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Green synthesis of benzyl esters mediated by enzymatic biocatalysts using different reactor configurations

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Be less curious about people and more curious about ideas. (Marie Curie)

RESUMO

Este trabalho visou a síntese dos ésteres aromáticos propionato de benzila, butirato de benzila e benzoato de benzila mediada por enzimas imobilizadas como biocatalisadores. A enzima Novozym 435 apresentou o melhor desempenho para a síntese dos ésteres propionato e butirato benzila em sistemas livres de solvente enquanto a enzima Lipozyme TL IM mostrou a melhor performance na síntese do benzoato de benzila. Um planejamento experimental 2^2 foi empregado para maximizar a conversão dos ésteres propionato e butirato de benzila utilizando ácido e álcool como substratos da reação de esterificação. A síntese do propionato de benzila em batelada atingiu uma conversão máxima de 44 % e quando a configuração batelada alimentada foi utilizada para mesma razão molar de substratos (1:1) a conversão aumentou para 90 %, indicando que a alimentação controlada do ácido durante as primeiras horas de reação reduziu os seus efeitos inibitórios. Além disso, as micrografias ópticas do suporte da Novozym 435 mostraram que o ácido propiônico agiu como um forte detergente em todas as razões molares ácido: álcool utilizadas, rompendo as ligações lipase-suporte, e consequentemente, causando a lixiviação da Cal B e impossibilitando o reuso da enzima. A síntese do éster butirato de benzila atingiu conversões acima de 80 % na configuração batelada considerando a menor razão molar (1:1) de substratos, mas os ensaios de reuso da enzima mostraram que houve uma perda acentuada da atividade da enzima após o primeiro ciclo de uso. A configuração batelada alimentada se mostrou eficiente para superar os problemas de inibição do ácido butírico e mesmo utilizando uma razão molar de substratos 1:1 a enzima garantiu a conversão máxima (~ 80 %) nos três reciclos testados. Um aumento de volume foi realizado e a elevada conversão foi mantida viabilizando a síntese do éster via enzimática na ausência de solventes. A síntese do éster benzoato de benzila foi conduzida utilizando o ácido benzoico e o anidrido benzoico como doadores acila para as reações de esterificação e acilação, respectivamente. No entanto, o ácido benzoico é um sólido de difícil solubilização e mesmo utilizando diferentes solventes a reação de esterificação não ocorreu. Por outro lado, a reação de acilação se mostrou eficiente na conversão do anidrido benzoico e produção do éster benzoato de benzila garantindo elevadas conversões mesmo na ausência de solventes e considerando apenas um excesso de álcool benzílico. Diferentes configurações de reatores, condições de reação e métodos de agitação foram testados afim de viabilizar a síntese do éster via biocatálise e a configuração em modo batelada foi a mais viável alcançando conversão máxima de 91 % em 6 h considerando uma razão molar ácido: álcool de 1:3. Embora alguns testes ainda sejam necessários para melhorar a performance do biocatalisador na síntese do propionato de benzila via biocatálise, os resultados indicam que as enzimas Novozym 435 e Lipozyme TL IM são biocatalisadores promissores para a síntese dos ésteres butirato/propionato de benzila e benzoato de benzila, respectivamente, com possível ampliação de escala.

Palavras-chave: Ésteres aromáticos. Ésteres benzílicos. Biocatálise. Esterificação. Acilação. Batelada. Batelada alimentada.

RESUMO EXPANDIDO

Introdução

Os ésteres de aroma que possuem um anel aromático na sua estrutura também são conhecidos como ésteres aromáticos. Muitos ésteres estão inclusos nesse grupo, como os ésteres de benzila, cresila, anisila, eugenila, cinamila, feniletila, benzoato e cinamato. Estes ésteres podem ser encontrados na natureza, no entanto, a produção industrial de muitos deles ainda é estritamente via rota química, utilizando catalisadores ácidos ou básicos, condições processo extremas e elevada geração de resíduos devido à necessidade de lavagens para remoção dos catalisadores. Alguns trabalhos na literatura vêm mostrando que a biocatálise enzimática é uma boa alternativa para síntese desses ésteres utilizando diferentes substratos na catálise de reações de esterificação, transesterificação e acilação. Os processos catalisados por enzimas têm diversas vantagens frente à catálise química uma vez que se encaixa nos conceitos da química verde, utilizando catalisadores inertes, biodegradáveis e reutilizáveis com elevada seletividade, condições amenas de processo, minimização da geração de resíduos e redução no consumo de energia. Algumas pesquisas já têm mostrado o potencial do uso de enzimas para síntese de importantes ésteres benzílicos como o butirato, propionato e benzoato de benzila, no entanto, muito ainda precisa ser feito para viabilizar a síntese desses ésteres aromáticos via esterificação almejando um futuro aumento de escala e aplicação industrial.

Objetivos

O objetivo geral dessa tese foi buscar alternativas para a síntese de ésteres benzílicos utilizando enzimas como biocatalisadores e diferentes sistemas de reação utilizando uma configuração livre de solvente em reatores em batelada e batelada alimentada. Os objetivos específicos foram: avaliar a síntese de ésteres benzílicos utilizando lipases imobilizadas como catalisador; avaliar a esterificação de diferentes doadores acila (ácido butírico, ácido propiônico, ácido benzoico e anidrido benzoico) com álcool benzílico como receptor acila para produção dos ésteres propionato de benzila, butirato de benzila e benzoato de benzila; estudar os efeitos das variáveis do processo temperatura, razão molar dos substratos, quantidade de enzima e tempo de reação para maximizar as conversões; estudar o efeito de reações conduzidas em batelada ou batelada alimentada na síntese dos ésteres e na performance da enzima; estudar o aumento do volume da reação na condição otimizada.

Metodologia

Inicialmente, a síntese dos ésteres foi conduzida em reatores em batelada utilizando quantidade fixa de enzima e temperatura. Diferentes enzimas imobilizadas foram testadas como catalisadores: Novozym 435 (*Candida antarctica* fração B lipase – Cal B), Lipozyme TL-IM (*Thermomyces lanuginosus* lipase), Lipozyme RM-IM (*Rhizomucor miehei* lipase), NS 88011 (enzima não comercial com a lipase Cal B imobilizada) e Cal B liofilizada (sem suporte), doadas pela Novozymes®. Testes foram conduzidos variando condições de processo como razão molar de substratos (doador acila: receptor acila), temperatura, quantidade de enzima e tempo de reação a fim de maximizar as conversões. Em todos os experimentos de esterificação foram utilizadas peneiras moleculares de 4Å (8-12 mesh, Sigma-Aldrich). Além dos testes em batelada, reações em batelada alimentada também foram conduzidas com a

adição controlada do doador acila durante as primeiras 5 horas de reação e a conversão foi acompanhada ao passar do tempo. As conversões foram determinadas através de Cromatografia Gasosa (Shimadzu GC 2010, coluna DB-5, detector por ionização de chama) utilizando uma curva de calibração específica para cada éster. Para o éster benzoato de benzila alguns solventes ou excesso de álcool benzílico (sistema livre de solvente) foram utilizados para solubilização dos doadores acila (ácido ou anidrido benzoico). A purificação do éster butirato de benzila foi realizada utilizando extração líquido-líquido e a pureza do éster foi aferida por Cromatografia Gasosa e ^{1}H RMN (Espectrofotômetro de Ressonância Magnética Nuclear, Bruker, 200 MHz).

Resultados e Discussão

A enzima Novozym 435 foi o melhor catalisador para esterificação do éster propionato de benzila utilizando o ácido propiônico e o álcool benzílico como substratos. A reação em batelada atingiu uma conversão máxima de 44 % considerando uma razão molar de substratos de 1:1. A baixa conversão está associada aos efeitos inibitórios causados pelo ácido de cadeia curta no sítio ativo da lipase. Desta forma, o emprego da configuração em batelada alimentada gerou um efeito positivo sobre a conversão do éster e a conversão subiu para 99 % após a alimentação controlada do ácido nas primeiras horas de reação. Apesar da redução das atividades inibitórias e aumento da conversão no primeiro ciclo de reação, os testes de reuso da enzima mostraram uma forte dessorção da enzima após a reação de esterificação e os resultados indicam que o ácido propiônico foi o responsável pela lixiviação. Por outro lado, a enzima Cal B liofilizada se mostrou eficiente na síntese do éster e pode ser utilizada para um futuro aumento de escala.

A enzima Novozym 435 também foi o melhor biocatalisador para síntese do éster butirato de benzila, atingindo conversões próximas a 75 % na melhor condição do planejamento experimental (10 % de enzima, 50 º C e razão molar de substratos 1:1) em batelada. No entanto, a atividade da enzima foi drasticamente reduzida após o primeiro ciclo de reação nessas condições operacionais, e testes de reuso com razões molares variando de 1:1 a 1:3 mostraram uma dependência de elevadas concentrações de álcool para garantir um bom desempenho do catalisador durante três ciclos de reação e conversões acima de 80 %. O ácido butírico, que também é um ácido de cadeia curta, mostrou ser o responsável pelos efeitos de inibição da lipase e uma mudança na configuração do reator para batelada alimentada com alimentação controlada do ácido nas primeiras horas de reação garantiu um bom desempeno da Novozym 435 e elevadas conversões nos três ciclos de reação testados mesmo em razão equimolar de substratos, mostrando que a abordagem é eficiente para garantir a diluição do ácido e eliminar os efeitos inibitórios. Um aumento no volume de reação foi testado e conversões próximas à 80 % em apenas 6h foram alcançadas após um aumento de 31 vezes. Uma pureza de 99 % foi atingida após a extração líquido-líquido e o espectro de RMN indicou ausência de substratos não reagidos e solventes no produto final.

O éster benzoato de benzila foi sintetizado utilizando o álcool benzílico como receptor acila e ácido benzoico ou anidrido benzoico como doadores acila para as reações de esterificação e acilação, respectivamente. A reação de esterificação não ocorreu devido a difícil solubilização do ácido mesmo utilizando diversos solventes no meio reacional. Por outro lado, a reação de acilação foi eficiente na síntese do éster e testes mostraram que a enzima Lipozyme TL-IM foi o catalisador mais adequado para o sistema. Foram realizados testes utilizando 2-propanol e terc-

butanol para auxiliar a solubilização dos substratos, no entanto, a razão molar 1:6 (anidrido: álcool) livre de solvente atingiu conversões acima de 70 % e foi selecionada como melhor condição de reação. A conversões alcançadas utilizando o reator em batelada foram elevadas e com taxas de reação aceleradas em comparação às reações realizadas em reator em batelada alimentada, indicando que o sistema em batelada é o mais adequado para a síntese do benzoato de benzila com condições de processo de 50 ºC, 6 % de enzima, 8 h de reação e razão molar de substratos de 1:6.

Considerações Finais

Este trabalho apresenta uma alternativa para síntese dos ésteres benzílicos via biocatálise utilizando enzimas imobilizadas como catalisadores na ausência de solventes orgânicos e baixa ou moderada razão molar de substratos. A configuração em batelada alimentada mostrou ser eficiente para melhorar a conversão dos ésteres e reduzir os efeitos inibitórios causados pelos ácidos de cadeia curta como os ácidos butírico e propiônico. O éster butirato de benzila foi sintetizado com sucesso via esterificação em uma razão equimolar de substratos através da adição controlada do ácido numa abordagem em batelada alimentada, garantindo uma ótima performance do biocatalisador. A síntese do éster propionato de benzila alcançou elevadas conversões utilizando a mesma abordagem, no entanto, o ácido propiônico foi responsável pela lixiviação da lipase do material de suporte, o que inviabiliza o uso da enzima imobilizada como catalisador. Por outro lado, a enzima Cal B liofilizada se mostrou eficiente na síntese do éster e pode ser utilizada para um aumento de escala. O éster benzoato de benzila foi sintetizado com sucesso utilizando anidrido benzoico e álcool benzílico como substratos e a configuração em batelada atingiu elevadas conversões em curtos períodos de tempo. As enzimas comerciais da empresa Novozymes foram eficientes na síntese dos ésteres propionato de benzila, butirato de benzila e benzoato de benzila e podem ser utilizadas para aumento de escala e produção industrial.

Palavras-chave: Ésteres aromáticos. Ésteres benzílicos. Biocatálise. Esterificação. Acilação. Batelada. Batelada alimentada.

ABSTRACT

This work aimed the synthesis of aromatic esters benzyl propionate, benzyl butyrate and benzyl benzoate mediated by immobilized enzymes as biocatalysts. The Novozym 435 showed the best performance for the benzyl propionate and benzyl butyrate synthesis in free-solvent system while the Lipozyme TL IM showed the best performance in the benzyl benzoate synthesis. A 2^2 experimental design was employed to maximize the conversion of both benzyl propionate and butyrate using acid and alcohol as substrates for the esterification. The benzyl propionate synthesis in batch reactor reached a conversion of 44 % and the fed-batch synthesis at same conditions (substrates molar ratio 1:1) reached 90 % of conversion, indicating the acid controlled fed during the first hours of reaction decreased its inhibition effects. Moreover, the optical micrographs of the Novozym 435 support showed the propionic acid acted as a lipase detergent in all substrates molar ratio tested, breaking the lipase-support bonds and, consequently, leaching out the Cal B from the support, making impossible the enzyme recycle. The benzyl butyrate synthesis reached conversions above 80 % in batch configuration considering the lower substrates molar ratio (1:1). However, the enzyme recycle assay showed a sharp decrease in the enzyme activity right after the first cycle of use. The fed-batch configuration showed to be efficient to overcome the butyric acid inhibition effects and the enzyme performance was maintained even at a substrates molar ratio of 1:1, reaching 80 % of conversion in the three recycles tested. The increase in the reaction volume showed a great ester conversion, which makes feasible the benzyl butyrate synthesis mediated by enzymes in the absence of organic solvents and substrate excess. The benzyl benzoate synthesis was conducted using the benzoic acid and benzoic anhydride as acyl donors to the esterification and acylation reactions, respectively. However, the benzoic acid is a solid of difficult solubilization and even using different solvents the esterification did not occur. On the other hand, the acylation showed to be very efficient in the benzoic anhydride conversion and occurred successfully in the absence of solvents, considering only an excess of benzyl alcohol. Different reactor configuration, reaction conditions, and agitation methods were tested in order to feasible the benzyl benzoate via biocatalysis and the batch configuration reached 91 % of conversion after 6 h in a molar ratio of 1:3. Although some tests are still needed to improve the biocatalyst performance in the benzyl propionate synthesis, the present results indicated the enzymes Novozym 435 and Lipozyme TL IM are promising biocatalysts for the benzyl propionate, butyrate and benzoate synthesis, respectively, with possible scale-up.

Keywords: Aromatic esters. Benzyl esters. Biocatalysis. Esterification. Acylation. Batch. Fed-batch.

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LIST OF SYMBOLS

ºC	Degrees Celsius
%	Percentage
wt%	Weight total percentage
v/v	Volume to volume ratio
w/w	Weight to weight ratio
m/m	Mol to mol ratio

LIST OF ABBREVIATIONS

CAGR	Compound Annual Growth Rate
CAS	Chemical Abstracts Service
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FEMA	Flavor and Extract Manufacturers Association
GC	Gas Chromatography
GRAS	Generally Recognized as Safe
JECFA	Joint FAO/WHO Expert Committee on Food Additives
NMR	Nuclear Magnetic Resonance
SFS	Solvent free system
WHO	World Health Organization

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1 INTRODUCTION

Flavor esters that possess an aromatic ring in the molecular structure are also known as aromatic esters. Many esters are included in this group, such as benzyl, cresyl, anisyl, eugenyl, cinnamyl, phenethyl, benzoate and cinnamate esters. These compounds are widely used in many food, cosmetics and pharmaceuticals industries to enhance taste and odor in ingredients because their aromatic potential including fruity, floral smell and taste (BERGER, 2009; YADAV; DHOOT, 2009; DHAKE et al., 2012; GAO et al., 2016; SÁ et al., 2017).

Besides the extraction from natural sources, many routes are available to synthesize these aromatic esters via esterification, transesterification, and acylation. However, most of them do not meet to the stringent specifications required to develop a clean and green chemical process, a very important concept for the chemical industry (YADAV; MUJEEBUR RAHUMAN, 2003).

Chemical synthesis is the most used route for the industrial production of flavor esters, using high temperature and acidic or a basic catalyst. The high temperature conditions generate poor quality product, which is not desirable for skin application, and require additional treatment and cost (KHAN; RATHOD, 2015; SÁ et al., 2017). In addition, the chemical route possesses several drawbacks related to the production and purification processes leading to environmental impacts due to the use of hazardous chemicals and catalysts, and high temperature and pressure. Long reaction times, excessive consumption of energy and possible corrosion of the equipment presents high costs and the aroma esters may contain traces of toxic impurities resulting in humans' health complications (KHAN; RATHOD, 2015; MOHAMAD et al., 2015; SILVA et al., 2015; WANG et al., 2015; MANAN et al., 2016; ISAH et al., 2017; SÁ et al., 2017).

In the past few years, there has been an increase of interest for alternative green processes capable of synthesizing aromatic esters (SHINTRE; GHADGE; SAWANT, 2002) and biocatalysis showed to be a great alternative for the biotechnological production of many products, and a very important route for the synthesis of natural flavors (BEN AKACHA; GARGOURI, 2015). The use of biocatalysts like enzymes present many advantages, like high specificity and chemo-, regio- and stereo-selectivity, high yields in mild reaction conditions, reduction of by-

products formation, biocatalyst reusability, low energy consumption and reduction of the overall production costs (KUO et al., 2014; FERRAZ et al., 2015; SÁ et al., 2017). Moreover, the esters synthesized by biocatalysis can be labeled as natural due to the agreement with green chemistry concepts, which emphasizes minimizing environmental impact by reducing waste and energy consumption (CHANG; BAE, 2011; KHAN; RATHOD, 2015).

Lipases constitute the most important group of biocatalysts for biotechnological applications. Many aromatic esters such as benzyl (SHINTRE; GHADGE; SAWANT, 2002; JEROMIN; ZOOR, 2008; SINGH et al., 2008; VOSMANN et al., 2008; BADGUJAR; BHANAGE, 2014, 2015; BADGUJAR; SASAKI; BHANAGE, 2015; WANG et al., 2015; ZHANG et al., 2016), cresyl (DHAKE et al., 2012; BADGUJAR; SASAKI; BHANAGE, 2015; BADGUJAR; PAI; BHANAGE, 2016), and anisyl esters (DHAKE et al., 2011, 2012; BADGUJAR; BHANAGE, 2015; BADGUJAR; SASAKI; BHANAGE, 2015; BADGUJAR; PAI; BHANAGE, 2016) have been successfully lipase-synthesized, by esterification and transesterification. Nonetheless, the production of aromatic esters via direct esterification mediated by lipases is still a poorly explored field and the presence of organic solvents or a stoichiometric excess of alcohol is usually necessary to improve enzyme performance, avoid acid inhibition, and facilitate the ester conversion. In this scenario, lipase-mediated synthesis of aromatic esters in solvent-free systems (SFS) at low substrates molar ratio has significant importance due to the absence of solvents in final products that reduces residues, in addition to the elimination of the need of recovery, downstream and purification processes (CHIARADIA et al., 2012; GENG et al., 2012; GARLAPATI; BANERJEE, 2013; SILVA et al., 2015; CIRILLO et al., 2018).

Although we can find in the literature some studies reporting the synthesis of aromatic esters by biocatalysis, this is still a poorly explored area, especially for the benzyl esters. There is also a lack of information in the literature regarding purification, reuse of the catalyst and scale-up of these processes. In this way, the present work proposes to determinate the best biocatalysis system for the synthesis of important benzyl esters, like benzyl propionate, benzyl butyrate and benzyl benzoate, aiming a future scale-up.

1.2 OBJECTIVES

1.2.1 Main objective

The general objective of this thesis is to investigate alternatives for the synthesis of benzyl esters using enzymes as biocatalysts and different reactions systems to allow a free-solvent configuration in batch and/or fed-batch reactors.

1.2.2 Specific objectives

- Evaluate the synhesis of benzyl esters using immobilized lipases as biocatalysts;
- Evaluate the esterification of benzyl alcohol as acyl acceptor and different acyl donnors (butyric acid, propionic acid, benzoic acid and benzoic anhydride) for the production of benzyl butyrate, benzyl propionate and benzyl benzoate;
- Study the effect of solvent-free configuration for the benzyl propionate and benzyl butyrate synthesis;
- Study the effect of different solvents and excess of benzyl alcohol (solvet free) for the benzoic anhydride and benzoic acid solubilization and synthesis of benzyl benzoate;
- Evaluate the effect of the process variables (temperature, molar ratio of substrates, enzyme amount and reaction time) to maximize the conversions to esters, as well as the study of the conversion over the time.
- Study the effect of batch and fed-batch reactor configurations in the benzyl esters sysnthesis and enzyme performance.
- Study of the reaction volume increase at the optimized condition.

2 LITERATURE REVIEW

This chapter will present a brief review of the available literature on the subjects pertinent to this work. Firstly, the main information about the aromatic esters and some prospects using biotechnological alternatives to their synthesis are presented. Then, some important concepts on enzymatic biocatalysis are described. Finally, the current state of the art of aromatic esters synthesis using immobilized enzymes as catalysts is presented.

2.1 AROMATIC ESTERS

Flavor and fragrances are very important and interesting ingredients in the cosmetic, food, chemical and pharmaceutical industries (OZYILMAZ; GEZER, 2010; ANSORGE-SCHUMACHER; THUM, 2013). Flavor esters are highly known due to the pleasant fruity smell and taste and have been widely used in many products, such as perfume, soap, cream, lotion, shampoo, washing up liquid, food, wine, cigarette, among others (MAHAPATRA et al., 2009; BEN AKACHA; GARGOURI, 2015). However, besides the fragrance they can also have other properties, such as emollient, surfactant and antioxidant, which make possible their application in many formulations of creams, shampoos and antiaging creams (BECKER et al., 2012; KHAN; RATHOD, 2015).

Commercial products and ingredients for personal care have an important impact on people's life worldwide and, consequently, the market for both consumer products and fine chemicals comprising their ingredients is considerable (ANSORGE-SCHUMACHER; THUM, 2013). In addition, the global market for flavors and fragrances was valued at $ 26.0 billion in 2015 and should increase from $ 27.1 billion in 2016 to $ 37.0 billion in 2021 at a compound annual growth rate (CAGR) of 6.4% and grow to $ 33.5 billion by 2019 (BBC, 2016), making the flavor and aroma syntheses interesting fields of research and development.

The aromatic esters are flavors that possess an aromatic ring in the structure and their low volatility and low molecular weight are responsible for a range of sensorial sensations (LONGO; SANROMÁN, 2006). In the past few years, there has been an increase of interest for alternative green tools capable of synthesizing the

aromatic esters, which are composed of aromatic and aliphatic acids and alcohols (SHINTRE; GHADGE; SAWANT, 2002).

These perspectives make the aromatic esters a potential area of research due to the added value of the final products and a remarkable attention has been given to the benzyl esters. The aroma of these esters are highly recognized due to the pleasant fruity smell and taste, and they are widely used in various food, cosmetics, and pharmaceutical industries (BADGUJAR; BHANAGE, 2014; LI et al., 2014; BEN AKACHA; GARGOURI, 2015; SÁ et al., 2017). They are found naturally in many vegetables and flowers, like jasmine, hyacinth, gardenia, azaleas, plums, melons, cloves, camellia, passion fruit, mint, honey, among others (VCF, 2017) and besides the natural extraction from plants and fruits the chemical synthesis still is the most common route to the industrial aromatic synthesis (OZYILMAZ; GEZER, 2010; ANSORGE-SCHUMACHER; THUM, 2013).

