescens,*

Rhizopus oryzae, Burkholderia cepacia, Aspergillus niger, Thermomyces lanuginosus and *Rhizomucor miehei*, being easy to obtain by fermentation and a basic purification step [71].

The selection of a lipase for a determined application must consider its specificity and stability in different solvent systems. For lipases to be applied for example in waste treatment, leather processing, essences extraction, detergent formulation, or biodiesel production, their exclusive properties and tolerance will aid in selecting and determining their effectiveness [67]. Lipases may be classified according their substrates specificities in three different groups: *sn*-1,3-specific (hydrolyze the ester bonds of the TAG at the positions R_1 or R_3 positions), *sn*-2-specific (hydrolyze the ester bonds of the TAG at the position R_2), and nonspecific. For a satisfactory production, lipases should be able to convert all three forms of glycerides – monoacylglycerols (MAG), diacylglycerols (DAG), and TAG – to biodiesel. Lipases with a limited specificity become inapplicable for biodiesel production, whereas lipases with *sn*-1,3-specific efficiently catalyze the process [72] and their yields exceed maximum theoretical yields under appropriate operating conditions [73]. Figure 4 shows how a *sn*-1,3-specific lipase-catalyzed alcoholysis with acyl migration develops. Although the application of these lipases leads to a maximum theoretical yield of 66.7 %, higher yields can be obtained due to the acyl migration (reactions 3 and 5 of the Fig. 4), which promotes the conversion of 1,2- to 1,3-DAG and 2- to 1-MAG, achieving an overall conversion of the TAG to glycerol and biodiesel [74].

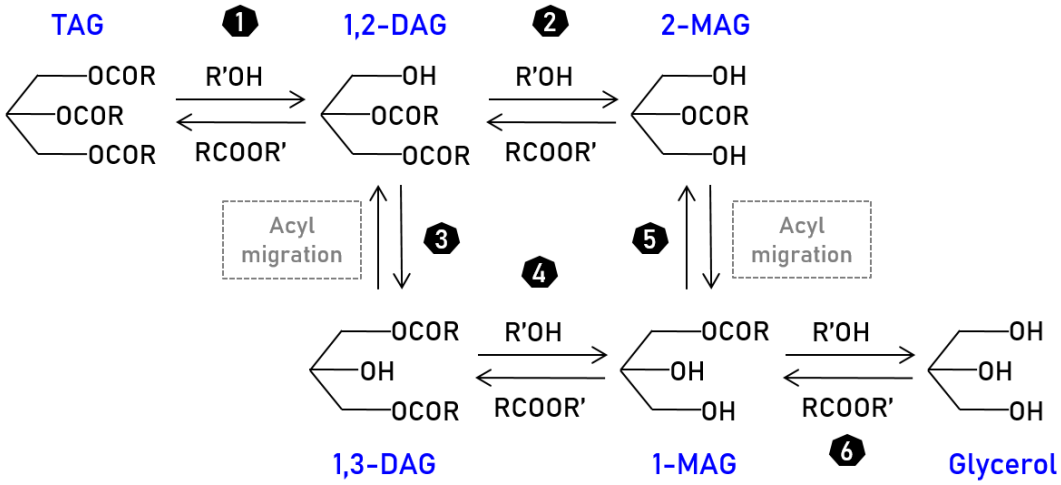


Figure 4. *sn*-1,3-specific lipase-mediated alcoholysis with acyl migration.

2.5.2 Enzymatic Transesterfication: Use of Immobilized Lipases

Researches published in recent years suggest that a technology that is apt of acting under the drawbacks of the alkaline route and producing biodiesel efficiently is the transesterification

using enzymes as a catalyst of the process [75–78].

Considering the biodiesel production, enzymatic transesterification has diverse advantages: process conduction under mild reaction conditions, which implies a lower energy consumption involved; wide range of possibilities for raw materials suitable to be employed in the reaction due to the ability of the lipases to convert both TAG and FFA present in the substrate in a single step; use of lower alcohol excesses in the process in relation to other techniques; lesser possibility of the occurrence of undesired reactions due to the specificity and selectivity of the biocatalysts; elimination of the stage and associated costs with the biodiesel washing for traces removal of catalyst; production of a high purity biodiesel; and the fact that the lipases have an environmental acceptability higher than chemical catalysts, making the enzymatic process "greener" [79–83].

Some published works explore the environmental benefits of the enzymatic process in comparison with the alkaline one in the biodiesel industries. Peñarrubia Fernandez et al. [84] investigated an industrial-scale simulation presenting a comparison for 1 ton of biodiesel produced by alkali-catalyzed process (sodium hydroxide as catalysts) and a novel enzyme-catalyzed process (immobilized lipase from *C. antarctica* B) developed by the researches employing *Life Cycle Analysis*. For the situation where alkali-catalyzed process was considered, refined soybean oil was used as feedstock; in the enzymatic one, waste cooking oil was the raw material employed. Based on the results obtained, the authors concluded that given the negligible environmental influence of the biocatalyst, low energy consumption, and absence of soaps or other wastes, the enzymatic process decreases the ecological impact considerably when compared with the alkaline process. Nevertheless, the efficiency of electric or thermal energy as well as the chemicals utilized should be appropriately managed to reduce the impacts as much as possible. In an analogous work, de Mello et al. [81] conducted a comparative environmental impact analysis of a biodiesel production by an alkali-catalyzed methylic transesterification and an enzyme-mediated ethylic transesterification, using the software Aspen HYSYS® for realize the simulation, approaching the *Principles of Green Chemistry* [85] and calculation of sustainability metrics. Results obtained by the authors indicated a better environmental performance of the enzymatic technique for practically all the metrics considered (excepting for the "*Total raw material used per kilogram of product*", once was considered a high lipase load of 15 wt% to reach a biodiesel yield comparable to the alkaline process). Anyway, this study demonstrates that the enzymatic route is an interesting alternative for future industrial applications, mainly in terms of sustainability and safety.

Despite the favorable points, the enzymatic technology also presents

problems/complications that must be considered: high cost of the biocatalysts in comparison with the alkaline ones; lower reaction rates, implying in higher reaction time required to achieve satisfactory conversions; and loss of catalytic activity when the lipase is under high temperatures and mainly by the denaturing action of the main reagent used in the reaction, the methanol [86, 87]. In a transesterification, at least the stoichiometric amount of 3 mol of alcohol for each mole of substrate is required to complete the conversion of the TAG of the feedstock to their corresponding FAAE, whereby an excess of alcohol is usually used in order to shift the reaction equilibrium toward the products. However, several papers report that the reaction rate of the enzymatic transesterification decreases significantly when the alcohol concentration in the system is high. Usually a methanol excess of 4.5:1 is utilized and an inhibition of the enzyme activity is already verified for a molar ration between methanol and substrate of 1.5:1 [55]. This decrease in the reaction rate is associated with the loss of the catalytic capacity of the lipase caused by the polarity of the short-chain alcohols applied in the process, being one of the main obstacles to the production of enzymatic biodiesel [88, 89]. The employment of an organic solvent is typically reported as a tool to overcome this problem; however, considering an industrial biodiesel production, solvents are undesirable from a financial and an environmental perspective [90].

The vast majority of the works published about the theme "enzymatic biodiesel" utilized lipases immobilized in chemical supports as catalysts of the transesterification process. The immobilized lipase Novozym 435 (NZ435) is the main biocatalyst chosen. Currently, researchers have focused efforts mainly on the development of alternative supports for the immobilization of the enzymes or improvement of the process productivity through of a modification in the reaction conduction in order to potentiate the catalytic capacity of the biocatalyst. In one of these works, Zhang et al. [91] evaluated a modified polyporous magnetic cellulose beads from an organic amine as a novel type of support to immobilize *C. antarctica* lipase B for biodiesel production. The authors justified that the synthesized catalyst presents a good biocompatibility, which is beneficial to preserve the lipase activity besides to allow to recover (and reuse) easily the enzyme from the reaction system. Using yellow horn seed oil under 60 °C, methanol to oil molar ratio of 6:1, a quite high catalyst load of 15 wt% and 2 h, 92.3 % of biodiesel yield was obtained, where the catalyst could be recycled by a magnet five times and still reached about 85 % of yield after the fourth cycle. In another work, Picó et al. [92] prepared and characterized a magnetic cross-linked enzyme aggregates of lipase B from *C. Antarctica* (which were covalently bound to magnetic nanoparticles) for application in a biodiesel synthesis of lipids extracted by saponification method from nitrogen-starved cells of

Chlorella vulgaris var L3. A biodiesel conversion of more than 90 % was obtained after 3 h. Though the magnetic character of the biocatalyst permitted its easy recovery and reuse for 10 consecutive cycles, retaining 90 % of the initial activity, concentrations of methanol at 15 vol% or higher caused a decrease in the lipase activity. Aiming to overcome the negative effect of the glycerol on the catalytic performance of the lipases, Tian et al. [93] suggests a novel process configuration by introducing of dimethyl carbonate into an enzyme-mediated methanolysis for biodiesel production, using the immobilized lipase NZ435. The dimethyl carbonate could react with glycerol and release methanol, which not only realizes the in situ removal of byproduct, but also achieves higher biodiesel yield at shorter reaction time compared to only employment of the carbonate as acyl acceptor. Also, dimethyl carbonate reacts with glycerol to produce glycerol carbonate, which it has been demonstrated that this component is a sort of good additive to fuels due to its high content of oxygen [94]. At 60 °C, 5 wt% of lipase, methanol to oil molar ratio of 0.5:1, dimethyl carbonate to glycerol molar ratio of 6:1, and 24 h, the authors could be achieved 95.3 % of biodiesel yield with a glycerol concentration of 0.05 wt% and with a negligible loss in the lipase's activity after six batches. Table 1 summarizes others researches recently published in which immobilized lipases were applied in the synthesis of biodiesel.

Many works defend the utilization of immobilized lipases in the process once the procedure would concede a higher operational stability to the catalyst, besides the fact of permitting its recovery from the reaction system and its reutilization [95, 96]. Luković et al. [97] report that the recycling of immobilized enzymes can considerably reduce production costs, making the process more competitive against the alkaline route. Nonetheless, Cesarini et al. [98] describe that the immobilization process of the enzyme is not simple, and the supports usually employed in the process have high costs, making expensive the total cost of the biocatalyst. Considering for example the lipase NZ435, the authors cite that the number of reuses of the enzyme should be higher than one hundred cycles for the process be feasible, something improbable considering the loss of lipase activity at each batch when in contact with the alcohol. Moreover, the glycerol generated in the reaction, which is hydrophilic and insoluble in oils, also causes inactivation of the biocatalyst due to its adsorption on the surface of the immobilized enzyme, leading to losses of activity and operational stability of the lipase [99]. In these circumstances, the employment of soluble/liquid lipases and the concept of hydroesterifi cation reaction for biodiesel production emerged as an alternative to the biotechnological process.

Table 1. Some relevant papers published recently which was employed an immobilized lipase-mediated for FAAE production.

(continued)

Ref.	Enzyme	Substrate	Reaction Conditions	Yield (%)	Remarks
[80]	Lipase from *T. lanuginosus* immobilized on hydrophobic polymer	Waste cooking oil	30 °C, MeOH:oil molar ratio of 3.0:1, 10 wt% lipase, 1 wt% water, 2 h	80.0	Different lipases were physically immobilized on hydrophobic supports via interfacial activation, where their properties was very depended on the immobilization support.
[86]	Lipase MAS1 immobilized on XAD1180	Waste cooking oil	35 °C, MeOH:oil molar ratio of 3.0:1, 5 wt% lipase, 24 h of reaction	95.5	Immobilized MAS1 showed high tolerance to MeOH.
[100]	Novozym 435	Macaúba oil	65 °C, EtOH:oil molar ratio of 9.0:1, 20 wt% lipase, system assisted by ultrasound (132 W), 30 min of reaction	70.0	The enzyme could be reused without significant losses in its activity up to 5 cycles.
[99]	Novozym 435	Soybean oil	50 °C, EtOH:oil molar ratio of 3.0:1, 5 wt% lipase, 4 h of reaction	~ 60.0	A washing of the biocatalyst with butanol led to a lower decrease in FAEE yield after the first batch and allowed a removal of more than 85 % of glycerol from the enzyme.
[101]	Lipase from *B. cepacia* immobilized on heterofunctional magnetic nanoparticles	Soybean oil	40 °C, MeOH:oil molar ratio of 4.0:1, 2.5 wt% lipase, 7 wt water, 50 wt% isooctane, 12 h of reaction	96.8	This work reports a novel heterofunctional carrier to avoid enzymatic activity loss and improve the operational stability of the biocatalyst.
[102]	Lipozyme TL IM and Novozym 435	Used frying oil	Hydrolysis: 40 °C, water:oil molar ratio of 20:1, 132 W (ultrasound), 10 wt% Lip. TL IM, 2 h. Esterification: 65 °C, EtOH:FFA molar ratio of 6.0:1, 132 W, 1 wt% NZ435, 9 min.	79.0	This paper reports an interesting strategy for continuous ethyl esters production through a two-step process where in a first step the substrate was hydrolyzed and after esterified using immobilized lipases to produce FAEE.
[95]	Eversa® Transform 2.0 immobilized on polymeric resin	Sunflower oil	40 °C, MeOH:oil molar ratio of 4.0:1, 10 wt% lipase, hexane as solvent, 3 h.	99.0	After 4 cycles, the biocatalyst kept 75 % of its enzymatic activity.
[103]	Lipase from *C. rugosa* immobilized on nanoparticles of Fe_3O_4	Refined soybean oil	40 °C, MeOH:oil molar ratio of 4.0:1, 25 wt% lipase, 60 h of reaction	92.8	Due to its magnetic characteristics, the immobilized lipase could be easily recovered using an external magnetic field, allowing the biocatalyst was reused five times without significant loss of activity.

(conclusion)

Ref.	Enzyme	Substrate	Reaction Conditions	Yield (%)	Remarks
[104]	Lipase from *T. lanuginosus* immobilized on Immobead 150	Blend of non-edible oils	36 °C, MeOH:oil molar ratio of 7.64:1, 3.55 wt% lipase, 10 wt% water, system assisted by ultrasound (35 W), 2 h	94.0	Good retention of activity by immobilized lipase after 6 consecutive reuse batches was reported.
[105]	Novozym 435	Waste fish oil	35 °C, 1.82 g of EtOH excess per gram of oil, 50 wt% lipase, 8 h of reaction	74.6	The biocatalyst could be reutilized by 10 cycles with a loss of enzymatic activity of 16 %.

2.5.3 Enzymatic Hydroesterification: Use of Soluble Lipases

Most of the researches focusing on enzymatic catalysis employ lipases immobilized on polymer supports as catalyst. However, the process of immobilization itself is not simple either cheap [98]. Consequently, recent researches have turned its attention to the use of lipases in liquid/soluble/free formulations for the biodiesel synthesis. Such formulations consist basically of a solution composed by water, glycerol, surfactants, and enzyme (approximately 3.5 wt%). Soluble lipases having a lower production and sales costs and higher specific activities compared with immobilized lipases [106]. Remonatto et al. [33] reports that the use of liquid lipases on biodiesel production can make the whole process more sustainable, cost effective, and competitive, once these commercial enzymes can be produced and sold to prices up to 50 times lower than the immobilized biocatalysts. Furthermore, it is important to cite that lipases are spontaneously soluble in aqueous solutions as a result of their globular protein nature [72].

Pedersen et al. [107] describe that the use of lipases in liquid formulation modifies the reaction system in diverse forms. The main change is the introduction of water into the system, which promotes the hydrolysis of the substrate and entails that there will always be a dispersed aqueous phase present in the reaction medium, once the lipase cannot be dissolved in the oil phase [91]. The presence of water in the system, leading to the formation of this second liquid phase, create an interface that is known to activate many lipases and also benefits the system diluting the alcohol present in the reaction, moderating its inhibitory effect on the lipase [107, 108]. Nevertheless, Nordblad et al. [90] describes that the catalytic activity of the soluble lipases is distributed restrictedly to the interface, meaning that the reaction rate can easily be limited by the available interfacial area, being fundamental an appropriate agitation to maximize the enzyme activity. Also, the hydrolysis of the substrate, forming FFA, implies that the final product will contain some acidity content that needs to be reduced after the reaction conclusion.

It is usual to denominate the soluble lipases-mediated biodiesel synthesis as a hydroesterification [28, 109, 110], since during the process occurs the hydrolysis of the raw material and the esterification of the FFA generated in the hydrolysis in FAAE. It should be noted that the use of an enzymatic hydroesterification process is not exclusive to lipases in liquid formulation. Zenevicz et al. [102] report a strategy for enzymatic fatty acid ethyl esters (FAEE) production through hydroesterification assisted by ultrasound using waste cooking oils, including two steps: enzymatic hydrolysis in batch mode employing the immobilized lipase Lipozyme TL IM, followed by esterification in continuous mode catalyzed by NZ435. A conversion of 79 % (in the esterification stage) was obtained after 9 Min. Despite the interesting

results, it cannot be ignored the fact that a high lipase load was applied by the authors: 10 wt% of Lipozyme TL IM and 1 wt% of NZ435. In addition, the energy demand required by the ultrasound would imply in a considerably onerous process.

An interesting point to note is that as is possible for immobilized enzymes, soluble lipases also enable their reuse after recovery from the reaction medium by simple decantation [87, 107, 111]. The enzyme being an amphiphilic molecule acting on the interface water/oil, remains concentrated, after separation by gravity, in an emulsion between a superior layer (rich in FAAE) and a clear inferior layer (rich in glycerol and water). Nielsen et al. [112] relate that more than 90 % of the enzyme activity is located in this emulsion phase. However, considering the fact that the lipase recuperation may be problematic depending on the raw material utilized in the process and considering that recent advances reduced the price of the enzyme at a level where a unique use of the biocatalyst is feasible, the lipase reutilization becomes unnecessary [28, 112].

Unconventional approaches have been considered in order to enhance the catalytic capacity of soluble lipases. In an interesting work, Fraga et al. [113] investigated the effects of a magnetic field on the enzymatic activity and structural conformation of the liquid lipase Eversa Transform 2.0®, aiming improve its stability and activity. Under a pH of 7.0, a magnetic field intensity of 1.34 T and 4 h of recirculation, the authors were able to obtain an enzyme activity 77 % higher than the control sample. Since the utilization of magnetic flux is simple, of low cost, and environmentally friendly, the application of this technique showed to be a favorable technique to increase the lipase activity for employing in a biodiesel production. In this context, several studies developed in recent years have proposed to investigate the influence of the different reaction parameters on the FAAE yield. Table 2 presents a general overview of researches that has been published to date about soluble lipase-catalyzed biodiesel synthesis.

The results presented in Table 2 show that the use of soluble lipases as biocatalysts of reactions for biodiesel synthesis is novel and promising. However, the FFA content present in the biofuel produced above the limit imposed by regulatory standards turns out to be a drawback of the process. High levels of FFA contained in the biodiesel can cause depositions within of engines, affecting the equipment performance, which encourage researchers to find an efficient and ecologically friendly alternative in order to circumvent the situation.

Table 2. Review of liquid/soluble enzyme-mediated production of FAAE (biodiesel) from different feedstock and lipases.

(continued)

Ref.	Enzyme	Substrate	Reaction Conditions	Yield (%)	Remarks
[114]	NS81020	Oleic acid	40 °C, MeOH:acid molar ratio of 2:1, 68 U/g of lipase, 10 wt% water, 8 h	93.0	Esterification rate improved with the increasing of lipase load as long as the maximum enzyme absorptive capacity on the reaction interface was not reached.
[115]	NS81006	Soybean oil	40 °C, 16 wt% MeOH, 1.5 wt% lipase, 10 wt% water, 7 h	90.0	Reaction mechanism model was hypothesized and the parameters were simulated by software and validated further.
[116]	Liq. lipase from *R. miehei* (RML) and *P. cyclopium* (MDL)	Soybean oil	30 °C, MeOH:oil molar ratio of 4:1, 188 U/g of RML lipase, 88.2 U/g of MDL lipase, 34 wt% water, 12 h	95.1	Using RML alone, the FAME yield was 68.5 %. When RML was assisted by MDL lipase, the FAME yield increased to > 95 % under the same reaction conditions.
[117]	NS81006	Soybean oil	45 °C, EtOH:oil molar ratio of 5.5:1, 1.5 wt% lipase, 20 wt% water, 16 h	> 90.0	Investigation of reaction parameters and lipase reuse in a soybean oil ethanolysis for FAEE production. No losses in lipase activity was observed after 5 reuses.
[98]	Callera™ Trans L	Crude soybean oil	35 °C, 16 wt% MeOH, 1 wt% lipase, 3 wt% water, 24 h	96.3	A good reduction of the FFA present in the feedstock could be achieved with a low water content added to the system.
[118]	NS81006	Oleic acid	45 °C, EtOH:FA molar ratio of 4.5:1, 4.95 U of lipase, 10 wt% water, 6 h	92.0	Liquid lipase kept its enzymatic activity after 5 cycles of reutilization.
[107]	Callera™ Trans L	Rapeseed oil	35 °C, EtOH:oil molar ratio of 4.5:1, 2 wt% lipase, 20 wt% water, 24 h	98.0	Addition of glycerol to the initial reaction mixture decreased the initial reaction rate. On the other hand, improved the final biodiesel yield suppressing the hydrolysis reaction.
[119]	NS81006	Soybean oil	55 °C, MeOH:oil molar ratio of 4.4:1, 1.5 wt% lipase, 10 wt% water, 8 h	~ 90.0	Both the catalytic performance and reuse stability of the lipase were inhibited with more than 5 wt% phospholipids contained in the oil feedstock
[90]	Callera™ Trans L	Rapeseed oil with 5 wt% FFA	35 °C, MeOH:oil molar ratio of 4.5:1, 0.5 wt% lipase, 5 wt% water, 24 h	95.4	The lipase was highly active in consuming the TAG of the substrate, and less for di- and monoglycerides.

(continued)

Ref.	Enzyme	Substrate	Reaction Conditions	Yield (%)	Remarks
[120]	Callera™ Trans L	Crude soybean oil	35 °C, MeOH:oil molar ratio of 4.5:1, 1 wt% lipase, 3.5 wt% water, 10 ppm NaOH, 24 h	> 95.0	Combination of a phospholipase with the soluble lipase allowed to reduce the phosphorus content of a high phospholipid substrate to less than 5 ppm.
[121]	NS81006	Crude soybean oil	55 °C, MeOH:oil molar ratio of 4.4:1, 1.5 wt% lipase, 10 wt% water, 8 h	95.1	Development of a two-step system that allowed to process a substrate with 10 wt% of phospholipids using only the soluble lipase, eliminating the inhibitory effect of the phosphorus on lipase.
[121]	NS81006	Crude soybean oil	55 °C, MeOH:oil molar ratio of 4.4:1, 1.5 wt% lipase, 10 wt% water, 8 h	95.1	Development of a two-step system that allowed to process a substrate with 10 wt% of phospholipids using only the soluble lipase, eliminating the inhibitory effect of the phosphorus on lipase.
[122]	Callera™ Trans L	Jatropha curcas oil	35 °C, MeOH:oil molar ratio of 1.5:1, 0.75 wt% lipase, 2 wt% water, 24 h	80.7	Biodiesel production using the liquid lipase presented higher yields compared to an immobilized enzyme from *Candida antarctica* also tested for the same raw material.
[123]	Free Lipase from *Candida* sp. 99-125	Waste cooking oil	40°C, MeOH:fatty acid molar ratio of 1:1, 1 wt% lipase, 2 wt% water, 30 h	> 90.0	Five different non-edible oils was tested. Conversion rate for oils with low acidity was improved by a high water content, even at low lipase load.
[124]	NS81006	Soybean oil	45 °C, 5.5 wt% MeOH, 1.5 wt% lipase, 10 wt% water, 1 h	-	Kinetic model based on *Ping Pong Bi Bi* mechanism were proposed for the alcoholysis process. It was found that the hydrolysis followed by esterification as well as the methanolysis occur simultaneously.
[125]	NS81006	Soybean oil with 10 wt% phopholipids	45 °C, MeOH:oil molar ratio of 4.4:1, 1.5 wt% lipase, 3 wt% water, 8 h	94.9	Application of a phospholipase acting together with the soluble lipase allowed to reduce the phosphorus content in the biodiesel to less than 4 ppm. Furthermore, both enzymes could be reused for 5 batches without significant losses of their activities.
[126]	NS81006	Microalgae lipid	45 °C, 20 vol% of MeOH, 3 wt% lipase, 6 h	78.0	Addition of a second stage (after the reaction using the soluble lipase) employing the lipase NZ435 increased the FAME yield to 97 %.

(continued)

Ref.	Enzyme	Substrate	Reaction Conditions	Yield (%)	Remarks
[30]	NS81006	Microalgae oil	45 °C, 16 wt% MeOH, 1.5 wt% lipase, 15 wt% phosphate buffer solution, 24 h	~ 60.0	Insertion of a reaction step (after the soluble lipase-catalyzed methanolysis), using the immobilized lipase NZ435, aiming to convert the polyunsaturated fatty acids of the feedstock, increased the FAME yield to 95 %.
[127]	NS81006	Microalgae oil	45 °C, 16 wt% MeOH, 3 wt% lipase, 10 wt% phosphate buffer solution, 21h	78.0	Combination of NZ435 immobilized enzyme in a subsequent reaction step allowed to increase the FAME yield from 78 % to 97 % and to reduce the AV from 25 to 0.86 mg $KOH \cdot g^{-1}$, in a total reaction time of 27 h.
[33]	Eversa® Transform	Soybean oil with 50 wt% FFA	35 °C, MeOH:oil molar ratio of 4.5:1, 1 wt% lipase, 2.5 wt% water, 16 h	97.3	Laboratory results served as starting point for a study aiming a scale-up production, where the results obtained were similar to those obtained in the lab.
[112]	Eversa® Transform	Refined soybean oil	35 °C, MeOH:oil molar ratio of 4.5:1, 0.2 wt% lipase, 3 wt% water, 24 h	95.0	Proposal of a two-step system consisting of a transesterification for FAME synthesis followed for a saponification aiming to reduce the biodiesel acidity.
[31]	Eversa® Transform	Rapeseed oil	35 °C, MeOH:oil molar ratio of 4.5:1, 1 wt% lipase, 3 wt% water, 24h	99.4	From the results obtained for the biodiesel production, a mathematical model of the process was designed.
[128]	Callera™ Trans L	Refined rapessed oil	35 °C, 1 wt% lipase, 10 wt% water, 77 W (ultrasound irradiation), 15 h	91.0	Reaction rate was enhanced up to 2-fold through the use of ultrasound irradiation compared with the process without any ultrasound treatment.
[106]	NS81006	Waste cooking oil	35 °C, MeOH:oil molar ratio of 4.2:1, 1.5 wt% lipase, 5 wt% water, 30 h	90.0	No significant differences in the FAME yield was observed varying the water content in the system from 3 to 10 wt%. However, the AV reduced considerably for lower water contents.
[109]	Eversa® Transform	Castor oil	35 °C, MeOH:oil molar ratio of 6:1, 5 wt% lipase, 3 wt% water, 8 h	94.2	A composition profile showed that the TAG of the substrate are almost consumed totally within the first two hours of reaction.
[129]	Liq. lipase from *C. rugosa* and *R. oryzae*	Rapessed oil deodorizer distillate	34 °C, 6.5 wt% of MeOH, *R. oryzae* ratio of 0.84, 46 wt% water, 6 h	98.2	Synergistic combination of two lipases in liquid formulation aiming enhance the biodiesel yield.

