Amanda Gomes Almeida Sá

LIPASE-CATALYZED PRODUCTION OF BENZYL ESTERS IN SOLVENT-FREE SYSTEM

Dissertação submetida ao Programa de Pós-Graduação em Engenharia Química da Universidade Federal de Santa Catarina para a obtenção do Grau de Mestre em Engenharia Química.

Orientador: Prof.ª Dr.ª Débora de Oliveira

Coorientador: Prof. Dr. Pedro Henrique Hermes de Araújo.

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Esta Dissertação foi julgada adequada para obtenção do Título de Mestre, e aprovada em sua forma final pelo Programa de Pós-Graduação em Engenharia Química da Universidade Federal de Santa Catarina.

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A todos a minha sincera gratidão.

“It is only with the heart that one can see rightly;
what is essential is invisible to the eye.”
(Antoine de Saint-Exupery)

ABSTRACT

Many sectors of industry, mainly food, cosmetics and pharmaceutics, have increased their interest in esters due to their flavor property. Flavor esters that possess an aromatic ring in their molecular structure are also known as aromatic esters. These esters are widely found in nature (fruits and plants) and the synthetic (i.e. via chemical) and natural routes (i.e. via direct extraction from nature or via biotechnology) are suitable for their biocatalysis. Enzyme-catalyzed reactions are the most economical approach to reach final green products with no toxicity and no harm to human health. The study of the process parameters and their interaction are very important to understand the system optimization and achieve the maximum reaction yield to scale up. Benzyl propionate and benzyl butyrate are aromatic esters that possess a fruity and floral odor and are usually found in nature in the composition of some fruits like plums and melons. This work aimed for the benzyl esters biotechnological synthesis by esterification of benzyl alcohol and acid using a new immobilized enzyme preparation with low-cost material from *Candida antarctica* fraction B (NS 88011) and three commercial immobilized lipases (Novozym 435, Lipozyme TL-IM and Lipozyme RM-IM) from Novozymes®. Novozym 435 had the best performance even when the solvent *tert*-butanol was absent of the reaction medium. Results from a 2^2 factorial design showed that an increase in the enzyme amount led

to a higher ester conversion, even when the temperature was kept at the low value. Currently, no research had synthesized successfully benzyl propionate via esterification mediated by lipases. Aromatic esters can present some biological activities, in addition to their fragrances. The antimicrobial activity of benzyl butyrate was tested, and the results showed a good performance, indicating that a low benzyl butyrate concentration was sufficient to prevent bacterial growth.

Keywords: Flavor; Ester; Enzyme; Biocatalysis; Benzyl propionate; Benzyl butyrate.

RESUMO

Muitos setores da indústria, principalmente alimentos, cosméticos e produtos farmacêuticos, aumentaram seu interesse em ésteres aromáticos devido à sua propriedade de aroma. Estes ésteres são amplamente encontrados na natureza (frutas e plantas) e as rotas sintéticas (isto é, via química) e naturais (ou seja, via extração direta da natureza ou via biotecnológica) são adequadas para sua biocatálise. As reações catalisadas por enzimas são a abordagem mais econômica para alcançar produtos finais verdes sem toxicidade e sem danos para a saúde humana. O estudo dos parâmetros do processo e sua interação são muito importantes para entender a otimização do sistema e alcançar o rendimento de reação máximo para aumentar a escala. O propionato de benzila e o butirato de benzila são ésteres aromáticos que possuem um odor frutado e floral e geralmente são encontrados na natureza na composição de algumas frutas como ameixas e melões. Este trabalho objetivou a síntese biotecnológica de ésteres de benzila por esterificação de álcool benzílico utilizando uma nova preparação enzimática imobilizada com material de baixo custo da fração B de *Candida antarctica* (NS 88011) e três lipases imobilizadas comerciais (Novozym 435, Lipozyme TL-IM e Lipozyme RM-IM) da Novozymes®. A enzima Novozym 435, uma enzima imobilizada comercial bem conhecida, teve o melhor desempenho mesmo quando o solvente tert-butanol estava ausente

do meio de reação. Os resultados do planejamento experimental fatorial 2^2 mostraram que um aumento na quantidade de enzima levou a uma maior conversão de éster, mesmo quando a temperatura foi mantida no menor valor. Atualmente, nenhuma pesquisa sintetizou com sucesso o propionato de benzila por esterificação mediada por lipases. Os ésteres aromáticos podem apresentar algumas atividades biológicas, além de suas fragrâncias. A atividade antimicrobiana do butirato de benzila foi testada e os resultados mostraram um bom desempenho, indicando que uma baixa concentração de butirato de benzila foi suficiente para prevenir o crescimento bacteriano.

Palavras-chave: Aroma; Éster; Enzima; Biocatálise; Propionato de benzila; Butirato de benzila.

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LIST OF SYMBOLS

° C	Degrees Celsius
%	Percentage
wt%	Weight total percentage
v/v	Volume-to-volume ratio

LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
BBC	British Broadcasting Corporation
COF	Council of Europe
CAGR	Compound Annual Growth Rate
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FEMA	Flavor and Extract Manufacturers Association
GRAS	Generally Recognized as Safe
JECFA	Joint FAO/WHO Expert Committee on Food Additives
MBC	Minimum Bactericidal Concentration
MIC	Minimum Inhibitory Concentration
WHO	World Health Organization

SUMÁRIO

CHAPTER I

1 INTRODUCTION

Flavor esters are significant and versatile compounds, also known as important ingredients in food, beverages, cosmetics, pharmaceuticals, chemicals and personal care products, like perfumes, body lotions, face creams, shampoos, soaps, shower-shaving gels and other toiletries, due to their flavor and fragrance properties (SHINTRE; GHADGE; SAWANT, 2002; LI et al., 2014; SILVA et al., 2014; AKACHA; GARGOURI, 2015; TODERO et al., 2015; BADGUJAR; PAI; BHANAGE, 2016).

Many aromatic esters are flavor esters that have an aromatic ring in their molecular structure. These esters are usually obtained via direct extraction from plant or fruit source; however, disadvantages make this technique inadequate for industrial applications including the seasonal and climatic dependency of the source, besides the low yield and high production costs needed for extraction and purification (BADGUJAR; SASAKI; BHANAGE, 2015; GAO et al., 2016; GUMEL; ANNUAR, 2016a; SANTOS et al., 2016).

The chemical production of aromatic esters also possesses several drawbacks and environmental impacts in the production and purification processes, such as the use of hazardous chemicals and catalysts, toxic solvents and high temperature and pressure. Moreover, chemical synthesis presents high costs due to the lack of substrate selectivity and by-products removal, long reaction times, excessive consumption of energy and possible corrosion of the equipment.

Furthermore, the final products cannot be legally labeled as natural and the synthesized esters contain traces of toxic impurities, which may result in humans' health complications. Therefore, all these factors make the process industrially disadvantageous (KHAN; RATHOD, 2015; MOHAMAD et al., 2015; SILVA et al., 2015; WANG et al., 2015; MANAN et al., 2016).

Biotechnology is a technique that uses living organisms (or parts of them) to make or modify products, to improve plants and animals or to develop microorganisms for specific uses (BICAS et al., 2010). Biotechnological transformations (i.e. microbial and enzymatic routes) are employed in ester production and among various biotechnological processes, the lipase-catalyzed reaction is economically viable for flavor and aromatic esters synthesis (GARLAPATI et al., 2013; STENCEL; LEADBEATER, 2014). Biocatalysis applied in ester production is a useful and promising alternative green tool, which offers several advantages, such as high specificity and chemo-, regio- and stereo-selectivity, high yields in mild reaction conditions (low temperature and pressure), 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). In addition, aromatic esters produced by microbial or enzymatic methods are 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).

1.1 OBJECTIVES

1.1.1 General Objective

This work aims to study the production of bioactive aromatic benzyl esters (benzyl propionate and benzyl butyrate) by enzymatic esterification with different lipases in a solvent-free system.

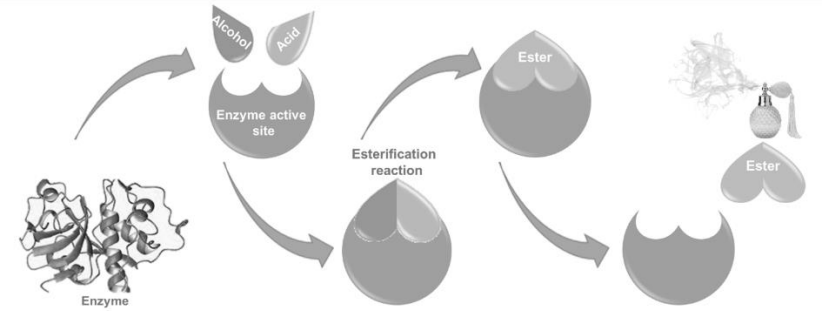
1.1.2 Specific Objectives

- Analyze the principal parameters of esterification reaction on the benzyl esters synthesis, like temperature, molar ratio, reaction kinetics and enzyme type and concentration of biocatalyst (Novozym 435, Lipozyme RM-IM, Lipozyme TL-IM and NS 88011).
- Study the utilization of molecular sieves in the esterification of benzyl esters.
- Evaluate the effect of organic solvent *tert*-butanol and the efficiency of ultrasound in the ester conversion.
- Evaluate the antimicrobial activity of benzyl butyrate against Gram-positive and Gram-negative bacteria.

CHAPTER II

This chapter is a review article entitled "**A review on enzymatic synthesis of aromatic esters used as flavor ingredients for food, cosmetics and pharmaceuticals industries**" published in 2017 in the periodic **Trends in Food Science & Technology**.

GRAPHICAL ABSTRACT



2 A REVIEW ON ENZYMATIC SYNTHESIS OF AROMATIC ESTERS USED AS FLAVOR INGREDIENTS FOR FOOD, COSMETICS AND PHARMACEUTICALS INDUSTRIES

2.1 INTRODUCTION

The present work presents an overview of the current state of the art of aromatic esters production by enzymatic route, considering the main effects in the reaction media conditions and enzymes used, providing knowledge about the latest advances and most relevant process parameters related to their synthesis. This study also reviews the recent techniques used, optimization and kinetics aiming to improve the reaction

yield and scale up. This review also describes applied trends in enzymatic-catalyzed reactions, pointing alternatives to production, like ultrasound-assisted reactions and process optimization of aromatic esters. Furthermore, this review presents perspectives concerning biological potential in ester production, modern progress in aromatic esters encapsulation and the future trends, challenges and prospects in this field.

2.2 BIOFLAVORS

Flavors are composed of different organic chemicals, such as hydrocarbons, alcohols, aldehydes, ketones, acids, esters or lactones. The low volatility and low molecular weight, usually lower than 400 Da, are responsible for a range of sensorial sensations attributed to the flavors (LONGO; SANROMÁN, 2006).

Flavor and aromatic esters are widely found in nature and confer pleasant organoleptic impact attributes, including fruity, floral, spicy, creamy or nutty aromas (BERGER, 2009; YADAV; DHOOT, 2009; GAO et al., 2016). These properties make possible a great variety of applications in the food sector in many beverages, candies, jellies, jams, wines and dairy products (BIAŁECKA-FLORJAŃCZYK et al., 2012; AKACHA; GARGOURI, 2015) and also in the cosmetic industry, as fragrances in perfumes, deodorants, creams and soaps and flavors in lip cosmetics. However, besides the fragrance they can also have others properties, such as emollient, surfactant and antioxidant, which make possible their application in many formulations of creams, shampoos and anti-aging creams (BECKER et al., 2012; KHAN; RATHOD, 2015).

The rapid absorption, metabolic excretion, low level of use and the lack of significant genotoxic and methanogenic potential make the aromatic esters Generally Recognized as Safe (GRAS) for use as flavor ingredient since 1965 by the Flavor and Extract Manufacturers Association (FEMA) Expert Panel (ADAMS et al., 2005a). Furthermore, some international regulations determine that aromatic esters have no safety concerns when used as flavoring agents. European Commission, Food and Drug Administration (FDA) and International Joint FAO/WHO Expert Committee on Food Additives (JECFA) approve these esters for food additives (COF, 2000; JECFA, 2001; FDA, 2013).

The global market for flavors and fragrances was valued at $ 26.0 billion in 2015. This market 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).

2.3 ENZYMES

Enzymes are non-toxic biocatalysts that accelerate the rate of reactions and are highly versatile in the catalysis of various types of reactions using mild conditions. Enzymes have attracted significant attention due to their 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; SHARMA; KANWAR, 2014; BADGUJAR; BHANAGE, 2015). Among the enzymes, lipases are important because of 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).

The global market for industrial enzymes reached nearly $ 4.9 billion in 2015. This market should increase from $ 5.0 billion in 2016 to $ 6.3 billion in 2021 at a compound annual growth rate (CAGR) of 4.7 % (BBC, 2017).

2.3.1 Lipases

Lipases (triacylglycerol hydrolases, EC 3.1.1.3) play an important role in 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) and transfer of acyl groups from esters to other nucleophiles (e.g. amines and thiols) (HORCHANI et al., 2010; MENDES; CASTRO; GIORDANO, 2014; PALUDO et al., 2015; TOMKE; RATHOD, 2015; HOANG; MATSUDA, 2016; NARWAL et al., 2016).

Lipases are abundant in nature and found in multiple organisms, but yeast and fungi are the main sources of lipases for industrial applications. Most commercially important lipase-producing yeasts belong to the class of ascomycetes like *Candida* sp. Novozymes® (Denmark), DuPont® (United States) and Roche® (Switzerland) are the main companies for the production and commercialization of lipases (GUPTA et al., 2015).

The practical use of homogeneous biocatalysis (free enzyme) has some disadvantages on industrial process economics because of low solvent, thermal, mechanical and operational stabilities and no recyclability, which leads to high production costs. To overcome these limitations, efforts have been taken by researchers to develop several advance skillful immobilization techniques (SHARMA; KANWAR, 2014; BADGUJAR; BHANAGE, 2015; FERRAZ et al., 2015), which offer many process advantages: lower production cost, increased activity, specificity and selectivity, 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).

