



**UNIVERSIDADE FEDERAL DO CEARÁ  
CENTRO DE TECNOLOGIA  
DEPARTAMENTO DE ENGENHARIA QUÍMICA  
PROGRAMA DE PÓS-GRADUAÇÃO EM ENGENHARIA QUÍMICA**

**RODOLPHO RAMILTON DE CASTRO MONTEIRO**

**DESIGN, CHARACTERIZATION AND APPLICATION OF NANOBIOCATALYSTS  
OF LIPASE A FROM *Candida antarctica***

**FORTALEZA**

**2020**

RODOLPHO RAMILTON DE CASTRO MONTEIRO

DESIGN, CHARACTERIZATION AND APPLICATION OF NANOBIOCATALYSTS OF  
LIPASE A FROM *Candida antarctica*

Dissertação apresentada à Coordenação do Programa de Pós-graduação em Engenharia Química da Universidade Federal do Ceará, como requisito parcial para obtenção do título de Mestre em Engenharia Química. Área de concentração: Processos químicos e Bioquímicos.

Orientador: Prof. Dr. José Cleiton Sousa dos Santos

Coorientadora: Prof<sup>ª</sup>. Dr<sup>ª</sup>. Maria Cristiane Martins de Souza

FORTALEZA

2020

Dados Internacionais de Catalogação na Publicação  
Universidade Federal do Ceará  
Sistema de Bibliotecas  
Gerada automaticamente pelo módulo Catalog, mediante os dados fornecidos pelo(a) autor(a)

---

M779d Monteiro, Rodolpho Ramilton de Castro.  
Design, characterization and application of nanobiocatalysts of lipase a from *Candida antarctica* /  
Rodolpho Ramilton de Castro Monteiro. – 2020.  
148 f. : il. color.

Dissertação (mestrado) – Universidade Federal do Ceará, Centro de Tecnologia, Programa de Pós-  
Graduação em Arquitetura e Urbanismo e Design, Fortaleza, 2020.

Orientação: Prof. Dr. José Cleiton Sousa dos Santos.

Coorientação: Profa. Dra. Maria Cristiane Martins de Souza.

1. Enzyme immobilization. 2. Lipase a from *Candida antarctica*.. 3. Magnetite. 4. Halloysite. I. Título.  
CDD 720

---

RODOLPHO RAMILTON DE CASTRO MONTEIRO

DESIGN, CHARACTERIZATION AND APPLICATION OF NANOBIOCATALYSTS OF  
LIPASE A FROM *Candida antarctica*

Master's Thesis presented to the Postgraduate Program in Chemical Engineering of the Federal University of Ceará, as a partial requirement to obtain a Master's degree in Chemical Engineering. Concentration area: Chemical and Biochemical processes.

Approved on: 10/02/2019.

EXAMINATION BOARD

---

Prof. Dr. José Cleiton Sousa dos Santos  
Universidade da Integração Internacional da Lusofonia Afro-Brasileira (UNILAB)

---

Prof.<sup>a</sup> Dr.<sup>a</sup>. Artemis Pessoa Guimarães  
Universidade da Integração Internacional da Lusofonia Afro-Brasileira (UNILAB)

---

Prof.<sup>a</sup>. Dr.<sup>a</sup>. Rita Karolinny Chaves de Lima  
Universidade da Integração Internacional da Lusofonia Afro-Brasileira (UNILAB)

## ACKNOWLEDGMENTS

To God, for the breath of life, the sacrifice on the cross, the irresistible Grace and the unconditional election.

To my mother, for the example of determination and altruism, for the effort and dedication placed in me, and especially for the daily love and companionship. To my friends and family, especially my maternal grandparents.

To my advisors, Professors Cleiton Sousa dos Santos and Maria Cristiane Martins de Souza, for the confidence, care and for the time and attention devoted to my academic and/or professional career.

To GENEZ (Grupo de Engenharia Enzimática), GPBio (Grupo de Pesquisa e Desenvolvimento de Processos Biotecnológicos) and GQMat (Grupo de Química de Materiais Avançados) for the exchange of knowledge, mutual growth and respect.