(conclusion)

Ref.	Enzyme	Substrate	Reaction Conditions	Yield (%)	Remarks
[130]	Eversa® Transform	Castor oil	35 °C, MeOH:oil molar ratio of 6:1, 5 wt% lipase, 5 wt% water, 8 h	-	Four different reaction models were compared to determine the mechanism that best fitted the experimental data. Results indicate that the methanolysis and hydrolysis reactions occurred simultaneously in the system.
[28]	Callera™ Trans L	Degummed soybean oil	35 °C, MeOH:oil molar ratio of 4.5:1, 1.45 wt% lipase, 6 wt% water, 24 h	96.9	Reaction parameters investigated presented different effects and influence on the FAME yield at distinct moments of the process.
[108]	Callera™ Trans L	Beef tallow	35 °C, MeOH:oil molar ratio of 4.5:1, 1.45 wt% lipase, 6 wt% water, 8 h	84.6	Use of the concept of "degree of unsaturation" of the raw material. Results showed that a decrease in the degree of unsaturation resulted in an increase on FAME yield.
[131]	NS40116	Abdominal chicken fat	30 °C, MeOH:oil molar ratio of 4.5:1, 0.3 wt% lipase, 2 wt% water, 24 h	77.0	Investigation of the influence of reaction parameters on the FAME yield obtained from a poultry industry waste.
[87]	Eversa® Transform	Beef tallow	35 °C, MeOH:oil molar ratio of 4.5:1, 1.0 wt% lipase, 6 wt% water, 8 h	85.1	Different ways for fed-batch MeOH and lipase to the reaction were examined: one-step addition of lipase and MeOH fed at a constant flow present the best results.
[29]	NS40116	Acid waste oil	35 °C, MeOH:acid molar ratio of 1.5:1, 4 wt% of lipase, 24 h	68.4	From an acid waste oil from soapstock of vegetable oil refining, was possible to reduce the initial acidity of the substrate by 80 % using the soluble lipase NS 40116.
[111]	NS40116	Degummed soybean oil	35 °C, MeOH:oil molar ratio of 4.5:1, 0.5 wt% lipase, 15 wt% water, 12 h	94.3	Investigation of water and lipase load as well as the enzyme reuse in a soybean oil hydroesterification for FAME production. The lipase kept 90 % of its catalytic activity after 5 cycles.
[132]	NS40116	Degummed soybean oil	35 °C, MeOH:oil molar ratio of 6.3:1, 0.7 wt% lipase, 8 wt% water, 8 h	97.1	Proposal of a two-step hydroesterification reaction system which allowed the reaction time to be reduced by two thirds of that usually is used in researches to achieve a similar FAME yield.

2.6 CHALLENGES OF THE BIODIESEL PRODUCTION CATALYZED BY SOLUBLE LIPASES

For being used in diesel engines, the biodiesel needs to meet a series of parameters and properties provided by regulatory standards. Many researches have been developed over the years aiming the purification of crude biodiesel using various techniques and materials such as wet washing (with deionizer water or with water and organic solvents), dry washing (by adsorption and ion-exchange process), separation by membranes, employing ionic liquids, silica gel, hydrogels, starch, cellulose, and so on [133–141]. Some examples of the biodiesel parameters, accordingly presented in Table 3, are the AV, responsible for determining the amount of FFA present in a determined biodiesel sample. A high AV can lead to the formation of precipitates which will cause the clogging of the fuel filter and the process of fuel degradation, as well as compromises the durability of the motor causing corrosion of metal parts and formation of deposits [142].

Table 3. Some specifications of biodiesel (B100) prior to use or blending with diesel fuel [143].

Property	**Limits**
Water and sediments	500 ppm
Acid value	0.05 mg $KOH \cdot g^{-1}$ (0.25 wt%)
Free glycerol	0.02 wt%
Total glycerol	0.24 wt%
Monoglycerides	0.70 wt%
Diglycerides	0.20 wt%
Triglycerides	0.08 wt%

In this context that the soluble lipase-catalyzed synthesis of biodiesel confront its greatest challenge: the development of the production of a biofuel with FFA content within the standards specified by regulatory norms. The use of a liquid formulation of lipase modify the system mainly by the introduction of water in the reaction that promotes the TAG hydrolysis generating FFA that are not completely esterified in biodiesel, needing to be removed later. Most of the published papers that used lipases in liquid formulation to catalyze the synthesis of FAAE report AV out of specification (usually higher than 4.0 mg $KOH \cdot g^{-1}$ or 2.0 wt% [33, 90, 98, 106, 109, 111] when no action is taken aiming to correct such parameter), making it

necessary a post-reaction stage of polishing/purification of the biofuel.

Recently, papers have been focused on the reduction of the biodiesel acidity produced by liquid lipase-mediated hydroesterification [144–147]. In one of these researches, Uliana et al. [144] proposed an alkaline wet washing using aqueous solution of sodium hydroxide/methanol as a technique for reducing the biodiesel acidity produced by enzymatic hydroesterification: under 35 °C, 1 wt% of methanol, molar ratio NaOH:FFA of 1:1 and 3 wt% of alkaline solution in relation to crude biodiesel, the authors reduced the FAME acidity from 3.94 to 0.19 wt% for a final product yield of 96 %. In a similar approach, Firdaus et al. [31] utilized a caustic washing to bring the biodiesel produced by soluble lipase-mediated hydroesterification to on-spec parameters: after the alkaline washing, the FFA content decreased from 2.0 to 0.04 wt%, with an increase in the biodiesel yield from 96.7 % to 99.4 %, where the residual MAG contained in the FAME sample could be transesterified, rising the process yield. Although the wet washing process is a simple and efficient procedure, the water content of the biofuel after the purification step is generally higher than the value predicted by the regulatory standards (0.05 wt%, see Table 3) and it is therefore necessary to introduce a posterior stage of drying under vacuum conditions [139]. Moreover, the alkaline wastewater generated in the procedure should be treated before the final discharge in order to reduce the environmental impact, causing one of the conventional problems faced for the biodiesel industry, where the alkaline homogeneous catalysis is used.

Uliana et al. [145] also reported in an another research results of a continuous-mode packed-bed column dry-washing for the polishing of biodiesel produced by enzyme-mediated process, using two ion-exchange resins (Lewatit SP112H and GF101), a commercial immobilized enzyme (NZ435), and a magnesium silicate adsorbent (Magnesol R600). According to the authors, an efficacy of the ion exchange resins and immobilized lipase on reducing the biodiesel acidity only was perceptible for a very low initial water content in the FAME samples (less than 0.03 wt% of water), where an AV around 0.8 wt% was possible to reach, value still higher than that provided by regulatory standards. However, the addition of Magnesol R600 showed to be efficient to remove FFA from the biodiesel charges, achieving values inferior that the upper standard limit (< 0.25 wt%).

Another alternative for acidity reduction of the biodiesel produced by liquid lipase catalysis was developed by Tian et al. [147]. The authors proposed a system combining the advantages of liquid and immobilized lipases in a two-stage process, with the introduction of dimethyl carbonate into the reaction. The reason of the addition of dimethyl carbonate into the system is for the online removal of the glycerol generated in the process, where the enzyme-

mediated reaction between these two composts produce glycerol carbonate, a good additive to fuels [148]. Using microalgae oil as substrate, in the first step the liquid lipase NS 81006 was used to catalyze the feedstock conversion in an oil/water system, where the glycerol generated in this stage was removed in order to avoid negative effects in the subsequent reaction, where an immobilized lipase was used. Thus, in a second step, the aforementioned authors utilized the immobilized lipase NZ435 for esterify the FFA generated in the first step (FAME acidity around 1.8 mg $KOH \cdot g^{-1}$) and also perform the conversion of the nonconverted TAG of the previous step, obtaining a FAME yield of 95 % after 36 h of reaction with a final AV of 0.5 mg $KOH \cdot g^{-1}$. However, a crucial point of this process is the need to reduce the water concentration (essentially < 500 ppm) in the NZ435-catalyzed reaction for a value that does not cause a thermodynamic interference in the acidity reduction of the FAME produced, making necessary the addition of 24 wt% of a 3 Å molecular sieve in the process, aiming to remove the water of the system.

As the use of soluble lipases in the biodiesel production is a quite new technology, still there are few published works where the focus is the biodiesel purification in relation to the FFA content present in the biofuel produced by this method. The high AV of the biodiesel produced by liquid lipase-catalyzed hydroesterification can be considered a bottleneck of the process, therefore, being a challenging point that will boost future researches to produce an on-spec biofuel.

2.7 CURRENT STATUS OF THE BIODIESEL CATALYZED BY SOLUBLE LIPASES

It is reported in distinct researches that evaluated costs involved in the production of biodiesel the substantial percentage relative to the use of certain raw materials on the final price of the biofuel produced [149, 150], regardless of the technological route chosen. Estimates put more than 350 different kinds of oil-bearing crops employed worldwide as sources for the biodiesel production [6], where around 95 % of the biodiesel originated from these substrates is derivative from edible oils [151], which have a higher aggregate value in comparison with nonedible oils or other waste materials. Logically, the utilization of low-priced feedstock tends to reduce production costs. For the enzymatic approach, the dilemma is no different. Always aiming at the search for the economic viability of the biodiesel synthesis catalyzed by soluble lipases in a scale higher than the pilot, it can be stated that the use of residual raw materials in the process plays a fundamental role in terms of general production costs and competitiveness of the enzymatic biodiesel within of an energy matrix [100]. There is a key point in the liquid

lipase-mediated hydroesterification for biodiesel production: the use of a feedstock in the reaction with low-cost, low-grade, high FFA, and high moisture content. At this point, as previously pointed out, that the enzymatic pathway excels in comparison with the alkaline technique industrially used to produce biodiesel, where the capacity to process residual raw materials may compensate the higher costs of the lipases (in relation to the chemical catalysts employed in the biodiesel facilities).

Although many papers about enzymatic biodiesel still focus on the use of lipases immobilized, it is perceptible that the cost of this biocatalyst per mass of biodiesel produced is so elevated that it hinders a possibility of production scaleup [55, 77, 152]. Budžaki et al. [153] published recently an extensive economic analysis composed of several scenarios for an enzymatic biodiesel production in a small-scaled packed-bed reactors, where the utilization of the lipase from *T. lanuginosus* immobilized on a polymeric support for production of 10,000 ton of biodiesel in a period of one year was considered. In the best scenario obtained in this research, 32 reactors in parallel would have to be used to achieve the desired annual production, in addition to the reutilization of the immobilized enzymes for 50 days to obtain the lowest cost of biodiesel production, which is very difficult considering the inhibitory caused by methanol on lipase activity.

Therefore, in view of the higher cost competitiveness of soluble lipase compared with immobilized form [33], the range of industrial applications for this kind of biocatalyst has stimulated the interest of the scientific community in expanding results obtained in laboratory scale for pilot scales. Nielsen and Rancke-Madsen [154] cite that the flexibility of soluble lipases in processing materials regardless of FFA or moisture content allows producers to use cheaper raw materials, reducing the overall cost of the biodiesel production process. Another interesting point is the possibility of combining the enzymatic process with the alkaline transesterification as a way of "feedstock pretreatment", bringing waste inputs with high acidity to parameters susceptible of processing in the chemical technique of biodiesel production [155], integrating the current biodiesel production facilities with the enzymatic technology.

Mibielli et al. [156] performed a study using the free lipase Eversa® Transform 2.0 (a liquid formulation of a modified lipase from *T. lanuginosus* microorganism) to process a residual raw material with high acid (around 91% FFA) for FAME production in a 1 m^3 pilot plant. After 15 h of reaction, the authors obtained a FAME yield of 96.6 % with a final acidity of 4.2 wt%, using a raw material with fixed cost 50 % lower than that conventionally used in the alkaline biodiesel production process on industrial scale, result that could contribute in a reduction of 30 % in the total cost of the biodiesel production process [155].

Price et al. [157] proposed a prediction model of performance of a 40 m³ enzymatic biodiesel production plant through data collected from a 4 m³ pilot plant employing used cooking oil (15 wt% FFA) and soluble lipase NS 40116 (obtained from *T. lanuginosus* microorganism also). The authors conclude that assuming a biodiesel selling price of US$ 0.6·kg^{-1} and one-time use of the enzyme (0.1 wt%); the lipase can be sold for US$ 30·kg^{-1}, which ensure that the biocatalyst cost is not more than 5 % of the biodiesel revenue. Wancura et al. [28] published a preliminary cost analysis carried out for an enzymatic biodiesel production facility (São Paulo State, Brazil), which operates with a 90 ton fed-batch reactor using the soluble lipase Callera^{TM} Trans L: today, the cost associated with the lipase is around US$ 12.0·kg^{-1} (about 10% of the total cost of the biodiesel production process, approximately US$ 0.18·L^{-1} of biodiesel), somewhat higher in comparison with the alkaline catalyst used industrially that has a total cost of around US$ 0.10·L^{-1} of biodiesel. However, the authors highlight that the economic gain of the bioprocess is on the final margin of the biodiesel because the alkaline route requires a feedstock with a maximum FFA content of 0.2 wt% (cost of approximately US$ 0.7·kg^{-1}) and with the enzymatic process usually using waste materials as feedstocks (FFA content generally between 30 and 97 wt%), the cost with raw materials drop to an average of US$ 0.3·kg^{-1}.

In a similar research, Andrade et al. [158] performed an extensive economic analysis comparing operating costs of an enzymatic biodiesel production facility, with capacity of 250,000 tons·year^{-1}, catalyzed by lipases in liquid (Eversa® Transform) and immobilized (NZ435) form. According to the authors, considering the process economic assessment, the system employing the liquid lipase resulted in a production cost of US$ 0.78·kg^{-1} of biodiesel, corresponding to a profit of almost US$ 52 million·year^{-1}, for the annual biodiesel production considered. In relation to the immobilized enzyme, due to the high price of the biocatalyst (according to the manufacturer around US$ 1,000·kg^{-1}), the authors concluded that the lipase NZ435 should be reutilized at least 300 times to achieve a profit similar to the soluble lipase-catalyzed process, something totally unlikely due to loss of the catalytic capacity of the lipase after each recovery [23, 66, 99].

In a simplified form, Table 4 presents a SWOT (*Strengths, Weaknesses, Opportunities,* and *Threats*) analysis, summarizing the main points addressed so far, which are fundamental for the prosperity of the technique of enzymatic biodiesel production catalyzed by lipases in liquid formulation.

Table 4. SWOT analysis for the soluble lipases-catalyzed biodiesel production.

S	Strengths	W	Weaknesses
- Possibility of processing feedstock with high levels of impurities (waste raw materials);		- FAAE produced usually present AV out of specs;	
- "Environmentally greener" route compared to the technique industrially used to produce biodiesel;		- Reaction rates are still considerably lower compared to the conventionally technique employed to synthesizing biodiesel industrially;	
- Lower cost and higher catalytic activities compared to immobilized lipases.		- Lipase denaturation caused by the alcohol;	
		- Higher costs of the biocatalyst compared to alkaline ones used on industrial biodiesel production.	
O	**Opportunities**	**T**	**Threats**
- World biodiesel production grows annually, arousing the interest of researchers to investigate alternative techniques for biofuel production;		- Government policies to encourage the use of biodiesel must to continue to evolve;	
- A considerable evolution has been observed in recent years in the development of soluble lipases.		- An effective and environmentally friendly technique for reducing the biodiesel AV needs to be developed in order to the technique continue to thrive.	

Currently, more than 10 industrial plants are using enzymatic technologies to produce biodiesel from different quality raw materials [159]. In 2008, Novozymes (Denmark), the main global supplier of lipases for application on biodiesel synthesis, in partnership with Piedmont Biofuels (North Carolina, USA) developed a large-scale soluble lipase-catalyzed production of biodiesel, which resulted in a patent application in 2012 (Patent WO2012/098114, 2012) [160]. Also in the North America, using waste cooking oil and brown grease in a continuous stirred tank reactor system, allowing a low-cost enzymatic biodiesel synthesis, Viesel Biofuel (Florida, USA) has been one of the pioneer companies in the implantation of lipases in liquid formulation for the production of enzymatic biodiesel in large scale. High Plains BioEnergy (Missouri, USA) is another company that uses enzymatic route to produce 28 million gallons of biodiesel annually. Both industrial biodiesel companies make use of Eversa Transform 2.0 from Novozymes D/K. The industrial production of enzymatic biodiesel is not exclusive for lipases in liquid form. On Asia, two Chinese companies produce biodiesel employing immobilized enzymes as catalysis [55]: Hunan Rivers Bioengineering Co. (Hunan, China) has a project capacity of 50,000 ton·year^{-1} using stirred tank reactors and waste oils as raw material; and Lvming and Environmental Protection Technology Co. (Shanghai, China) with a production of

10,000 ton·year^{-1}, uses waste cooking oil as feedstock. Obviously, the reutilization of the biocatalyst for several times is fundamental for the financial health of the companies.

2.8 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The search for a fuel able of replacing society's dependence in relation to fossil fuels and, at the same time, contributing to the reduction of emissions that impacts to the environmental pollution has consolidated the biodiesel as an alternative ecologically friendly energy source for the community. Industrially, biodiesel production globally advances at each year, according to its interconnected demand to governmental legislation that predict gradual increases in the blend of biofuel into the diesel. The route of biodiesel synthesis, in industrial scale, based on a homogeneous alkaline transesterification has wide acceptance. Nevertheless, drawbacks deriving of the waste generation from process bring up questions about how to make the process more sustainable. Another questionable point in the alkaline technique is the necessity to use a feedstock with a purity of edible degree, which gives rise to the discussion between groups that question if it would be correct to employ raw materials that could be destined to food industry in order to generate energy. Therefore, how to make the biodiesel production process be unanimously acceptable for the society? In view of the several studies published in recent years and presented in the previous sections of this review, the enzymatic route for the synthesis of the biofuel is the most promising considering how it overcomes the problems faced by the alkaline method. Although were the primary focus of these works, the use of immobilized lipases, due mainly to its high cost, makes the process feasibility highly dependent of the achievement of numerous reuses of the biocatalyst, which would make the process, on an industrial scale, economical and operably instable considering the agents that may cause the lipase inactivation. For these reasons, the application of lipases in liquid/soluble formulation, with production costs smaller than the immobilized one, becomes a real alternative to produce enzymatic biodiesel on large scale. For this, two points are fundamental:

1. Utilization in the process of waste and low-cost raw materials, with high impurity content (moisture, FFA, phospholipids, etc.). Such substrates are unviable to be processed via homogeneous alkaline route. On the other hand, by using such materials, full advantages are taken of the catalytic potential of lipases in working with this type of feedstock and, at the same time, costs of production associated to inputs are minimized;

2. Development of an efficient and environmentally attractive technique capable of

reducing the acidity of the biodiesel produced by hydroesterification mediated by liquid lipases. To be used in diesel engines, biodiesel needs to meet specifications defined by regulatory standards, and the high AV reported in researches about the subject is a problem. In this sense, it is assumed that future works should give higher attention to the investigation of an alternative process apt of overcoming this drawback.

The rapid advance in the researches using commercial lipases in liquid formulations on the biodiesel synthesis in terms of evolution of experiments in laboratory scale for productions in pilot units exemplifies that the use of these biocatalysts is promising. More examples in relation to this are due to the fact that already there are industrial plants producing biodiesel by this pathway. Associated with this, biocatalyst suppliers have always been focused on the production of more robust and cost-effective enzymes with high activity, thermostability, and resistance to the adverse conditions that the process required. All these factors can contribute to, in the future, the soluble lipase-mediated biodiesel production to become one of the main techniques for industrial synthesis of the biofuel.

2.9 ACKNOWLEDGMENTS

The authors would like to thank the CNPq (National Council of Technological and Scientific Development, 428180/2018-3) and FAPERGS (Research Support Foundation of the State of Rio Grande do Sul, 16/2551-0000522-2) for the financial support of this work, as well as CAPES (Coordination for the Improvement of Higher Education Personnel) for scholarships.

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3 ESTRATÉGIAS DE ALIMENTAÇÃO DE METANOL E LIPASE NA HIDROESTERIFICAÇÃO MEDIADA PELA LIPASE EVERSA® TRANSFORM PARA PRODUÇÃO DE FAME

Neste capítulo estão apresentados os resultados relacionados ao cumprimento do primeiro objetivo específico desta tese, que foi de avaliar como o rendimento do processo responde a diferentes estratégias de alimentação de insumos (álcool e lipase) ao meio reacional.

Feeding Strategies of Methanol and Lipase on Eversa® Transform-Mediated Hydroesterification for Fame Production

DOI: https://doi.org/10.1002/cjce.23404

João H. C. Wancura[a], Daniela V. Rosset[a], Gustavo A. Ugalde[b], J. Vladimir Oliveira[c], Marcio A. Mazutti[a], Marcus V. Tres[d], Sérgio L. Jahn[a]

[a] Department of Chemical Engineering, Federal University of Santa Maria, 1000, Roraima Avenue, Santa Maria, 97105-900, Brazil;

[b] Department of Crop Protection, Federal University of Santa Maria, 1000, Roraima Avenue, Santa Maria, 97105-900, Brazil;

[c] Department of Chemical and Food Engineering, Federal University of Santa Catarina, Florianópolis, SC, 88040-900, Brazil;

[d] Laboratory of Agroindustrial Processes Engineering (LAPE), Federal University of Santa Maria, 1345, Ernesto Barros Street, Cachoeira do Sul, 96506-322, Brazil.

ABSTRACT

This work reports the influence of different feeding strategies of methanol and catalysts on fatty acid methyl esters (FAME) yield in a beef tallow hydroesterification reaction for biodiesel production catalyzed by liquid lipase. Soluble lipase from the *Thermomyces lanuginosus* microorganism, named Eversa® Transform, was selected for the reactions in this work. The feeding of methanol and lipase into the system was evaluated varying the methanol to fat molar ratio of 4.0:1 and 4.5:1 and the lipase load of 1.0 wt% and 1.45 wt% in relation to the mass of beef tallow utilized in each assay. The highest yield (85.08 %) of FAME was reached at 35 °C, methanol to fat molar ratio of 4.5:1, 1.0 wt% of lipase, 6.0 wt% of water in only 8 h of reaction, with a one-step addition of lipase and methanol fed to the process at a constant flow of 3.0 $g \cdot h^{-1}$. Moreover, it was evaluated the reutilization process of the liquid

lipase after four cycles, which is a point rarely addressed in similar works available in the open literature. The findings of this paper demonstrate that the adoption of an appropriate strategy to feed inputs to the system is fundamental in order to take advantage of the maximum catalytic capacity of the lipase and consequently to obtain high yields in the process.

Keywords: FAME; Eversa® Transform; Liquid lipase; Methanol; Tallow.

3.1 INTRODUCTION

The use of lipases as biocatalyst for biodiesel (fatty acid methyl esters (FAME)) production from the enzymatic transesterification of oleaginous sources proved to be an interesting alternative to alkaline methanolysis, which is the method most often used on an industrial scale.[1] Mild reactive conditions, ability to process high-acid, low grade, low-cost feedstock, production of a high purity biofuel, and high selectivity of the lipases, which prevents the occurrence of secondary reactions, are some factors that have aroused the interest of researchers about the enzymatic route.[2] These characteristics enable the biotechnological process to act in a way to circumvent (and even eliminate) some of the inconveniences of the chemical technique, such as production of alkaline wastewater generated in biodiesel purification stages, high energy demand, and the need to process a high quality raw material to avoid saponification reactions.[3,4]

However, the lipase inactivation in the presence of methanol, the main acyl acceptor used in the reaction, remains one of the main drawbacks of the process.[5] Transesterification reaction requires a stoichiometric molar ratio between methanol and triglycerides of 3:1 and considering that the reaction has a reversible character, an excess of alcohol is necessary to shift the equilibrium toward the formation of FAME. This increase in alcohol concentration is attributed to the main agent that causes the enzyme denaturation, where the low solubility of methanol in the oil source forms insoluble drops of alcohol on system that, in contact with the lipase, causes its inactivation.[6] Some strategies have been adopted in an attempt to overcome this inconvenience such as the use of other alcohols (ethanol, propanol, and n-butanol) and solvents (hexane, t-butanol) or the stepwise addition of methanol to the reaction.[7,8]

Recently, mutagenesis approaches have been applied to improve the lipases resistance to presence of methanol.[5] Nevertheless, since methanol is the alcohol that presents the highest reactivity for use on reaction, and since the utilization of solvents is a cost-additive to the process and the mutagenesis still needs to consolidate its effectiveness, the stepwise dosage of

methanol to the process is the most appropriate tool to minimize the problem of lipase denaturation.[8]

Another drawback associated with the enzymatic process for FAME synthesis is the high cost of the lipases.[9] Most of the published works about this subject use immobilized enzymes in a carrier for enable its reuse.[10–12] Nonetheless, the most appropriate supports for this purpose are costly.[13] In this sense, the use of enzymes in liquid formulation, such as Eversa® Transform lipase, arises as an interesting option for production of enzymatic biodiesel. However, to date, only a few papers address this issue. The use of a liquid formulation of lipase instead of an immobilized one entails changes on reaction medium through the introduction of water in the process. The presence of water implies that, firstly, the triglycerides are hydrolyzed by three steps to generate free fatty acids (FFA) and posteriorly these FFA are esterified into FAME.[14] Moreover, water promotes alcohol dilution in the system, moderating its denaturing effect on the enzyme and leading to the formation of a second liquid phase in the reaction, creating an interface that is known to activate many lipases.[15]

A point that has been explored in research that uses the enzymatic route for biodiesel production is the use of waste feedstock in the process, such as animal tallow.[16] The non-sensitivity of the enzymatic technique in processing these materials, most of the time with a high moisture and acidity content, is another favourable point that encourages the utilization of this kind of oleaginous source in the biotechnological route.[14] In countries like Brazil, it is estimated that 40 million of cattle are slaughtered annually, generating approximately 800 million kg of beef tallow, half of this amount bring destined for the production of biodiesel, which makes this material the second main feedstock utilized for synthetize biofuel in this country.[17,18]

Considering all of the aforementioned factors, feeding strategies of inputs (methanol and lipase) on enzymatic biodiesel production are a key point for process development, in order to potentiate the catalytic capacity of the lipase, minimizing the inactivation effects caused by methanol. So far, only a few studies have been reported in the literature concerning the influence and efficiency of the gradual dosage of methanol and enzyme on a hydroesterification reaction for biodiesel production catalyzed by a soluble lipase. In addition, few works, to date, have evaluated the process yield after recovery and reuse of the Eversa® Transform lipase in the reaction. Therefore, the objective of this work is to evaluate the efficiency of the stepwise dosage of inputs and, consequently, the influence of FAME yield on a hydroesterification reaction catalyzed by the liquid lipase Eversa® Transform using beef tallow as a raw material.

Additionally, the enzyme recovery was also evaluated with the objective of investigate the catalytic activity of the lipase after reuse in the process.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Olfar Alimento e Energia S/A (Erechim, Brazil) kindly supplied the deacidified beef tallow (BT) utilized in the assays. The tallow contained 3.2 mg/g (0.32 wt%) of free fatty acids, 8 mg/g (0.8 wt%) phosphorus, 95 $mg \cdot L^{-1}$ of soaps, and 0.9 mg/g (0.09 wt%) water, with the following fatty acids composition: 0.13 g/g (13.0 wt%) palmitic acid; 0.043 g/g (4.3 wt%) palmitoleic acid; 0.054 mg/g (5.4 wt%) stearic acid; 0.0354 g/g (35.4 wt%) oleic acid; 0.0388 g/g (38.8 wt%) linoleic acid; and 0.031 g/g (3.1 wt%) linolenic acid. The enzyme employed in the reactions was the liquid lipase named Eversa® Transform, obtained from the *Thermomyces lanuginosus* (TLL) microorganism and was provided by Novozymes (Denmark), with an enzymatic activity of approximately 100 $kLU.g^{-1}$. Methanol (analytically pure), isooctane, and methyl tricosanoate (chromatographically pure) were purchased from Sigma-Aldrich (Germany). The pump utilized to feed reagent to the system was an infusion pump purchased from Samtronic Indústria e Comércio Ltda (São Paulo, Brazil), model ST670.

3.2.2 Hydroesterification Reaction

The reaction system used to perform the experiments consisted of a 250 mL 3-way round bottom flask equipped with a reflux condenser, water bath, temperature controller, and magnetic stirrer. In all experiments, 100 g of BT, a reaction temperature of 35 °C, an amount of water added to the reaction system of 0.06 g/g (6.0 wt% relative to the mass of tallow), and a stirring speed of 300 rpm was utilized. The molar ratios of methanol to BT used in the experiments were 4.0:1 and 4.5:1 and the lipase load of 0.01 g/g (1.0 wt%), and 0.0145 g/g (1.45 wt% relative to the mass of tallow used). It should be noted that the molar excess of methanol of 4.5:1 and lipase load of 1.45 wt% were values previously optimized in a preliminary work of this research group.[19] The suggested values utilized in the tests, lower than the optimized ones (4.0:1 for the molar excess of methanol and 1.0 wt% of lipase by weight of raw material), were adopted since it is intended to show that not only the stepwise addition of methanol but also the lipase will allow the use of smaller amounts of these inputs in the

hydroesterification reaction. All experiments were finished after 8 h of reaction.