Many research groups have been investigating different alternatives for the synthesis of these esters in order to replace the natural and chemical pathways (GRYGLEWICZ; JADOWNICKA; CZERNIAK, 2000; TEWARI et al., 2004; MAJUMDER et al., 2006; CHEN; MILLER; GROSS, 2007; JEROMIN; ZOOR, 2008; SENGUPTA; BASU, 2009; DHAKE et al., 2012; GARLAPATI; BANERJEE, 2013; CHANDANE et al., 2017). However, little has been made for the synthesis of these benzyl esters by direct esterification of alcohol and acid using biotechnological catalysts, targeting future industrial application.

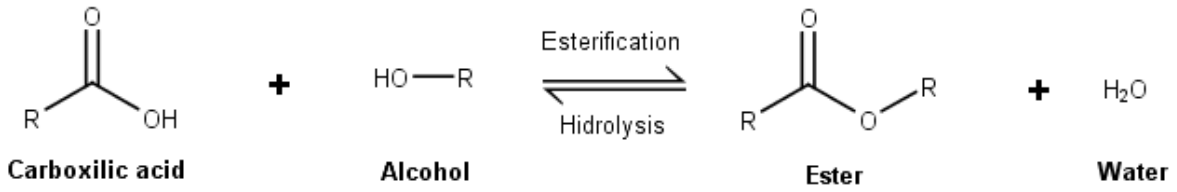
In this way, the next topics of this literature review will approach concepts and prospects of esterification, biocatalysis and aromatic esters synthesis focusing on important benzyl esters, like benzyl butyrate, benzyl propionate, and benzyl benzoate, highlighting the main scenarios and difficulties in their syntheses using lipases as biocatalysts.

2.2 ESTERIFICATION AND CATALYSTS

The esterification is the main reaction for the esters production and occurs between the alcohols (acyl acceptor) and acids (acyl donor) (Figure 1). In general, the esterification has slow reaction rates and possesses a limited chemical equilibrium. Thereby, the use of a catalyst, homogeneous, heterogeneous, or enzymatic, is desirable to enhance the reaction rate (YADAV; MUJEEBUR RAHUMAN, 2003; CHANDANE et al., 2017).

The homogeneous catalysts include strong mineral acids such as HF, H_3PO_4, H_2SO_4, HCl, p-toluenesulfonic acid, dihydroxyfluoroboric acid, and methanesulfonic acid. These catalysts have strong catalytic activity but also have many drawbacks, like equipment corrosion, side reactions and the need of product purification. In addition, at the end of the reaction the catalysts must be neutralized, leading to large salt generation, loss of yield, corrosion problems and a large waste generation (YADAV; MUJEEBUR RAHUMAN, 2003; CHANDANE et al., 2017).

Figure 1 – General scheme of esterification between a carboxylic acid and an alcohol.



Source: Author.

The heterogeneous catalysts include many solid acids and bases, zeolites, ion exchange resins, supported catalysts, and clay catalysts. In a different way, they have several advantages when compared to the homogenous ones due to the elimination of equipment corrosion and side reactions, plus easy separation from the reaction mixture, potential for reuse in several cycles and high product purity, which makes the heterogeneous catalyst most adequate for the esterification reactions (CHANDANE et al., 2017).

The biocatalysts comprise a variety of enzymes and whole cells that act as catalysts in organic synthesis. Lipases are remarkable biocatalysts for esterification,

biodegradable and operate under mild reaction conditions of pH and temperature, as well as high activities and chemo-, regio- and stereo-selectivities. In analogy to chemical processes, the lipases may be used in the immobilized form (binding or inclusion in polymeric materials) as heterogeneous catalysts that can be recovered and reused. The immobilization of the enzymes provides a well-balanced overall performance, low mass transfer limitations, reuse over many cycles, and high operational stability, making the immobilized lipases an important group of biocatalysts in organic chemistry (TISCHER; WEDEKIND, 1999; SCHMID et al., 2001; SHELDON, 2007).

The enzymatic heterogeneous biocatalysts are prepared by the immobilization of unique enzymes, such as lipases, onto solid supports and are very successful catalysts in the ester synthesis at low temperature. The heterogeneous enzymatic processes can be conducted in batch, semi-batch or continuous modes and fully satisfy the requirements of “green” chemistry, so they are promising for implementation into the organic synthesis industry (KOVALENKO; PERMINOVA; BEKLEMISHEV, 2019).

Green Chemistry is defined as the ‘‘design of chemical products and processes to reduce or eliminate the use and generation of hazardous substances” that involves sustainability at the molecular level and supplies the need for more environmentally acceptable chemical processes (ANASTAS; EGHBALI, 2010; PARAVIDINO; HANEFELD, 2011). The Twelve Principles of Green Chemistry were designed to comprise the criteria or guidelines to provide the framework for sustainable design, safer chemicals, and chemical transformations (ANASTAS; WARNER, 1998; ANASTAS; EGHBALI, 2010) and comprises:

1. Prevention. It is better to prevent waste than to treat or clean up waste after it is formed.
2. Atom economy. Synthetic methods should be designed to maximize the incorporation of all materials used in the process into the final product.
3. Less Hazardous Chemical Synthesis. Whenever practicable, synthetic methodologies should be designed to use and generate substances that pose little or no toxicity to human health and the environment.
4. Designing Safer Chemicals. Chemical products should be designed to preserve efficacy of the function while reducing toxicity.

5. Safer Solvents and Auxiliaries. The use of auxiliary substances (e.g. solvents, separation agents, ect.) should be made unnecessary whenever possible and, when used, innocuous.
6. Design for Energy Efficiency. Energy requirements of chemical processes should be recognized for their environmental and economic impacts methods and should be minimized. If possible, synthetic methods should be conducted at ambient temperature and pressure.
7. Use of Renewable feedstock. A raw material of feedstock should be renewable rather than depleting whenever technically and economically practicable.
8. Reduce Derivatives. Unnecessary derivatization (use of blocking groups, protection/ deprotection, temporary modification of physical/ chemical processes) should be minimized or avoided if possible, because such steps require additional reagents and can generate waste;
9. Catalysis. Catalytic reagents (as selective as possible) are superior to stoichiometric reagents.
10. Design for Degradation. Chemical products should be designed so that at the end of their function they break down into innocuous degradation products and do not persist in the environment.
11. Real-Time Analysis for Pollution Prevention. Analytical methodologies need to be further developed to allow for real-time, in-process monitoring and control prior to the formation of hazardous substances.
12. Inherently Safer Chemistry for Accident Prevention. Substances and the form of a substance used in a chemical process should be chosen to minimize the potential for chemical accidents, including releases, explosions, and fires.

Biocatalysis performs well in the context of Green Chemistry, offering an environmentally benign catalyst that usually refers to the direct use of purified enzymes or living microorganisms. Reaction conditions usually are mild and the enzymes are proven to have chemo-, regio-, and stereo-selectivity (ANASTAS; EGHBALI, 2010; PARAVIDINO; HANEFELD, 2011).

The enzyme-catalyzed systems can be carried out as an equilibrium-controlled process (thermodynamically controlled) or as a kinetically controlled process (KASCHE; HAUFLER, 1987). In equilibrium-controlled processes, the enzyme only accelerates the rate with which the equilibrium is obtained (e.g. hydrolysis or esterification). In kinetically controlled processes, an activated acyl donor is necessary and the enzyme acts as a transferase. The maximum and transient yields depend on the thermodynamics of the process come from the properties of the catalyst (hydrolysis of activated acyl donor and of the product, versus rate of product production) (KASCHE, 1986; KASCHE; HAUFLER, 1987).

Generally, in controlled acyl-transfer esterifications that are diluted in aqueous media, the reaction equilibrium is largely favored to hydrolysis, while, in nonpolar organic media with low water amount the enzyme remains active and the reaction equilibrium is shifted in favor of synthesis (HALLING, 1984, 1990; KASCHE, 1986). Commonly, organic solvents are used to favor esterification reaction over hydrolysis (HALLING, 1984, 1990; MARTY et al., 1991; CLAON; AKOH, 1994; HUANG; CHANG; GOTO, 1998; HARI KRISHNA; PRAPULLA; KARANTH, 2000; HARI KRISHNA et al., 2001; WU et al., 2014).

Regarding enzymatic-catalyzed esterification the use of organic solvents in the media presents some advantages, such as increased substrates and products solubility and the shifting of the thermodynamic reaction equilibrium to favor esterification over hydrolysis (BEN AKACHA; GARGOURI, 2015; PATEL et al., 2015). The nature of the solvent influences the activity, selectivity and stability of the enzymes. In general, lipases are more stable when suspended in non-polar solvents that have low solubility in water. The choice of the organic solvent for the enzymatic reactions is essential to offer good solubility of the substrate in the reaction media, without affecting the catalytic power of the enzyme (SHINDE; YADAV, 2014; BADGUJAR; PAI; BHANAGE, 2016), and respecting the Green Chemistry concepts.

On the other hand, the use of solvents may have some drawbacks associated with separation costs and lipase-mediated synthesis of aromatic esters under SFS has showing a good alternative to the industry, eliminating the need of recovery, downstream, and purification processes and, consequently, reducing the environment hazards (CHIARADIA et al., 2012; GENG et al., 2012; GARLAPATI; BANERJEE, 2013; SILVA et al., 2015). In this scenario, many studies have been

reporting solvent-free systems (SFS) for ester synthesis and molecular sieves showed to be a suitable water adsorbent (reaction product of the esterification), favoring the ester synthesis and ensuring high conversions (CLAON; AKOH, 1994; SANDEKEROGANDLU; FADLOGANDLU; IBANOGANDLU, 2002; KHAN; PRATAP, 2013; BANSODE; RATHOD, 2014; KHAN; RATHOD, 2015; PALUDO et al., 2015).

2.2.1 Acylation

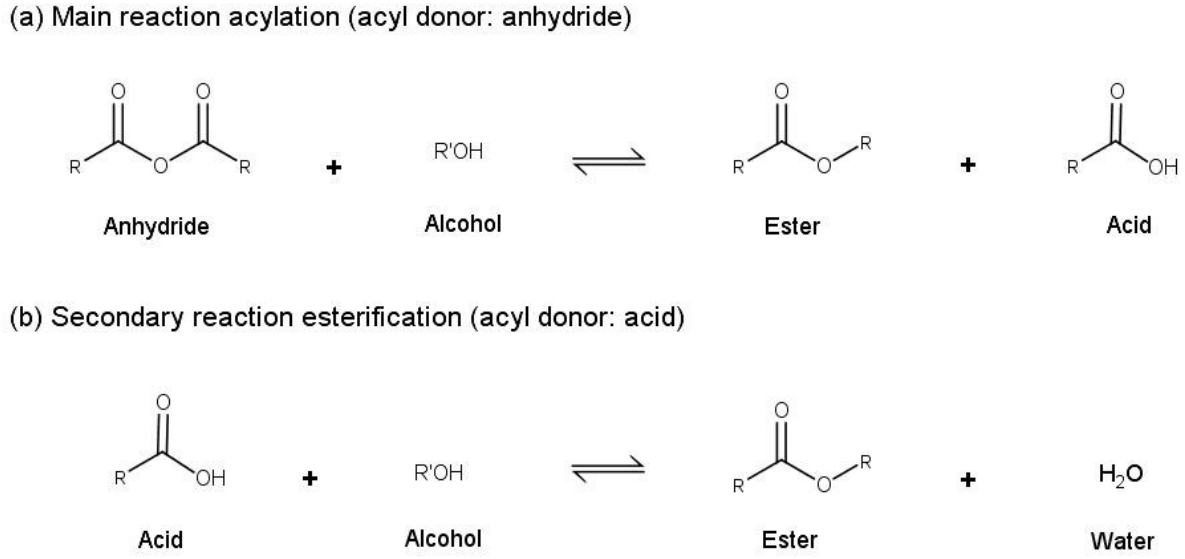
The acylation reaction is frequently used to prepare aromatic ketenes that are very interesting compounds for the chemical and pharmaceutical industries (HASAN; YOON; JHUNG, 2015). This reaction is commonly used for the synthesis of acetyl salicylic acid (aspirin) using salicylic acid and acetic anhydride as substrates and concentrated H_2SO_4 or H_3PO_4 as catalysts (TYAGI; MISHRA; JASRA, 2010; HASAN; YOON; JHUNG, 2015). Some works have been reporting the use of acylation on the aroma esters synthesis using alcohol (acyl acceptor) and anhydride (acyl donor) as substrates and enzymes as biocatalysts (HARI KRISHNA et al., 2001; ROMERO et al., 2005, 2007; CHIARADIA et al., 2012; SILVA et al., 2015; DOS SANTOS et al., 2016; ZARE; GOLMAKANI; NIAKOUSARI, 2019). Most of the authors name the reaction between the anhydride and alcohol as esterification, which generates some conflict of nomination and identification.

The use of an anhydride as acyl donor for aroma ester synthesis is a complex reaction as shown in Figure 2. The anhydride is very reactive and possesses two acyl groups, consequently, one acyl group is employed to form the ester and the other one leads to the formation of one acid molecule. The acid generated as byproduct may react with the remaining alcohol and both acylation and esterification occur simultaneously (ROMERO et al., 2007).

Hari Krishna et al. (2001) studied the enzymatic synthesis of isoamyl acetate (banana flavor) using acetic acid and acetic anhydride as acyl donors for transesterification, esterification, and acylation, respectively. The Novozym 435 was employed as biocatalyst in organic media and high conversions were found for both transesterification and acylation. The low ester conversion reached for the esterification was related to the accumulation of acetic acid in the aqueous microenvironment of the enzyme at a concentration level sufficient to cause protein

denaturation. Zare et al. (2018) reported a similar behavior using a novel microwave assisted-esterification in the isoamyl acetate synthesis. The short chain acid negative effects on the enzyme catalyzed esterification was reported in several works (CLAON; AKOH, 1994; HARI KRISHNA et al., 2001; GÜVENÇ; KAPUCU; MEHMETOĞLU, 2002; ROMERO et al., 2005; ZARE; GOLMAKANI; NIAKOUSARI, 2019). In this way, in enzymatic catalyzed aroma ester reactions the anhydrous acyl donors may possess several benefic effects in comparison to the acidic acyl donors. The main two positive effects include that (i) the anhydride does not acidify the microaqueous layer present in the lipase active site, reducing the problems of enzyme deactivation and (ii) the undissociated anhydride is a source of acyl groups, leading to high ester yields (HARI KRISHNA et al., 2001).

Figure 2 - General scheme for enzymatic synthesis of esters using anhydride as acyl donor and alcohol as acyl acceptor. Main reaction: acylation; Secondary reaction: esterification.



Source: Adapted from Romero et al. 2007.

However, for both esterification and acylation reaction the efficiency is highly dependent on the choice of the right catalyst. Some attributes are required to qualify a good catalyst, like a high selectivity for production of the desired products with minimal production of undesirable side-products and achieving adequate rates of reaction. The catalyst must have stable performance at the proposed reaction conditions (temperature as a function of time) in addition to the reusability in other

cycles of reaction, and the reactants and products may have good accessibility to access the active sites with high rates of diffusion (DUMESIC; HUBER; BOUDART, 2008).

2.3 IMMOBILIZED LIPASES AS BIOCATALYSTS

Enzymes are very important catalysts due to the high specificity, high chemo-, regio-, and stereo-selectivity, ease of processing, broad substrate array and ability to succeed organic transformations in various reaction media (GRYGLEWICZ; JADOWNICKA; CZERNIAK, 2000; BADGUJAR; BHANAGE, 2015; ORTIZ et al., 2019). In addition, enzymes are the most efficient biological catalysts found in nature and they are promising biocatalysts for the aromatic ester synthesis, non-toxic, and accelerate the rate of the reactions under mild conditions of temperature and pressure (ORTIZ et al., 2019). Among the enzymes, lipases are important due to the large number of reactions which they can catalyze in organic systems, high stability, versatility and low commercial cost (PAROUL et al., 2011; JAKOVETIĆ et al., 2013b; GIUNTA; SECHI; SOLINAS, 2015)

Lipases (triacylglycerol hydrolases, EC 3.1.1.3) play an important role in the organic synthesis and flavor biotechnology (DUBAL et al., 2008; WU et al., 2014). These enzymes are responsible for the hydrolysis of lipids to fatty acids and glycerol, and possess the ability to catalyze several reactions, such as esterification (alcohol and carboxylic acid), transesterification (ester and alcohol), interesterification (ester and acid), acylation (anhydride and alcohol), and transfer of acyl groups from esters to other nucleophiles (e.g. amines and thiols) (ROMERO et al., 2005; HORCHANI et al., 2010; MENDES; DE CASTRO; GIORDANO, 2014; PALUDO et al., 2015; TOMKE; RATHOD, 2015; HOANG; MATSUDA, 2016; NARWAL et al., 2016). Many aromatic esters such as benzyl (SHINTRE; GHADGE; SAWANT, 2002; JEROMIN; ZOOR, 2008; SINGH et al., 2008; VOSMANN et al., 2008; BADGUJAR; BHANAGE, 2014, 2015; BADGUJAR; SASAKI; BHANAGE, 2015; WANG et al., 2015; ZHANG et al., 2016), cresyl (DHAKE et al., 2012; BADGUJAR; SASAKI; BHANAGE, 2015; BADGUJAR; PAI; BHANAGE, 2016), and anisyl esters (DHAKE et al., 2011, 2012; BADGUJAR; BHANAGE, 2015; BADGUJAR; SASAKI; BHANAGE, 2015;

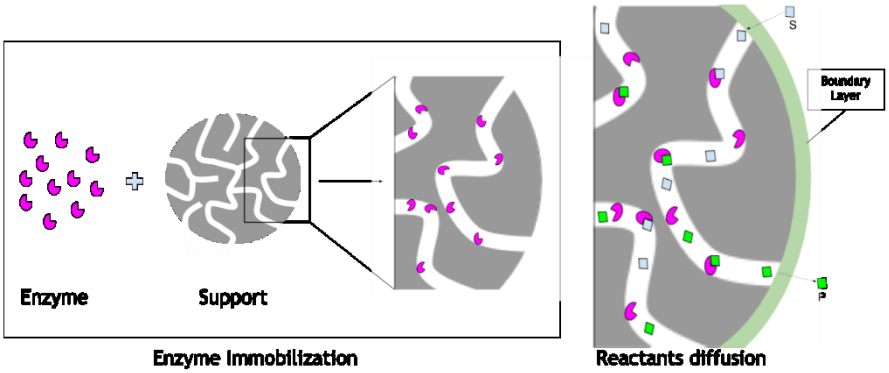
BADGUJAR; PAI; BHANAGE, 2016) have been successfully lipase-synthesized, by esterification and transesterification.

The enzyme immobilization was the first tool to improve the enzymes properties since the free enzyme (homogeneous form) possess some disadvantages on industrial process economics because of high solubility in water, low solubility in organic and inorganic solvents, leading to aggregation. The low thermal, mechanical, and operational stabilities, leading to deactivation, and no recyclability, which cause an increase in the production costs (OZYILMAZ; GEZER, 2010; ORTIZ et al., 2019). The immobilization of these enzymes on inert and resistant supports can to overcome these limitations and offers many process advantages, like lower production cost, increased activity, specificity and selectivity. In addition to improved structural stability, reduction of inhibition, ease of separation, recovery and further reuse of the biocatalyst due to the heterogeneous characteristic (DHAKE et al., 2011; BANSODE; RATHOD, 2014; KUO et al., 2014; NARWAL et al., 2016).

On the other hand, as seen in Figure 3, the immobilization of an enzyme means a deliberate restriction of its mobility, which can also affect mobility of the solutes. Various phenomena referred to as mass transfer effects, can lead to a reduced reaction rate, in other words to a reduced efficiency as compared to the free enzyme (TISCHER; WEDEKIND, 1999). However, in a common overall catalytic reaction catalyzed by a heterogeneous catalyst, the reactants and products undergo a series of steps over the catalyst (DUMESIC; HUBER; BOUDART, 2008), including (Figure 4):

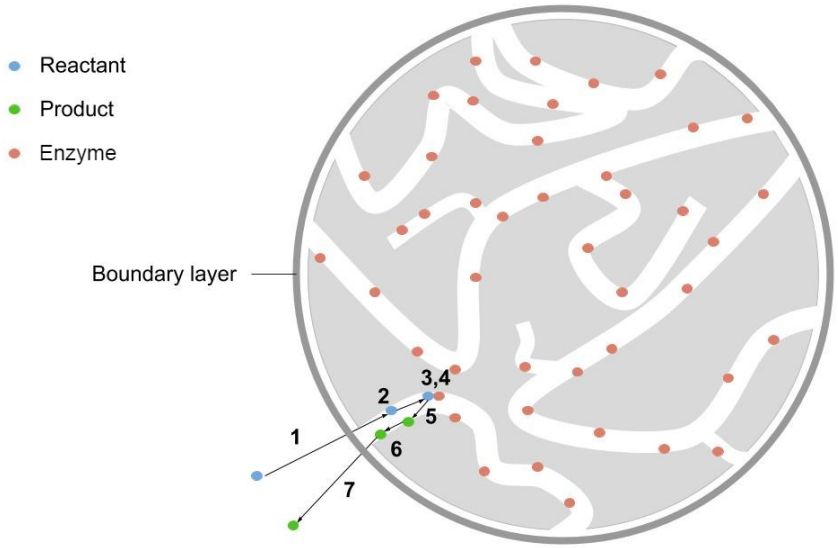
1. Diffusion of the reactants through a boundary layer surrounding the catalyst particle.
2. Intraparticle diffusion of the reactants through the catalyst pores to the active sites.
3. Adsorption of the reactants onto the active sites.
4. Surface reactions involving formation or conversion of various adsorbed intermediates, possibly including surface diffusion steps.
5. Desorption of products from catalyst sites.
6. Intraparticle diffusion of the products through the catalyst pores.
7. Diffusion of the products across the boundary layer surrounding the catalyst particle (DUMESIC; HUBER; BOUDART, 2008).

Figure 3 - Restriction of the enzyme mobility after the immobilization in a support material and description of the substrate (S) and product (P) diffusion through the catalyst particle.



Source: Author.

Figure 4 - Overall steps the reactants and products undergo over the catalytic reaction catalyzed by a heterogeneous catalyst.



Source: Author.

Based on this mechanism, the catalytic rate can be controlled by different regimes including: (i) film diffusion control (Steps 1 and 7); (ii) pore diffusion control (Steps 2 and 6); and (iii) intrinsic reaction kinetics control (Steps 3 to 5) and those regimes affect directly the catalyst performance (DUMESIC; HUBER; BOUDART, 2008).

The most important effects that must be controlled in a reaction that uses a commercial immobilized enzyme as catalyst are the external and internal diffusion

limitations. The external limitations are associated with the formation of a static film (composed by the reactants) around the particle, which difficult the diffusion of both substrate and products. Generally, a stirred reactor must be efficient to avoid the reactants stagnation around the catalyst particle. The internal diffusion limitations are usually associated with the pore size and substrate size, thereby, it is desirable a catalyst that possesses a large pore size in order to avoid any restriction in substrate diffusion within the catalyst (TISCHER; WEDEKIND, 1999).

The most used commercial immobilized lipases are supplied by Novozym ®, and they are described in Table 1. All these immobilized enzymes have demonstrated satisfying activity for esterification, and among these commercial preparation Novozym 435 was predominantly applied and regarded as more active and stable under operation conditions compared to the other preparations (BEZBRADICA et al., 2017).

Table 1 - Physical properties and enzyme source of some commercial immobilized lipases.