2.4 AROMATIC ESTERS

Flavor esters that possess an aromatic ring in the molecular structure are also known as aromatic esters. These esters are broadly present in nature and confer pleasant organoleptic attributes, such as fruity and floral smell and taste, which make possible a great variety of applications in the food, pharmaceuticals and cosmetic industries (BERGER, 2009; YADAV; DHOOT, 2009; DHAKE et al., 2012; GAO et al., 2016). The main aromatic esters are benzyl, cresyl, anisyl, eugenyl, cinnamyl, phenethyl, benzoate and cinnamate esters. The applications and natural occurrences of these esters are shown in Table 1 (ADAMS et al., 2004, 2005a, 2005b; BELSITO et al., 2012; MCGGINTY; LETIZIA; API, 2012; MCGINTY; LETIZIA; API, 2012a; API et al., 2015, 2016a).

The biotechnological production of these aromatic esters is an alternative to natural source and has the possibility of scale up to industrial applications. Some studies have been made in this area, with different immobilized lipases as the heterogeneous biocatalysts in the synthesis of benzyl acetate (GARLAPATI et al., 2013), cinnamyl acetate (BADGUJAR; SASAKI; BHANAGE, 2015) and 2-methyl benzyl acetate (DHAKE et al., 2012). Tables 2 to 6 present the main reaction parameters for the synthesis of benzyl, cresyl, anisyl, eugenyl, cinnamyl, phenethyl, benzoate and cinnamate esters, respectively.

Aromatic esters production via enzymatic route is a green process alternative and the process variables are important for the final conversion and reaction yield. The molar ratio of substrates, type and amount of enzyme, nature of substrates, temperature, reaction time, agitation speed and use of organic solvents have a great impact on the process production. The optimization of the process by the study of the individual effects and interactions is useful to achieve high product yield and conversion (GENG et al., 2012; PAROUL et al., 2012; STENCEL; LEADBEATER, 2014).

Table 01 – Applications and natural occurrences of aromatic esters.

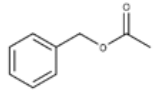
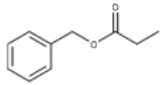
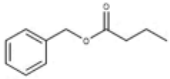
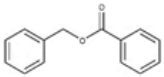
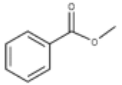
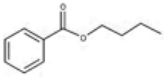
Aromatic Ester		Application in Food, Flavour-Fragance	Natural occurrence in plants and fruits
Benzyl acetate		Jasmine, green apple, strawberry, banana, orchid	*Acacia farnesiana, Hyacinthus sp., Jasminum*
Benzyl propionate		Almond, apple, banana, coconut, grape, cherry, strawberry, plum	*Michelia champaca, Prunus species, Tanacetum parthenium* and melon
Benzyl butyrate		Cherry, apple, berry, grape, plum, pear, peach, papaya, pineapple, raspberry, orchid	*Spondias mombins, Osmanthus fragrans, Vasconcellea pubescens, Grape cherimoya, Michelia champaca*
Benzyl benzoate		Apple, apricot, banana, blueberry, cherry, cranberry, grape, jasmin, lily, melon, pineapple, raspberry, violet	*Michelia champaca, Jasminum*
Methyl benzoate		Herb, lettuce, prune, violet, cherry, clove, cranberry, jasmin, mint, pineapple, raspberry, strawberry, vanilla	*Michelia champaca, Jasminum sambac, Cananga odorata, Ribes nigrum*|
Butyl benzoate		Banana, cherry, geranium, orchid, papaya, pineapple, rose, strawberry	*Michelia champaca, Jasminum* and strawberry

Table 1 (continue) - Applications and natural occurrences of aromatic esters.

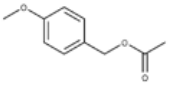
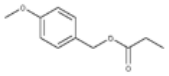
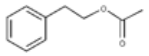
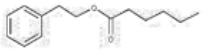
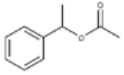
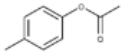
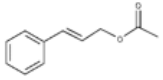
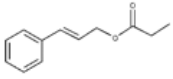
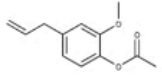
Aromatic Ester		Application in Food, Flavour-Fragance	Natural occurrence in plants and fruits
Anisyl acetate		Juniper berry, raspberry, vanilla, fig, coconut, cherry, balsam, plum,	*Cananga odorata, Clavija euerganea, Jacquinia keyensis, Jacquinia sprucei*
Anisyl propionate		Sweet, fruity, floral, vanilla	*Pimpinella anisum L.*
2-phenethyl acetate		Apricot, peach vanilla, tutti-frutti	*Freesia magnolia, Hyacinth reseda*
2-phenethyl hexanoate		Pineapple, rose, apple, banana, fruity	*Michelia champaca*
1-phenethyl acetate		Rose, honey, fruity, floral	*Michelia champaca, Eschweilera coriacea*
p-cresyl acetate		Apple blossom, cherry blossom	*Prangos uechtritzii, Cananga odorata*
Cinnamyl acetate		Cinnamon, oriental, rose, apricot, guava	*Psidium guajava, Laurus nobilis, Cinnamomum verum*
Cinnamyl propionate		Apricot, almond, apple, banana, coconut, grape, cherry, blackberry, vanilla, strawberry, plum	*Cinnamon, Michelia sp., Prunus sp., Tanacetum sp.* and melon
Eugenyl acetate		Clove, cinnamom	*Syzygium aromaticum, Cinnamomum verum*

Table 2 – Synthesis of benzyl esters with benzyl alcohol as acyl acceptor.

Aromatic Ester	Acyl Donor	Molar Ratio Acyl Acceptor: Acyl Donor	Enzyme	Solvent	Temperature	Time	Conversion	References
Benzyl acetate	Vinyl acetate	2 : 3.5	Immobilized *Pseudomonas* fluorescens lipase, 36 mg	Heptane	50 °C	4 h	99 %	(Badgujar et al., 2015)
Benzyl acetate	Vinyl acetate	1 : 1	*Pseudomonas aeruginosa* lipase, 50 mg	Heptane	50 °C	3 h	88.8 ± 0.2 %	(Singh et al., 2008)
Benzyl propionate	Vinyl propionate	2 : 5	Immobilized *Pseudomonas cepacia* lipase, 54 mg	Isooctane	50 °C	2.5 h	99 %	(Badgujar & Bhanage, 2014b)
Benzyl butyrate	Vinyl butyrate	1 : 2.7	Immobilized *Pseudomonas cepacia* lipase, 300 mg	Isooctane	52 °C	3 h	99 % with ultrasound technique	(Badgujar & Bhanage, 2015)
Benzyl butyrate	Butiric acid	1 : 1	Novozym 435®, 200 mg	Methyl tert-butyl ether	52 °C	24 h	82 %	(Jeromin & Zoor, 2008)
Benzyl laurate	Lauric acid	5 : 1	*Aspergillus oryzae* lipase	Solvent-free	30 °C	6 h	80 %	(Shintre et al., 2002)
Benzyl oleate	Oleic acid	1 : 1	Novozym 435®, 12.5 mg	Solvent-free	80 °C	1 h	94 %	(Vosmann et al., 2008)
Benzyl cinnamate	Cinnamic acid	3 : 1	Lipozyme TL-IM®, 30 mg	Isooctane	40 °C	24 h	97.3 %	(Wang et al., 2015)
Benzyl cinnamate	Cinnamic acid	2.6 : 1	Lipozyme TL-IM®, 31 mg.mL^{-1}	Isooctane	40 °C	27 h	97.7 %	(Zhang et al., 2016)

Table 3 – Synthesis of cresyl and anisyl esters with cresol and anisyl alcohol as acyl acceptor, respectively.

Aromatic Ester	Acyl Donor	Molar Ratio Acyl Acceptor: Acyl Donor	Enzyme	Solvent	Temperature	Time	Conversion	References
p-cresyl acetate	Vinyl acetate	2 : 3.5	Immobilized *Pseudomonas* fluorescens lipase, 36 mg	Heptane	50 °C	12 h	69 %	(Badgujar et al., 2015)
p-cresyl acetate	Vinyl acetate	1 : 5	Steapsin lipase, 70 mg	Hexane	55 °C	60 h	81.0 ± 1.3 %	(Dhake et al., 2012)
p-cresyl propionate	Vinyl propionate	1 : 2	Immobilized *Pseudomonas cepacia* lipase, 20 mg	Heptane	40 °C	1.5 h	99 %	(Badgujar et al., 2016)
o-cresyl butyrate	Vinyl butyrate	1 : 2.7	Immobilized *Pseudomonas cepacia* lipase, 300 mg	Isooctane	52 °C	3.5 h	99 % with ultrasound technique	(Badgujar & Bhanage, 2015)
Anisyl acetate	Vinyl acetate	2 : 3.5	Immobilized *Pseudomonas* fluorescens lipase, 36 mg	Heptane	50 °C	4.5 h	99 %	(Badgujar et al., 2015)
Anisyl acetate	Vinyl acetate	1 : 5	Steapsin lipase, 70 mg	Hexane	55 °C	48 h	99.0 ± 0.3 %	(Dhake et al., 2012)
Anisyl acetate	Vinyl acetate	1 : 5	Immobilized *Rhizopus oryzae* lipase, 50 mg	Hexane	45 °C	48 h	99 %	(Dhake et al., 2011)
Anisyl butyrate	Vinyl butyrate	1 : 2.7	Immobilized *Pseudomonas cepacia* lipase, 300 mg	Isooctane	52 °C	3 h	99 % with ultrasound technique	(Badgujar & Bhanage, 2015)
Anisyl propionate	Vinyl propionate	1 : 2	Immobilized *Pseudomonas cepacia* lipase, 20 mg	Heptane	40 °C	1.5 h	99 %	(Badgujar et al., 2016)

Table 4 – Synthesis of eugenyl and cinnamyl esters with eugenol and cinnamyl alcohol as acyl acceptor, respectively.

Aromatic Ester	Acyl Donor	Molar Ratio Acyl Acceptor: Acyl Donor	Enzyme	Solvent	Temperature	Time	Conversion	References
Eugenyl acetate	Acetic anhydride	1 : 5	Novozym 435®, 1 wt %	Supercritical CO_2, 10 MPa	40 °C	1 h	33.2 ± 0.3 %	(Santos et al., 2016)
Eugenyl acetate	Acetic anhydride	1 : 5	Lipozyme 435®, 10 wt %	Supercritical CO_2, 10 MPa	40 °C	1 h	20.3 ± 1.0 %	(Santos et al., 2016)
Eugenyl acetate	Acetic anhydride	1 : 5	Lipozyme TL-IM®, 5 wt %	Solvent-free	70 °C	2 h	94.3 %	(M. J. A. Silva et al., 2015)
Eugenyl acetate	Acetic anhydride	1 : 5	Novozym 435®, 10 wt %	Solvent-free	60 °C	6 h	99.9 %	(Vanin et al., 2014)
Eugenyl acetate	Acetic anhydride	1 : 3	Novozym 435®, 5.5 wt %	Solvent-free	50 °C	6 h	99.86 %	(Chiaradia et al., 2012)
Eugenyl benzoate	Benzoic acid	4 : 1	Immobilized *Rhizomucor miehei* lipase, 15 mg	Chloroform	60 °C	6 h	56.1 %	(Manan et al., 2016)
Eugenyl benzoate	Benzoic acid	1 : 1.22	Immobilized *Staphylococcus aureus* lipase, 240 UI	Chloroform	41 °C	6 h	75 %	(Horchani et al., 2010)
Eugenyl caprylate	Caprylic acid	1.13 : 1	Novozym 435® and Lipozyme TL-IM®	Hexane	56.8 °C	4 h	> 80 %	(Radzi et al., 2016)
Eugenyl caprylate	Caprylic acid	2 : 1	Lipozyme TL-IM®, 100 mg	Solvent-free	65 °C	4.3 h	72 %	(Chaibakhsh et al., 2012)

Table 4 (continue) – Synthesis of eugenyl and cinnamyl esters with eugenol and cinnamyl alcohol as acyl acceptor, respectively.

Aromatic Ester	Acyl Donor	Molar Ratio Acyl Acceptor: Acyl Donor	Enzyme	Solvent	Temperature	Time	Conversion	References
Cinnamyl acetate	Vinyl acetate	1 : 2	Esterase, 10 $g.L^{-1}$	Hexane	40 °C	12 h	99 %	(Gao et al., 2016)
Cinnamyl acetate	Vinyl acetate	1 : 2	Novozym 435®, 0.2 %	Solvent-free	40 °C	20 min	99.99 %	(Tomke & Rathod, 2015)
Cinnamyl acetate	Vinyl acetate	1 : 5	Immobilized *Rhizopus oryzae* lipase, 50 mg	Hexane	45 °C	24 h	99 %	(Dhake et al., 2011)
Cinnamyl acetate	Ethyl acetate	1 : 15	Novozym 435®, 2.67 $g.L^{-1}$	Solvent-free	40 ° C	3 h	90 %	(Geng et al., 2012)
Cinnamyl acetate	Acetic acid	1 : 2	Immobilized procine pancreatic lipase, 3 g	Hexane	35 °C	10 h	62.6 %	(Wu et al., 2014)
Cinnamyl acetate	Vinyl acetate	1 : 2	Novozym 435®, 10 mg	Toluene	40 °C	1 h	96 %	(Yadav & Devendran, 2012)
Cinnamyl propionate	Vinyl propionate	1 : 2	Immobilized *Pseudomonas cepacia* lipase, 20 mg	Heptane	40 °C	1.5 h	99 %	(Badgujar et al., 2016)
Cinnamyl laurate	Lauric acid	1 : 1	Novozym 435®, 50 mg	Toluene	30 °C	2 h	60 %	(Yadav & Dhoot, 2009)

Table 5 – Synthesis of phenethyl esters with phenethyl alcohol as acyl acceptor.