Feeding strategies were developed to investigate the influence of input dosage on FAME yield. For this five combinations of methanol and lipase addition at reaction were used, as shown in Table 1. The dosages ranged from the situation in which the entire amount of methanol and enzyme were added in a single step at the beginning of the reaction (strategy 1) to the situation in which the addition of methanol was divided in five fractions (strategy 5) and the lipase in three fractions (strategies 3, 4, and 5). After considering the response obtained in feeding strategy 5 of the Table 1, further variations of methanol and lipase dosages were performed originating new experiments, as presented in Table 2.

The experimental procedure consisted of preheating the BT until the reaction temperature and then connecting the reactor to the condensation and temperature control system. Subsequently, the amounts of methanol and lipase (plus water) were added gradually to the process, as presented in Tables 1 and 2.

Samples were taken from the reaction and centrifuged at 4 000 rpm for 5 min at a temperature of 15 °C for phases separation, collecting only the upper layer (FAME) for GC analysis. The calculation of FAME yield obtained in the assays was determined through the methodology proposed by EN14103.[20]

3.2.3 Acid Value (AV) for FAME Samples

The acid value for FAME samples was determined using Equation (1): $AV = V \cdot c \cdot (56.1/m)$, expressed in milligrams of KOH required to neutralize the acidity of one gram of substrate. In the Equation (1) *AV* is the acid value of the FAME sample, *V* is the volume of KOH solution utilized in the titration (mL), *c* is the concentration of the KOH solution ($mol \cdot L^{-1}$), *m* is the weight of the FAME sample (g) and *56.1* is the molecular weight of KOH. Following the methodology proposed by EN14104 [21], 0.01 g of FAME was dissolved in 10 mL of anhydrous ethyl alcohol and titrated with potassium hydroxide solution of 0.01 N in the presence of phenolphthalein as indicator (0.01g/g or 1 wt%).

3.2.4 Reuse Assays of the Lipase

In order to investigate the soluble lipase reutilization in the process, it was carried out with new assays under the best strategy of input feeding (lipase and methanol) that was previously obtained. After the end of the test, the reaction medium was placed in a separator

Table 1. Methanol and lipase feeding strategies used in the assays.

	Feeding strategies	t = 0.0 h of reaction		t = 0.5 h of reaction		t = 1.0 h of reaction		t = 2.0 h of reaction		t = 3.0 h of reaction		t = 4.0 h of reaction	
		MeOH (wt%)	**Lipase (wt%)**	**MeOH (wt%)**	**Lipase (wt%)**	**MeOH (wt%)**	**Lipase (wt%)**	**MeOH (wt%)**	**Lipase (wt%)**	**MeOH (wt%)**	**Lipase (wt%)**	**MeOH (wt%)**	**Lipase (wt%)**
Mass percentage dosed of each input	1	100.0	100.0	-	-	-	-	-	-	-	-	-	-
	2	50.0	50.0	-	50.0	50.0	-	-	-	-	-	-	-
	3	50.0	33.3	-	33.3	30.0	33.4	20	-	-	-	-	-
	4	40.0	33.3	-	33.3	25.0	33.4	20.0	-	15.0	-	-	-
	5	40.0	33.3	-	33.3	20.0	33.4	15.0	-	15.0	-	10.0	-

Table 2. Variations of the input feeding strategy 5.

Assay	Dosage of lipase	Dosage of methanol (wt%)				
		t = 0.0 h	t = 1.0 h	t = 2.0 h	t = 3.0 h	t = 4.0 h
A	1/3 at 0.0 h, 0.5 h and 1.0 h of reaction	40.0	20.0	15.0	15.0	10.0
B		20.0	20.0	20.0	20.0	20.0
C		10.0	15.0	15.0	20.0	40.0
D	1/1 at 0.0 h of reaction	40.0	20.0	15.0	15.0	10.0
E		20.0	20.0	20.0	20.0	20.0
F		10.0	15.0	15.0	20.0	40.0
G	1/3 at 0.0 h, 0.5 h and 1.0 h of reaction	20.0	35.0 (4.6 $g \cdot h^{-1}$)	20.0 (3.6 $g \cdot h^{-1}$)	15.0 (2.6 $g \cdot h^{-1}$)	10.0 (1.6 $g \cdot h^{-1}$)
H		20.0	20.0 (3.0 $g \cdot h^{-1}$)	20.0 (3.0 $g \cdot h^{-1}$)	20.0 (3.0 $g \cdot h^{-1}$)	20.0 (3.0 $g \cdot h^{-1}$)
I		20.0	10.0 (1.6 $g \cdot h^{-1}$)	15.0 (2.6 $g \cdot h^{-1}$)	20.0 (3.6 $g \cdot h^{-1}$)	35.0 (4.6 $g \cdot h^{-1}$)
J	1/1 at 0.0 h of reaction	20.0	35.0 (4.6 $g \cdot h^{-1}$)	20.0 (3.6 $g \cdot h^{-1}$)	15.0 (2.6 $g \cdot h^{-1}$)	10.0 (1.6 $g \cdot h^{-1}$)
L		20.0	20.0 (3.0 $g \cdot h^{-1}$)	20.0 (3.0 $g \cdot h^{-1}$)	20.0 (3.0 $g \cdot h^{-1}$)	20.0 (3.0 $g \cdot h^{-1}$)
M		20.0	10.0 (1.6 $g \cdot h^{-1}$)	15.0 (2.6 $g \cdot h^{-1}$)	20.0 (3.6 $g \cdot h^{-1}$)	35.0 (4.6 $g \cdot h^{-1}$)
N		20.0	40.0 (6.3 $g \cdot h^{-1}$)	40.0 (6.3 $g \cdot h^{-1}$)	-	-
O		20.0	26.5 (4.1 $g \cdot h^{-1}$)	26.5 (4.1 $g \cdot h^{-1}$)	27.0 (4.1 $g \cdot h^{-1}$)	-

[1] All experiments were performed at 35°C, 0.06 g/g (6 wt%) of water added to the reaction, methanol to BT molar ratio of 4.5:1 and 0.01 g/g (1.0 wt%) of lipase.

[2] Dosage of methanol of the experiments A to F was performed in batch

[3] Dosage of methanol of the experiments G to O was performed using a pump, being the values shown in the table the percentage of MeOH added until the corresponding reaction time with the respective flow rate.

[4] Dosage of lipase was performed manually for all experiments without exception.

funnel for phases separation, where a three-layer system was formed: upper (rich in FAME); intermediate (rich in lipase); and lower layer (rich in glycerin, methanol, and water). In the second stage of settling, the lower and intermediate phases were destined to the centrifugation process, 4,000 rpm for 8 min at 15 °C, for lipase recovery. The Eversa® Transform lipase recovered was directly reutilized (without any kind of treatment) on a new batch where fresh reactants were added to the reaction. It was possible to evaluate the catalytic capacity of the lipase after 4 cycles of reutilization with this procedure. After each reaction it was determined the FAME yield for the cycle.

3.2.5 GC Analysis

Gas chromatography for FAME quantification was performed using a GC-2010 Plus gas chromatography (Shimadzu, Japan), equipped with an auto injector AOC-20i, an auto sampler AOC-20s, and a flame ionization detector. For the compound separations, a Zebron ZB-WAX plus (United States) with 30.0m x 0.32mm I.D. x 0.25 μm film thickness capillary column was used. Helium 5.0 (99.999 % of purity) with a column flow rate of 1.24 $mL \cdot min^{-1}$ and at a split ratio of 1:50 was used as carrier gas. Injector and detector temperature was set to 242 and 250 °C, respectively. The oven temperature was started at 150 °C, increased at 10 $°C \cdot min^{-1}$ to 200 °C and then increased at a rate of 1 $°C \cdot min^{-1}$ to 242 °C, according with temperature program established for FAME analysis in previous works. [19] As internal standard and solvent, methyl tricosanoate (C23:0) and isooctane were utilized, respectively.

3.3 RESULTS AND DISCUSSION

This work proposes to investigate different methods to feed inputs in an enzymatic hydroesterification reaction for biodiesel production in order to explore the maximum catalytic capacity of the enzyme, optimizing the amounts of reagent and catalyst utilized. Figure 1 presents the FAME yields for different input dosage strategies in the reaction. Among the five strategies tested, the highest FAME yield was obtained with the strategy 5, in which the highest number of stepwise of methanol was employed. The maximum FAME yield obtained was 82.99 % with 0.0145 g/g (1.45 wt%) of enzyme added in three steps and methanol to tallow molar ratio of 4.5:1 added in five steps. However, the five-step addition of methanol with a molar ratio of 4.5:1 and 0.01 g/g (1.00 wt%) of lipase achieved a FAME yield of 82.23 %, very close to that cited above. On the other hand, for the same enzyme load, the FAME yield of the process

was always lower for an excess of methanol of 4.0:1 than 4.5:1, regardless of the number of steps utilized to add the acyl acceptor to the system. Evaluating the effect of lipase load, it was verified that for the same molar ratio of MeOH:BT, naturally the FAME yield was always slightly higher for a lipase loading of 0.014 5 g/g (1.45 wt%) in comparison to 0.01 g/g (1.00 wt%) of Eversa® Transform lipase. Wancura et al.[19] verified that an increase of 3.5 times in the reaction lipase load resulted in an increment of only 3.5 % on biodiesel yield from a hydrolysis-esterification of soybean oil catalyzed by soluble lipase Callera™ Trans L (from TLL microorganism) using methanol as an acyl acceptor. In this sense, the authors of that work suggested that besides the stepwise dosage of methanol to the process, the stepwise addition of soluble lipase on reaction could also be interesting since this would enable a reduction in the required amount of biocatalyst. Indeed, results depicted in Figure 1 show that for a molar ratio of methanol and tallow of 4.5:1, the FAME yield when using 0.01 g/g (1.00 wt%) and 0.0145 g/g (1.45 wt%) of enzyme, with the stepwise addition of biocatalyst, was practically the same (82.23 and 82.99 %, respectively, for feeding strategy 5 of Table 1 after 8 h of reaction).

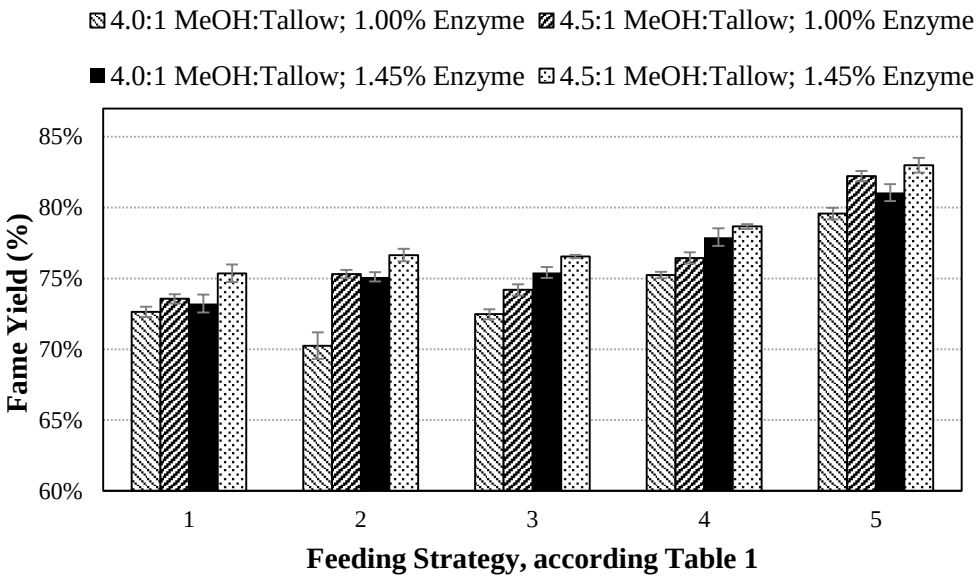

Figure 1. FAME yield for different feeding strategies of inputs in the process. Reaction conditions: 8 h of reaction; 35 °C; and 0.06 g/g (6 wt%) of water.

From Figure 1 it is also possible to observe that for the same amounts of catalyst and alcohol utilized in the process, an increase in the number of steps to feed methanol to the system was beneficial to the FAME yield. For example, for an enzyme load of 0.01 g/g (1.00 wt%) and MeOH:BT molar ratio of 4.5:1, the FAME yield obtained when all inputs were added at the reaction beginning was 73.56 %, increasing to 76.45 % when was used a four-step addition of

methanol and achieving 82.23 % for a five-step addition of alcohol. Published works about the subject have shown that the stepwise addition of methanol to the enzymatic reaction of biodiesel production is more effective in preserving the lipase activity, since the concentration of alcohol in the medium becomes smaller, contributing to minimize the inhibitory effects of alcohol on lipase which provides more satisfactory yields.[6,22]

Although the gradual dosage of lipase allows using a smaller amount of enzyme in the system (enzyme load can be reduced from 1.45 to 1.00 wt% without significant losses in system performance), in these tests it is perceived that there was a limitation of the reaction rate, which prevented the obtaining of higher FAME yields. Such a limitation could be associated with the fact that the soluble lipase obtained from the microorganism TLL has a high catalytic activity in the initial moments of the reaction where the stepwise dosing of the biocatalyst at this stage of the process may have impaired the reaction evolution precisely at instants where the enzyme has a high performance.[16] Since one of the aims of this work is to investigate different forms of feeding inputs (reagent and catalyst) in the reaction, besides the stepwise dosage of enzyme, it is necessary to evaluate how the process responds by adding all the lipase at the beginning of the reaction. Therefore, a one-step addition of lipase at the beginning of the reaction should be considered for posterior assays.

Lotti et al.[8] conducted a study about the effects of methanol on lipases. The authors cited the problem of the low solubility of methanol in oils and fats on solvent free system, mainly during the initial moments of the reaction when the triglyceride (TAGs) content is still high, which is an important factor of enzyme inactivation caused by alcohol. An alternative course of action would be start the process with an addition of lower amounts of methanol, increasing the alcohol dosage according the reaction progress and FAME being formed, since the solubility of methanol is higher in FAMEs than in TAGs. In this sense, some variations were accomplished in the inputs feeding strategy number 5 of the Table 1 (which presented better performance in relation to the others) in order to evaluate the process yield. Besides the dosage originally adopted, where most of the methanol was added to the reaction mixture during the initial moments of the reaction (Table 1), assays were performed in which the amount of methanol was added equally at each step and others in which the amount of methanol was increased according to the reaction development, according to Table 2. In addition, soluble lipase was added to the reaction mixture in one (all of the lipase at the beginning of the reaction) and three steps. Figure 2 presents the FAME yields for the experiments described. There was a little variation in the FAME yield for the experiments during which the amount of methanol added to the process changed with the reaction progress, either decreasing (assays A = 82.23 %

and D = 79.37 %) or increasing the amount of alcohol with time (assays C = 71.16 % and F= 70.65 %), both in the one-step addition of lipase at the beginning of the reaction (D and F) and on three-step biocatalyst dosing (A and C). Nonetheless, the dosage of a higher amount of methanol at the start of the process presented better performance in relation to the tests where the alcohol load at the beginning of the reaction was low and increased with the reaction advancement. These results may be related to the fact that the lipase from the TLL microorganism presents a high reaction rate at the initial moments of the reaction, where a higher amount of alcohol added to the system would benefit the reaction advance, pushing the reaction equilibrium towards the products.[16] On the other hand, in the experiments where the mass of methanol added in the medium was the same in each step (assays B and E), an increase of almost 11 % in the FAME yield was observed in the situation where all the biocatalyst was added at the beginning of the process (E: yield of 83.95 %) compared to the experiment where the lipase was added in three steps (B: yield of 72.98 %). This combination, utilized in the assay named E (Figure 2), proved to be a strategy favourable to the reaction rate of the system in comparison to the others.

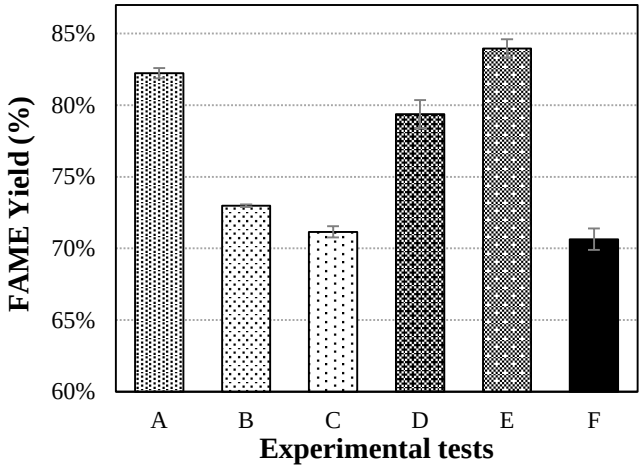

Figure 2. Evaluation of input dosage at system with lipase added in three steps (assays A, B, and C) and lipase added in a single step at reaction beginning (assays D, E, and F). A and D: higher amount of alcohol at the reaction beginning and decreasing with the reaction progress; B and E: amount of alcohol added equally in each step; and C and F: lower amount of alcohol at the reaction beginning and increasing with the reaction progress. Conditions: 8 h of reaction; 35 °C; 0.06 g/g (6 wt%); methanol to BT molar ratio of 4.5:1 (added in five steps); and 0.01 g/g (1 wt%) of lipase.

Results shown in Figures 1 and 2 demonstrated that an increase in the number of steps in the methanol addition to the reaction medium were beneficial to the process yield. Consequently, a system for methanol dosage was developed using a pump for low flowrates in order to perform a high number of small stepwise additions of alcohol to the system. Similar to the assays presented in the Figure 2, was performed tests with variations in the methanol addition gradient to the system: increase (positive gradient) and decrease (negative gradient) in the amount of methanol fed with the reaction progress, besides the dosage of alcohol with constant flow to the system (null gradient), according presented in Table 2. To start the reaction, 0.2 g/g (20 wt%) of methanol was added in each test at t = 0 h, the same amount of acyl acceptor added to assay E, which presented better performance than the other experiments. Figure 3 presents the FAME yields obtained for these experiments.

Assays with decreasing gradients of alcohol feed (G and J) started with a flow rate of 4.6 $g \cdot h^{-1}$ fed over 1 h, decreasing 1.0 $g \cdot h^{-1}$ at each hour of reaction, finishing the pumping in 4.0 h of process and thus leaving more 4 h of reaction so that the added methanol reacted with the substrate, where 3.3 g (20 wt%) were added to the system at t = 0 min to initiate the reaction. The summation of this amount of methanol added to the system totalize the alcohol to BT molar ratio of 4.5:1 utilized in the other tests, where Table 2 shows the mass percentage of MeOH added until the corresponding reaction time. The experiments with an increasing gradient of alcohol dosage (I and M) started with a flow rate of 1.6 $g \cdot h^{-1}$ fed over 1 h, increasing 1.0 $g \cdot h^{-1}$ at each hour of reaction, finishing the pumping in 4.0 h of process and again leaving more 4 h of reaction so that the added methanol reacted with the substrate, with 3.3g (20 wt%) added to the system at t = 0 min to initiate the reaction. The tests in which the methanol flow rate was null with the reaction advancement (H and L) was used a constant flow rate of 3.0 $g \cdot h^{-1}$ of alcohol fed over 4.0 h, where also was added 0.2 g/g (20 wt%) of methanol for start the reaction. The results of Figure 3 showed similar behaviour to what was observed on stepwise addition of methanol in the feeding strategy 5 analyzed in Figure 2. No relevant variations were observed in the FAME yield in situations where the methanol gradient increased or decreased when lipase was added to the reaction in three steps (G and I) or in one step (J and M). For the tests with increasing gradient of alcohol addition, the FAME yield reached 80.10 % (G) when was utilized the stepwise addition of enzyme against 77.10 % (J) when the biocatalyst was added in one single step. In the assays with a decreasing gradient of methanol feeding, the FAME yield reached 68.66 % (I) for the stepwise addition of lipase versus 69.35 % (M) for the single dosage of lipase at the beginning of the reaction. For the tests with a flow gradient equal to zero, a leap of approximately 12 % in the FAME yield was observed again for the situation where lipase

was added in three steps to the system (H, yield of 72.93 %) relative to the tests where the lipase was added in a single step to the reaction (L, yield of 85.08 %). The assay named L (Figure 3) was the experiment with the highest FAME yield obtained in this study. Among the tests performed and results obtained, it is possible to state that the strategy of feed methanol with a constant flow, adding all the lipase load in the reaction beginning was the strategy that presented the best conciliation between minimize the lipase denaturation and not limit the FAME production rate.

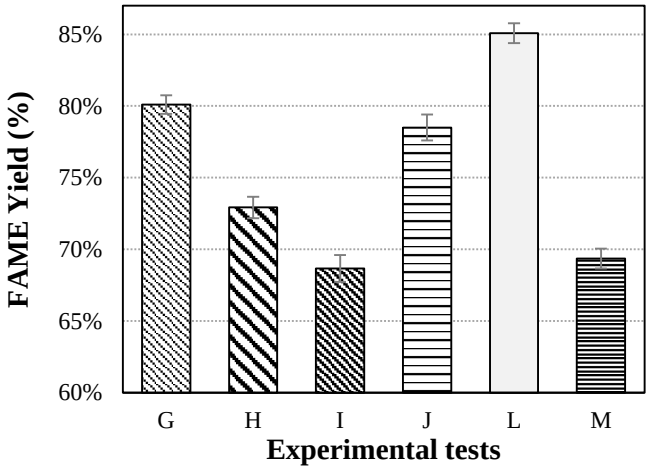

Figure 3. Evaluation of input dosage at system with lipase added in three steps (assays G, H, and I) and lipase added in a single step at reaction beginning (assays J, L, and M). G and J: higher amount of alcohol at the reaction beginning and decreasing with the reaction progress; H and L: amount of alcohol added equally with the reaction progress; and I and M: lower amount of alcohol at the reaction beginning and increasing with the reaction progress. Conditions: 8 h of reaction; 35 °C, 0.06 g/g (6 wt%) of water; methanol to BT molar ratio of 4.5:1 (fed with a pump); and 0.01 g/g (1 wt%) of lipase.

The obtained results demonstrated that the form in which the inputs of the FAME production via enzymatic catalysis are inserted in the reaction medium influence the process yield. The utilization of a pump for the feeding of alcohol, which simulated a large number of small stepwise additions of methanol to the system, and the addition of lipase in one step showed to be the most appropriate way of working with the process inputs, considering the enzyme used in this research. However, among all the variations in ways of adding these items to the system investigated, there is the inevitable questioning of what would be the behaviour of the system if methanol was fed in a shorter interval of time instead of being added during 4 h of reaction. Figure 4 presented the results for FAME yield with a one-step addition of lipase

as well as methanol pumped to the reaction system. As before, Table 2 presents the mass percentages of alcohol added to the system until the corresponding reaction time. The constant flow rates that were utilized were 6.3 $g \cdot h^{-1}$ (assay named "N", which means that the amount of alcohol corresponding to a molar ratio of 4.5:1 was pumped in 2 h), 4.1 $g \cdot h^{-1}$ (assay named "O" with a flow rate corresponding to 3 h of reaction to pump the amount of methanol), and 3.0 $g \cdot h^{-1}$ (assay named "L" in which methanol was added to the reaction over 4 h). From Figure 4, it is possible to verify that the decrease of the methanol dosage time (increasing the pump flow) reduced the FAME yield of the process. With the methanol dosage performed during 2 h of reaction, the process yield reached 71.08 %, compared to 76.97 % for 3 h and 85.08 % with the dosage done in 4 h. In the first situation there was an increase of 14 % for a reaction time of only 8 h. This behaviour can be explained by the fact that with the reduction of the alcohol dosage time, the equilibrium between the lipase denaturation and favouring of the reaction rate factors is undone since there is an increase in the methanol concentration in the first instants of the reaction. Wancura et al.[19] reported that the TLL liquid lipase presented elevated catalytic capacity in the first 45 min of reaction and stated that having the enzymatic activity of the lipase compromised precisely during this period negatively affected the global yield of the process. Such conclusions can be compared with the time course of the beef tallow hydroesterification of this study, presented in Figure 5. Utilizing the reaction conditions of the assay that achieved the best yield among all tests, the time course for the FAME yield shows that the process develops with a good productivity until 45 and 60 min of reaction, where with the process advancement, the inhibitory effects of the methanol act under the lipase activity. It is possible to observe that from 2 h of reaction, the increments in FAME yield with the development of the process dropped considerably: in 2 h of reaction, the FAME yield reached around 65 % and the final yield of the process after 8 h was 85 %. In other words, in 6 h of reaction, there was an increase in the FAME yield of only 20 %, less than one-third of the value obtained in the first 2 h of reaction. This may be associated with inhibition of lipase activity caused by the methanol. In relation to the acid values of the samples (presented in the Figure 5), an important factor in the evaluation of the efficiency of the biodiesel production, it is possible to note that it presents a considerable elevation in the first hour of reaction (reaching 21.5 mg $KOH \cdot g^{-1}$) decreasing considerably with the reaction progress, where at the end of the assay the acid value was of 5.6 mg $KOH \cdot g^{-1}$, which was a reduction of 74 %. This behaviour can be explained by the fact that in the first stages of the reaction, the hydrolysis of the substrate has predominance, which elevates the acid value of the FAME. With the reaction development, the free fatty acids formed with the hydrolysis of the triglycerides presented in the feedstock are esterified to fatty acid

methyl esters, explaining the decay of the acid values of the FAME samples according the process progress.

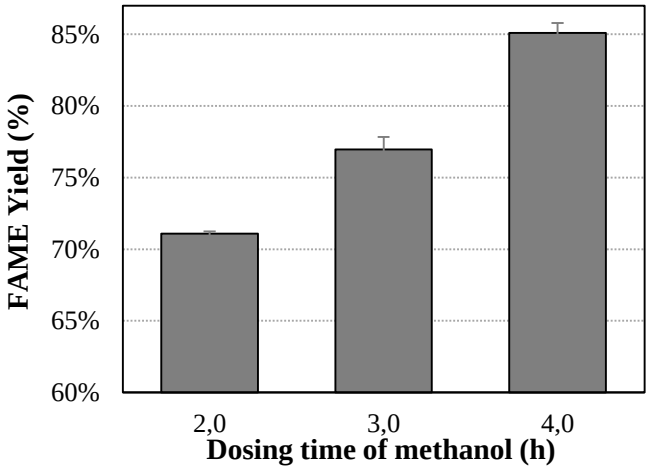

Figure 4. Influence of the FAME yield with the time spent for dosing methanol in the system. Reaction conditions: 8 h of reaction; 35 °C; 0.06 g/g (6 wt%) of water; methanol to BT molar ratio of 4.5:1 added using a pump; and 0.01 g/g (1 wt%) of lipase added at single step.

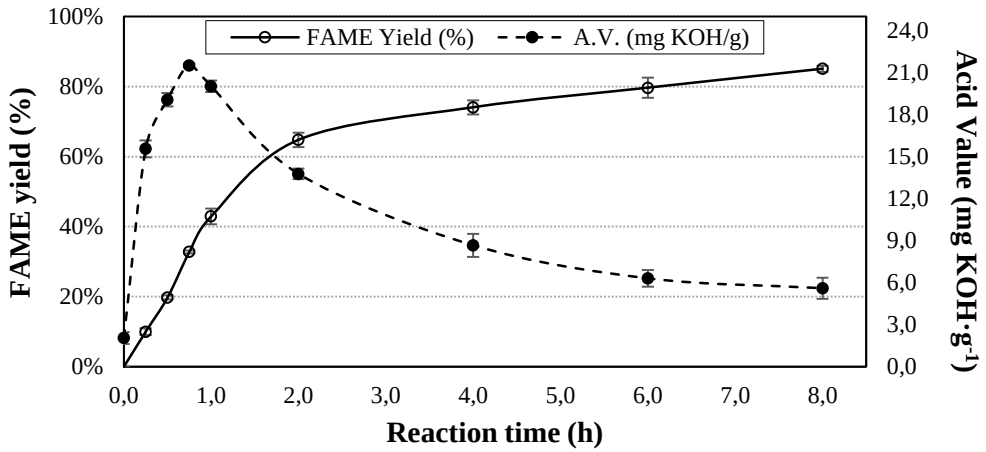

Figure 5. Time course of hydroesterification of beef tallow catalyzed by Eversa® Transform lipase. Reaction conditions: 35 °C; alcohol to BT molar ratio of (4.5:1); 0.01 g/g (1 wt%) enzyme; water concentration of 0.06 g/g (6 wt%); and 8 h of reaction.

