UNIVERSIDADE FEDERAL DE ALFENAS

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PRODUÇÃO E CARACTERIZAÇÃO DE CÉLULAS ÍNTEGRAS DE *Rhizopus oryzae* CCT3759 PARA SER APLICADO COMO BIOCATALISADOR NA HIDRÓLISE DE ÓLEOS VEGETAIS

Alfenas/MG

2021

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Dissertação apresentada como parte dos requisitos para obtenção do título de Mestre em Biotecnologia pela Universidade Federal de Alfenas. Área de concentração: Biomoléculas.

Orientador: Prof. Dr. Ernandes Benedito Pereira

Coorientadora: Profª. Drª. Heizir Ferreira de Castro

Alfenas/MG

2021

Dados Internacionais de Catalogação-na-Publicação (CIP)
Sistema de Bibliotecas da Universidade Federal de Alfenas
Biblioteca Central – Campus Sede

Reis, Willian de Souza Matias
R375p Produção e caracterização de células íntegras de *Rhizopus oryzae* CCT3759 para ser aplicado como biocatalisador na hidrólise de óleos vegetais / Willian de Souza Matias Reis – Alfenas, MG, 2021.
47 f.: il. –

Orientador: Ernandes Benedito Pereira.
Dissertação (Mestrado em Biotecnologia) – Universidade Federal de Alfenas, 2021.
Bibliografia.

1. Caracterização. 2. Células Íntegras. 3. Fermentação Submersa. 4. Hidrólise. 5. Óleos Vegetais. 6. *Rhizopus oryzae*. I. Pereira, Ernandes Benedito. II. Título.

CDD- 572

Ficha Catalográfica elaborada por Marlom Cesar da Silva
Bibliotecário-Documentalista CRB6/2735

WILLIAN DE SOUZA MATIAS REIS

"PRODUÇÃO E CARACTERIZAÇÃO DE CÉLULAS ÍNTEGRAS DE Rhizopus oryzae CCT3759 POR FERMENTAÇÃO SUBMERSA PARA SER APLICADO COMO BIOCATALISADOR NA HIDRÓLISE DE ÓLEOS VEGETAIS"

A Banca examinadora abaixo-assinada aprova a Dissertação apresentada como parte dos requisitos para a obtenção do título de Mestre em Biotecnologia pela Universidade Federal de Alfenas. Área de concentração: Biomoléculas.

Aprovado em: 24 de fevereiro de 2021

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Documento assinado eletronicamente por **Ernandes Benedito Pereira**, **Professor do Magistério Superior**, em 24/02/2021, às 10:50, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

Documento assinado eletronicamente por **Adriano Aguiar Mendes**, **Presidente**, em 24/02/2021, às 10:53, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

Documento assinado eletronicamente por **LARISSA DE FREITAS**, **Usuário Externo**, em 24/02/2021, às 10:55, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

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AGRADECIMENTOS

A minha família, especialmente minha mãe, Elba, minha irmã, Laís e minhas tias, Isamara e Lismara, por todo incentivo e sacrifício que fizeram para que sempre tivesse a minha educação como prioridade.

Ao Prof. Dr. Ernandes B. Pereira, agradeço por dar início ao meu conhecimento científico. Obrigado pelo companheirismo, amizade, incentivo, orientação, paciência, correções necessárias e, além disso a boa amizade desenvolvida durante a graduação e mestrado.

Ao Prof. Dr. Adriano A. Mendes, agradeço por toda a ajuda cedida para a finalização deste trabalho, a qual foi de grande importância para a conclusão deste trabalho. Obrigado também pela boa amizade, conselhos e as boas trocas de conversas.

Aos meus amigos de laboratório, Nicole e Wagner, agradeço pelos bons momentos compartilhados e ainda mais por deixarem as matérias complicadas mais fáceis e os perrengues do laboratório mais divertidos. Desejo o melhor a vocês.

Ao técnico do Laboratório de Bioprocessos, Gustavo e a auxiliar Alessandra, por toda a ajuda concedida.

Gostaria de agradecer, especialmente a Prof ª Dr.ª Heizir F. de Castro, por todo o tempo em que esteve como minha coorientadora, sendo sempre prestativa, atenciosa, tendo grande participação neste trabalho, sem ela este trabalho não teria sido possível.

Agradeço ao apoio e ensino dado pela prof. Dr. Ana Karine Furtado de Carvalho no começo dos experimentos, que deu início a todo o trabalho desenvolvido, além de toda assistência dada no decorrer do mestrado. Também agradeço ao Dr. Heitor Buzetti Simões Bento pelo apoio e tempo cedido para análises de amostras no Laboratório de Biocatálise da Escola de Engenharia de Lorena - EEL/USP.

Agradeço a CAPES pela bolsa concedida. O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001.

RESUMO

O presente estudo teve como objetivo a produção de lipase ligada ao micélio do fungo *Rhizopus oryzae* CCT3759 por fermentação submersa para ser aplicada como biocatalisador na hidrólise de diferentes óleos vegetais. Condições ótimas de cultivo foram alcançadas em meio contendo azeite de oliva como indutor por 72 h de fermentação, obtendo 30,5 g/L de concentração de biomassa seca e atividade hidrolítica de 389,1 U/g, o que corresponde a uma produtividade de 12.322,2 U/L. Máxima atividade hidrolítica foi observada em pH 6,0 e 40 ºC. Os parâmetros cinéticos, constante de Michaelis-Menten (K_m = 50,5 mM) e velocidade máxima de reação (V_{max} = 815,4 µmol/g.min) foram determinados na hidrólise da emulsão de azeite de oliva. Teste de estabilidade térmica revelou que a enzima reteve 75 % de sua atividade inicial após 4 h de incubação a 50 ºC, com constante de inativação térmica (K_d) e tempo de meia-vida ($t_{1/2}$) de 0,073 e 9,4 h, respectivamente. O efeito da concentração do biocatalisador, expresso como o número de unidades de atividade – U (200 e 400 U) na hidrólise de óleos vegetais foram investigadas sob condições fixas de: porcentagem de óleo 25% m/m, tampão fosfato 100 mM pH 6,0, 40 ºC e a frequência de agitação mecânica de 600 rpm. O aumento de atividade oferecida aumentou os valores de velocidades iniciais e porcentagem de hidrólise. Entretanto, os valores de velocidades iniciais obtidos foram similares para todos os óleos empregados devido à alta acessibilidade da lipase ao substrato nas condições experimentais adotadas. A hidrólise completa dos óleos de oliva, algodão, girassol e canola foi alcançado após 26 – 30 h de reação usando 400 U de atividade. Estes resultados sugerem a promissora aplicação do biocatalisador produzido na produção de ácidos graxos livres (AGL), uma importante classe de compostos para as indústrias oleoquímicas.

Palavras-chave: Caracterização. Células Íntegras. Fermentação Submersa. Hidrólise. Óleos Vegetais. *Rhizopus oryzae*.