Aromatic Ester	Acyl Donor	Molar Ratio Acyl Acceptor: Acyl Donor	Enzyme	Solvent	Temperature	Time	Conversion	References
1-phenethyl acetate	Vinyl acetate	0.4 : 1.1	Novozym 435®, 10 mg	Liquid CO_2, 7 MPa	20 °C	1 h	32 %	(Hoang & Matsuda, 2016)
2-phenethyl acetate	Vinyl acetate	2 : 3.5	Immobilized *Pseudomonas* fluorescens lipase, 36 mg	Heptane	50 °C	4 h	99 %	(Badgujar et al., 2015)
2-phenethyl acetate	Vinyl acetate	1 : 3.65	Immobilized *Candida rugosa* lipase	Hexane	35.85 °C	38.8 h	95.3 ± 2.6 %	(Kuo et al., 2014)
2-phenethyl acetate	Vinyl acetate	1 : 1	Novozym 435®, 122.5 mg	Hexane	57.8 °C	79 min	85.4 ± 0.4 %	(Kuo et al., 2012)
1-phenethyl acetate	Vinyl acetate	0.5 : 2	Steapsin lipase, 70 mg	Hexane	55 °C	24 h	48.0 ± 0.5 %	(Dhake et al., 2012)
2-phenethyl propionate	Vinyl propionate	1 : 2	Immobilized *Pseudomonas cepacia* lipase, 20 mg	Heptane	40 °C	1.5 h	98 %	(Badgujar et al., 2016)
2-Phenethyl butyrate	Butter oil[a]	3 : 1	Lipozyme TL-IM®, 20 wt%	-	40 °C	8 h	50.8 ± 1.9 %	(Li et al., 2014)
2-Phenethyl hexanoate	Butter oil[a]	3 : 1	Lipozyme TL-IM®, 20 wt%	-	40 °C	8 h	66.6 ± 2.7 %	(Li et al., 2014)
2-phenethyl caffeate	Caffeic acid	1:71	Novozym 435®, 2938 PLU	Isooctane	70 °C	9.6 h	93.1 ± 0.4 % with ultrasound technique	(Chen et al., 2011)

[a] Fatty acids from butter oil: butyric 10%; caproic 5%, caprylic 2.6%; capric 5%; lauric 5%; myristic 12%; palmitic 27%; stearic 10%; and oleic 23%.

Table 6 – Synthesis of benzoate and cinnamate esters with benzoic acid and cinnamic acid as acyl donor, respectively.

Aromatic Ester	Acyl Acceptor	Molar Ratio Acyl Acceptor: Acyl Donor	Enzyme	Solvent	Temperature	Time	Conversion	References
Methyl benzoate	Methanol	12 : 7	Lecitase Ultra™, 123 $U.g^{-1}$	Dichloromethane	40 °C	Continuous flow rate 0.1 mL/min	41 %	(Gumel & Annuar, 2016)
Methyl benzoate	Methanol	6.2 : 5	*Candida rugosa* lipase, 10 $mg.mL^{-1}$	Hexane and Toluene	37 °C	75 h	100 %	(Leszczak & Tran-Minh, 1998)
Hexyl benzoate	Hexanol	19 : 1	Immobilized *Bacillus subtilis* esterase, 6 wt %	Solvent-free	40 °C	2.7 d	24 %	(Krause, Hilterhaus & Liese, 2009)
Heptyl benzoate	N-heptanol	1 : 1	Novozym 435®, 50 mg	Cyclohexane	60 °C	24 h	100 %	(Giunta et al., 2015)
Benzyl benzoate	Benzyl alcohol	2 : 1	Novozym 435®, 300 mg	Solvent-free	55 °C	100 h	90 %	(Gryglewicz et al., 2000)
Ethyl cinnamate	Ethanol	3 : 1	Lipozyme TL-IM®, 30 mg	Isooctane	50 °C	24 h	99 %	(Wang et al., 2016)
Butyl cinnamate	Butanol	15 : 1	Novozym 435®, 3 wt%	Isooctane	55 °C	72 h	60.7 %	(Jakovetić, Jugović, et al., 2013)
Oleyl cinnamate	Oleyl alcohol	6 : 1	Novozym 435®, 20 mg	Isooctane and 2-butanone	55 °C	12 d	100 %	(Lue et al., 2005)
Geranyl cinnamate	Geraniol	2 : 1	Novozym 435®, 60 mg	Hexane	65 °C	15 min	88 ± 2 %	(Shinde & Yadav, 2015)

2.4.1 Effect of acyl donors

The main reaction for the esters production is the esterification, which occurs between the alcohols (acyl acceptor) and acids (acyl donor). A disadvantage of the enzymatic esterification is the possibility of low yield production, because of a severe enzyme activity deactivation due to the high acid concentration in the reaction medium (BADGUJAR; BHANAGE, 2014a). Therefore, the choice of the substrates can affect directly the conversion and yield results. Meanwhile, researchers have presented that it is possible to reach good results using acid and alcohol for ester synthesis, as shown in a study of esterification between benzyl alcohol and butyric acid (molar ratio 1:1) to produce benzyl butyrate, reaching 82 % of yield in 24 h (JEROMIN; ZOOR, 2008).

Transesterification is an alternative to avoid the drawbacks found in the esterification and improve the ester conversion and yield. The transesterification occurs between alcohols (acyl acceptor) and esters (acyl donor). Vinyl esters are usually employed in the transesterification and thanks to the isomerization of the vinyl alcohol, that prevents the opposite reaction, it is able to promote higher conversions in the synthesis process. However, the co-product acetaldehyde may have unfavorable deactivation effect on some enzymes, so another alkyl esters can be used in order to overcome this drawback, such as ethyl acetate and methyl butyrate (KUO et al., 2012, 2014; BADGUJAR; BHANAGE, 2015).

Some researchers have shown this behavior related for esterification and transesterification in the aromatic ester production, like the synthesis of benzyl propionate with propionic acid as acyl donor. The results showed lower initial reaction rate and conversion (8 % in 2.5 h)

for the enzymatic catalysis, probably due to the acting of acid as a potent inhibitor of enzyme activity. On the other hand, tests made with methyl propionate and vinyl propionate as acyl donor indicated that the reaction with vinyl propionate had the conversion increased substantially (99 %) than that with methyl propionate (17 %) in the same conditions. The low conversion reached when methyl propionate was used as acyl donor was probably due to the presence of methyl alcohol that is a side product of the transesterification reaction and was able to compete with benzyl alcohol for nucleophilic attack on carbonyl ester and inhibits the reaction rate to yield (BADGUJAR; BHANAGE, 2014a). Other study presented the benzyl butyrate synthesis using butyric acid, ethyl butyrate and vinyl butyrate for esterification and transesterification, respectively. The results showed that the reaction with vinyl butyrate had a better conversion (~99 %) than that with butyric acid (~ 40 %) and ethyl butyrate (~ 50 %) in similar reaction parameters (BADGUJAR; BHANAGE, 2015).

2.4.2 Effect of enzyme

The utilization of immobilized enzymes in the synthesis of many products aims high process specificity, selectivity, productivity and easy recuperation. Several aspects might influence the activities of these biocatalysts, such as the enzyme source and the nature of immobilization support. As the main source, microorganisms can produce many enzymes and naturally present strong influence in the final structural characteristics, which modify the biocatalysts properties and activities even in similar reaction parameters (MARTINS et al., 2014).

Moreover, the nature of the immobilization support can alter the lipases properties making difficult the access of the substrate to the enzyme active site. The impediment to access the active site may occur due to the hydrophilic or hydrophobic characteristic of the support material, and can cause partition of the substrates and products or blocking of the active site during the immobilization process. The immobilization protocol is also an important aspect because it changes the enzyme activities even when it is immobilized on the same support (MARTINS et al., 2014).

Many commercial lipases are available for purchased and widely used in a range of catalysis. The commercial immobilized lipases most used are Novozym 435®, Lipozyme TL-IM® and Lipozyme RM-IM®, from Novozymes®, and each one has a different lipase source, support material and immobilization method. Novozym 435® is the *Candida antarctica* B lipase, immobilized in Lewatit VP OC 1600, a macro-porous resin with a hydrophobic surface. Lipozyme TL-IM® is a *Thermomyces lanuginosus* lipase, immobilized in a hydrophilic gel silicate and Lipozyme RM-IM® is a *Rhizomucor miehei* lipase, immobilized in a support of Duolite ES562, a weak anion-exchange resin based on phenol-formaldehyde copolymers. Regarding the immobilization protocols, Novozym 435® is prepared via interfacial activation on the hydrophobic surface of the support, while Lipozyme RM-IM® and Lipozyme TL-IM® are immobilized via anionic exchange (MARTINS et al., 2014). All these differences of the biocatalysts make the comprehension of the substrates interactions on the enzymes activities in different systems more complex.

In laboratory scale, studies easily show how these influences (i.e. lipase source, immobilization method and type of immobilization support) affect the reaction conversion results. Researchers evaluated the efficacy of Novozym 435®, Lipozyme RM-IM® and Lipozyme TL-IM® to synthesize cinnamyl laurate in similar conditions. The final conversion of 60, 16.5 and 9 % in 2 h, respectively, showing the Novozym 435® is the most adequate immobilized enzyme to this reaction system. These results indicate that the different lipase sources and immobilization material can really affect the conversion results (YADAV; DHOOT, 2009).

In addition, another important factor is the enzyme amount, which regards some attention for economic and industrial viability. In industrial applications, there is a great interest in the study of the process parameters to achieve lower production costs and to obtain the maximum productivity. Enzyme amount affects significantly the economic feasibility of the entire process. The higher biocatalyst amount assisted rapid enzyme-substrate complex formation, which resulted in a higher conversion, but a very high quantity of the enzyme leads to their agglomeration, which can block the substrate sites for enzyme attack (KHAN; RATHOD, 2015; WANG et al., 2015; BADGUJAR; PAI; BHANAGE, 2016). Thereby, the excess of the enzyme amount has no contribution to the reaction rate and must be controlled in order to guarantee high conversions and low cost.

2.4.3 Effect of molar ratio

The molar ratio is the ratio of acyl donor (i.e. acids, esters or anhydrides) and acyl acceptor (i.e. alcohols) used in the ester biosynthesis. The quantity of the substrates is an important factor to determine their influence on enzyme activity and subsequent reaction rate (BADGUJAR; SASAKI; BHANAGE, 2015). Thereby, the study of the best molar ratio is a very important parameter for any catalysis system that aims to reach great conversions, no loss of the enzyme activity and no waste of reagents.

The acid, at high concentrations, may present an inhibitory effect and cause a reduction in the enzyme catalytic activity (BADGUJAR; BHANAGE, 2014a, 2015; BADGUJAR; PAI; BHANAGE, 2016). In order to counterbalance the inhibitory influence of the acid in the enzyme activity on the esterification, some researchers recommend the use of a higher concentration of alcohol. However, a large increase of alcohol amount may also decrease the conversion due to the polar character of this reactant, which shows hydrophilic interaction with the water layer present on the enzyme surface and causes changes in the protein structure of the enzyme with consequent inhibition and reduction of the activity (SHINDE; YADAV, 2014; BADGUJAR; BHANAGE, 2015; WANG et al., 2015; GUMEL; ANNUAR, 2016a).

The high quantity of alcohol in the formulation also leads to higher costs, from the economic point of view, due to the need of purification. Therefore, researchers have been studying different substrates molar ratio to avoid unnecessary amounts of alcohol. The production of benzyl acetate (MAJUMDER; GUPTA, 2010) and ethyl

cinnamate (SHARMA; CHAUHAN; KANWAR, 2011) with 1:1 molar ratio (alcohol/acid) reaches 82 % and 54.1 % of conversion, respectively, showing the possibility to reach good conversion results with no need of a higher concentration of alcohol.

Another study showed the effect of the substrates molar ratio on the enzyme activity in the biocatalysis of benzyl propionate. Results indicated a conversion decrease with the increase of benzyl alcohol quantity. This behavior present the inhibitory effect of the benzyl alcohol, which may form a dead-end inhibition complex with the biocatalyst at higher concentrations, reducing the enzymes binding sites and consequently decreases the reaction rate (BADGUJAR; BHANAGE, 2014b).

Some researchers investigated the effect of the molar ratio in the conversion of the subtrates for the eugenyl acetate and phenylethyl acetate synthesis. For eugenyl acetate production, using various molar ratios (1:1, 1:3, 1:5 eugenol/acetic anhydride), the maximum yield was 99.86 % after 6 h of reaction at 50 ºC with 1:3 molar ratio (CHIARADIA et al., 2012). The 2-phenylethyl acetate synthesis, with two different molar ratios of substrates (2:1 and 4:1 vinyl acetate/2-phenylethyl alcohol), presented the best conversion of 94.81 % at 36 h with 4:1 molar ratio (KUO et al., 2014).

2.4.4 Effect of reaction media

Esterification is generally a water-limited reaction because the equilibrium by hydrolytic enzymes is in favor of the hydrolysis (inverse reaction). The esterification resulted in the formation of water as a

reaction by-product and its removal using molecular sieves might enhance the synthesis of the ester by pushing the reaction equilibrium in the forward direction (SHARMA; CHAUHAN; KANWAR, 2011).

The use of organic solvent media for enzymatic aromatic ester synthesis presents some advantages, such as increased substrates and products solubility and the shifting of the thermodynamic reaction equilibrium to favor esterification over hydrolysis (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 reaction media, without affecting the catalytic power of the enzyme (SHINDE; YADAV, 2014; BADGUJAR; PAI; BHANAGE, 2016).

The log P concept (the partition coefficients between water and solvent, which correlated with solvent hydrophobicity) determines the effect of the organic solvents on the reaction rate and the yield. Solvents with low log P values are more hydrophilic and tend to strip away the water present on the surface of the enzyme. Solvents with intermediate log P (> 4) are more suitable for the aromatic esters synthesis (SINGH et al., 2008; YADAV; DEVENDRAN, 2012; WANG et al., 2015, 2016). Some studies have shown good results by the use of isooctane for benzyl propionate, benzyl butyrate, benzyl cinnamate, anisyl butyrate and oleyl cinnamate (LUE et al., 2005; BADGUJAR; BHANAGE, 2014a, 2015; WANG et al., 2015; ZHANG et al., 2016), heptane for 3-phenylpropyl acetate, 2-methylbenzyl acetate, phenyl acetate and hexyl benzoate (SHINDE; YADAV, 2014; BADGUJAR; SASAKI; BHANAGE, 2015),

hexane for benzyl acetate, 2-methylbenzyl acetate, 2-phenoxybenzyl acetate and *p*-cresyl acetate (DHAKE et al., 2011, 2012).

On the other hand, the use of solvents may have some drawbacks associated with the separation costs and presence of harmful residual substances at the final product, which could be prejudicial to human health. In this scenario, lipase-mediated synthesis of aromatic esters under solvent-free systems has significant importance due to the absence of toxic solvents, which eliminates the need of recuperation, downstream and purification processes and reduces the environment hazards (GENG et al., 2012; PAROUL et al., 2012; GARLAPATI; BANERJEE, 2013; SILVA et al., 2015). The most important advantage is the possibility of high reaction conversions, as shown in benzyl acetate synthesis study under a solvent-free system with final conversion of 100 % (MAJUMDER et al., 2006).