Table 3 presents an overview about the catalytic capacity of different lipases in relation to several strategies of adding alcohol to the reaction system. Lee et al.[23] reported a three-step production of biodiesel under supercritical fluid (SC-CO2) where a biodiesel yield of 98.9 % was achieved after 6 h of reaction with 0.2 g/g (20 wt%) of lipase added to the process. Although

Table 3. Comparison of the catalytic capacity of various lipases for biodiesel production with several methanol dosage strategies.

Substrate	Lipase	Reaction Conditions	Methanol Dosage Strategy	Yield (%)	Reference
Beef tallow	*Thermomyces lanuginosus* (soluble form)	35 °C, 1 wt% catalyst, methanol:fat molar ratio of 4.5:1, 6 wt% water, 8 h of reaction	Dosed equally over 4 h of reaction using a pump	85.0	Present work
Crude soybean oil	*Thermomyces lanuginosus* (soluble form)	35 °C, 1 wt% catalyst, 16 wt% methanol, 3 wt% water, 24 h of reaction.	Four steps: 4 wt% each time at 0 h, 3 h, 5 h and 7 h of reaction	96.3	[13]
Soybean oil	Immobilized *Candida antartica* lipase B	45 °C, 20 wt% catalyst, methanol:oil molar ratio of 4.5:1, 10 wt% water. Process under supercritical fluid (SC-CO_2), pressure of 130 bar, 6 h of reaction.	Three steps: 90mmol at 0 h, 2 h and 4 h of reaction	98.9	[23]
Lard	Immobilized *Candida* sp. 99-125	40 °C, 20 wt% catalyst, methanol:fat molar ratio of 3:1, 20 wt% water, *n*-hexane as solvent, 30 h of reaction.	Three steps: 1/3 of the methanol amount at 0 h, 10 h and 20 h of reaction	87.4	[24]
Waste cooking oil	Immobilized marine *Streptomyces* sp (MAS1 lipase)	30 °C, 5 wt% catalyst, methanol:oil molar ratio of 3:1, 24 h of reaction.	One-step addition of methanol	95.5	[25]
Refined soybean oil	Immobilized *Thermomyces lanuginosus* lipase	30 °C, 15 wt% catalyst, ethanol:oil molar ratio of 3:1, 4 wt% water, *n*-hexane as solvent, 10 h of reaction.	Three steps: 1/3 of the ethanol amount at 0 h, 3 h and 6 h	85.0	[27]

supercritical assistance increases the reaction mass transference, which benefits the system, this process requires not only a large amount of energy but also a large amount of lipase (around twenty times higher than the load used in our work), which makes this route expensive. Lu et al.[24] utilized a three-step strategy of methanol dosage to obtain 87.4 % of FAME yield from lard after 30 h of reaction with 0.2 g/g (20 wt%) of catalyst, 0.2 g/g (20 wt%) of water, and n-hexane as solvent. Despite the use of organic solvents, such as n-hexane and tert-butanol, which ensure the homogenous mixture of the reaction system and minimize the inhibitory effects of methanol on lipase, their addition to the process necessitates a later stage of bioproduct purification to remove the solvent, increasing the cost of the process. On the other hand, a considerable amount of research is currently being carried out with the objective of developing lipases that are tolerant to the inhibitory action of methanol. Wang et al.[25] reports the action of a novel immobilized lipase obtained from a marine *Streptomyces* sp strain W007, where despite the high enzyme load used (0.05 g/g (5 wt%)) a biodiesel yield of 95.5 % was obtained after 24 h of reaction, with a single-step addition of methanol in a solvent-free system. This shows the promising behaviour of this biocatalyst for biodiesel production. The strategy proposed in this study reached a FAME yield of 85.08 % from BT after 8 h using 0.01 g/g (1 wt%) of catalyst, which is a technique that not only improved the catalytic activity of the Eversa® Transform lipase but also made it possible to obtain a satisfactory bioproduct yield even with one third of the reaction time that is normally employed in similar research.

Moreover, an investigation of the biocatalyst reuse was performed under the best conditions found previously: 8 h of reaction; 35 °C; 0.06 g/g (6 wt%) of water added to reaction; 0.01 g/g (1 wt%) of lipase with a single-step addition at the beginning of the reaction; and methanol to fat molar ratio of 4.5:1 pumped equally during 4 h of reaction. According occurs with immobilized enzymes, it is possible to recover the liquid enzyme for further reutilization, another economic advantage of soluble lipases. Since the enzyme is an amphiphilic molecule working on the interface between water and oil, the lipase stays concentrated in an emulsion between a clear heavy phase (rich in glycerin) and the FAME phase and after the separation process where it has been found that 95 % of the enzyme activity is concentrated in this emulsion.[26] For each cycle, the soluble lipase was recovered from the reaction mixture and was directly utilized in the next batch, without any treatment. From Figure 6 it is possible to observe that Eversa® Transform lipase maintained its catalytic capacity for two cycles, and a biodiesel yield of 85.08 % and 76.32 % were obtained respectively. For more than two cycles, a decay in the FAME yield of approximately 35 % (yield of 50.19 % after 3 batches) and 50 % (yield of 35.54 % after four batches) was observed, which can be associated with the denaturing

action of the methanol on the enzyme after each cycle. He et al.[27] cited that it is evident that the number of reusable liquid lipases is significantly lower than immobilized lipase since immobilized enzymes are easier to recover from the system and the immobilization process by itself offers more catalytic stability for lipase. In this sense, considering the lower cost of the soluble lipases in comparison to the immobilized lipases and considering catalytic loss after few cycles, in addition to the operational costs involved with the separation process, the use of soluble enzymes in a single cycle seems to be appropriate.

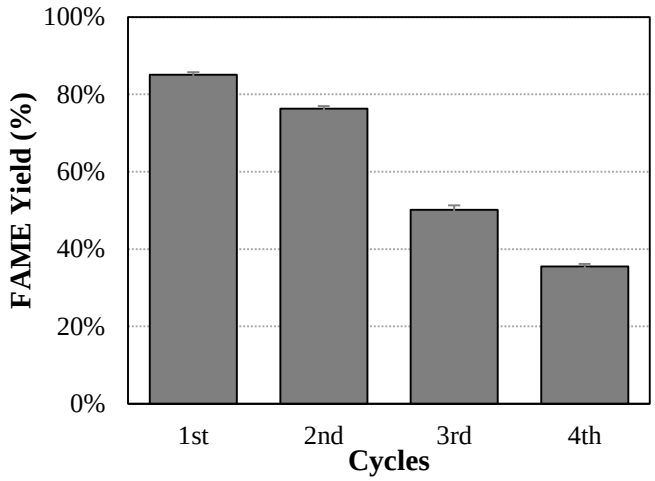

Figure 6. Lipase reutilization in the hydroesterification of BT with methanol using Eversa® Transform as catalyst. Experimental conditions: 35 °C; alcohol to BT molar ratio of (4.5:1); 0.01 g/g (1 wt%) enzyme; water concentration of 0.06 g/g (6 wt%); and 8 h of reaction time.

3.4 CONCLUSIONS

The addition of the acyl acceptor and the lipase to the hydroesterification of beef tallow to produce fatty acid methyl esters showed a significant influence on the enzymatic process. The utilization of a stepwise dosage of lipase in the reaction does not cause a significant improvement in the FAME yield when the enzyme load is increased, allowing the use of a smaller amount of lipase to obtain the same process yield. However, for the same enzyme load, the single dosage of biocatalyst at the beginning of the process was shown to be more efficient. The stepwise addition of methanol to the reaction only proved to be more efficient based on the increase in the number of dosage steps of alcohol. In this sense, the use of a pump with low flow rates of methanol to the system was beneficial to the system, and a biodiesel yield of 85.08 % was achieved after 8 h of reaction, which was 12 % more than the one-step addition of inputs

at the beginning of the reaction for a same amount of acyl acceptor (4.5:1) and catalyst (1 wt%). The soluble lipase used in this study proved to be a versatile catalyst, making it possible for it to be recovered from the process to be used with a new batch. Otherwise, the catalytic capacity of the liquid lipase was considerably reduced after four cycles, and a decay of 50% in the FAME yield was observed, which indicates that it would be more appropriate to use the biocatalyst for only one cycle. Nevertheless, the results of this work will provide efficient alternatives for the manipulation the biocatalytic process, permitting individuals to choose the best feeding strategy of inputs that contribute to the overall performance of the Eversa® Transform lipase.

3.5 ACKNOWLEDGMENTS

The authors would like to thank the National Council of Technological and Science Development (CNPq) and Research Support Foundation of the State of Rio Grande do Sul (FAPERGS) for the financial support for this project.

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4 MELHORAMENTO DA PRODUÇÃO DE BIODIESEL CATALISADA POR LIPASES SOLÚVEIS ATRAVÉS DE UM SISTEMA REACIONAL DE HIDROESTERIFICAÇÃO DE DUAS ETAPAS

Neste capítulo estão apresentados os resultados relacionados ao cumprimento do segundo objetivo específico desta tese. Para isto, realizou-se uma otimização, via planejamento estatístico, das variáveis de processo "concentração de água adicionada a reação", "carga de catalisador" e "razão molar entre metanol e óleo" para uma proposta de configuração de duas etapas reacionais em série de hidroesterificação enzimática, catalisada pela lipase solúvel NS 40116 (Eversa® Transform 2.0).

Improving the Soluble Lipase-catalyzed Biodiesel Production through a Two-step Hydroesterification Reaction System

DOI: https://doi.org/10.1007/s00253-019-10075-y

João H. C. Wancura[a], Daniela V. Rosset[a], Gustavo A. Ugalde[b], J. Vladimir Oliveira[c], Marcio A. Mazutti[a], Marcus V. Tres[d], Sérgio L. Jahn[a]

[a] Department of Chemical Engineering, Federal University of Santa Maria, 1000, Roraima Avenue, Santa Maria, 97105-900, Brazil;

[b] Department of Crop Protection, Federal University of Santa Maria, 1000, Roraima Avenue, Santa Maria, 97105-900, Brazil;

[c] Department of Chemical and Food Engineering, Federal University of Santa Catarina, Florianópolis, SC, 88040-900, Brazil;

[d] Laboratory of Agroindustrial Processes Engineering (LAPE), Federal University of Santa Maria, 1345, Ernesto Barros Street, Cachoeira do Sul, 96506-322, Brazil.

ABSTRACT

The application of lipases in liquid formulation instead of immobilized forms in the enzymatic biodiesel synthesis can make the process cost-efficient, more competitive and sustainable. However, despite the benefits, the long reaction times required to achieve satisfactory yields is still a drawback of this biotechnological process. In this sense, employing the novel low-cost soluble NS40116 lipase, this paper proposes an innovative two-step hydroesterification reaction (TSHR) system as a technique of improving the reaction rate of an enzymatic biodiesel production. With the employment of two Central Composite Statistical

Design to optimize the parameters of each of the reactions involved, it was investigated the influence of the parameters “water concentration added to the reaction”, “methanol to oil molar ratio” and “lipase load” on the process yield, besides the acid value of the samples. After only 8 hours of reaction, the highest fatty acid methyl ester yield reached was 97.1 % with an acid value of 4.62 mg $KOH \cdot g^{-1}$ utilizing a total of 8 wt% water, methanol to oil molar ratio of 6.3:1 and 0.70 wt% of lipase. Furthermore, the statistical models for both reactions indicated to be significant with 95 % of reliability. Considering that the papers published using soluble lipases in an one-step batch process normally reach similar yields to those obtained in this research after 16 h to 24 h of reaction, the proposed system demonstrated to be a promising option of process configuration for the enzymatic production of biodiesel.

Keywords: Biodiesel; FAME; Soluble lipase; NS40116; Enzymatic hydroesterification.

4.1 INTRODUCTION

Biodiesel (fatty acid methyl esters - FAME) is a non-toxic, biodegradable and renewable fuel derived from vegetable oils or animal fats (Röttig et al. 2010; Vieitez et al. 2010; Selvaraj et al. 2016). The main technique established for biodiesel production in industrial scale is based on a homogeneous alkaline methanolysis, using a molar ratio between alcohol and oil of 6:1, 60 °C, 1 h of reaction with 0.5 wt% of sodium methoxide or 1.0 wt% sodium hydroxide as catalyst of the process when methanol (MeOH) is utilized as acyl acceptor (Azócar et al. 2010; Knothe and Razon 2017). However, considering that when dealing with biodiesel necessarily there is an environmental appeal related to its utilization, disadvantages of the homogeneous alkali-catalysis have directed recent researches to develop environmental-friendly routes for synthesis of the fuel in question. Such disadvantages, of environmental order, are mainly associated to the generation of considerable volumes of alkaline wastewater from the washing step to remove traces of catalyst in the purification process of the biodiesel (Fjerbaek et al. 2009; Michelin et al. 2015).

In this scenario, the employment of lipases emerges as an attractive alternative for biodiesel synthesis (Takaya et al. 2011). Lipases (*triacylglycerol acylhydrolase,* EC 3.1.1.3) are enzymes that have the capability to catalyze hydrolysis, alcoholysis, esterification, interesterification and transesterification reactions, having a wide spectrum of biotechnological applications (Luković et al. 2009). The main advantages of the enzymatic method for biodiesel synthesis are elimination of the biofuel washing stages, bland reaction conditions, versatile

lipase activity for simultaneous catalysis of triglycerides (TG) and free fatty acids (FFA), production of a biofuel with high purity and lipase selectivity, which prevents the occurrence of secondary reactions (Hama et al. 2018; Aguieiras et al. 2019). Nevertheless, drawbacks like low reaction rates and consequently long reaction times (in comparison to the homogeneous alkali-catalysis) and lipase deactivation due the contact with alcohols still are problems that the enzymatic process faces (Christopher et al. 2014).

The enzymatic process for biodiesel production can be classified as heterogeneous (where immobilized lipases are used in the process) or homogeneous (liquid or soluble lipases are used in the reaction) (Norjannah et al. 2016). The application of liquid (free or soluble) lipases for biodiesel synthesis can make the process cost-efficient, more competitive and sustainable, once soluble lipases can be produced and sold at until 50 times lower than the immobilized ones, present higher reaction rates and can also be reused after recovery from the system providing a high competitive to the technique in relation to the others technologies (Cesarini et al. 2013; Wancura et al. 2019). Utilization of soluble lipases in the process implies in the addition of water to reaction, which, through the action of the alcohol of the system, makes the enzyme catalyze simultaneously both the hydrolysis of the TG (generating FFA), and the esterification of these FFA in fatty acid acyl esters. Therefore, the process is often referred to as hydrolysis-esterification reaction or hydroesterification.

Works published employing soluble lipases in biodiesel production still represents a small number in the literature when compared to the use of lipases in immobilized forms (Nielsen et al. 2016; Andrade et al. 2017; Wancura et al. 2018b; Wancura et al. 2018a). Most of the published works involving the enzymatic production of biodiesel employed lipases immobilized on polymeric supports as biocatalyst of the process (Verma et al. 2013; Matte et al. 2014; Facin et al. 2018). In terms of enzymatic hydroesterification is not different. The majority of the studies in the literature employ immobilized lipases in a hydrolysis and esterification systems working in two reactions separately (firstly the substrate is hydrolyzed and the FFA obtained are then destined to an esterification reaction) (Pourzolfaghar et al. 2016). In one of these researches that soluble lipases were employed, Wancura et al. (2018b) evaluated the influence of the reaction parameters on hydrolysis-esterification process catalyzed by the Callera^{TM} Trans L lipase to produce biodiesel, obtaining 96.9 % of biodiesel after 24 h of reaction in a single-batch process. Results obtained in this aforementioned research demonstrated that the liquid lipase utilized had a high catalytic activity in the first hours of reaction, achieving a biodiesel yield around 72 % after only 4 h requiring, however, another 20 h of process to increase the biodiesel yield in approximately 25 %. Such decrease in the process

productivity (after 4 h of reaction) would be associated, according these authors, the loss of lipase activity due to the denaturant action of the reaction temperature and mainly by the methanol under the biocatalyst.

Considering this scenario, the present research suggests a new configuration of process aiming to increase the system productivity: a process conducted with two reactions of hydroesterification in series employing the novel, low-cost and thermostable liquid lipase NS40116, recently launched by Novozymes. The effect of the parameters “water concentration added to the reaction”, “molar ratio between methanol and oil” and “lipase load” on the biodiesel yield was evaluated using statistical design, where variables were optimized for both reactions that compose the system proposed.

4.2 MATERIALS AND METHODS

4.2.1 Materials

The biocatalyst employed on reactions was the free formulation of the enzyme produced from *Thermomyces lanuginosus* microorganism, named NS40116 (100 LCLU·g^{-1} of enzymatic activity), supplied by Novozymes Brasil (Araucária, Brazil). The degummed soybean oil utilized in the assays (0.20 wt% of FFAs, 134 ppm of soaps and 0.15 wt% of moisture) was provided by Olfar Alimento e Energia S/A (Erechim, Brazil). Methanol and ethanol (both analytical grade), isooctane and methyl tricosanoate (both chromatographic grade) were acquired from Sigma-Aldrich (São Paulo, Brazil).

4.2.2 Two-step Hydroesterification Reaction

The system proposed is represented in the Figure 1. The hydroesterification on two reactions in series, separating the light phase of the first reaction (rich in FAME) and sending it to the second reaction with the addition of fresh inputs, aims to move the reaction equilibrium towards the products, besides taking full advantage of the catalytic ability of the lipase in each reaction. The system utilized to accomplish the tests involved a 250 mL 3-way round bottom flask, which was employed as reactor. Process temperature was kept through of a temperature controller, whit the reactor immersed in a water bath. A reflux condenser connected in the top of the flask avoided the loss of methanol. In all assays it was used 50 g of oil, 35 °C and 500 rpm of stirring speed. For each reaction, a reactional time of 4 h was adopted, according to

previous studies that demonstrated that after this period, there is a considerable and gradual decrease on lipase activity and consequently in the FAME production rate, where no substantial raise on FAME yield was verified (Wancura et al. 2018a).

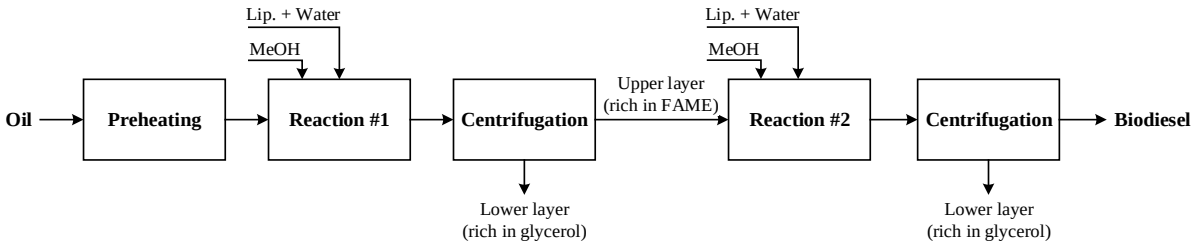

Figure 1. Flowchart of the two-step hydroesterification system.

Firstly, the oil was heated until the temperature defined and then adding the amounts of methanol and lipase diluted in distilled water established for each assay. After the *Reaction #1* to be finished, the reactional medium is conducted to the first stage of centrifugation for separate the FAME phase from the glycerin phase, being the FAME phase directed for the second hydroesterification reaction, where fresh inputs were added to the system in amounts according is presented hereafter. After the process conclusion, the reaction medium of the *Reaction #2* was conducted to a second stage of centrifugation, where the biodiesel phase was separated from the glycerin phase and finally subjected to analysis for calculate the process yield. The MeOH dosage in both reactions was performed fractionally, according to a previous study (Wancura et al. 2019): 40 wt% in t = 0 h, 30 wt% in t = 0.75 h and 30 wt% in t = 1.50 h, seeking to keep low the methanol concentration in the medium and consequently minimize the lipase denaturation. Samples were taken from each reaction and centrifuged at 4,000 rpm for 5 min at 20 °C for phases separation, where the upper layer (FAME) was collected and directed for gas chromatography analysis. Procedure defined by EN 14103 (2015) was employed to determine the FAME yield achieved in the experiments, according the equation 1:

$$\text{FAME Yield (\%)} = \left(\frac{\sum \text{A} - \text{A}_\text{I}}{\text{A}_\text{I}}\right) \cdot \left(\frac{\text{c}_\text{I} \cdot \text{V}_\text{I}}{\text{c}_\text{S} \cdot \text{V}_\text{S}}\right) \quad (1)$$

where A is the area of the major peak of methyl ester detected in the analysis; A_I is the area of the peak corresponding to the standard (C23:0); c_I and V_I indicate the concentration of the internal standard solution and the volume of this solution utilized in each sample, respectively; and c_S and V_S corresponds to the concentration of solution of the FAME sample prepared and

the volume of solution that was used in the analysis, respectively.

The optimization of each reaction was performed individually in order to obtain a satisfactory biodiesel yield. The reaction conditions applied for *Reaction #1* are presented in Table 1 (levels based according previous studies, where the data are not shown). For *Reaction #2*, the levels employed are presented in the Table 3, where these values were selected according to the results verified from the optimization of the first reaction. The feedstock for evaluation of the *Reaction #2* was the product obtained from the *Reaction #1*. After the investigation of each reaction, a validation test was performed, in triplicate, using the parameters obtained from each experimental design, following each step of the flowchart shown in the Figure 1.

A central composite rotational design (CCRD) was adopted aiming to optimize the biodiesel synthesis optimization via the TSHR. Seventeen (*Reaction #1*) and eleven (*Reaction #2*) experiments was performed. The independent variables chosen for the first reaction were water concentration (x_1, wt%), molar ratio between methanol and oil added to the system (x_2, $mol \cdot mol^{-1}$) and lipase load in relation to the amount of oil (x_3, wt%). For the second reaction, the independent variables selected were methanol to oil molar ratio (x_4, $mol \cdot mol^{-1}$) and lipase concentration (x_5, wt%). For *Reaction #2*, the variable "water concentration" was fixed in 2.0 wt% and added in the second reaction for all assays, since it was verified that the hydrolysis of the substrate occurs predominantly in the first reaction, therefore it was not necessary to add more distilled water in the second step of the hydroesterification system. The response variable (dependent variable) was FAME yield (Y, %).

4.2.3 Gas Chromatography

Gas chromatography for methyl esters quantification and FAME yield determination was performed using a GC-2010 Plus gas chromatography (Shimadzu, Japan), equipped with a flame ionization detector. A Zebron ZB-WAX plus with 30.0m × 0.32mm I. D. × 0.25μm film thickness capillary column was employed for compounds separation. The method of analysis was the same developed and applied in previous work (Wancura et al. 2018b).

4.2.4 Acid Value Analysis

Procedure defined by EN 14104 (2003) was applied to acid value (AV) determination for the FAME samples, according equation 2, expressed in milligrams of potassium hydroxide (KOH) required to neutralize the acidity of one gram of substrate:

$$AV = V \cdot c \cdot \left(56.1/m\right) \quad (2)$$

where *AV* is the acid value of the sample, *V* is the volume of KOH solution consumed on the titration (mL), *c* is the concentration of the KOH solution ($mol \cdot L^{-1}$), *m* is the mass of sample (g) and *56.1* is the molecular weight of KOH.

4.3 RESULTS

4.3.1 Optimization of the First Reaction of the System

The results for the biodiesel yield obtained in the assays and predicted by the statistical model for the first reaction are presented in Table 1. The quadratic regression for the response variable "biodiesel yield" (for the *Reaction #1*) considering the coded variables that presented significance in this situation is represented by the equation (3):

$$Y_1\ (\%) = 83.120 + 7.213x_1 - 6.777x_1^2 + 2.469x_2 - 5.747x_2^2 + 6.301x_3 - 4.864x_1x_3 \quad (3)$$

where Y_1 is the biodiesel yield, x_1 is water concentration added to the system, x_2 is the MeOH to oil molar ratio and x_3 is the lipase load. Coefficients with a positive sign express a synergic influence and the negative coefficients express an antagonistic effect.

Table 2 presented the analysis of variance (ANOVA), utilized for evaluate and confirm the significance of equation (3), determining also the fitness of the regression model established and the influence of the parameters considered under dependent variable. The coefficient of determination ($R^2 = 0.9734$) obtained for the *Reaction #1* model presented an excellent fit, since 97.34 % of variability in the situation considered can be justified by the model. Moreover, the quadratic regression for the results of the first reaction, having a F-value of 29.49 and a *p*-value less than 0.0001, indicated with 95 % of reliability level that the model is significant, since the tabulated F_{value} is F_{tab} (9, 7, 5 %) = 3.68 ($F_{value} > F_{tab}$). From the ANOVA, it was concluded that, for the first reaction of the system composed by a two-step hydroesterification process, the parameter "water concentration", "MeOH to oil molar ratio" and "lipase load" had a positive effect on biodiesel yield. On the other hand, the quadratic parameter for "water concentration" and "molar ratio between MeOH and oil" besides of the interaction "water concentration and lipase load" presented a negative influence under the response variable. Notwithstanding, did

Table 1. CCRD for the *Reaction #1* with the results observed and predicted for the FAME yield and AV measured.

Assay	Independent variables			FAME yield, Y (%)		Acid value, AV (mg KOH·g^{-1})	Productivity (g FAME·g enz^{-1})
	Water concentration (wt%), x_1	Methanol to oil molar ratio (mol·mol^{-1}), x_2	Lipase load (wt%), x_3	Obtained	Predicted		
1	3.6 (-1)	3.6:1 (-1)	0.26 (-1)	53.0	51.4	8.9 ± 0.2	203.1
2	8.4 (+1)	3.6:1 (-1)	0.26 (-1)	75.7	74.3	10.6 ± 0.4	290.5
3	3.6 (-1)	5.4:1 (+1)	0.26 (-1)	54.8	53.7	6.3 ± 0.4	210.3
4	8.4 (+1)	5.4:1 (+1)	0.26 (-1)	79.9	79.1	8.2 ± 0.5	306.6
5	3.6 (-1)	3.6:1 (-1)	0.44 (+1)	74.1	71.6	8.0 ± 0.3	168.7
6	8.4 (+1)	3.6:1 (-1)	0.44 (+1)	77.3	75.1	10.1 ± 0.4	176.0
7	3.6 (-1)	5.4:1 (+1)	0.44 (+1)	79.1	77.1	7.9 ± 0.2	180.1
8	8.4 (+1)	5.4:1 (+1)	0.44 (+1)	84.9	83.0	7.5 ± 0.3	193.2
9	2.0 (-1.68)	4.5:1 (0)	0.35 (0)	49.2	51.9	5.4 ± 0.7	140.6
10	10.0 (+1.68)	4.5:1 (0)	0.35 (0)	74.0	76.1	8.6 ± 0.2	211.4
11	6.0 (0)	3:1 (-1.68)	0.35 (0)	59.6	62.6	11.7 ± 0.4	170.3
12	6.0 (0)	6:1 (+1.68)	0.35 (0)	69.4	71.2	7.1 ± 0.5	198.3
13	6.0 (0)	4.5:1 (0)	0.20 (-1.68)	71.9	73.2	8.3 ± 0.4	359.5
14	6.0 (0)	4.5:1 (0)	0.50 (+1.68)	90.0	91.8	7.4 ± 0.3	179.9
15	6.0 (0)	4.5:1 (0)	0.35 (0)	83.1	83.1	7.9 ± 0.7	237.5
16	6.0 (0)	4.5:1 (0)	0.35 (0)	83.3	83.1	7.9 ± 0.4	237.9
17	6.0 (0)	4.5:1 (0)	0.35 (0)	83.8	83.1	7.9 ± 0.5	239.4

not present a significant effect under the FAME yield the interactions between “water concentration and MeOH:oil molar ratio”, “MeOH:oil molar ratio and lipase load”, likewise the quadratic interactions for “enzyme load”.

Table 2. ANOVA for the FAME samples at the conclusion of the first reaction.

