Universidade Estadual de Maringá
Programa de Pós-graduação em Ciências Biológicas
Área de concentração: Biologia Celular e Molecular

FAUSTO FERNANDES DE CASTRO

CARACTERIZAÇÃO DA LIPASE LIGADA AO MICÉLIO E DA ESTERASE SECRETADA DE *ASPERGILLUS WESTERDIJKIAE*

Maringá
2018

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Tese apresentada ao Programa de Pós-graduação em Ciências Biológicas (Área de Concentração - Biologia Celular e Molecular) da Universidade Estadual de Maringá, para obtenção do grau de Doutor em Ciências Biológicas.

Orientadora: Profa. Dra. Ione Parra Barbosa-Tessmann

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BIOGRAFIA

Fausto Fernandes de Castro nasceu em Paranavaí, Paraná, em 08/03/1989. Possui graduação em Ciências Biológicas pela Universidade Estadual de Maringá – Paraná (2010) e mestrado em Ciências Biológicas – Biologia Celular e Molecular pela Universidade Estadual de Maringá, Paraná (2013). Em março de 2014 iniciou o curso de Doutorado em Ciências Biológicas - Biologia Celular e Molecular pela mesma instituição. Tem experiência na área de Bioquímica e Biologia Molecular de Microrganismos.

DEDICATÓRIAS

Dedico este trabalho a minha família, razão de minha existência.

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How many oceans are there still in me?
How many can I not see?
How many more shall I sail?
Which are just waiting for the next gale?

Oh, my relentless thirsty waters!
Tempestuous florid leas,
trite vain brooks,
fetid muddy seas,
forgotten nooks
are all your own makers!

You reap the humidity no one placed,
the drop which very few faced,
from fields where blistering suns cracked the soil,
from streams thrown into turmoil

The seas of whom must I fear
whilst you blend others' waters,
creating excruciating bores inside?
What will remain after their final clatter,
after curbing their dusk, taming their tides?
How much will dry below my placid veneer?

Whom will I leave ashore?
Who will ever comprehend my many a bore?
Many will try their dams
Very few will only sail across them
Even fewer will dive
into these oceans, into these oceans of mine

Quantos oceanos ainda existem em mim?
Quantos não consigo ver?
Quantos mais devo navegar?
Quais estão somente esperando o próximo vendaval?

Ah, minhas implacáveis águas sedentas!
Prados tempestuosos floridos,
ribeiros banais vãos,
mares fétidos enlameados,
recantos esquecidos
são todos os seus próprios criadores!

Você colhe a umidade que ninguém colocou,
a gota que muito poucos enfrentaram,
de campos onde sóis intensos racharam o solo,
de córregos jogados em turbulência

Os mares de quem devo temer,
enquanto você mistura as águas dos outros,
criando macaréus excruciantes?
O que permanecerá após o seu ruído final,
depois de refrear suas escuridões, domesticar suas marés?
Quanto vai secar abaixo de minha aparência plácida?

Quem eu deixarei na praia?
Quem alguma vez entenderá os meus muitos macaréus?
Muitos tentarão suas barragens
Muito poucos navegarão neles
Menos ainda mergulharão
nesses oceanos, nesses oceanos meus

Fausto Fernandes de Castro

APRESENTAÇÃO

Esta tese é composta por dois artigos científicos. O primeiro concerne a descoberta do fungo lipolítico *Aspergillus westerdijkiae* e a caracterização de uma lipase ligada ao seu micélio, publicado na revista *Biocatalysis and Agricultural Biotechnology*. O segundo artigo concerne a otimização da produção, purificação e caracterização de uma esterase secretada pelo mesmo fungo, publicado na revista *Journal of Basic Microbiology*.

Castro, F.F.; Pinheiro, A.B.P.; Nassur, C.B.; Barbosa-Tessmann, I.P. Mycelium-bound lipase from a locally isolated strain of *Aspergillus westerdijkiae*. **Biocatalysis and Agricultural Biotechnology**, 10, 321-328, 2017. DOI.org/10.1016/j.bcab.2017.04.009

Castro, F.F.; Pinheiro, A.B.P.; Gerhardt, E.C.M.; Oliveira, M.A.S.; Barbosa-Tessmann, I.P. Production, purification, and characterization of a novel serine-esterase from *Aspergillus westerdijkiae*. **Journal of Basic Microbiology**, 58, 131–143, 2018. DOI: 10.1002/jobm.201700509.

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CAPÍTULO II

LISTA DE ABREVIAÇÕES

µg – Microgram(s).

µL – Microliter(s).

AN – GenBank Acession Number.

bp – Base pairs

CTAB – Cetyltrimethylammonium bromide.

CV - Coefficient of variation.

DNA – Deoxyribonucleic Acid.

EDTA – Ethylenediaminetetraacetic acid

g – Gram(s).

g – Gravitacional force

h – Hour(s).

ITS4 e ITS5 – Primers targeted to the internal transcribed sequence of fungi rDNA.

kb - Kilobases

K_M – Michaelis-Menten Constant.

LED – Lipase Engineering Database.

M – Molar.

M.M. – Molecular markers

m/v – Mass/volume.

mg – Miligram(s).

min – Minute(s).

mL – Mililiter(s).

mM – Milimolar.

MS – Mass spectrometry.

ng – Nanogram(s).

PCR – Polymerase Chain Reaction.

PDA – Potato Dextrose Agar.

PMSF – Phenylmethylsulfonyl fluoride.

p-NP – *p*-nitrophenol.

p-NPA – *p*-nitrophenyl acetate.

p-NPB – *p*-nitrophenyl butyrate.

p-NPD – *p*-nitrophenyl dodecanoate.

p-NPP – *p*-nitrophenyl palmitate.

RNAase – Ribonuclease.

SD – Standard Deviation.

SDS – Sodium Dodecyl Sulphate.

SDS-PAGE – Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis.

s – Second(s).

TAE – Tris-Acetate-EDTA Buffer.

TE – Tris-EDTA Buffer.

U – International Units.

UEM – Universidade Estadual de Maringá.

USA – United States of America.

UV – Ultraviolet.

V_{max} – Maximal Reaction Velocity.

w/v – Weight/volume.

^{o}C – Celsius degree.

RESUMO GERAL

Introdução. Esterases e lipases são enzimas lipolíticas que atuam sobre triglicerídeos. Elas podem hidrolisar as ligações éster ou formá-las nas reações de interesterificação, transesterificação e esterificação. Não só essas α/β hidrolases compartilham os tipos de reações que podem catalisar, mas também compartilham muitas propriedades estruturais. Apesar destas semelhanças, as esterases hidrolisam ésteres simples e triglicerídeos contendo ácidos graxos com menos de seis carbonos, enquanto que as lipases preferem triglicerídeos com ácidos graxos de cadeia longa, insolúveis em água. Devido ao fato de que estas enzimas possuem alta regio- e estereoespecificidade, não requerem cofatores e são estáveis e muitas vezes ativas em solventes orgânicos, elas são muito valorizadas, especialmente na síntese orgânica. As lipases ganharam atenção especial devido à sua maior estabilidade em solventes orgânicos e especificidade de substrato mais ampla. No entanto, muitas aplicações de lipases, entre elas a produção de biodiesel, são impedidas pelo seu alto custo. O emprego de esterases na síntese de compostos opticamente puros colocou este grupo em evidência, mas seu uso também enfrenta problemas, principalmente devido à escassez de disponibilidade comercial e à enantioseletividade moderada. Esses obstáculos podem ser contornados de muitas maneiras diferentes e a descoberta de novos microrganismos lipolíticos e suas enzimas é uma das soluções mais diretas. **Objetivos.** Em busca da descoberta de novos fungos lipolíticos, um produtor foi isolado de tanques de coleta para a reciclagem de óleo de cozinha usado. Foi verificado que este fungo é produtor de uma esterase e uma lipase ligada ao micélio. Os objetivos deste trabalho foram caracterizar sua lipase ligada ao micélio e avaliar seu possível uso na síntese orgânica. Além disso, este trabalho também teve como objetivo otimizar a produção de uma nova esterase, purificá-la e caracterizá-la. **Materiais e métodos.** Uma bioprospecção de novos fungos lipolíticos foi realizada em amostras de óleo de cozinha usado coletadas de tanques para reciclagem e gordura coletada de uma caixa de gordura de uma casa localizada na cidade de Maringá, Paraná, Brasil. As amostras foram distribuídas em placas de ágar rodamina B contendo antibióticos para isolar fungos e avaliar a produção de lipase concomitantemente. Os fungos assim obtidos foram isolados por meio de isolamento monospórico e a sua capacidade de produzir esterases foi avaliada em placas de ágar tributirina. Os isolados foram identificados por meio de DNA *barcoding*, na qual a região do rDNA 5.8S-ITS foi amplificada por PCR e sequenciada, e a sequência obtida foi comparada com sequências em bancos de dados. Um produtor lipolítico não descrito foi identificado, o fungo *Aspergillus westerdijkiae*. Foi verificado que a fluorescência nas placas de ágar de rodamina B estava associada ao micélio devido à presença de uma lipase. Para estudar esta lipase ligada ao micélio, o fungo foi cultivado em meio basal contendo óleo de oliva para obter um micélio fresco. O micélio foi coletado por filtração sob vácuo em papel de filtro e desengordurado com acetona. A acetona foi removida por evaporação e o micélio foi liofilizado e moído a um pó. A atividade enzimática foi avaliada medindo a taxa de hidrólise do *p*-nitrofenil palmitato (*p*-NPP) ou titulação. Várias propriedades da lipase ligada ao micélio foram avaliadas, a saber, inducibilidade por óleo de oliva, efeito da concentração do *p*-NPP na velocidade inicial, reciclabilidade, temperatura ótima, estabilidade térmica, pH ótimo, estabilidade em diferentes pHs, especificidade de substrato, potenciais inibidores e ativadores, e capacidade de esterificar o ácido oleico com etanol. Foi verificado nas placas de ágar tributirina que o fungo também produz esterase. Assim, em outro trabalho, uma esterase secretada foi estudada. Em primeiro lugar, uma curva de crescimento e produção de esterase foi realizada em meio basal contendo óleo de oliva. Após a separação do micélio do meio por filtração sob vácuo em papel de filtro, a atividade da esterase

foi avaliada no filtrado. A atividade enzimática foi avaliada pela hidrólise do *p*-nitrofenil butirato (*p*-NPB) ou titulação. Vários parâmetros, a saber, diferentes óleos, tamanho de inóculo, pH inicial, adição de carboidratos e íons, presença e ausência de agitação, luz e emulsificantes foram avaliados passo a passo para otimizar a síntese de esterase. A purificação da esterase foi realizada com o filtrado de uma cultura de 10 frascos nas condições otimizadas. As proteínas do filtrado foram precipitadas com sulfato de amônio, dialisadas e aplicadas numa coluna cromatográfica Sephacryl S-200 HR. Foram analisadas diferentes características da esterase purificada, isto é, K_M e $V_{máx}$, pH ótimo, estabilidade em diferentes pHs, temperatura ótima, estabilidade térmica, especificidade de substrato, potenciais inibidores e ativadores, e resistência a solvente orgânico. Além disso, a esterase foi analisada por espectrometria de massa MALDI-TOF/TOF. **Resultados e Discussão.** A presença de uma lipase ligada ao micélio de *A. westerdijkiae* foi confirmada pela dependência da velocidade inicial em relação à concentração de *p*-NPP (rendimento médio de atividade de 40.000 U/g de micélio) e sua atividade contra o óleo de oliva (52 U/g de micélio) e tributirina (78 U/g de micélio). Além disso, a produção de lipase ligada ao micélio foi induzida por óleo de oliva. Foi verificado que a enzima também pode ser usada repetidamente, mantendo 35,8% de sua atividade após a terceira rodada de reação contra *p*-NPP. A temperatura ótima encontrada foi de 40 ^{o}C, permanecendo estável a 50 ^{o}C por 6 horas, mantendo 81% de sua atividade, e apresentou uma T_{50} calculada de 62 ^{o}C. Além disso, mostrou um pH ótimo de 7,0, mas pôde atuar com mais de 50% de atividade na faixa de pH de 5,0 a 9,0. A lipase ligada ao micélio foi inibida pelos íons Na^{+}, Ca^{+2}, Hg^{+2}, Ni^{+2}, pelos compostos EDTA e PMSF e pelos detergentes não iônicos Tween 20, Tween 80 e Triton X-100. A enzima apresentou atividade contra diferentes ésteres de ácidos graxos de *p*-nitrofenil, além do palmitato, a saber, *p*-nitrofenil acetato (C2), butirato (C4) e dodecanoato (C12). A capacidade da lipase ligada ao micélio de esterificar em solvente orgânico foi verificada em *n*-heptano, ao qual foram adicionados ácido oleico e etanol. A formação de oleato de etila foi confirmada por TLC e um rendimento máximo de conversão de 35% foi obtido. A curva de crescimento e de produção da esterase de *A. westerdijkiae* mostrou um pico de produção após três dias de cultura. Após testar vários parâmetros, foram encontradas as melhores condições para a produção da esterase, com as quais uma atividade máxima de 24 U/mL de filtrado foi alcançada. O tamanho da esterase purificada foi estimado em 32 kDa por SDS-PAGE. De todos os substratos testados, a esterase mostrou apenas atividade contra os ésteres de cadeia curta, o *p*-nitrofenil butirato (100% da atividade relativa) e *p*-nitrofenil acetato (6,1%). A enzima apresentou uma temperatura ótima de 40 ^{o}C, manteve 60% de sua atividade após 8 horas de incubação na mesma temperatura e a T_{50} calculada foi de 49 ^{o}C. A enzima mostrou um perfil alcalino, com a melhor atividade na faixa de pH de 7,0 a 9,0 e pH ótimo de 8,0. A enzima permaneceu estável em uma ampla gama de pHs (5,0 a 9,0) após 6 horas de incubação. O K_M e a $V_{máx}$ foram de 638 μM para *p*-NPB e 5,47 μM de *p*-nitrofenol liberado $\cdot$ $min^{-1} \cdot \mu g^{-1}$ de proteína, respectivamente. A enzima foi parcialmente ativa na presença de 25% de acetona, etanol, metanol, isopropanol e acetonitrila. A atividade da esterase foi fortemente inibida por Hg^{+2}, Ni^{+2}, PMSF, EDTA e pelos detergentes não iónicos Triton X-100, Tween 20 e Tween 80, enquanto foi ativada por K^{+}, Na^{+} e SDS. Sequências de peptídeos da esterase obtidas por espectrometria de massa permitiram que a proteína fosse encontrada no genoma anotado de *A. westerdijkiae* e seu modelo estrutural revelou que a proteína é de fato um membro da superfamília das α/β-hidrolases. **Conclusões.** Um novo fungo lipolítico foi identificado neste estudo. Duas enzimas lipolíticas, i.e., uma lipase ligada ao micélio e uma esterase secretada, foram caracterizadas e ambas as enzimas apresentaram propriedades que podem se revelar muito úteis em diferentes aplicações biotecnológicas.