ABSTRACT

The present study aims to produce mycelium-bound lipase of the fungus *Rhizopus oryzae* CCT3759 by submerged fermentation in order to be applied as biocatalyst in the hydrolysis of different vegetable oils. Optimal cultivation conditions have been achieved in a medium containing olive oil as inducer for 72 h of fermentation, thus obtaining 30,5 g/L of dry biomass concentration and hydrolytic activity of 389,1 U/g, which corresponds to a productivity of 12.322,2 U/L. Maximum hydrolytic activity was observed at pH 6,0 and 40 ºC. Kinetic parameters concerning apparent Michaelis-Menten constant (K_m = 50,5 mM) and maximum reaction rate (V_{max} = 815,4 µmol/g.min) have been determined in olive oil emulsion hydrolysis. Thermal stability tests revealed that the enzyme retained 75% of its initial activity after 4 h at 50 ºC, whose thermal inactivation constant (K_d) and half-life ($t_{1/2}$) was 0,073 h^{-1} and 9,4 h, respectively. The effect of biocatalyst concentration, expressed as activity units – U (200 and 400 U), on the hydrolysis of vegetable oils was investigated under fixed conditions: oil percentage 25% m/m, buffer sodium phosphate (100 mM, pH 6,0), 40 ºC and the mechanical stirring frequency of 600 rpm. Increased activity leads to higher values of initial reaction rates and hydrolysis percentage. However, initial velocity values were similar for six different vegetable oils due to the high accessibility of the lipase to the substrate under such experimental conditions. A complete hydrolysis of olive, cottonseed, sunflower and canola oils was achieved after 26–30 h of reaction using 400 U of activity. These results suggest a promising application of the produced biocatalyst in the production of FFA, an important class of compounds for oleochemical industries.

Keywords: Characterization. Hydrolysis. Mycelium-bound lipase. *Rhizopus oryzae*. Submerged fermentation. Vegetable oils.

SUMÁRIO

1 INTRODUÇÃO

O consumo mundial de óleos vegetais cresce a cada ano, tendo em 2020/2021 atingindo uma produção de 200 milhões de toneladas globalmente e sendo o Brasil responsável por 10 milhões de toneladas, segundo o *United States Department of Agriculture* (USDA). Estes valores referem-se a produção dos principais óleos vegetais como óleo de coco, algodão, palma, palmiste, amendoim, colza, soja e girassol [1].

Apesar de a indústria de alimentos ser o principal setor de consumo de óleos vegetais (triacilgliceróis - TAG), foram hidrolisados para a obtenção de AGL e glicerol, importantes precursores para as indústrias farmacêutica, cosmética e oleoquímica; para produzir biodiesel [2–5], biossurfactantes [6–8], sabores ésteres [9,10], biolubrificantes [11–15] e lipídios estruturados [16–18].

Um conhecido processo comercial para a obtenção de concentrados de AGL é o Processo Colgate-Emery, no qual é conduzido em altas temperaturas (250ºC) e pressão (50 bar). Nestas condições bruscas de operação, reações indesejáveis de oxidação, desidratação e interesterificação de TAG, exigindo etapas de separação e purificação do produto final [19–21].

Para superar as problemáticas envolvidas no processo termoquímico, a hidrólise enzimática de óleos vegetais vem apresentando resultados satisfatórios. O uso de lipases como biocatalisadores apresenta diversas vantagens em relação aos métodos termoquímicos, pois podem atuar em condições moderadas de temperaturas e pressão, reduzindo o custo com energia e facilitando a recuperação e purificação do produto final [19,22,23].

As lipases (triacylglycerol acylhydrolases EC 3.1.1.3) são enzimas que atuam na hidrólise de ligações éster-carboxílicas presentes em TAG, resultando na liberação de AGL e glicerol. No entanto, podem atuar em reações de esterificação, interesterificação e transesterificação em meios não aquosos. Além disso, apresentam características importantes como quimio-, régio-, enantiosseletividade e alta versatilidade em uma variedade de substratos - ésteres naturais e não naturais [24–27]. Propriedades tão atrativas indicam que as lipases podem ser aplicadas em segmentos industriais diferentes, como detergentes, têxteis, cosméticos, farmacêuticos [28,29].

As lipases podem ter origem animal, vegetal ou microbiana, contudo aquelas obtidas de origem microbiana são as mais aplicadas industrialmente devido às suas características favoráveis ao setor industrial [30,31]. Dentre as características atrativas ao setor industrial, destacam-se: a alta atividade e estabilidade em uma ampla faixa de valores de temperatura, pH

e em solventes orgânicos. Além disso, apresentam maior rendimento de produção, possibilidade de manipulação genética e rápido crescimento celular em meios de baixo custo [32,33].

As lipases produzidas por microrganismos podem ser extracelulares ou lipases intracelulares [30,33,34]. Dentre as lipases intracelulares têm-se as lipases ligadas ao micélio, definidas como as lipases que se encontram associadas à biomassa fúngica, sendo assim naturalmente imobilizadas. Ainda que ligadas ao micélio, as lipases ainda são ativas, por isso podem ser utilizadas como biocatalisadores, eliminando parcialmente etapas de alto custo como as de purificação, recuperação e imobilização por diferentes protocolos [35–37].

O fungo *Rhizopus oryzae* já foi relatado como bom produtor de lipase. O *Rhizopus orizae* é um fungo filamentoso do gênero *Rhizopus* sp., capaz de produzir diferentes substâncias, como enzimas (celulases, lipases, proteases, tanases), ácidos orgânicos (ácido lático e fumárico), compostos aromáticos e corantes, além de ter a vantagem de ser categorizado como um fungo GRAS (Generally Recognized as Safe), sendo assim seguro para a aplicação na indústria alimentícia [38,39].

As cepas de *Rhizopus oryzae*, contém dois tipos de lipase, sendo uma lipase de 34 kDa encontrada ligada à parede celular e uma lipase de 31 kDa, ligada à membrana e parede celular [40]. A maioria das suas lipases requerem um valor de pH entre 6.0 e 8.5 e tem uma temperatura ideal entre 30 °C e 45 °C para expressar máxima atividade hidrolítica. Estas enzimas exibem elevada atividade catalítica para ésteres contendo ácidos graxos com 8 a 18 átomos de carbono [41].

Na literatura ainda há poucos estudos sobre a aplicação da lipase ligada ao micélio do fungo *Rhizopus oryzae* na hidrólise de óleos vegetais para a produção de ácidos graxos de grande relevância na indústria oleoquímica, tornando este, um nicho promissor para o desenvolvimento novas pesquisas [42]. Nesse contexto, o presente trabalho tem como objetivo investigar o potencial de uma cepa de *Rhizopus oryzae* CCT3759 como produtora de lipase ligada ao micélio, sua caracterização bioquímica e cinética e aplicação na hidrólise de diferentes óleos comerciais afim de se obter concentrados de AGL.

2 OBJETIVOS

2.1 Objetivo Geral

O presente trabalho teve como objetivo investigar o potencial de uma cepa de *Rhizopus oryzae* CCT3759 como produtora de lipase ligada ao micélio, sua caracterização bioquímica e cinética e aplicação na hidrólise de diferentes óleos comerciais afim de se obter concentrados de AGL.

O objetivo geral do projeto foi alcançado mediante à execução dos seguintes objetivos específicos:

2.2 Objetivos Específicos

a) Obtenção da lipase ligada ao micélio por meio do cultivo submerso do fungo *Rhizopus oryzae*;
b) Determinação das condições otimizadas de cultivo submerso afim de se obter maior atividade hidrolítica*;*
c) Determinação das propriedades bioquímicas (pH; temperatura; estabilidade térmica) e cinéticas (K_m e V_{max}) da lipase de *Rhizopus oryzae* visando maximizar o grau de hidrólise dos lipídios*;*
d) Avaliação da aplicabilidade da lipase em reações de hidrólise de diferentes óleos comerciais e o efeito da atividade inicial fornecida na hidrólise.