To the examination board, Professors Artemis Pessoa Guimarães and Rita Karoliny Chaves de Lima, for analyzing this work and for the valuable suggestions.

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001.

Receive my instruction, and not silver, and knowledge rather than choice gold; for wisdom is better than rubies, and all the things one may desire cannot be compared with her. (Proverbs 8:10-11, New King James Bible)

## ABSTRACT

In this communication, lipases from *Candida antarctica* were immobilized onto magnetite or halloysite through chemical or physical adsorption. Firstly, lipase A from *Candida antarctica* (CALA) was immobilized by covalent bonding onto magnetite ( $\text{Fe}_3\text{O}_4$ ) functionalized with chitosan (CHI) and activated with glutaraldehyde (GLU), labelled  $\text{Fe}_3\text{O}_4@\text{CHI-GLU-CALA}$  or CALA-MNPC for short, ( $84.1\% \pm 1.0$  for immobilization yield and  $208.0 \pm 3.0$  U/g for derivative activity). CALA-MNPC was characterized by X-Ray Diffraction (XRD), Fourier Transform Infrared (FTIR) spectroscopy, Thermogravimetry (TG) and Scanning Electron Microscope (SEM), indicating the incorporation of magnetite and the immobilization of CALA onto the chitosan matrix. At  $85^\circ\text{C}$ , CALA-MNPC showed a half-life 8–11 times higher than that of CALA at pH 5–9. For CALA, the highest activity was at pH 7, whereas for CALA-MNPC, the highest activity was at pH 10. CALA-MNPC was applied to the production of a biolubricant ester from Tilapia oil, maintaining half of its activity after 7 consecutive cycles of esterification. Then, CALA or lipase B from *Candida antarctica* (CALB) were immobilized by covalent bonding onto  $\text{Fe}_3\text{O}_4$  functionalized with 3-aminopropyltriethoxysilane (APTES) and activated with GLU, labelled  $\text{Fe}_3\text{O}_4@\text{APTES-GLU-CALA}$  (CALA-MNPA) or  $\text{Fe}_3\text{O}_4@\text{APTES-GLU-CALB}$  (CALB-MNPA), respectively, ( $100 \pm 1.2\%$  and  $57.6 \pm 3.8\%$  for immobilization yield;  $198.3 \pm 2.7$  U/g and  $52.9 \pm 1.7$  U/g for derivative activity, respectively). XRD and Raman spectroscopy analysis indicated the production of a magnetic nanomaterial with a diameter of 13.0 nm, whereas FTIR indicated functionalization, activation and enzyme immobilization. CALA-MNPA and CALB-MNPC were applied to the synthesis of ethyl butyrate; under optimized conditions (1:1,  $45^\circ\text{C}$  and 6 h) by a Central Composite Design (CCD), it was possible to achieve  $99.2 \pm 0.3\%$  of conversion for CALA-MNPA (10mg) and  $97.5 \pm 0.8\%$  for CALB-MNPA (12.5mg), which retained approximately 80% of their initial activity after 10 consecutive cycles of esterification; under ultrasonic irradiation, similar conversions were achieved but at 4 h of incubation. Finally, the immobilization of CALA through ionic adsorption onto halloysite (HNT) was optimized by the Taguchi method (CALA-HNT); under optimized conditions (pH 5, 5 mM,  $5^\circ\text{C}$  and 4 hours), it was possible to achieve  $97.1 \pm 0.1\%$  for immobilization yield and  $83.81 \pm 0.5$  U/g for derivative activity. Moreover, at pH 7, CALA-HNT showed a half-life 2–8 times higher than that of CALA at  $50\text{--}90^\circ\text{C}$ . For CALA, the highest activity was at pH 7, whereas for CALA-HNT, the highest activity was at pH 9. HNT and CALA-HNT were characterized by XRD, FTIR, SEM, TG, elemental analysis (CHNS) and Differential Scanning Calorimetry (DSC), proving the immobilization of CALA on HNT and maintenance of the nanotubes structure even after immobilization.