Properties	**Novozym 435**	**Lipozyme TL IM**	**Lipozyme RM-IM**
Lipase source	*Candida antarctica* B lipase	*Thermomyces lanuginosus* lipase	*Rhizomucor miehei* lipase
Support material	Lewatit VP OC 1600/ macro-porous acrylic resin with a hydrophobic surface.	Hydrophilic gel silicate.	Duolite ES562/ weak anion-exchange resin based on phenol-formaldehyde copolymers.
BET surface area (m^2/g)	81.5	50	165
Total Pore Volume (cm^3/g)	0.45		-
Average pore diameter (nm)	17.7		30
Porosity (-)	0.349		-
True Density (g/cm^3)	1.19		-
Temperature (º C)	30-60	50-75	30-60
pH	5-9	6-8	7-10

Source: (HAIGH et al., 2013; BASSO; HESSELER; SERBAN, 2016; SÁ et al., 2017).

Furthermore, many biotechnological applications have been successfully established using these immobilized lipases for the synthesis of many products, including flavor compounds (YADAV; DHOOT, 2009; GIUNTA et al., 2013; MARTINS et al., 2013; GIUNTA; SECHI; SOLINAS, 2015), biopolymers (COMIM ROSSO et al., 2013; GUMEL; ANNUAR; HEIDELBERG, 2013; ROSSO COMIM et al., 2015; POLLONI et al., 2017) and broadly employed in biodiesel production (SHIEH; LIAO; LEE, 2003; RATHORE; MADRAS, 2007; HERNÁNDEZ-MARTÍN; OTERO, 2008; TALUKDER et al., 2009b; RODRIGUES et al., 2011; COLOMBO et al., 2015).

2.4 AROMATIC ESTER SYNTHESIS USING IMMOBILIZED ENZYMES

The enzymatic synthesis of aromatic esters is a very interesting area for the pharmaceutical and food industries and lipases play an important role since the aroma esters produced by microbial or enzymatic methods may be labeled as natural in accordance with the United States and European Legislations, thereby satisfying the consumer trend towards natural products in various industries (LESZCZAK; TRAN-MINH, 1998; VANIN et al., 2014; TOMKE; RATHOD, 2015; SÁ et al., 2017). Lipases are an important group in the organic chemistry, biodegradable and responsible for catalyzing many organic reactions, operating under mild conditions of pH and temperature in homogeneous or heterogeneous form, and have shown to be promising biocatalysts in the aromatic ester synthesis with potential industrial applications (TISCHER; WEDEKIND, 1999; GRYGLEWICZ; JADOWNICKA; CZERNIAK, 2000; SCHMID et al., 2001; SHELDON, 2007; BADGUJAR; BHANAGE, 2015; SÁ et al., 2017).

In the enzymatic synthesis of aromatic esters, the process variables are important for the final conversion and reaction yield. The temperature, reaction time, agitation speed, use of organic solvents and/or excess of substrates (to shift the thermodynamic reaction equilibrium to favor esterification over hydrolysis) have a great impact on the process production, and must be determined as the first step (BADGUJAR; BHANAGE, 2014; SÁ et al., 2017).

The investigation of the ideal substrates type and amount are also very important parameters as the some acids in high concentrations can deactivate the enzyme and some alcohols may cause some inhibition effects. Moreover, the type and amount of enzyme is also a critical issue and, in this way, an investigation with lipases from different sources is recommended to select the most adequate biocatalyst. In addition, the amount of the substrates and enzyme used must ensure high conversions, but no waste of reagents. All these parameters are very important, and the process optimization by the study of the individual effects and interactions is useful to achieve high product yield and conversion (CHIARADIA et al., 2012; GENG et al., 2012; STENCEL; LEADBEATER, 2014; SÁ et al., 2017).

As mentioned above, high concentrations of acid in the reaction medium may cause severe decrease of enzyme activity and consequently low yield for ester synthesis, mainly for esterification-mediated by lipases. Many efforts have been dedicated to overcome this drawback, and the transesterification (alcohol and ester) and acylation (anhydride and alcohol) are good alternatives to ensure high conversions (ROMERO et al., 2005, 2007; SINGH et al., 2008; DHAKE et al., 2012; BADGUJAR; BHANAGE, 2014; BADGUJAR; SASAKI; BHANAGE, 2015; GAO et al., 2016). However, the esterification is still the most adequate, simple and less expensive reaction to produce aromatic esters and many researchers have been investigating this synthesis using immobilized enzymes as biocatalyst. Table 2 shows all the aromatic esters synthesized via esterification using acids as acyl donors.

Table 2 - Aromatic esters synthesized via esterification using immobilized enzymes as biocatalyst and acid as acyl donors.

Aromatic ester	**MR [1]**	**Enzyme**	**Solvent/ Temperature (ºC)/ Time (h)**	**Conv (%)[2]**	**Reference**
Benzyl cinnamate	2.6: 1	Lipozyme TL IM	Isooctane/ 40 ºC / 27 h	97.7%	(ZHANG et al., 2016)
Benzyl cinnamate	3: 1	Lipozyme TL IM	Isooctane/ 40 ºC/ 24 h	97.3%	(WANG et al., 2015)
Benzyl oleate	1: 1	Novozym 435	Solvent-free/ 80 ºC/ 1 h	94%	(VOSMANN et al., 2008)
Benzyl butyrate	1: 1	Novozym 435	Methyl tert-butyl ether/ 52º C/24 h	82%	(JEROMIN; ZOOR, 2008)
Benzyl cinnamate	3:1	Novozym 40086 (lipase *Rhizomucor miehei*)	Isooctane/ 46.3 ºC/ 11.3 h	96 %	(SUN; TIAN, 2018)
Benzyl cinnamate	4.1	Porcine lipase immobilized in nanoporous metal organic framework supports	Isooctane/ 8h/ 55 ºC	96 %	(NOBAKHT et al., 2018)
Eugenyl benzoate	4: 1	Immobilized *Rhizomucor miehei* lipase	Chloroform/ 60 ºC/ 6 h	56.1%	(MANAN et al., 2016)
Eugenyl benzoate	3: 1	Chitosan-chitin nanowhiskers supported *Rhizomucor miehei* lipase	Chloroform/ 50 ºC/ 5 h	66 %	(MANAN et al., 2018)
Eugenyl benzoate	3: 1	Chitosan–chitin nanowhiskers *Rhizomucor miehei* lipase	Chloroform/ 50 ºC / 5 h	62 %	(ABDUL MANAN et al., 2018)
Eugenyl benzoate	1: 1.22	Immobilized *Staphylococcus aureus* lipase	Chloroform/41º C/ 6 h	75%	(HORCHANI et al., 2010)
Eugenyl caprylate	1.13: 1	Novozym 435 and Lipozyme TL IM	Hexane/ 56.8º C/ 4h	80%	(RADZI; HANIF; SYAMSUL, 2016)
Eugenyl caprylate	2: 1	Lipozyme TL IM	Solvent-free/ 65º C/ 4.3 h	72%	(CHAIBAKHSH et al., 2012)
Phenethyl acetate	1: 1	Novozym 435	n-Hexane/ 30 ºC/ 30 min	69 %	(KIM; PARK, 2017)
Cinnamyl acetate	1: 2	Immobilized procine pancreatic lipase	Hexane/ 35º C/ 10 h	62.6%	(WU et al., 2014)
Cinnamyl laurate	1: 1	Novozym 435	Toluene/ 30º C/ 2 h	60%	(YADAV; DHOOT, 2009)
Geranyl cinnamate	3: 1	NS88011 (lipase *Candida antactica B*)	n-Heptane/ 80 ºC/ 30 min	97 %	(ZANETTI et al., 2017)
Ethyl cinnamate	3: 1	Lipozyme TL IM	Isooctane/ 50º C/ 24 h	99%	(WANG et al., 2016)
Butyl cinnamate	15: 1	Novozym 435	Isooctane/ 55º C/ 72 h	60.7%	(JAKOVETIĆ et al., 2013a)
Oleyl cinnamate	6: 1	Novozym 435	Isooctane and 2-butanone/ 55º C/ 12 d	100%	(LUE et al., 2005)

[1] M:R = Molar ratio (alcohol: acid)
[2] Conv (%) = conversion of ester synthesis in percentage

The main aromatic esters are benzyl, cresyl, anisyl, eugenyl, cinnamyl, phenethyl, benzoate and cinnamate esters, and as possible seen, some of them were synthesized with immobilized enzymes as biocatalyst and good conversions were reached. The presence of toxic organic solvents is usually necessary to improve and facilitate the substrate conversion, which requires fine downstream processes for solvent recovered, increasing the costs, and possibility of harmful residual substances at the final product.

2.4.1 Benzyl butyrate

The benzyl butyrate is a clear colorless to very pale yellow liquid with odor suggesting plum, melon and peach. It is a fragrance ingredient present in the composition of many decorative cosmetics, fine fragrances, shampoos, toilet soups, other toiletries, and may be present in non-cosmetic products such as detergents and household cleaners (MCGINTY; LETIZIA; API, 2012a). Table 3 presents the identification and main physical properties of benzyl butyrate.

Table 3 - Identification and main physical properties of benzyl butyrate (MCGINTY; LETIZIA; API, 2012a; VCF, 2017).

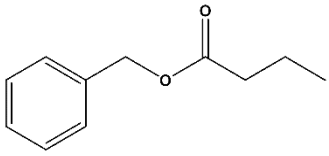
Synonyms	**Benzyl butyrate; Benzyl butanoate; phenylmethyl ester**
CAS registry number	103-37-7
EINECS number	203–105–1
Formula	$C_{11}H_{14}O_2$
Structure	
Molecular weight	178.23
Boiling point	240 ºC
Water solubility	136 mg/l
UV spectra	Peaks at 200–220 nm and minor absorption noted from 250 to 260 nm.
Refractive index:	1.492–1.496 (20 ºC)
Council of Europe (2000)	COE No. 277
FDA	21 CFR 172.515
FEMA	GRAS 3 (2140)

Table 4 - Literature review of the benzyl butyrate, propionate and benzoate synthesis.

Ester/ Substrates	Reaction	Biocatalyst	Best condition /conversion	Reference
Benzyl butyrate/ benzyl alcohol, butyric acid	Esterification	Novozym 425	MR[1] 1:1 (acid:alcohol); solvent methyl tertbutyl ether; 4 g of catalyst, 52 ºC; 24 h. 82 %	(JEROMIN; ZOOR, 2008)
Benzyl benzoate/ benzyl alcohol, methyl benzoate	Transesterification	Novozym 435	MR 2:1 of benzyl alcohol to methyl benzoate, 0.3 g of catalyst, 55◦C. 90 %	(GRYGLEWICZ; JADOWNICKA; CZERNIAK, 2000)
Benzyl propionate/ benzyl alcohol, propionic acid	Esterification	*Pseudomonas cepacia* immobilized in a biodegradable support made by chitosan (CHI) and polyvinyl alcohol (PVA)	Catalyst 54 mg; MR alcohol to acid 2:3; solvent iso-octane; Temperature 50 °C; 160 rpm; time 2.5 h, 8%.	(BADGUJAR; BHANAGE, 2014)
Benzyl propionate/ benzyl alcohol, Vinyl propionate	Transesterification	*Pseudomonas cepacia* immobilized in a biodegradable support made by chitosan (CHI) and polyvinyl alcohol (PVA)	Catalyst 54 mg; MR alcohol to ester 2:5; solvent iso-octane; Temperature 50 °C; 160 rpm; time 2.5 h, 99%.	(BADGUJAR; BHANAGE, 2014)

[1] MR – Molar ratio

Benzyl butyrate is a safe ingredient included by Council of Europe in the list of substances granted A that may be used in foodstuff and considered generally recognized as safe (GRAS) by Food and Drug Administration (FDA) and by Flavor and Extract Manufacturers Association (FEMA). The worldwide volume of use for benzyl butyrate as fragrance ingredient is in the region of 1– 10 metric tons per year (MCGINTY; LETIZIA; API, 2012a).

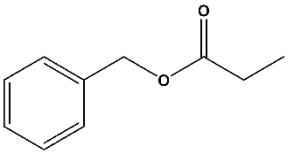
The natural occurrence of this benzyl ester is usually observed at highest quantities in hog plum (*Spondias mombins L.*), but also found in acerola (*Malpighia*), babaco fruit (*Carica pentagona Heilborn*) and cherimoya (*Annona cherimolia Mill.*) (VCF, 2017).

There is just one report in the literature about the enzymatic synthesis of benzyl butyrate, as may be seen in Table 4. Despite the promising result of conversion (~90 %), there is still a lack of information about its synthesis and viability, including tests in SFS, substrate molar ratio evaluation, reusability of the catalyst, kinetics and ester purification.

2.4.2 Benzyl propionate

Benzyl propionate is a fragrance ingredient used in many decorative cosmetics, fine fragrances, shampoos, toilet soaps, other toiletries, and non-cosmetic products such as household cleaners and detergents. Its appearance is a clear colorless to very pale yellow liquid with a fruity odor of considerable tenacity (MCGINTY; LETIZIA; API, 2012b). Table 5 presents the identification and main physical properties of benzyl propionate.

Table 5 - Identification and main physical properties of benzyl propionate (MCGINTY; LETIZIA; API, 2012b; VCF, 2017).

Synonyms	**Benzyl propionate; benzyl propanoate; phenylmethyl ester.**
CAS registry number	122-63-4
EINECS number	204-559-3
Formula	$C_{10}H_{12}O_2$
Structure	
Molecular weight	164.2
Boiling point	222 ºC
Water solubility	416.4 mg/l
UV spectra	Peaks at 200–220 nm and minor absorption noted from 250–260 nm.
Refractive index:	1.496–1.50 (20 ºC)
Council of Europe (2000)	COE No. 413
FDA	21 CFR 172.515
FEMA	GRAS 3 (2150)
JECFA	JECFA No. 842

The benzyl propionate was included in the list of substances granted A by the Council of Europe, considered GRAS by the FDA and FEMA as a flavor ingredient. In addition, the Joint FAO/WHO Expert Committee on Food Additives (JECFA No. 842) concluded that the substance does not present any safety concern at current levels

of intake when used as a flavoring agent. Based on this, the worldwide volume of use for benzyl propionate as fragrance ingredient is in the region of 10–100 metric tons per year (MCGINTY; LETIZIA; API, 2012b; VCF, 2017).

The benzyl propionate occurs naturally in plums (*prunus* species), passion fruits (*passiflora* species) and melons (VCF, 2017). Regarding the synthesis of this ester using different routes besides the natural, just a few studies are available in literature, as may be seen in Table 4.

As shown there is only one report in the literature about the benzyl propionate synthesis. Badgujar and Bhanag (2014) studied the synthesis of benzyl propionate using three different propanoyl donors, propionic acid, vinyl propionate and methyl propionate using a new biodegradable preparation of *Pseudomonas cepacia*. The authors concluded that the low conversions found for the acid (8 %) and methyl propionate (17 %) were associated with inhibition effects. In the esterification, the acid can act as an inhibitor of the enzyme activity, and the methyl alcohol that is as a side product of the transesterification competes with benzyl alcohol for the nucleophilic attack on the carbonyl ester and inhibits the reaction rate. Only the vinyl propionate was effective as propanoyl donor at the fixed conditions reaching conversions of 99 %.

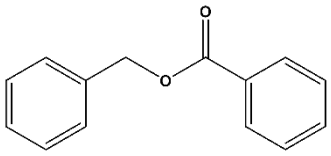
The esterification for the synthesis of benzyl propionate has shown, until the present moment, low conversions to ester, and this behavior has been associated with the use of the short-chain acids as esterification substrate, which leads to enzyme limitations as inhibition and inactivation events (CLAON; AKOH, 1994; HARI KRISHNA et al., 2001; GÜVENÇ; KAPUCU; MEHMETOĞLU, 2002; ROMERO et al., 2005; BADGUJAR; PAI; BHANAGE, 2016; MENESES et al., 2019a; ZARE; GOLMAKANI; NIAKOUSARI, 2019). However, more studies are needed to investigate the viability of the benzyl propionate synthesis by direct esterification of the propionic acid as acyl donor using different and well stablished immobilized enzymes, with focus on SFS and reusability of the catalyst.

2.4.3 Benzyl benzoate

Benzyl benzoate is an aromatic ester that is found in traces in some plants like babaco fruit (*Carica pentagona Heilborn*), caper (*Capparis spinosa*), and celery (*Apium graveolens*), and possesses a balsamic, herb, oily smell (VCF, 2017). Table 6 presents the identification and main physical properties of benzyl benzoate.

Benzyl benzoate is one of the oldest medicines used for the treatment of scabies, a highly contagious skin infection. The mite *Sarcoptes scabiei hominis* burrows into the skin and consumes the epidermis, resulting in inflammation, allergic reactions and pruritic lesions (SHARMA et al., 2016). In the 1930s the topical application of this compound treated successfully thousands of scabies cases in Denmark, since then, the benzyl benzoate body lotion (25 %) has been used in emerging countries for scabies treatment due to the lower cost as compared to other topical treatments like monosulfiram, malathion, lindane, crotamiton, and sulphur in petrolatum, and drugs like permethrin and ivermectin (HEUKELBACH; FELDMEIER, 2006; LY et al., 2009).

Table 6 - Identification and main physical properties of benzyl benzoate (VCF, 2017).

Synonyms	**Benzyl benzoate; Benylate Benzoic acid, phenylmethyl ester Benzyl phenylformate, Phenylmethyl benzoate, Benzoic acid benzyl ester**
CAS registry number	120-51-4
EINECS number	204-402-9
Formula	C14H12O2
Structure	
Molecular weight	212.12
Boiling point	323.5 °C
Melting point	21 ºC
Water solubility	None
UV spectra	427 nm
Refractive index	-
Council of Europe (2000)	COE No. 262
FDA	21 CFR 172.515
FEMA	2138
JECFA	24

In addition, a study realized by Ly and coworkers (2009) compares the effectiveness of oral (ivermectin) and topical (benzyl benzoate) approaches for treating scabies in a community setting in Dakar, Senegal. The results showed that the benzyl benzoate application was more effective in the scabies treatment than the ivermectin oral usage. The group of patients that made two applications of benzyl benzoate per day had 33 patients cured (68.8%) versus 37 (54.4%) in the group that made only one local application per day, on the other hand, only 16 (24.6%) patients were cured in the ivermectin group.

Besides the effective action against the scabies mite, the benzyl benzoate is also used as bioactive component in the formulations of some acaricidal cleaning products like Acarosan. Some studies showed that the active powder was able to kill 100 % of dust mite in culture within 24 hours (HAYDEN et al., 1992), and the application of the Acarosan in carpets and upholsteries led to a significant decrease in major mite allergens, considering a group with proven *Dermatophagoides pteronyssinus* (Dp) asthma (DIETEMANN et al., 1993).

The benzyl benzoate has little availability in nature, and only the synthetic compound is available commercially being produced generally via transesterification or condensation in presence of chemical or inorganic catalysts (THARP et al., 1947). There is a lack of work and information about the benzyl benzoate enzymatic-catalyzed synthesis in the literature. The only one report presented in Table 4 using transesterification mediated by lipases did not show a viable reaction condition (90 % of conversion in 100 h) (GRYGLEWICZ; JADOWNICKA; CZERNIAK, 2000).

On the other hand, the benzoic acid that is a substrate to the benzyl benzoate esterification is a colorless crystalline solid of difficult solubilization and some research has been conducted to understand its solubility in different organic solvents (BEERBOWER; WU; MARTIN, 1984; OLIVEIRA et al., 2007; LONG et al., 2010; THATI; NORDSTRÖM; RASMUSON, 2010; WANG et al., 2014). The use of this compound as a substrate for enzymatically catalyzed reactions may present some drawbacks related to the solubility and diffusion problems. In this way, studies are needed to investigate the viability of the benzyl benzoate synthesis via enzymatically-catalyzed reaction using different acyl donors and well established immobilized enzymes.

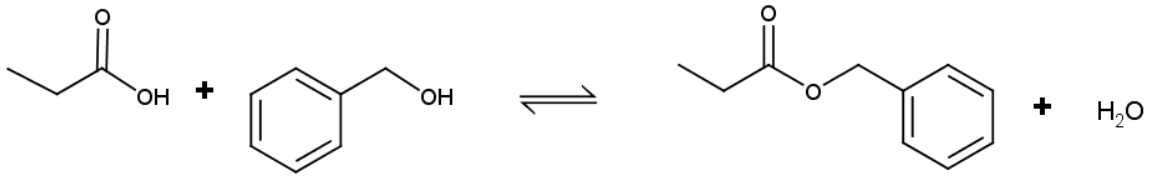
2.5 FINAL CONSIDERATIONS REGARDING THE STATE OF THE ART

Based on the literature review exposed in this chapter, the esterification of aromatic esters mediated by lipases using short chain acids as substrates is still a poorly explored field. Generally, the reaction is an unfeasible alternative from the economic point of view since the presence of organic solvents or a stoichiometric excess of alcohol are necessary to improve enzyme performance, avoid acid inhibition, and facilitate the ester synthesis. The acid inhibition problems limit the enzyme performance defaulting the esterification of many esters like benzyl propionate and benzyl butyrate that use short chain acids as substrate. On the other hand, solid acids like benzoic acid turn the substrates solubilization a difficult issue in the esterification of benzyl benzoate. In this way, an investigation is necessary to find alternatives that enable the synthesis of these esters in solvent free systems considering different reactor configurations to ensure good biocatalyst performance and process efficiency, aiming scale-up and industrial production.

3 BENZYL PROPIONATE SYNTHESIS BY FED-BATCH ESTERIFICATION

The benzyl propionate esterification (Figure 5) mediated by lipases is still a challenge as shown in a previous work (SÁ et al., 2018) that studied the ester synthesis in batch reactors using different immobilized enzymes to catalyze the esterification in absence of organic solvents. After an experimental design, a maximized condition led to 44 % of conversion considering 15 % of Novozym 435, 50 ºC and a substrate molar ratio of 1:1. Some tests were conducted varying the substrates molar ratio from 1:1 to 1:5 and even at high amount of benzyl alcohol, the conversion remained below 35 %. These results strongly indicate a lipase inhibition by acid as the propionic acid possesses a short chain and other authors already reported that these acids might lead to enzyme acidic deactivation (CLAON; AKOH, 1994; HARI KRISHNA et al., 2001; GÜVENÇ; KAPUCU; MEHMETOĞLU, 2002; ROMERO et al., 2005; MENESES et al., 2019a; ZARE; GOLMAKANI; NIAKOUSARI, 2019).

Figure 5 - Esterification of benzyl alcohol and propionic acid.



Based on the enzyme deactivation/inhibition problems, more studies are necessary to ensure high conversion using the esterification reaction that still is the simplest alternative for producing aromatic esters targeting future industrial applications. In order to overcome the drawbacks related to the use of the short chain acids (like propionic acid) as acyl donors, the present work adopted a fed-batch approach for the synthesis of benzyl propionate via esterification, using a controlled feed of the acid during the initial hours of reaction.

Here, a maximized condition from our previous work (SÁ et al., 2018) was used and adapted to the fed-batch strategy using Novozym 435 (the commercial

immobilized preparation of the *Candida antarctica* fraction B lipase - Cal B) and the lyophilized Cal B, as biocatalysts in a SFS using molecular sieves to adsorb water. The fed-batch esterification using both biocatalysts was tested under different molar ratios of propionic acid and benzyl alcohol, and the immobilized enzyme was also tested in the recycle. The initial reaction rate was determined, and the enzyme activity and protein content of the immobilized enzyme before and after the reaction cycles were measured in order to determine the best biocatalyst to enhance the viability of the benzyl propionate synthesis via esterification.

3.1 MATERIAL AND METHODS

3.1.1 Enzymes and chemicals

The esterification substrates were propionic acid (Neon, 99.5 %) and benzyl alcohol (Neon, 99.5 %). The biocatalysts Novozym 435, a commercial immobilized *Candida antarctica* fraction B lipase (Cal B), and Sorbitol solution of free Cal B (Novozymes NZL-102, CALB) were kindly donated by Novozymes®. Molecular sieves (4 Å, beads 8-12 mesh, Sigma-Aldrich) were used as water adsorbent. The solvents acetone (Quemis, 99.5 %) and dichloromethane (Quemis, 99.8 %) were employed to wash the immobilized lipase for recycling and for gas chromatography quantification, respectively.