3 ARTIGO 1 - Production and characterization of whole-cell *Rhizopus oryzae* CCT3759 to be applied as biocatalyst in vegetable oils hydrolysis

3.1 Artigo submetido em Periódico Indexado

Os resultados obtidos durante o mestrado foram submetidos para avaliação no periódico indexado *Catalysis Letters*. O trabalho consistiu na investigação do potencial de uma cepa de *Rhizopus oryzae* CCT3759 como produtora de lipase ligada ao micélio, sua caracterização bioquímica e cinética e aplicação na hidrólise de diferentes óleos comerciais afim de se obter concentrados de AGL. Pelos resultados obtidos, as lipases produzidas pela cepa de *Rhizopus oryzae* CCT3759 demonstraram alta atividade hidrolítica, além de alta eficiência na aplicabilidade como biocatalisador na hidrólise dos óleos vegetais avaliados.

PRODUCTION AND CHARACTERIZATION OF WHOLE-CELL *Rhizopus oryzae* CCT3759 TO BE APPLIED AS BIOCATALYST IN VEGETABLE OILS HYDROLYSIS

Running head: **Production and characterization of whole-cell** *Rhizopus oryzae* CCT3759

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Abstract

The present study aims to produce a mycelium-bound lipase of the fungus *Rhizopus oryzae* CCT3759 by submerged fermentation in order to be applied as biocatalyst in the hydrolysis of different vegetable oils. Optimal cultivation conditions have been achieved in a medium containing olive oil as inducer for 72 h of fermentation, thus obtaining 30.5 g/L of dry biomass concentration and hydrolytic activity of 389.1 U/g, which corresponds to a productivity of 12,322.2 U/L. Maximum hydrolytic activity was observed at pH 6.0 and 40 ºC. Kinetic parameters concerning apparent Michaelis-Menten constant (K_m = 50.5 mM) and maximum reaction rate (V_{max} = 815.4 µmol/g.min) have been determined in olive oil emulsion hydrolysis. Thermal stability tests revealed that the enzyme retained 75% of its initial activity after 4 h at 50 ºC, whose thermal inactivation constant (K_d) and half-life ($t_{1/2}$) was 0.073 h^{-1} and 9.4 h, respectively. The effect of biocatalyst concentration, expressed as activity units – U (200 and 400 U), on the hydrolysis of vegetable oils was investigated under fixed conditions: oil percentage 25% m/m, buffer sodium phosphate (100 mM, pH 6.0), 40 ºC and the mechanical stirring frequency of 600 rpm. Increased activity leads to higher values of initial reaction rates and hydrolysis percentage. However, initial velocity values were similar for six different vegetable oils due to the high accessibility of the lipase to the substrate under such experimental conditions. A complete hydrolysis of olive, cottonseed, sunflower and canola oils has been achieved after 26–30 h of reaction using 400 U of activity. These results suggest a promising application of the produced biocatalyst in the production of FFA, an important class of compounds for oleochemical industries.

Keywords: Mycelium-bound lipase. *Rhizopus oryzae*. Submerged fermentation. Characterization. Hydrolysis. Vegetable oils.

1 INTRODUCTION

The worldwide consumption of vegetable oils has been increasing yearly and their production has reached 200 million tons in 2020/2021 globally, and Brazil is responsible for 10 million tons according to the United States Department of Agriculture (USDA). These data refer to the production of main vegetable oils, such as coconut oil, cottonseed, palm, palm kernel, peanut, rapeseed, soybean and sunflower [1].

Despite the fact that the food industry is the main sector of consumption of vegetable oils (triacylglycerols – TAG) have been hydrolyzed to obtain FFA and glycerol, which are important precursors for the pharmaceutical, cosmetic and oleochemical industries; to produce biodiesel [2–5], biosurfactants [6–8], flavors esters [9,10], biolubricants [11–15] and structured lipids [16–18].

A well-known commercial process for obtaining FFA is the Colgate-Emery Process, which is carried out at high temperatures (250 ºC) and pressures (50 bar). In these abrupt operating conditions, undesirable reactions of oxidation, dehydration and interesterification of TAG require steps of separation and purification of the final product [19–21].

In order to overcome problems involved in the thermochemical process, an enzymatic hydrolysis of vegetable oils has been yielding satisfactory results. The use of lipases as biocatalysts has several advantages over thermochemical methods, as they can act under moderate conditions of temperatures and pressure, thus reducing energy costs and facilitating recovery and purification of the final product [19,22,23].

Lipases (triacylglycerol acylhydrolases EC 3.1.1.3) are enzymes that act in the hydrolysis of ester-carboxylic bonds present in TAG, which result in releasing FFA and glycerol. However, they can act in esterification, interesterification and transesterification reactions in non-aqueous media. In addition, they have important features such as chemo-, regio-, enantioselectivity and high versatility towards a variety of substrates – natural and non-

natural esters [24–27]. Such very attractive properties indicate that lipases can be applied in different industrial segments, such as detergents, textiles, cosmetics, pharmaceuticals [28,29].

Lipases can be obtained from animal, vegetable or microbial origins, however those obtained from microbial origins are the most commonly used by industries due to their favorable characteristics to the industrial sector [30,31]. Among their attractive characteristics to the industrial sector, the following can be highlighted: high activity and stability over a wide range of temperature and pH values, and in organic solvents. In addition, they have higher production yield, possibility of genetic manipulation and rapid cell growth in low-cost media [32,33].

Lipases produced by microorganisms can be either extracellular or intracellular [30,33,34]. Among intracellular lipases, there are those bound to the mycelium, which are defined as lipases that are associated with fungal biomass, thus being naturally immobilized. Although being mycelium-bound, lipases are still active, therefore they can be used as biocatalysts so as to partially eliminate costly steps, such as purification, recovery and immobilization by different protocols [35–37].

The fungus *Rhizopus oryzae* has already been reported as good lipase producer. It is a filamentous fungus of the genus *Rhizopus* which is capable of producing different substances, such as enzymes (cellulases, lipases, proteases, tannases), organic acids (lactic and fumaric acid), aromatic compounds and dyes, in addition to having the advantage of being categorized as a GRAS fungus (Generally Recognized as Safe), thus it is safe for applications in the food industry [38,39].

Rhizopus oryzae strains contain two types of lipase, a 34 kDa lipase bound to the cell wall and a 31 kDa lipase bound to the membrane and cell wall [40]. Most of its lipases require a pH value ranging between 6.0 and 8.5 and their ideal temperature ranges between 30 °C and

45 °C to express the high hydrolytic activity. These enzymes are most active for esters containing fatty acids with 8 to 18 carbon atoms [41].

There are still few studies in literature on the application of mycelium-bound lipase of the fungus *Rhizopus oryzae* in the hydrolysis of vegetable oils for producing FFA of great relevance to the oleochemical industry, which makes it a promising niche for the development of new research [42]. Therefore, the present work aims to investigate the potential of a strain of *Rhizopus oryzae* CCT3759 as a producer of mycelium-bound lipase, its biochemical and kinetic characterization and application in the hydrolysis of different commercial oils in order to obtain FFA.

2 MATERIALS AND METHODS

2.1 Microorganism

The used strain was that of the fungus *Rhizopus oryzae* CCT3759 obtained from the André Tosello Tropical Research and Technology Foundation (Campinas/SP/Brazil). In order to obtain and maintain culture spores, fungal cells had been previously inoculated on Sabouraud agar medium under aseptic conditions. The culture was incubated at 30 ºC and 72 h, or until they reached the highest sporulation status. Cells were washed with 10 mL sterile distilled water to obtain spore suspension under aseptic conditions.