Factor	Sum of Squares	Degree of Freedom	Mean Square	F-value	*p*-value
Model	2,381.49	9	264.61	29.49	< 0.0001[a]
Water concentration, x_1	709.96	1	709.955	79.12	< 0.0001[a]
$x_1 \cdot x_1$	516.45	1	516.447	57.55	0.000128[a]
MeOH, x_2	90.07	1	90.071	10.04	0.015748[a]
$x_2 \cdot x_2$	371.27	1	371.272	41.38	0.015748[a]
Lipase load, x_3	496.25	1	496.251	55.30	0.000356[a]
$x_3 \cdot x_3$	0.06	1	0.0568	0.006	0.000145[a]
$x_1.x_2$	2.99	1	2.989	0.33	0.93883[b]
$x_1.x_3$	189.25	1	189.249	21.09	0.581923[b]
$x_2.x_3$	5.20	1	5.200	0.58	0.002507[a]
Residual	62.82	7	25.03		
Total	2,356.84	16			

[a] Significant

[b] Non-significant

Coefficient of variation = 2.35 %; standard deviation = 1.72; $R^2 = 0.9734$

To investigate the effects for the interactivity between the variables for the *Reaction #1*, contour surfaces were utilized, reporting the behavior of the system for the values of the dependent variable obtained. Figure 2a represents the response of the FAME yield according to the variation of the water concentration and MeOH to oil molar ratio, Figure 2b expounds the influence of the water concentration and the enzyme load under the response variable, Figure 2c presenting the FAME yields achieved according to the variation of the parameters “MeOH to oil molar ratio” and “enzyme load”. The worst reaction condition, which led to the lowest FAME yield and productivity at the end of the *Reaction # 1*, was employing 2 wt% water to the medium, a MeOH to oil molar ratio of 4.5:1 and 0.35 wt% lipase – FAME yield of 49.2 % and 140.6 g FAME·(g enzyme)$^{-1}$. On the other hand, 90.0 % of yield was obtained increasing the water concentration in the first reaction to 6 wt% and the lipase load to 0.5 wt%, keeping the molar ratio of MeOH:oil in 4.5:1.

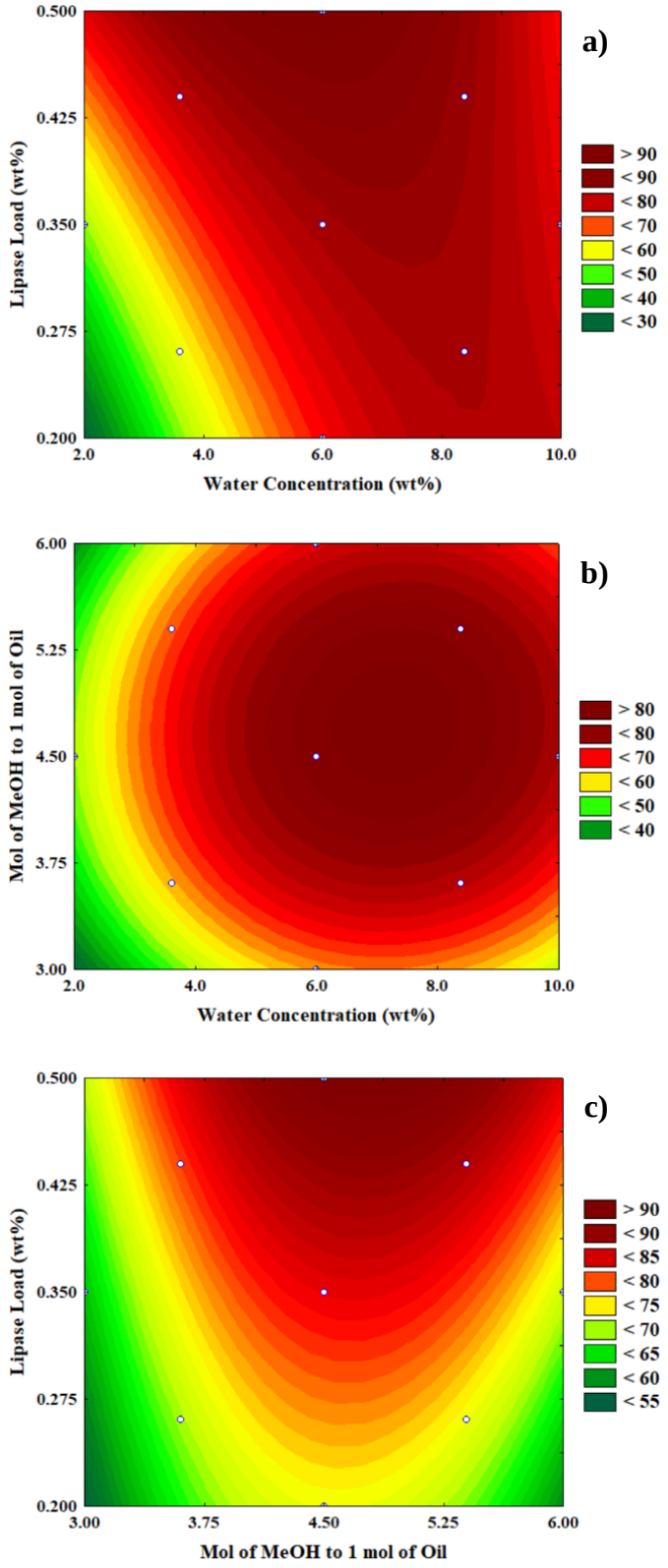

Figure 2. Response of the biodiesel yield after the first reaction of the system in relation to the variation of (a) the mass of water added and of the MeOH to oil molar ratio, (b) the “water concentration” and “lipase load” and (c) the “MeOH to oil molar ratio” and “enzyme load”.

An interesting result obtained concerns about the amounts of lipase added into the *Reaction #1*. Analyzing the axial points of the statistical design for the parameter "lipase load (x_3)" (according Table 1), it was achieved a FAME yield of 71.9 % for 0.20 wt% of NS40116 lipase and 90.0 % for 0.50 wt% of lipase added to the reaction, keeping fixed the parameters x_1 (6.0 wt%) e x_2 (molar ratio of 4.5:1) at the center point, resulting in a productivity of 359.5 g FAME per gram of enzyme and 179.9 g FAME per gram of enzyme, respectively. Therefore, considering the satisfactory FAME yield obtained and the higher productivity per gram of biocatalyst for the inferior axial point of the variable x_3, the reaction condition x_1 = 6.0 wt% of water, x_2 = 4.5:1 of methanol to oil molar ratio and x_3 = 0.20 wt% of lipase was chosen as parameters for the accomplishment of the *Reaction #2* optimization of the TSHR for biodiesel production suggested in this research. It should be noted that these reaction conditions were utilized for synthesize the volume of raw material needed to perform the experiments for evaluation of the second reaction. Nonetheless, aiming to validate the results obtained for this situation, new assays (in triplicate) were performed, obtaining a FAME yield of 70.8 ± 1.6 % after 4 h of reaction, a value close to that predicted by the quadratic regression, indicating that the model suggested is apt to describe the data collected.

4.3.2 Optimization of the Second Reaction of the System

To investigate the parameters of the second reaction of the system proposed in this work, the feedstock utilized as substrate was produced using pre-established reaction conditions obtained from the results for *Reaction #1*, where a FAME yield of 70.8 % was obtained after 4 h of reaction with an AV of 8.3 mg $KOH \cdot g^{-1}$. Consequently, in the second reaction of the system, there is around 30 % of material that still needs to be esterified into fatty acids methyl esters. In this sense, in order to not use unnecessary amounts of reagent, the amount of methanol added in each assay performed of the *Reaction #2* optimization is equivalent to 30 % of the MeOH to oil molar ratio shown in each level of the experimental design proposed (Table 3). For the lipase load applied in the reaction, the mass percentage added refers to the total mass of feedstock that is being processed. In time, it should be noted that the reaction parameter "water concentration added to the system" was fixed at 2 wt% for second experimental design since it was observed, through preliminary tests, that this parameter has substantial influence only in the *Reaction #1*. The addition of an amount of water higher than 2 wt% in the *Reaction #2*, besides being unnecessary since the substrate theoretically would already be hydrolyzed in its totality, would lead to the formation of a product with a high acid value.

According results obtained experimentally, the model proposed for Reaction #2 is presented in the equation (4), considering only the significant variables:

$$Y_2\ (\%) = 90.137 + 2.678x_4 + 1.213x_5 \quad (4)$$

where Y_2 is the biodiesel yield for the *Reaction #2*, x_4 is the methanol to oil molar ratio used for this reaction and x_5 is the lipase load, where coefficients with a positive sign designate a synergic influence under the response variable.

The biodiesel yields experimentally obtained and predicted by the regression are presented in Table 3. The fit of the model suggested for the *Reaction #2* of the system in order to determine the statistical significance of the achieved results was determined by ANOVA, presented in Table 4. For the FAME yields obtained for samples collected at the end of the second reaction, 95.70 % of the variability of these results can be explained by the regression, showing a good fit, since the coefficient of determination for this situation was 0.9570 and the coefficient of variation was 0.51 %, indicating a good accuracy, reliability and reproducibility of the tests. The quadratic regression suggested for the response variable had a F_{value} of 22.22 and a *p*-value less than 0.0001, indicating with a reliability level of 95 %, that the model is significant, since the tabulated F-value is F_{tab} (5, 5, 5 %) = 5.05 ($F_{value} > F_{tab}$). From the ANOVA presented in Table 4, it was concluded that, for the second reaction of the TSHR system proposed, both the "molar ratio between MeOH:oil" applied to the *Reaction #2* and the "enzyme load" had a positive influence on biodiesel yield; while the quadratic parameters of the independent variables and the interaction between "MeOH to oil molar ratio and lipase load" did not present a significant effect under the response variable.

An interesting point to note from the ANOVA is the considerable significance of the linear parameter for the MeOH to oil molar ratio (x_4), where according presented in the previous section, this parameter had not presented a significant effect on the response variable Y_1 (FAME yield at the end of *Reaction #1*). In the other hand, for the second reaction, the MeOH to oil molar ratio showed significance comparable to the linear parameter of the variable x_1 ("water concentration") at the end of *Reaction #1*, evidencing that for the TSHR system proposed, the hydrolysis of the substrate has a more substantial role in the *Reaction #1* and the esterification process in the *Reaction #2*. Nevertheless, according to results presented, a considerable proportion of the hydrolyzed substrate in *Reaction #1* was esterified in FAME at the end of the first reaction.

Table 3. CCRD applied on the second reaction for the system proposed, with the results obtained and predicted by the model besides the acid value measured for each assay.

Assay	**Independent variables**		**FAME yield, Y (%)**		**Acid value, AV (mg KOH·g^{-1})**	**Productivity (g FAME·g enz^{-1})**
	Methanol to oil molar ratio (mol/mol), x_4	Lipase load (wt%), x_5	Obtained	Predicted		
18	3.4:1 (-1)	0.24 (-1)	85.6	86.3	5.7 ± 0.3	351.5
19	5.6:1 (+1)	0.24 (-1)	91.5	91.9	5.0 ± 0.2	375.7
20	3.4:1 (-1)	0.46 (+1)	88.4	89.0	5.7 ± 0.3	193.7
21	5.6:1 (+1)	0.46 (+1)	93.8	94.1	5.3 ± 0.2	205.4
22	3.0:1 (-1.41)	0.35 (0)	86.9	86.2	6.3 ± 0.4	248.4
23	6.0:1 (+1.41)	0.35 (0)	94.1	93.8	4.6 ± 0.2	268.9
24	4.5:1 (0)	0.20 (-1.41)	89.5	89.0	5.4 ± 0.2	447.7
25	4.5:1 (0)	0.50 (+1.41)	92.8	92.4	5.1 ± 0.2	185.7
26	4.5:1 (0)	0.35 (0)	90.9	90.1	5.8 ± 0.2	259.7
27	4.5:1 (0)	0.35 (0)	89.7	90.1	5.8 ± 0.3	256.2
28	4.5:1 (0)	0.35 (0)	89.8	90.1	5.8 ± 0.2	256.8

Table 4. ANOVA for the FAME samples at the conclusion of the second reaction.

Factor	Sum of Squares	Degree of Freedom	Mean Square	F-value	p-value
Model	69.47	5	13.89	22.22	< 0.0001[a]
MeOH, x_4	57.22	1	57.22	91.52	0.000211[a]
$x_4.x_4$	0.022	1	0.022	0.04	0.858582[b]
Lipase load, x_5	11.73	1	11.73	18.76	0.007489[a]
$x_5.x_5$	0.406	1	0.406	0.65	0456791[b]
$x_4.x_5$	0.084	1	0.084	0.13451	0.728812[b]
Residual	3.126	5	0.625	-	-
Total	72.691	10	-	-	-

[a] Significant at "Prob > F" less than 0.05

[b] Insignificant at "Prob > F" more than 0.05

Coefficient of variation (CV) = 0.51 %; standard deviation = 0.46; R^2 = 0.9570

In the same manner that was performed for the first reaction, it was utilized contour surfaces to investigate the effect of the considered reaction parameters on the FAME yield of the process. Figure 3 describes the behavior of the *Reaction #2* for the variations of the parameters "methanol to oil molar ratio" (x_4) and "lipase load" (x_5). For the second reaction stage of the TSHR system, the FAME yields varied between 85.6 and 94.1 %, where the lowest value obtained when it was use simultaneously a low molar ratio of MeOH:oil (3.4:1) and biocatalyst load (0.24 wt%). The yield of 94.1 % was reached when the maximum molar ratio between alcohol and substrate was used (6.0: 1) together with an intermediate lipase load (0.35 wt%), resulting in a productivity of 268.9 gram of FAME produced by gram of lipase in the *Reaction #2*.

The optimized reaction conditions for *Reaction #2* of the TSHR system employing the NS40116 lipase, considering the response variable "FAME yield", were: methanol to oil molar ratio (x_4) of 6.0:1 and 0.50 wt% of lipase (x_5), providing a predicted FAME yield of 95.8 %. New experiments (in triplicate) were accomplished to validate the result: 97.1 ± 1.1 % of FAME yield was obtained after 8 h of reaction, a value close to that predicted by the model, indicating a good accuracy and representativeness.

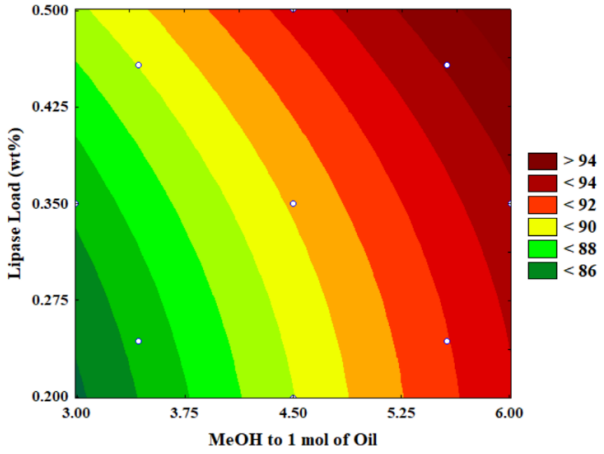

Figure 3. Response of the biodiesel yield after the *Reaction #2* varying the parameters "MeOH to oil molar ratio" and "lipase load".

4.3.3 Time Course of the Two-step Hydroesterification System

After obtaining of the adequate reaction conditions, new experiments using these parameters was performed, aiming to analyze the conversion of the raw material in FAME and the behavior of the FFA reduction according to the reaction development. For *Reaction #1*, 6.0 wt% of water, 0.2 wt% of NS40116 lipase and a methanol to oil molar ratio of 4.5:1 were used; and for *Reaction #2* were employed 2.0 wt% of water, 0.5 wt% of NS40116 lipase and 30 % of the stipulated amount of methanol for a molar ratio between alcohol and oil of 6.0:1, according to previously analyzed to complete the substrate conversion in FAME. Figure 4 presents the time course of the two-step hydroesterification reaction system obtained. The experiments followed the steps of the process shown in Figure 1, where a FAME yield of 97.1 % was obtained with the biofuel samples presenting an average AV of 4.6 mg KOH·g^{-1} after 8 h of reaction.

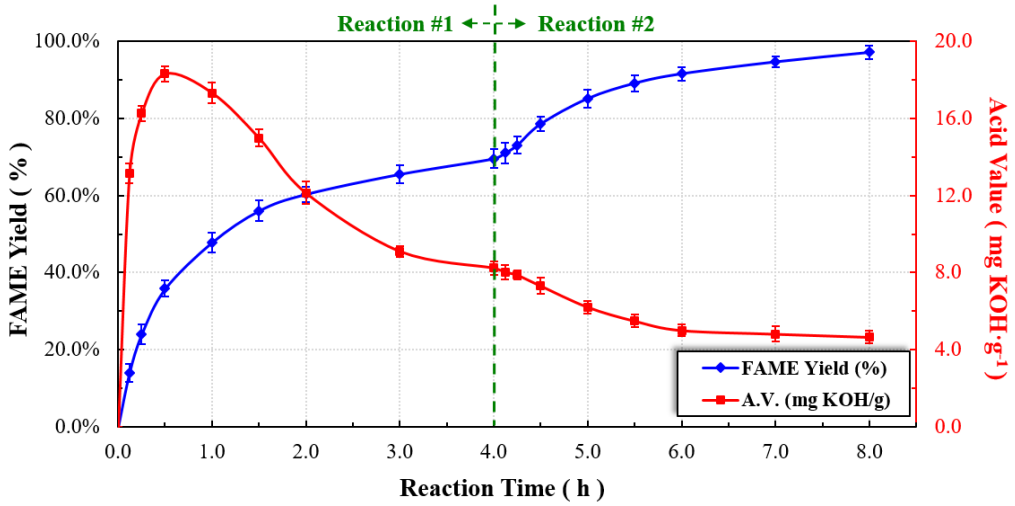

Figure 4. Time course of the two-step hydroesterification reaction system proposed. Reaction conditions: 0.2 wt% lipase, 6.0 wt% distilled water and 4.5:1 MeOH to oil molar ratio in the first reaction; in the second reaction was utilized 0.5 wt% lipase, 2.0 wt% distilled water 6.0:1 MeOH to non-esterified feedstock from *Reaction #1*.

4.4 DISCUSSION

4.4.1 Influence of the Reaction Parameters on the Yield of the *Reaction #1*

This research proposes a reaction system that aims to increase the reaction rate of an enzymatic production of FAME using commercial lipases in liquid formulation as process biocatalyst and, consequently, enhance the productivity of the biodiesel production for the process. The first reaction of the TSHR system demonstrated to be the key to obtaining a satisfactory yield at the end of the process, since the substrate hydrolysis (conversion of the TG in FFA) require to be effective in order that the free fatty acids generated are, in as much as possible, converted into FAME.

Results showed that the employment of low concentrations of water to the first reaction (below 3.0 wt%) was not beneficial to the system (Figure. 2a). Such results are associated with the circumstance that using a low concentration of water simultaneously with a high molar ratio between MeOH and substrate, the enzyme are being submitted to a critical situation to cause its denaturation and inhibition (Amini et al. 2017), impairing the catalytic capacity of the lipase and prejudicing the process yield. In relation to the low FAME yields achieved for the experiments where was utilized the combination of low water concentrations (below 3.0 wt%) with low MeOH:oil molar ratios (below 3.5:1), it is possible to associate these results to the fact that for this range, both variables are very close or below of the values stoichiometrically required to hydrolyze the TG of the substrate and esterify the FFA produced in this hydrolysis (stoichiometric amounts: 6.0 wt% of water and 3.0:1 molar ratio of MeOH:oil). Thereby, being these reversible reactions, an “excess” of these inputs are necessary to dislocate the reaction equilibrium towards the products (Wancura et al. 2018b). In this sense, high FAME yields (above 75 %) could be obtained for a narrow range of MeOH to oil molar ratio (between 4.5:1 and 5.3:1) and a wide range for the variable x_1 (between 6.0 wt% and 9.0 wt% of water). For water concentrations higher than 9.0 wt%, a decrease on FAME yields was observed due to a favoring of the substrate hydrolysis reaction (Cesarini et al. 2013).

Analyzing the Figure 2b, it was observed again that low concentrations of water in the

medium (below 3.0 wt%) do not lead to satisfactory FAME yields, unless that a high amount of enzyme be applied on *Reaction #1* (at least 0.50 wt% of NS40116 lipase). With the increase of the amount of water added to the medium and consequently increasing the hydrolysis reaction rate, an improvement in the system response was observed, where satisfactory results were obtained for water concentrations between 6.0 and 8.0 wt% (FAME yields > 78 %). Evidently, with the increase of the lipase load within of the range where the parameter "water concentration" provided satisfactory results (6.0 to 8.0 wt%), it was observed an increase in the FAME yield, insofar as lipases acting as biocatalysts, reduce the activation energy required to form the product of interest, rising the velocity of the reaction. Notwithstanding, with the increment of the lipase load applied to the system, the process productivity (mass of FAME produced by mass of lipase employed) decreases. Furthermore, interesting FAME yields (around 70 %) can be obtained with low lipase loads (less than 0.25 wt% of lipase) since at least 6.0 wt% of water be added to the reaction, providing an interesting productivity of approximately 360 g FAME·(g enzyme)$^{-1}$. However, it is observed that even with high lipase loads (above 0.43 wt%), there was a decayment in the values of FAME yield when more than 9.0 wt% of water was added to *Reaction #1*, which is related with the disequilibrium of the reaction system in favor of the substrate hydrolysis in detriment of the esterification, according reported by Antczak et al. (2009).

Evaluating the results presented in Figure 2c, it was noted that the utilization of a molar ratio between MeOH and oil inferior to 3.5:1 on *Reaction #1* do not provide acceptable results (FAME yield < 60 %) even with the application of high biocatalyst loads. On the other hand, interesting FAME yields (> 80 %) can be achieved for a low quantity of lipase (below 0.35 wt%) since intermediate amounts of water (around 6.0 wt%) and MeOH (molar ratio of 4.5:1) are employed, where a gradual increase in the lipase load leads to a rise in the FAME yield, reaching values greater than 85 % when a lipase load of 0.50 wt% is utilized. An explanation for this situation is that with the addition of an appropriate amount of water in the process, there is the formation of a suitable interfacial area for the action of the enzyme, not compromising its catalytic function within the reaction (Pedersen et al. 2014). When high molar ratios between MeOH and oil are utilized (higher than 5.4:1), satisfactory yields only are obtained with the application of a high lipase load (more than 0.45 wt%). The combination of low enzyme loads (less than 0.30 wt%) with high MeOH to oil molar ratios (more than 5.4:1) also did not result in satisfactory yields compared to the other assays. Such results would be associated with the high MeOH concentration in the medium, affecting the lipase activity (causing its denaturation) and consequently harming the FAME yield (Guldhe et al. 2015).

4.4.2 Influence of the Reaction Parameters on the Yield of the *Reaction #2*

Evaluating the results presented (Figure 3), it is observed that for MeOH to oil molar ratio less than 3.75:1 it was not possible to obtain FAME yields higher than 90 % even for a high lipase load added to the reaction. The combination of low amounts of lipase (less than 0.28 wt%) with low MeOH to oil molar ratio added to the reaction medium (less than 3.5:1) also did not result in satisfactory yields compared to the other assays. Satisfactory FAME yields (Y_2 > 92 %) could be obtained using an intermediate lipase load of 0.35 wt% since the MeOH molar excess higher than 5.25:1 were used in the reaction. Since for *Reaction #2* was adopted the strategy of adding only the amount of methanol enough for esterify the non-converted material into biodiesel in the first reaction, the higher FAME yields were obtained for the higher MeOH to oil molar ratio employed. This behavior is the opposite of was observed for the first reaction, where high molar ratios of methanol and oil inactivated the biocatalyst and consequently leading to unsatisfactory FAME yields. Lotti et al. (2015) report that the problem of the inactivation of lipases by methanol can be associated to the fact of the low alcohol solubility in TGs, where insoluble drops of methanol denature the enzyme, harming its catalytic capacity, where only for mixtures with less than 4 % of biodiesel in methanol, some solubilization of biodiesel in water and methanol takes place (Santos et al. 2018). According the reaction progress and with the formation of FAME, higher methanol concentrations can be added to the process since the alcohol solubility is higher in FAME than in TG (Salihu and Alam 2015). Therefore, since the amount of alcohol utilized in the system is low (just enough to esterify the 30 % of non-converted substrate from Reaction #1), 2 wt% of water added in the beginning of *Reaction #2* seems to be sufficient to maintain the concentration of MeOH in the medium to a non-detrimental point to the lipase activity.

According Table 3, a higher FAME yield does not necessarily entail a high productivity of the system. Even though assays where a higher amount of lipase has been used result in higher FAME yields, the productivity was considerably lower. Concerning the variable “lipase load, x_5”, an increase in the amounts of lipase added to the system led to higher FAME yields. However, if the productivity of the system per gram of lipase utilized is evaluated, keeping the variable “methanol excess” at the center point (x_4 = 4.5:1), 447.7, 257.6 and 185.7 g of FAME was produced by gram of lipase for 0.20, 0.35 and 0.50 wt% of biocatalyst respectively. In other words, a productivity 2.4 times higher when 0.2 wt% of enzyme was added compared to the assay where 0.5 wt% was utilized. Also, evaluating the results of productivity obtained when 0.35 wt% of NS40116 lipase was used (center point of the parameter x_5), it has been 248.4 g,

257.6 g and 268.9 g FAME produced per gram of lipase at a methanol to oil molar ratio of 3:1, 4.5:1 and 6:1 respectively, that is, similar productivities. Notwithstanding, even though clearly the process productivity is interestingly superior when 0.2 wt% of lipase was utilized in both reactions of the system, it cannot be ignored the fact that the FAME yield in the end of the process was of 89.54 % (for a MeOH to oil molar ratio of 4.5:1 added in both reactions), an inferior value to the required by regulatory standards. Consequently, larger amounts of inputs (values closer to the superior extreme of the experimental design shown in Table 3) need to be added to *Reaction #2* aiming a higher conversion of the feedstock. From an economic point of view, the only hindrance would be in relation to the biocatalyst, since methanol can be recovered from the biodiesel stream by evaporation and reutilized in the process.

4.4.3 AV for the FAME Samples

When soluble/liquid lipases is used in the biodiesel production and necessarily with the addition of water in the system, the acid value evaluation of the FAME generated in the process becomes needed, since the hydrolysis of the TG that constitute the substrate forms FFA, which are not converted into FAME in their totality. High AV detected in biodiesel samples indicate an acceleration in the deterioration of the biofuel, besides leading to the formation of deposits and soap in diesel engines (Xue et al. 2011; Rajkumar and Thangaraja 2019). Nevertheless, it should be emphasized that the water added to the process responsible for the feedstock hydrolysis is fundamental to maintain an active reaction interface, which benefits the biocatalyst action, in addition to minimizing the denaturing of the lipase caused by the methanol (Rosset et al. 2019). In this sense, it was determined the AV for each assay accomplished on the reaction parameters investigation of the TSHR proposed.

In relation to the first reaction of the process, the acid values of the FAME samples are presented in the Table 1. Considering the central point of the variable “water concentration” (x_1 = 6 wt%, corresponding to the stoichiometric amount required to hydrolysate the TG of the substrate), the results for the acid value were in the order of 8.0 mg KOH·g^{-1}. For an amount of water added to the process of 6.0 wt%, AV lower than aforementioned only were reached when a high lipase load or a high molar ratio of MeOH:oil was employed in the reaction (assays 12 and 14). In the same trend, even considering a water concentration of 6.0 wt% added to *Reaction #1*, low lipase loads and methanol to oil molar ratios presented acid values of 8.3 (assay 13) and 11.7 mg KOH·g^{-1} (assay 11) respectively, values higher than that obtained for the central point of the experimental design (assay 15 to 17). Analyzing the lower extreme point of the

variable x_1 (assay 9), it was observed that the addition of 2.0 wt% of water to the system resulted in the lowest acid value among the assays (5.4 mg KOH·g^{-1}) but the lower FAME yield (49.2 %) also, an expected result which may be associated with the lipase inhibition caused by high methanol concentration in the experiment. The assays 1, 3, 5 and 7, where the water was added in amounts lower than 6.0 wt%, it was observed that the acid values were slightly lower compared to the central point of the experimental design (assay 15 to 17). An exception for this trend was the assay 1, with an acid value of 8.9 mg KOH·g^{-1} and a modest FAME yield of 53.9 %, which may be associated with the lower amounts of alcohol and lipase added to the system for this test. Experiments that the water concentration added was higher than 6.0 wt% (assays 2, 4, 6, 8 and 10) presented interesting FAME yields, however, high acid values (between 7.5 and 10.6 mg KOH·g^{-1}), confirming that an excess of water added to the system can increasing the hydrolysis of the raw material however, at the same time, producing a biodiesel with high acidity. The exception for this situation was the assay 8, where the acid value was of 7.5 mg KOH·g^{-1} and the FAME yield of 83.5 %, which may be associated with the relative high MeOH to oil molar ratio and lipase load utilized (5.4:1 and 0.44% respectively).