3.1.2 Benzyl propionate quantification

The benzyl propionate obtained after acid and alcohol esterification was analyzed based on a calibration curve previously prepared using the ester standard. The analyses were carried out in a gas chromatograph (Shimadzu GC 2010) with auto-injector (Shimadzu AOC 5000), equipped with a DB-5 column (27 m length x 0.25 mm internal diameter x 0.25 μm film thickness). The samples were diluted in dichloromethane and injected into the column with an initial temperature of 100 °C for 2 min, then the temperature was raised from 100 to 230 °C with a rate of 10 °C/min

and kept at 230 °C for 10 min. Injector and detector were set to 250 °C (SÁ et al., 2018).

3.1.3 Benzyl propionate fed-batch synthesis using immobilized lipase

Initially, a study of the benzyl propionate fed-batch synthesis via esterification was made at the same conditions of temperature, enzyme type and amount, and acid: alcohol molar ratio (MR) described in a previous work that maximized the ester synthesis in batch mode (SÁ et al., 2018). The fed-batch reaction involved the addition of 35 mmol of benzyl alcohol in a glass-jacketed reactor previously heated at 50 ºC, containing molecular sieve and 5, 10 or 15 wt.% (related to the amount of substrates) of Novozym 435. The propionic acid was added to the medium during the first five hours, totalizing 35 mmol after six additions (5.83 mmol/h). The reaction was magnetically stirred at 150 rpm during all reaction time, and aliquots were taken in 0, 1, 2, 3, 4, 5, 6, 8, 10 and 24 h of reaction and adequately diluted for quantification. All experiments were carried out in duplicate.

The molecular sieve used had a water holding capacity of 20 % (m/m), according to fabricant. The amount of molecular sieve in each system was calculated based on the molar amount of the limiting substrate (acid) and the total water amount generated as a secondary product of esterification.

3.1.4 Effect of substrates molar ratio in the reuse of immobilized enzyme

The performance of Novozym 435 as biocatalyst in the fed-batch synthesis of the benzyl propionate was evaluated using different substrate molar ratios. The esterification reaction involved the addition of the adequate amount of benzyl alcohol, molecular sieve and 10 wt.% of Novozym 435 in a pre-heated (50 ºC) jacketed glass reactor. The propionic acid was added to the medium during the first five hours with a feed rate of 5.83, 2.91 and 1.83 mmol/h, ensuring the adequate final MR of 1:1, 1:3 and 1:5 (acid: alcohol), respectively. The reaction was magnetically stirred at 150 rpm during all reaction time, and aliquots were taken after 0, 1, 2, 3, 4, 5, 6, 8, 10 and 24

h of reaction and adequately diluted for quantification. All experiments were carried out in duplicates.

The enzyme reuse study involved the use of the same immobilized enzyme (Novozym 435) in a new reaction cycle using the same reaction conditions. After the first cycle, the enzyme was recovered from the medium by vacuum filtration using a glass funnel and filter paper. The molecular sieve beads were collected, and the enzyme was washed with acetone (m/v ratio of 1:10) three times to remove the product or substrate residues (TOMKE; RATHOD, 2015). In sequence, the immobilized enzyme was dried overnight (50 ºC) and reused in a new fresh fed-batch reaction.

3.1.5 Benzyl propionate fed-batch synthesis using lyophilized Cal B lipase

The enzymatic liquid containing the free Cal B lipase was concentrated using the cellulose membrane method described elsewhere (CHIARADIA et al., 2016) and then, the enzyme was recovered by lyophilization using a LIOTOP Lyophilizer, Model L101. The lyophilized Cal B was stored under refrigeration (4 ºC).

The benzyl propionate fed-batch synthesis using 50 mg of lyophilized Cal B lipase as biocatalyst involved the use of the same reaction conditions and the procedure described above. The quantity of the lyophilized lipase used for these esterification reactions was based on the amount of Cal B physically immobilized onto the macroporous acrylic polymer resin Lewatit VP OC 1600 from Novozym 435 (SHELDON, 2007), which is around 8% (CHEN; MILLER; GROSS, 2007). The amount of lyophilized Cal B was 8 wt.% related to the total amount of the supported Novozym 435 used in the previous fed-batch reactions.

3.1.6 Initial reaction rate

The initial reaction rate (*r*) was calculated as described by Equation 1:

$$r = \left(\frac{\Delta Cester}{\Delta t} \right) \quad (1)$$

where r is the initial rate of reaction (h^{-1}); C_{ester} is the ester conversion (wt.%) at time t; and t is the reaction time (h) (ZENEVICZ et al., 2016).

3.1.7 Immobilized lipase activity

The enzyme activity (U/g) was determined by the lauric acid and n-propanol esterification technique described elsewhere with minor modification (CENI et al., 2010). The esterification reaction was conducted in a substrate MR of 1:1, enzyme amount of 5 wt.% (related to the substrates) for 40 min at 60 ºC. The remaining lauric acid content was determined by titration with NaOH 0.01 M. A unit of activity (U) was defined as the amount of enzyme necessary to consume 1 µmol of lauric acid per minute. All enzymatic activity determinations were carried out in triplicates.

3.1.8 Qualitative protein content determination by optical microscopy

The qualitative analysis of the protein immobilized in the Novozym 435 support, and the content of lipase that remained on the support after the fed-batch cycle was performed using optical microscopy. The samples were prepared for analysis on glass microscope slides. The immobilized enzymes were marked using lugol iodine solution, the excess of solution was removed, and immersion oil was dropped directly under the Novozym 435 support beads. In sequence, the samples were analyzed in an Olympus BX 41 microscope using bright-field illumination with magnifications of 40 and 100 times and the images were recorded using a digital camera Q-imaging (3.3 Mpixel).

3.2 RESULTS AND DISCUSSION

3.2.1 Fed-batch esterification of benzyl propionate ester using Novozym 435 as biocatalyst

The benzyl propionate natural production via lipase-mediated esterification is still a challenge due to the propionic acid short chain characteristics that generally

implies in enzyme inhibitory/inactivation effects, as already reported for other short chain acids (CLAON; AKOH, 1994; HARI KRISHNA et al., 2001; GÜVENÇ; KAPUCU; MEHMETOĞLU, 2002; ROMERO et al., 2005; MENESES et al., 2019a; ZARE; GOLMAKANI; NIAKOUSARI, 2019). Researchers have reported that the main reason for inhibition by acids is associated with acidification of the microaqueous interface leading to enzyme inactivation (GÜVENÇ; KAPUCU; MEHMETOĞLU, 2002; ROMERO et al., 2005; ZARE; GOLMAKANI; NIAKOUSARI, 2019).

In our previous work, the benzyl propionate was synthesized by batch esterification using Novozym 435 as biocatalyst, a commercial and very well-known lipase, and after the experimental design the maximum ester conversion was around 44%, considering the immobilized enzyme highest level (15 wt.% related to the total amount of substrates) (SÁ et al., 2018). The low conversion values were related to enzyme acidic inactivation, and in order to overcome the drawbacks related to the propionic acid, a fed-batch approach was adopted in this work and the acid was added to the reaction along the initial 5 hours.

Figure 6 - Conversion to benzyl propionate during fed-batch reactions at different Novozym 435 amounts of 5, 10 and 15 wt.% related to the substrates total weight. The reaction conditions were substrates molar ratio of 1:1, 50 ºC and 150 rpm.

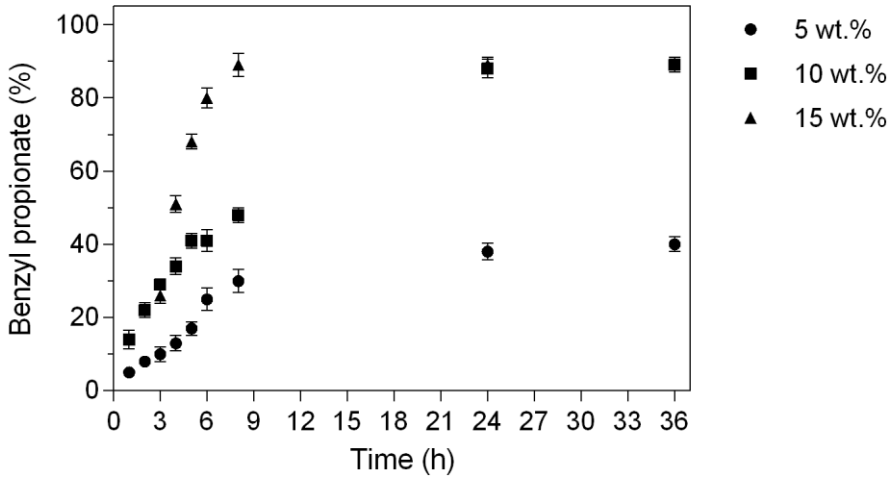
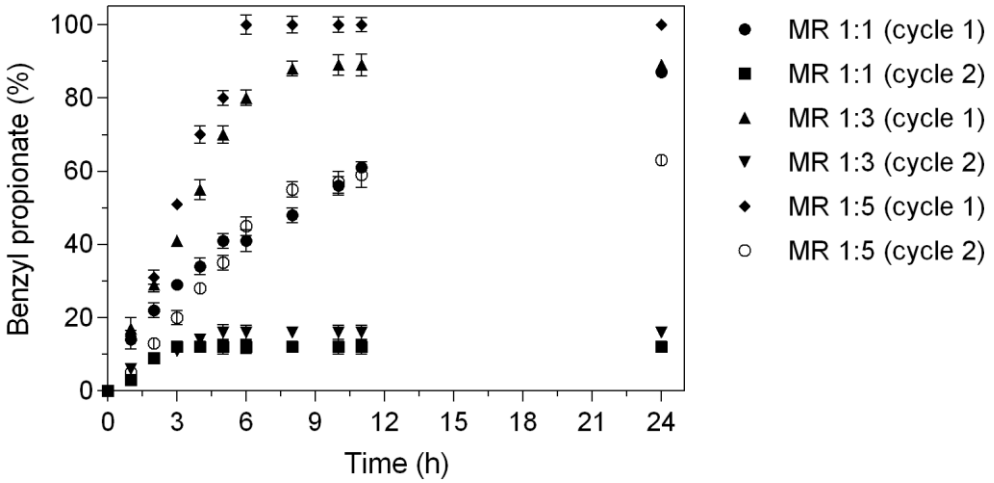


Figure 6 shows the results of the fed-batch esterification between the benzyl alcohol and propionic acid using three different enzyme amounts of Novozym 435 in

presence of molecular sieve and reaction conditions set at 50 ºC, 150 rpm, and substrates molar ratio of 1:1. The molecular sieve is able to adsorb the water present in the reaction media (reaction product) reducing the amount of water in the lipase microaqueous environment and avoiding the protein deactivation (MARTY et al., 1991; BANSODE; RATHOD, 2014; PALUDO et al., 2015; MENESES et al., 2019a). A great increase in the conversion to ester was found (~ 90%) compared with the previous results in batch mode (~44%) (SÁ et al., 2018), showing that the fed-batch approach is able to overcome the acid inhibitory effects, visualized in batch reactor configuration. The maximum conversion was ensured after 8 h of reaction with 15 wt.% of biocatalyst. However, the reaction medium was saturated due to the high amount of enzyme during the first 4 h, making difficult the fed-batch process. The enzyme amount of 5 wt.% was not able to increase the ester conversion to the maximum levels. The enzyme amount of 10 wt.% was enough to ensure a high conversion (~ 90%) after 24 h, and good fed-batch performance with lower enzyme amount in the reaction medium. Based on these ester conversions results, the immobilized enzyme amount was set at 10 wt.% for the next experimental step.

The effect of the substrate molar ratio was investigated in the benzyl propionate synthesis in fed-batch esterification reactions. Figure 7 shows the results of the conversion as a function of time for the molar ratios of 1:1, 1:3 and 1:5 (acid: alcohol). All molar ratios tested reached in the first cycle of reaction high conversions to benzyl propionate in different periods of reaction. As the alcohol amount was increased, the global conversion was also increased, and the MR of 1:5 led to the maximum conversion (~99%) after 6 hours of reaction, i.e., the maximum conversion was reached right after the last acid addition, showing a rapid and effective performance. These results may be related to the capacity of high amounts of benzyl alcohol to dilute the propionic acid present in the reaction media, together with the slower addition of the acid in the medium, indicating that the enzyme inactivation effects were reduced in the fed-batch conditions, ensuring high conversions. A similar increase in ester conversion was found for a study of oleic acid ethyl ester synthesis using crude rice bran (*Oryza sativa*) lipase in a fed-batch system. The alcohol acted as a lipase inhibitor, and the fed-batch configuration was able to overcome the negative effects and increase the conversion (HIDAYAT et al., 2014).

Figure 7 - Reusability of Novozym 435 for benzyl propionate synthesis using the fed-batch approach and different acid: alcohol molar ratios (1:1 to 1:5).



The recycle of the immobilized enzyme was performed under the same fed-batch reaction conditions as shown in Figure 7. The conversion was sharply reduced in the second cycle of the Novozym 435 for the MR 1:1 and 1:3, remaining around 12 and 16%, respectively. The higher amount of alcohol present in the medium (MR acid to alcohol 1:5) allowed a slight improvement in the enzyme performance, but still, a great reduction of conversion was observed after 24 h of reaction (conversion of ~ 63%). Similar results were found in the benzyl butyrate esterification using Novozym 435 as biocatalyst in a fed-batch configuration with the feed of the butyric acid. The performance of the enzyme was improved in larger amounts of benzyl alcohol (MENESES et al., 2019a).

This strong negative effect on enzyme performance was probably accentuated with the use of a SFS configuration, which requires high amounts of alcohol to dilute the propionic acid. Other researchers synthesized many aroma esters using, commonly, organic solvents like *n*-hexane for the enzymatic syntheses with short-chain acids as acyl donors (MARTY et al., 1991; CLAON; AKOH, 1994; HUANG; CHANG; GOTO, 1998; HARI KRISHNA; PRAPULLA; KARANTH, 2000; HARI KRISHNA et al., 2001; WU et al., 2014). The presence of a great amount of a nonpolar solvent favors the reaction equilibrium to the ester synthesis and increases

substrates and products solubility, reducing the acid damaging effects against the enzyme active site (HALLING, 1984; MARTY et al., 1991; SÁ et al., 2017).

Some investigation around the enzyme activity was also conducted in order to understand this sharp reduction in the conversion even at high amounts of alcohol in the reaction medium in SFS. Table 7 shows the Novozym 435 activity before the reaction, after the second reaction cycle for all acid: alcohol molar ratios tested, and after contact with acid and alcohol separately. Before the reaction, the immobilized enzyme showed an activity of 54.6 U/g, and a similar value was observed after the alcohol contact, showing that neither the enzyme support, nor the enzyme active site were harmed by the contact with the alcohol. On the other hand, the lipase activity was sharply reduced after the contact with the acid and the same behavior was observed after the reactions with molar ratio acid: alcohol 1:1 and 1:3 (with an increased amount of acid). This behavior indicates the propionic acid is responsible for activity loss of the enzyme. The enzyme activity after the reaction with molar ratio 1:5 still presented some activity (~ 18 U/g) which explains the lower conversion showed in Figure 7. However, it is worth mentioning that the mixtures/reactions visual appearance showed that the Novozym 435 support was not dissolved after the reaction or substrates contact.

Table 7 - Novozym 435 activity before, and after fed-batch esterification (cycle 2) and after substrates contact.

Condition	Novozym 435 esterification activity (U/g)
Before reaction	54.6 ± 0.7
After esterification reaction MR 1:1	1.0 ± 0.2
After esterification reaction MR 1:3	1.1 ± 0.4
After esterification reaction MR 1:5	18.5 ± 1.2
After acid contact	1.7 ± 0.7
After alcohol contact	46.2 ± 0.4

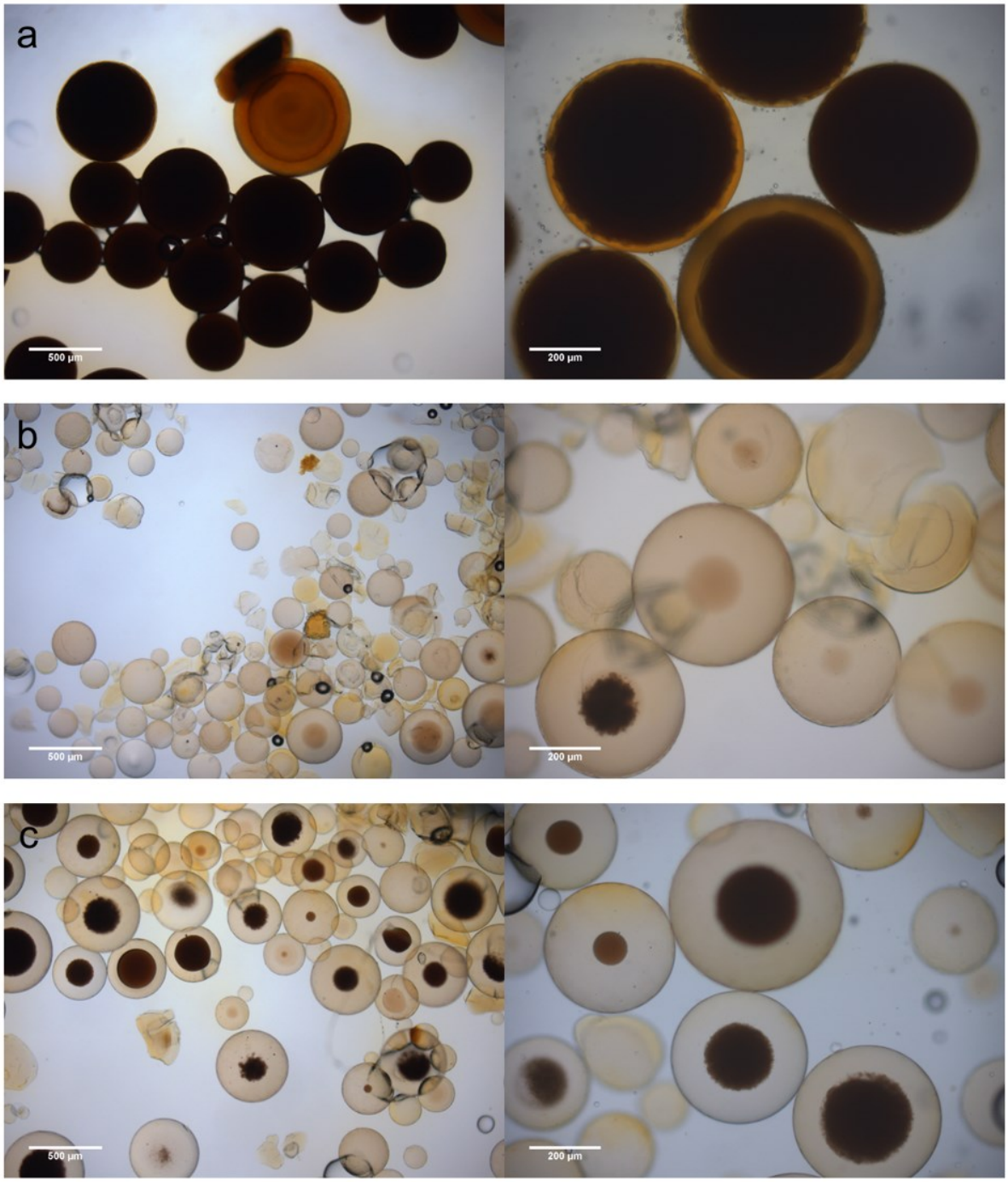
In order to understand the relationship between the propionic acid and the Novozym 435 loss of activity, a qualitative investigation about the protein content in the polymeric support of the biocatalyst was also performed using an optical microscopy assay with lugol iodine solution as protein marker. Figure 8 shows the micrographs of the Novozym 435 before and after the reaction cycles. The protein

content (brown filling) in the support of the Novozym 435 was large before the esterification (Figure 8a), all polymeric support is filled with lipase. Figure 8b shows the support spheres (clear yellow spheres) almost unfilled after the enzyme recycle (molar ratio acid: alcohol 1:3), and a similar behavior was observed in Figure 8c that shows the immobilized enzyme used in the reaction with substrate molar ratio of 1:5. However, in this case, some protein (brown filling) remains inside the support spheres.

Novozym 435 is a commercial immobilized enzyme obtained by immobilization via interfacial activation of the lipase on a moderately hydrophobic macroporous resin (SÁ et al., 2018; ORTIZ et al., 2019). The interfacial activation is used very often for lipase immobilization, however, as reported in the literature, the main drawback related to this protocol is the enzyme desorption during high-temperature exposure and in contact with surfactants or organic solvents (PANAHI et al., 2014; RUEDA et al., 2016; VIRGEN-ORTÍZ et al., 2017).

Although Novozym 435 is generally very resistant and used to catalyze successfully many organic reactions (RATHORE; MADRAS, 2007; TALUKDER et al., 2009a; GUMEL; ANNUAR; HEIDELBERG, 2013; ROSSO COMIM et al., 2015; POLLONI et al., 2017; ORTIZ et al., 2019) the micrographs showed that the presence of the lipase inside the support was strongly reduced after the reaction, and the protein leaching was pronounced at higher amounts of propionic acid in the medium (molar ratio of 1:1 and 1:3). These results corroborate with the previously shown results in Figure 7 and Table 7, in which the Novozym 435 had lost almost completely its activity after the first cycle of use in the presence of high amounts of propionic acid (molar ratio of 1:1 and 1:3), but some activity remained after the reaction with substrates molar ratio of 1:5. This behavior is visibly related to the inactivation/desorption of the Cal B immobilized in the Novozym 435 support after the acid contact, leading to the breakdown of the physical bonds between the enzyme and the polymer support. The absence of covalent bonds is the main disadvantage of the interfacial activation immobilization technique. The lipase adsorbed on the macroporous acrylic resin possesses a weak bond and if the enzyme is irreversibly deactivated and/or desorbed, both the enzyme and the support are rendered unusable (SHELDON, 2007).

Figure 8 - Optical micrographs of the enzyme support beads showing the protein content (brown filling) in the polymeric support (clear yellow sphere) of the (a) Novozym 435 before use and (b) after cycle 2 with substrates molar ratio of 1:3 and (c) molar ratio of 1:5 in the fed-batch esterification to synthesize benzyl propionate.



A similar result was observed for Cal B lipase immobilized in onoctyl agarose (CALB-OA) using the same immobilization technique (interfacial activation)

(VIRGEN-ORTÍZ et al., 2017). The CALB-OA beads used in the first cycle of 100 mM tributyrin hydrolysis exhibited a linear reaction course. However, a drastic drop in the enzyme activity was observed after the second cycle. SDS-PAGE (Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis) gels of the supernatant and biocatalyst showed significant enzyme desorption after the reaction. Similar results were found using triacetin and sunflower oil, suggesting the substrates and products may act as a lipase detergent causing massive enzyme desorption.

Some researchers have already reported that other highly polar short-chain aliphatic acids may cause enzyme inhibition and inactivation like acetic acid (CLAON; AKOH, 1994; HARI KRISHNA et al., 2001; ROMERO et al., 2005) and butyric acid (HARI KRISHNA; PRAPULLA; KARANTH, 2000; MENESES et al., 2019a). However, there are not any other works showing, until the present moment, that these acids can act as a lipase detergent, causing the lipase desorption from the hydrophobic support during the esterification reaction even at increased alcohol molar ratio.