2.2 Materials

Olive oil (Carbonell™), cottonseed oil, canola oil (Vitalliv™), corn oil, (Sinhá™), sunflower oil, and soybean oil (Liza™) were purchased from local stores (Alfenas, MG, Brazil). Sabouraud agar medium and soybean peptone were acquired from HiMedia Laboratories (Mumbai, MH, India). Gum Arabic, monobasic potassium phosphate, monobasic sodium

phosphate, bibasic sodium phosphate were acquired from Dinâmica Química (Indaiatuba, SP, Brazil); and magnesium sulfate heptahydrate, sodium hydroxide, sodium nitrate, ethanol solution (70% v/v) from Vetec Química (São Paulo, SP, Brazil). All other reagents and organic solvents of analytical grade were purchased from Vetec Química.

2.3 Culture medium and conditions

The culture medium consisted of 30 g/L of vegetable oil, 70 g/L of soybean peptone, 1 g/L of $NaNO_3$, 1 g/L of KH_2PO_4 and 0.5 g/L of $MgSO_4.7H_2O$, and all of which have been previously autoclaved (121 °C and 15 min). Commercial oils (cottonseed, olive oil, canola, sunflower, corn and soybean oils) were added afterwards under aseptic conditions. Cultures were performed in 250 mL Erlenmeyer flasks containing 100 mL of autoclaved medium were inoculated with a suspension of $1x10^6$ spores at 30 °C and orbital shaking at 180 rpm. Spore concentration was determined by counting cells in a Neubauer chamber using an Olympus® binocular microscope. At the end of the culture process, the produced biomass was separated from the medium by vacuum filtration, washed with water and acetone and quantified for hydrolytic activity and humidity by drying the wet biomass (0.25 g) in a microwave oven (180 W per 5 min) [43]. Subsequently, the fungal biomass were stored at 4 °C prior to use.

2.4 Determination of optimized submerged culture conditions of *Rhizopus oryzae* CCT3759 for mycelium-bound lipase production

In each culture cycle, the mycelium-bound lipase production was evaluated in terms of dry biomass concentration (g/L) and hydrolytic activity (U/g) by the method of olive oil emulsion hydrolysis described by Marotti et al [37], and enzymatic productivity (U/L) that was defined as the amount of lipolytic activity produced per liter of submerged culture in accordance with WANG et al [44]. To determine optimal conditions for producing whole-cells with high

catalytic activity, six vegetable oils with different fatty acid compositions such as olive oil, cottonseed oil, canola, sunflower, corn and soybean oils (Table 1) have been evaluated. In this study, the influence of the submerged cultivation time was also evaluated for each studied carbon source at every 24, 48, 72 and 96 h of submerged cultivation. Enzyme productivity was calculated according to Equation 1, where *HA* is the hydrolytic activity and *DBC* is the dry biomass concentration. Enzymatic activity (U) is defined as the amount of dry biomass or culture broth required for the release of 1 μmol of free fatty acids per minute under experimental conditions (0.1 g of biomass or 1 mL of culture broth at 37 ºC reaction temperature, 100 mM sodium phosphate buffer and pH of 7.0 in 5 minutes of reaction).

$$\text{Productivity} = HA * DBC \qquad \text{Equation 1}$$

2.5 Characterization of biochemical and kinetic properties of mycelium-bound lipase

Biochemical and kinetic properties of lipase bound to the mycelium were characterized by olive oil emulsion hydrolysis. The effect of temperature was evaluated in the range of 25-60 ºC using a 100 mM phosphate buffer at pH 6.5, while the pH effect was investigated in the range of 4-5.5 (100 mM buffer sodium citrate) and from 6.0 to 8.0 (100 mM buffer sodium phosphate) at 40 ºC. The influence of substrate concentration (olive oil) was investigated in the range of 5 and 40% m/m (corresponding to 186 to 1488 mM of FFA) under optimal conditions (100 mM buffer sodium phosphate pH 6.0 and 40 °C). Apparent Michaelis-Menten kinetic constants (K_m) and maximum reaction rate (V_{max}) were determined according to a non-linear model using the software Origin Pro version 5.0. Thermal stability tests were performed by incubating the biomass in a phosphate buffer (100 mM pH 6.0) and a thermostatic bath at 50 ºC by 4 h. Samples were removed periodically to determine residual hydrolytic activity. The thermal denaturation constant (K_d) and half-life time ($t_{1/2}$) were respectively determined as follows (Equations 2 and 3):

$$\ln A = \ln A_o - \mathrm{K_d}.\mathrm{t} \qquad \text{Equation 2}$$

$$t_2^1 = \frac{0{,}693}{K_d} \qquad \text{Equation 3}$$

Where: ln *A* is the residual activity after the heat treatment during a incubation period and ln A_0 is the initial enzyme activity.

Table 1. Fatty acids composition and molecular mass of the vegetable oils used in this study [45].

Fatty acid	Composition (% m/m)					
	Cottonseed	Olive	Canola	Sunflower	Corn	Soybean
C16:0 – Palmitic	25.2	11.3	4.5	6.3	11.9	11.5
C18:0 – Stearic	1.8	2.8	2.0	3.9	2.1	4.1
C18:1 - Oleic	16.5	74.5	60.4	20.9	27.2	23.5
C18:2 - Linoleic	54.8	9.8	21.2	67.6	57.7	53.3
C18:3 - Linolenic	0.2	0.5	9.4	0.2	0.6	6.8
Average molecular mass of FFA (g/mol)	274.7	279.3	280.5	279.5	278.2	276.0

2.6 Hydrolysis reactions of vegetable oils in stirred-tank reactors

In 250 mL glass jacketed reactors, 100 mL of the substrate composed by the emulsion of 25 g of vegetable oil in buffer sodium phosphate (100 mM, pH 6.0), using Gum Arabic as an emulsifier (3 % m/v). The tests were carried out at 40 ºC and the ratio of enzyme units was set at 200 and 400 U (which corresponds to an average mass of approximately 0.9 and 1.8 g of dry biomass, respectively) and 600 rpm of mechanical stirring, which was performed by using an overhead motor stirrer with a steel helical impeller. A 50:50 (v/v) mixture of acetone and ethanol was added to the aliquots (0.5 g) that have been removed periodically, and fatty acid

content was quantified by titration with a 20 mM sodium hydroxide solution (NaOH) using phenolphthalein as indicator. Hydrolysis percentage (%), according to [46], was calculated by Equation 4.

$$\text{Hydrolysis (\%)} = \frac{(V_a - V_b)\,.\,C_{NaOH}\,.\,10^{-3}\,.\,M}{m\,.\,f} \qquad Equation\ 4$$

Where: V_a is the volume of NaOH (mL) in the sample; V_b is the volume of NaOH in the control (mL); C_{NaOH} is the molar concentration of NaOH (20 mM); M is the average molecular mass of fatty acids in the vegetable oil (Table 1); m is the sample mass (0.5 g); f is the oil fraction (0.25).

Initial reaction rates have been analyzed by the formation of FFA (mM) in the first 12 h of reaction. The results were plotted using the software Origin Pro version 5.0 to obtain a linear equation for initial hydrolysis reaction rates of each vegetable oil. The calculation of free fatty acids is performed as described in Equation 5.

$$FFA\ (mmol/L) = \frac{V_a - V_b.C_{NaOH}.10^3}{m} \qquad Equation\ 5$$

Where: V_a is the volume of NaOH in the sample (mL); V_b is the volume of NaOH in the control (mL); C_{NaOH} is the molar concentration of NaOH (20 mM); m is the sample mass (0.5 g).