ABSTRACT

Introduction. Esterases and lipases are lipolytic enzymes that act on triglycerides. They can either hydrolyze ester bonds or form them in interesterification, transesterification, and esterification reactions. Not only do these α/β hydrolases share the reactions types they can catalyze, but also many structural properties. Despite these similarities, esterases hydrolyze simple esters and triglycerides containing fatty acids shorter than six carbons, whereas lipases prefer triglycerides with long-chain fatty acids, insoluble in water. Owing to the fact that these enzymes have high regio- and stereospecificity, do not require cofactors, and are stable and often active in organic solvents, they are greatly valued, especially in organic synthesis. Lipases have gained special attention because of their greater stability in organic solvents and broader substrate specificity. Nonetheless, many applications of lipases, among which is the production of biodiesel, are impeded by their high cost. The employment of esterases in the synthesis of optically pure compounds has brought this group into focus, but their use also faces problems, mainly due to their scarce commercial availability and moderate enantioselectivity. These obstacles can be circumvented in many different ways and the discovery of new lipolytic microorganisms and their enzymes is one of the most straightforward solutions. **Objectives**. In pursuance of the discovery of new lipolytic fungi, a producer has been isolated from collection recipients for the recycling of cooking oil. This fungus was found to produce an esterase and a mycelium-bound lipase. The objectives of this work were to characterize its mycelium-bound lipase and assess its possible use in organic synthesis. Furthermore, this work also intended to optimize the production of a new esterase, purify, and characterize it. **Materials and Methods**. A bioprospection for new lipolytic fungi was carried out in samples of used cooking oil collected from recycling tanks and fat collected from a private house grease trap in the city of Maringá, Paraná, Brazil. The samples were distributed on rhodamine B agar plates containing antibiotics in order to concomitantly isolate fungi and evaluate lipase production. The fungi thus obtained were isolated by means of monosporic isolation and their ability to produce esterases were assessed with tributyrin agar plates. The isolates were identified by dint of DNA barcoding, in which the 5.8S-ITS rDNA region was amplified by PCR, sequenced, and the obtained sequence was compared with sequences in data banks. An unreported lipolytic producer was identified, o fungo *Aspergillus westerdijkiae*. Fluorescence in Rhodamine B agar plates was found to be associated with its mycelium due to the presence of a lipase. In order to study this mycelium-bound lipase, the fungus was cultivated in a basal medium containing olive oil so as to obtain fresh mycelium. The mycelium was collected by vacuum filtration on filter paper and defatted with acetone. The acetone was removed by evaporation and the mycelium was freeze-dried and ground to a powder. The enzymatic activity was evaluated by measuring the hydrolysis rate of *p*-nitrophenyl palmitate (*p*-NPP) or by titration. Various properties of the mycelium-bound lipase were assessed, namely, inducibility by olive oil, effect of *p*-NPP concentration on initial velocity, potential recyclability, optimal temperature, thermal stability, optimal pH, pH stability, substrate specificity, effect of potential inhibitors and activators, and its ability to esterify oleic acid with ethanol. In tributyrin agar plates, the fungus was also found to produce esterase. Thus, in another work, a secreted esterase was studied. First, a five-day time course of fungi growth and esterase production was performed in a basal medium olive oil. After separation of the mycelium from the medium by vacuum filtration on filter paper, the esterase activity was assessed in the filtrate. The enzymatic activity was evaluated by the hydrolysis of *p*-nitrophenyl butyrate (*p*-NPB) or titration. Various parameters, viz. different oils, inoculum size, initial pHs, addition of carbohydrates and ions,

presence and absence of agitation, light, and emulsifiers were assessed stepwise in order to optimize the esterase synthesis. The esterase purification was achieved with the filtrate of a 10-flask culture in the optimized conditions. The proteins of the filtrate were precipitated with ammonium sulphate, dialyzed, and applied on a Sephacryl S-200 HR chromatographic column. Different characteristics of the purified esterase were analyzed, viz., its K_M and V_{max}, optimal pH, pH stability, optimal temperature, thermal stability, substrate specificity, potential inhibitors and activators, and organic solvent resistance. What is more, the esterase was analyzed by MALDI-TOF/TOF mass spectrometry. **Results and Discussion.** The presence of a *A. westerdijkiae* mycelium-bound lipase was confirmed by the dependency of the initial velocity on *p*-NPP (average activity yield of 40,000 U/g of mycelium) and its activity against olive oil (52 U/g of mycelium) and tributyrin (78 U/g of mycelium). Furthermore, the mycelium-lipase production was confirmed to be induced by olive oil. The enzyme could also be used repeatedly, retaining 35.8% of its activity after the third round of reaction against *p*-NPP. The enzyme optimal temperature found was 40 ºC. The enzyme remained stable at 50 ºC for 6 hours, retaining 81% of its activity, and presented a calculated T_{50} of 62 ºC. In addition, it showed an optimal pH of 7.0, but could act with more than 50% of activity in the range of 5.0 to 9.0. The mycelium-bound lipase was inhibited by the ions Na^{+}, Ca^{+2}, Hg^{+2}, Ni^{+2}, the compounds EDTA and PMSF and the non-ionic detergents Tween 20, Tween 80, and Triton X-100. The enzyme presented activity against different fatty acid esters of *p*-nitrophenol besides palmitate, namely, *p*-nitrophenyl acetate (C2), butyrate (C4), and dodecanoate (C12). The mycelium-bound lipase ability to esterify in organic solvent was verified in *n*-heptane, to which oleic acid and ethanol were added. The formation of ethyl oleate was confirmed by TLC and a maximum conversion yield of 35% was obtained. The *A. westerdijkiae* esterase biosynthesis and growth curve showed a production peak after three days of culture. After testing various parameters, it was found the best conditions for the esterase production, with which a maximum activity of 24 U/mL of filtrate was achieved. The size of the purified esterase was estimated to be 32 kDa by SDS-PAGE. From all the substrates tested, the esterase only showed activity against the short-chain esters *p*-nitrophenyl butyrate (100% of relative activity) and acetate (6.1%). The enzyme presented an optimum temperature of 40 ºC, retained 60% of its activity after 8 hours of incubation at the same temperature and its calculated T_{50} was 49 ºC. It showed an alkaline profile, with best activity in the pH range from 7.0 to 9.0 and optimum pH of 8.0. The enzyme was stable over a wide range of pHs (5.0 tp 9.0) after 6 hours of incubation. The K_M and V_{max} were 638 µM for *p*-NPB and 5.47 µmol of released *p*-nitrophenol $\cdot$ min^{-1} $\cdot$ μg^{-1} of protein, respectively. The enzyme was partially active in the presence of 25% acetone, ethanol, methanol, isopropanol, and acetonitrile. Esterase activity was strongly inhibited by Hg^{+2}, Ni^{+2}, PMSF, EDTA, and the non-ionic detergents Triton X-100, Tween 20, and Tween 80, while it was activated by K^{+}, Na^{+}, and SDS. Sequences of the enzyme peptides obtained mass spectrometry allowed the protein to be found in the *A. westerdijkiae* genome and its structure model revealed that the protein is indeed a member of the α/β-hydrolase fold superfamily. **Conclusions.** A new lipolytic fungus has been identified in this study. Two new lipolytic enzymes, i.e., a mycelium-bound lipase and a secreted esterase, have been characterized and both enzymes showed properties that may prove to be very useful in different biotechnological applications.

CAPÍTULO I
MYCELIUM-BOUND LIPASE FROM A LOCALLY ISOLATED STRAIN OF *ASPERGILLUS WESTERDIJKIAE*

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ABSTRACT

Lipases can hydrolyze ester bonds in triacylglycerols and can be used, for instance, in detergent industries and in bioremediation. They are also able to esterify in organic interface. A strain of *Aspergillus westerdijkiae* was isolated from used cooking oil. The fungus was cultivated at 25 °C, 100 rpm, for 3 days, in a liquid medium containing olive oil as carbon source. The obtained mycelium was acetone washed, freeze dried, and macerated. The average mycelium yield production was 0.115 g of dried mycelium/25 mL of liquid media. The average enzyme yield was 40,000 U and 52 U per g of dry mycelium, evaluated by the release of *p*-nitrophenol from *p*-nitrophenyl palmitate and by titration of fatty acids released from olive oil, respectively. The enzyme optimum temperature was 40 °C and it was stable at 50 °C for 30 min, presenting a T_{50} of 61.62 °C. The enzyme remained stable after 6 h of incubation at 40 °C or 50 °C. The enzyme optimum pH was 7.0–8.0 and it remained active after incubation of the mycelia for 6 h at pHs 5.0–8.0. Several ions, EDTA, PMSF, and non-ionic detergents inhibited the enzyme, but SDS did not. The enzyme activity also could use other short and medium chain esters as substrates. In addition, the lipase was able to synthesize ethyl oleate from oleic acid and ethanol. In conclusion, the mycelium bound lipase of *A. westerdijkiae* was characterized and this naturally immobilized enzyme could be useful in the biodiesel industry.

Keywords: Lipase; Biodiesel; Mycelium; *Aspergillu*s.

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1. INTRODUCTION

True lipases (EC 3.1.1.3, triacylglycerol hydrolases) are enzymes that can hydrolase ester bonds that connect glycerol and long-chain fatty acids (C≥10) (Singh and Mukhopadhyay, 2012; Gupta et al., 2015). They possess a myriad of properties that ultimately make them biotechnologically important, including their production in large amounts by bacteria and fungi; chemo-, region-, and stereo-selectivity; absence of side reaction catalysis; no cofactor requirement; and stability in water-restricted environments (Singh and Mukhopadhyay, 2012; Gupta et al., 2015). Due to these characteristics, these hydrolases have been used in many fields, such as food technology, detergent, chemical and biomedical sciences industry, synthesis of optically active compounds, and lipid modification (Gupta et al., 2015).

All living organisms are producers of lipases, including plants, microorganisms, and animals, which is in accordance with the fact that these enzymes have a role in the turnover of lipids in nature. Nonetheless, for biotechnological and industrial uses, microorganisms have long been the preferable source of enzymes, owing to the fact that they can be easily genetically modified and their enzymes are more stable, are produced regularly, affordably, and in high yields. A plethora of bacteria and fungi have been reported as good producers of lipases, among which, for example, are the genera *Pseudomonas*, *Burkholderia*, *Bacillus*, *Candida*, *Geotrichum*, *Penicillium*, *Aspergillus*, and *Rhizomucor* (Singh and Mukhopadhyay, 2012; Gupta et al., 2015).