Recently, there is an increase of interest in the use of green solvents for enzymatic ester production, such as dense gases like supercritical carbon dioxide, due to the low cost, non-toxicity, non-flammability, inertness, recyclable, environmentally friendly and moderate critical properties (Pc = 7.38 MPa and Tc = 304.2 K). Supercritical fluids are defined as fluids above their critical temperature and pressure, having liquid-like densities and gas-like diffusivities. Therefore, they appear as suitable solvents for enzymatic reactions. The low viscosity and high diffusivity of supercritical CO_2 also serve to provide favorable mass transfer properties (LONGO; SANROMÁN, 2006; CENI et al., 2010b; SANTOS et al., 2016). There are a few studies using supercritical fluids (CO_2, 10 MPa) for aromatic esters synthesis, such as benzyl acetate (TEWARI et al., 2004) and eugenyl acetate

(SANTOS et al., 2016). The experimental apparatus used for the synthesis of eugenyl acetate is a high-pressure stirred-bath reactor unit, using Novozym 435® as the biocatalyst and eugenol and acetic anhydride as substrates. After 1 hour of reaction under CO_2 pressurized the samples reached 33.2 % of conversion (SANTOS et al., 2016). However, there is a lack of investigation around the use of supercritical fluids for aromatic ester synthesis.

2.4.5 Effect of temperature

Temperature is a crucial key parameter for biocatalysis, which facilitates reactants solubility in reaction media, reduces mixture viscosity, boosts the molecular collision interface, reduces mass transfer limitations and assists the interactions between enzyme particles and substrates. Temperature increases the molecular collision interaction and causes a decrease in the energy barrier between reacting molecules and enzyme-substrate complex formation, which causes improvement in initial reaction rate and conversion. Enzymes are biocatalysts, stables at an optimal range of temperature, however, some modification on the catalytic activity and stability can occur when the temperature increases beyond the optimum temperature, leading to enzyme thermal deactivation (CENI et al., 2010a; BADGUJAR; SASAKI; BHANAGE, 2015; GUMEL; ANNUAR, 2016a; MANAN et al., 2016).

In aromatic ester production, many researchers showed that the optimum temperature of an enzyme might change depending on the system used. The utilization of Novozym 435® in the synthesis of 2-phenethyl caffeate and cinnamyl acetate with different temperatures (70

and 40 ºC, respectively) showed that the large range of temperature did not present great effect in the final conversion for both esters, which remained 93.1 and 96% for 2-phenethyl caffeate and cinnamyl acetate, respectively (CHEN et al., 2011; YADAV; DEVENDRAN, 2012).

2.5 ULTRASOUND-ASSISTED SYNTHESIS TECHNOLOGY

Ultrasound is an emergent energy-efficient technique and recently has been used for flavor and aromatic ester synthesis. The technique is able to increase the initial rate and enhance mass transfer of the reactions. Furthermore, ultrasound method is a green technology with high efficiency, economic performance and low instrumental requirement (ZHENG et al., 2013; KHAN; RATHOD, 2015; PALUDO et al., 2015; TOMKE; RATHOD, 2015).

Ultrasound is the sound energy at frequencies above the range that is audible to human beings (> 16 kHz). The cycles of compression and rarefaction of the sound waves can generate a phenomenon known as cavitation, which comprises the formation, enlargement and collapse of bubbles, increasing the rate of enzymatic reactions. When cavitation bubbles collapse near the phase boundary of two immiscible liquids, it can provide a very efficient stirring. The collapse of the bubbles produces localized supercritical conditions (high temperature and high pressure). However, ultrasound reactions at very high intensity can lead to the disruption of the enzyme structure and low intensity of ultrasound will not result in the desirable cavitation effect (CENI et al., 2011; CHEN et al., 2011; BANSODE; RATHOD, 2014). Researchers produced benzyl butyrate, anisyl butyrate and *o*-cresyl butyrate with ultrasound technique

and reached 99 % of yield, in 3 h at 52 ºC, for these aromatic esters. On the other hand, the reactions performed without the use of ultrasound at the same conditions presented low conversions of 57 %, 55 % and 43 % for benzyl butyrate, anisyl butyrate and *o*-cresyl butyrate, respectively (BADGUJAR; BHANAGE, 2015). These results show the efficiency of the utilization the ultrasound technique in aromatic esters production, being a promising technology to increase the initial rate of reaction and yields.

2.6 SYSTEM OPTIMIZATION

Optimization plays a significant role in the commercial success of the biotechnological industry based on quality, cost and the process performance. The conventional method of optimization requires screening of a large number of variables, many experiments, with plenty time and resources. Experimental design approach provides an easy and efficient evaluation of the main reaction variables, such as temperature, time reaction, enzyme amount and the molar ratio of substrates, which improves the biocatalyst activity and the conversion in the esters synthesis (CHAIBAKHSH et al., 2012; SHINDE; YADAV, 2014, 2015; NARWAL et al., 2016).

The response surface methodology (RSM) technique is an innovative powerful tool for the aromatic esters synthesis, able to determine the optimum reaction conditions necessary to scale up the process and to reduce the number and cost of experimental tests need to provide statistically acceptable results (RADZI; HANIF; SYAMSUL, 2016; ZHANG et al., 2016). The statistical method of RSM was employed

to optimized the synthesis of eugenyl benzoate, by 5-level-4-factor central composite design (CCD) with the parameters of incubation time (2 - 18 h), temperature (30 - 70 ºC), molar ratio of acid to alcohol (1:1 - 1:5) and enzyme loading (5 - 45 mg). The highest yield of eugenol benzoate was 56.1 %, at 60ºC, 6h of reaction, 15 mg of enzyme loading and molar ratio 1:4 (MANAN et al., 2016). The same method was used for the synthesis of benzyl cinnamate; the reaction variables were time (18 - 30 h), temperature (30 - 50 ºC), molar ratio of acid to alcohol (1:1 - 1:5) and enzyme loading (20 - 40 mg). Under optimized conditions (40 ºC, 31 mg of enzyme loading, 1:2.6 molar ratio and 27 h) the yield reaches 97.7% (ZHANG et al., 2016).

2.7 ENZYMATIC KINETICS

Kinetic modeling and mechanistic study of a reaction are important aspects for reactor designing and scale up. Several mechanisms are available to explain lipase-catalyzed reactions and usually follows Ping-Pong Bi-Bi or ternary complex (order Bi-Bi) mechanism (YADAV; DHOOT, 2009; KUO et al., 2014).

In the Ping-Pong Bi-Bi mechanism, the reaction occurs between two substrates and a product is released. For the aromatic ester synthesis, firstly the lipase (E) combines with the acyl donor (Ad) (i.e. acid, ester or anhydride) and forms an enzyme-acyl donor complex (E-Ad). E-Ad is transformed into an intermediate complex (E-Ac) by molecular isomerization. Then, the acyl acceptor (Aa) (i.e. alcohol) combines with E-Ac to form an enzyme-acyl-alcohol complex (E-Ac-Aa), which isomerizes into another enzyme-product ester complex (E-Q)

and releases the desired product (Q) and frees the enzyme. Equation 1 defines the reaction rate (V) of the Ping-Pong Bi-Bi mechanism (GENG et al., 2012; JAKOVETIĆ et al., 2013a).

$$V = \frac{V_{máx}.[Ad]_0.[Aa]_0}{K_{Aa}.[Ad]_0 + K_{Ad}.[Aa]_0 + [Ad]_0.[Aa]_0} \quad (1)$$

Sometimes the acyl acceptor (Aa) acted as a competitive inhibitor of lipase, forming a dead-end complex (E-Aa), which impeded the lipase to combine with the acyl donor (Ad). An additional parameter is necessary to describe the reaction rate with inhibition and Equation 2 describes adequately the kinetic expression (GENG et al., 2012; JAKOVETIĆ et al., 2013a).

$$V = \frac{V_{máx}.[Ad]_0.[Aa]_0}{K_{Aa}.[Ad]_0 + K_{Ad}.[Aa]_0(1+\frac{[Aa]_0}{K_{I,Aa}}) + [Ad]_0.[Aa]_0} \quad (2)$$

The Ping-Pong Bi-Bi mechanism describes the enzymatic synthesis of some aromatic esters, such as cinnamyl acetate with ethyl acetate (Ad) and cinnamyl alcohol (Ac). The increase of cinnamyl alcohol concentration decelerated the initial rate of the reaction and shows that cinnamyl alcohol acted as a competitive inhibitor of the lipase due to the formation of a dead-end complex, which stops the lipase to combine with ethyl acetate (GENG et al., 2012).

The Lineweaver-Burk graph can determine the inhibition from substrates, by plotting initial rates at different concentration of substrates (BADGUJAR; PAI; BHANAGE, 2016). In the study of the benzyl propionate synthesis using vinyl propionate as acyl donor, Lineweaver-Burk plot showed a decrease in the initial rate with a high concentration of benzyl alcohol, which reveals that the alcohol acts as an inhibitor of the enzyme for the benzyl propionate synthesis (BADGUJAR; BHANAGE, 2014a).

In the ternary complex mechanism (order Bi-Bi), the lipase (E) binds with the acyl donor (Ad) to form enzyme-acyl donor complex (E-Ad). Then, the acyl acceptor (Ac) combines with E-Ad to form a ternary complex (E-Ad-Aa). This ternary complex isomerizes to another ternary complex (E-Q-W), which releases the desired product (Q) and water (W) and frees the enzyme (YADAV; DHOOT, 2009; BADGUJAR; PAI; BHANAGE, 2016). Some studies describes the ternary complex mechanism for the enzymatic synthesis of aromatic esters, such as cinnamyl laurate using cinnamyl alcohol (Ac) and lauric acid (Ad) (YADAV; DHOOT, 2009). Other research showed the kinetic parameters calculated for the benzyl propionate production, suggesting that the ternary complex mechanism fits the data (BADGUJAR; BHANAGE, 2014a). The same mechanism explains the cinnamyl propionate synthesis with cinnamyl alcohol and vinyl propionate; however, with a high concentration of cinnamyl alcohol, there is the formation of a dead-end binary inhibition complex between the enzyme and the cinnamyl alcohol, instead of the enzyme and vinyl propionate, reducing the reaction rate and conversion (BADGUJAR; PAI; BHANAGE, 2016).

2.8 BIOLOGICAL ACTIVITIES OF FLAVOR ESTERS

Emerging studies demonstrate that aromatic esters may present biological activities, such as antioxidant, antimicrobial and larvicidal properties (BERGER, 2009). Studies have shown that benzyl and methyl benzoate, benzyl and cinnamyl acetate have larvicidal properties against larvae of *Aedes aegypti* mosquitoes (PAVELA, 2015). Eugenyl acetate, an ester derived from eugenol, the main compound of clove essential oil,

also had antimicrobial and larvicidal properties (CHIARADIA et al., 2012; SILVA et al., 2015).

The phenolic compounds (e.g. flavonoids) present advantageous biological and physiological properties, such as antimicrobial, pest repellents, anti-allergenic, anti-inflammatory, anticarcinogenic and antimutagenic properties. The modification of these compounds, via esterification to produce useful derivatives with a great commercial importance, has been the subject of increased interest aiming to improve the solubility and stability of these compounds (LUE et al., 2005; VOSMANN et al., 2008; YADAV; DHOOT, 2009; CHEN et al., 2011; ZHENG et al., 2013).

Benzyl benzoate is one of the oldest drugs used for the treatment of scabies, a highly contagious skin infection. It is common worldwide, but is more conspicuous in the areas with poor sanitation and overcrowding. The mite *Sarcoptes scabiei* burrows into the skin and consumes the epidermis, resulting in inflammation, allergy reactions and pruritic lesions. There are adversities associated with the use of benzyl benzoate, like severe burning sensation, itching and scaling of the skin, after repeated use. Therefore, the topical use of the benzyl benzoate is not recommended and a suitable carrier system can be a good alternative to protect the skin against the adverse effects. Thereby, the encapsulation technique can revitalize the usage of this compound (SHARMA et al., 2015).

2.9 FUTURE TRENDS

The use of flavor and aromatic esters produced from green and safety technology will increase, mainly for cosmetic and food industries, due to the market and consumers preferences for natural products. Academic studies are in constant development around the world to find novel biotechnological routes for flavor synthesis. There is an immense progress in genomics and metabolic engineering for modification and transformation of new biocatalysts to use in the ester production. These new applications with innovative strategies will certainly play an important role in enhancing the quality of the new products and protection of the environment for generations to come.

This review paper presents the current use of biocatalysis for the aromatic esters production by esterification and transesterification studied in the academic area. There are many prospects for expansion the biotechnological route to the industry in the future. However, more investigation would be helpful to understand better the potential of ultrasound, the optimization process, reaction kinetics and scale up for well assessment of economic and industrial viability in aromatic ester synthesis.

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CHAPTER III

3 PRIMARY STUDIES

3.1 INTRODUCTION

The purpose of this work was to study of the effect of enzyme type on the synthesis of benzyl propionate and benzyl butyrate by esterification, taking into account the industrial importance of these products. This research evaluated the efficiency of a new commercial immobilized preparation with low-cost support from *Candida antarctica* fraction B (NS 88011) and three commercial immobilized lipases (Novozym 435, Lipozyme TL-IM and Lipozyme RM-IM). This study also investigated the effect of various reaction parameters on ester conversion, like organic solvent presence and system assisted by ultrasound.

3.2 MATERIAL AND METHODS

3.2.1 Enzymes and chemicals

The substrates utilized in the experimental tests were propionic acid (Neon), butyric acid (Neon) and benzyl alcohol (Neon). Molecular sieves (4 Å, beads 8-12 mesh, Sigma-Aldrich) was used as the water adsorbent and the solvent employed for gas chromatography quantification was dichloromethane (Quimis). Novozymes® kindly

donated the immobilized biocatalysts: Novozym 435 (*Candida antarctica* fraction B lipase), Lipozyme TL-IM (*Thermomyces lanuginosus* lipase), Lipozyme RM-IM (*Rhizomucor miehei* lipase) and NS 88011 (*Candida antarctica* fraction B lipase).

3.2.2 Lipase esterification activity

The enzyme esterification activity (U/g) was determined following an adapted methodology (CENI et al., 2010a), in the reaction between lauric acid and n-propanol at a molar ratio of 1:1, a temperature of 60 ºC and enzyme amount of 5 % in relation to the substrates. At time 0 and 40 min, samples of the reaction medium were collected and titrated with NaOH 0.01 N to pH 11. One unit of activity (U) is the amount of enzyme necessary to consume 1 µmol of lauric acid per minute at the established experimental conditions. All enzymatic activity determinations were carried out in triplicate.