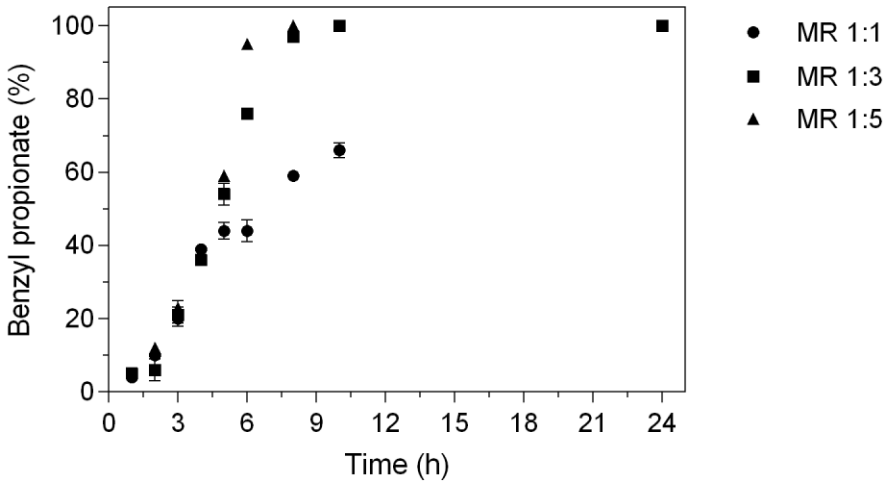
These results suggest that the use of an immobilized enzyme as catalyst in this specific reaction should possess irreversible bonds between the support and enzyme, as in hetero-functional supports, to prevent the lipase desorption (ABAHÁZI; BOROS; POPPE, 2014; RUEDA et al., 2015; VIRGEN-ORTÍZ et al., 2017). Another alternative is the use of a crosslinking agent as glutaraldehyde, polyethylenimine and aldehyde dextran to reinforce the enzyme-support bonds, preventing the enzyme release (FERNANDEZ-LORENTE et al., 2011; ZAAK et al., 2017; ORTIZ et al., 2019).

3.2.2 Fed-batch synthesis of benzyl propionate using lyophilized Cal B

Novozym 435 is one of the most used immobilized lipases, extensively employed in different reactions and processes (ORTIZ et al., 2019). The immobilized form of Cal B is preferred for laboratory and industrial applications due to the improved stability besides the traditional advantages of immobilized enzyme systems, as reusability and products high purity related to the easier downstream processing.

However, although high conversions were obtained only in the first reaction cycle of the Novozym 435, the recycle results hamper the use of an immobilized enzyme to the present benzyl propionate synthesis, since the recycling is the most important advantage of the immobilized enzyme usage. Based on the results, some tests were conducted using the lyophilized Cal B as biocatalyst of the propionic acid and benzyl alcohol esterification. Figure 9 shows the results of conversion for the fed-batch esterification using substrates in molar ratio of 1:1, 1:3 and 1:5. As we can seen, the lyophilized enzyme showed a similar performance to Novozym 435, ensuring high conversions for all molar ratios tested in the same period of reaction. These results may be related to the lyophilized lipase form, which did not ensure a free enzyme configuration due to the presence of aggregates in the reaction medium.

Figure 9 - Conversion to Benzyl propionate over 24 h of reaction using lyophilized Cal B as biocatalyst in fed-batch system varying substrate molar ratio from 1:1 to 1:5 (acid: alcohol). The reaction conditions were fixed in 50 ºC, 8 wt.% of lyophilized Cal B (related to the Novozym 435 initial weight), and 150 rpm.

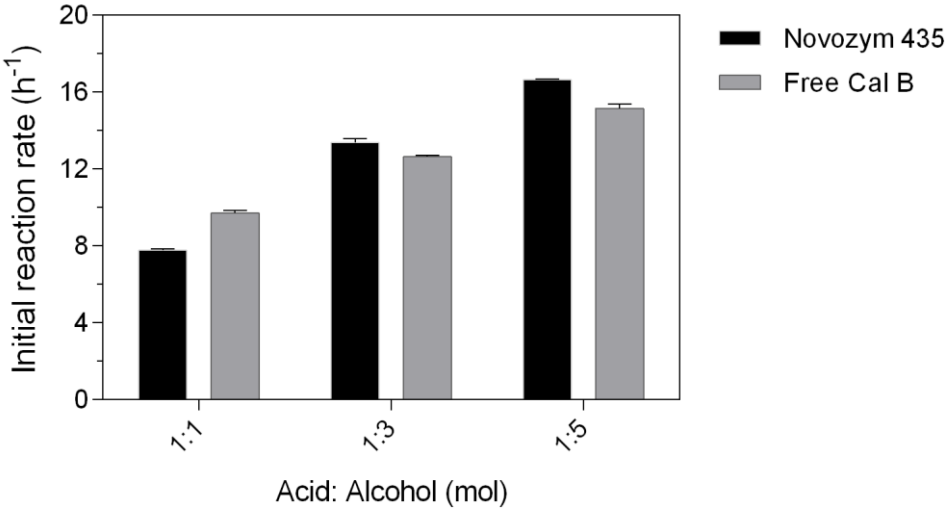


The initial fed-batch reaction rate was determined for both lyophilized and immobilized biocatalysts and Figure 10 displays the results for the three MR tested. As the amount of alcohol was increased, an increase in the reaction rate was observed for both Cal B forms, showing that the lyophilized enzyme had the same performance of the immobilized enzyme. It is worth noting that alcohol plays an

important role in the reaction rate and was able to dilute the propionic acid present in the medium, reducing the inhibition effects and ensuring a high conversion to ester.

Based on these results, the lyophilized Cal B could be used to catalyze the benzyl propionate reaction. However, a proper immobilization system may improve enzyme stability and some techniques as crosslinking and hetero-functional supports might prevent the lipase desorption problems and enable the use of a heterogeneous catalyst for the benzyl propionate synthesis. The heterofunctional supports possess several distinct functionalities on its surface able to interact covalently with the lipase (BARBOSA et al., 2013) and generally are suitable for the CALB immobilization ensuring enhancement in the results and increase enzyme stability as already reported by some authors (GARCIA-GALAN et al., 2011; BARBOSA et al., 2013; RUEDA et al., 2015; HIRATA et al., 2016; PEIRCE et al., 2016; DE MELO et al., 2017; ARANA-PEÑA; LOKHA; FERNÁNDEZ-LAFUENTE, 2018).

Figure 10 - Initial reaction rate of the fed-batch esterification between benzyl alcohol and propionic acid using Novozym 435 and lyophilized Cal B as biocatalysts with different acid: alcohol molar ratios.



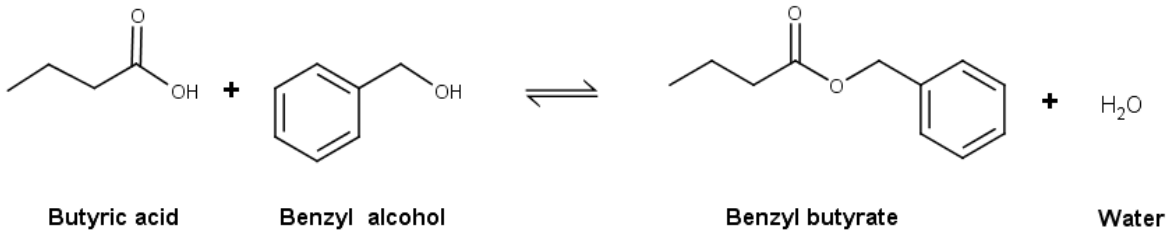
3.3 CONCLUSION

The present work showed a natural alternative for the esterification of the propionic acid and benzyl alcohol using a biotechnological approach. The use of a fed-batch configuration allowed to overcome the inhibitory effects related to the use of propionic acid in the direct esterification, with an increase from 44% of conversion to benzyl propionate in batch mode to 99% in fed-batch mode. The Novozym 435 was effective in the first cycle of conversion ensuring high conversion; however, the immobilized lipase was desorbed from the support due to the presence of propionic acid. Although high amounts of alcohol were used to dilute the propionic acid, all substrates molar ratios tested showed enzyme desorption, hampering the use of an immobilized enzyme for the benzyl propionate synthesis. The lyophilized Cal B led to similar conversions and initial reaction rates to those found for Novozym 435. These results indicated the possibility of using a free enzyme as biocatalyst to ensure a high conversion and a feasible process for the esterification of benzyl alcohol and propionic acid. However, more studies are needed in order to find a suitable support material to ensure a good immobilized enzyme process performance, considering an immobilization technique that avoids the enzyme desorption since the propionic acid in solvent-free conditions acted as a lipase detergent.

4 BENZYL BUTYRATE SYNTHESIS MEDIATED BY IMMOBILIZED LIPASES

The main objective of the present work was to synthesize benzyl butyrate in a solvent-free system (SFS) via esterification mediated by immobilized lipases (Figure 11) using batch and fed-batch approaches in order to eliminate the excess of substrates in the medium. Initially, the biocatalyst type and amount, temperature, time of reaction, molar ratio of substrates, presence of molecular sieves, and enzyme recycle were evaluated aiming to improve the conversion of benzyl butyrate in batch mode. In the sequence, a study about the use of fed-batch esterification was carried out, feeding the acid during the first five hours of reaction to improve the performance of the biocatalysts. Finally, an increase in reaction volume was performed using the best experimental condition.

Figure 11 - Benzyl butyrate synthesis via esterification of benzyl alcohol and butyric acid.



4.1 MATERIAL AND METHODS

4.1.1 Enzymes and chemicals

Butyric acid (Neon, 99 %) and benzyl alcohol (Neon, 99.5 %) were utilized as substrates. Molecular sieve beads (4 Å, 8-12 mesh, Sigma-Aldrich) were utilized to remove possible residues of water from esterification reaction. Enzymatic catalysts were the commercial immobilized lipases Novozym 435 (immobilized Cal B lipase), Lipozyme TL-IM (immobilized *Thermomyces lanuginosus* lipase), Lipozyme RM-IM (immobilized *Rhizomucor miehei* lipase), and NS 88011 a non-commercial

immobilized lipase prepared from Cal B lipase, donated by Novozymes®. Dichloromethane (Quemis, 99.8 %) was used in gas chromatography injections. Acetone (Dinâmica, 99.5 %) was utilized to wash the enzyme for reuse tests. Sodium hydroxide (NaOH 1 M, Dinâmica), ethyl acetate (Quemis, 99.5 %) and sodium sulfate (Vetec, 99 %) were employed for ester purification.

4.1.2 Biocatalyst type and molecular sieves

The esterification between benzyl alcohol and butyric acid was tested with four biocatalysts to determine the best enzyme for benzyl butyrate synthesis. The reaction involved the addition of 10 wt% of Lipozyme TL-IM, Lipozyme RM-IM, Novozym 435 or NS 88011 in 2 mL tube reactors containing 3.5 mmol of butyric acid and 3.5 mmol of benzyl alcohol (molar ratio 1:1). After 24 h of reaction at 65 ºC under mechanical agitation, aliquots of the product from each reactor were taken and adequately diluted for quantification.

The presence of molecular sieves 4Å at the reaction media was also studied to investigate its potential as water adsorbent. The molecular sieve used had a water holding capacity of 20 % (m/m), according to fabricant. The amount of molecular sieve in each system was calculated based on the molar amount of the limiting substrate (acid) and the total water amount generated as a secondary product of esterification.

4.1.3 Benzyl butyrate quantification

All reaction samples were diluted in dichloromethane and analyzed in a gas chromatograph (Shimadzu GC 2010) with auto-injector coupled (Shimadzu AOC 5000), equipped with a DB-5 column (27 m length x 0.25 mm internal diameter x 0.25 µm film thickness) and flame ionization detector (FID). Then, the samples were injected into the column with an initial temperature of 100 °C for 2 min, this temperature was raised to 230 °C at a rate of 10 °C/min and maintained for 10 min; injector and detector were kept at 250 °C (SÁ et al., 2018). The benzyl butyrate corresponding peak of each chromatogram was analyzed by a previously prepared

benzyl butyrate (99% purity) calibration curve (R^2 = 0.99) for the determination of the conversion to benzyl butyrate (%).

4.1.4 Experimental design

The benzyl butyrate synthesis was maximized by Response Surface Methodology (RSM) in batch mode using two immobilized enzymes from Cal B with different support materials (NS 88011 or Novozym 435) as biocatalysts. A 2^2 factorial experimental design was employed considering two levels (+1, -1) and two central points (0) totaling 12 experiments; the independent variables involved were the amount of enzyme (E) and temperature (T), and the responses were related to the conversion (in percentage, %) to each biocatalyst type (Novozym 435 and NS 88011). The independent variables, their levels with real and coded values are presented in Table 8.

Table 8 - Parameter levels and coded values used in the experimental design.

Factors	**Range and level**		
	-1	0	+1
Temperature (ºC)	50	60	70
Enzyme amount (wt %)	2	6	10

Statistica 7.0 (Statsoft Inc.) was used to analyze the influence of the independent variables on the response. The, data were fitted to a linear model and Pareto charts were plotted to verify the reproducibility of the fitted model with the experimental data.

The benzyl butyrate synthesis involved the use of butyric acid and benzyl alcohol with no excess of reactants in order to reduce operational costs. Therefore, a fixed molar ratio of 1:1 (5 mmol each) was used for the entire experimental design assay. The substrates were placed in 2 mL batch reactors containing molecular sieves and different amounts of biocatalyst (Novozym 435 or NS 88011), and different reactions T were employed, as presented in Table 8. The reactors were stirred using mechanical agitation in a thermal bath over 24 h. At the end of the

reaction time, an aliquot of the product was centrifuged and adequately diluted for quantification.

The investigation of the behavior of conversion as a function of time was conducted under the best condition of T and E determined by the experimental design for conversion responses for both enzymes (Novozym 435 and NS88011). Destructive experiments, without sampling, were prepared in 2 mL reactors with substrates at the molar ratio of 1:1 (5 mmol), and submitted to mechanical agitation at the adequate temperature. After the time periods of 0, 1, 2, 4, 6, 8, 12, 24, 36 and 48 h, a destructive reaction was removed, and an aliquot was centrifuged and adequately diluted for quantification.

4.1.5 Effect of substrates molar ratio and enzyme reuse in batch mode

The absence of solvents may directly influence the enzyme performance, and an excess of alcohol may be required to ensure a good enzyme performance. In this way, a study involving the performance of the enzymes in the ester synthesis was carried out varying the molar ratio of substrates (acid: alcohol) from 1:1 to 1:3 using the immobilized lipases Novozym 435 and NS 88011 as biocatalysts. The esterification reaction involved the addition of butyric acid and benzyl alcohol at adequate molar concentrations into a 2 mL batch reactor containing molecular sieves beads and the enzyme amount previously determined in the experimental design. The batch reactors were placed under mechanical agitation at the best temperature and time of reaction previously determined. At the end of reaction time, an aliquot of the product was centrifuged and adequately diluted for quantification.

The enzyme recycles involved the use of the same immobilized enzyme over three reaction cycles at the same reaction parameters. The recovery of the immobilized enzyme after each reaction was made by vacuum filtration using glass funnel and filter paper. The molecular sieve beads were collected using a granulometric sieve, and the enzyme was washed three times with acetone (m/v ratio of 1:10) to remove the product or subtract residues (TOMKE; RATHOD, 2015). Then, the enzyme was dried for 20 h at 50ºC and reused in a new fresh reaction batch. This procedure was repeated for each reuse cycle.

4.1.6 Benzyl butyrate synthesis in fed-batch mode and enzyme reuse

The fed-batch configuration was also employed in the synthesis of benzyl butyrate using the best condition (T and E) determined in the experimental design. The fed-batch synthesis involved a jacketed glass reactor previously heated and prepared with 35 mmol of benzyl alcohol, the respective immobilized enzyme amount (NS 88011 and Novozym 435) and molecular sieves. The butyric acid was added to the medium during the first five hours, totalizing 35 mmol after six additions (5.83 mmol/h). The reaction was maintained under magnetic agitation at 150 rpm during the whole reaction time, and aliquots were taken at 0, 1, 2, 3, 4, 5, 6, 8, 10 h of reaction and adequately diluted for quantification.

The reaction volume was increased 31 times and the reaction was performed with the fed-batch configuration using the best reaction condition determined in the experimental design and the biocatalyst that had the best performance in the recycling tests. For that, a 500 mL jacketed glass reactor was used, and a similar procedure as earlier described was employed with fixed reaction parameters and molar ratio of 1:1, 1.1 mol.

4.1.7 Benzyl butyrate purification

Firstly, the reaction media was mixed with ethyl acetate and NaOH 1 M (v/v ratio of 1:4 related to product volume) for the removal of remaining butyric acid. Then, the mixture was homogenized with distilled water (v/v ratio of 1:4 related to the organic phase) and, the organic phase was recovery from liquid-liquid extraction and filtered under vacuum with sodium sulfate to remove the remaining water. Finally, the organic phase was submitted to evaporation for the removal of ethyl acetate and not reacted benzyl alcohol using a rotary evaporator (R3 BUCHI) with a vacuum pump coupled (V-700 BUCHI). After evaporation, the purified benzyl butyrate was analyzed by gas chromatography (Shimadzu GC 2010). In addition, the purified benzyl butyrate ^{1}H NMR analyses were recorded in deuterated chloroform ($CDCl_3$), using a Bruker Ascend spectrophotometer at 200 MHz. Chemical shifts (δ) are reported in

part per million (ppm) relative to internal standard tetramethylsilane (TMS, δ = 0 ppm) which is used to calibrate the chemical shift.

4.1.8 Lipase esterification activity

The enzyme activity (U/g) was determined following a modified technique (CENI et al., 2010) with the esterification between lauric acid and n-propanol. A unit of activity (U) was defined as the amount of enzyme necessary to consume 1 μmol of lauric acid per minute. Therefore, the esterification reaction was conducted in a substrates molar ratio of 1:1 (alcohol:acid), enzyme amount of 5 wt% (related to the substrates) at 60 ºC for 40 min. The remaining lauric acid content was determined by titration with NaOH 0.01 M. All enzymatic activity determinations were carried out in triplicates.

The enzyme activity was measured for Novozym 435 before and after esterification recycle in batch mode (cycle 3), and after its contact with acid and alcohol separately at the same reaction condition (50 ºC, 24 h, and brand mechanical stirring).

4.2 RESULTS AND DISCUSSION

4.2.1 Effect of the biocatalyst type and molecular sieve presence

In order to find the best biocatalyst for benzyl butyrate synthesis, some tests were conducted using four different immobilized lipases from Novozymes®. Figure 12 shows the conversion to benzyl butyrate using Novozym 435, NS 88011, Lipozyme TL-IM and Lipozyme RM-IM as biocatalysts. From the results, the immobilized lipases from Cal B had the best performances in the esterification of benzyl alcohol and butyric acid at the conditions studied, reaching 52% and 19% of conversion for Novozym 435 and NS 88011, respectively. On the other hand, the lipases from *Thermomyces lanuginosus* (Lipozyme TL-IM) and *Rhizomucor miehei* (Lipozyme RM-IM) had very low conversions, showing not to be adequate as biocatalysts.

Figure 12 - Influence of the molecular sieves in the benzyl butyrate biosynthesis using Novozym 435, NS 88011, Lipozyme TL-IM and Lipozyme RM-IM as catalysts and in the blank sample (only substrates), using an acid:alcohol 1:1 molar ratio, at 65 ºC, and during 24 h.

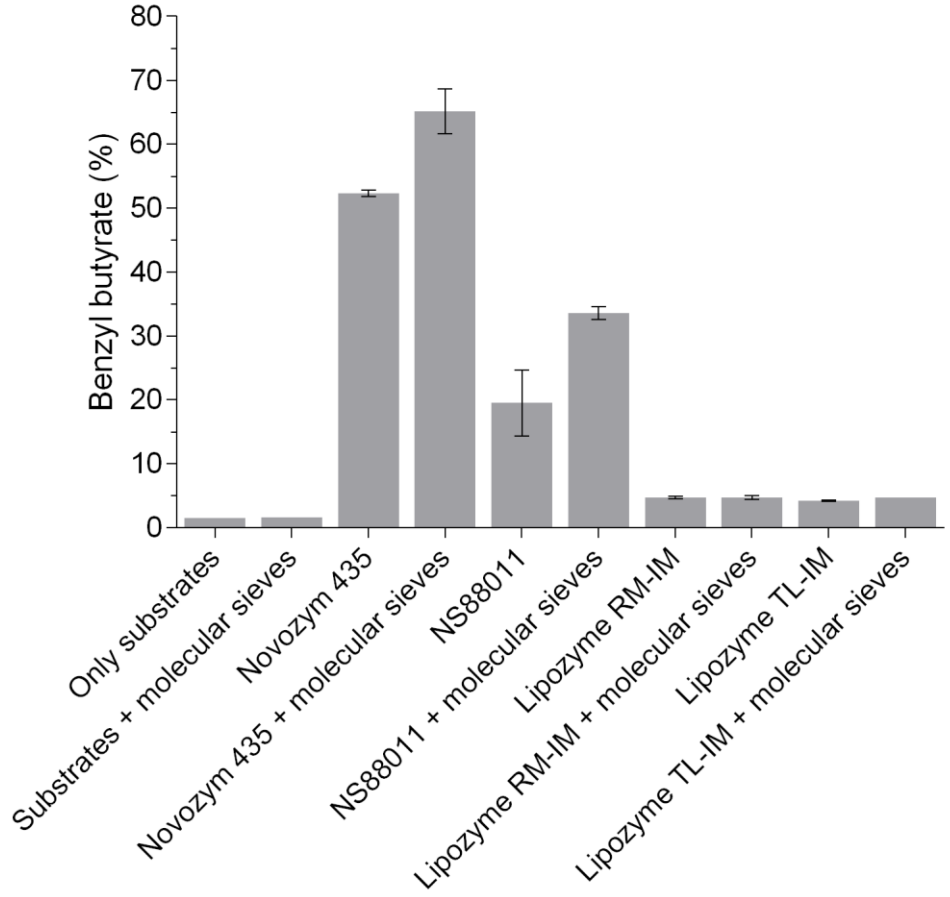


Figure 12 also shows the conversion to benzyl butyrate in presence or absence of molecular sieves 4Å, once these are commonly used as water adsorbent for esterifications (BANSODE; RATHOD, 2014; PALUDO et al., 2015), but in some cases, it can also be a catalyst (LAROQUE et al., 2015). The molecular sieves by themselves were ineffective as catalyst for benzyl butyrate esterification, but they was very effective in the water removal (a side product of the esterification), increasing the conversion for both Novozym 435 and NS88011, due to the reduction of product hydrolysis (reverse reaction). Based on the results, all experiments were conducted in the presence of molecular sieves to ensure the maximum levels of conversion.

4.2.2 Experimental factorial design

Conventional optimization methods require screening of a large number of variables, many experiments, with plenty of time and resources. On the other hand, the response surface methodology (RSM) technique is a great tool for the aromatic esters synthesis, able to determine the optimum or maximized reaction conditions necessary to scale-up and to reduce the number and cost of experimental tests needed to provide statistically acceptable results (MANAN et al., 2016; SÁ et al., 2017).