**Keyword:** Enzyme immobilization; Lipase a from *Candida antarctica*; Magnetite; Halloysite.

## RESUMO

Nesta comunicação, lipases de *Candida antarctica* foram imobilizadas em magnetita ou haloisita por adsorção física ou química. Primeiramente, a lipase A de *Candida antarctica* (CALA) foi imobilizada por ligação covalente à magnetita ( $\text{Fe}_3\text{O}_4$ ) funcionalizada com quitosana (CHI) e ativada com glutaraldeído (GLU), rotulada como  $\text{Fe}_3\text{O}_4@CHI\text{-GLU-CALA}$  ou CALA-MNPC, ( $84,1\% \pm 1,0$  para rendimento de imobilização e  $208,0 \pm 3,0$  U/g para atividade do derivado). CALA-MNPC foi caracterizada por Difração de Raios X (DRX), Espectroscopia no Infravermelho por Transformada de Fourier (FTIR), Termogravimetria (TGA) e Microscopia Eletrônica de Varredura (MEV), indicando a incorporação da magnetita e a imobilização da CALA na matriz de quitosana. A  $85^\circ\text{C}$ , a CALA-MNPC mostrou uma meia-vida 8 a 11 vezes maior que a da CALA a pH 5-9. Para CALA, a atividade mais alta foi em pH 7, enquanto para CALA-MNPC, a atividade mais alta foi em pH 10. CALA-MNPC foi aplicado à produção de um éster biolubrificante a partir de óleo de Tilápia, mantendo metade de sua atividade após 7 ciclos consecutivos de esterificação. Em seguida, a CALA ou a lipase B *Candida antarctica* (CALB) foram imobilizadas por ligação covalente em  $\text{Fe}_3\text{O}_4$  funcionalizada com 3-aminopropiltriethoxissilano (APTES) e ativada com GLU, rotulada como  $\text{Fe}_3\text{O}_4@APTES\text{-GLU-CALA}$  (CALA-MNPA) e  $\text{Fe}_3\text{O}_4@APTES\text{-GLU-CALB}$  (CALB-MNPA), respectivamente, ( $100 \pm 1,2\%$  e  $57,6 \pm 3,8\%$  para o rendimento de imobilização;  $198,3 \pm 2,7$  U/g e  $52,9 \pm 1,7\%$  para a atividade do derivado, respectivamente). A análise por DRX e espectroscopia Raman indicou a produção de um nanomaterial magnético com diâmetro de 13,0 nm, enquanto o FTIR indicou funcionalização, ativação e imobilização enzimática. CALA-MNPA e CALB-MNPA foram aplicadas na síntese de butirato de etila; sob condições otimizadas (1:1,  $45^\circ\text{C}$  e 6 h) por um Planejamento Composto Central (PCC), foi possível alcançar  $99,2 \pm 0,3\%$  de conversão para a CALA-MNPA (10mg) e  $97,5 \pm 0,8\%$  para a CALB-MNPA (12,5mg), que retiveram aproximadamente 80% de suas atividades iniciais após 10 ciclos consecutivos de esterificação; sob irradiação ultrassônica, conversões semelhantes foram alcançadas, mas às 4 h de incubação. Finalmente, a imobilização da CALA por adsorção iônica na haloisita (HNT) foi otimizada pelo método de Taguchi (CALA-HNT); sob condições otimizadas (pH 5, 5 mM,  $5^\circ\text{C}$  e 4 horas), foi possível atingir  $97,1 \pm 0,1\%$  para o rendimento de imobilização e  $83,81 \pm 0,5$  U/g para atividade do derivado. Além disso, no pH 7, a CALA-HNT mostrou uma meia-vida 2-8 vezes maior que a da CALA a  $50\text{-}90^\circ\text{C}$ . Para CALA, a atividade mais alta foi em pH 7, enquanto para CALA-HNT, a atividade mais alta foi em pH 9. HNT e CALA-HNT foram caracterizados por DRX, FTIR, MEV, TGA, análise elementar (CHNS) e calorimetria exploratória diferencial (DSC), comprovando a imobilização da CALA em HNT e a manutenção da estrutura dos nanotubos mesmo após a imobilização.