3.2.3 Support material characterization

Novozym 435 and NS 88011 are immobilized fraction B lipases from *Candida antarctica,* however, the support material of each enzyme is different. Novozym 435 is commercially obtained by immobilization via interfacial activation of the enzyme on a moderately hydrophobic resin, Lewatit VP OC 1600 (SÁ et al., 2017). In contrast, the NS 88011 is a new immobilized preparation with a low-cost material, with no more information from the fabricant. The characterization of the immobilized material supports of Novozym 435 and NS 88011 was

determined by the Autorsorb-1C equipment (Quantachrome Instruments). Samples were previously dried (100 – 300 ºC) under vacuum ($9.2x10^{-3}$ - $1.3x10^{-2}$ atm). The gas nitrogen adsorption was conducted at -196 ºC with relative pressure in a range of 0 to 1. The isotherm data were used to determine the total pore volume and average pore diameter of the samples. The Brunauer-Emmett-Teller (BET) model evaluated the specific surface area of the enzyme supports.

3.2.4 Effect of the biocatalysts and reaction media

Experiments screened four different immobilized lipases to evaluate their efficacy on the benzyl propionate and benzyl butyrate synthesis, under a similar set of reaction conditions to find out the best suitable biocatalyst. The esterification involves the mixture of 2.5 mmol of acid and 12 mmol of benzyl alcohol (molar ratio 1:5), with the addition or not of 1 mL of solvent *tert*-butanol. The esterification resulted in the formation of water as a reaction by-product (Figures 1 and 2) and molecular sieves were used for its removal, enhancing the synthesis of the ester by pushing the reaction equilibrium in the forward direction (reversible) (SÁ et al., 2017). The molecular sieves were previously dried at 200 ºC in muffle for 2 h and then stored in a desiccator until its use.

Figure 1 - Esterification of benzyl alcohol and propionic acid.

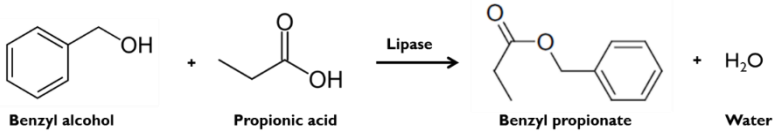
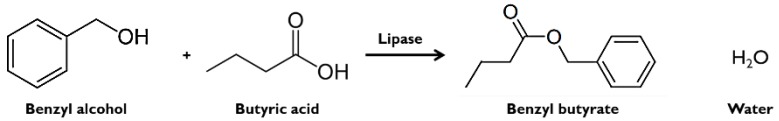


Figure 2 - Esterification of benzyl alcohol and butyric acid.



In sequence, 10 wt% (total weight of substrates %) were added to the medium. Finally, the reaction flasks were submitted to 50 °C during 24 hours with mechanical agitation and after this period, the centrifuged samples were stored for further quantification. All tests were realized in duplicate.

3.2.5 Effect of ultrasound

The esterification reactions were carried out in an ultrasonic bath (Unique Inc.). The ultrasonic unit has an operating frequency of 37 kHz and a maximum rated power output of 132 W. The advantage of using this system is the offer of larger effective cavitational area as compared to conventional thermal bath. The mixture of substrates (molar ratio 1:5) and enzyme (10 wt%) were placed in the ultrasonic bath at the desired temperature (50 ºC) for 6 h. All tests were realized in duplicate and the centrifuged samples were stored for further quantification.

3.2.6 Benzyl esters quantification

The benzyl propionate conversion was analyzed by gas chromatography (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). The temperature program tested and chosen was 100 ºC (2 min), 100-230 ºC (10ºC/min) and 230 ºC (10 min). The injector and detector temperatures were kept at 250 °C. The injection volume was 10 µL of the reaction sample diluted in dichloromethane and hydrogen (H_2, 56 kPa) was the carrier gas. The quantification of the benzyl propionate conversion was calculated according to the Equation 3:

$$Conversion\ (\%) = [1 - (BAA_f / BAA_i)] \times 100 \qquad (3)$$

where, BAA_f is the final Benzyl Alcohol Area, after the esterification, and BAA_i is the initial Benzyl Alcohol Area, before the esterification reaction.

3.3 RESULTS AND DISCUSSION

3.3.1 Lipase and reaction media effects

Four immobilized enzymes were used to perform the esterification of propionic acid and butyric acid with benzyl alcohol, with the addition or not of 1 mL of solvent *tert*-butanol. Novozym 435, NS 88011, Lipozyme RM-IM and Lipozyme TL-IM presented the enzymatic activity of 54.6 ± 0.7 U/g, 54.2 ± 0.5 U/g, 58.8 ± 0.9 U/g, and 19.3 ± 0.4 U/g, respectively. The benzyl propionate conversions after 24 h of reaction are listed in Table 7.

Table 7 - Benzyl propionate conversion of the esterification reaction in the presence or not of *tert*-butanol.

Lipase	Benzyl Propionate Conversion (%)	
	With solvent *tert*-butanol	**Solvent-free**
Novozym 435	25.6 ± 3.9	31.4 ± 4.5
Lipozyme RM-IM	6.8 ± 0.1	5.6 ± 1.6
Lipozyme TL-IM	0.79 ± 0.01	1.8 ± 0.1
NS 88011	14.3 ± 0.4	8.1 ± 1.0

The only research around the synthesis of benzyl propionate by esterification, using propionic acid as acyl donor, reached a very low conversion (8 %) at 50 ºC in 2.5 h, molar ratio 2:5 (alcohol:acid) with solvent isooctane and immobilized *Pseudomonas cepacia* lipase as the catalyst (BADGUJAR; BHANAGE, 2014a).

Novozym 435 showed good results of ester conversion (around 30 %) for both systems (solvent presence or not) at the conditions studied. The results suggest that Novozym 435 was the most efficient enzyme on the esterification of propionic acid and benzyl alcohol, with no need of solvent to improve the ester conversion, eliminating the need for recuperation and purification processes and reducing the environment hazards.

On the other hand, Lipozyme TL-IM and Lipozyme RM-IM led to very low conversions. This great difference in conversion, compared to Novozym 435, can be attributed to different factors that may affect the efficiency of these biocatalysts, such as the enzyme source and the immobilization method and support, modifying the enzyme properties

and activities even in similar reaction conditions (SÁ et al., 2017). Lipozyme RM-IM and Lipozyme TL-IM are immobilized *Rhizomucor miehei* lipase and *Thermomyces lanuginosus* lipase, respectively, the enzyme source in this specific case was probably responsible for the low activity of these lipases on the specific esterification of propionic acid and benzyl alcohol.

Novozym 435 and NS 88011 are lipases from the same source, *Candida antarctica* fraction B, however, they also presented very different results in the ester conversion. This great difference in the catalysis of the same system might be explained due to the different immobilization methods employed for obtaining each immobilized lipase and the very different support material. Novozym 435 is commercially obtained by immobilization via interfacial activation of the enzyme on a moderately hydrophobic resin, Lewatit VP OC 1600. In contrast, NS 88011 is a new immobilized preparation with a low-cost material, with no more information by the fabricant.

The support characterization of Novozym 435 and NS 88011 was performed to better explain the possible influence of the immobilization material on the reaction conversion. Analyses of specific surface area (BET), total pore volume and average of pore volume were realized (Table 8).

The immobilization supports had very different characteristics and the Novozym 435 material showed a larger surface area, total pore volume and average pore volume than NS 88011, showing that the support material might be the responsible aspect for the reduced reaction conversion. The low total pore volume and average pore diameter of the NS 88011 support reduce the mass transfer of the substrates, making

difficult the diffusion of the substrates through the pores and the access to the lipase active sites.

Table 8 - Immobilization support characteristics for Novozym 435 and NS 88011.

Enzyme	Surface Area	Total Pore Volume	Average Pore Diameter
Novozym 435	78.69 m²/g	0.2837 cc/g	144.2 Å
NS 88011	15.16 m²/g	0.02613 cc/g	68.96 Å

The benzyl butyrate conversions are listed in Table 9. Results showed that the immobilized lipase from *Candida antarctica* fraction B had the best performance in the esterification of benzyl alcohol and butyric acid at the conditions studied, reaching similar results for both Novozym 435 and NS 88011 (~ 40 % of conversion).

Table 9 - Benzyl butyrate conversion of the esterification reaction in the presence or not of *tert*-butanol.

Lipase	Benzyl Butyrate Conversion (%)	
	With solvent *tert*-butanol	Solvent-free
Novozym 435	23.2 ± 3.3	30.2 ± 3.3
Lipozyme RM-IM	7.5 ± 0.1	8.1 ± 1.6
Lipozyme TL-IM	0.5 ± 0.1	1.1 ± 0.1
NS 88011	22.6 ± 1.6	40.9 ± 2.5

On the other hand, the lipases from *Thermomyces lanuginosus* (Lipozyme TL-IM) and *Rhizomucor miehei* (Lipozyme RM-IM) had a very low conversion (less than 9 %) showing do not be adequate for the benzyl butyrate synthesis.

Based on the results, the following investigations were conducted only with Novozym 435 and NS 88011, for benzyl butyrate, and Novozym 435 for benzyl propionate synthesis.

3.3.2 Effect of ultrasound

The results of the benzyl propionate and benzyl butyrate synthesis with ultrasound technique are presented in Table 10. These conversions showed that the method using the ultrasonic bath was not effective to increase the conversion of the esterification reaction to produce the benzyl esters.

Table 10 - Benzyl propionate and benzyl butyrate conversion of the esterification reaction in the ultrasound bath.

Lipase	**Ultrasound Conversion (%)**	
	Benzyl Propionate	**Benzyl Butyrate**
Novozym 435	41.5 ± 5.1	28.1 ± 3.3
Lipozyme RM-IM	4.0 ± 0.1	3.0 ± 1.6
Lipozyme TL-IM	1.0 ± 0.1	0.7 ± 0.1
NS 88011	31.5 ± 1.7	42.2 ± 4.1

Based on the Table 10 results, where ultrasonic and conventional thermal stirring techniques had similar results, the thermal bath was used for further experiments.

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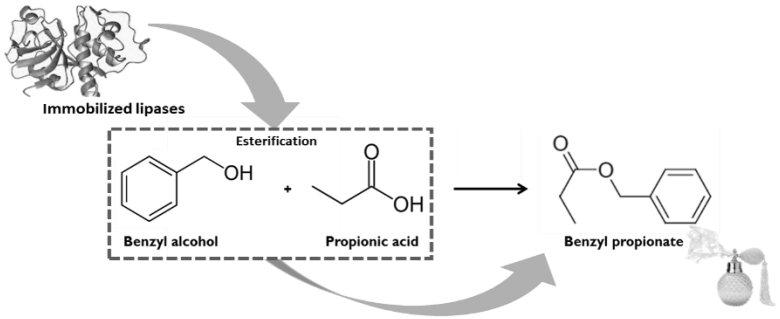
CHAPTER IV

This chapter is a part of publication entitled "**Biocatalysis of aromatic ester benzyl propionate by different immobilized lipases**" published in 2018 in the periodic **Bioprocess and Biosystems Engineering**.

GRAPHICAL ABSTRACT



4 BIOCATALYSIS OF AROMATIC ESTER BENZYL PROPIONATE BY DIFFERENT IMMOBILIZED LIPASES

4.1 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 that includes fruity

and floral smell and taste (BERGER, 2009; YADAV; DHOOT, 2009; DHAKE et al., 2012; GAO et al., 2016; SÁ et al., 2017).

Biocatalysis is a useful green tool for the synthesis of these aromatic esters and collects many advantages like high selectivity, mild reaction conditions and environmental compatibility. Furthermore, esters produced via biocatalysis can be labeled as "natural", which is an alternative to natural sources with the possibility of scale up to industrial applications (BIAŁECKA-FLORJAŃCZYK et al., 2012; SILVA et al., 2014; VANIN et al., 2014; FERRAZ et al., 2015; KHAN; RATHOD, 2015; TODERO et al., 2015; WANG et al., 2015; SÁ et al., 2017). Studies have been developed in this area and the use of many immobilized lipases seems to be promising for synthesis of many aromatic esters like eugenyl acetate (CHIARADIA et al., 2012; VANIN et al., 2014; SILVA et al., 2015; SANTOS et al., 2016), benzyl acetate (SINGH et al., 2008; BADGUJAR; SASAKI; BHANAGE, 2015), anisyl acetate (DHAKE et al., 2012; BADGUJAR; SASAKI; BHANAGE, 2015), cinnamyl propionate (BADGUJAR; PAI; BHANAGE, 2016), *p*-cresyl propionate (BADGUJAR; PAI; BHANAGE, 2016) among others.

Inside of the benzyl ester group, the benzyl propionate is a clear colorless to pale-yellow liquid that possesses a fruity odor of considerable tenacity. It is recognized as a safe compound by European Legislations and Food and Drug Administration (FEMA, 1965; ADAMS et al., 2005a; FDA, 2013). Usually, benzyl propionate is found naturally in the composition of some foods, like in plums and melons and can be used in fragrances for decorative cosmetics, antiperspirants, shampoos, body lotions, toilet soaps, household cleaners and detergents (MCGINTY;

LETIZIA; API, 2012b; API et al., 2015). Nonetheless, there is a lack of studies of enzymatic synthesis of this ester in literature.

Currently, only one research had studied the synthesis of benzyl propionate by lipase-mediated catalysis, using *Pseudomonas cepacia* immobilized in an eco-friendly support material as the biocatalyst. The authors found satisfactory results for transesterification of vinyl propionate with benzyl alcohol, however, the esterification between propionic acid and benzyl alcohol reached a low conversion of 8% (BADGUJAR; BHANAGE, 2014a).

Based on the mentioned aspects, taking into account the industrial importance of the product, the purpose of the work was the maximization of the synthesis of benzyl propionate by esterification between benzyl alcohol and propionic acid. This study investigated the effect of various reaction parameters on ester conversion, like enzyme amount, acid:alcohol molar ratio, reaction time and temperature.

4.2 MATERIAL AND METHODS

4.2.1 Enzymes and chemicals

The substrates utilized in the experimental tests were propionic acid (Neon) and benzyl alcohol (Neon). Molecular sieves (4 Å, beads 8-12 mesh, Sigma-Aldrich) was used as the water adsorbent and the solvent employed for gas chromatography quantification was dichloromethane (Quimis). Novozymes® kindly donated the immobilized biocatalyst: Novozym 435 (*Candida antarctica* fraction B lipase).