Lipases are also able to synthesize esters in water-restricted environment, catalyzing esterification, inter-esterification, and transesterification reactions (Gupta et al., 2015). They have a pronounced stability in organic solvents, a broad range of substrates, and the ability to catalyze the synthesis reaction as efficiently as the hydrolysis reaction (Singh and Mukhopadhyay, 2012;

Gupta et al., 2015). Thus, lipases can be applied in the synthesis of biodiesel, by the transesterification or esterification of triglycerides or free fatty acids, respectively, with short chain alcohols. Biodiesel, which is an ester of a long chain fatty acid with ethanol or methanol, is an interesting alternative to the inevitable scarcity of fossil fuels in the near future and its wider employment would benefit the globe temperatures, by reducing global warming and emissions of harmful particles, such as sulfur and hydrocarbons, and also by reducing carbon monoxide release (Poppe et al., 2015). Enzymatic catalysis presents advantages over chemical catalysis in that it is more adequate for the raw material quality variations; is reusable; is performed in fewer steps, thereby reducing energy costs and waste; can improve product separation; and yield a higher quality glycerol. Nonetheless, many setbacks hinder the lipases applicability in the biodiesel production, such as the low reaction rate, their cost, the natural loss of activity that happens in the process, and the chemical catalysts still are the preferred choice (Poppe et al., 2015).

Therefore, it is imperative to discover new lipase-producer organisms and/or methods that will ultimately make the enzymatic processes affordable enough to be preferable to the chemical catalyzed biodiesel production (Poppe et al., 2015). One of these methods is the enzyme immobilization, which permits its reutilization, and different methods have been devised so as to immobilize lipases for biodiesel production (Narwal and Gupta, 2013). Mycelium-bound lipases from different fungi are naturally immobilized, very stable, and have been proposed as an inexpensive alternative to overcome the hurdles involved in the use of lipases in biodiesel production and other areas, such as flavor synthesis (Long et al., 1996; Romero et al., 2007; Colin et al., 2011). It is reported in this paper a new mycelium-bound lipase from the fungus *Aspergillus westerdijkiae*, which has the ability to catalyze the synthesis of ethyl oleate.

2. MATERIAL AND METHODS

2.1. Microorganism isolation and maintenance

Initially, a bioprospection for lipase-producing fungi was performed in used cooking oil collected from recycling tanks and in fat collected from a private house grease trap, in the city of Maringá, Paraná, Brazil. The oil was collected in the following places: at the main cafeteria at the Maringá State University, at the food store Cidade Canção located at 903 Tamandaré Avenue, and at the Santa Isabel de Portugal Church located at 188 Jalbas Rodrigues Alves Street. The private residence was located at 54 Pioneiro Raul Parandiuc Street. Ten microliter of the used cooking oil samples or a loopful of fat were streaked on Rhodamine B agar plates (Kouker and Jaeger, 1987). This medium was prepared with 2% olive oil, 1% Tween 80, 0.125 g/% yeast extract, 0.3 g/% peptone, 0.5 g/% tryptone, 0.4 g/% NaCl, agar 2 g/%, pH 5.0 adjusted with lactic acid. Rhodamine B (1.0 mg/L), penicillin (320 UI/mL), and streptomycin (125 μg/mL) were aseptically added after sterilization in autoclave and cooling to 60 °C. After inoculation, the plates were incubated at 25 °C for up to 5 days, with a photoperiod of 12 h, before evaluation. Lipase production was assayed by the formation of a pink fluorescent halo under UV light (312 nm).

Colonies with fluorescent pink color were submitted to monosporic isolation, as described by Nelson et al. (1983), and the monosporic isolates were tested again for lipase production by culture in the Rhodamine B agar medium, as described above. Macro and micro morphological characteristics were used to evaluate the isolates in traditional classification keys (Pitt and Hocking, 2009). The best lipase producer isolates were maintained in Potato Dextrose Agar (PDA) slants, at 4 °C, with trimestral transfer.

2.2. Molecular identification

A total of seven fungi monosporic isolates were obtained, but only three produced fluorescent pink color in Rhodamine B medium, after re-culture. These three isolates were identified by DNA extraction, amplification of the 5.8S-ITS-rDNA region, purification of the amplicon, sequencing, and comparison with other sequences deposited in data banks. For the DNA extractions, an approximately 1 cm^3 fragment of a monosporic culture in inclined PDA was smashed and shaken in 5 mL of distilled water. Two milliliters of the obtained suspension were used as inoculum in 125 mL Erlenmeyer flasks containing 25 mL of liquid potato dextrose medium. The flasks were incubated for 5 days without shaking at 25 °C, with a photoperiod of 12 h. The mycelium was collected by filtration in sterile gauze or cells were collected by centrifugation (4000*g*, 10 min). The mycelium or cells were macerated in a mortar with liquid nitrogen, and transferred to a microtube. The genomic DNA was extracted from the macerated mycelium using a protocol based in the method described by Koenig et al. (1997), with modifications. For that, 700 μL of extraction buffer were added for each 300 μL of macerated material. The extraction buffer contained nuclear lyses buffer (0.2 M Tris, pH 7.5; 50 mM EDTA (ethylenediaminetetraacetic acid); and 2% CTAB (cetyltrimethylammonium bromide); pH 7.5), DNA isolation buffer (0.35 M sorbitol; 0.1 M Tris, pH 7.5; and 5 mM EDTA; pH 7.5), and 5% Sarkosyl, in the proportion of 1:1:0.4. The extraction buffer was combined right before use and was then added of 3.8 mg/mL of sodium bisulfite. The tubes were incubated in a dry bath at 65 °C for 60 min. After that, 500 μL of phenol-tris, pH 8.0, were added and the mixture was agitated by gentle inversion several times. The tubes were centrifuged at room temperature (12,000*g*, 10 min), and the supernatant fraction was transferred to clean tubes. Next, 500 μL of a mixture of chloroform: isoamylic alcohol (24:1) were added to the supernatant and the mixture was agitated

again by gentle inversion. The tubes were centrifuged at room temperature (12,000*g*, 10 min), and the supernatant fraction was transferred to clean tubes. Samples were treated with 5 μL of RNAse A (20 mg/mL), for 30 min, at 37 °C, and next with 5 μL of proteinase K (20 mg/mL), for 30 min, at 56 °C. The DNA was then precipitated with an equal volume of isopropanol and was incubated at −20 °C overnight. The precipitated DNA was collected by centrifugation at room temperature (12,000*g*, 10 min), and the DNA pellet was washed three times with cold 70% ethanol. The final DNA pellet was dried at room temperature and suspended in 50 μL of TE buffer (10 mM Tris, pH 8.0; 1 mM EDTA). The whole process was carried out under aseptic conditions. The DNA was quantified by spectrophotometry at 260 nm and by fluorometry, using the Qubit Quantitation Fluorometer and the Quant-it™ dsDNA HS Assay Kit (Life Technologies, USA). The DNA final concentration was adjusted to 100 ng/μL in TE buffer, and the DNA was kept frozen at −20 °C.

The amplification reactions were performed in a termocycler Techne TC-312 (Techne, Cambridge, UK) in PCR tubes containing 50 μL of the following reaction mixture: 1 × enzyme buffer; 1.5 mM $MgCl_2$; 3.0 U of Platinum *Taq* DNA polymerase (Life Technologies, USA); 0.2 mM of each dNTP (Life Technologies, USA); 50 pmol of each ITS4 and ITS5 primers (White et al., 1990); and 400 ng of the DNA sample. The PCR reaction consisted of 25 cycles of 1 min and 30 s at 94 °C, 1 min and 30 s at 50 °C, and 2 min at 72 °C. Prior to the cycles, the samples were heated for 5 min at 94 °C, and after the cycles the samples were incubated for 10 min at 72 °C and frozen at −20 °C until use. Negative controls (no DNA template) were used in each experiment to test for the presence of DNA contamination of reagents and reaction mixtures. Ten microliters of each PCR reaction were analyzed in a 1.5% agarose gel containing ethidium bromide (0.25 μg/mL). The PCR products were visualized and photographed under UV light.

Amplicons of approximately 500 bp were obtained. The rest of the PCR reaction was purified with the PureLink™ PCR purification kit (Life Technologies, USA), and the amplified DNA was sequenced in the Center for the Human Genome Studies (CEGH) in the University of São Paulo (USP), Brazil. One of the primers used in the PCR amplification was used in the sequencing reaction and one strand of each amplicon was sequenced. After trimming the 5′ and 3′ extremities, the resulting sequences were compared with sequences deposited in data banks (GenBanK, Mycobank, and CBS) using pairwise alignment. The obtained rDNA gene partial sequences were deposited in GenBank (Table 1).

2.3. Submerse culture

Among the three molecularly identified strains, *A. westerdijkiae* has never been reported as a lipase producer, and it was used in the rest of this study. A basal medium was developed based on different media used for the production of lipases by isolates of the *Aspergillus* genera (Xia et al., 2011; Abrunhosa et al., 2013). The media consisted of three solutions prepared separately. Solution A: 3 g/L peptone, 2 g/L yeast extract, 10 g/L anhydrous KH_2PO_4, pH 6.0, and 1% olive oil. Solution B: 10 g/L $MgSO_4$. Solution C: 10 g/L $(NH_4)_2SO_4$. After sterilization and before inoculation, the solutions were aseptically blended, with 22.5 mL of solution A, 1.0 mL of solution B, and 1.0 mL of solution C, totalizing a final volume of de 25 mL, including the inoculum, in 125 mL Erlenmeyer flasks, giving the final concentration above mentioned. The inoculum consisted of 0.5 mL of sterile deionized water containing 1.8×10^6 spores, prepared by smashing and agitating a 1 cm^3 of the inclined PDA monosporic culture in 20 mL of sterile distilled water. The flasks were incubated at 25 °C, with agitation of 100 rpm, in an orbital shaker for 72 h. After that, the culture was vacuum filtered on filter paper. In order to ascertain if the

production of the lipase by *A. westerdijkiae* was inducible by the vegetable oil used as carbon source, an experiment with and without olive oil in the medium was carried out.

2.4. Mycelium preparation

When lipase activity was assayed in the culture supernatant of *A. westerdijkiae*, no activity was found. Considering that a strong fluorescence was seen inside the *A. westerdijkiae* colony in the Rhodamine B media (Fig. 1), it was hypothesized that the lipase activity could be bound to the mycelia. To test that, the mycelium of 12 flasks of 125 mL containing 25 mL of medium was collected by vacuum filtration. The obtained mycelium was defatted with acetone and the remaining acetone was evaporated by incubation at room temperature. The mycelium was then frozen at −20 °C, for 24 h, before being freeze dried overnight at −40 °C. The freeze-dried mycelium was then macerated with a mortar and pestle to a powder and this power was used to test lipase activity.

To evaluate if the mycelium-bound lipase could be released by sonication, the mycelium cultured in ten 125 mL flasks containing 25 mL of medium was collected by vacuum filtration, washed with 50 mM phosphate buffer, pH 7.0, and suspended in 40 mL of this same buffer. The suspension was kept in an ice bath and was sonicated three times in a Fischer Scientific (USA) sonicator (32 cycles of 10 s on and 5 s off, with 40% of amplitude). The obtained homogenate was centrifuged (14,*000g*, 5 min) and the supernatant was analyzed by the enzyme assay.

To assess if the mycelium-bound lipase could be released by detergent, the freeze-dried mycelium was suspended, in a proportion of 1:10 (w:v), in extraction buffer (50 mM phosphate buffer, pH 7.0, 1 mM EDTA, 1 mM PMSF (phenylmethane sulfonyl fluoride), and 1% SDS (sodium dodecyl sulphate)) and was agitated for 30 min in an ice bath and centrifuged (14,000*g*,

10 min). The supernatant was used in the enzyme assay. Neutral detergents, Tween 80 and Triton X-100, and a cationic detergent, CTAB, were also used in the extraction buffer, at 1%, in substitution of the anionic detergent SDS. In the treatments with SDS and CTAB, in order to remove the detergent, the supernatant was dialyzed twice in 50 mM phosphate buffer, pH 7.0, containing 1 mM EDTA, and once more in 50 mM phosphate buffer, pH 7.0, without EDTA, for a total of 24 h, before the enzyme assay.

2.5. Enzyme assay

The lipase activity was assayed by the hydrolysis of *p*-nitrophenyl palmitate (*p*-NPP), following the methodology described by Winkler and Stuckmann (1979), with modifications. For that, 5 mg of freeze dried mycelium powder or 100 μL of the sample (culture supernatant, or sonicated supernatant, or detergent treatment supernatant) were added to 100 μL of 0.5 M phosphate/citrate buffer, pH 5.0, and 900 μL of the substrate solution. The substrate solution was prepared with 10 mL of isopropanol containing 30 mg of *p*-NPP mixed with 90 mL of 0.1% arabic gum and 0.4% Triton X-100, resulting in a final concentration of 0.8 mM of *p*-NPP. The reaction was carried out in capped microtubes incubated in a water bath at 40 °C, with inversion each 30 s, for 8 min. After centrifugation (14,000*g*, 2 min), the released *p*-nitrophenol was read at 410 nm, unless otherwise stated. One lipase unit was defined as the amount of enzyme that liberated 1 μmol $mL^{-}1.min^{-}1$ of *p*-nitrophenol. A blank without enzyme was used to subtract the natural hydrolysis of the *p*-NP derivatives.