**Palavras-chave:** Imobilização enzimática; Lipase a de *Candida antarctica*; Magnetita; Haloisita.



## CONTENTS

<b>CHAPTER 1 – INTRODUCTION AND OBJECTIVES</b>	13
<b>1.1 Introduction</b>	14
<b>1.2 Objectives</b>	
<b>1.2.1 General objective</b>	17
<b>1.2.1 Specific objectives</b>	17
<b>REREFENCES</b>	18
<b>CHAPTER 2 – REVIEW: BIOTECHNOLOGICAL RELEVANCE OF THE LIPASE A FROM <i>Candida antarctica</i></b>	22
<b>2.1 Abstract</b>	23
<b>2.2 Introduction</b>	24
<b>2.2.1 Biocatalysis</b>	24
<b>2.2.2 Lipases</b>	26
<b>2.2.3 Lipase A from <i>Candida antarctica</i> (CALA)</b>	28
<b>2.3 Enzyme Engineering</b>	33
<b>2.3.1 Directed evolution and rational design</b>	33
<b>2.3.2 Immobilization of CALA</b>	35
<b>2.4 Applications</b>	43
<b>2.4.1 Hydrolysis</b>	43
<b>2.4.2 Ammonolysis</b>	44
<b>2.4.3 Esterification, interesterification and transesterification</b>	44
<b>2.5 Conclusion</b>	46
<b>REREFENCES</b>	47
<b>CHAPTER 3 – IMMOBILIZATION OF LIPASE A FROM <i>Candida antarctica</i> ONTO CHITOSAN-COATED MAGNETIC NANOPARTICLES</b>	67
<b>3.1 Abstract</b>	68
<b>3.2 Introduction</b>	69
<b>3.3 Materials and Methods</b>	72
<b>3.3.1 Materials</b>	72
<b>3.3.2 Methods</b>	72
<b>3.3.2.1 Synthesis of iron magnetic nanoparticles (<math>Fe_3O_4</math>) functionalized with CHI</b>	72
<b>3.3.2.2 Activation of <math>Fe_3O_4@CHI</math> with glutaraldehyde (GLU)</b>	73
<b>3.3.2.3 Covalent immobilization of CALA on <math>Fe_3O_4@CHI-GLU</math></b>	73
<b>3.3.2.4 Immobilization of CALA on <math>Fe_3O_4@CHI</math></b>	73
<b>3.3.2.5 Determination of enzymatic activity and protein concentration</b>	73
<b>3.3.2.6 Immobilization parameters</b>	74
<b>3.3.2.7 Thermal and pH inactivation</b>	74