4.2.2 Effect of alcohol molar ratio

Tests investigated the influence of different concentrations of alcohol in ester conversion, with different acid:alcohol molar ratios (1:1, 1:2, 1:5 and 1:6), using the most effective biocatalyst. After the mixture of the substrates, molecular sieves and 10 wt% of enzyme were added to the reaction medium. The reaction occurred at 65 ºC in a thermal bath for 24 h with mechanical agitation. Then, the centrifuged samples were stored for later analysis. The experiments were realized in duplicate.

4.2.3 Experimental design and statistical analysis

Response Surface Methodology (RSM) was employed to maximize the benzyl propionate synthesis, based on a three-level and two variable factorial design, including two central points (12 trials). The process variables studied were enzyme concentration (E) and temperature (T) and the response variable was the amount of ester converted in percentage (Conversion %). The software Statistica® 7.0 (Statsoft Inc.) was used to assist the design and the statistical analysis of experimental information, adopting in all cases studied a confidence level of 95% ($p < 0.05$). The model proposed for the experimental data was validated by the analysis of variance (ANOVA).

The benzyl propionate synthesis for the experimental design data occurred by the reaction between propionic acid and benzyl alcohol (molar ratio 1:1), using the best biocatalyst verified in previous experiments and molecular sieves. The reaction flasks were placed in a thermal bath at the adequate temperature and mechanical agitation. After

24 hours of reaction, samples were centrifuged for removal of residual enzyme and stored for further quantification.

4.2.4 Benzyl propionate quantification

The benzyl propionate conversion was analyzed by gas chromatography (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). The temperature program tested and chosen was 100 ºC (2 min), 100-230 ºC (10 ºC/min) and 230 ºC (10 min). The injector and detector temperatures were kept at 250 °C. The injection volume was 10 µL of the reaction sample diluted in dichloromethane and hydrogen (H_2, 56 kPa) was the carrier gas. The quantification of the benzyl propionate conversion was calculated according to the Equation 4:

$$Conversion\ (\%) = [1 - (BAA_f / BAA_i)] \times 100 \qquad (4)$$

where, BAA_f is the final Benzyl Alcohol Area, after the esterification, and BAA_i is the initial Benzyl Alcohol Area, before the esterification reaction.

4.2.5 Esterification kinetics

After the analysis of the results obtained in the experimental design, kinetic experiments were performed at the best reaction condition, in terms of conversion, for the benzyl propionate production with Novozym 435. Destructive samples were prepared in 2 mL flask, kept at

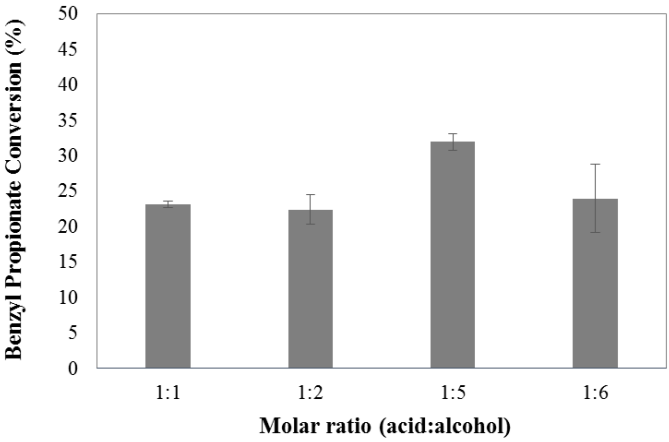
thermal bath with mechanical agitation, taken at 0, 2, 4, 6, 8, 12, 24 and 36 h, centrifuged and stored for later quantification.

4.3 RESULTS AND DISCUSSION

4.3.1 Effect of molar ratio

The study of the molar ratio of the substrates is an important factor to determine the influence of the substrates on enzyme activity. This work studied the effect of substrate molar ratio on lipase catalytic efficiency in the esterification by fixing acid concentration and varying the molar quantity of benzyl alcohol (Figure 3).

Figure 3 - Benzyl propionate conversion at different acid:alcohol molar ratios using 10 wt% of Novozym 435, at 65 ºC.



A nonhomogeneous behavior was observed for the esterification results when the benzyl alcohol concentration varied,

showing a slight increase from 1:2 to 1:5 and a small decrease from 1:5 to 1:6, suggesting that any of these molar rations could be employed in the benzyl propionate synthesis ensuring good results of conversion. Based on this, to keep the minimum waste of reagents and avoid unnecessary amounts of alcohol, the acid:alcohol molar ratio of 1:1 was selected as the best condition and fixed for all the further experiments.

4.3.2 Experimental factorial design on maximization of benzyl propionate conversion

The 2^2 experimental factorial design analyzed the effect of each independent variable (E and T) through the conversion of benzyl propionate ester, as the main objective of improving the ester conversion by determining the statistical model and response functions. Table 11 present the results obtained for the benzyl alcohol and propionic acid esterification using Novozym 435.

The conversion varied from 13.76 to 44.82 % and the conditions of the highest conversion were 15 wt% of enzyme and temperature 50 ºC indicating a better performance at the high and low levels of E and T, respectively. The statistical analysis of the data presented in Table 11 was validated by the polynomial model of first order related to the T and E presented by Equation 5:

$$Conversion\ (\%) = 28.64 - 3.39T + 10.76E - 0.49TE \quad (5)$$

The predicted values associated with the fitted model satisfactorily correlated to the observed values with low residues (Table

11). The linear model permitted to build the response surface presented in Figure 4, showing the influence of E and T on benzyl propionate production.

Table 11 - Design matrix of 2^2 complete factorial and response in terms of conversion of benzyl propionate, using Novozym 435 lipase as the biocatalyst.

Run	Variable levels		Novozym 435		
	T (ºC)	E (wt%)	Conversion (%)	Predict (%)	Residues
1	-1 (50)	-1 (5)	22.27	20.78	1.49
2	1 (70)	-1 (5)	16.51	14.98	1.53
3	-1 (50)	1 (15)	44.82	43.28	1.54
4	1 (70)	1 (15)	37.68	35.52	2.16
5	0 (60)	0 (10)	27.08	28.64	-1.56
6	0 (60)	0 (10)	32.10	28.64	3.46
7	-1 (50)	-1 (5)	19.60	20.78	-1.18
8	1 (70)	-1 (5)	13.76	14.98	-1.22
9	-1 (50)	1 (15)	42.04	43.28	-1.24
10	1 (70)	1 (15)	33.67	35.52	-1.85
11	0 (60)	0 (10)	29.59	28.64	0.95
12	0 (60)	0 (10)	24.50	28.64	-4.14

The conversion of the benzyl propionate was highly dependent on the E variable, reaching the highest conversion at the high E level, and low dependent of the T that can be maintained at the low level for industrial economic viability. The effectiveness of the fitted linear model determined by the analysis of variance (ANOVA) is shown in Table 12.

Figure 4 - Response surface for benzyl propionate synthesis related to the temperature (T, ºC) and enzyme amount (E, wt%).

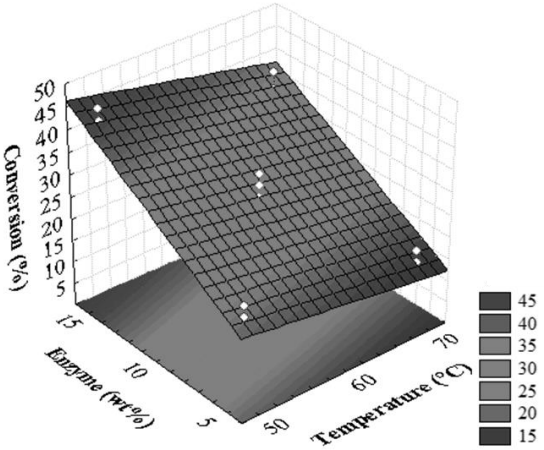


Table 12 - Variance analysis (ANOVA) for the fitted model in the system using Novozym 435.

Factor	SS	DF	MS	p	F-ratio	F-value[a]	R
(1) T (L)	91.87	1	91.87	0.0055			
(2) E (L)	926.01	1	926.01	0.0000			
1L by 2L	1.91	1	1.91	0.6020			
Lack of Fit	0.60	1	0.60	-			
Pure Error	51.28	7	7.33	-			
Regression	1019.79	3	339.93	-	52.41	4.07	0.98
Residual	51.89	8	6.49	-			
Total SS	1071.67	11	-	-			

SS: sum of square; DF: degree of freedom; MS: mean square; p: probability; R: correlation coefficient;

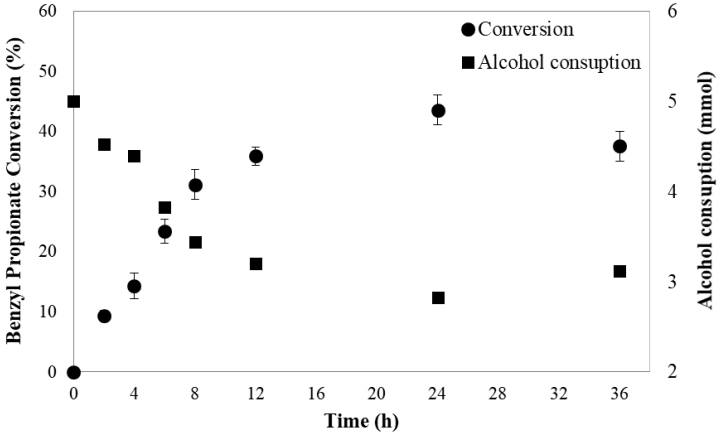
[a] F-value $_{(0.95;\ 3,8)}$.

The results showed that the model for benzyl propionate conversion (Equation 5) was predictive inside the range of the conditions studies since the calculated F-ratio for regression (52.41) was almost thirteen times higher than the F-value at a 95 % of confidence ($F_{0.95;\ 3,8} = 4.07$). The mathematical model is statistically significant and adequate to represent the relationship between the response (conversion %) and the variables. A suitable coefficient of determination (R^2) of 0.952 illustrated that the model can explain approximately 95 % of results variability and the values of $p < 0.05$ implies that the model terms are significant.

4.3.3 Benzyl propionate kinetics

The influence of the reaction time on the ester conversion and consumption of the benzyl alcohol was verified on the kinetics of the benzyl propionate production (Figure 5).

Figure 5 - Influence of the time of reaction on benzyl alcohol consumption and benzyl propionate conversion.



The system of esterification reached the maximum conversion ~44 % in 24 h of reaction, which validates the linear model presented in Equation 5. The consumption of alcohol was accentuated in the first hours, with the estimated initial velocity of consumption of 0.07 mmol/h.

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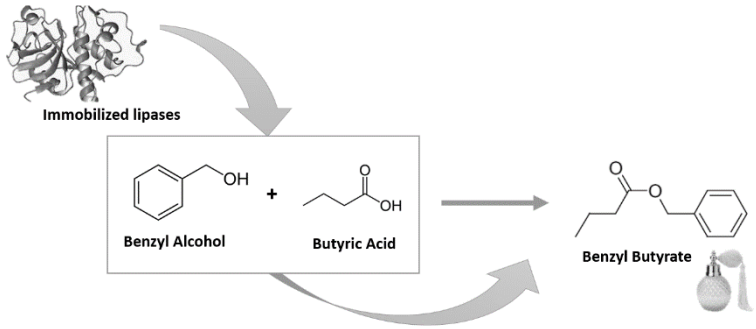
CHAPTER V

This chapter is a part of publication entitled "**Benzyl butyrate synthesis from lipase-catalyzed esterification of butyric acid in solvent-free system**" submitted to the periodic **Process Biochemistry**.

GRAPHICAL ABSTRACT



5 BENZYL BUTYRATE SYNTHESIS FROM LIPASE-CATALYZED ESTERIFICATION OF BUTYRIC ACID IN SOLVENT-FREE SYSTEM

5.1 INTRODUCTION

In the past few years, there was an increase of interest for alternative green tools capable of synthesizing aromatic esters, which are composed of aromatic and aliphatic acids and alcohols (SHINTRE; GHADGE; SAWANT, 2002). Flavor and aromatic esters are highly

known due to the pleasant fruity smell and taste and they are widely used in various food, cosmetics and pharmaceuticals industries (BADGUJAR; BHANAGE, 2014a; LI et al., 2014; SÁ et al., 2017).

Biocatalysis is a great alternative to the biotechnological production of natural flavors, with 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).

Lipases constitute the most important group of biocatalysts for biotechnological applications. Many aromatic esters have been successfully lipase-synthesized, such as benzyl (SHINTRE; GHADGE; SAWANT, 2002; JEROMIN; ZOOR, 2008; SINGH et al., 2008; VOSMANN et al., 2008; BADGUJAR; BHANAGE, 2014a, 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), anisyl (DHAKE et al., 2011, 2012; BADGUJAR; BHANAGE, 2015; BADGUJAR; SASAKI; BHANAGE, 2015; BADGUJAR; PAI; BHANAGE, 2016) 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, generally, is necessary to improve and facilitate the ester conversion.

Benzyl butyrate is a benzyl ester with a fruity odor (suggesting plum) of considerable tenacity (MCGINTY; LETIZIA; API, 2012c; API et al., 2016b) very interesting to the food and cosmetic industries. However, there is only one report of benzyl butyrate synthesis via

esterification mediated by lipases (JEROMIN; ZOOR, 2008) and the solvent methyl tertbutyl ether was used in order to avoid the formation of water that may cause hydrolysis (backward reaction) of the product.

The main objective of the present work was to synthesize benzyl butyrate in absence of organic solvents to ease downstream processing via esterification mediated by immobilized lipases. Thereby, an investigation was made to improve the benzyl butyrate conversion evaluating the biocatalyst type and amount, molar ratio of butyric acid:benzyl alcohol, temperature, time of reaction and enzyme recycle. In addition, an evaluation of the antimicrobial activity of this ester was made, since there are no reports in the literature until the current moment, therefore a study testing relevant Gram-positive and Gram-negative bacteria was investigated.