3 RESULTS AND DISCUSSION

3.1 Selection of culture conditions for the mycelium-bound lipase produced from *Rhizopus oryzae* CCT3759

The growth and regulation of metabolic activities of microorganisms are directly influenced by physical-chemical conditions of the culture medium and the characteristics of each microorganism, for example, TAG substrates can be applied as inducers in lipase production [47]. The first stage of this work consisted in investigating the best culture conditions for obtaining a mycelium-bound lipase with high catalytic activity. Six vegetable oils with different fatty acid compositions (Table 1) have been studied as inducers for producing

a lipase bound to the mycelium of *Rhizopus oryzae* CCT3759. These vegetable oils were selected due to their different fatty acid compositions that can be easily obtained in our country (Brazil).

Table 2 shows the average values of dry biomass concentration and the hydrolytic activity of the biomass produced. According to the results, all oils showed greater lipase retention onto the mycelium of fungal biomass, since the lipase produced was mostly retained onto the fungus mycelium due to lower values of lipolytic activity in the fermentation liquid extract (< 30.0 U/mL, see Table 3).

The results obtained in cultures with olive oil and canola oil showed the best results regarding biomass concentration (dry biomass), reaching 35.0 ± 2.0 and 17.2 ± 0.6 g/L, respectively. In fact, the results obtained by using olive oil as inducer showed higher values than other vegetable oils in all studied cultivation times (24, 48, 72 and 96 h), with its lowest biomass concentration being 24.5 ± 4.0 g/L, that is higher than maximum values obtained for the other evaluated vegetable oils (see Table 2). Olive oil and canola oil also reached high values of lipolytic activity (389.1 ± 16.2 and 364.5 ± 13.2 U/g, respectively), but lower than the results provided by the cottonseed oil (764.3 ± 36.0 U/g) after 72 h of cultivation.

Both vegetable oils (olive oil and canola oil) showed results that suggest greater activity retention onto the mycelium after reaching maximum activity after 72 h of culture. After this fermentation period, an activity reduction of 17.0% was observed for olive oil (from 389.1 to 322.9 U/g) and 32.5% for canola oil (from 364.5 to 245.9 U/g) for 96 h of culture. A drastic activity reduction of 72.5% was also observed for the lipase produced using cottonseed oil (764.3 U/g in 72 h to 209.8 U/g at the end of 96 h of culture). These results may be due to the need for a new carbon source after substrate consumption (TAG and derivatives), in which there was a consumption of primary metabolite (enzyme) to maintain cell growth.

Table 2. – Influence of cultivation time of *Rhizopus oryzae* CCT3759 on biomass concentration (g/L) and hydrolytic activity of the mycelium-bound lipase (U/g).

Vegetable Oil	24 h		48 h		72 h		96 h	
	Hydrolytic Activity (U/g)	Dry Biomass (g/L)	Hydrolytic Activity (U/g)	Dry Biomass (g/L)	Hydrolytic Activity (U/g)	Dry Biomass (g/L)	Hydrolytic Activity (U/g)	Dry Biomass (g/L)
Cottonseed	450.0 ± 20.6	3.2 ± 1.0	764.3 ± 36.0	5.6 ± 0.3	715.0 ± 57.8	7.9 ± 1.2	209.8 ± 11.0	13.1 ± 4.5
Olive	291.9 ± 9.5	24.5 ± 4.0	275.2 ± 30.6	30.9 ± 1.9	389.1 ± 16.2	30.5 ± 2.1	322.9 ± 18.0	35.0 ± 2.0
Canola	267.8 ± 4.9	1.6 ± 0.1	336.8 ± 13.4	5.8 ± 1.0	364.5 ± 13.2	17.2 ± 0.6	245.9 ± 16.7	13.2 ± 3.0
Sunflower	252.1 ± 15.1	3.0 ± 0.6	388.2 ± 30.7	6.7 ± 0.6	263.7 ± 12.1	10.1 ± 0.9	181.4 ± 20.1	10.3 ± 0.4
Corn	335.0 ± 13.0	3.1 ± 0.9	285.7 ± 56.0	4.3 ± 0.6	233.2 ± 20.2	5.7 ± 0.6	189.5 ± 20.6	7,8 ± 0.2
Soybean	385.4 ± 35.0	4.5 ± 3.8	251.4 ± 36.1	3.1 ± 0.2	287.8 ± 37.1	6.1 ± 0.1	195.8 ± 7.4	7,0 ± 0.7

Table 3. – Influence of cultivation time of *Rhizopus oryzae* CCT3759 on hydrolytic activity in the fermentation liquid extract (U/mL).

Vegetable Oil	Hydrolytic activity (U/mL)			
	24 h	48 h	72 h	96 h
Cottonseed	25.0 ± 0.5	20.1 ± 1.0	21.0 ± 0.9	19.8 ± 0.7
Olive	23.9 ± 0.4	27.4 ± 0.6	23.3 ± 0.4	25.4 ± 0.3
Canola	24.5 ± 0.8	28.0 ± 3.1	28.7 ± 1.3	24.9 ± 0.3
Sunflower	26.2 ± 1.2	25.7 ± 1.1	26.2 ± 1.8	19.3 ± 3.7
Corn	23.2 ± 1.6	25.8 ± 1.4	23.1 ± 1.7	20.6 ± 1.4
Soybean	22.8 ± 4.1	28.2 ± 1.2	23.3 ± 0.6	19.6 ± 0.9

In this study, enzyme productivity for the selection of vegetable oil as a carbon source was also evaluated and evidenced greater microorganism selectivity for olive oil, as shown in Figure 1. According to these results, a maximum enzyme productivity of 12,322.2 U/L was observed after 72 h of culture using olive oil as inducer.

According to Table 2 and Fig. 1, the difference in enzymatic activities and productivity presented by lipases produced over culture time can be explained by the different composition of fatty acids present in all oils. Studies suggest that the culture of *Rhizopus oryzae* cells using vegetable oils with higher concentration of oleic and linoleic acids in their composition efficiently obtained a fungal biomass with high catalytic activity [48,49]. The same behavior was observed in this study, once olive oil has the highest percentage of oleic acid (74.5% m/m), in addition to palmitic acid (11.2% m/m), which provides whole-cell lipases with greater activities [37,47]. In this sense, while olive oil and canola oil are rich in oleic acid, over 60%,

the other studied oils present percentages below 28% (Table 1), thus explaining the low yields of hydrolytic activity obtained using the other oils.