For the *Reaction #2* of the system, the acid values of the FAME samples are shown in the Table 3. According aforementioned in the previous section, the feedstock of the second reaction of the system presented an AV of 8.3 mg KOH·g^{-1}. Analyzing the assays for the center point of the statistical design of the *Reaction #2*, it is observed that the acid value of the FAME samples remained around of 5.8 mg KOH·g^{-1}, a reduction of almost 30 % of the initial value. In this sense, when evaluating the parameter "methanol excess" used in the second reaction, it is possible to observe a clear coherence and influence of the variable x_4 on the acid value of the FAME samples: with the increase of the amount of methanol added to the reaction, lower the FAME acidity, showing the advance of the esterification reaction according to the higher alcohol excess added to the system. For example, maintaining the lipase load at the center point (x_5 = 0.35 wt%), the highest AV obtained in the assays was for the assay 22 (6.3 mg KOH·g^{-1}), where the MeOH to oil molar ratio was the lowest tested among the experiments (3.0:1). On the other hand, the lowest AV obtained was for the assay 23 (4.6 mg KOH·g^{-1}, a reduction of approximately 45 % of the initial value), which was utilized the highest methanol excess in the second reaction of the system (6.0:1), where this test presented the highest FAME yield (94.1%), evidencing a higher conversion of the FFA in FAME, according to a higher excess of alcohol utilized. Regarding the parameter "lipase load, x_5", it was observed that this variable had little effect on the AV of the biodiesel samples: for example, for a MeOH to oil molar ratio of 4.5:1 (center point), an increase in the lipase load of 0.2 wt% (assay 24) to 0.5 wt% (assay

25) caused a reduction of only 0.31 mg KOH·g^{-1} in the acid value of the product formed. Such behavior is corroborated even for a higher MeOH excess added to the system: considering a MeOH to oil molar ratio of 5.6:1, the increase in the lipase load from 0.24 wt% (assay 19) to 0.46 wt% (assay 21) caused a reduction in the acidity of the FAME samples of only 0.3 mg KOH·g^{-1}.

Once this present research is an improvement of the process evaluated previously, is important to cite that the acid value for the biodiesel samples in that previous work, where the one-step reaction was employed, was around 5.8 mg KOH·g^{-1} after 8 hours (Wancura et al. 2018b). However, it should be highlighted that another commercial liquid enzyme was investigated, with a catalytic capacity inferior than the NS40116 lipase used in this work, which was reflected in the higher acid value obtained in the previous work for the same reaction time and water concentration.

High acid values are commonly reported in studies using soluble lipases in the FAME synthesis. Lv et al. (2017) for example, employing 3 wt% of water, a MeOH to oil molar ratio of 4.2:1 and 1.5 wt% of NS81006 lipase found an AV of 4.9 mg KOH·g^{-1} for a FAME yield of 90 % after 8 h of reaction. Cesarini et al. (2013), after 24 h of process, using 1 wt% of CalleraTM Trans L soluble lipase, 16 wt% of MeOH and 3 wt% of water, achieved an AV of 2.5 wt% for a FAME yield of 96.3 %. Although for the best result obtained, it was observed a reduction in the AV of the FAME samples at the end of the Reaction #2 of 8.3 to 4.6 mg KOH·g^{-1}, these numbers are still much higher than those required by regulatory standards. In this sense, researches recently published has focused on alternatives to reduce the FFA content present in the biodiesel originated from these systems. Uliana et al. (2017) proposed an alkaline washing using aqueous solution of NaOH/MeOH as a way of reducing the biodiesel acidity produced by enzymatic hydroesterification. Employing 35 °C, 1.0 wt% of methanol, molar ratio MeOH:FFA of 1:1 and 3.0 wt% of alkaline solution, the authors aforementioned reduced the acidity of the FAME samples from 3.94 wt% to 0.19 wt% for a final FAME yield of 96 %. In a similar research, Tian et al. (2017) developed a process combining the advantages of soluble and immobilized lipases in a two-step process, converting microalgae oil to FAME: in the first step, the authors utilized the liquid lipase NS81006 to catalyze the methanolysis of the substrate in an oil/water system, where the glycerol generated in this step was removed in order to avoid negative effects in the subsequent reaction, where an immobilized lipase was used; thus, in a second step, the authors utilized the immobilized lipase Novozym 435 to esterify the FFAs generated in the first step and perform the conversion of the non-converted TG of the previous stage also, obtaining a FAME yield of 95 % after 36 h of reaction with a final acid value of 0.5

mg KOH·g^{-1}.

Thereupon, although the TSHR system proposed in this research has shown efficiency in improve the reaction rate of the enzymatic FAME synthesis, a subsequent step to the reaction system aiming to reduce the final acid value of the product obtained is necessary in order to produce a biodiesel within of the required technical specifications, mainly in terms of acidity.

4.4.4 Behavior of the FAME Yield and AV with the Reaction Development

After investigation of the reaction parameters, an analysis of the substrate conversion as well as the acidity of the FAME samples tend to decrease with the development of the process is fundamental to conclude if the process efficiency was satisfactory. Evaluating the Figure 4, it is possible to observe that the conversion rate of the feedstock in the first hours of process is considerably elevated, where a FAME yield of 60.3 % was achieved after 2 h and 70 % at the end of the *Reaction #1* (after 4 h). Between 2 and 4 h of process, it is possible to verify a reduction in the increment of FAME yield in comparison to the first two hours of reaction. In the second reaction of the system, with the separation of the heavy phase (rich in glycerol and water) and forcing a displacement in the reaction equilibrium toward the formation of methyl esters, besides the addition of fresh inputs, a rise in the FAME production rate is observed between 4.0 and 4.5 h of reaction, achieving a FAME yield of 91.6 % after 6 h and finishing the process with a FAME yield of 97.1 % after 8 h of reaction. In relation to the acid values of the FAME samples, it is possible to observe that the values increase considerably in the first hour of reaction, reaching a peak around 18.3 mg KOH·g-*1*, since the presence of water in the system is responsible for hydrolyzing a part of the TG of the feedstock in FFA. From the first hour of reaction, the acidity of the samples decreased quickly, according the FFA generated are esterified in FAME, reaching an acid value around 8.25 mg KOH·g^{-1} after 4 h with the conclusion of the *Reaction #1*. On the second reaction, a slow decrease in the acidity was observed in the first half hour of reaction, which may be associated to a new insertion of distilled water into the system. From this moment, at a lower rate of decrease than was verified in the first reaction, the acidity of the samples declined gradually, reaching a final value of 4.6 mg KOH·g^{-1} after 8 h of process, with the reaction equilibrium achieved.

Only a few part of the papers published in the literature addresses the use of lipases in soluble/liquid/free formulation as a catalyst for a synthesis of biodiesel. In a study conducted by our research group using the lipase NS40116 (Rosset et al. 2019), a FAME yield of 94.30 % was achieved at 35 °C, applying 0.50 wt% of enzyme, 15 wt% of water, and a methanol to oil

molar ratio of 4.5:1 in an one-step-12 h hydroesterification. Another work performed by our group (Wancura et al. 2018b), using another commercial free enzyme, 96.9 % of FAME yield was achieved using soybean oil as feedstock at 35 °C, MeOH to oil molar ratio of 4.5:1, 6 wt% of water added to the system and 1.45 wt% of Callera^{TM} Trans L soluble lipase in an one-step-24 h hydroesterification reaction. In the present research, using the system suggested of two hydroesterification reactions in series, the highest FAME yield reached was 97.1 % at 35 °C, a total of 8 wt% water, 6.3:1 of molar excess of methanol and 0.70 wt% of NS40116 lipase applied to the two reactions in only 8 h of the process. In other words, with the TSHR proposed, it was possible to reduce in 4 h the total reaction time in comparison with our previous work and achieve a two-thirds reduction in the reaction time to obtain a similar FAME yield in relation to the Callera Trans L-mediated hydroesterification process. Such efficacy can be associated with the strategy of adding at least 6 wt% of water into the first reactor of the system, in order to accelerate the hydrolysis of the raw material, initiating, still in this reactor, the esterification of the substrate, achieving satisfactory FAME yields already in the first reaction of the system.

In another research, Remonatto et al. (2016) obtained 97 % of FAME yield using 2.5 wt% of water, methanol to substrate molar ratio of 4.5:1 and 1 wt% of Eversa® Transform soluble lipase after 16 h of reaction. Comparing the results of these authors with the obtained in our present research, it is possible to observe that the system of reactions suggested was able to achieve a similar FAME yield with half of the reaction time and lipase load added to the process. A reaction rate similar to the results presented in our present research was reached by Andrade et al. (2017): 94 % of FAME yield from castor oil in 8 h of reaction at 35 °C, MeOH to oil molar ratio of 6:1, 5 wt% water and, however, 5 wt% of Eversa® Transform lipase added to the reaction, an amount of catalyst seven times higher than what was utilized in the best conditions found in our research.

Considering that one of the main drawbacks for a future scale-up of the enzymatic biodiesel production is the low reaction rates of the process (resulting in long reaction times), the suggested system in this research appears as an interesting alternative. Since the reactions involving the hydroesterification process has a reversible character, the removal of the glycerol formed in the *Reaction #1* of the system induced a shift of the reaction equilibrium towards the biodiesel (FAME) formation, favoring the process yield. In addition, results of researches which presented time curves for the FAME yield show that after 4 to 6 hours of reaction, the catalytic capacity of the lipase begins to be affected due to the inhibitory effects of the alcohol. Therefore, the process being share in two steps of 4 h instead of a batch of 16 or even 24 h, adding fresh

lipase in the beginning of the second reaction, demonstrated to be a positive factor for the system. However, the high acid values of the FAME samples at the end of the process showed to be necessary the insertion of a subsequent stage to the suggested reaction system exclusively to reduce the acidity of the bioproduct. Nevertheless, the results obtained demonstrated that the novel low-cost NS40116 lipase is an efficient biocatalyst able to be applied in the enzymatic synthesis of biodiesel.

4.5 ACKNOWLEDGMENTS

The authors thank CNPq (National Council of Technological and Scientific Development, 428180/2018-3) and FAPERGS (Research Support Foundation of the State of Rio Grande do Sul, 16/2551-0000522-2) for the financial support of this work, as well as CAPES (Coordination for the Improvement of Higher Education Personnel) for scholarships. M. A. Mazutti and M. V. Tres (308936/2017-5) thank CNPq for the productivity grants.

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5 PRODUÇÃO SEMI-CONTÍNUA DE BIODIESEL EM ESCALA PILOTO VIA HIDROESTERIFICAÇÃO ENZIMÁTICA DE MATERIAL RESIDUAL: CONSIDERAÇÕES DE PROCESSO E ECONÔMICAS

Neste capítulo estão apresentados os resultados relacionados ao cumprimento do terceiro objetivo específico desta tese. Através de todos dados e resultados levantados durante o período desta pesquisa, pode-se realizar uma ampliação de escala de produção de biodiesel via hidroesterificação enzimática catalisada por diferentes lipases em formulação líquida. No entanto, nesta oportunidade, utilizou-se óleo de cozinha residual como matéria-prima do processo.

Semi-continuous Production of Biodiesel on Pilot Scale via Enzymatic Hydroesterification of Waste Material: Process and Economics Considerations

DOI: https://doi.org/10.1016/j.jclepro.2020.124838

João H. C. Wancura[a], Antonio L. Fantinel[b], Gustavo A. Ugalde[a], Filipe F. Donato[c], J. Vladimir Oliveira[d], Marcus V. Tres[e], Sérgio L. Jahn[a]

[a] Department of Chemical Engineering, Federal University of Santa Maria, Santa Maria, RS, Brazil;

[b] Center of Studies and Researches in Agribusiness (CEPAN), Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil;

[c] Department of Chemical and Food Engineering, Federal University of Santa Catarina, Florianópolis, SC, Brazil;

[d] Laboratory of Agroindustrial Processes Engineering (LAPE), Federal University of Santa Maria, Cachoeira do Sul, RS, Brazil.

ABSTRACT

Nowadays, the use of edible oils with elevate purity in the biodiesel production represents around 70 % of the chain of feedstocks applied in the process by the main producing countries at world level. This occurs due, mainly, to reaction limitations of the traditional alkaline route employed industrially. Bypassing drawbacks of the chemical process, enzymatic technique is an effective alternative for the biodiesel synthesis. In this sense, this paper reports results to semi-continuous production of biodiesel on pilot scale, from waste cooking oil, *via* hydroesterification mediated by different commercial lipases in liquid formulation: Eversa® Transform and its novel thermostable successor Eversa® Transform 2.0. Data for yields,

residual acidity, productivities, biocatalysts performance, kinetics and economic analysis are presented in this comprehensive research. Using the best reaction conditions found (40 °C, 0.7 wt% of Eversa® Transform 2.0, 4.0 wt% of water, methanol to oil molar ratio of 6.3:1 and 8 h at a two-step reaction system), 96.2 % of FAME yield was achieved. A pseudo-first order model presented a good fit of the experimental data, where a k_{App} of $0.373 \cdot h^{-1}$ was obtained for the process with the best performance. Moreover, for this process, an economic analysis indicated the feasibility of the system through a positive net return and operating cost of US$ $0.50 \cdot kg^{-1}$ of biofuel. This information served as foundation to conclude that enzymatic hydroesterification catalyzed by liquid lipases has the necessary tools to be implemented on industrial biodiesel production.

Keywords: Biodiesel; Liquid Lipase; Waste Cooking Oil; Enzymatic Hydroesterification; Pilot Scale; Costs Analysis.

5.1 INTRODUCTION

Report disclosed by the World Meteorological Organization (WMO) at the 25th Climate Change Conference (Madrid, December 2019) highlights that 2019 ends an alarming decade in terms of historic increase in the average temperature of the planet, melting of polar ice caps and record in the elevation of the seas level (WMO, 2019). This environmental problems were boosted mainly by greenhouse gases expelled by human activities, among them the burning of fossil fuels in diesel engines. In this context, biodiesel emerged in the global energy matrix as an alternative to his “concurrent” derived from petroleum, becoming an indispensable component of the diesel. Biodiesel (fatty acid methyl esters, FAME) is a fuel composed of mono-alkyl esters, biodegradable, non-toxic, sustainable and environmentally friendly (Galadima and Muraza, 2020). Governmental policies proposed by different countries in recent years aiming encourage its use are mainly supported on the fact that biodiesel can be obtained from renewable sources and to act reducing the emission of gases that accelerate the greenhouse effect (Zheng et al., 2020; Hatzisymeon et al., 2019). In Brazil for example, the second largest producer of biodiesel in the world, resolution moved by the government in October 2018 established schedule for a gradual increase in the volume of biodiesel to be mixed in the diesel. According this program, the mixture will reach 15 vol% in 2023, which will entail growth of 85 % in the domestic demand and an annual production that will exceed 10 billion of liters (Brazilian Council of Energy Policies, 2018).

To obtain biodiesel, an oleaginous source react with a short-chain alcohol (usually methanol, MeOH) in the presence of a catalyst. Industrially, the main route of the biofuel synthesis is through homogeneous alkaline transesterification, where under 60 °C, 0.5 wt% of sodium methoxide (catalyst), methanol to oil molar ratio of 6:1 and 1 h of reaction, biodiesel yields superior to 97 % can be obtained (Knothe and Razon, 2017). However, to achieve such performance, it is necessary to use a raw material with high purity (acidity and moisture preferably less than 0.5 wt% and 400 ppm, respectively) in order to avoid side reactions of saponification (Christopher et al., 2014). Feedstock with indexes of purity so rigorous and consequently with a high aggregate value, fit in a grade close to alimentary, increasing production costs. The choice of the raw material plays fundamental role in the process viability, once this input can correspond to up 75 % of the production total cost (Gebremariam and Marchetti, 2018). For this reason, the option of using a waste raw material with high level of impurities, low grade and consequently low cost has crucial importance for the process (Coppini et al., 2019). Data released by Brazilian Association of Vegetable Oils Industries highlights that the use of soybean oil reached 67.5 % of the amount of raw material destined for the biofuel production in 2019 for this country, with the use of waste materials inferior to 28 %, as presented in Figure 1 (Brazilian Association of Vegetable Oils Industries, 2019).

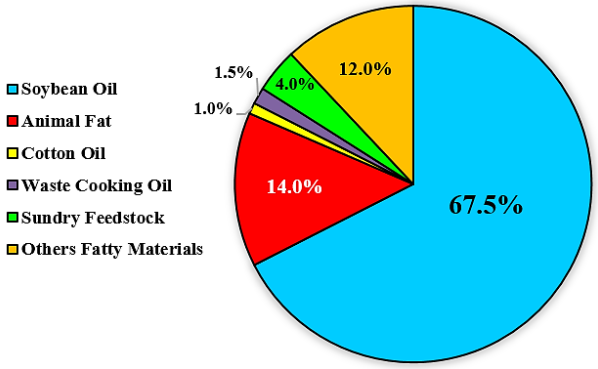

Figure 1. Distribution of the raw materials applied in the Brazilian biodiesel production in 2019.

In this scenario, the enzymes-catalyzed process emerges as an interesting alternative for the biodiesel preparation. Acting under milder reaction conditions, the employment of lipases (*triacylglycerol ester hydrolases*, EC.3.1.1.3) allows the use of low-purity inputs to produce a high-quality biofuel, since these biocatalysts are tolerant to raw materials with high acidity and moisture, having the ability of simultaneously convert the triglycerides and free fatty acids (FFA) of the substrate in mono-alkyl esters (Sun and Li, 2020; Wancura et al., 2019c).

Currently, researches have been focused on improving the lipase properties through

different techniques. Microbiology has made interesting advances on the development of new enzymes, some of them of unusual origin, however with new and interesting particularities (Facin et al., 2019). Facin et al. (2019) and Hama et al. (2018) describe that genetic tools, such as mutagenesis and directed evolution have enhanced the enzyme properties for specific problems, mainly stability in aggressive systems. For this reason, obsolete techniques for preparation of biocatalysts such as immobilization and chemical modification have slowly losing relevance, opening ways for the use of lipases in free formulation for application in the biodiesel synthesis.

Although the vast majority of the papers about "enzymatic biodiesel" still apply immobilized enzymes as reaction catalyst (Moazeni et al., 2019; Ortiz et al., 2019), lipases in free/liquid/soluble formulation, notwithstanding little explored by scientific researches, can make the whole process more competitive. These type of biocatalysts have a sale price up to 50 % lower than the immobilized form, besides that presenting higher reaction rates, reducing the process time required to obtain a satisfactory yield (Hama et al., 2018; Remonatto et al., 2016). In previous works published by our research group, tests performed on lab scale investigated the soluble lipases-catalyzed biodiesel production (named enzymatic hydroesterification), under different reaction configurations and substrates to define conditions favorable to obtain satisfactory yields (Wancura et al., 2019b, 2019a, 2018b, 2018a). With these parameters in hand, arises the possibility to evaluate the process on a scale superior than laboratory, aiming to expand this alternative pathway for biodiesel synthesis.

This work reports results for a semi-continuous production of biodiesel from waste cooking oil (WCO), on pilot scale, *via* enzymatic hydroesterification using two commercial lipases in liquid formulation – Eversa® Transform (ET) e Eversa® Transform 2.0 (ET 2.0). Data aiming evaluate the processes' performance as well as reaction kinetic and economic considerations are presented proposing to contribute for the consolidation of the soluble lipase-mediated enzymatic route as a promising alternative for biodiesel preparation.

5.2 MATERIALS AND METHODS

5.2.1 Materials

Two commercial enzymes were used in the runs: the lipases in liquid formulation Eversa® Transform and Eversa® Transform 2.0 (also named as NS 40116), both obtained from *Thermomyces lanuginosus* microorganism, with a nominal enzymatic activity of 100 LCLU·g^{-}

[1] and kindly provided by Novozymes Latin America LTDA (Araucária, Brazil). Liquid lipases consist of a solution composed by approximately 3.5 wt% of enzyme, water, glycerol and surfactants. Considering the lipases ET and ET 2.0, the main difference between them is the higher thermostability of the "version 2.0", allowing the system to operate at higher temperatures without affecting overly the lipase activity (Wancura et al., 2019c). Waste cooking oil with 15 wt% of acidity and 0.9 wt% of moisture was collected from local restaurants and used as feedstock. Table 1 shows the distribution of fatty acids for the WCO utilized in the tests and its average molar mass calculated from this composition. Methanol (99.4 % purity, Quimicar, Curitiba, Brazil), ethanol (analytical grade, Sigma-Aldrich, São Paulo, Brazil), sodium hydroxide (analytical grade, Dinâmica, Indaiatuba, Brazil), isooctane and methyl tricosanoate (both chromatographic grade, Sigma-Aldrich, São Paulo, Brazil) are the chemicals utilized in the process and analysis of the samples.

Table 1. Distribution of fatty acids and average molar mass for the waste cooking oil used in the biodiesel production.

Fatty Acid Distribution (wt%)								**Average Molar Mass ($g \cdot mol^{-1}$)**
C16:0	C16:1	C17:0	C18:0	C18:1	C18:2	C18:3	C20:0	
11.6	0.5	0.9	4.1	29.6	48.5	4.3	0.6	873.1

5.2.2 Operations on the Pilot Unit of Biodiesel Production

A pilot plant with a production capacity of 60 L of biodiesel (CheckMatic Automação Industrial, São Paulo, Brazil) was utilized in the research. Figure 2 shows a flowchart with each step and equipment of the unit. The WCO stored in containers (1), before being sent for reaction, goes through a fine mesh screen (2) for removal of sediments. The starting of equipments as well as the adjustment and temperature control of the tanks is performed through a control panel (3). The screened feedstock is pumped into a heating tank (4) where the WCO is heated to the reaction temperature by coil to thus be sent to the reactor-tank (6). Inputs (biocatalyst plus water and alcohol) are added to the process by a secondary tank (5), allowing the semi-continuous addition of methanol to the process in order to minimize the lipase inhibition caused by the alcohol. In the reactor, agitators with 0.25 kW operating at 1,700 rpm mix the inputs at a controlled temperature maintained by coils. After the reaction finishing, the mixture is sent to a tank for evaporation of the unreacted methanol and water (7). The wet methanol is condensed (8) and collected in a container (10). With the evaporation of the alcohol and water, the reaction

medium is sent to a decanter (11) for separation of the biodiesel and glycerol phases. The heavy phase containing glycerol is collected (12) and the light phase rich in FAME is pumped to a biodiesel purification filter (13), containing Amberlite BD10^{TM} Dry organic adsorbent, capable of removing biodiesel impurities such as residual moisture and methanol, acidity, free and bound glycerol (Faccini et al., 2011; Stojković et al., 2014). According to the supplier, 1 kg of Amberlite BD10^{TM} Dry can typically treat between 900 to 1600 kg of biodiesel depending on its impurity level. Finally, the biodiesel produced is stored in containers.

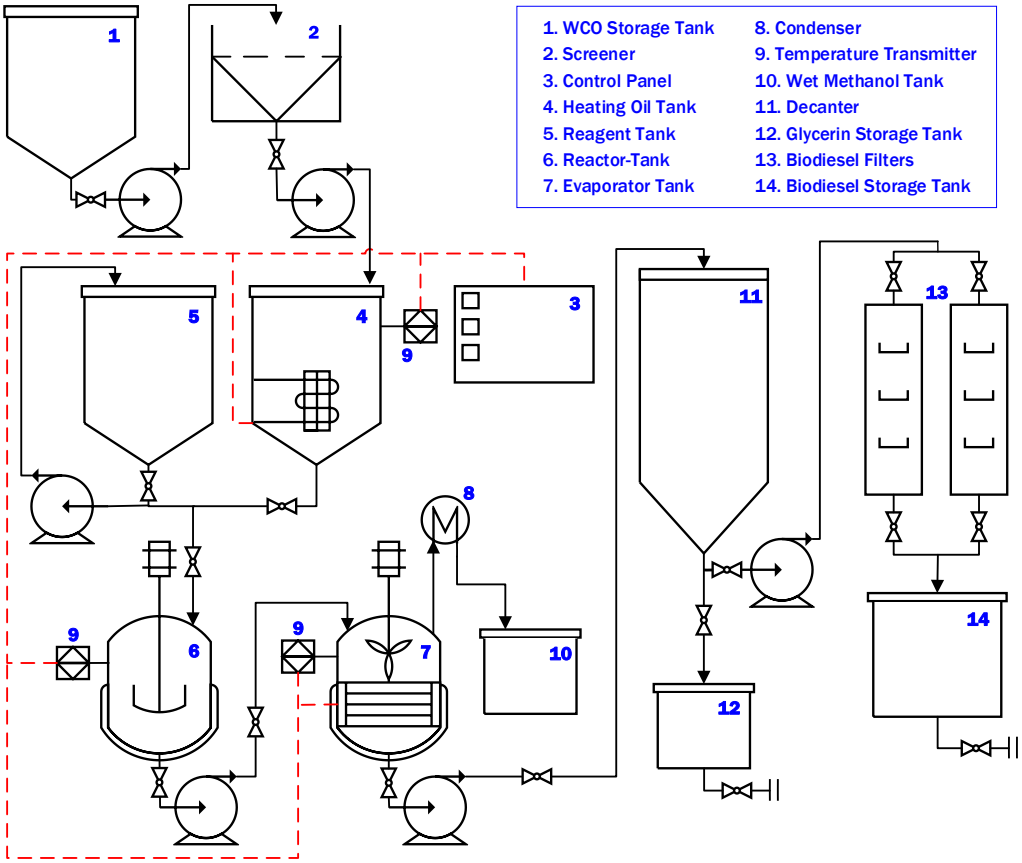

Figure 2. Flowchart of the pilot unit of biodiesel production utilized in the research.

Table 2 presents the reaction conditions employed used in each operation. These parameters were established in tests previously performed on lab scale by our research group (Rosset et al., 2019; Wancura et al., 2019b, 2018b). The process named "*Process nº 1*" represents the evaluation of the soluble lipase Eversa® Transform, while "*Process nº 2*" and "*Process nº 3*" represent the application of the lipase Eversa® Transform 2.0 in the reaction. *Process nº 1* and *nº 2* are conducted in a one-step reaction, while *Process nº 3* configures an enzymatic hydroesterification reaction in two steps. Regarding *Process nº 3*, it is emphasized that the reaction mixture was directed to decantation after the reaction finishing, where after the

Table 2. Reaction conditions adopted to perform the tests of biodiesel production in pilot scale *via* enzymatic hydroesterification.

Reaction Conditions	**Process nº 1**	**Process nº 2**	**Process nº 3**	**Remarks**
Type of reaction system	One-step reaction	One-step reaction	Two-step reaction	
Lipase utilized	ET	ET 2.0	ET 2.0	
Lipase load (wt%) [a]	1.0	0.5	0.7	For *Process nº 3*, 0.5 wt% of lipase was added in the first step and 0.2 wt% in the second one.
Temperature (°C)	40.0	40.0	40.0	
MeOH ($mol \cdot mol^{-1}$) [b]	4.5:1	4.5:1	6.3:1	For *Process nº 3*, 4.5:1 of MeOH was added in the first step and more 1.8:1 in the second one.
MeOH addition [b]	40 % in t = 0 h, 30 % in t = 1.5 h and 30 % in t = 3 h	40 % in t = 0 h, 30 % in t = 1.5 h and 30 % in t = 3 h	40 % in t = 0 h, 30 % in t = 0.75 h and 30 % in t = 1.5 h	For *Process nº 3*, the MeOH stepwise addition is described for each reaction.
Water load (wt%) [a]	2.5	2.5	4.0	For *Process nº 3*, 2.0 wt% of water was added in each reaction.
NaOH (ppm) [a]	100	100	100	Aiming neutralizing possible mineral acids present in the raw material
Reaction time (h)	8.0	8.0	8.0	For *Process nº 3*, was utilized 4.0 h for each reaction.