Lipase activity was also determined by titrating the fatty acids liberated from triacilglycerol with alkali according to the methodology described by Hama et al. (2006), with modifications, as follows. Briefly, the assay mixture contained 2.0 g of olive oil or tributyrin, 9

mL of 50 mM phosphate buffer, pH 7.0, 1.2 g of arabic gum, and 0.1 g of dry mycelia ressuspended in 1.0 mL de distilled water. The control reaction consisted of the same mixture, but the mycelia ressuspended in distilled water was heat inactivated at 100 °C, for 30 min, before being added to the reaction mixture. The sample and control reactions were incubated for 1 h, at 40 °C, with agitation of 100 rpm. After this incubation, the reaction was stopped with addition of 40 mL of 99.5% ethyl alcohol. Five drops of 1% phenolphtalein (in 99.5% ethyl alcohol) were added to the reactions, wich were then titrated with 0.1 M NaOH. One unit of lipase activity was defined as the activity that has liberated one micromol of free fatty acid, per minute of reaction, under the above conditions.

2.6. Mycelium-bound lipase properties

2.6.1. Effect of p-NPP concentration on initial velocity

The effect of *p*-NPP concentration on the initial velocity was measured by varying the *p*-NPP concentration from 0.08 mM to 0.8 mM in the substrate solution, using 5 mg of dried powder mycelium. The reaction was carried out at room temperature, for 15 min, with agitation (100 rpm).

2.6.2. Potential recyclability

The lipase activity was assayed by the hydrolysis of *p*-nitrophenyl palmitate (*p*-NPP), following the methodology described above, with 5 mg of the freeze-dried mycelium powder. After centrifugation (14,000*g*, 2 min) at the end of the reaction, the *p*-nitrophenol released in the supernatant was read at 410 nm. The collected mycelia was washed twice with 1 mL of the

reaction buffer (0.5 M phosphate/citrate buffer, pH 5.0) and incubated again in a new reaction. After this second reaction, the collected mycelia was washed again in the same way and used in a third reaction.

2.6.3. Optimal temperature and thermal stability

The optimal temperature of the mycelium-bound lipase was found by ranging the reaction temperature from 20 °C to 80 °C. In order to evaluate the protective effect of the natural immobilization on the mycelium-bound lipase thermal stability, 5 mg of the mycelium powder was incubated in 100 μL of 0.5 M phosphate/citrate buffer, pH 5.0, for 30 min at 20, 30, 40, 50, 60 °C, before the enzyme assay. The mycelium powder was also incubated at 40, 50, and 60 °C for 0.5, 1, 2, 4, and 6 h, in 100 μL of 0.5 M phosphate/citrate buffer, pH 5.0, before the enzyme assay. The relative activity was calculated against a control without any incubation.

2.6.4. Optimal pH and pH stability

The optimal pH was discovered by varying the reaction pH using McIlvaine buffer (0.1 M acid citric, 0.2 M sodium phosphate dibasic) for pHs 4.0–8.0 and Britton and Robinson buffer (0.1 M boric acid, 0.1 M acetic acid, 0.1 M phosphoric acid) for pHs 9.0–11. Mycelium powder (1 mg) was added of 100 μL of the reaction buffer and of 900 μL of the substrate solution containing 0.08 mM of pNPP, in a capped tube. The tubes were incubated at 40 °C with intermitent agitation for 8 min. After centrifugation (14,000g, 2 min), the released *p*-nitrophenol in the supernatant was read at its isosbestic point, 348 nm (the pH independent wavelength for *p*-nitrophenol).

The pH stability was assayed by incubating the mycelium (5 mg) in 500 µL of McIlvaine buffer for pHs 5.0–8.0 and Britton and Robinson buffer for pH 9.0. After 2, 4, and 6 h, the mycelium was collected by centrifugation (14,000g, 5 min) and washed twice with 500 µL of the enzyme assay reaction buffer. Finally, the mycelium was suspended in 100 µL in 0.5 M phosphate citrate buffer, pH 5.0, and was submitted to the enzyme assay with 0.8 mM of *p*-NPP, which was read at 410 nm.

2.6.5. Substrate specificity

The substrate specificity was verified by measuring the enzyme activity against *p*-nitrophenyl acetate (*p*-NPA), *p*-nitrophenyl butyrate (*p*-NPB), *p*-nitrophenyl dodecanoate (*p*-NPD), and *p*-nitrophenyl palmitate (*p*-NPP) at 0.8 mM, in the standard conditions. The relative activity was measured considering the highest activity as 100%.

2.6.6. Effect of potential inhibitors and activators

The reagents $HgCl_2$, NaCl, $NiCl_2 \cdot 6H_2O$, EDTA, SDS, $CaCl_2 \cdot H_2O$, PMSF, and CTAB were added to the standard enzyme assay, at the concentrations of 5 mM and 10 mM. Tween 20, Tween 80, and Triton X-100 were also added to the standard enzyme assay, at the concentrations of 1% and 2%. The relative activity was calculated as the percentage of the remaining activity observed in a control without any compound.

2.7. Esterification reaction

The mycelium ability to esterify oleic acid with ethanol was verified by incubating 20 mg of mycelium powder with 100 mM oleic acid and 200 mM ethanol (ratio 1:2, oleic acid:ethanol) in n-heptane with a final volume of 1.0 mL. The reaction was also performed with 100 mM oleic acid and 300 mM or 400 mM ethanol (ratios 1:3 and 1:4, oleic acid:ethanol, respectively). A control reaction omitting the ethyl alcohol was also performed. All reactions were performed in capped tubes with four ceramic beads of 8–12 mesh molecular sieves (Sigma, 70955-01-0, USA), at 40 °C, for 24 h, in a rotatory shaker (100 rpm). After that, the mixture was centrifuged (14,000g, 5 min). The reactions supernatants were diluted 1:2 with *n*-heptane and were applied in a Silica gel TLC (Fluka, 60505, USA) together with the oleic acid and ethyl oleate standards (Sigma, 75100, USA) also diluted 1:2 with n-heptane. The mobile phase consisted of *n*-hexane/ethylic ether/acetic acid (70:29:1 by volume). The spots were revealed in a saturated iodine chamber.

To quantify the yield of ethyl oleate synthesis, the residual oleic acid in the reactions were titrated following the methodology described by Kartal et al. (2011). For that, 500 μL of each reaction supernatant were added of 10 mL ethyl alcohol:acetone (1:1) and of 5 drops of 1% phenolphthalein (in 99.5% ethyl alcohol) and were titrated with 0.01 M NaOH.

3. RESULTS AND DISCUSSION

3.1. Microorganism isolation

The bioprospection for lipase-producing fungi in used cooking oil and fat from a grease trap resulted in the finding of three producing isolates. After searching sequence databanks for DNA homologous with the obtained 5.8S-ITS-rDNA sequences, a range of identity from 89% to 100% was determined (Table 1). In the identity analysis, only sequences with coverage higher than 92% with the obtained sequences were considered. The three fungal identified species were *Candida cylindracea* (*Candida rugosa*), a Saccharomycetales (probably a *Geotrichum* sp.), and *A. westerdijkiae*.

Candida rugosa lipases are examples of the enzymes most frequently used in biotransformations and their production were firstly described as early as in the sixties (Gupta et al., 2015). The species of the Saccharomycetales isolate could not be determined, because of the low level of identity (maximum of 85%) with sequences of identified species in databanks. However, the lipase production by species of the *Geotrichum* genus, a Saccharomycetales, such as *Geotrichum candidum*, is well known (Hlavsová et al., 2009). Lipase production by *A. westerdijkiae* has never been reported, to the best of our knowledge. The species *A. westerdijkiae* Frisvad & Samson was described as a new member of the *Aspergillu*s section Circumdati (Frisvad et al., 2004). To this section belong several fungi well-known for their production of ochratoxin A, a mycotoxin, named after the producer *Aspergillus ochraceus*. The species *A. westerdijkiae* is the main ochratoxin-producing species found in Brazilian Arabica coffee beans (Morello et al., 2007). The northwestern region of the Paraná State, where the city of Maringá is located, is a traditional coffee crop production region in Brazil.

3.2. Enzyme production

The Rhodamine B test agar has confirmed the production of lipase by *A. westerdijkiae* (Fig. 1). However, the enzyme assay performed with the culture filtrate has shown no enzyme activity. The enzyme assay performed with fresh or freeze-dried mycelium has yielded enzyme activity, being the reactions with the freeze-dried mycelium better to perform because of the reproducibility. The enzyme was, therefore, defined as mycelium-bound. An attempt to release the enzyme from the mycelium by sonication or by detergent washes has recovered less than 15% of the initial activity, even after detergent removal by dialysis.

When the fungus was grown in the established medium with 1% olive oil as the carbon source, the lipase activity yield was an average of 40,000 U/g of dry mycelium, using *p*-NPP as substrate. This yield was much higher than the yield obtained for mycelium-bound lipase activities of other fungi, also evaluated by using *p*-NPP as substrate, such as 1.8 U/g of dry mycelium for *Aspergillus niger* (Colin et al., 2011). When the titration method was used to determine enzyme activity, it was found 52 U/g and 78 U/g of dry mycelium with olive oil and tributyrin as substrates, respectively. Comparing with the titration yield of other mycelium-bound lipases, these yields were higher than the yield of 22.59 U/g of dry mycelium for the enzyme of *G. candidum* (Loo et al., 2014), but were lower than the yield of 4,230 U/g of dry mycelium for the enzyme of *Rhizopus oryzae* (Essamri et al., 1998).

When the fungus was cultured without oil, the yield was 12,000 U/g of dry mycelium, assayed by using *p*-NPP. This low level of *A. westerdijkiae* mycelium-bound lipase production when olive oil was omitted from the medium indicates that the enzyme biosynthesis is inducible. Similar results of substrate induction was verified in mycelium-bound lipases of *A. niger* (Colin

et al., 2011), *A. flavus* (Long et al., 1996), *Rhizopus chinensis* (Teng and Xu, 2008), and *R. oryzae* (Essamri et al., 1998).

The yield of *A. westerdijkiae* mycelium in each 125 mL flask with 25 mL of liquid culture media, cultured for 3 days, in the stablished conditions, was 0.115 g of dried mycelium, which is equivalent to 4,600 U of enzyme, assayed by using *p*-NPP.

3.3. The mycelium-bound lipase properties

3.3.1. Effect of p-NPP concentration on initial velocity

The presence of a mycelium-bound lipase activity has been confirmed by the initial velocity dependence on the *p*-NPP concentration, in the enzyme assay (Fig. 2). In the first few minutes after enzyme addition, the lipolysis rate was directly proportional to the bulk substrate concentration. Similar results were found in the effect of tricaprylin concentration on intracellular lipase activity of *R. oryzae* (Essamri et al., 1998).

3.3.2. Potential recyclability of the mycelial-bound lipase

When the enzyme assay was conducted with fresh mycelia, it was obtained an activity of 52,000 U/g of mycelium. When the enzyme assay was conducted with the mycelium that was used in the first reaction, it was obtained an activity of 30,000 U/g of mycelium (corresponding to 57% of the initial activity). In the third round of reaction, with the mycelium used in the second reaction, it was obtained an activity of 18,600 U/g of mycelium (corresponding to 35.8% of the initial activity). These results demonstrate that the mycelium-bound lipase from *A. westerdijkiae* has a great potential of recyclability, which is very useful in industrial technology application.

3.3.3. Optimum temperature and thermal stability

The hydrolytic activity showed best activity in the interval of 30–40 °C, with an optimum temperature of 40 °C (Fig. 3A). The cell bound lipase was stable after 30 min at 50 °C, with a calculated T_{50} (the temperature at which the enzyme loses 50% of its activity) of 61.62 °C, calculated from the obtained second order polynomial regression curve equation (Fig. 3B). The enzyme was completely inhibited after 30 min at 70 °C. Regarding the thermal stability over time, the enzyme retained around 80% of its activity after 6 h at 40 or 50 °C, but was inactivated at 60 °C, after the same period of incubation (Fig. 3C). The thermal stability of *A.westerdijkiae* mycelium-bound lipase for several hours at 50 °C is important, because many industrial processes have to be heated around 50 °C, due to the necessity of liquefying lipids with high melting points.