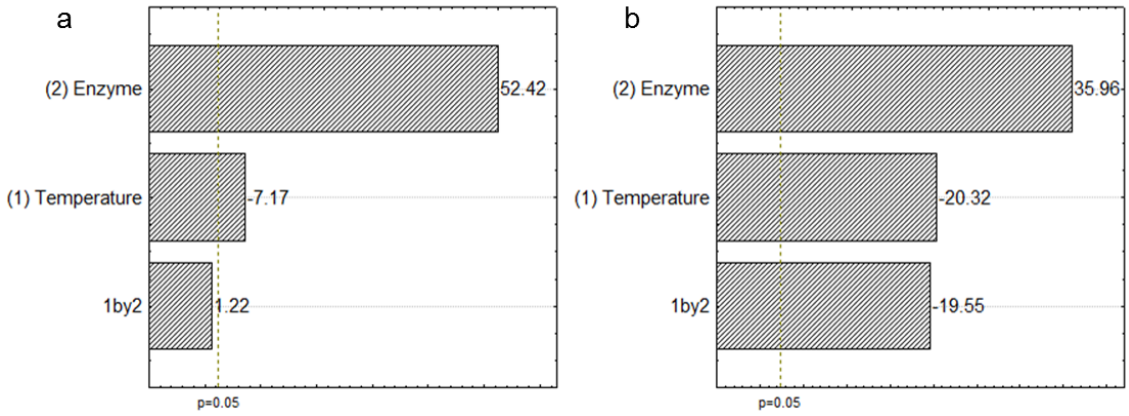
In this way, a 2^2 factorial design with 12 trials was conducted to improve the conversion as much as possible using the Cal B lipase immobilized in two different support materials: Novozym 435, a well-known commercial immobilized lipase, and NS 88011, a non-commercially prepared lipase developed with a low-cost support material. The reaction time and molar ratio acid: alcohol were fixed, respectively in 24 h and 1:1, evaluating the effect of temperature and enzyme amount on conversion and the results are shown in Table 9. Novozym 435 showed the best performance in the benzyl butyrate esterification and the highest conversion was found at 50 ºC and 10% of enzyme.

The experimental data from Table 9 were statistically treated and Figure 13 presents the standard Pareto charts that show the effects of each reaction variable (enzyme amount and temperature) on conversion. A similar behavior was visualized for both biocatalysts (Figure 13a and b) and the enzyme amount showed a positive significant effect ($p < 0.05$) on the conversion, which indicates that an increase in the enzyme amount leads to an increase in conversion. On the other hand, the temperature showed a negative significant effect ($p < 0.05$) on the conversion, indicating that a decrease in the temperature leads to an increase in conversion.

Table 9 - 2² full factorial design variables (coded and real) and responses in terms of predicted and experimental benzyl butyrate conversion in percentage using the Novozym 435 and NS 88011 as biocatalysts. All experiments were conducted at a molar ratio of acid to alcohol fixed in 1:1.

Run	Real variables		Novozym 435	NS 88011
	T (º C)	E (wt %)	Conversion after 24 h (%)	Conversion after 24 h (%)
1	50	2	27.8	4.6
2	70	2	20.7	4.5
3	50	10	76.9	45.6
4	70	10	71.2	15.9
5	60	6	50.3	9.7
6	60	6	49.2	8.9
7	50	2	27.0	5.6
8	70	2	18.1	4.7
9	50	10	75.1	42.3
10	70	10	69.5	16.1
11	60	6	49.8	8.4
12	60	6	47.2	9.1

Figure 13 - Pareto chart of the effects of enzyme amount and temperature on the benzyl butyrate production ($p < 0.05$) using (a) Novozym 435 and (b) NS 88011 as biocatalyst. Experimental data and conditions shown in Table 9.



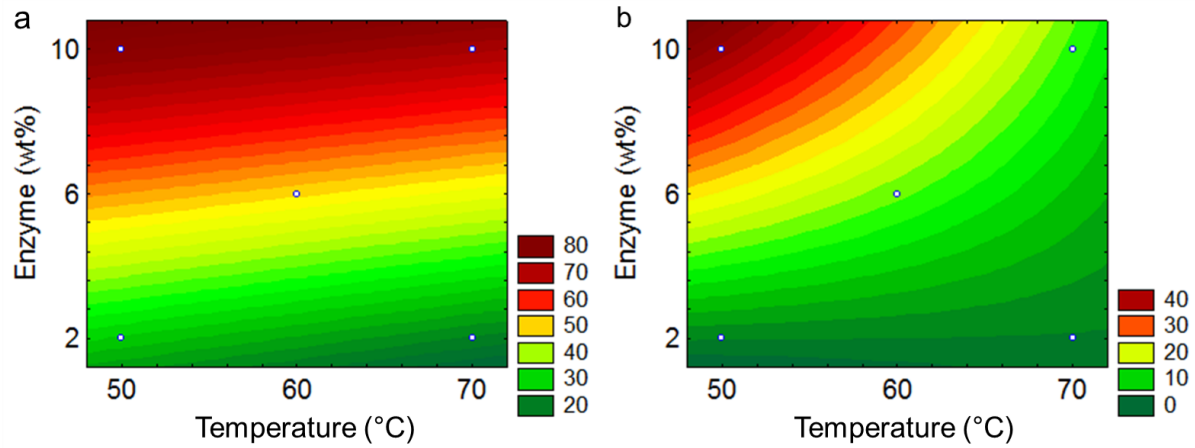
The fitted models obtained from the range of data (Table 9) are presented by first order polynomials related to T and E, and interaction between both. Equations 2 and 3 represent the systems with Novozym 435 and NS 88011, respectively.

$$Conversion = 48.57 - 3.41T + 24.91E + 0.58TE \quad (2)$$

$$Conversion = 14.62 - 7.11T + 12.58E - 6.84TE \quad (3)$$

The quality of the fitted models was checked with the analysis of variance (ANOVA) (data not shown). The R-value and F-test for regression showed that the models (Eq. 2 and 3) were able to represent well the experimental data. Figure 14a and 14b show the 2D-contour plots from Equations 2 and 3, respectively for Novozym 435 and NS 88011. The conversion to benzyl butyrate was highly dependent on the enzyme amount in both cases and the increase in the enzyme amount led to a higher conversion, corroborating with the Pareto chart. The biocatalyst Novozym 435 had the best performance for the batch esterification process even though the same lipase (Cal B) is immobilized in the NS 88011. However, the method of lipase immobilization and support material are different (non-commercial product; no information was provided by the fabricant) and may be responsible for the negative effects on the biocatalyst reaction performance. In addition, the NS 88011 and Novozym 435 BET surface area were determined in our previous work (SÁ et al., 2018), and great differences in the surface area and pore diameter of both biocatalysts may also be responsible for the large difference in the ester conversion, as Novozym 435 had a surface area and total pore volume ten times greater than NS 88011.

Figure 14 - 2D-Contour plot of the conversion to benzyl butyrate (%) as function of enzyme (wt%) and temperature (ºC) for (a) Novozym 435 and (b) NS 88011 as biocatalyst after 24 h of esterification in batch mode.



The T variation between the range studied presented low influence in Novozym 435 system (Figure 14a) and all the three T showed to be adequate to reach high conversions. On the other hand, when NS 88011 (Figure 14b) was used as biocatalyst, the lowest T (50 ºC) showed to be most adequate for the esterification reaction.

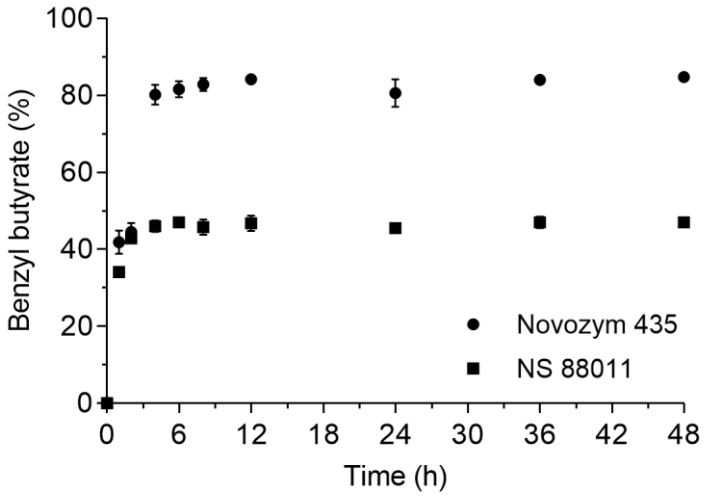
During the last decade, there has been an increasing number of works that have used the experimental design to maximize/optimize the conversion of aromatic esters. Manan and co-workers synthesized eugenol benzoate using *Rhizomucor miehei* lipase reinforced nano-bioconjugates using the response surface methodology by employing the central composite design (CCD) based on four parameters (incubation time, temperature, substrate molar ratio, and enzyme loading) (MANAN et al., 2016). In other works, the same method was employed to obtain high yield of benzyl cinnamate and ascorbyl palmitate (LERIN et al., 2011b; ZHANG et al., 2016) and high conversion of eugenol caprylate (RADZI; HANIF; SYAMSUL, 2016).

A study of conversion as a function of time was conducted with both biocatalysts Novozym 435 and NS 88011 under maximized condition (50 ºC and 10 wt%) during 48 h of reaction and the results are shown in Figure 15. Both biocatalysts showed maximum conversion to benzyl butyrate in only 4 h of reaction, but Novozym 435 was the most adequate biocatalyst for benzyl butyrate synthesis, reaching 80% of conversion. Novozym 435 is one of the most used biocatalysts for biotechnological applications, and a range of flavor esters were synthesized successfully using this enzyme, such as butyl acetate (MARTINS et al., 2013), eugenyl acetate (CHIARADIA et al., 2012), isoamyl acetate (GÜVENÇ; KAPUCU; MEHMETOĞLU, 2002), ascorbyl palmitate (LERIN et al., 2011b) among others.

The single study of benzyl butyrate synthesis by esterification of benzyl alcohol and butyric acid (molar ratio alcohol to acid to solvent of 1:1:1) also used Novozym 435 and reached similar results for conversion (JEROMIN; ZOOR, 2008). Methyl tertbutyl ether was employed as the solvent to favor the reaction equilibrium on the esterification side and to avoid the hydrolysis of the product. However, SFSs are very interesting for the food and pharmaceutical industries, reducing the use of toxic

reactants and allowing the synthesis of more natural products without hazardous traces.

Figure 15 - Evolution of conversion to benzyl butyrate (%) during reactions using Novozym 435 and NS 88011 as biocatalysts, considering the best experimental design condition (50ºC, 10 wt% of enzyme, molar ratio 1:1).



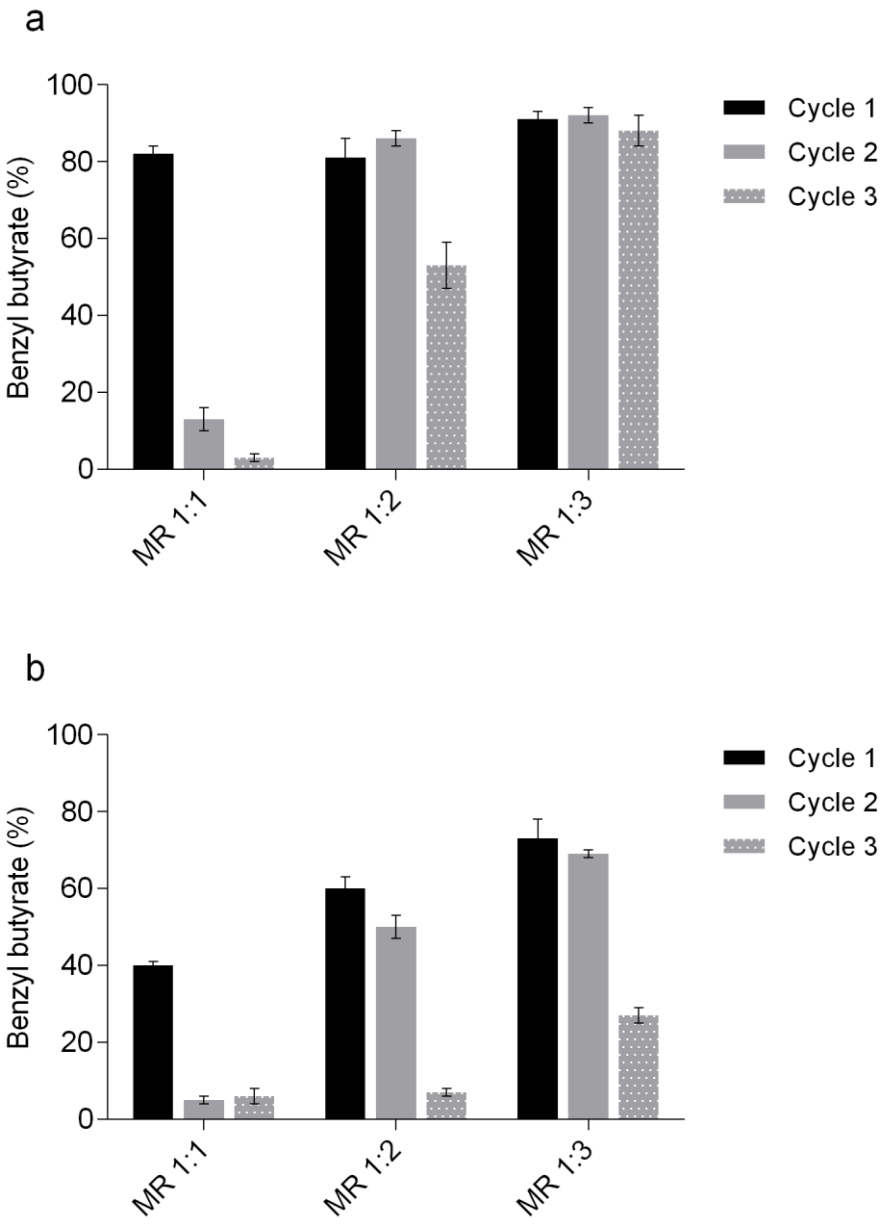
4.2.3 Influence of the substrates molar ratio in the enzyme performance and reuse in batch mode

An increase in the alcohol amount may be interesting, as no solvent was used in the reaction, to ensure a good substrates' solubilization and to improve the conversion, without affecting the catalytic activity of the enzyme. In this way, the reuse cycle of the immobilized enzymes, Novozym 435 and NS 88011, was tested in different acid: alcohol molar ratios in the batch esterification between benzyl alcohol and butyric acid. After each enzyme recycle, the immobilized enzymes were recovered and washed with acetone to remove residual water and acid from the environment of the immobilized biocatalyst.

Figure 16a shows that the molar ratio 1:1 (used in the experimental design) ensured 76% of conversion in the first cycle of Novozym 435; however, the conversion was sharply reduced in the second and third enzyme cycles. The increase in alcohol molar ratio in the reaction medium was positive, ensuring a great conversion over the three cycles at molar ratio 1:3 and still a slight increase in the

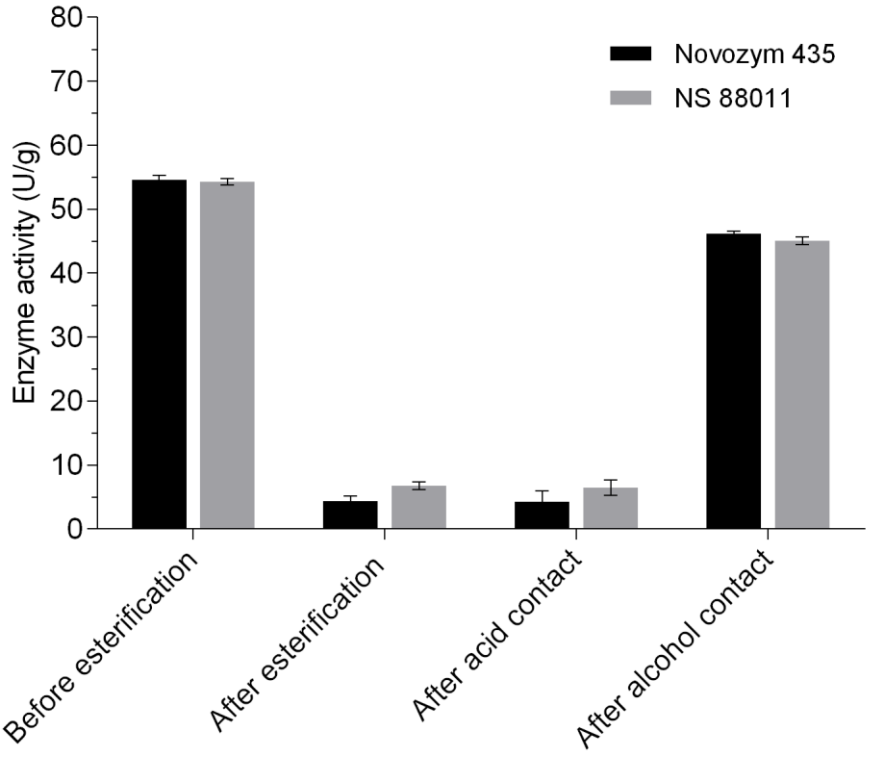
global conversion. The system containing the NS 88011 as biocatalyst (Figure 16b) showed a similar behavior with improved enzyme performance and increased conversion as the alcohol molar ratio was increased in the medium, showing an accentuated loss of enzyme activity at low substrate molar ratios.

Figure 16 - Performance of the (a) Novozym 435 and (b) NS88011 in the conversion to benzyl butyrate (%) over three enzyme recycles in batch reactor, considering an increase in the substrates molar ratio from 1:1 to 1:3 (acid: alcohol) and reaction conditions fixed in 50 ºC, 10% of enzyme, and 24 h of reaction.



Some tests were performed to better explain this loss of the activity of the immobilized enzymes. The enzymes, Novozym 435 and NS 88011, were submitted to the same conditions of the batch reaction with the substrates butyric acid and benzyl alcohol, separately. As shown in Figure 17, a large reduction in the enzyme activity (U/g) was observed after contact with the acid, the same trend was observed after the batch esterification (molar ratio 1:1), indicating an acid inhibition/deactivation behavior. Researchers have reported that the main reason for enzyme acid inhibition is associated to the fact that acids may cause acidification of the micro-aqueous interface leading to enzyme inactivation (GÜVENÇ; KAPUCU; MEHMETOĞLU, 2002; ROMERO et al., 2005), usually associated with aliphatic acid that possesses a very short chain (CLAON; AKOH, 1994; HARI KRISHNA et al., 2001; GÜVENÇ; KAPUCU; MEHMETOĞLU, 2002; ROMERO et al., 2005).

Figure 17 - Enzyme activities (U/g) for Novozym 435 and NS 88011 before esterification, after batch esterification reaction (Cycle 3, MR 1:1), and after contact with both substrates acid and alcohol separately.

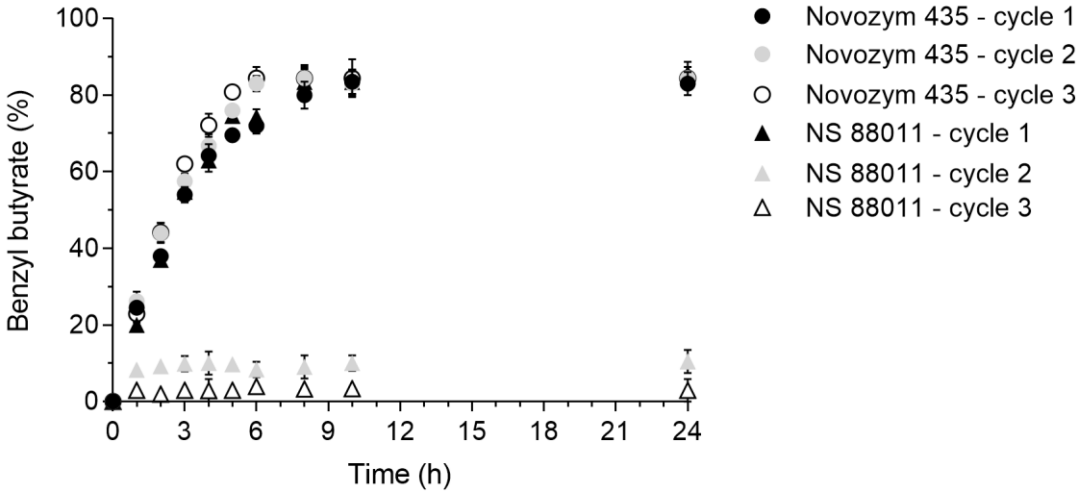


Based on the results, we can conclude the butyric acid was the main responsible to deactivate the Cal B lipase from Novozym 435 and NS 88011. However, an increase in the alcohol molar ratio can dilute the acid and improve the performance of the immobilized enzymes over the recycles in batch mode, showing the need for a high amount of alcohol in the medium to ensure a good SFS process condition.

4.2.4 Immobilized enzymes performance in fed-batch mode

The fed-batch mode was adopted in this work as a strategy to overcome the drawbacks associated with the use of excess alcohol, such as downstream process and high costs. The best experimental design condition of E and T was employed (10 wt% and 50 ºC), and the acid was fed in the first five hours with a final substrates molar ratio of 1:1, ensuring no excess of substrates. The results of benzyl butyrate conversion using the fed-batch approach over three enzyme cycles are shown in Figure 18 for both biocatalysts Novozym 435 and NS 88011. Both biocatalysts presented a high conversion in the first enzyme cycle with maximum conversion after last acid addition (between 6 and 8 hours of reaction). It is worth noting a positive effect in the NS 88011 system with a considerable increase in the global conversion compared to the batch mode. However, the NS 88011 lost the activity after the first cycle. This result may be associated with the inhibition effects related to butyric acid as previously shown in Figure 16, but it may still be related to the unknown information about the lipase immobilization and material support that could negatively affect the enzyme performance. The Novozym 435 was the best biocatalyst for the fed-batch system with a good performance over the three cycles used, ensuring a high conversion to benzyl butyrate in a SFS process condition, eliminating the need for excess alcohol.

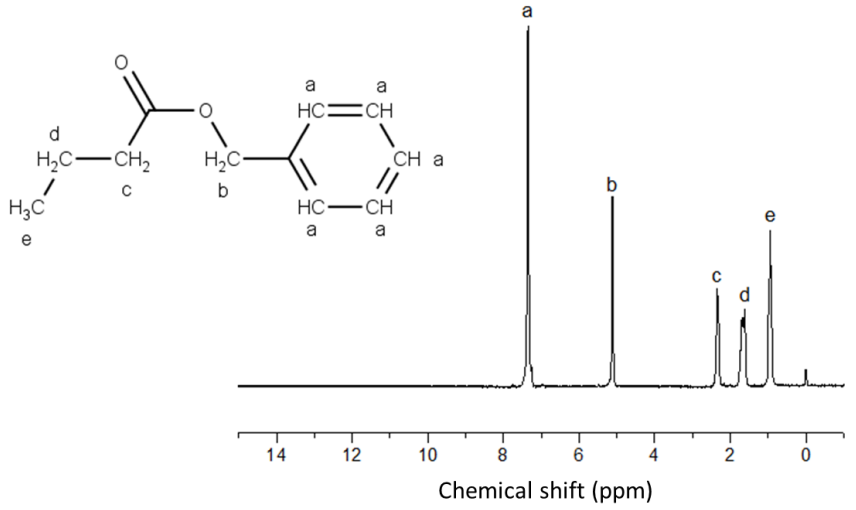
Figure 18 - Performance of the Novozym 435 and NS 88011 in the benzyl butyrate synthesis, evolution of conversion (%) during three enzyme recyles in a fed-batch reactor, considering the acid fed in the first 5 h of reaction and the best experimental design condition of 50 ºC, 10 wt% of enzyme, molar ratio 1:1).



The influence of an increase in the reaction volume was studied using the fed-batch strategy with reference to the benzyl butyrate synthesis. The reaction volume was increased 31 times with substrates molar ratio of 1:1 (1.1 mol), Novozym 435 as biocatalyst at 10 wt%, and temperature at 50 ºC (best condition). The conversion to benzyl butyrate was 78% the tested conditions indicating that the results found in Figure 18 are reproducible in a large scale. The present results show that the fed-batch configuration has the potential for the biotechnological industrial synthesis of SFS benzyl butyrate ensuring a molar ratio acid: alcohol of 1:1.

The benzyl butyrate obtained from fed-batch esterification was purified by liquid-liquid extraction to remove the unreacted acid and alcohol. The purified final product (> 99%) was analyzed by ^{1}H NMR as shown in Figure 19. As we can see, the ^{1}H NMR (200 MHz, $CDCl_3$, δ) showed chemical shifts in 7.37 (m, 5H, aromatic), 5.12 (s, 2H, $-CH_2-O$), 2.32 (t, 2H, $CH_2-C=O$), 1.65 (sextet, 2H, CH_2), and 0.94 (t, 3H, CH_3) that correspond to the specific chemical shifts of the benzyl butyrate molecule. The result indicates the high purity of the final product.