[a] By weight of WCO processed;

[b] Amounts expressed in methanol to oil molar ratio;

[c] By weight of MeOH added to each process.

phase separation, the upper layer was sent back to the reactor-tank for a new stage of reaction with addition of fresh inputs, according process configuration developed in lab scale by Wancura et al. (2019a). After the conclusion of the second reaction stage, alcohol and water are evapo rated from the mixture biodiesel/glycerol. Each operation presented in Table 2 was performed in duplicate.

5.2.3 Analysis of the FAME Samples

Gas chromatography was employed for FAME quantification. For this, a chromatograph GC-2010 Plus (Shimadzu, Japan), equipped with flame ionization detector and a column Zebron ZB-WAX plus with 30.0 m × 0.32 mm of intern diameter × 0.25 μm of film capillary thickness was used. The methodology of analysis with the temperature program applied is described in our previous work (Wancura et al., 2018b). Samples collected from the reactor-tank were centrifuged at 4,000 rpm, 20 °C and 5 min for separation of the phases FAME/glycerol, where the light phase (FAME) was conducted for analysis. Equation 1 was used to determine the biodiesel yield of each sampled, according provided by EN 14103 (2015):

$$\text{Biodiesel Yield (\%)} = \left(\frac{\sum \text{A} - \text{A}_{\text{IS}}}{\text{A}_{\text{IS}}}\right) \cdot \left(\frac{\text{c}_{\text{IS}} \cdot \text{V}_{\text{IS}}}{\text{c}_{\text{S}} \cdot \text{V}_{\text{S}}}\right) \quad (1)$$

where A is the area detected to the peak of methyl ester; A_{IS} is the area of the peak corresponding to the internal standard (methyl tricosanoate, C23:0); c_{IS} and V_{IS} are the concentration and volume of the solution of internal standard used in each injected sample, respectively; and c_S and V_S are the concentration and volume of FAME solution that was employed in the analysis, respectively.

Determination of the FFA content (wt%) for the FAME samples, an important parameter to be considered in the case of the production of biodiesel *via* enzymatic hydroesterification, was measured according equation 2, defined by EN 14104 (2003):

$$\text{FFA (wt\%)} = \frac{(\text{V} - \text{V}_{\text{B}}) \cdot \text{c} \cdot \text{MM}_{\text{FFA}}}{10 \cdot \text{m}_{\text{S}}} \quad (2)$$

where FFA is content of free fatty acids on the biodiesel sample (wt%), V is the volume of KOH solution consumed on the titration required to neutralize the acidity of one gram of substrate (mL), V_B is the volume of the blank (mL); c is the concentration of the KOH solution ($mol \cdot L^{-1}$); MM_{FFA} is the average molar mass of the free fatty acids ($g \cdot mol^{-1}$) and m_S is the mass of

sample titrated (g).

5.2.4 Kinetic Model

In the kinetic approach, three considerations were assumed:

(i) The reaction of converting triglycerides from an oleaginous source into FAME could be safely considered as obeying a pseudo-first order kinetics (Birla et al., 2012; Freedman et al., 1986; Price et al., 2016; Zhang et al., 2010). As described by Freedman et al. (1986), the overall reaction follows a second order reaction rate law. However, as methanol is usually used in sufficient excess in the process, changes in alcohol concentration can be considered constant. That is, it do not alter the reaction order, behaving, therefore, like a first order reaction. In this sense, it is convenient to assume that the process obeys a pseudo-first order kinetics;

(ii) Aiming to simplify the analysis, it was assumed that the process of converting the triglycerides of the raw material into methyl esters occurred in a single step. Thus, the presence of intermediates (mono- and diglycerides) formed on consecutive reverse reactions was disregard;

(iii) Regarding the inputs, it was assumed that the amounts utilized of biocatalyst and MeOH were sufficient to shift the reaction equilibrium towards the formation of FAME. In this sense, the main reverse reaction could be ignored. Thereby, equation to the reaction rate can be described according proposed by Birla et al. (2012) and Zhang et al. (2010):

$$-r_A = -\frac{d[TG]}{dt} = k_{App} \cdot [TG] \quad (3)$$

where k_{App} is the apparent rate constant of the reaction (h^{-1}) and *[TG]* the concentration of triglycerides in an interval of time *dt*. Assuming that the initial concentration of triglycerides in *t = 0 h* is *[TG,0]* and *[TG,x]* at *t = x h*, the integration of the Eq. (3) gives:

$$\ln[TG,0] - \ln[TG,x] = k_{App} \cdot t \quad (4)$$

Using mass balance, we have that:

$$X_{FAME} = 1 - \frac{[TG]}{[TG,0]} \quad (5)$$

where X_{FAME} is the conversion of the raw material into methyl esters. The equation for the reaction rate could also be expressed in terms of conversion to methyl esters:

$$\frac{dX_{FAME}}{dt} = k_{App} \cdot (1 - X_{FAME}) \quad (6)$$

where after an operation of integration, it is possible to reach the Eq. (7) and obtain the apparent rate constant of the reaction through a linear fit:

$$-\ln(1 - X_{FAME}) = k_{App} \cdot t \quad (7)$$

5.3 RESULTS AND DISCUSSION

5.3.1 Process Using the Soluble Lipase Eversa® Transform

Figure 3 presents results for the yield and FFA content of the FAME samples for the hydroesterification reaction using the lipase ET. In terms of FAME yield, it is possible to notice that the enzyme exhibited interesting catalytic capacity in the first two hours of reaction, reaching 59.3 % of FAME yield. However, after more six hours, an increase of little more than 20 % in the yield was observed and, after 8.0 h of reaction, a final FAME yield of 80.9 % was obtained. The loss in the catalytic activity of the lipase over the reaction course is mainly associated with the denaturing effects of the methanol under the enzyme (Lotti et al., 2015). According research conducted by Tong et al. (2016), the alcohol molecules cause substantial chemical perturbations on enzyme structure, indicating that alcohol-induced inactivation of the lipase is directly linked to the conformational change in its actives sites. Anyway, the conversion curve for the enzymatic hydroesterification using the lipase ET showed a behavior similar to that previously observed in lab (Wancura et al., 2019b).

When a residual substrate is utilized as feedstock for a biodiesel synthesis, an important analysis to be considered is the FFA content of the FAME samples, since this category of oleaginous source has usually high acidity. The analysis of the acidity content indicates the tendency of biodiesel to corrode metals, besides that to being related to the biofuel degradation. Another key point: in the biodiesel preparation *via* enzymatic hydroesterification, the addition of water to the system entails the hydrolysis of the triglycerides forming free fatty acids that, even though in grand part they be converted to acyl esters, still remain amounts that need to be removed later. Moreover, the water addition is essential to maintain the three-dimensional structure of the enzyme, preserving its catalytic capacity and assisting the biocatalyst activation. As can be seen in Figure 3, after 4.0 h of reaction the FFA content of the raw material was reduced from 15 wt% to 3.6 wt% and then to a final value of 2.8 wt% after 8.0 h, a reduction

of more than 80 % of the initial acidity. It is important to note that even though the reaction has been finished, the reaction medium contained at least 2.5 wt% of water which, due to thermodynamic circumstances, prevent the reduction of the FFA content to values below than 2.0 wt%. A similar result, nevertheless on lab scale, was obtained by Remonatto et al. (2016): 3.1 wt% of FFA content (and 97.3 % of FAME yield obtained from waste cooking oil) after 16 h of process using 1 wt% of ET and 2.5 wt% of water added to the system.

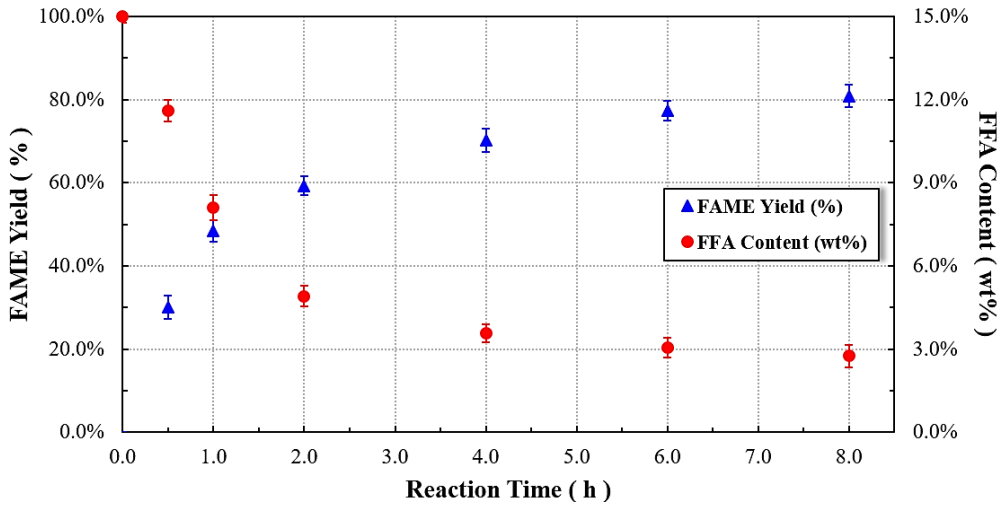

Figure 3. FAME yield and FFA content for the pilot scale biodiesel production using the soluble lipase ET.

5.3.2 Processes Using the Soluble Lipase Eversa® Transform 2.0

Figure 4 presents results for the FAME yield obtained through hydroesterification using the lipase ET 2.0 in two different reaction configurations. For the process named “one-step reaction”, according observed when using the lipase ET, the enzymatic activity of the lipase ET 2.0 in the first two hours of reaction is satisfactorily interesting, reaching a yield of approximately 60 %. Reaction conditions naturally adverse (contact with methanol and temperature action) impact on the catalytic capacity of the biocatalyst, gradually reducing the increments in the FAME yield throughout the process, where after 8 h the yield reached 84.5 %. In other words, in the final four hours of reaction there was an increase in the FAME yield of only 11.5 % against the 73.0 % obtained in the first half of the process. This behavior was also described by us when tests in lab scale were performed (Rosset et al., 2019) and presented by da Silva et al. (2018), where on lab scale, assays were accomplished in order to investigate a hydroesterification catalyzed by the soluble lipase NS 40116 to obtain methyl esters from abdominal chicken fat: 77 % of conversion of was obtained after 24 h of reaction and 0.3 wt%

of enzyme.

In this sense, it has been suggested a reaction configuration modulated in two-steps with potential to circumvent this inconvenience: after 4 h, the reaction is concluded, the FAME and glycerol phases are separated in a decanter vessel and the phase rich in methyl esters is sent to another reaction stage with the addition of fresh inputs (lipase plus water and methanol). This reaction configuration also acts on another limitation of the enzymatic process: the low reaction rates and consequent long intervals of time to achieve a satisfactory biodiesel yield, compared to the conventional alkaline route. The application of this configuration allows to significantly reduce the reaction time necessary to obtain yields comparable to those achieved *via* the chemical route (Wancura et al., 2019a). Also, another advantage of this design is the possibility of inserting a step of phase separation for disposal byproducts with the potential to inhibit the reaction. As can be observed in Figure 4, the process configuration in two-step had a better performance and a final FAME yield of 96.2 % could be achieved after 8 h, that is, the process presented a FAME yield 11.7 % higher than the reaction performed in a single-stage using the same biocatalyst. A similar result was obtained by our research group on laboratory: 97.1 % of FAME yield after 8 h of reaction, however using degummed soybean oil as feedstock (Wancura et al., 2019a).

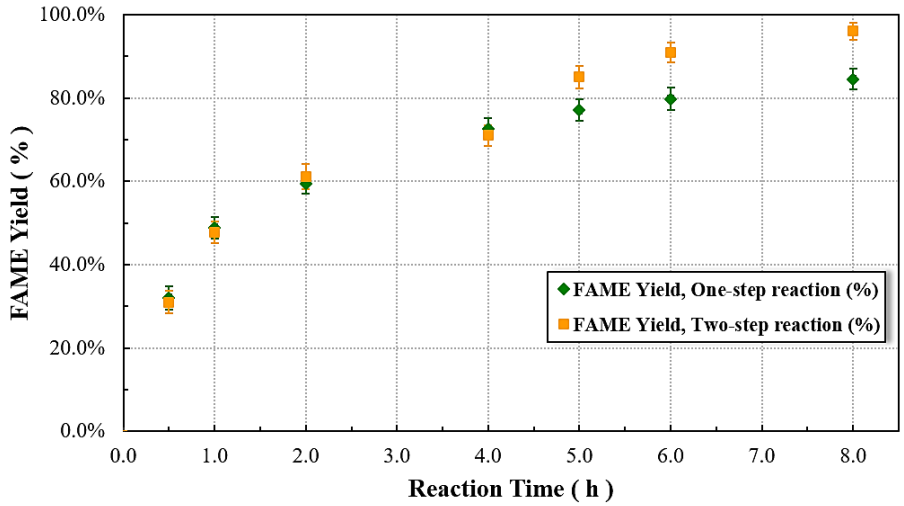

Figure 4. Methyl esters yield for the pilot scale biodiesel production using the lipase in liquid formulation ET 2.0 for two distinct reaction systems.

On the other hand, in terms of reducing the FFA content, the *Process nº 3* (performed in two stages) did not show results significantly better compared to the *Process n º 2* (single-step reaction), according presented in Figure 5: 2.3 wt% against 2.7 wt% of FFA content, respectively. These results may be associated with the fact that in the first reaction, water is

generated according the FFA of the substrate are esterified. Part of this water is conducted to the second reaction, becoming a kind of "barrier" to the acidity reduction to values lower than those obtained. Nonetheless, the values were still slightly lower than that obtained with the lipase ET (*Process nº 1*). Also, such results indicate that a posterior stage aiming the removal of this material is necessary to purify the biofuel.

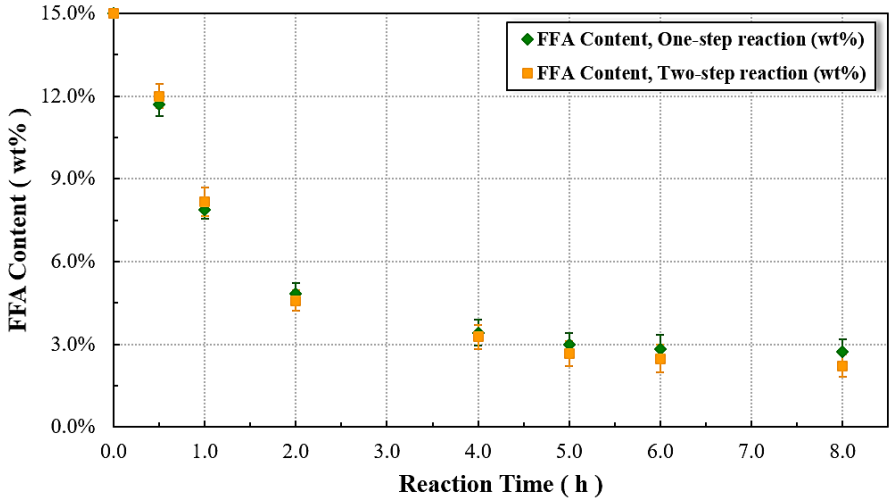

Figure 5. Free fatty acids content for the pilot scale biodiesel production using the liquid lipase ET 2.0 in two distinct reaction systems.

Papers focusing on the production of biodiesel catalyzed by lipases in liquid formulation on a larger scale than the laboratory are rare. Mibielli et al. (2019) evaluated the FAME synthesis in pilot scale using FFA charge from the physical soybean oil refining with 91.4 wt% of acidity and a 1,000 L-batch reactor operating under recirculation. As catalyst, the soluble lipase Eversa® Transform 2.0 was employed in the process. At 45 °C, 2 wt% of water addition, 100 ppm of sodium hydroxide, methanol to oil molar ratio of 6.0:1, 0.2 wt% of lipase load and 24 h of reaction, these authors obtained 96.6 % of FAME yield with 4.2 wt% of FFA content. Despite the satisfactory yield achieved, an overly reaction time was utilized by the authors, once it is possible to notice through the curve of conversion presented that after 13 h of reaction, there is no further reduction of the feedstock acidity, i.e. 11 h of reaction longer than necessary were used, indicating an energy waste in the process. In a similar work, Price et al. (2016) presented results of reaction kinetics regarding a process scale-up for a continuous enzymatic biodiesel production (4 m^3 of capacity) mediated by liquid lipase NS 40116 using WCO as raw material (FFA < 15 wt%): under 35 °C, 20 wt% of methanol excess, 0.1 wt% of lipase load, 2 wt% of water addition and over 60 h of reaction, 97.5 % of biodiesel yield was obtained. Although the biocatalyst load utilized by these authors is interestingly low, it is perceptible that

the reaction time adopted of more than 60 h is approximately 8 times longer than the used in our research to obtain a similar biodiesel yield.

5.3.3 Reactor Productivities and Biocatalysts Performance

The definition of a reactor configuration for an enzymatic production of biodiesel depends on mainly of the biocatalyst properties that will be employed in the process and of possible technical problems that may eventually happen: homogeneity of the reaction mixture, product solubility in alcohol, enzyme stability, enzyme recovery, limitations in the diffusion and the effect of glycerol (Poppe et al., 2015). In this sense, conducting semi-continuous processes employing stirred tank reactors offers some operational advantages such as the high degree of substrate dispersion, simple equipment structure, simplicity in handling/operation and easy reaction control (Christopher et al., 2014).

Laosuttiwong et al. (2018) highlight that to produce economical biodiesel, FAME yield cannot be the only parameter considered to conclude about the operation feasibility. Other parameters such as reaction time, energy demand (directly affected by the reactor productivity) and catalyst performance are crucial factors that must be required to evaluate the costs of the biodiesel production. In this sense, reactor productivity and biocatalysts performance were used to evaluate the enzymatic operation presented in this research. Figure 6 shows results for reactor productivity and biocatalysts performance for the tests accomplished. According could be observed in Figure 3 and 4, the conversion curves of the substrate into FAME exhibited a similar behavior in the first 4 hours of reaction. It is worth remembering that, logically, the same reactor was used in each process, providing to the system an agitation of 1,700 rpm. In this way, according to the results presented in Figure 6a, the reactor productivity for both liquid lipases was basically the same for this interval of time. In the second-half of the reaction, where the inevitable denaturing effects of the parameters “temperature” and “alcohol” affected the enzyme stability, a slight and clearer difference in the reactor productivity can be observed favorable to the reaction configuration performed in two-steps (Figure 6a). For the biodiesel production though enzymatic hydroesterification, an adequate agitation in the reactor has particular importance due to its critical influence on mixing degree between two phases that are immiscible: alcohol and oil (Tabatabaei et al., 2019). However, agitation in excess impacts in the economic feasibility of the process by increasing equipment and operation costs. Moreover, the application of lipases in liquid formulation on biodiesel synthesis implies that, with the necessary addition of a certain amount of water to the medium, there will always be an aqueous

phase disperse in the reaction, since the enzyme does not dissolve in the oil-phase. In this sense, the catalytic action of the enzyme is not distributed throughout the system bulk but restricted to the interface, meaning that the reaction rate may be limited by the available interfacial area (Nordblad et al., 2014). Therefore, it is crucial that an adequate agitation be provided by the reactor to potentialize the lipase activity and to benefit economics questions of the process.

For each run, the performance of the biocatalysts was also analyzed. The results obtained are presented in Figure 6b. For this analysis, was considered as "biocatalyst performance" the amount of FAME that was produced, in kilograms, per kilogram of enzyme used in the process per hour. The lipase ET (*Process nº 1*) presented a lower performance compared to the "version 2.0" since a higher concentration of lipase was applied to the process to even so achieve inferior yield compared to the others runs. Regarding the lipase ET 2.0, besides the clear superior performance in relation to the another enzyme evaluated, it should be noted that *Process nº 2* and *3* presented a similar biocatalyst performance up to 4 h of reaction due to the fact that the same catalyst load has been used until then (0.5 wt%). However, for *Process nº 3*, was observed a slight drop in the biocatalyst performance once more 0.2 wt% of lipase was added to the second reaction. Notwithstanding, according described previously, this addition of lipase was necessary in order to allow, among other improvements, to obtain the minimum concentration of esters that is typically required by regulatory standards. At the same time, this additional amount of lipase, which reduced the biocatalyst performance for *Process nº 3*, also impacts in the economic balance of the operation (discussed in detail in Section 3.5) since more lipase is needed to the process.

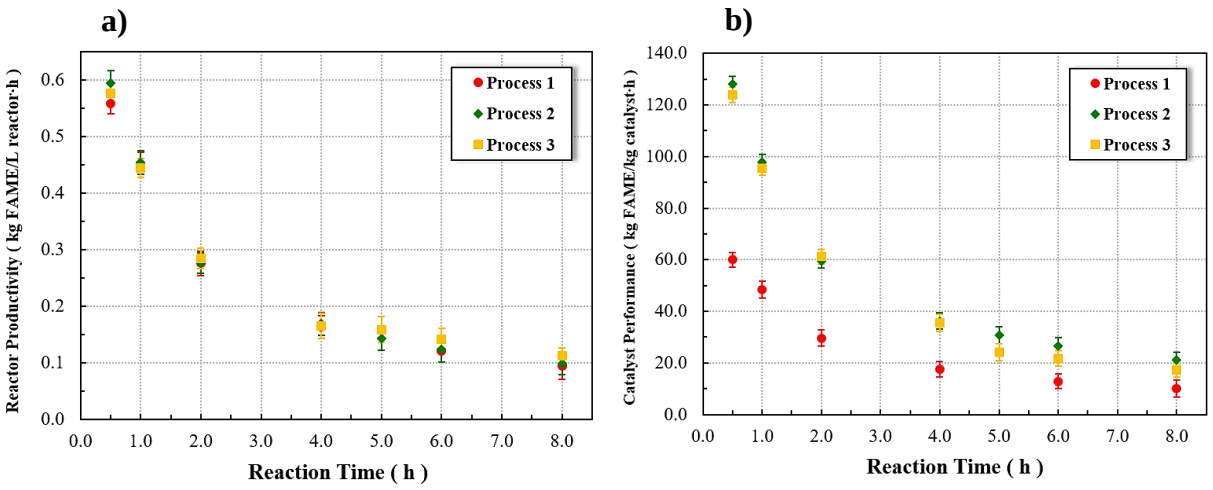

Figure 6. Reactor productivity (a) and catalyst performance (b) for the runs performed.

5.3.4 Kinetics Analysis

Is well-established that the enzymatic reaction to biodiesel production can be described by a Ping-Pong Bi Bi mechanism where the lipase reacts with the substrate forming an active intermediate complex (Facin et al., 2019). This complex can be decomposed back to substrate and enzyme or broken down into products, releasing the enzyme that then stay free to form a new "enzyme/substrate" complex (Andrade et al., 2017). Notwithstanding, for this work, a more objective approach was suggested and adopted, making it possible in a simple way to estimate kinetics parameters of the process.

Figure 7 presents the fitting of the data for $-ln\ (1-X_{ME})$ versus t. The results obtained suggest a linear fit for the considered values, corroborating the hypothesis adopted that the reaction presents a behavior of pseudo-first order. In a similar research, Price et al. (2016) evaluated the reaction kinetics for biodiesel synthesis from used cooking oil assisted by the soluble lipase NS 40116, previously assuming a first order behavior for the system. However, these authors concluded that the plotting of the data indicated that the reaction appeared to have a behavior of pseudo-first order according the bounds glycerol were consumed. The results obtained through of Figure 7 allow to easily estimate the apparent rate constant (k_{App}) by determining the slope of the plotted lines. The estimation of the k_{App} enable characterize the apparent rate of the reaction for different types of feedstock in relation to a specific degree of mixing (Price et al., 2016).

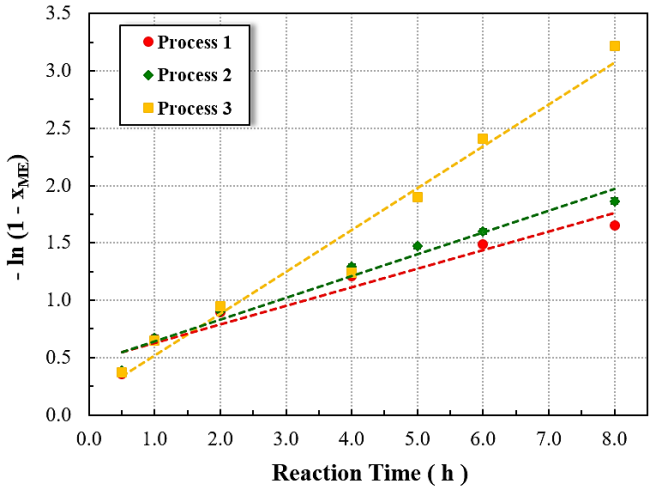

Figure 7. Prediction of "-ln(1 - x_{ME})" as a function of reaction time at 40 °C for the different processes. Reaction conditions: *Process nº 1* – methanol to oil molar ratio of 4.5:1, 1 wt% of lipase, 2.5 wt% of water; *Process nº 2* – methanol to oil molar ratio of 4.5:1, 0.5 wt% of lipase, 2.5 wt%; *Process nº 3* – methanol to oil molar ratio of 6.3:1, 0.7 wt% of lipase, 4.0 wt%.

The results of k_{App} *f*or each process with the respective coefficient of determination are presented in Table 3. According can be verified, the reaction rate for *Process nº 3* was approximately the double that the obtained for *Process nº 1* and *2*. The main difference from *Process nº 3* to the others that can explain the results achieved, once the power delivered to the reaction system *via* mixing was the same for the processes, is due to the slight superiority of the amount of inputs used in this system, where the reaction configuration adopted for this situation was a two-step hydroesterification. It should also be remembered that for *Process nº 1*, soluble lipase ET was utilized, while version 2.0 was used in the other two runs. Considering the lipase ET 2.0, apparently the slight superiority of enzyme load used on *Process nº 3* was interestingly effective in leveraging the reaction rate of the system if be compared the value obtained for the k_{App} on *Process nº 2*. The reactor agitation acting under a higher lipase load appeared to be adequately enough to let the average droplet size of the reaction system smaller. This implied that there were more lipases at the reaction interface apt to catalyzing the process and, being the soluble enzymes actived interfacially, a faster reaction rate could be achieved.

Table 3. Reaction rate constants for the enzymatic hydroesterification of waste cooking oil at 40 °C for the different processes evaluated.

	k_{App} (h^{-1})	**R^2**
Process nº 1	0.1622	0.9480
Process nº 2	0.1896	0.9680
Process nº 3	0.3731	0.9748

Price et al. (2016) described that the apparent rate constant of the reaction presents behavior directly proportional to the increase in the lipase load employed in the reaction. On the other hand, a k_{app} of 0.067 h^{-1} was obtained by these authors in an ET 2.0-mediated hydroesterification, using used cooking oil as feedstock and a pilot reactor of 4 m^3. This value 5.5 times lower than the obtained in our research for the *Process nº 3* can be associated with diverse factors. Among the most evident are the higher lipase load applied in our research (0.7 wt% against 0.1 wt%), the two-step reaction configuration proposed by our research, which enhanced the reactor' productivity; and the higher reaction time used by Price et al. (a reaction time of 60 h, 8 times longer than the adopted in our research to achieve a similar FAME yield, according discussed in Section 3.2).

5.3.5 Economics Considerations

Currently, the main feedstock applied in the biodiesel production in Brazil is soybean oil (degummed), representing around of 75 % of the total cost of operation (Manaf et al., 2019). Waste cooking oil corresponded to less than 2 % of the 5.9 million m³ of biofuel produced in 2019 (Brazilian Agency of Petroleum, Natural Gas and Biofuels, 2019). Even though our society is experiencing a period of alert in terms of the importance of environmental awareness for the ecosystem preservation, there is still little exploration of the potential of using WCO on biofuel preparation due to the reaction limitations imposed by the alkaline route applied industrially. The Brazilian Association of Vegetable Oils Industries (2019) estimates that the annual consumption of vegetable oils in the country for food preparation is around three million cubic meters per year. Of this volume, it is believed that potentially more than 700,000 m³ per year is released into the environment without management. Considering only this amount inadequately destined and based on the results presented in our research, it would be possible to produce at least 694,000 m³ of enzymatic biodiesel.