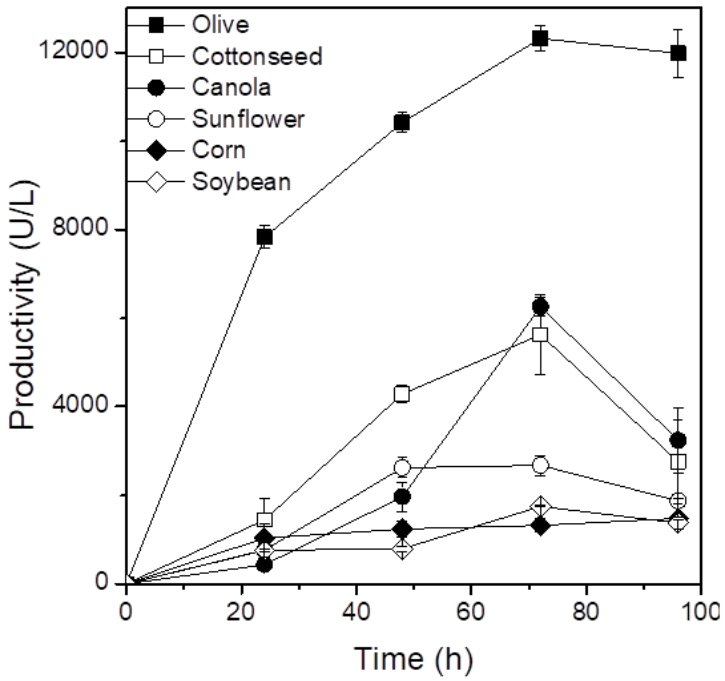

Figure 1. Effect of vegetable oil and cultivation time of *Rhizopus oryzae* CCT3759 on the enzyme productivity (U/L).

Similar results have been achieved in other studies involving the production of whole cells with the lipolytic activity of *Rhizopus oryzae* lipolytic [44,50–52]. Hama et al [48] demonstrated that, with the use of oleic acid or olive oil as inducer to produce *Rhizopus oryzae* lipases, it was found a strong inhibition of lipase secretion and a high amount of lipase located in the cell wall and membrane. Andrade et al [53] and Lima et al [54] evaluated the effect of different vegetable oils on cell growth and the lipolytic activity of *Mucor circinelloides*, moreover, it was observed that the highest values of hydrolytic activity and cell growth have been achieved in both studies by using olive oil as carbon source due to a high concentration of oleic acid in its composition. Marotti et al. [37], under the same cultivation conditions selected in this work, carried out a selection of species of fungi belonging to the genus *Penicillium* that

produce a mycelium-bound lipase and the highest values of lipolytic activity and cell concentration have also been obtained from different strains using olive oil as carbon source.

Based on the achieved results, the culture of subsequent experiments was carried out using 30 g/L of olive oil, and a submerged culture has been performed for 72 h.

3.2 Biochemical characterization of mycelium-bound lipase

Biochemical and kinetic characteristics are presented in Figure 2. The effect of temperature on the mycelium-bound lipase activity was in the range of 25 ºC to 60 ºC and pH 6.5 (Figure 2a) which showed that, in these experimental conditions, the produced lipase has greater catalytic activity at 40 ºC with hydrolytic activity of 738.1 ± 11.8 U/g (relative activity of 100%). Lipase has been proved capable of acting in a wide temperature range (25–60 ºC) with relative activity above 80%. Temperatures above 40 ºC resulted in a progressive reduction of hydrolytic activity due to the enzyme thermal inactivation. Similar results have been observed in previous studies [55,56].

After determining optimal hydrolysis temperature, the effect of pH on the hydrolytic activity of the mycelium-bound lipase was also evaluated (Figure 2b). Under experimental conditions, it is observed that the produced lipase showed higher values of catalytic activity at pH 6.0 (maximum activity of 869.5 U/g ± 8.8 U/g) and a slight increase in pH promoted a slight decay of enzyme, maintaining 72% of its maximum activity at pH 6.5 (631.4 ± 8.2 U/g). Similar results have been reported for whole-cell *Rhizopus oryzae* S3 lipase [56].

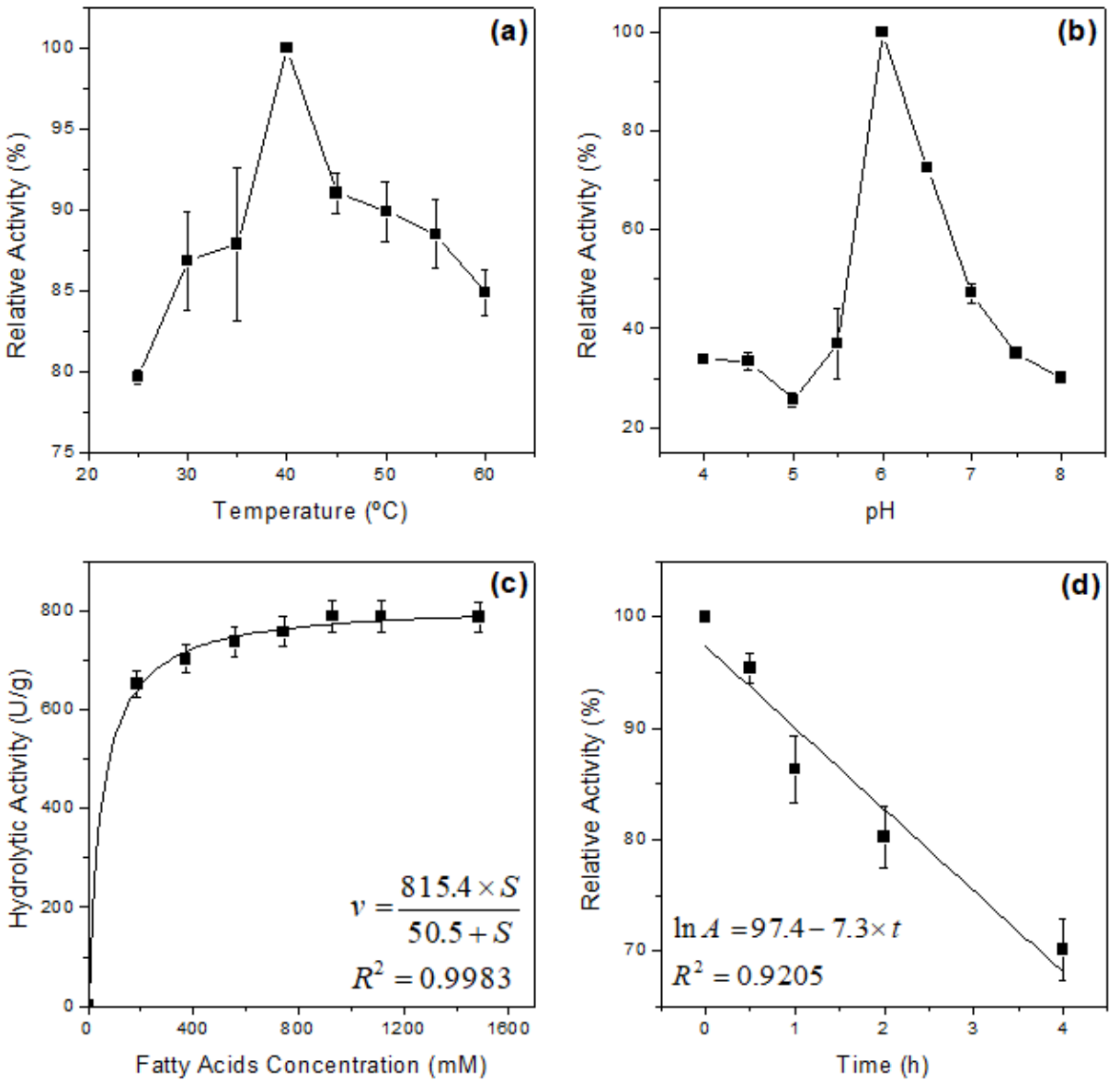

Figure 2. Biochemical and kinetic characterization of the lipase bound to the mycelium of *Rhizopus oryzae* CCT3759 in the hydrolysis of olive oil emulsion. (a) Effect of reaction temperature (maximum activity of 738.1 ± 11.8 U/g, defined as 100% relative activity); (b) Effect of pH (maximum activity of 869.5 ± 8.8 U/g, defined as 100% relative activity); (c) Effect of olive oil concentration on the hydrolytic activity and estimation of apparent kinetic parameters; (d) Thermal stability tests at 50 ºC and estimation of denaturation parameters.