The optimum temperature and thermal stability found for the mycelium-bound lipase of *A. westerdijkiae* are superior to the mycelium-bound lipase of other fungi such as from the *R. oryzae* enzyme, with optimum temperature of 30 °C and thermal stability at 40 °C for 30 min (Essamri et al., 1998). Nonetheless, these data are comparable with the data of other mycelium-bound lipase, such as the one from *G. candidum*, with optimum temperature of 35 °C and recovery of more than 85% of the activity after 5 h at 30–53 °C (Hlavsová et al., 2009); and from the enzyme of *A. niger* with optimal activity in the temperature range of 8–35 °C and retention of 14–54% of its activity after 1-h incubation in the temperature range of 4–55 °C (Romero et al., 2007).

3.3.4. Optimum pH and stability in different pHs

The mycelium-bound lipase from *A. westerdijkiae* has a broad range of optimum pH, from 7.0 to 8.0 (Fig. 4A), and was approximately 60% active in lower pHs, as 5.0 and 6.0, and in higher pHs, as 9.0 (Fig. 4A). The capability to act in a broad range of pH was also found in the mycelium-bound lipase of *R. oryzae* and *A. niger* (Essamri et al., 1998; Romero et al., 2007). In addition, the enzyme from *A. westerdijkiae* was stable for 6 h when incubated in buffers with pH 5.0, 6.0, 7.0, and 8.0 (Fig. 4B).

It is possible that the lipase immobilization in the *A. westerdijkiae* mycelium environment may have conferred the ability to act in extremes of temperatures and pHs. The environment in which the lipase is inserted is complex and scarce data are found in the literature pertaining to cell-wall associated enzymes, but it is known that some enzymes are entrapped in the fabric of the wall in fungi, aiding in the conversion of nutrients into metabolisable monomers before they enter into the protoplasm, besides their role in the metabolism of the cell wall components (Rast et al., 2003; Free, 2013).

3.3.5. Substrate specificity

The mycelium-bound lipase specific hydrolytic activity against short, mid, and long-chain fatty acid esters is shown in Table 2. The enzyme could hydrolyze all used substrates with different activities. This broad substrate activity may be due to the presence of different esterases/lipases or isoenzymes immobilized in the mycelium (Colin et al., 2011). Furthermore, lipases often have broad substrate specificity (Singh and Mukhopadhyay, 2012; Gupta et al., 2015). It is worth mentioning that the *A. westerdijkiae* mycelium-bound enzyme activity against *p*-NPB was higher than against *p*-NPP what is in accordance with the obtained tributyrin lipolysis

activity, which was higher than the olive oil lipolysis activity (above). In another work (Castro et al., 2017), the authors have studied an esterase secreted by *A. westerdijkiae*, which can primarily use *p*-NPB as substrate, but cannot use long chain fatty acid esters as substrates. It is possible that this high mycelium activity with *p*-NPB is related to this enzyme in its secretion pathway.

3.3.6. Inhibitors and activators

Table 3 summarizes the inhibitors and activators of lipolytic activity of the lyophilized mycelium. Calcium has slightly inhibited the mycelium-bound lipase. The effect of calcium as an inhibitor of secreted fungal lipases is also reported for the enzyme of *Yarrowia lipolytica* (Yadav et al., 2011). However, calcium has been generally known as an activator of secreted and purified lipases, such as those from the fungi *Aspergillus terreus* and *Talaromyces thermophiles* (Yadav et al., 1998; Romdhane et al., 2010). The calcium effect could be explained by the complexation with released fatty acids forming fatty acids salts, which have low solubility, and/or with charged groups of the enzyme, changing its properties. Lipase inhibition by heavy metals, such as mercury and nickel, are well documented (Singh and Mukhopadhyay, 2012; Gupta et al., 2015) and the *A. westerdijkiae* mycelium-bound lipase is not an exception, being strongly inhibited by both of these ions. It is worth emphasizing that some of the tested ions did not inhibit the mycelium-bound lipase from *A. westerdijkiae* and this may due to the fact that immobilized enzymes are less prone to inhibition and such protection may have partly masked the effects of the tested substances (Rodrigues et al., 2013).

EDTA has also inhibited the mycelium-bound lipase of *A. westerdijkiae* (Table 3) and this inhibition could be due to the chelation of a metal ion that could act as a cofactor or could influence on the interfacial area between substrate and enzyme. The *A. westerdijkiae* mycelium-

bound lipase inhibition by PMSF may indicate the presence of an essential serine in the catalytic site. Accordingly, lipases are generally regarded as serine hydrolases, and filamentous fungi lipases have a common sequence around this active site composed of Gly-XSer-X-Gly, where X can be any amino acid (Gupta et al., 2015).

The non-ionic detergents Tween 20, Tween 80, and Triton X-100 have partially inhibited the mycelium-bound lipase of *A. westerdijkiae* (Table 3). Those detergents may have caused modification in the lipase tertiary conformation by hydrophobic interactions, and, in the case of Tween-20 and 80, also may have acted as competitors for the catalytic site. The anionic surfactant SDS, whose interactions are mainly electrostatic, presented almost no impact on the enzyme. Similar results were found for the secreted lipase of Talaromyces thermophiles (Romdhane et al., 2010). The cationic detergent CTAB, however, has precipitated the reaction solution and the enzyme inhibition could not be determined. Surfactants can induce significant changes in the partitioning of compounds between the water phase and lipid-water interfaces. Lipases are peculiar catalysts in that they can act on lipid water interfaces, presenting the phenomenon called ‘interfacial activation’, where activity is low on monomeric substrates and becomes greatly enhanced by aggregated substrates, such as emulsions and micellar solutions, that are formed above their saturation limit. Interfacial activation is often accompanied by structural changes, in which a surface loop (generally known as ‘lid’), that covers the active site, is displaced (Gupta et al., 2015). The action of surfactants, in the oil-water interface, or soluble in the form of micellar aggregates and monomers, combined with the emulsification and dispersion of insoluble substrates and inhibitors, can either promote or decrease the activity of lipases (Delorme et al., 2011).

3.4. Esterification reaction

The TLC analysis results have shown the production of ethyl oleate from oleic acid and ethanol when they were incubated in the presence of the lyophilized mycelium (Fig. 5), but not when oleic acid alone was incubated with the mycelium. Thus, the mycelium bound lipase of *A. westerdijkiae* represents a simple alternative for biodiesel synthesis, without the necessity of isolation, purification, and immobilization of the enzyme, which are processes of considerable cost (Narwal and Gupta, 2013; Poppe et al., 2015). Furthermore, the mycelium can be regarded as a heterogeneous catalyst that ultimately facilitates the purification of biodiesel and the byproduct glycerol.

The quantitative analysis have shown that a 35% conversion yield of oleic acid to ethyl oleate was achieved when a ratio of 1:2, oleic acid:ethyl alcohol, was used. Increases in this ratio of oleic acid:ethyl alcohol to 1:3 or 1:4 resulted in 20% and 17% conversion yield, respectively. The alcohol/fatty acid molar ratio is a parameter that has to be optimized in esterification reactions catalyzed by enzymes, as other variables, such as temperature and catalyst concentration (Garcia et al., 1999). It has been observed in this work that increases in the alcohol concentration in relation to oleic acid inhibited the reaction.

4. CONCLUSIONS

A new mycelium-bound lipase from a locally isolated strain of *A. westerdijkiae* has been described in this work. The naturally immobilized lipase main properties have been characterized and it represents an inexpensive alternative for biodiesel production.

DECLARATION OF INTEREST STATEMENT

We declare no conflict of interest.

ACKNOWLEDGEMENTS

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Table 1. Fungal isolates and molecular identification.

Genera and species	Isolate	Source	Amplicon (bp)	GenBank Accession Number	Coverage	GenBank ID or CBS Accession Number of similar sequences (percentage of identity)
Candida cylindracea *Candida cylindracea* *Candida cylindracea*	MGA 3	Grease	503	KY264739	100% 100% 100%	• CBS 6330_ex21990_18538_ITS (99.6%) • CBS_7869-13740_ITS4_ G5_035_13740_ITS5_G5_035 (99.6%) • cr - CBS 8604 (99.4%)
Saccharomycetales *Geotrichum* sp. *Geotrichum* sp.	MGA 4	Grease	348	KY264740	99.7% 89% 91%	• KC310808.1 (99%) • AB287313.1 (85%) • AY805559.1 (83%)
Aspergillus westerdijkiae *Aspergillus westerdijkiae* *Aspergillus westerdijkiae*	MGA 6	Oil	492	KY264741	100% 100% 100%	• KJ599601.1 (100%) • FR733837.2 (100%) • HQ843035.1 (100%)

Table 2. Substrate specificity of the mycelium-bound lipase of *A. westerdijkiae*.

Substrate	**Concentration**	**Relative activity (%)[a]**
p-Nitrophenyl palmitate	0.8 mM	88
p-Nitrophenyl butyrate	0.8 mM	100
p-Nitrophenyl acetate	0.8 mM	16
p-Nitrophenyl dodecanoate	0.8 mM	53

[a]The results are the average of duplicates.

Table 3. Impact of activators and inhibitors on the mycelium-bound lipase of *A. westerdijkiae*.

Substance	Concentration	Relative activity (%)[a]	Concentration	Relative activity (%)[a]
Control	-	100	-	100
$HgCl_2$	5 mM	51	10 mM	63
NaCl	5 mM	93	10 mM	86
$NiCl_2$	5 mM	60	10 mM	35
$CaCl_2$	5 mM	79	10 mM	64
EDTA	5 mM	82	10 mM	68
SDS	5 mM	109	10 mM	91
PMSF	5 mM	76	10 mM	-[b]
CTAB	5 mM	-[b]	10 mM	-[b]
Tween 20	1%	79	2%	16
Tween 80	1%	66	2%	28
Triton X-100	1%	74	2%	35

[a]The results are the average of duplicates.
[b] Reading was not possible due to the formation of precipitate

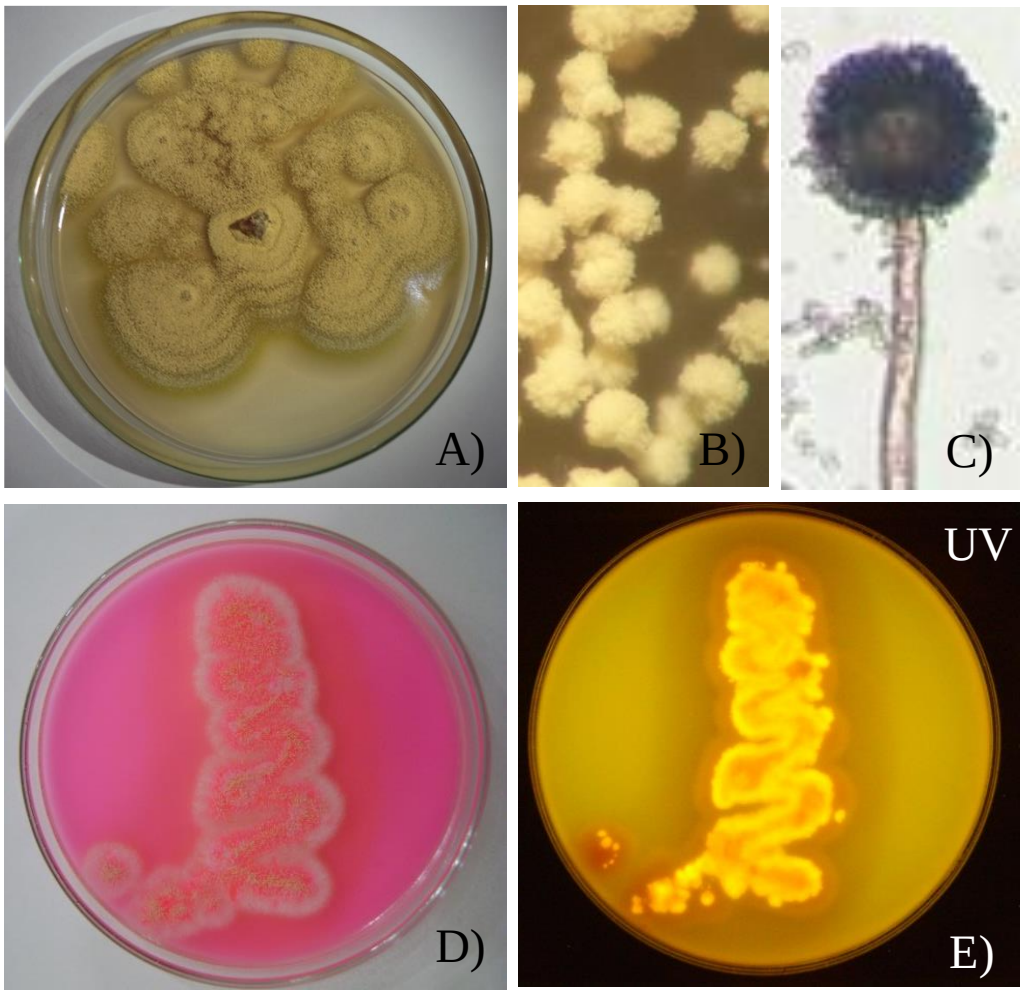

Fig. 1. *A. westerdijkiae* morphology and analysis of lipase production in selective media. A) A five-day old culture at 25 °C of *A. westerdijkiae* in PDA. B) Conidiophores of the *A. westerdijkiae* isolate in PDA seen in a stereoscope microscrope. C) A conidiophore of the *A. westerdijkiae* isolate in PDA seen in an optical microscope. D) A Rhodamine B agar dish with the isolate of *A. westerdijkiae* (front). E) The same dish in D) photographed under UV light using a yellow filter (reverse).