5.2 MATERIAL AND METHODS

5.2.1 Enzymes and chemicals

Butyric acid (Neon) and benzyl alcohol (Neon) were used as subtracts. Molecular sieves beads (4Å, 8-12 mesh, Sigma-Aldrich) were utilized to remove possible residues of water from esterification reaction. Enzymatic catalysts were Novozym 435 and NS 88011 (immobilized *Candida antarctica* fraction B lipase), kindly donated by Novozymes®. Dichloromethane (Quemis) was used in gas chromatography injections. Acetone (Dinâmica) was utilized to wash the enzyme for reuse tests. Sodium hydroxide (NaOH 1 M, Dinâmica), ethyl acetate (Quemis) and sodium sulfate (Vetec) were employed for ester purification.

5.2.2 Effect of alcohol molar ratio in benzyl butyrate synthesis

The variation of acid:alcohol molar ratio from 1:1 to 1:6 was studied to investigate the influence of the different alcohol molar concentrations in ester conversion using two lipases Novozym 435 and NS 88011. The reaction of esterification involved the addition of butyric acid and benzyl alcohol at adequate molar concentrations into a 2 mL reactor containing molecular sieves beads. In sequence, 10 wt % (weight total of substrates %) of the enzyme was added to the reaction medium. Reactors were placed in a thermal bath with mechanical agitation at 65 ºC for 24 hours. At the end of reaction time, an aliquot of the product was centrifuged and adequate diluted for quantification. All experiments were carried out in duplicate.

5.2.3 Benzyl butyrate quantification

The quantitative analysis of benzyl butyrate synthesized via lipase-catalyzed reaction was carried out 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). A volume of 10 µL of the reaction sample diluted in dichloromethane was injected to the column with an initial temperature at 100 °C for 2 min, raised from 100 to 230 °C with a rate of 10 °C/min and the final temperature maintained for 10 min. Injector and detector temperature were kept at 250 °C. Hydrogen gas (H_2, 56 kPa) was used as carrier gas. The determination of the benzyl butyrate conversion was defined according to the Equation 6:

$$Conversion\ (\%) = [1 - (BAA_f / BAA_i)] \times 100 \qquad (6)$$

where, BAA_f is the final Benzyl Alcohol Area, after the esterification reaction, and BAA_i is the initial Benzyl Alcohol Area, before the esterification reaction.

5.2.4 Experimental design

The factorial design was utilized to identify optimum parameters levels for the synthesis of benzyl butyrate using two immobilized enzymes from *Candida antarctica* with different support materials (NS 88011 or Novozym 435). A 2^2 factorial experimental design with three level and two central points was used; the independent variables involved were the amount of enzyme (E) and temperature (T), and the responses were related to the ester 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 13.

All experiments were carried out in duplicate, totalizing 12 experiments. Statistica 7.0 (Statsoft Inc.) was utilized to analyze which effect has more influence on the response variable. Afterwards, data were fitted to a linear model and variance analysis (ANOVA) was considered to verify if the fitted model proposed is able to reproduce the experimental data.

The benzyl butyrate synthesis involved butyric acid and benzyl alcohol as substrates, at a molar ratio fixed in 1:1, followed by the addition of different amounts of the biocatalyst (Novozym 435 or NS 88011), at

different T according to Table 13. The mixture was placed in a 2 mL reactor and mechanically stirred in a thermal bath for 24 h. At the end of reaction time, an aliquot of the product was centrifuged for removal of residual enzyme and adequate diluted for quantification.

Table 13 - Experimental factorial design and responses for Novozym 435 and NS 88011 lipases.

Run	Coded and real variables		Novozym 435			NS 88011		
	T (°C)	E (wt%)	Conv. (%)[a]	Predicts	Residues	Conv. (%)[a]	Predicts	Residues
1	-1 (50)	-1 (2)	27.79	27.65	0.14	4.60	2.31	2.29
2	1 (70)	-1 (2)	20.73	19.67	1.06	4.47	1.77	2.70
3	-1 (50)	1 (10)	76.91	76.31	0.60	45.59	41.14	4.45
4	1 (70)	1 (10)	71.21	70.65	0.56	15.94	13.25	2.69
5	0 (60)	0 (6)	50.34	48.57	1.77	9.71	14.61	-4.90
6	0 (60)	0 (6)	49.24	48.57	0.67	8.90	14.61	-5.71
7	-1 (50)	-1 (2)	26.95	27.65	-0.70	5.60	2.31	3.29
8	1 (70)	-1 (2)	18.06	19.67	-1.61	4.65	1.77	2.88
9	-1 (50)	1 (10)	75.14	76.31	-1.17	42.28	41.14	1.14
10	1 (70)	1 (10)	69.51	70.65	-1.14	16.14	13.25	2.89
11	0 (60)	0 (6)	49.79	48.57	1.22	8.43	14.61	-6.18
12	0 (60)	0 (6)	47.18	48.57	-1.39	9.07	14.61	-5.54

[a] Conv. (%) = Conversion after 24h of reaction.

5.2.5 Esterification kinetics

The esterification kinetics was conducted with the best condition (related to the percentage of ester conversion) determined by the experimental design. Tests were made to evaluate a reduction in production costs and two other conditions were considered, one at 40 ºC

and 5 % of biocatalyst and the other at 40 ºC and 10 %. The most efficient enzyme (determined from experimental design) was used as the biocatalyst. Destructive samples were prepared in 2 mL reactor with substrates at molar ratio of 1:1, submitted to the mechanical agitation at adequate temperature and in each period of 0, 1, 2, 4, 6, 8, 12, 24, 36 and 48 h a sample was removed, an aliquot was centrifuged and adequate diluted for quantification. All experiments were carried out in duplicate.

5.2.6 Enzyme reuse

The reusability study was conducted just with the best reaction parameters determined by the experimental design. After each reaction cycle, the recuperation of enzyme and molecular sieves beads were made by vacuum filtration using glass funnel and filter paper. The molecular sieves beads were collected, and the enzyme was washed with acetone (m/v ratio of 1:10) for three times to remove any product or subtract. Then, the enzyme was dried for 20 h at 50 ºC and reused in a new fresh reaction batch. This procedure was repeated for each reuse cycle.

5.2.7 Benzyl butyrate purification

The benzyl butyrate purification was conducted to remove the remaining chemicals after the esterification reaction. The final product of the esterification was washed two times with ethyl acetate and NaOH 1 M (v/v ratio of 1:4 related to product volume) to remove completely the remaining acid of the mixture. A final wash was realized with distilled water (v/v ratio of 1:4 related to the organic phase) and then, the organic

phase was recuperated from liquid-liquid extraction and filtered under vacuum with sodium sulfate to remove the remaining water. Finally, the organic phase was submitted to evaporation using a rotaevaporator (R3 BUCHI) with a vacuum pump coupled (V-700 BUCHI) for the ethyl acetate and benzyl alcohol removal. After evaporation, the benzyl butyrate purified was analyzed by gas chromatograph (Shimadzu GC 2010).

5.2.8 Antimicrobial activity

The Minimum Inhibitory Concentration (MIC) is the lowest concentration of the active compound that allows no visible growth and the Minimum Bactericidal Concentration (MBC) is defined as the lowest concentration of the active compound that kills nearly 99.9% of the original inoculum. The MIC and MBC were determined in this work to investigate the antimicrobial potential of the benzyl butyrate.

The MIC was determined using the indirect method of bacterial growth by the determination of the optical density in liquid culture medium (PIEROZAN et al., 2009). Tests with bacteria Gram positive *Listeria monocytogenes*, *Staphylococcus aureus* and *Bacillus cereus* and Gram negatives *Klebsiella pneumoniae*, *Escherichia coli* and *Salmonella choleraesuis* were performed. After the growth period, 10 µL of pre-inoculum (108 UFC.mL^{-1}) were inoculated in microtubes with 1 mL of Luria Bertani (LB) (1:20 v/v) broth in different concentrations obtained with dilutions of the samples of benzyl butyrate and the control dimethylsulphoxide (DMSO) (from 15 to 0.01 %).

An electromagnetic mixer (60 Hz) was used for incubation of the microtubes for 24 h at 37 °C. Before (0 h) and after (24 h) incubation period, bacterial broth aliquots of 100 µL were removed to flat bottom microplates and absorbance was measured in 490 nm in an automatic microplates reader (Bio-Tec Instruments Inc., EL800). The inhibition of growth was determined by the difference between 24 and 0 h measurements. The MIC was considered as the minor concentration of the agent, which the optical density after 24 h was not different from the initial, in a 95 % confidence interval (Tukey test), i.e., the concentration able to inhibit the microbial growth after 24 h of incubation. All experiments were carried out in triplicate.

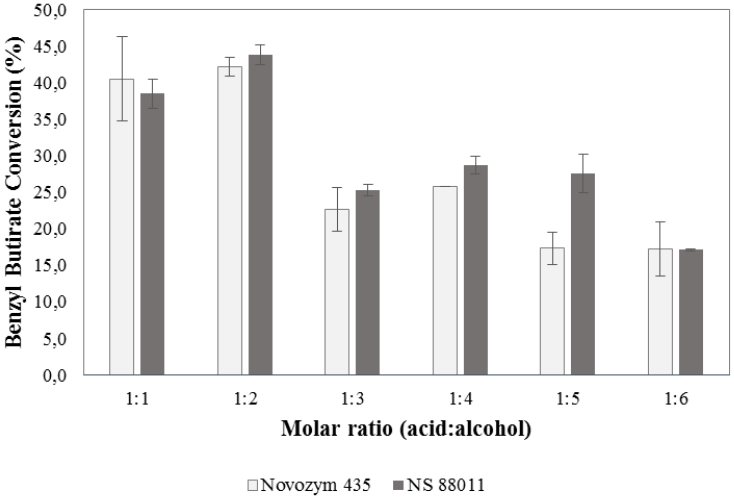
The MBC was determined for the same microorganisms described above. After previous growth period, they were submitted to dilutions (15 % to 0.01 %) of benzyl butyrate and DMSO during 1 h. Then, 10 µL of each treatment was transferred to 100 µL of LB sterile broth and incubated for 24 h at 37 ºC. The optical density was measured (490 nm) after and before the incubation period. The MBC was determined as the minor concentration of the agent, in which the optical density after 24 h was not statistically different (Tukey test, 95 % confidence interval) from the initial, i.e., the concentration able to kill the microorganism after 1 h of exposition. All experiments were carried out in triplicate.

5.3 RESULTS AND DISCUSSION

5.3.1 Effect of substrates molar ratio

The influence of the alcohol amount on the esterification reaction was investigated varying the acid:alcohol molar ratio from 1:1 to 1:6 using NS 88011 and Novozym 435 as biocatalysts and the results are shown in Figure 6.

Figure 6 - Benzyl butyrate conversion at different acid: alcohol molar ratio using 10 wt% of Novozym 435 and NS 88011, at 65ºC.



There was an increase in conversion when alcohol was maintained at low quantities, for both immobilized enzymes at molar ratio 1:1 and 1:2, reaching conversions up to 40 %. However, increasing the molar ratio of acid:alcohol above 1:3 resulted in a decrease of the

conversion for all reactions, do not exceed 28 % of conversion. These results suggest that a large amount of alcohol in the system is probably limiting the lipase activity. Similar behavior was found in the synthesis of methyl butanoate and methyl benzoate by esterification with methanol (GUMEL; ANNUAR, 2016b), and in the synthesis of benzyl propionate by transesterification with benzyl alcohol (BADGUJAR; BHANAGE, 2014a). A large increase in methanol and benzyl alcohol concentration may form a dead-end inhibition complex at higher alcohol concentration (BADGUJAR; BHANAGE, 2014a; GUMEL; ANNUAR, 2016b).

Based on these results and aiming a reduction of expenses with reagents, the molar ratio of acid:alcohol was fixed in 1:1 for all the factorial design experiments.

5.3.2 Experimental factorial design on the ester conversion

The 2^2 factorial design was conducted to improve the ester conversion as maximum as possible using the *Candida antarctica* fraction B lipase immobilized in two different support materials. One is the Novozym 435, a well-known commercial immobilized lipase, and the other is NS 88011, developed with a low-cost support material.

The response functions for the fitted models from the range of data presented in Table 13 are presented by polynomials of first order related to T and E, and interaction between both. Equations 7 and 8 are representing the systems with Novozym 435 and NS 88011, respectively.

$$Conversion = 48.57 - 3.41T + 24.91E + 0.58TE \quad (7)$$

$$Conversion = 14.62 - 7.11T + 12.58E - 6.84TE \qquad (8)$$

The quality of the fitted models was checked with the analysis of variance (ANOVA) as shown in Table 14. The pure error was very low indicating a good reproducibility of the fitted results in both cases. The correlation coefficient for the system using Novozym 435 was 0.99 and the F-test had an F-ratio 227 times bigger than the F-value $_{(0.95;\ 3,8)}$.

Table 14 - Variance analysis (ANOVA) for both fitted models using Novozym 435 and NS 88011.

System	Factor	SS	DF	MS	p	F-ratio	F-value[a]	R
Novozym 435	(1) T (L)	93.03	1	93.03	0.0001			
	(2) E (L)	4962.07	1	4962.07	0.0000			
	1L by 2L	2.67	1	2.67	0.2607			
	Lack of Fit	1.93	1	1.93	-			
	Pure Error	12.64	7	1.81	-			
	Regression	5057.77	3	1685.92	-	925.8	4.07	0.99
	Residual	14.57	8	1.82	-			
	Total SS	5072.33	11	-	-			
NS 88011	(1) T (L)	1040.82	1	1040.82	0.0023			
	(2) E (L)	2278.46	1	2278.46	0.0002			
	1L by 2L	992.13	1	992.13	0.0026			
	Lack of Fit	187.315	1	187.315	0.0000			
	Pure Error	6.852	7	0.979	-			
	Regression	2044.17	3	681.39	-	28.07	4.07	0.96
	Residual	194.17	8	24.271	-			
	Total SS	2238.34	11	-	-			

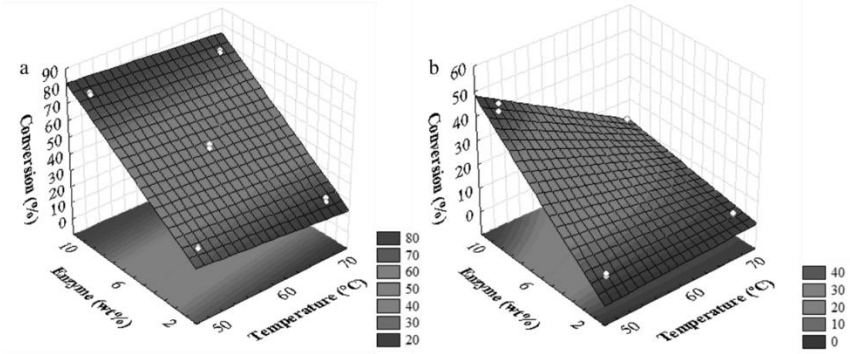
SS: sum of square; DF: degree of freedom; MS: mean square; p: probability; R: correlation coefficient

[a] F-value $_{(0.95;\ 3,8)}$

When NS 88011 was used for esterification the correlation coefficient was 0.96 and F-ratio 6.9 times bigger than F-value $_{(0.95;\ 3,8)}$. The correlation coefficient and F-ratio were lower for NS 88011 than for Novozym 435, however, these results suggest that the models presented in Equation 7 and 8 are good to represent the relation between the ester conversion and variables E and T on this interval of data for both biocatalysts studied.