Data to the economic analysis presented in this section are for *Process nº 3*, once it presented the best performance in relation to the others tested. Information referring to unit costs of inputs, total cost of inputs, total cost per kg of biodiesel produced, unit price of the products obtained, total price of the products and net return of the process are presented in Table 4. The monetary values considered refer to July 2020 and may vary from country to country due to exchange differences. Prices of the chemicals, biocatalyst, WCO and other inputs were obtained from local companies. Therefore, the results have higher representativeness and applicability for Brazil. Operation costs of the biodiesel production and its steps were computed while pilot unit purchase/labor costs were not included so that the analysis does not become complex, which is beyond the scope of this research. Also, data presented in Table 4 correspond to one run. However, befits highlight that at least three daily runs can be performed using the reaction configuration considered, since while a system is on methanol recovery stage, a new load of inputs can be fed to the reactor, starting a new lot of production. Process costs per kg of biodiesel were in the order of US$ 0.50 and the process presenting a positive net return, indicating its feasibility. In a similar approach, Andrade et al. (2019) accomplished an economic analysis for the synthesis of biodiesel obtained from castor oil and catalyzed by Eversa® Transform, reaching a production cost of US$ 0.78·kg^{-1} for a facility with production capacity of 250 kton·year^{-1}. However, it should be note that these authors considered in the analysis an estimate of costs beyond the process operation itself, such as maintenance, labor and quality

analysis, which could justify the cost per kilogram of biodiesel produced around 1.5 times higher than that obtained in our research. In another interesting study, Mohammadshirazi et al. (2014) perform an energy and economic analyses for a biodiesel production from WCO by the conventional alkaline transesterification method: using a pilot unit of 2,000 L of capacity and potassium hydroxide as catalyst, the authors obtained a net return of US$ 1.47·kg^{-1} of biodiesel for a total operation cost of US$ 1.06·kg^{-1}. It is should be noted that these authors also realized a full economic analysis, considering costs involving human labor, maintenance, machinery and rant land. Even so, the estimate of operational cost found in our research is comparative to the conventional chemical technique of biofuel preparation for a same feedstock, highlighting the competitive power of the liquid lipase-catalyzed biodiesel preparation.

Table 4. Data for economic evaluation per run of the operation costs to the *Process nº 3*.

Inputs	**Required amounts (kg)**	**Unit cost (US$)**	**Total cost (US$)**
WCO	54.0	150·ton^{-1} [a,b]	8.1
Methanol	12.5	400·ton^{-1} [b]	5.0
ET 2.0	0.38	12·kg^{-1} [c]	4.6
Water	2.16	1.1·ton^{-1} [b]	-
Utilities			
Electricity	90 kWh	0.1·kWh^{-1} [b]	9.0
		Total operating costs:	**US$ 26.7**
		Cost per kg biodiesel:	**US$ 0.50·kg^{-1}**
Products	**Produced amounts (kg)**	**Unit price (US$)**	**Total price (US$)**
Biodiesel	53.4	850·ton^{-1} [b]	45.4
Crude Glycerin	5.1	280·ton^{-1} [b]	1.4
		Total products price:	**US$ 46.8**
		Net Return	**+ US$ 20.1**

[a] Although the WCO used in the tests has been collected from local restaurants as waste, a market cost per ton was associated to the analysis in order to make it as realistic as possible;

[b] Values obtained from a local biodiesel manufacturer in Brazil;

[c] Value obtained from the local supplier in Brazil.

It is perceptible through the analysis of the Table 4 that the highest unit cost of inputs is associated with the biocatalyst. However, when verifying the gross participation by input relative to the total cost of operation, the lipase load had the least weight (17 %), since the amount used in the reaction was not high. The highest share of cost were for electricity and

WCO, with 34 and 30 % respectively. The initial cost related to methanol represented 19 % of the total. However, this percentage tends to decrease for subsequent runs since 90 to 95 % of the unreacted alcohol excess can be easily recovered using an efficient evaporation and condensation system. Thus, only complementary amounts to the recovered MeOH should be added to the reaction for following processes. Considering a recovery of 90 % of the MeOH excess, for prolonged operations, around 10 % of fresh alcohol (plus the complementary amount of this reagent) should be added to the system, reducing the cost associated to this input to 11 % of the total cost of operation, resulting in an estimate of retraction of the costs to US$ 0.45·kg^{-1} of biodiesel (a reduction of 10 % in relation to the original value), applying the units costs presented in Table 4. Moreover, the recovered methanol, usually having 5 to 10 wt% of moisture, can be directly employed in another runs, since the water will not harm the system, implying in energy savings for the process. Contrary to what occurs, for example, for the conventional alkaline process, where the complete drying of wet methanol is recommended to be possible recycle and reuse the alcohol in a new reaction. Nevertheless, it should be pay attention that the water concentration in the enzymatic system does not exceed the previously stipulated load.

Regarding to possible residues that will be generated by the unit after successive runs, it can mention the organic adsorbent Amberlite BD10^{TM} Dry that composes the filters. This will occur when the material be overused and to lose its polishing capacity. However, the cost of this input (US$ 4.0·kg^{-1}) is not enough to compromise the process feasibility because according described in Section 2.2, 1 kg of this adsorbent is capable of treating up to 1,600 kg of biodiesel. Thus, this expenditure is diluted throughout the many operations that the material will be able to support considering the production capacity of the pilot unit.

A favorable economic balance for the process logically depends on the sale price of biodiesel and the cost of the oleaginous source that will be used in the system. According simulation shown in Figure 8, it is possible to verify that the process will present a positive net return for a biodiesel unit price up to US$ 475 per ton, considering the unit costs of the inputs described in Table 4 (for a raw material – WCO – costing US$ 150·ton^{-1}). As the cost of the substrate increases, the sale value of biodiesel must increase so that the system does not become unviable. For a raw material costing US$ 300·ton^{-1} for example, keeping the unit costs of reagents and lipase shown in Table 4, it is necessary to sell the ton of biodiesel at least US$ 630 in order to the process feasibility has been not affected. With the cost per ton of raw material increasing to US$ 450 and US$ 600, the sale price of the ton of biodiesel must be at least US$ 780 and US$ 930 respectively, impacting significantly the process viability. For this reason, is

critical for the economic health of the enzymatic biodiesel preparation to apply a waste and low-purity feedstock, with low aggregate value. In this respect that the enzymatic pathway has the potential to compete with the traditional homogeneous alkaline technique where, despite chemical route having a cost per kg of biodiesel associated with the catalyst lower than the liquid lipases-mediated process, it requires a raw material with high purity (maximum 0.5 wt% of FFA) and consequently high cost (around US$ 700·ton^{-1}) for be used in the reaction in order to avoid side reactions of saponification (Wancura et al., 2018b). It should be made clear that the colors used in the Figure 8 aim to illustrate that an economic balance below than US$ 5.00 put the process in a "red zone", detrimental to system's feasibility. According the economic balance becomes more positive, the system enters in a "blue zone", with higher probabilities of being viable.

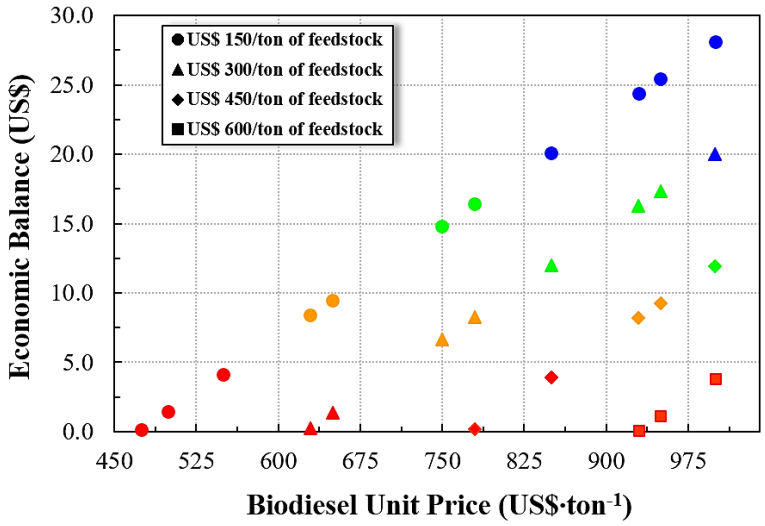

Figure 8. Economic balance for the process according to the biodiesel unit price for different costs of feedstock.

5.4 CONCLUSIONS

With the results achieved in this research, the following conclusions were drawn:

(i) The increase of scale for an enzymatic biodiesel production from WCO and catalyzed by different liquid lipases was successfully performed. Lipase ET 2.0 presented better performance than its predecessor ET (FAME yield of 84.5 % against 80.9 %, respectively);

(ii) It was possible to enhance the FAME yield and biocatalyst performance applying a two-step reaction configuration: 96.2 % of FAME yield was achieved after 8 h under 40 °C,

0.7 wt% of ET 2.0, 4.0 wt% of water and methanol to oil molar ratio of 6.3:1;

(iii) For both biocatalysts, it was not observed significant differences in the FFA content of the samples. Final acidity contents around 2.3 to 2.7 wt% were obtained. A polishing step is necessary in order to reduce the FFA content to values on spec;

(iv) The consideration that the system behaves as a pseudo-first order reaction mechanism presented a good fit for the three processes analyzed. The two-step reaction configuration catalyzed by ET 2.0 had the highest k_{App}: 0.3731 h^{-1};

(v) Data for the costs involved in the operation of the *Process #3* presented a positive net return. However, the employment of a low-grade and low-cost feedstock is fundamental for the enzymatic technique be able to economically compete with the conventional transesterification alkaline;

(vi) The operating cost obtained of US$ 0.50 kg^{-1} of biodiesel can be reduced to US$ 0.45 kg^{-1} considering that, for long operations, the unreacted methanol can be recovered and reused in the process. Thus, considering the operation simplicity and the possibility of using residual raw materials of easy access, the liquid lipase-catalyzed biodiesel preparation can be considered an attractive technique to be applied on biofuel production in a scale higher than lab.

Competing Interests

Declarations of interest: none.

5.5 ACKNOWLEDGMENTS

The authors thank CNPq (National Council of Technological and Scientific Development, 428180/2018-3) and FAPERGS (Research Support Foundation of the State of Rio Grande do Sul, 16/2551-0000522-2) for the financial support of this work, as well as CAPES (Coordination for the Improvement of Higher Education Personnel) for scholarships.

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6 DISCUSSÃO DOS RESULTADOS

Ao longo do período de doutoramento, cinco artigos foram publicados em periódicos internacionais, escritos a partir de resultados obtidos através dos experimentos realizados. Foram quatro artigos escritos pelo discente, os quais estão apresentados nesta tese, e um sob sua supervisão direta (*Enzyme-Catalyzed Production of FAME by Hydroesterification of Soybean Oil Using the Novel Soluble Lipase NS 40116,* publicado no periódico *Applied Biochemistry and Biotechnology, 2019* – ISSN 1559-0291). Os resultados apresentados nestes artigos foram fundamentais para fomentar o embasamento previsto no objetivo geral da tese.

Distintos insumos sob diferentes configurações reacionais foram testados para o desenvolvimento do processo de produção de biodiesel enzimático em escala piloto. Como biocatalisadores, foram empregadas as lipases solúveis Eversa® Transform e Eversa® Transform 2.0 (também conhecida por NS 40116), ambas obtidas do microrganismo *Thermomyces lanuginosus* e comercializadas pela Novozymes A/K. Ainda, três substratos foram usados como matéria-prima: óleo de soja degomado e sebo animal desacidificado, principais matérias-primas empregadas em usinas de biodiesel no Brasil; e óleo de cozinha residual, um material com altos índices de impurezas e baixo valor agregrado que permite a rota enzimática vislumbrar viabilidade econômica frente a convencional rota industrial alcalina. Como reagente, optou-se por trabalhar com metanol devido a sua maior reatividade e menor custo de manufatura dentre os principais álcoois testados em pesquisas similares.

Estudos conduzidos durante o período de mestrado do discente demonstraram que manter a atividade catalítica da lipase ilesa pelo maior intervalo de tempo possível durante a reação é fundamental para se alcançar um rendimento de biodiesel satisfatório. Isto não é uma tarefa simples pois a presença de metanol no sistema age sobre a estrutura molecular da enzima desnaturando-a e inibindo gradativamente sua capacidade de catalisar a reação. Uma das formas de minimizar tal inconveniente é alimentar de forma gradual o álcool ao sistema, mantendo sua concentração baixa no meio reacional. Por se tratar de um ponto crucial no desenvolvimento do processo, o artigo *"Feeding Strategies of Methanol and Lipase on Eversa® Transform-Mediated Hydroesterification for FAME Production"* se propôs a analisar diferentes formas de alimentar metanol e lipase na reação, discutindo como a adoção de determinada estratégia de adição destes insumos ao sistema impacta no rendimento de FAME a ser alcançado. Durante as primeiras horas de processo, observou-se que a capacidade catalítica da lipase era elevada, observando-se interessantes rendimentos de FAME e produtividades. Assim, se faz necessário que quantidades de metanol não inferiores à estequiométrica devam ser adicionadas ao meio

durante este período de forma a se aproveitar ao máximo da performance do biocatalisador. Dentre as estratégias testadas de alimentação de metanol e lipase ao sistema, o maior rendimento obtido de 85,08 % (em 8 h de processo) foi alcançado quando toda a carga de lipase Eversa® Transform (1,0 m%) foi adicionada no início da reação (em t = 0 h) e o metanol alimentado de forma semi-contínua à um fluxo constante de 3,0 $g \cdot h^{-1}$ durante às primeiras 4 h de reação.

Embora interessantes rendimentos tenham sidos obtidos até então, a queda de produtividade catalítica da lipase Eversa® Transform a partir de determinado tempo de processo devido a ação de agentes inibidores intrínsecos à reação (temperatura e metanol) ainda eram visíveis. Além disso, os rendimentos de FAME (concentração de ésteres) estavam aquém do mínimo previsto por normas regulamentadoras. Neste sentido, uma ação tomada foi a substituição da enzima Eversa® Transform pela sua versão mais recente e termoestável Eversa® Transform 2.0 (NS 40116). Assim, embora a NS 40116 ainda apresentasse sensibilidade à desnaturação causada pelo metanol, o efeito da temperatura reacional sob a capacidade catalítica do biocatalisador pode ser minimizado. Testes preliminares utilizando a NS 40116 – os quais geraram resultados que foram publicados no artigo de Rosset, Wancura, Ugalde et al. (2019) – demonstraram resultados promissores: 94,30 % de rendimento de FAME foi obtido após 12 h de reação utilizando uma carga de lipase de apenas 0,5 m% e óleo de soja degomado como matéria-prima do processo. Além disso, a enzima manteve 90 % da sua atividade catalítica após cinco ciclos de reuso em reações subsequentes. Ainda assim, mesmo com tais resultados satisfatórios de rendimento, uma melhora na produtividade do processo, reduzindo principalmente o tempo de reação, era necessária a fim de viabilizar um aumento de escala de produção, pois ainda foi observada uma perda da capacidade catalítica da lipase após algumas horas de reação. Neste contexto, sugeriu-se uma configuração reacional disposta de dois estágios de hidroesterificação em série.

O artigo *"Improving the soluble lipase-catalyzed biodiesel production through a two-step hydroesterification reaction system"* reporta resultados de rendimentos de FAME e valor de acidez para o preparo de biodiesel produzido através de uma inovadora configuração reacional composta de duas hidroesterificações em série, catalisadas pela lipase NS 40116. O intuito de separar o processo em duas reações, separando a fase pesada dos produtos (rica em glicerol) na primeira reação provocou um deslocamento do equilíbrio reacional em direção a formação de FAME, melhorando consideravelmente a produtividade do sistema. Utilizando-se de dois delineamentos compostos centrais rotacionais para otimizar as variáveis da reação, 97,1 % de rendimento de FAME à 35 °C, 8 m% de água, 6,3:1 de excesso de metanol e 0,70 m% de

lipase foi alcançado após apenas 8 h de reação. Isto representa uma redução de até dois terços dos habituais tempos reacionais utilizados em pesquisas similares que adotam um sistema reacional de batelada única. Embora a concentração de ésteres metílicos tenha atingido o valor mínimo usualmente previsto em normas regulamentadoras, a configuração reacional de duas etapas não foi eficaz em reduzir o valor de acidez das amostras de biodiesel para valores dentro do exigido/especificado (obteve-se valores de acidez na ordem de 4,6 mg $KOH \cdot g^{-1}$). Este deverá ser o foco de futuras pesquisas envolvendo a produção de biodiesel via hidroesterificação enzimática: desenvolver um processo que possibilite a obtenção de uma concentração mínima de ésteres acima do previsto em normas mas também valores de acidez especificados, inferiores à 0,5 mg $KOH \cdot g^{-1}$.

Embora os valores de acidez das amostras de biodiesel estarem acima do especificado, a otimização dos parâmetros reacionais foi consistente, estatisticamente significativa e confiável, permitindo aplicar tais variáveis em um processo de aumento de escala de produção. Entretanto, ao se trabalhar com escalas de produção superiores à laboratorial, convém observar cuidadosamente questões econômicas do processo, de forma que possibilite-se estimar a viabilidade do sistema. Neste sentido, ao dar-se início a investigação da produção de biodiesel em escala piloto, optou-se por utilizar uma matéria-prima residual, com altos teores de impurezas e baixo valor agregado como matéria-prima da reação, aproveitando-se da capacidade das enzimas em processar este tipo de material. O substrato escolhido foi óleo de cozinha usado, coletado de restaurantes presentes no Campus da UFSM.

O artigo *"Semi-continuous production of biodiesel on pilot scale via enzymatic hydroesterification of waste material: Process and economics considerations"* apresenta os resultados alcançados no procedimento de expansão da escala de produção. Utilizando-se de uma unidade piloto de 60 L de capacidade, foi possível comparar o desempenho das duas lipases solúveis usadas nesta tese, obtendo-se dados de rendimento de FAME, acidez residual, produtividade, performance dos biocatalisadores além de dados cinéticos e considerações econômicas a respeito da operação do sistema. Utilizando-se dos parâmetros previamente otimizados, 96,2 % de rendimento de FAME foi obtido após 8 h de reação utilizando-se da lipase Eversa® Transform 2.0 em uma configuração reacional de duas etapas, um valor próximo ao obtido em laboratório. Aplicando um modelo cinético simplificado de pseudo-primeira ordem, este mesmo processo retornou um k_{App} de $0{,}373 \cdot h^{-1}$ (aproximadamente o dobro do valor obtido quando se utilizou as lipases em um processo de batelada simples), com um bom ajuste dos dados experimentais. Além disso, uma análise econômica do processo indicou sua viabilidade econômica através de um retorno líquido positivo e custo de operação na ordem de

US$ 0,50·kg^{-1} de biodiesel. Considerando a redução de custos com a recuperação de metanol em operações subsequentes, este custo pode ser reduzido para até US$ 0,45·kg^{-1} de biodiesel. No entanto, tal viabilidade está estritamente relacionada ao custo do substrato utilizado na operação, sendo de fundamental importância o uso de uma matéria-prima residual de baixo valor agregado na reação a fim de permitir um retorno econômico positivo do processo.

Convém salientar que na avaliação econômica do processo, apenas custos restritos à operação do sistema foram estimados, sendo que custos fixos como relacionados à compra da unidade piloto ou manutenção da unidade não foram considerados. No entanto, utilizando-se como referência a convencional rota alcalina homogênea para produção de biodiesel, pode-se concluir que estes mesmos custos desconsiderados na análise estariam presentes no processo químico, com o adicional de despesas com equipamentos e gastos energéticos que o processo alcalino teria com a necessária unidade de pré-tratamento do óleo residual para remoção de impurezas. Conforme vastamente discutido ao longo desta tese, o processo enzimático não é sensível a presença destas impurezas da matéria-prima, sendo possível minimizar estes custos associados a uma unidade de pré-tratamento. Embora à nível piloto, esta economia pode não parecer tão significativa, em termos de produção em escala industrial, tais custos de projeto e equipamentos seriam consideravelmente elevados, uma vez que a unidade de pré-tratamento de matéria-prima representa uma complexa planta industrial à parte da unidade de transesterificação, contendo colunas de destilação, filtros, tanques trocadores de calor, centrífugas e bombas de elevados valores. Todas estas despesas com a unidade de pré-tratamento não estariam presentes em um processo enzimático, destacando o potencial da rota biotecnológica em ser aplicada à produção de biodiesel em grandes escalas.

7 CONCLUSÕES

A busca por um combustível capaz de substituir a dependência da sociedade por combustíveis de origem fóssil consolidou o biodiesel como uma fonte alternativa de energia ecologicamente favorável. Anualmente, a produção de biodiesel aumenta de acordo com políticas governamentais que preveem acréscimos graduais do biocombustível ao diesel. A principal rota industrial de síntese de biodiesel baseia-se em uma transesterificação alcalina homogênea. No entanto, desvantagens do processo químico decorrentes da geração de resíduos levantam questões sobre como tornar o processo mais sustentável. Dentro deste cenário que o preparo de biodiesel catalisado por enzimas ganhou destaque em pesquisas científicas.

Os resultados demonstraram que a forma como os principais insumos do processo (metanol e lipase) são adicionados à reação afetam o rendimento de FAME passível de ser alcançado. Tal fato pode ser associado a estreita e instável relação entre álcool e biocatalisador: ambas as lipases empregadas nessa pesquisa apresentaram uma elevada atividade catalítica nas primeiras horas de reação, sendo que a partir de determinado momento, a sua capacidade de catalisar a reação é afetada, principalmente pela presença de moderadas concentrações de álcool no sistema e a consequente inibição da enzima. Porém, ao mesmo tempo, um excesso de metanol se faz necessário para deslocar o equilíbrio da reação em direção a formação de FAME, principalmente nestes instantes iniciais da reação onde a atividade catalítica da lipase é nitidamente alta. Assim, encontrar uma estratégia de alimentar os insumos à reação de forma a potencializar o rendimento do processo, mas ao mesmo tempo não prejudicar a atividade enzimática da lipase, é um ponto-chave para o sucesso do processo. Avaliando-se diferentes estratégias de alimentação de insumos ao processo verificou-se que o fracionamento da adição da lipase à reação não se mostrou efetiva em incrementar o rendimento de FAME do processo, ao contrário da dosagem fracionada de metanol que conforme o número de etapas de dosagem do álcool aumentava, observou-se um incremento no rendimento de FAME. Cabe salientar que uma quantidade de metanol não inferior a 40 m% (em relação a massa da fonte oleaginosa utilizada) deve ser adicionada no início da reação visando, conforme mencionado, aproveitar a capacidade catalítica da enzima.

Uma proposta de sistema reacional composto por duas reações de hidroesterificação em série mediadas pela lipase NS 40116, com tempo reacional de 4 h cada uma e com separação do glicerol gerado na primeira reação, foi sugerida visando melhorar a produtividade do processo. Através da aplicação de dois planejamentos estatísticos para otimizar os parâmetros reacionais de cada reação, foi possível obter 97,1 % de rendimento de FAME após apenas 8 h

de reação. Além da utilização de quantidades razoáveis de insumos, foi possível ocasionar uma redução de até 16 h do tempo total de processo comparando resultados de pesquisas similares publicadas sobre o tema (CHRISTOPHER et al., 2014; NIELSEN et al. 2016). No entanto, os elevados valores de acidez das amostras de FAME detectados após o fim da reação mostram que para a obtenção de um biocombustível com características dentro de especificações exigidas por normas regulamentadoras, uma etapa posterior ao sistema reacional exclusiva para redução desta acidez se faz necessária. Nestes ensaios, os modelos estatísticos para ambas reações indicaram ser significativos com 95 % de confiabilidade. Nesse sentido, conclui-se que a otimização dos parâmetros reacionais foi satisfatória, permitindo que o projeto de expansão de escala de produção possa ser enfim realizado.

Utilizando-se de óleo de cozinha residual como matéria-prima, o aumento de escala de produção de biodiesel utilizando-se de uma hidroesterificação enzimática foi realizado com sucesso. As diferenças de performance das lipases solúveis aplicadas nos ensaios ficaram evidenciadas: empregando a lipase Eversa® Transform 2.0, 84,5 % de rendimento de FAME foi obtido contra 80,9 % quando usou-se da lipase Eversa® Transform. Ao utilizar-se da configuração reacional em duas etapas, o processo catalisado pela Eversa® Transform 2.0 melhorou ainda mais em termos de rendimento de FAME: 96,2 %, confirmando a efetividade do processo com sequências de reações de hidroesterificação em série.

Com um balanço positivo para os custos associados a operação em escala piloto do processo que desempenhou melhor performance dentre as configurações reacionais avaliadas, há indicação de que o sistema possa ser considerado viável desde que um substrato de baixo valor agregado seja utilizado como matéria-prima do processo, permitindo o processo enzimático competir economicamente com a convencional transesterificação alcalina.

7.1 SUGESTÕES PARA TRABALHOS FUTUROS

Embora os resultados alcançados ao longo desta pesquisa sejam promissores, ainda há pontos à serem explorados. A seguir, estão descritas sugestões para pesquisas futuras:

(i) Apesar da consideração de que o sistema se comporte como um mecanismo reacional de pseudo-primeira ordem tenha retornado um bom ajuste dos dados experimentais, ainda há margem para uma abordagem mais profunda deste tópico. Utilizando-se de um software de programação com mais recursos, é possível realizar a modelagem do comportamento reacional do sistema com exatidão, prevendo curvas de consumo de insumos e geração de produtos, além do levantamento de dados termodinâmicos

escassos na literatura sobre um tema ainda pouco explorado;

(ii) Ficou evidente que um dos gargalos do processo é a acidez final do biodiesel produzido. Uma técnica sugerida por pesquisadores é a lavagem alcalina do biocombustível com uma solução de NaOH. Embora eficaz, entende-se que este caminho não é o mais apropriado pois traz para a rota enzimática um dos inconvenientes da convencional transesterificação química: a geração de resíduos alcalinos que necessitam ser posteriormente tratados. Outra alternativa sugerida é o uso de resinas à base de silicato como o Magnesol® e resinas de troca iônica GF101 e SP112H. No entanto, para atingir uma eficiência satisfatória utilizando esses materiais, se faz necessária a redução da umidade do biodiesel para teores inferiores à 300 ppm, impactando severamente na demanda energética do processo de forma geral. Deste modo, o desenvolvimento de um processo que seja eficiente em reduzir a acidez do biocombustível e ao mesmo tempo seja ambientalmente favorável sem demandar muito energia é um desafio que pesquisas futuras devem abordar. Uma sugestão de processo seria o uso de uma lavagem alcalina utilizando, no entanto, uma solução de cálcio. Os ácidos graxos livres seriam convertidos em sabão, precipitando no sistema, facilitando a separação destes materiais do meio reacional. No entanto, esta é uma proposta de processo que necessita ser testada;

(iii) Com o biodiesel atendendo a parâmetros previstos em normas regulamentadoras, seria possível realizar testes de aplicação do biocombustível em motores. O desenvolvimento de um processo eficiente, capaz de reduzir a acidez do biodiesel, permitiria seu uso em motores à diesel. Assim, uma investigação sobre as emissões geradas por este combustível oriundo de diferentes matérias-primas seria pertinente, por exemplo. Além disso, seria interessante analisar a eficiência energética e de queima do motor quando o biodiesel produzido provém de materiais contendo elevados teores de ácidos graxos insaturados (como o sebo) ou com uma acidez inicial elevada (como o óleo de cozinha usado). Tais observações seriam interessantes, principalmente pelo fato de que o biodiesel obtido exclusivamente deste tipo de matérias-primas possuem o parâmetro "ponto de entupimento à frio" de veras elevado.

Portanto, os dados apresentados nesta tese evidenciam que a rota enzimática através do uso de lipases em formulação líquida para a síntese de metil ésteres de ácidos graxos é uma técnica promissora. Fatores tais como facilidade de manueseio e operação, possibilidade de processar matérias-primas residuais e sua capacidade em contornar os inconvenientes presentes na convencional transesterificação alcalina servem como incentivo para um factível uso industrial destes biocatalisadores na produção de biodiesel.

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