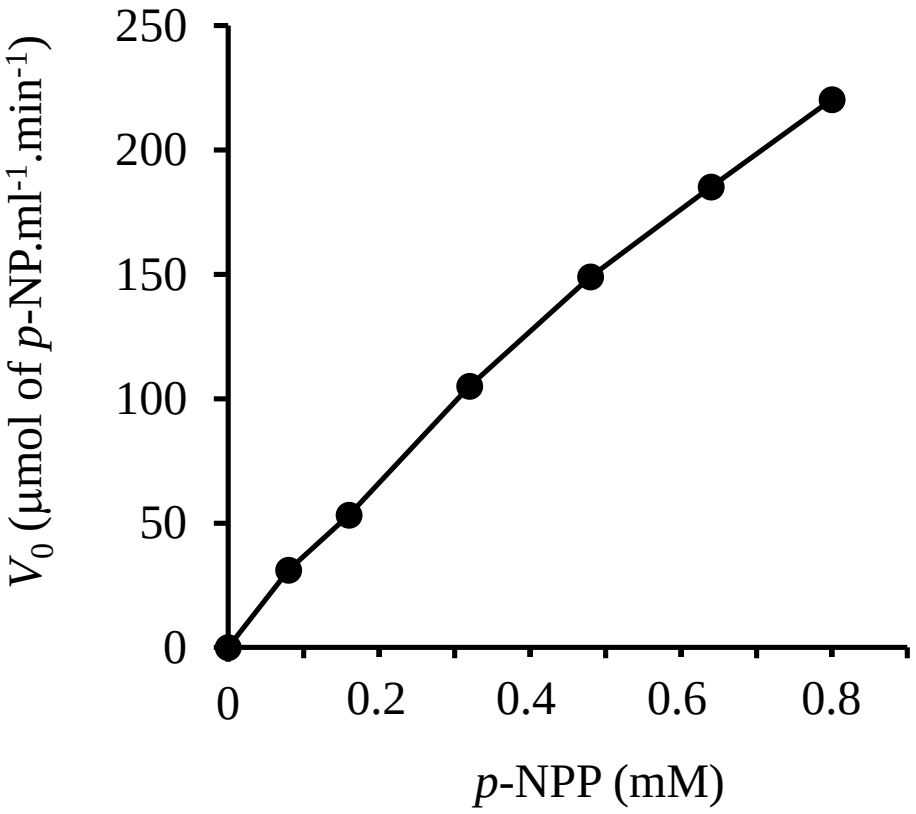

Fig. 2. Effect of *p*-NPP concentration on the mycelium-bound lipase activity. Five micrograms of dried mycelium were incubated with *p*-NPP in the concentrations varying from 0.08 to 0.8 mM at 30 °C, in phosphate buffer, pH 5.0. The results are the average of duplicates.

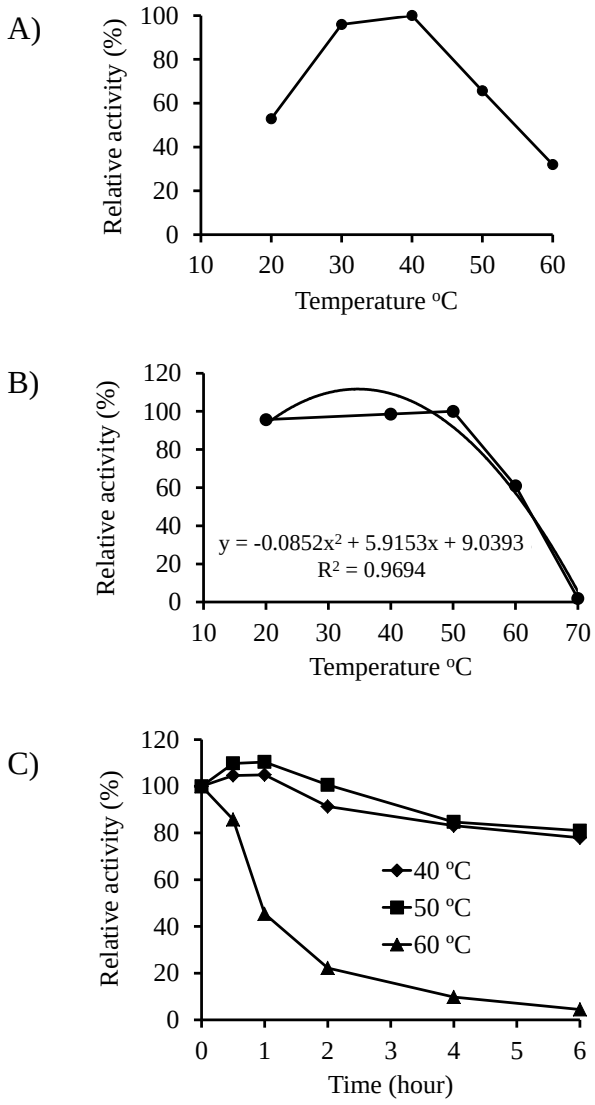

Fig. 3. Effect of temperature on the lipase activity. A) Optimum temperature, which was found by varying the reaction temperature of the enzyme assay from 20 °C to 60 °C (pH 5.0). B) Thermal stability at different temperatures (pH 5.0), with 30 min of incubation before the enzyme assay, which was carried out at 30 °C (pH 5.0). C) Thermal stability curve of the enzyme incubated at 40 °C, 50 °C, and 60 °C for several hours (pH 5.0) prior to the reaction assay at 30 °C (pH 5.0). *p*-NPP at 0.8 mM was used as substrate in all experiments. The results are the average of duplicates.

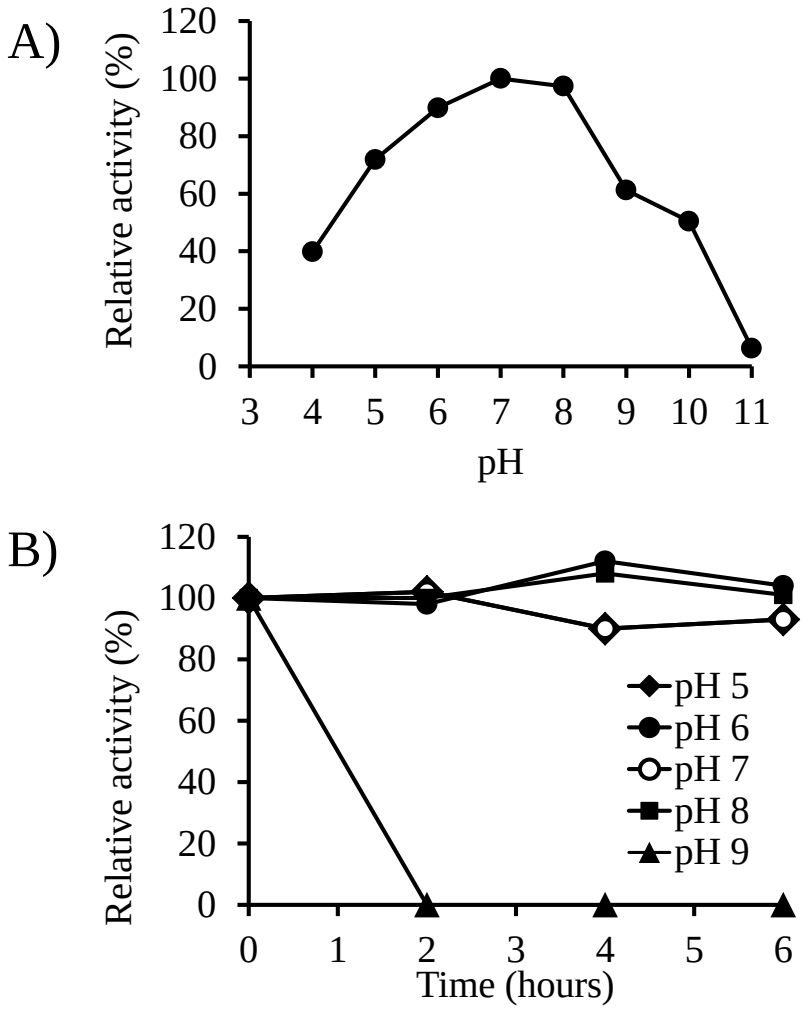

Fig. 4. Effect of pH on the lipase activity. A) The optimum pH was assessed by calculating the relative activity of the mycelium-bound lipase in different reaction pHs (4.0 to 11.0). B) The stability in different pHs was assayed by incubating the mycelium in buffers with different pHs, at room temperature, and assaying the activity after 2, 4, and 6 h of incubation, in the enzyme reaction, pH 5.0, at 40 °C. The results are the average of duplicates.

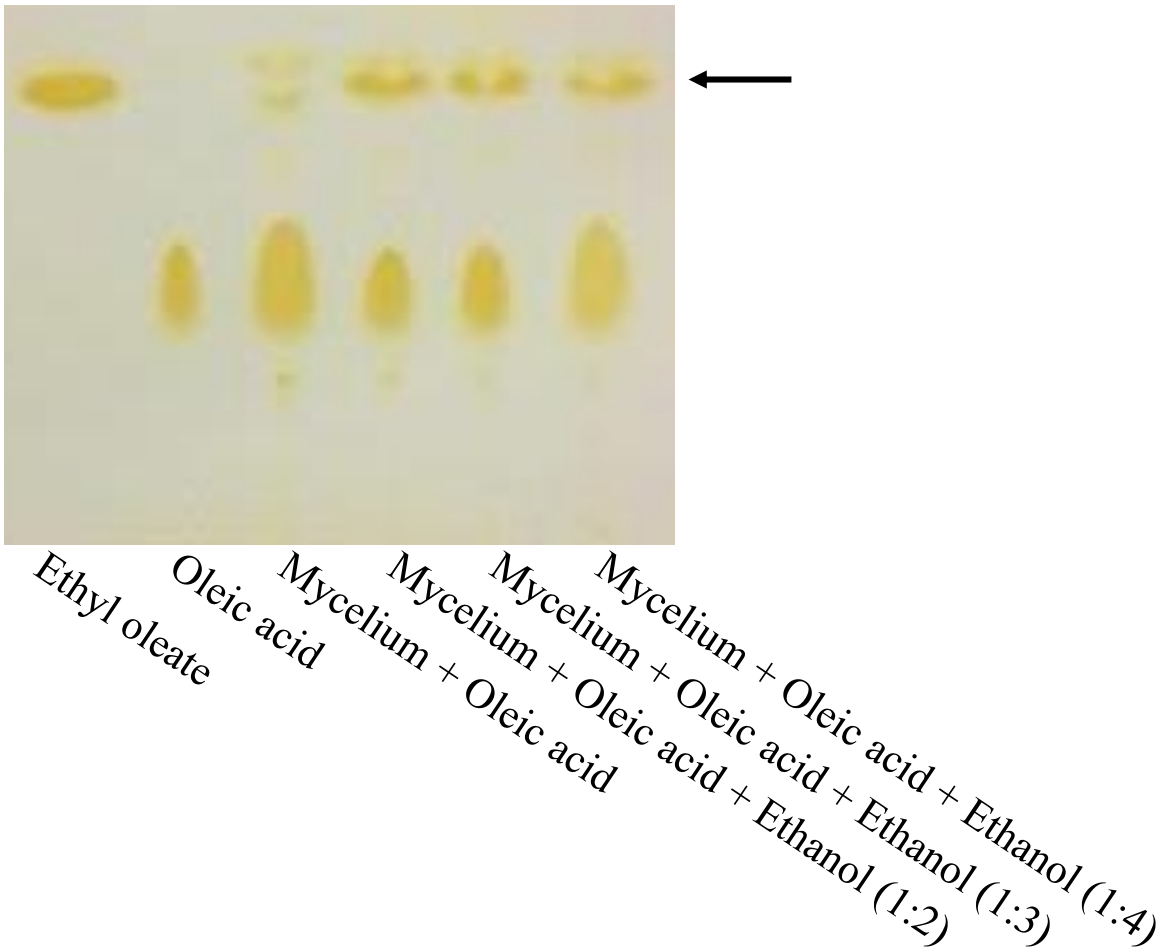

Fig. 5. The esterification ability of the mycelium-bound lipase of *A. westerdijkiae*. The mycelium (20 mg) was incubated with oleic acid and ethyl alcohol, in the following proportions 1:2, 1:3, and 1:4. The reactions were performed as described in the materials and methods section in *n*-heptane. The supernatant reactions were diluted 1:2 with *n*-heptane and spotted in a Silica gel TLC together with control reactions performed without ethyl alcohol and with the ethyl oleate standard also diluted 1:2 with *n*-heptane. The spots were revealed in a saturated iodine chamber.