3.3.2.8. <i>Effect of pH on biocatalyst activity</i>	74
3.3.2.9 <i>X-Ray Powder Diffraction (XRPD)</i>	74
3.3.2.10 <i>Fourier Transform Infrared (FTIR) spectroscopy</i>	75
3.3.2.11 <i>Thermogravimetry (TG)</i>	75
3.3.2.12 <i>Scanning Electron Microscope (SEM)</i>	75
3.3.2.13 <i>Extraction and purification of tilapia oil</i>	75
3.3.2.14 <i>Production of Free Fatty Acids (FFAs) from tilapia oil</i>	75
3.3.2.15 <i>Production of biolubricant ester</i>	76
3.3.2.17 <i>Operational stability</i>	76
<b>3.4 Results and Discussion</b>	77
3.4.1 <i>Immobilization parameters</i>	77
3.4.2 <i>Effect of pH on the thermal stability of CALA biocatalysts</i>	78
3.4.3 <i>Effect of pH on CALA biocatalysts activity</i>	78
3.4.4 <i>Characterization of the nanoparticles and biocatalysts</i>	79
3.4.5 <i>Operational stability</i>	83
<b>3.5 Conclusion</b>	84
<b>REFERENCES</b>	85
<b>CHAPTER 4 – ETHYL BUTYRATE SYNTHESIS CATALYZED BY LIPASES A AND B FROM <i>Candida antarctica</i> IMMOBILIZED ONTO MAGNETIC NANOPARTICLES. IMPROVEMENT OF BIOCATALYSTS' PERFORMANCE UNDER ULTRASONIC IRRADIATION</b>	92
<b>4.1 Abstract</b>	93
<b>4.2 Introduction</b>	94
<b>4.3 Materials and Methods</b>	97
4.3.1 <i>Materials</i>	97
4.3.2 <i>Methods</i>	97
4.3.2.1 <i>Ultrasound equipment setup</i>	97
4.3.2.2 <i>Synthesis of iron magnetic nanoparticles (<math>Fe_3O_4</math>) functionalized with 3-aminopropyltriethoxysilane (APTES)</i>	97
4.3.2.3 <i>Activation of <math>Fe_3O_4@APTES</math> with glutaraldehyde (GLU)</i>	98
4.3.2.4 <i>Covalent immobilization of CALA or CALB onto <math>Fe_3O_4@APTES</math>-GLU</i>	98
4.3.2.5 <i>Adsorption immobilization of CALA or CALB onto <math>Fe_3O_4@APTES</math></i>	98
4.3.2.6 <i>Determination of enzymatic activity and protein concentration</i>	99
4.3.2.7 <i>Immobilization parameters</i>	99
4.3.2.8 <i>X-Ray Diffraction (XRD)</i>	99
4.3.2.9 <i>Raman spectroscopy</i>	100
4.3.2.10 <i>Fourier-transform Infrared spectroscopy (FTIR)</i>	100
4.3.2.11 <i>Enzymatic esterification</i>	100

4.3.2.12. <i>Central Composite Design (CCD)</i>	100
4.3.2.13 <i>Statistical analysis</i>	101
4.3.2.14 <i>Operational stability</i>	101
4.3.2.15 <i>Thermodynamic properties</i>	101
<b>4.4 Results and Discussion</b>	102
4.4.1 <i>Immobilization performance</i>	102
4.4.2 <i>Characterization of Fe<sub>3</sub>O<sub>4</sub> NPs</i>	103
4.4.3 <i>Fourier-Transform Infrared Spectroscopy (FTIR)</i>	104
4.4.4 <i>Model fitting and ANOVA</i>	105
4.4.5 <i>Time course of esterification</i>	109
4.4.6 <i>Thermodynamics of the enzymatic esterification</i>	110
4.4.7 <i>Operational stability</i>	112
<b>4.5 Conclusion</b>	113
<b>REFERENCES</b>	115
<b>CHAPTER 5 – IMMOBILIZATION OF LIPASE A FROM <i>Candida antarctica</i> ONTO HALLOYSITES NANOTUBES</b>	123
<b>5.1 Abstract</b>	124
<b>5.2 Introduction</b>	125
<b>5.3 Materials and Methods</b>	127
5.3.1 <i>Materials</i>	127
5.3.2 <i>Methods</i>	127
5.3.2.1 <i>Optimization of the immobilization of lipase A from <i>Candida antarctica</i> (CALA) onto Halloysite Nanotubes (HNT)</i>	127
5.3.2.2 <i>Determination of enzymatic activity and protein concentration</i>	128
5.3.2.3 <i>Immobilization parameters</i>	129
5.3.2.4 <i>pH profile</i>	129
5.3.2.5 <i>Thermal deactivation</i>	129
5.3.2.6 <i>Loading capacity of HNT for CALA</i>	130
5.3.2.7 <i>Immobilization course of CALA onto HNT</i>	130
5.3.2.8 <i>Materials characterization</i>	130
<b>5.4 Results and Discussion</b>	131
5.4.1 <i>Optimization of the immobilization of CALA onto HNT</i>	131
5.4.3 <i>pH profile</i>	135
5.4.4 <i>Thermal deactivation</i>	136
5.4.4 <i>Materials characterization</i>	137
<b>5.5 Conclusion</b>	137
<b>REFERENCES</b>	143
<b>CHAPTER 6 – FINAL CONSIDERATIONS</b>	147