Figure 19 - ^{1}H NMR spectrum for benzyl butyrate in $CDCl_3$ after purification by liquid-liquid extraction.



4.3 CONCLUSION

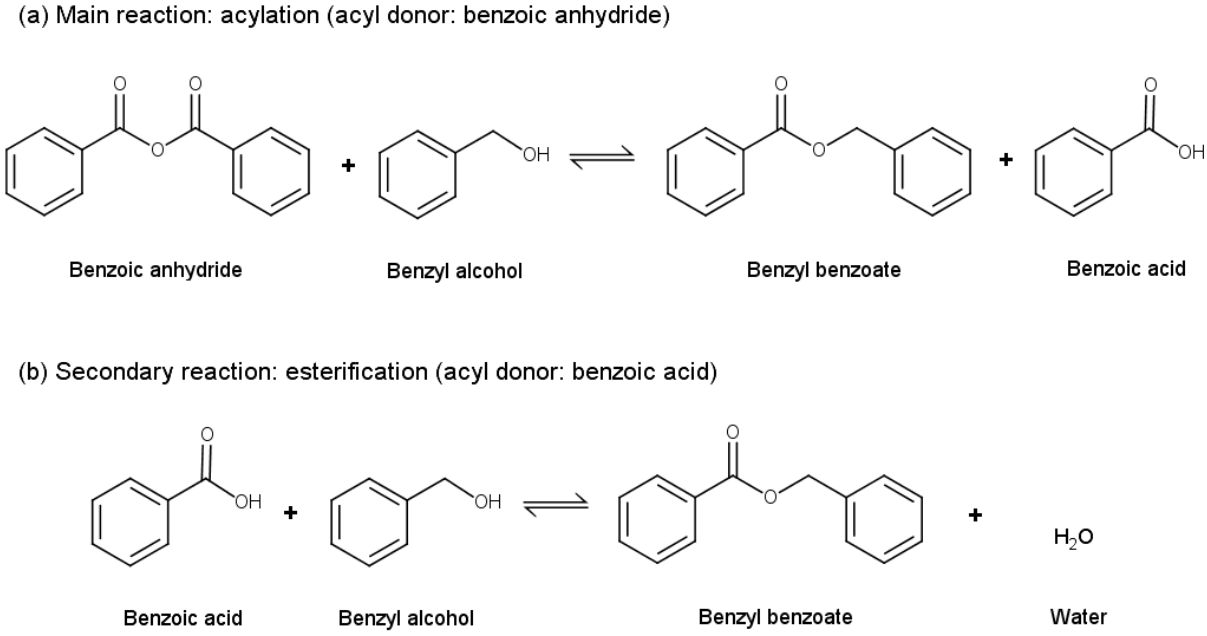
This work showed for the first time the biotechnological synthesis of benzyl butyrate using an efficient approach via lipase-mediated esterification of benzyl alcohol and butyric acid in a solvent-free system. Conversions above 80% were reached in absence of organic solvents and at a low molar ratio, with an assurance in costs reduction. The experimental design showed to be adequate to correlate the batch experimental data. The support material of the biocatalysts Novozym 435 and NS 88011 revealed to have great influence on the esterification reaction, and Novozym 435 was the best biocatalyst for the system. The loss of enzyme activity was accentuated in the batch esterification at lower benzyl alcohol amount, suggesting that the butyric acid may influence the acidification of the microaqueous interface leading to enzyme deactivation. The amount of alcohol present in the reaction media plays an important role in the Novozym 435 activity and reusability, and a high amount of alcohol was essential to improve the butyric acid solubilization and increase the enzyme performance. However, in order to overcome the drawbacks associated with excess alcohol in the reaction media, the fed-batch configuration was employed with the molar ratio of substrates fixed in 1:1. The

strategy showed to be adequate for benzyl butyrate synthesis ensuring no loss of enzyme activity over three cycles of use and no substrates expended. The increased in the reaction volume using the best fed-batch condition indicated a good reproduction of the conversion. The present results showed a natural alternative for natural synthesis of benzyl butyrate.

5 BENZYL BENZOATE SYSNTHESIS VIA BIOCATALYSIS

At present, no information is available in the literature depicting the benzyl benzoate lipase-mediated synthesis using benzoic anhydride or benzoic acid as acyl donor. The use of an anhydride as acyl donor for aroma ester synthesis is a complex reaction (ROMERO et al., 2007), as shown in Figure 20. The anhydride possesses two acyl groups, consequently, one acyl group is employed to form the ester and the other one leads to the formation of one acid molecule. The acid generated as a byproduct may react with the remaining alcohol and both acylation and esterification can occur simultaneously.

Figure 20 - Benzyl benzoate enzymatic synthesis using benzoic anhydride as acyl donor and benzyl alcohol as acyl acceptor. Main reaction: acylation; Secondary reaction: esterification.



The present study deals with the benzyl benzoate synthesis using two different acyl donors (benzoic anhydride and benzoic acid) mediated by different immobilized enzymes (Novozym 435, Lipozyme RM-IM and Lipozyme TL IM) in the presence or absence of solvents. In order to find the best reaction configuration, tests were conducted using ultrasound, conventional stirring, and batch and fed-batch operation

regimes. Finally, process variables like substrate molar ratio (acyl donor to acyl acceptor), amount of catalyst and reaction temperature were investigated to ensure high global productivity.

5.1 MATERIAL AND METHODS

5.1.1 Enzymes and chemicals

Benzyl alcohol (Neon, 99.5 %), benzoic acid (Lafan, 99.5 %) and benzoic anhydride (Acros, 95 %) were used as substrates. Enzymatic catalysts were the commercial immobilized lipases Novozym 435 (*Candida antarctica* fraction B lipase), Lipozyme TL IM (*Thermomyces lanuginosus* lipase), Lipozyme RM IM (*Rhizomucor miehei* lipase), kindly donated by Novozymes®. Tert-butyl alcohol (Sigma-Aldrich, 99.8 %) and 2-propanol (Neon, 99.5 %) were used as solvents of the reaction system. Dichloromethane (Quemis, 99.8 %) was used in gas chromatography injections and acetone (Dinâmica, 99.5 %) was utilized to wash the enzyme for reuse tests.

5.1.2 Enzymatic synthesis of benzyl benzoate in the presence and absence of organic solvents

Initially, the solubility of the substrates (benzoic acid and benzoic anhydride, as acyl donors, and benzyl alcohol, as acyl receptor) was tested using tert-butyl alcohol, cyclohexane, n-heptane, n-octane, n-pentane, 2-propanol, and isooctane as solvents. In addition to these solubility tests, excess of benzyl alcohol was used to enable the reaction in a solvent-free system (SFS), which would represent a possible economic advantage for the industrial applications.

The substrates benzoic acid: benzyl alcohol (esterification) or benzoic anhydride: benzyl alcohol (acylation) in a molar ratio of 1:9 were added in 2 mL reactors. In sequence, when used, 1 mL of solvent was added in each reactor (1:9:30 molar ratio of anhydride: alcohol: solvent). The enzyme Novozym 435 was used as biocatalyst (10% w/w) and at the end of 24 h of reaction at 50 ºC, the reaction media

were centrifuged (10,000 rpm for 5 min), aliquots of the supernatant were withdrawn and suitably diluted in dichloromethane for quantification analysis. All experiments were carried out in duplicate.

5.1.3 Variation of the molar ratio of the acylation substrates

The synthesis of benzyl benzoate by enzymatic acylation was performed under different molar ratios of benzoic anhydride: benzyl alcohol from 3:1 to 1:9 using 2-propanol as solvent for solubilization of the substrates. The solvent-free system (SFS) was also evaluated using a high excess of benzyl alcohol (1:9) that plays the role of solvent for the solubilization of benzoic anhydride. The biocatalysts tested in this assay were Lipozyme RM-IM, Lipozyme TL IM, and Novozym 435. The reaction parameters temperature, amount of catalyst and reaction time were fixed at 50 °C, 10 w/w% catalyst and 24 h, respectively. The reactions were conducted by adding the substrates in the adequate proportion of 3:1, 1:1, 1:3, and 1:9, when used, 1 mL of 2-propanol was added to the reactions with solvent. Reactions were performed under mild mechanical stirring and with controlled temperature and at the end of the reaction time, an aliquot of the product was centrifuged (10,000 rpm for 5 min) and adequately diluted for quantification. All experiments were carried out in duplicate.

5.1.4 Effect of ultrasound on the enzymatically-catalyzed synthesis of benzyl benzoate

In order to investigate a nonconventional method of homogenization, reactions performed in an ultrasonic bath were compared with those performed with conventional mechanical stirring. The SFS containing benzoic anhydride: benzyl alcohol (1:9) and 10 w/w% of catalyst (Lipozyme RM-IM, Lipozyme TL IM or Novozym 435) were added into glass reactors of 50 mL and conducted either in an ultrasonic bath (Unique, Model USC-1800A, 40 kHz, 132 W) or in a conventional heating bath with mechanical agitation (Dist), both previously heated at 50 °C. Aliquots of the reaction were collected at times 0, 1, 2, 4, 6 and 8 h. Subsequently, the samples were centrifuged (10,000 rpm for 5 min), aliquots of the supernatant

were properly diluted in dichloromethane and stored under refrigeration for further analysis.

5.1.5 Benzyl benzoate synthesis in batch and fed-batch mode

The batch and fed-batch syntheses of benzyl benzoate were performed with conventional stirring. Different substrate molar ratios (1:3, 1:6 and 1:18, anhydride: alcohol) and 10 w/w% of enzyme (Lipozyme TL IM) were added to glass batch reactors of 100 mL. The reactions were carried out at 150 rpm and 50 ºC, and at reaction times of 0, 1, 2, 3, 4, 5, 6, 8, 10 and 24 h samples were taken and properly diluted for further quantification.

For fed-batch reactions only benzyl alcohol and 10 w/w% of enzyme (Lipozyme TL IM) were added as initial charge in a jacketed glass reactor of 100 mL previously heated at 50 ºC. Benzoic anhydride was fed into the reaction media every 1 h during the first 5 h, totaling six additions. The final benzoic anhydride: benzyl alcohol molar ratios were 1:3, 1:6 and 1:18. The remaining conditions were the same as those described in the previous paragraph for the respective batch reactions.

5.1.6 Effect of temperature

The effect of reaction temperature on the enzymatically-catalyzed synthesis of benzyl benzoate was studied at 50, 60 and 70 °C. The reactions involved the addition of 10 w/w% of Lipozyme TL IM as biocatalyst, benzoic anhydride and benzyl alcohol (molar ratio of 1:6) in glass reactors and placed in a mechanical shaker under mild agitation. Aliquots were taken at times 0, 1, 2, 4, 6, 8, 10 and 24 h, then, the samples were centrifuged (10,000 rpm for 5 min) and the supernatant was collected, properly diluted and properly stored under refrigeration for further analysis. All experiments were carried out in duplicate.

5.1.7 Effect of enzyme amount

The effect of enzyme amount in benzyl benzoate synthesis was evaluated at different Lipozyme TL IM amounts of 6, 8 and 10 w/w% with the temperature fixed at 50 ºC. The reactions involved the addition of an adequate amount of biocatalyst, benzoic anhydride and benzyl alcohol (molar ratio of 1:6) in glass reactors and placed in a mechanical shaker under mild agitation. The sample collection and preparation were the same as those described in the previous paragraph.

5.1.8 Benzyl benzoate quantification

The ester quantification was carried out as described previously elsewhere with some modifications (MENESES et al., 2019a). Gas chromatography (GC, Shimadzu 2010), with the automatic coupled injector (Shimadzu AOC 5000) was used. A DB-5 column (27 m length × 0.25 mm internal diameter × 0.25 µm thickness) was employed for the separation of the analytes and the detection was performed by flame ionization detector (FID). The column temperature ramp was programmed to 100 °C (2 min), 100-135 °C (3 °C/min), 135-250 °C (10 °C/min) and 250 °C for 15 min to ensure proper separation of all components. The temperatures of the injector and detector were kept at 250 ºC and a split ratio of 1:100 was maintained to sample injection, the injection volume was 10 µL and the carrier gas was nitrogen (N_2, 56 kPa).

Conversion was calculated based on the benzyl benzoate corresponding peak of each chromatogram and a previously prepared benzyl benzoate (99% purity) calibration curve ($R^2 = 0.99$).

5.1.9 Enzyme reuse

The reusability study was conducted to investigate the enzyme performance on the benzyl benzoate synthesis following the washing procedure described elsewhere with minor modifications (TOMKE; RATHOD, 2015). After each reaction cycle, the enzyme was recovered by vacuum filtration using glass funnel and filter paper. The enzyme was washed with acetone two times to remove any product or

substrate. Then, the enzyme was dried during 24 h at 50 °C and reused in a new reaction cycle. This procedure was repeated for each reuse cycle.

5.1.10 Initial reaction rate

The initial reaction rate (r) was calculated as Equation 4:

$$r = \left(\frac{\Delta Cester}{\Delta t} \right) \quad (4)$$

Where *r* is the initial rate of reaction (h^{-1}); C_{ester} conversion of benzoic anhydride into ester (wt.%) at time *t*; and *t* is the reaction time (h) (ZENEVICZ et al., 2016).

5.2 RESULTS AND DISCUSSION

5.2.1 Effect of solvents

Benzoic acid is the simplest aromatic carboxylic acid that can be produced in large quantities, with high purity and low-cost. It is a colorless crystalline solid of difficult solubilization and research has been conducted to understand its solubility in different organic solvents (BEERBOWER; WU; MARTIN, 1984; OLIVEIRA et al., 2007; LONG et al., 2010; THATI; NORDSTRÖM; RASMUSON, 2010; WANG et al., 2014). The use of this compound as a substrate for enzymatically-catalyzed reactions may present some drawbacks related to the solubility and diffusion problems. Lipases are stable in many solvents, however, some organic solvents may reduce the lipase catalytic activity (HARI KRISHNA et al., 2001; WU et al., 2007; BATISTELLA et al., 2012; YADAV; DEVENDRAN, 2012), and the choice of a suitable solvent for solid substrates like benzoic acid is fundamental in biocatalysis to ensure good performance of the catalyst.

Initially, a solubilization test was conducted with organic solvents like cyclohexane, heptane, octane, pentane, isooctane, tert-butyl alcohol, 2-propanol, and benzyl alcohol and the benzoic acid was visually soluble in tert-butyl alcohol and 2-propanol, solvents usually employed for enzyme-catalyzed reactions (HALLING, 1984, 1990; MARTY et al., 1991; CLAON; AKOH, 1994; HUANG; CHANG; GOTO,

1998; HARI KRISHNA; PRAPULLA; KARANTH, 2000; HARI KRISHNA et al., 2001; LERIN et al., 2011a; BATISTELLA et al., 2012; WU et al., 2014). Table 10 shows the conversion into ester using Novozym 435 as catalyst and benzoic acid as acyl donor and both solvents tested were inefficient in the intermolecular attractions between solute-solvent, reaching very low conversion. On the other hand, benzoic anhydride showed positive results as acyl donor for the benzyl benzoate synthesis in presence of the solvents tert-butyl alcohol and 2-propanol. The results also indicate that an excess of benzyl alcohol in the reaction media was efficient in the anhydride solubilization and presented good conversion as a solvent-free system (SFS).

Table 10 - Effect of organic solvents on the synthesis of benzyl benzoate using different acyl donors and benzyl alcohol as acyl acceptor. Reaction conditions fixed at molar ratio acyl donor: acceptor 1:9, 50 ºC, 10 w/w% of Novozym 435 and 24 h of reaction.

Acyl donor	**Solvent**	**Log P**	**Conversion %**
Benzoic acid	Tert- butyl alcohol	0.35	1.2
	2-propanol	0.05	0.4
Benzoic anhydride	Tert- butyl alcohol	0.35	30.6
	2-propanol	0.05	71.3
	Benzyl alcohol	1.10	60.3

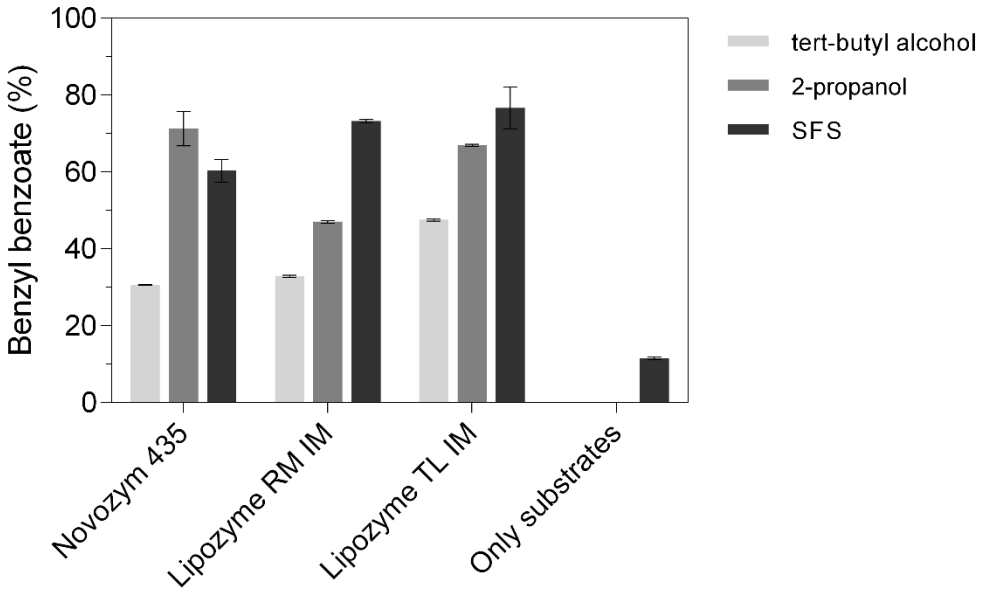
The synthesis of benzyl benzoate using benzoic anhydride is a complex reaction since it possesses two acyl groups. The first acyl donor is employed to form the benzyl benzoate and the second one leads to the formation of benzoic acid. The acylation is the main reaction and the acid formed as a byproduct may react with the excess of alcohol and, consequently, a secondary reaction of esterification may occur (Figure 20). The benzoic acid was not soluble in the benzyl alcohol, and the 2-propanol and tert-butyl alcohol were not good solvents for the esterification reaction between both substrates. The results strongly indicate that the acylation is the main and only reaction occurring in the benzyl benzoate synthesis mediated by lipases.

The benzyl benzoate synthesis using solvents with log P>4, like isooctane and heptane, 4.5 and 4, respectively, reached no conversion (data not shown) and the solvents tert-butanol, 2-propanol and benzyl alcohol that possess very low log P (Table 10) showed the best conversion results using anhydride as substrate. The log P represents the partitioning of a given solvent between water and octanol in a two-phase system and values above 4 are associated to higher enzyme activity and

better ester synthesis (HARI KRISHNA; PRAPULLA; KARANTH, 2000; HARI KRISHNA et al., 2001; TODERO et al., 2015; SÁ et al., 2017). The benzoic anhydride is a white solid, soluble in more hydrophilic solvents (low Log P) (HOU; XIE; ZHOU, 2015) and the conversion results strongly indicate a correlation between solute solubilization and diffusion problems, i.e., if an inadequate solvent is used the poor solubilization of the anhydride prevents its diffusion through the Novozym 435 pores and the substrates never reach the enzyme active site.

Based on these results, the commercial immobilized enzymes Novozym 435, Lipozyme TL IM and Lipozyme RM-IM were tested under a fixed condition using 2-propanol, tert-butyl alcohol and SFS (benzyl alcohol in excess) as organic media for the benzyl alcohol acylation (Figure 21). The solvent tert-butyl alcohol showed the lowest ester conversions probably due to the weak solute-solvent intermolecular attractions associated to the reduced benzoic anhydride solubility. Both SFS and 2-propanol showed to be efficient in the substrates and products solubilization, reaching conversions above 60, 47, and 66% respectively for Novozym 435, Lipozyme RM IM and Lipozyme TL IM. These enzymes are broadly used in the biocatalytic synthesis of aroma esters (VOSMANN et al., 2008; YADAV; DHOOT, 2009; GENG et al., 2012; KUO et al., 2012; TOMKE; RATHOD, 2015; WANG et al., 2015; ZHANG et al., 2016; MANAN et al., 2016; RADZI; HANIF; SYAMSUL, 2016; SÁ et al., 2018; MENESES et al., 2019a) and showed an excellent performance (conversions close to 100%) in other acylation reactions mediated by these lipases for eugenyl acetate (CHIARADIA et al., 2012; SILVA et al., 2015), isoamyl acetate (HARI KRISHNA et al., 2001; ROMERO et al., 2007), isoamyl butyrate (HARI KRISHNA; PRAPULLA; KARANTH, 2000) synthesis.

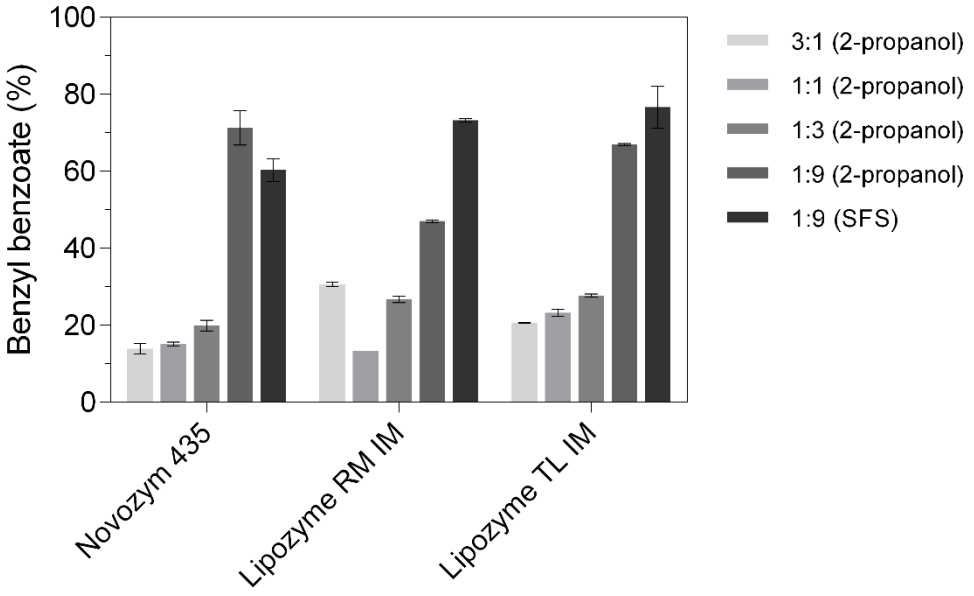
Figure 21 - Benzyl benzoate synthesis using different biocatalysts (Novozym 435, Lipozyme RM IM and TL IM) in solvent-free system (SFS) or tert-butyl alcohol and 2-propanol as solvents, using fixed reaction conditions of substrate MR 1:9 (anhydride :alcohol), 50 ºC, 10 w/w% of enzyme and 24 h of reaction.



5.2.2 Effect of substrate molar ratio

The equilibrium of esterification reactions can be pushed forward by either using an excess of the nucleophile (alcohol) or by removing products from the reaction mixture (HARI KRISHNA; PRAPULLA; KARANTH, 2000; HARI KRISHNA et al., 2001) and reports showed that the excess of alcohol reduces the negative effects of the acid associated with inhibition and lipase inactivation (MENESES et al., 2019a, 2019b). The acylation generally occurs successfully in equimolar concentrations of substrates since the anhydride substrates are not associated to lipase inhibition reaching conversions around 100% (HARI KRISHNA et al., 2001; ROMERO et al., 2007) and an excess of alcohol stimulates the secondary reaction of esterification between the acid generated as by-product of the main reaction of acylation with the alcohol resulting in ester and water (ROMERO et al., 2007).