CAPÍTULO II
PRODUCTION, PURIFICATION, AND CHARACTERIZATION OF A NOVEL SERINE-ESTERASE FROM *ASPERGILLUS WESTERDIJKIAE*

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ABSTRACT

Esterases hydrolyze water soluble short chain fatty acids esters and are biotechnologically important. A strain of *Aspergillus westerdijkiae* isolated from cooking oil for recycling was found to secrete an esterase. The best enzyme production (19-24 U/ml of filtrate) culture conditions were stablished. The protein was purified using ammonium sulphate precipitation, dialysis, and a chromatographic step in Sephacryl S-200 HR. The 32 kDa purified protein presented an optimal temperature of 40°C, with a T_{50} of 48.9 °C, and an optimal pH of 8.0. K_M and V_{max} were 638.11 µM for *p*-NPB and 5.47 µmol of released *p*-NP $\cdot$ min^{-1} $\cdot$ $\mu g^{-1}$of protein, respectively. The purified enzyme was partially active in the presence of 25% acetone. PMSF inhibited the enzyme, indicating that it is a serine hydrolase. MS enzyme peptides sequences were used to find the protein in the *A. westerdijkiae* sequenced genome. A structure model demonstrated that the protein is a member of the α/β-hydrolase fold superfamily.

Keywords: *Aspergillus westerdijkiae*, esterase, identification.

Abbreviations: CV, coefficient of variation; EDTA, ethylenediaminetetraacetic acid; K_M, Michaelis-Menten constant; LED, lipase engineering database; M.M., molecular markers; MS, mass spectrometry; PMSF, phenylmethylsulfonyl fluoride; *p*-NP, *p*-nitrophenol; *p*-NPA, *p*-nitrophenyl acetate; *p*-NPB, *p*-nitrophenyl butyrate; *p*-NPD, *p*-nitrophenyl dodecanoate; *p*-NPP, *p*-nitrophenyl palmitate; SD, standard deviation; SDS, sodium dodecyl sulphate; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; V_{max}, maximal reaction velocity.

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1 INTRODUCTION

Esterases (Carboxylesterase EC 3.1.1.1) compose a group of hydrolases capable of degrading and forming ester bonds [1]. Esterases mainly catalyze the ester bonds hydrolysis of short-chain triglycerides or esters (<10 carbon atoms) [1,2]. The majority of the esterases detain the α/β hydrolase fold, which consists of a domain composed of a core of eight, mainly parallel β sheet, surrounded by α helices [1,3]. Their active sites usually have the consensus sequence Gly-X-Ser-X-Gly (where X can be any amino acid) [1,3]. According to the Lipase Engineering Database (LED), lipases fall into three classes, namely, the GGGX-, GX-, and Y-class, on the basis of structural and sequence analysis of the oxyanion hole, and are further divided into superfamilies on the basis of the conserved pentapeptide GXSXG.

The mechanism of action centers on the serine residue, which through its nucleophilic oxygen forms a tetrahedral hemiacetal intermediate with the substrate. The hydrolysis of the ester bond releases the diacylglycerol and the active enzyme is renewed by the hydrolysis of the acyl group esterified with the serine residue [1,2]. In the presence of anhydrous organic solvent systems, esterases can catalyze esterification, as well as transesterification and interesterification [1,2]. In addition, they usually exhibit enantioselective, regioselective, and chemioselective properties [1,2]. Carboxyl esterases can be used in the synthesis of optically pure compounds such as the non-steroidal anti-inflammatory (S)-naproxen [1]. They can also be employed in the release of ferulic acid from plant cell wall polysaccharides. Ferulic acid has excellent anti-oxidative properties and can be enzymatically converted into vanillin, a major flavor compound [4].

Esterases are found in plants, animals, and microorganisms [2]. Nonetheless, microbial esterases have gained considerable attention from the industry, because they are more stable and much easier to produce on a large scale [1]. Several genera of bacteria and archaea are reported to produce esterases [1,2]. Furthermore, several esterase genes have been isolated and cloned frommetagenomics libraries [5,6]. However, fewer investigations have been reported on fungal esterases [7,8]. In this work, we describe the production, the purification, and the characterization of a secreted esterase from *Aspergillus westerdijkiae*.

2 MATERIALS AND METHODS

2.1 Microorganism and maintenance

The isolate of *A. westerdijkiae* Frisvad and Samson was previously obtained from cooking oil for recycling in the city of Maringá, Paraná, Brazil [9]. This isolate has been maintained in PDA slants, at 4°C, with trimestral transfer, and also in PDA slants under mineral oil.

2.2 Evaluation and optimization of the esterase production

Esterase production was evaluated in 10 cm diameter Petri dishes containing 20 ml of tributyrin agar (Sigma–Aldrich, USA: 12 g/L agar, 5 g/L special peptone, 3 g/L yeast extract, and 1%(v/v) tributyrin added after sterilization and cooling), in triplicate. Those plates were inoculated in the center with a 0.5 cm^3 diameter disk, cut with a sterile cork borer, from a *A. westerdijkiae* PDA plate culture. The dishes were incubated at 25 °C for 4 days, with a photoperiod of 12 h. Esterase production was assayed by the formation of a hydrolysis halo.

A basal medium was developed based on different media used for the production of lipases by isolates of *Aspergillus* spp. [10,11]. The media was prepared with three solutions: Solution A: 3 g/L peptone, 2 g/L yeast extract, 10 g/L anhydrous KH_2PO_4, pH 6.0, and 1% olive oil; Solution B: 10 g/L $MgSO_4 \cdot 7H_2O$; Solution C: 10 g/L $(NH_4)_2SO_4$. After sterilization, these solutions were aseptically combined: 22.5 ml of solution A, 1.0 ml of solution B, and 1.0 ml of solution C, in 125 ml Erlenmeyer flasks, giving the final concentration above mentioned. The inoculum consisted of 0.5 ml of sterile distilled water containing 1.8×10^6 spores, prepared by smashing and agitating a 1 cm^3 piece of a PDA slant culture in 20 ml of sterile distilled water. The flasks were incubated at 25 °C, with agitation of 75 rpm, in an orbital shaker. The mycelia

were harvested by vacuum filtration in filter paper, washed with hexane, and then dried overnight at 50 °C. The mycelia dry weight was used to evaluate fungi growth. The filtrate was used as the enzyme source.

Initially, a time course of fungi growth and esterase production was performed. Next, different medium components, concentrations, and conditions were tested stepwise to enhance esterase production. The data represent the average and the SD of the results obtained in three culture flasks.

In order to ascertain the role of oil in inducing the esterase biosynthesis, the fungus was cultured with and without olive oil. Next, different vegetable oils and tributyrin were added to the medium at the concentration of 1% (v/v). Different concentrations of corn oil were also used to evaluate enzyme production.

To test if carbohydrate addition to the medium, together with oil, could improve enzyme secretion, the medium was supplemented with different sugars at a final concentration of 1% (v/v). To avoid caramelization, sugars were decontaminated by continuous steam, for 20 min, and aseptically added to the sterile medium, with the exception of CMC, which was added directly in the medium before autoclaving. To investigate the effect of ions on the esterase secretion, different salts were added to the medium at the final concentration of 20 μM. The salts were all prepared in more concentrated solutions, sterilized in autoclave (with exception of $FeCl_3$, which was decontaminated by continuous steam for 20 min), and aseptically added to the medium. Different initial medium pHs, inoculum size, effect of light, and agitation were also evaluated on the esterase secretion.

In order to analyze the role of the detergent Tween 80 as emulsifier, this substance was added to the medium containing 1% corn oil, at the concentration of 0.2%, at the beginning of the

culture or before the last 30 min of the culture, just before filtration. Next, Tween 80 and Tween 20 were added to the medium at different concentrations, together with 1% corn oil, at the beginning of the culture. Higher concentrations of Tween 80 were also tested with or without 1% corn oil.

To verify if detergents could release more esterase from the mycelia, the mycelium was harvested by filtration in paper filter and washed with acetone. After evaporation or the acetone at room temperature, the humid mycelium (0.1 g) was suspended in 10 ml of 50mM phosphate buffer, pH 7.0, containing 0.5% SDS or 0.5% Tween 80 and was agitated for 3 h, at 100 rpm, at room temperature. After centrifugation (5000×*g*, 10 min), the supernatant was used in the enzyme assay.

2.3 Enzyme assay

The esterase activity was assayed by the hydrolysis of *p*-NPB as described by Winkler and Stuckmann [12], with modifications. For that, 100 µl of the sample (culture filtrate or the purified enzyme) were added of 100 µl of 0.1M phosphate buffer, pH 7.0, and of 900 µl of substrate solution (10 ml of isopropanol containing 2 µl of *p*-NPB mixed with 90 ml of 0.1% arabic gum and 0.4% Triton X-100) containing 113 µM of *p*-NPB. The reaction was carried out at 30 °C, for 30 min. The released *p*-NP was read at 410 nm, unless otherwise stated. One esterase unit was defined as the amount of enzyme that liberated 1 µmol of *p*-NP per minute.

2.4 Enzyme purification

The filtrates of ten 125 ml cultures flasks containing 25 ml of the basal medium, pH 6.0, with 1% corn oil, and inoculum of 3.6×10^6 spores, which were incubated at 25 °C, with

agitation of 75 rpm, for 72 h, under natural light, were combined and added of ammonium sulphate at 90% of saturation, at 4 °C. Tween 80 was omitted from the culture medium to allow protein precipitation with ammonium sulphate. Precipitated proteins were collected by centrifugation (12,000 × *g*, 10 min), resuspended in 3 ml of 50 mM phosphate buffer, pH 7.0, and dialyzed twice against the same buffer. The dialysate was applied onto a Sephacryl S-200 High Resolution (GE Healthcare Life Sciences, Chicago, IL, USA) column (100 cm × 1.5 cm), equilibrated with the same buffer. The elution was also performed with the same buffer with a flow rate of 0.6 ml/min. Fractions of 4 ml were collected. The protein elution profile was evaluated by absorbance reading at 280 nm and the enzyme elution profile was assayed by the enzymatic assay. Fractions containing the highest amount of the enzyme were pooled and frozen at −20 °C until use. Protein concentration was determined according to Bradford [13].

2.5 Electrophoresis analysis

An eluate aliquot (15 μg of protein) was precipitated with trichloroacetic acid, washed with absolute ethanol, air dried, resuspended in sample dilution buffer (20% glycerol, 1% SDS, 0.03 mg/ml bromophenol blue, 125 mM tris-base, pH 6.8, 0.72 M β-mercaptoethanol) and loaded into a discontinuous denaturing 7.5% polyacrylamide gel (SDS-PAGE), pH 8.9, with a 4.5% stacking polyacrylamide gel, pH 6.8, which was run in the Tris-glycine buffer system [14]. A dialysate total protein sample (30 μg) was treated in the same way. All samples were boiled for 10 min before being applied in the gel. M.M. (ThermoFisher Scientific, Waltham, MA, USA) were used to determine the approximate size of the esterase.

2.6 Enzyme analysis

K_M and V_{max} were determined by linear regression from a double-reciprocal plot, varying the *p*-NPB concentration from 113 μM to 1130 μM and using a constant amount of the purified enzyme, in the enzyme assay.

The optimal pH was estimated by performing the enzyme assay using the Britton and Robinson buffer (0.1M boric acid, 0.1M acetic acid, 0.1M phosphoric acid), instead of phosphate buffer, and reading the released *p*-NP at its isosbestic point, 348 nm. The pH stability was evaluated by diluting the enzyme 1:3 (v/v) in Britton and Robinson buffer with different pHs, and incubating it for several hours at room temperature. Aliquots were removed at intervals up to 26 h to estimate the enzyme activity, in the standard conditions. The released *p*-NP was also measured at 348 nm.

The optimal temperature was found by changing the enzyme assay temperature. The thermal stability was evaluated by incubating the purified enzyme for 30 min at different temperatures, in 50mM phosphate buffer, pH 7.0, before the enzyme assay. The temperature, at which the esterase of *A. westerdijkiae* loses 50% of its activity (T_{50}), after incubation of 30 min, was calculated using the second order equation from the obtained polynomial regression curve. The enzyme was also incubated at 4 and 40°C, for up to 48 h, and the activity was measured at intervals. The substrate specificity was verified by measuring the enzyme activity against different substrates at 113 μM, in the standard conditions.