The apparent Michaelis constant (K_m) and maximum reaction rate (V_{max}) of the reaction were determined through olive oil hydrolysis emulsified with Gum Arabic in the range of 5 to 40% m/m, which is equivalent to a concentration of 186 fatty acids at 1488 mM (Fig. 2C). The reactions have been carried out under the optimum conditions determined above (100 mM buffer sodium phosphate pH 6.0 and 40 °C). These parameters were determined by a non-linear adjustment of the Michaelis-Menten model, thus obtaining a high correlation coefficient (R^2) of 0.9983. The apparent values of K_m and V_{max} were 50.5 mM and 815.4 μmol/g.min, respectively. The produced lipase showed higher V_{max} and greater affinity (K_m) to the substrate (olive oil) than those produced using different fungus species, such as *Penicillium italicum* (539.1 μmol/min and 151.3 mM); *Penicillium janthinellum* (387.6 μmol/min and 123.6 mM); *Penicillium purpurogenum* (493.8 μmol/min and 141.4 mM) [37]; *Penicillium citrinum* (123.2 μmol/g.min and 158.1 mM) [36]; *Penicillium citrinum* (267.3 μmol/g.min and 136.5 mM) [54]; *Mucor circinelloides* (186.9 μmol/g.min and 115.7 mM) [57].

The enzyme inactivation profile is shown in Figure 2d, in which the lipase was maintained at pH 6.0 (100 mM buffer sodium phosphate) and 50 ºC. After 4 h, the lipase showed retention of approximately 75% of its initial activity (866.87 ± 5.4 U/g). The linear decay model fitted well to the experimental data ($R^2 = 0.9202$), in which it was possible to determine thermal inactivation constant (K_d) and half-life ($t_{1/2}$) - 0.0733 h^{-1} and 9.4 h, respectively. This result is of great industrial interest, because the longer the enzyme remains active and stable, the lower the number of required replacements of the biocatalyst and therefore have to reduce the costs involved. The produced lipase showed greater thermostability than other studies found in literature. Essamri and Deyris and Comeau [55] reported that lipase was inactivated at 40 ºC for 30 min of incubation time. Razak et al [56] reported that there was retention of 70% of initial activity after incubation at 50 ºC for 3 h.

3.3 Vegetable oils hydrolysis in a tank-stirred reactor

After determining the best cultivation conditions and evaluating the parameters that maximize the hydrolysis reaction such as pH, reaction temperature and substrate concentration, as described above, it was evaluated the performance of *Rhizopus oryzae* CCT3759 lipase in the hydrolysis of six different vegetable oils using two different amounts of biocatalyst (200 U and 400 U) in order to maximize the FFA production. Enzymatic hydrolysis reactions of vegetable oils were carried out under the best reaction conditions obtained for olive oil emulsion described above (100 mM buffer sodium phosphate pH 6.0 and 40°C). These tests were carried out using an initial vegetable oil concentration of 25% m/m, since a good dispersion of whole-cells in the reaction medium was observed in this condition, which resulted in maximum enzyme activity (see Figure 2C). In preliminary tests performed at a concentration of vegetable oils at 50% m/m that is maximum concentration assessed in tests for determining apparent kinetic parameters (see Figure 2d), it was observed a strong aggregation of oil droplets to the mycelium under such experimental conditions. The enzymatic hydrolysis profiles of vegetable oils and the determination of initial reaction rate values obtained using 200 U and 400 U of enzymatic activity are shown in Figure 3.

According to Figures 3a,b, the achieved increase in activity from 200 U to 400 U has resulted in higher initial reaction rates in the first 12 h of reaction using canola, sunflower and soybean oils. As it can be observed in Table 4, such higher activity increased the initial reaction rate values obtained from the hydrolysis of canola oil (36.8 – 46.9 mM/h), sunflower (33.1 – 51.1 mM/h) and soybean (26.2 – 40.7 mM/h), as expected. On the other hand, similar values of initial reaction rates for cottonseed oil (42.2 – 42.5 mM/h), olive oil (43.6 – 47.7 mM/h) and corn oil (42.5 – 44.9 mM/h) have been obtained in the same conditions. These results could be due to high selectivity of this lipase to hydrolyze preferentially vegetable oils containing high concentration of oleic and linoleic acids in their compositions.