The response functions of the fitted models relating the E and T on ester conversion were plotted from data presented in Table 13. The Figure 7a and 7b show the response functions from Equations 7 and 8, respectively for Novozym 435 and NS 88011. The conversion of the benzyl butyrate was highly dependent on the enzyme amount in both cases and the increase of the quantity of enzyme led to a higher ester conversion.

Figure 7 - Response functions for benzyl butyrate synthesis with (a) Novozym 435® and (b) NS 88011 in 24 h of reaction.



The T variation between the range studied presented low influence for the system using Novozym 435 (Figure 7a) and all the three

T showed to be adequate to reach high conversions when the high level of E was employed (10 wt%). On the other hand, when NS 88011 (Figure 7b) was used as biocatalyst, the lowest level of T (50 ºC) showed to be adequate to the esterification reaction.

Therefore, for both systems studied, E must be kept at the highest levels for great conversion of benzyl butyrate by esterification of benzyl alcohol and butyric acid, while the T can be kept at the lowest levels, in order to costs reduction.

5.3.3 Influence of the support material

Temperature and enzyme were varied in three different levels and results of the benzyl butyrate conversions are presented in Table 11. The best conversions were achieved in the low T level (50ºC) and the high E level (10 %) for both systems, with ~77 % and ~46 % of ester conversion for Novozym 435 and NS 88011, respectively. The biocatalyst Novozym 435 had the best performance for the esterification process even though the same lipase is immobilized in NS 88011, however, the method of immobilization and support material are different (no information was provided by the fabricant), which in this case can be responsible for the negative effects on the reaction.

Some studies were conducted in order to explain this great difference in conversion between both enzymes used, like the determination of BET area, volume, and size of the pores for both supports, showed in Table 8. Novozym 435 had a great surface area, a total pore volume ten times bigger and double average pore diameter comparing to NS 88011. These results suggest the substrates can easily

diffuse through the pores of the Novozym 435 material support to find the enzyme active site, where esterification will occur, moreover, the product can also go easily from the support to reaction media.

There may be more restriction for NS 88011 on the diffusion of the substrate due to the reduction of pore size and volume, hindering the access of the acid and alcohol to the enzyme active site, reaching a low ester conversion relating to Novozym 435. Novozym 435 was an adequate biocatalyst to the benzyl butyrate synthesis, reaching almost 80 % of conversion due to the great material support characteristics. Novozym 435 is one of the most used biocatalysts for biotechnological applications, and a range of other flavor esters was already synthesized reaching high conversions, such as butyl acetate (MARTINS et al., 2013), eugenyl acetate (CHIARADIA et al., 2012), isoamyl acetate (GÜVENÇ; KAPUCU; MEHMETOĞLU, 2002), among others.

The good results of conversion were also ensured by the use of molecular sieves that were efficient in the water removal (the side product of the esterification reaction) and they can be dried and reused in other cycles of reaction. The single study of benzyl butyrate synthesis by esterification of benzyl alcohol and butyric acid (molar ratio alcohol:acid:solvent of 1:1:1) also used Novozym 435 and reached similar results for ester conversion (JEROMIN; ZOOR, 2008). Methyl tertbutyl ether was employed as the solvent in order to favor the reaction equilibrium on the esterification side and to avoid the hydrolysis of the product. However, the use of solvent has significant importance in different food and pharmaceutical industries due to the need for toxic solvent elimination during the operation and ensuring no traces in the

final product. Based on these results, the kinetic study and enzyme reuse (present in next topics) were conducted only for the Novozym 435.

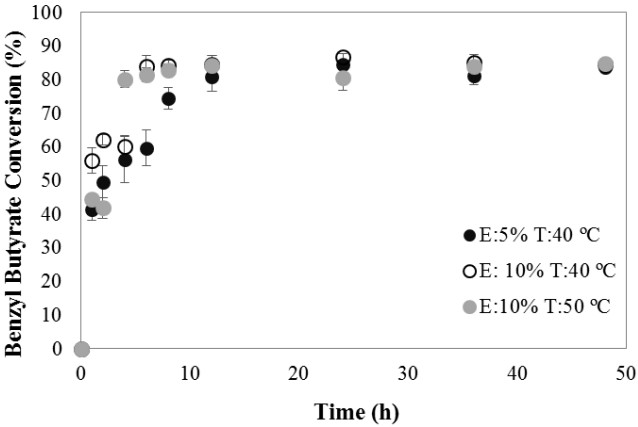
5.3.4 Kinetic study

The kinetic study was conducted with Novozym 435 as biocatalyst, the temperature was kept at the low level and enzyme amount at the high level (50 ºC and 10 wt%), following the best condition of the experimental design. Since the temperature could be kept at the low level for the esterification reaction, a strategic investigation for cost reduction was performed. Therefore, the comportment of conversion was also evaluated at a temperature lower than the experimental design (40 ºC) with enzyme amount between 5 and 10 wt%. The comportment of benzyl butyrate conversion versus time using Novozym 435 at different T and E is shown in Figure 8.

The values for the initial velocity of reaction were 1.9 mmol/h, 2.8 mmol/h and 2.2 mmol/h, respectively, for 40 ºC/5 wt%, 40 ºC/10 wt% and 50 ºC/10 wt%, indicating that even with a decrease in temperature and enzyme amount the initial velocity of reaction remained similar for all conditions studied. The benzyl butyrate conversion was efficient and reached high conversions (~80 %) in very different times of reaction for the three conditions. Results indicated that the esterification reaction was slower when temperature and enzyme amount were reduced, consequently, more time was needed to reach the highest ester conversion. The condition with 50 ºC/10 wt% had the maximum benzyl butyrate conversion in just 4 hours. However, considering the high

conversion results, any of the condition studied could be used to benzyl butyrate production with no excess of reagents and equivalent spending.

Figure 8 - The comportment of benzyl butyrate conversion versus time for reaction systems using Novozym 435 at different temperatures (T) and enzyme concentration (E).



5.3.5 Enzyme reuse

The reuse cycle of Novozym 435 was tested for the esterification between benzyl alcohol and butyric acid, showed in Figure 9. The first cycle of use presented high conversion, however, the enzyme has lost the activity, almost completely, in the second cycle of reuse. Some tests were performed in order to better explain the loss of activity of the immobilized enzyme. The enzyme Novozym 435 was submitted at the same conditions of reaction with the substrates butyric acid and benzyl alcohol, separately (Table 15). A huge reduction in the enzyme activity

was observed after the contact with the acid, the same found after the esterification reaction.

Figure 9 - Cycles of Novozym 435 reuse in esterification reaction between benzyl alcohol and butyric acid.

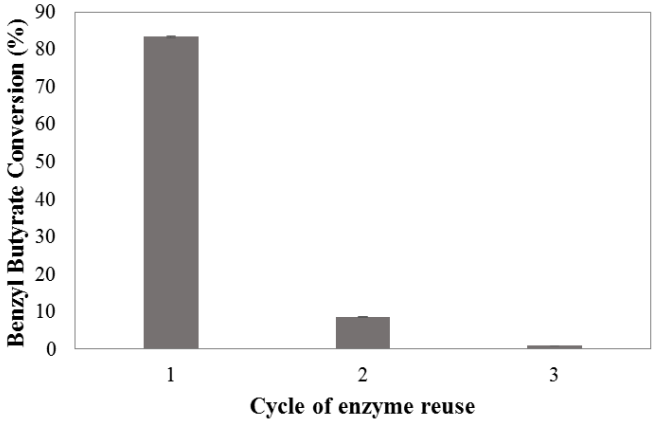


Table 15 - Enzyme activity (U/g) of Novozym 435 before and after esterification reaction, and contact with both substrates (acid and alcohol) separately.

Condition	Novozym 435 Activity (U/g)
Before reaction	54.6 ± 0.7
After esterification reaction	4.4 ± 0.8
After acid contact	4.3 ± 1.7
After alcohol contact	46.2 ± 0.4

Researchers had reported that the main reason for acid inhibition is associated to the fact that acids may cause acidification of the microaqueous 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). Based on the displayed results, butyric acid was able to deactivate the *Candida antarctica* fraction B lipase from Novozym 435, however, the ester conversion of ~80 % in the first cycle was ensured in these conditions of reaction.

5.3.6 Antimicrobial activity

The investigation about the antimicrobial property from the purified ester was conducted. The results of analyses of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) in vitro assays for benzyl butyrate for bacteria Gram positive *Listeria monocytogenes*, *Staphylococcus aureus* and *Bacillus cereus* and Gram negatives *Klebsiella pneumoniae*, *Escherichia coli* and *Salmonella choleraesuis* are presented in Table 16.

The results shows that all the microorganisms were susceptible to the benzyl butyrate. The lowest MIC was found for *S. aureus*, however, the MIC did not exceed 151.4 mg/mL for any of the microorganisms tested. The MBC was 151.4 mg/mL for all the microorganisms studied.

This study was the first evaluation of the product benzyl butyrate about antimicrobial activity and tests were carried out aiming at providing a comparison in terms of antimicrobial activity to other esters.

Table 16 – Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) for benzyl butyrate against Gram positive and Gram negative bacteria.

Bacteria		**Benzyl butyrate**	
Gram Positive	**ATCC***	**MIC (mg/mL)**	**MBC (mg/mL)**
Listeria monocytogenes	7644	100.9	151.4
Staphylococcus aureus	25923	80.7	151.4
Bacillus cereus	11778	151.4	151.4
Gram Negative	**ATCC***	**MIC (mg/mL)**	**MBC (mg/mL)**
Escherichia coli	25922	151.4	151.4
Salmonella choleraesuis	10708	151.4	151.4
Klebsiella pneumoniae	10031	151.4	151.4

*ATCC: American Type Culture Collection (USA)

Researchers studied the MIC and MBC of ester derivatives of tocopherol (vitamin E), such as α-tocopheryl acetate and α-tocopheryl phosphate, against *S. aureus*. The results showed potential antimicrobial activity for both esters. The MIC and MBC for α-tocopheryl acetate were 200 mg/mL. The α-tocopheryl phosphate presented 100 mg/mL of MIC and 200 mg/mL of MBC (BIDOSSI et al., 2017). The benzyl butyrate synthetized and purified, in the present study, had similar values for MIC and MBC for *S. aureus*. The antimicrobial activity can be associated to

the interaction of the bioactive compound with the cell membrane of the microorganisms with consequent alterations of membrane levels. These results could be attributed to the affinity of lipid matrix of the samples tested with the membrane of bacteria facilitating the transport of the products with consequent disruption of the plasmatic membrane. Therefore, a lower benzyl butyrate concentration was sufficient to prevent bacterial growth promoting a lower MIC and MBC for both Gram-positive and Gram-negative bacteria.

These results reinforce the relevance of the present work, besides the benzyl butyrate natural flavor potential, it still has potential as a natural antimicrobial agent. Although, further studies are needed to better investigate the ester action on the unfeasibility of Gram-positive and Gram-negative bacteria.

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CHAPTER VI

6 FINAL CONSIDERATIONS

6.1 CONCLUSIONS

The production of aromatic esters by the lipase-catalyzed reaction has shown to be a great alternative with several advantages over production via chemical route, such as high specificity, high yields in mild reaction conditions, reduction of by-products formation, biocatalyst reusability and reduction of the overall production costs. In addition, aromatic esters produced by enzymatic reactions are labeled as natural in accordance with the United States and European Legislations, thereby satisfying the consumer trend towards natural products in various industries.

The study of the process parameters (type of substrates, substrates molar ratio, reaction temperature, type and amount of enzyme and presence of organic solvents) and their interaction is very important. The process optimization is a great tool to achieve the best reaction conditions with maximum yield. The knowledge of reaction kinetics and mechanism is extremely important for lipase-catalyzed reactions to scale up and usually follows Ping-Pong Bi-Bi or ternary complex (order Bi-Bi) mechanism. In addition to its fragrance, aromatic esters can present some biological activities, further increasing the interest in the encapsulation of these compounds.

This work presents a biotechnological alternative to the production of benzyl propionate via esterification of benzyl alcohol and

propionic acid using Novozym 435 as the biocatalyst. There was no need of solvent to improve the ester conversion and the equimolar ratio of the substrates could be fixed to ensure the reduction in reagent costs. The factorial experimental design was able to evaluate the effect of each variable on the conversion and the surface response showed that it was greatly dependent on the high enzyme amount. This work showed for the first time a successfully report of benzyl propionate production by enzymatic esterification, presenting promising results of ester conversion of ~44 % in a solvent-free system.

This study also showed a green approach for the synthesis of benzyl butyrate via lipase-mediated esterification of benzyl alcohol and butyric acid. Conversions above 80 % were reached with no need of solvent and at a low molar ratio with an assurance of no expend in costs. The experimental design showed to be adequate to correlate the experimental data. The material support 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 synthesis of benzyl butyrate was effective in the first cycle of enzyme use ensuring a great ester conversion, nonetheless, the butyric acid induced an acidification of the microaqueous interface leading to enzyme inactivation after the first cycle.

The antimicrobial effect of benzyl butyrate was noticed for all bacteria tested and exhibited a good performance when compared to tocopherol esters. These results reinforce the relevance of the present work, finding an alternative of a natural antimicrobial for products in food industry.

6.2 FURTHER WORK

- Synthesize the benzyl esters testing others catalysts, like resins, to improve the reaction conversion.
- Evaluate the water retention capacity of the molecular sieves using the Karl Fisher methodology.
- Study other methods of water removal from the esterification reaction.
- Determine the kinetics model of the esterification and the kinetics parameters of the model (Km and Vmax) for both benzyl esters.
- Test other biological properties, like antitumoral, larvicide and anti-inflammatory.
- Evaluate different encapsulation techniques to encapsulate the aromatic benzyl esters.