Figure 22 - Impact of the substrate molar ratio on the benzyl benzoate synthesis using Novozym 435, Lipozyme RM IM and TL IM as biocatalysts in a system containing 2-propanol as solvent (anhydride: alcohol: solvent 3:1:30, 1:1:30, 1:3:30, 1:9:30) and in a solvent-free system (SFS) for the condition 1:9 (excess of alcohol). The experiments were conducted at a fixed reaction condition of 50 ºC, 10 w/w% of enzyme, and 24 h of reaction.



In order to investigate the effect of substrate molar ratio on benzyl benzoate synthesis using benzoic anhydride as acyl donor, tests were carried out varying the substrate molar ratio from 3:1 to 1:9 (anhydride: alcohol) using 2-propanol as solvent and the lipases Lipozyme TL IM, Lipozyme RM-IM and Novozym 435 as biocatalysts. A higher concentration of alcohol (acyl acceptor/nucleophile) usually leads to higher levels of conversion due to the availability of nucleophile in excess for acyl transfer and commonly both reactions of acylation and esterification may occur due to the use of anhydride substrate (HARI KRISHNA et al., 2001). However, the previous results showed that the benzoic acid did not react with benzyl alcohol in esterification using 2-propanol as solvent (Table 10). As acylation was the only reaction in progress, the excess of benzyl alcohol could be beneficial for the benzoic anhydride solubilization and Figure 22 shows the dependency of great amounts of alcohol to ensure high conversions. In addition, the SFS with benzyl alcohol in excess (same molar ratio conditions 1:9) also ensures high conversions for all enzymes tested, which indicates that strong solute-solvent intermolecular attractions lead to improved benzoic

anhydride solubility and diffusion through the pores of the Lipozyme TL IM and RM-IM and, consequently, to the highest results of conversion.

Thus, results showed that the excess of benzyl alcohol is necessary to ensure a good solubilization of the benzoic anhydride and to assist the diffusion of the substrates and products molecules through the enzyme pores even in the presence of 2-propanol as solvent. Based on the results, only SFS was employed to the next experiments.

5.2.3 Effect of conventional and non-conventional stirring methods

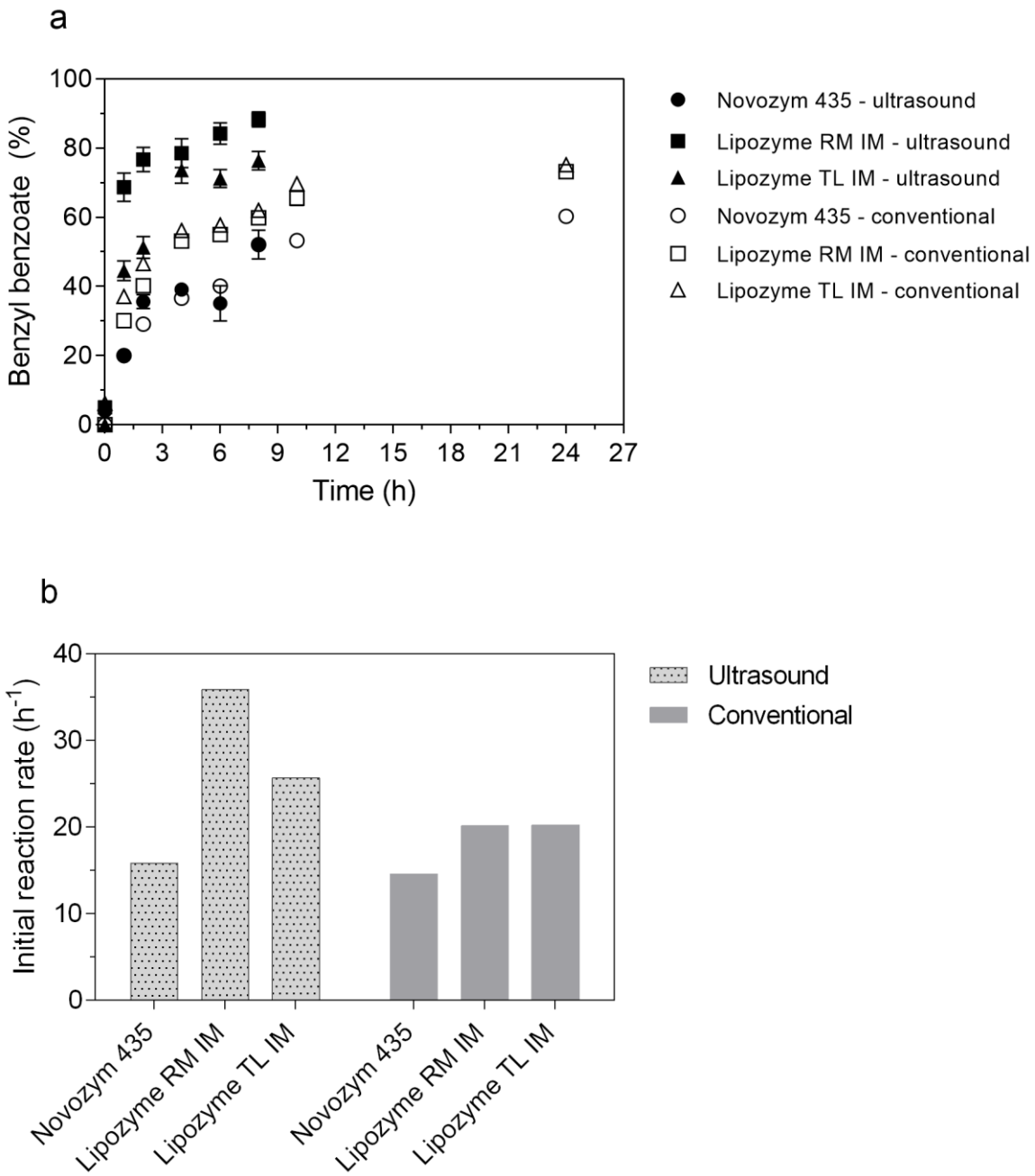
The immobilization of enzymes to an inert and resistant polymeric support offers many process advantages, like lower production cost, increased activity, specificity and selectivity, improved structural stability, reduction of inhibition, and the heterogeneous characteristic of the biocatalyst facilitates the separation, recovery and further reuse (DHAKE et al., 2011; BANSODE; RATHOD, 2014; KUO et al., 2014; NARWAL et al., 2016). On the other hand, the immobilization of an enzyme means a deliberate restriction of its mobility, which can also affect the solutes mobility (TISCHER; WEDEKIND, 1999) and various phenomena associated to mass transfer effects can lead to a reduced reaction rate, and an efficient stirring is crucial to avoid diffusion limitations and improve catalyst performance (DUMESIC; HUBER; BOUDART, 2008).

Generally, conventional stirring (mechanical or orbital) is efficient to avoid internal or external diffusion drawbacks associated to the immobilized enzymes. However, some emergent techniques like ultrasound have been successfully used for flavor and aromatic ester synthesis (MARTINS et al., 2013; BANSODE; RATHOD, 2014; PALUDO et al., 2015; TOMKE; RATHOD, 2015). Ultrasound is a high-energy technique considered as a green technology with high efficiency, economic performance and low instrumental requirement (MARTINS et al., 2013; SÁ et al., 2017). The application of ultrasound to an aqueous solution or suspension increases mixing, shearing, and mass transfer rate, reducing process time when compared with other conventional mixing techniques (SÁ et al., 2017).

Tests were conducted using conventional (mechanical agitation) and non-conventional (ultrasound) agitation methods using Novozym 435, Lipozyme TL IM and Lipozyme RM IM as biocatalysts of the benzyl alcohol acylation under SFS (molar ratio anhydride: alcohol 1:9). Figure 23a shows the anhydride conversion over time and the enzyme Lipozyme RM IM showed the highest conversions (88%) after 8 h of reaction in ultrasound-assisted acylation, followed by Lipozyme TL IM (76%). Both enzymes allowed to reach high conversions after 24 h under conventional agitation 73 and 75%, respectively, for Lipozyme RM IM and TL IM. Novozym 435 presented the lowest conversion (above 60%) for both homogenization methods showing not to be the best choice for the enzymatic synthesis of benzyl benzoate. Novozym 435 is one of the most used biocatalysts for aroma ester synthesis and other works reported that the enzyme had enhanced activity reaching 100% of conversion in ester and operational stability in ultrasound over several cycles (MARTINS et al., 2013; TOMKE; RATHOD, 2015).

The influence of the ultrasound on the performance of the enzymes can also be seen in Figure 23b that shows an increase in the initial reaction rate of the acylation when the non-conventional method was employed, as already reported by other authors (CENI et al., 2011; BATISTELLA et al., 2012; MARTINS et al., 2013; PALUDO et al., 2015). Despite the positive effects of conversion and initial reaction rates, and reports indicating that Lipozyme TL IM is resistant to the ultrasound cavitation (BATISTELLA et al., 2012; PALUDO et al., 2015), the enzyme support was destroyed under the presented conditions of reaction with no possibility of recovery and reuse. The same behavior was found for Lipozyme RM-IM. These results hamper the use of ultrasound in the benzyl benzoate synthesis and the conventional agitation was employed in the next reactions using Lipozyme TL IM as biocatalyst. The enzyme showed to be efficient in the benzyl alcohol acylation in all the systems studied and is the cheapest enzyme as compared to the other two immobilized lipases, justifying its use.

Figure 23 - Benzyl benzoate synthesis (a) and initial reaction rate (b) under conventional (mechanical agitation) and non-conventional (ultrasound) agitation using three different biocatalysts, considering fixed reaction condition in solvent-free system with molar ratio anhydride to alcohol of 1:9, 10 w/w% of enzyme and 50 ºC.



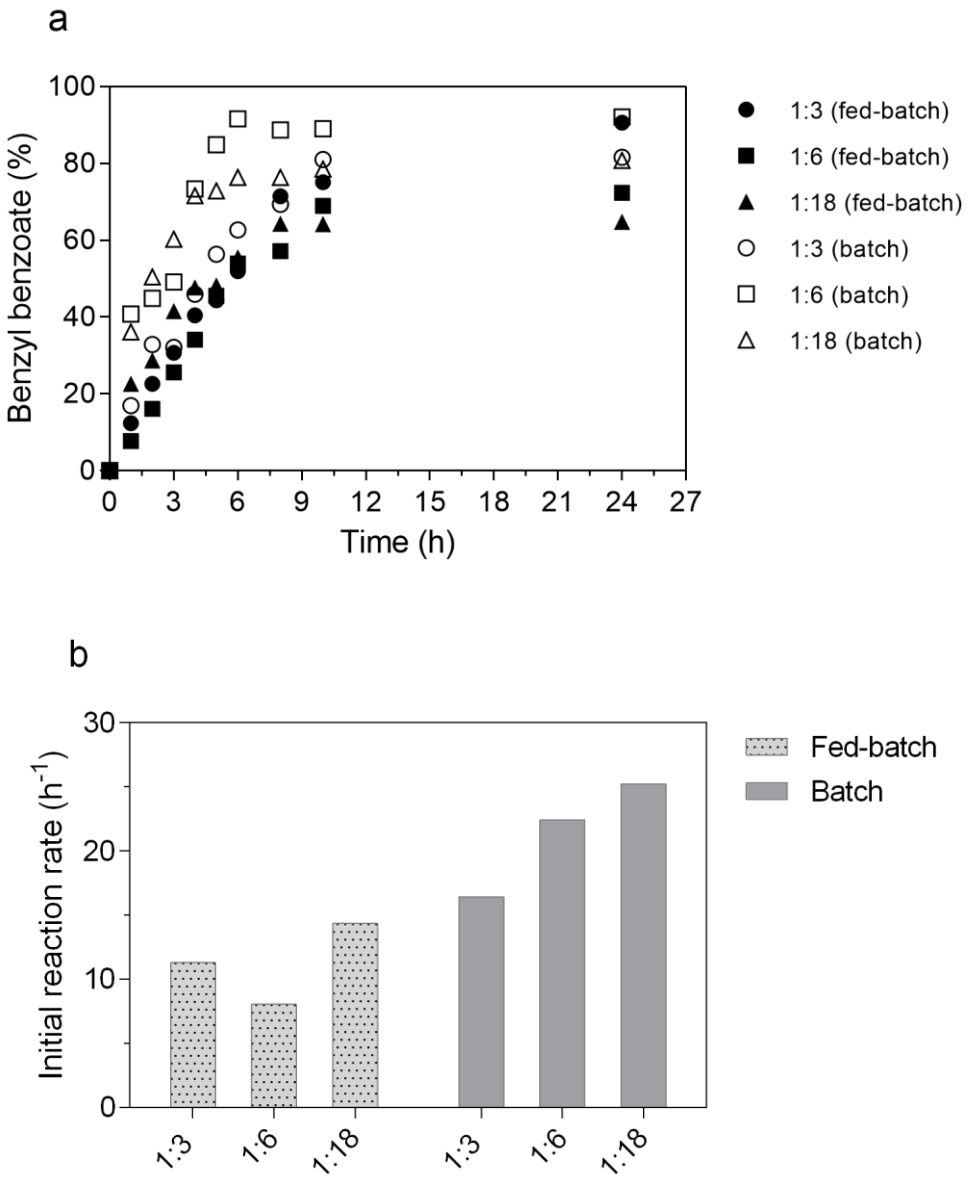
5.2.4 Effect of batch and fed-batch operation regimes

Generally, aromatic esters are successfully synthesized in batch reactions. However, a fed-batch configuration (controlled addition of the acyl donor) may directly affect the drawbacks related to the solubility of solid substrates. In this way, both configurations were tested with different anhydride: alcohol molar ratios using Lipozyme TL IM as biocatalyst for the benzyl alcohol acylation. Figure 24a shows good conversion for both reactor configurations. The effect of the molar ratio of the substrates on the final conversions (24 h) did not follow a clear tendency as the concentration of the alcohol was increased in the reaction media. The molar ratio of 1:3 in fed-batch mode reached a similar conversion to 1:6 in batch mode. The results showed that the slow addition of the anhydride during the initial hours improved its solubilization in the reaction media, improving the final conversion at lower molar ratios (anhydride: alcohol).

Several reports in literature show that the lipase-catalyzed acylation under conventional stirring occurs at moderate reaction rates and reach maximum conversion of 100% within 6 h (ROMERO et al., 2005, 2007; CHIARADIA et al., 2012; SILVA et al., 2015). The drawbacks related to the benzoic anhydride solubilization may be responsible to the incomplete acyl donor consumption and, consequently, conversions around 90%.

The operational stability of Lipozyme TL IM was tested under the benzyl alcohol acylation for both batch and fed-batch modes. The enzyme reusability was carried out using the recovered enzyme over three subsequent cycles. After each cycle, the enzyme was filtered out, washed with fresh solvent and allowed to drain out the solvent before reuse. The enzyme showed good performance over three reaction cycles with loss of 20 w/w% of the enzyme activity after de third cycle (data not shown).

Figure 24 - Benzyl benzoate synthesis (a) and initial reaction rate (b) of a solvent-free system under batch and fed-batch reactions at substrates molar ratio of 1:3, 1:6 and 1:18 using 10 w/w% of Lipozyme TL IM as biocatalyst and 50 ºC.



The high conversions reached and the operational stability of the enzyme showed that both configurations could be used for benzyl alcohol acylation despite the lower initial reaction rates found for the fed-batch reactors (Figure 24b) that can be associated to the controlled addition of the anhydride during the first five hours of reaction, limiting the reaction rate. The batch reactions presented higher reaction

rates and conversions with maximum values reached in only 6 h (molar ratio 1:6), which improved the global productivity and simplified the process conditions. Based on the results, the batch configuration and molar ratio 1:6 were employed for the next experiments.

5.2.5 Effect of temperature and enzyme amount

The impact of the reaction temperature is crucial for lipase-mediated reactions since each enzyme has an optimal range of temperature and an adequate increase in the temperature may lead to an increase in the kinetic constants, consequently, higher conversions. On the other hand, the treatment with high temperatures may lead to a negative effect as the indiscriminate increase can lead to the modification in the tertiary structure resulting in enzyme inactivation (ROMERO et al., 2005). Furthermore, temperature is a key parameter as it assists the solubilization of reactants and boosts the molecular-collision interface (BADGUJAR; BHANAGE, 2014; SÁ et al., 2017).

Experiments were performed to find out the most suitable temperature for the benzyl alcohol acylation considering similar reaction conditions. Figure 25 shows that the increase in the temperature from 40 to 50 °C led to an increase in the initial reaction rates but the final conversions (after 24 h) remained similar. A further increase in the temperature from 50 to 60 °C also led to an increase in the initial reaction rate while global conversions (91% after 6 h of reaction) were similar for both temperatures (Figure 25), and all temperatures showed maxima conversions between 90 and 100% after 24 h of reaction. Thus, to keep the minimum possible temperature and reaction time, the temperature of 50 °C was set for the next experiments.

The amount of biocatalyst is another crucial parameter from the industrial economical point of view and is important to achieve high conversion at low enzyme concentration. The effect of different Lipozyme TL IM concentrations on the benzyl anhydride conversions with fixed substrate molar ratio and temperature was investigated. Figure 26 shows that the conversion and initial reaction rate increased with enzyme concentration. The higher amount of biocatalyst increased the lipase

active sites available to the substrates and accelerated the reaction rate, reaching a value of 92% of conversion in just 6 h (10 w/w% of biocatalyst and 50 °C).

Figure 25 - Effect of temperature in the benzyl benzoate synthesis using the Lipozyme TL IM as biocatalyst in a solvent-free system with substrate molar ratio of 1:6 (anhydride to alcohol) and 10 w/w % of enzyme.

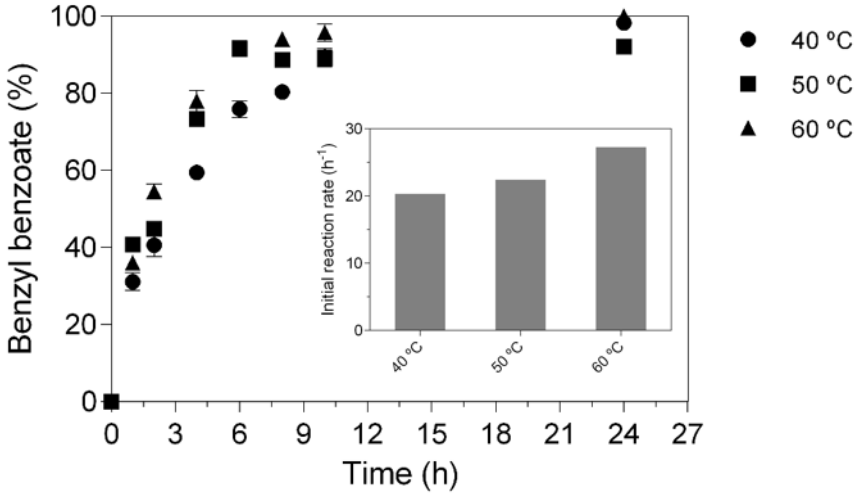
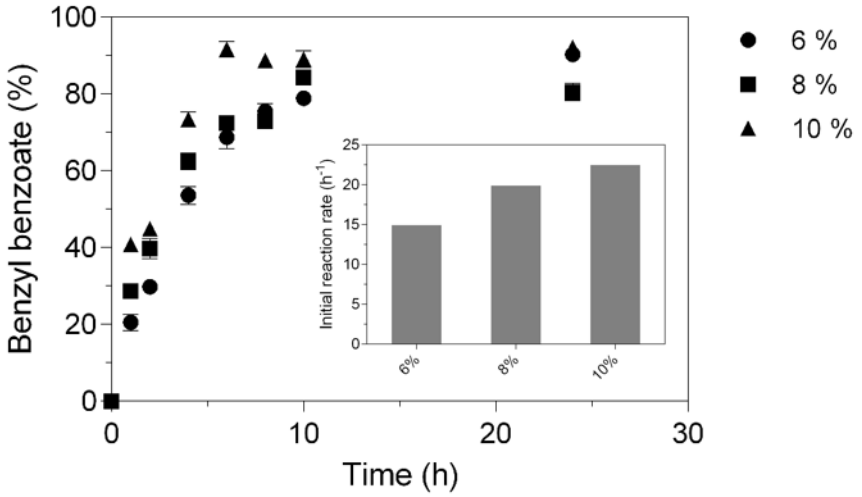


Figure 26 - Effect of Lipozyme TL IM concentration (6, 8 and 10 w/w%) in the production of benzyl benzoate, considering substrates molar ratio of 1:6 (anhydride to alcohol) and 50 ºC.



5.3 CONCLUSION

In the present work, benzyl benzoate was successfully synthesized via enzymatic catalysis in a solvent-free system. The benzoic acid is a solid of difficult solubilization and the synthesis of benzyl benzoate by esterification did not occur under the studied conditions. Controversially, the acylation was very effective in the benzyl benzoate synthesis using benzoic anhydride as acyl donor. The results showed the acylation was the main and only reaction occurring at the present conditions, reaching high conversions. The solvent 2-propanol was efficient in the substrates solubilization at increased benzyl alcohol molar ratios. However, the free solvent system with benzyl alcohol in excess was very efficient in the benzoic anhydride solubilization and it was the most adequate system for the synthesis of benzyl benzoate by acylation using the Lipozyme TL IM as biocatalyst. A great amount of acyl acceptor was essential to ensure good solubilization of the benzoic anhydride. The use of ultrasound damaged the Lipozyme TL IM and RM-IM supports hampering the enzyme reuse under the tested conditions. The controlled addition of benzoic anhydride to the reaction medium under conventional homogenization was efficient at lower benzyl alcohol molar ratios but the reaction rate was decreased due to the fed-batch approach and the maximum conversions were reached at the end of reaction period. On the other hand, the batch reactions showed an increased initial reaction rate reaching maximum conversion at the initial hours. The benzyl alcohol acylation mediated by lipases in batch reactors had high reaction rates and conversions using an excess of benzyl alcohol, allowing the ester synthesis in the absence of organic solvents. In addition, low temperature and a moderate amount of enzyme were required to ensure good global productivity and rather simple process conditions.

6 FINAL CONCLUSION

This work presented a green alternative for the synthesis of benzyl esters via biocatalysis using immobilized enzymes as catalysts in absence of organic solvents and low or moderate substrates molar ratio. The fed-batch configuration showed to be efficient to improve conversion to form esters and reduce acid inhibition effects associated to the short chain acids like butyric and propionic acid. The benzyl butyrate was successfully synthesized via direct esterification in an equimolar substrates ratio ensuring great enzyme reusability. The immobilized enzyme performance was only ensured in the presence of high amounts of benzyl alcohol and the fed-batch reactor was efficient to synthesize the benzyl butyrate and prevent the butyric acid inhibition effects. The benzyl propionate synthesis mediated by immobilized enzymes showed great conversions in fed-batch reactors and the addition of the acid during the first hours of reaction decreased the acid inhibition effects. However, the results strongly suggest the propionic acid acted as a lipase detergent causing the lipase leaching from the polymeric support, making impossible the enzyme recycling. The benzyl benzoate was successfully synthesized via enzymatic-catalyzed acylation ensuring great conversions in batch reactors showing higher reaction rate and short reaction time. The benzyl esters are very interesting compounds for the pharmaceutical and chemical industries and their biotechnological synthesis presents a green alternative to the chemical and natural extraction routes. Further investigation is necessary to improve the biocatalyst performance and ensure a feasible condition for the benzyl propionate synthesis by esterification mediated by immobilized enzymes. Nevertheless, the commercial immobilized enzymes from Novozymes were very efficient in the benzyl butyrate and benzyl benzoate synthesis and may be an interesting route for scale-up and industrial production.

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