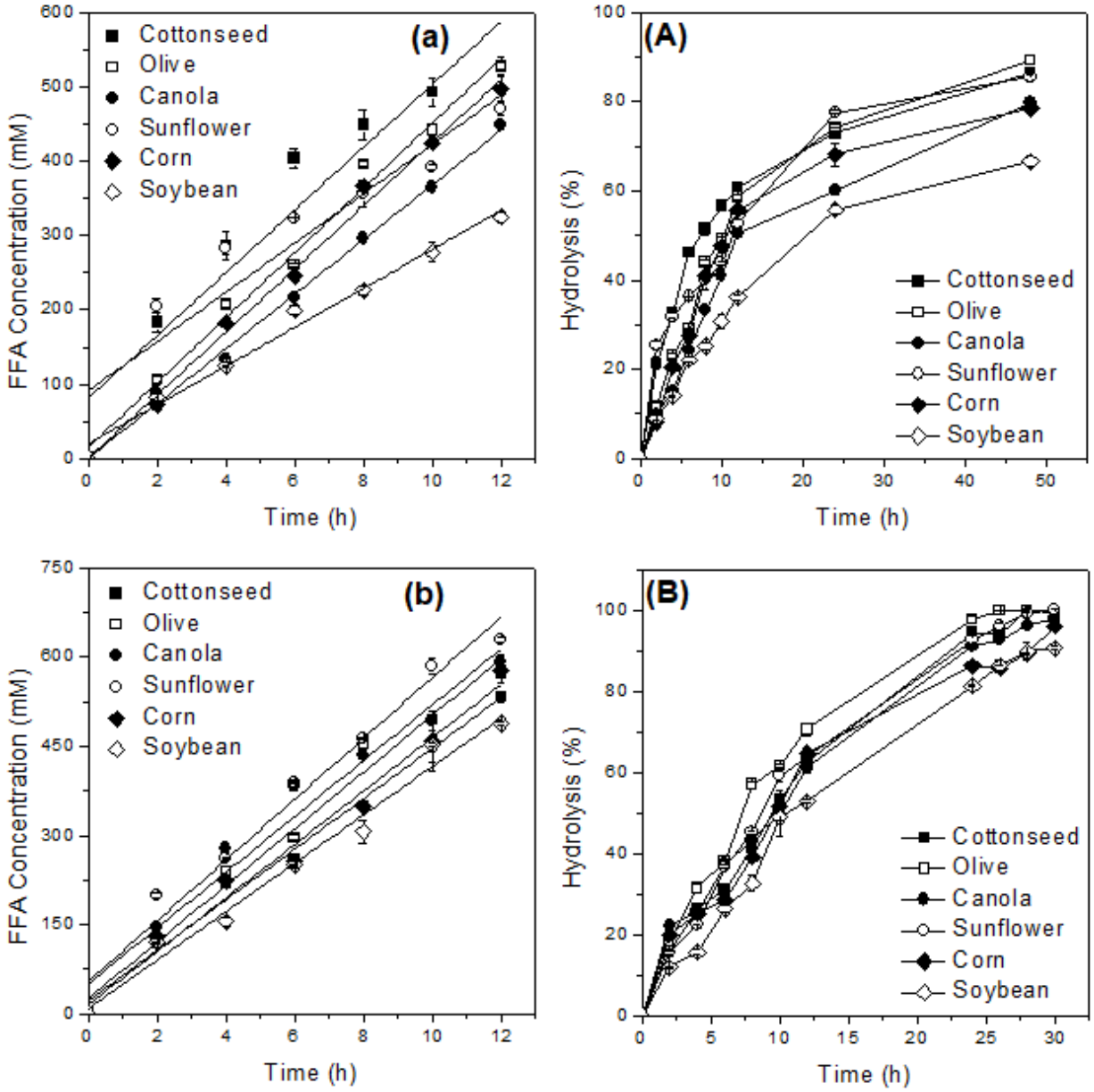

Figure 3. Effect of mycelium concentration (200 U and 400 U) on the initial reaction rate (FFA concentration versus reaction time) and hydrolysis percentage of vegetable oils. The reactions were carried out at 25% m/m of oil containing 3% m/v of Gum Arabic, 40 ºC, pH 6.0 (100 mM buffer sodium phosphate) and mechanical stirring frequency of 600 rpm.

Table 4. Effect of initial activity (U) on initial reaction rate (v) and hydrolysis percentage of vegetable oils performed in stirred-tank reactors using whole-cell *Rhizopus oryzae* CCT3759 as biocatalyst.

Vegetable oil	Activity (U)	Hydrolysis (%)	Time (h)	Linearized equation	R^2	v (mmol/h)
Cottonseed	200	86.4 ± 0.4	48	$y = 81.23 + 42.23x$	0.9027	42,2
	400	99.7 ± 0.3	28	$y = 21.70 + 42.55x$	0,9876	42.5
Olive	200	89.2 ± 1.4	48	$y = 14.87 + 43.65x$	0.9886	43.6
	400	~100	26	$y = 25.57 + 47.67x$	0.9791	47.7
Canola	200	79.5 ± 0.8	48	$y = 0.368 + 36.79x$	0.9960	36.8
	400	98.0 ± 2.0	30	$y = 50.84 + 46.92x$	0.9616	46.9
Sunflower	200	85.5 ± 0.9	48	$y = 91.10 + 33.13x$	0.8576	33.1
	400	99.0 ± 1.0	28	$y = 54.57 + 51.14x$	0.9703	51.1
Corn	200	78.5 ± 0.6	48	$y = 0.351 + 42.50x$	0.9924	42.5
	400	96.1 ± 0.7	30	$y = 15.36 + 44.93x$	0.9805	44.9
Soybean	200	66.7 ± 1.3	48	$y = 19.48 + 26.18x$	0.9827	26.2
	400	90.1 ± 2.0	28	$y = 8.81 + 40.73x$	0.9783	40.7

According to Figure 3A, hydrolysis conducted with 200 U has achieved hydrolysis percentages ranging from 66.7 ± 1.3% to 86.4 ± 0.4% after 48 h of reaction. As expected, higher amounts of enzyme in the reaction (400 U) also increased the percentage of hydrolysis and reduced reaction time (Figure 3B), and a complete hydrolysis of olive, cottonseed, canola and sunflower oils was achieved after 26-30 h reaction. Under these same conditions, hydrolysis

percentage of sunflower and soybean oil of 96% and 90%, respectively, was achieved after 30 h of reaction.

Based on these results, olive oil was the one that achieved the highest hydrolysis percentage (Figure 3B). This indicates that the obtained lipase has high selectivity for TAG containing high concentration of oleic acid ($C_{18:1}$) in their composition as olive oil, about 74.5% m/m. In fact, high hydrolysis percentage was also achieved for cottonseed and sunflower oils due to their high concentration of oleic acid and linoleic acid ($C_{18:2}$), since both present a similar concentration of this fatty acid, as aforementioned. The results obtained in the hydrolysis of soybean oil suggest that *Rhizopus oryzae* CCT3759 lipase has less activity for oils composed of higher proportions of linolenic acid ($C_{18:3}$). These results corroborate the previous results of lipase production, since the microorganism produces lipase to enable the assimilation of vegetable oil as a carbon source for energy production and, consequently, cell growth, production of enzymes and other compounds.

4 CONCLUSION

An application of whole-cells in biocatalysis reactions consists in using microbial biomass with high catalytic activity as biocatalyst, which is a technology that offers advantages such as low production costs, ease of operation, reduced recovery costs, purification or immobilization of lipases. The best culture conditions for obtaining catalytic cells were evaluated in 72 h of submerged culture using olive oil as a carbon source. Under optimal reaction conditions, hydrolysis percentages greater than 90% after 26-30 h of reaction in a stirred tank reactor for all evaluated oils have been obtained. Therefore, this study has demonstrated that whole-cell *Rhizopus oryzae* CCT3759 is an interesting biocatalyst to produce FFA due to its high catalytic activity in mild reaction conditions and selectivity to catalyze ester bonds hydrolysis containing high monounsaturated fatty acids in their composition as oleic acid. Moreover, a more in-depth study is currently being performed by the present research

group using residual oils (frying oil) as inductor to produce whole-cell *Rhizopus oryzae* to be subsequently used as biocatalysts in non-aqueous media, in addition to industrial esters production (emollient esters and biolubricants) via esterification.

Authors' contributions

Willian de S. M. Reis and Alexandre B. Matias carried out the experimental work. Willian de S. M. Reis, Adriano A. Mendes and Ernandes B. Pereira carried out the final editing of the manuscript and the writing of the article. Adriano A. Mendes, Ernandes B. Pereira and Heizir F. de Castro were responsible for conceptualization, supervision and funding acquisition. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001. W.S.M.R. thanks CAPES for the student fellowship. A.A.M. thanks Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq – Brazil) for the research fellowship (Process 310633/2020-6).

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4 CONCLUSÃO

A aplicação de células íntegras em reações de biocatálise consiste em utilizar a biomassa microbiana com alta atividade catalítica como biocatalisador, sendo uma tecnologia que oferece vantagens como baixo custo de produção, facilidade de operação, redução dos custos de recuperação, purificação ou imobilização das lipases. As melhores condições de cultivo para obtenção de células catalíticas foram avaliadas em 72 h de cultivo submerso utilizando azeite de oliva como fonte de carbono. Em condições ótimas de reação, porcentagens de hidrólise superiores a 90% após 26-30 h de reação em reator de tanque agitado para todos os óleos avaliados foram obtidas. Portanto, este estudo demonstrou que células integras de *Rhizopus oryzae* CCT3759 é um biocatalisador interessante para a produção AGL devido à sua alta atividade catalítica em condições de reação moderadas e seletividade para catalisar a hidrólise de ligações éster contendo ácidos graxos monoinsaturados em sua composição como o ácido oleico. Além disso, um estudo mais aprofundado está sendo realizado pelo presente grupo de pesquisa usando óleos residuais (óleo de fritura) como indutor para produzir células integras de *Rhizopus oryzae* para serem posteriormente usados como biocatalisadores em meios não aquosos, além da produção de ésteres industriais (ésteres emolientes e biolubrificantes) via esterificação.

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