To test for potential inhibitors and activators, salts, EDTA, SDS, and PMSF were added to the standard enzyme assay, at the concentrations of 5 and 10 mM. The detergents Tween 20, Tween 80, and Triton X–100 were also added to the standard enzyme assay, at the concentrations of 1% and 2%. The ability of the purified esterase to promote the hydrolysis of *p*-NPB in a water-

restricted environment was estimated by adding 25% of different organic solvent in the enzyme assay. In all these analysis, the relative activity was monitored compared with the control without any addition.

In order to test the activity of the *A. westerdijkiae* esterase or its dry mycelia against Tween 80, a preparation of partially purified esterase was carried out as described above until obtaining the dialysate, without the chromatographic step, and the dry mycelia was obtained as described previously [9]. Activity against Tween 80 or tributyrin as a control was measured by titrating the fatty acids released with alkali, according to the methodology described by Hama et al. [15], with modifications. The Tween 80 assay mixture consisted of 11 ml of 50mM phosphate buffer, pH 7.0, 1% (v/v) Tween 80, and 1 ml of the partially purified esterase (65 U) or 0.1 g of dry mycelia ressuspened in 1.0 ml of water. The tributyrin assay mixture consisted of 2 g of tributyrin, 9 ml of 50 mM phosphate buffer, pH 7.0, 1.2 g of arabic gum and the same amount of partially purified enzyme or dry mycelia. The control reaction consisted of the same mixture, but the mycelia ressuspended in distilled water and the partially purified esterase were inactivated with an incubation of 30 min at 100 °C, before being added to the reaction mixture. The reaction capped vials were incubated for 1 h and 30 min, with agitation of 120 rpm, at 40°C for the dry mycelia tests, or at 30 °C for the partially purified esterase tests. After this incubation, the reaction was stopped by the addition of 40 ml of 99.5% ethyl alcohol. Five drops of phenolphthalein 1% (in 99.5% ethyl alcohol) were added to the reactions, which were then titrated with 0.01M NaOH. One unit of activity was defined as the activity which liberated one micromol of free fatty acid, per minute of reaction, under the above conditions.

2.7 MS analysis

MALDI-TOF/TOF MS was performed using SDS-PAGE protein bands, which were cut and discolored with a solution containing 75mM ammonium bicarbonate and 40% ethanol. Then, the gel was dehydrated with 100%acetonitrile, dried in a vacuum chamber, and rehydrated with a 10mg/ml trypsin solution in 40mM ammonium bicarbonate and 10% acetonitrile. This mixture was incubated overnight at 37°C to generate the protein band peptides. Two microliter of the resulting peptide solution was mixed with an equal volume of saturated HCCA (α-Cyano-4-hydroxycinnamic acid) matrix solution. Then, the mixture was placed on a metal plate for analysis in a MALDI-TOF/TOF mass spectrometer. The peak acquisition was carried out with the positive polarity ion reflector mode. The spectrometer was calibrated following the protocol provided by the manufacturer (Bruker Daltonics, Billerica, MA, USA). The generated mass spectra were analyzed using the FlexAnalysis 3.0 software (Bruker Daltonics, Billerica, MA, USA). The de novo sequence of protein fragment was deduced by manual identification of the −b and −y ions mass peaks and assignment of the mass difference between peaks of each series.

2.8 Bioinformatics analysis programs

BLASTp was used to search for proteins in data banks. Protein sequences were aligned using the CLUSTAL Omega. Peptide Mass was used to predict the mass of the protein trypsin digestion peptides. SWISS-MODEL, Phyre2 and MODELLER were used to predict the protein structure. PyMol was used to analyze the enzyme structure model and active site. SignalIP and NetNGlyc were used to search for the presence of secretion signal peptide and of N-glycosylation sites, respectively. MEGA 7.0 was used to construct a protein phylogenetic tree. CDD was used to search for conserved domains. Augustus was used for gene prediction. Phobius and TMHMM

were used to predict transmembrane segments. WoLF PSORT and Phobius were used for subcellular localization prediction.

2.9 Statistical analysis

Statistical analyses were carried out by calculation of the means and SDs results. The data were submitted to ANOVA and compared using the Tukey test, using the SASM–Agri program [16]. Unless otherwise stated, identical letters indicate no difference among averages ($\alpha = 0.01$).

3 RESULTS

3.1 Evaluation and optimization of esterase production

When a disk from a *A. westerdijkiae* PDA culture (Fig. 1A) was transferred and cultured in a tributyrin agar plate, a hydrolytic halo around the colony was observed (Fig. 1B). The obtained growth and esterase production profile in the submerse culture are shown in Fig. 1C. Although there appears to be a tendency towards increased esterase secretion after 72 h of culture, no statistical difference was observed and, hence, in all following experiments the enzyme activity was measured after 72 h of culture.

A higher esterase secretion was perceived when the fungus *A. westerdijkiae* was cultured in the established basal liquid medium with 1% olive oil (Fig. 2A). Nonetheless, the lipids used as carbon sources enhanced esterase secretion equally, with exception of tributyrin, even though the fungus growth varied (Fig. 2B). Since the addition of corn oil to the medium produced more homogeneous esterase secretion, it was selected as the carbon source. Albeit increases in oil concentration in the culture medium enhanced the fungi growth, enzyme production did not differ (Fig. 2C), and therefore, the concentration of 1% was maintained.

The effect of carbohydrate and ions addition to the medium on the esterase secretion and *A. westerdijkiae* growth are shown in Fig. 3A and B. The enzyme production was the same in all tested medium initial pHs, despite their considerable impact on the fungi growth (Fig. 4A). The initial pH of 6.0 was maintained because in this pH there were fewer medium precipitation problems during its preparation. An increase in the inoculum size intensified the esterase synthesis by *A. westerdijkiae*, but greater inoculums did not produce better results (Fig. 4B). The

mycelial growth was not affected by the inoculum size (Fig. 4B). The effect of culture agitation and presence of light on the esterase production by *A. westerkijkiae* are also shown in Fig. 4B.

Addition of 0.2% Tween 80 at the end of the culture had no effect on enzyme secretion, but improved enzyme secretion when it was added to the medium containing 1% corn oil, at the beginning of the culture (Fig. 5A). Tween 20 did not present the same effect (Fig. 5B). However, higher concentrations of Tween 80 in the medium containing 1% corn oil, at the beginning of the culture, did not further improve the enzyme production (Fig. 5B). Fungi growth was only affected by addition of Tween 80 at the concentration of 2% and 5% to the medium containing 1% corn oil (Fig. 5B and 5C). Interestingly, when 0.5% Tween 80 was used as the carbon source, without any corn oil addition, an increased enzyme production was observed (Fig. 5C). Better results, nonetheless, were not achieved with higher or lower concentrations of Tween 80, despite its impact on improving the fungus growth (Fig. 5D). An attempt to release more enzyme from the mycelium by SDS or Tween 80 washes has recovered 8.8 and 15%, respectively, of the activity present there.

A maximum of 24.1 U/ml of esterase was obtained with 0.5% of Tween 80 as the main carbon source, with medium initial pH 6.0, agitation of 75 rpm, at 25 °C, in the presence of natural light, after a 3-day culture.

3.2 Enzyme purification and analysis

The enzyme purification data are presented in Table 1 and Fig. 6A. The discontinuous SDS-PAGE shows the purified enzyme of 32 kDa (Fig. 6B). The Michaelian purified enzyme presented a K_M of 638.11 μM for *p*-NPB and V_{max} of 5.47 μmol of released *p*-NP/min·μg of protein, at 30 °C, and pH 7.0 (Fig. 7A). The purified enzyme had an optimum pH of 8.0 (Fig. 7B)

and it was almost totally active when incubated for 26 h at pHs 5.0–9.0, at room temperature (Fig. 7C). The purified esterase presented an optimum temperature of 40 °C (Fig. 7D) and lost activity at temperatures above 40°C, with a calculated T_{50} of 48.95 °C (Fig. 7E). The enzyme was recovered almost completely active after 48 h of incubation at 4°C, but the activity was gradually lost when incubated at 40°C (Fig. 7F).

The purified enzyme showed to be highly specific for *p*-NPB, presenting also a small relative activity against *p*-NPA (6.1% in comparison with the activity against *p*-NPB). No significant activity was detected with *p*-NPD and *p*-NPP. When the partially purified enzyme was essayed for activity against Tween 80 and tributyrin using titration, no activity was observed for Tween 80 but there was activity against tributyrin (4.5 U/mg). However, a high activity against Tween80 was found to be associated with the dry mycelia of *A. westerdijkiae* (17U/g).

The results of potential inhibitors and activators on the esterase activity are summarized on Table 2. PMSF, EDTA, Hg^{2+}, Zn^{2+}, Ni^{2+}, Tween 80, Tween 20, and Triton X–100 inhibited the enzyme at both used concentrations, while Ca2+ inhibited it only at the highest concentration. Na^{+} and K^{+} activated the enzyme at the lowest concentration. The anionic detergent SDS slightly improved the enzyme activity at 5 mM, but also inhibited it at the highest concentration. The purified esterase of *A. westerdijkiae* was able to hydrolysate *p*-NPB in the presence of 25% organic solvent, being more active in the presence of acetone (Table 2).

3.3 MS and bioinformatics analysis

The MS analysis revealed a peptide with the sequence TWGLG and other small peptide sequences. A search in the *A. westerdijkiae* sequenced complete genome [17] found a protein containing those sequences (awe06028), which had one identical mass peak and two other very

close in size with our experimental MS data (not shown). This serine hydrolase protein (sequence deposited in GenBank as AN BK010323) has 303 amino acids with a predicted mass of 33.9 kDa (Fig. 8A). A BLASTp search for high identity proteins in other *Aspergillus* species sequenced genomes discovered other proteins, which aligned well with the *A. westerdijkiae* esterase (Fig. 8A).

A protein structural model was obtained with the Swiss model program, using as an identity template the putative esterase YHR049W/FSH1 (PDB 1ycd.1.A) from *Saccharomyces cerevisiae*, which has 19.31% of identity with the *A. westerdijkiae* esterase (Fig. 8B) [18]. Another similar structure model was also predicted with the program Phyre2 using the same template (not shown). The MODELLER program was not able to generate any model.

Esterases sequences from *Aspergillus* spp. were retrieved from databanks and were aligned. Conserved sequences were used to search for putative esterases in the *A. westerdijkiae* sequenced genome [17]. Six other serineα/β-hydrolases of similar size were found (awe04148, awe01598, awe01975, awe07644, awe04453, and awe019431 deposited inGenBank as BK010324, BK010325,BK010326, BK010327,BK010328, andBK010329, respectively). These proteins were classified using the ESTHER (ESTerases and alpha/beta-Hydrolase Enzymes and Relatives), LED, and Pfam (Protein Families Database) databases. Aprotein tree was generated with those proteins along with putative esterases from other *Aspergillus* species (Fig. 9).

4 DISCUSSION

The hydrolytic halo observed in tributyrin agar plates indicated the esterase production by *A. westerdijkiae*. The obtained growth and enzyme production time course shows that the esterase production was dependent on growth until the exponential phase was reached, after which it remained stable. In consonance with our results, the highest lipase production was also reached after 72 h of culture for the fungi *Penicillium restrictum* and *Fusarium solani* [19,20]. However, lipase production peak was also found as early as after 48 h of culture for *Aspergillus nidulans* [21] and as late as 96 h of culture for *Aspergillus* sp. [22]. These variances may reflect dissimilarities in the used culture medium and culture conditions, and in the differences among fungi physiology.

The esterase production by *A. westerdijkiae* is regulated by oil presence. Similarly, oil was found to modulate lipase secretion by *P. restrictum* and *Candida rugosa* [19,23] and the production of a mycelium-bound lipase by *A. westerdijkiae* [9]. Accordingly, the co-production of extracellular esterase and cell-bound lipase by *C. rugosa* [24] was dependent on oil presence in the culture medium.

Although the lipid source in the culture medium had no effect on the esterase production, the fungus growth was higher when the liquid medium was supplemented with oils containing greater amounts of unsaturated and long chain fatty acids. Similar results were found for lipase production by *F. solani* and *C. rugose* [20,23]. Addition of higher amounts of corn oil in the culture medium did not improve esterase secretion by *A. westerdijkiae*. Conversely, increasing concentrations of oil improved lipase secretion by *P. restrictum* and *Aspergillus terreus*, however, in both cases too much oil diminished the associated lipase secretion [19,25].

In spite of the fact that all carbohydrates added to the medium bolstered *A. westerdijkiae* growth, they presented a negative effect on the esterase secretion. The same was observed in the lipase production by *A. nidulans* [21]. Correspondingly, the media supplemented with ions decreased the esterase production by *A. westerdijkiae*. In regard to growth, calcium was found to stimulate it, while selenium decreased it. Ions can form complexes with fatty acids and this may have inhibited the esterase secretion, as it was reported for the *Penicillium camembertii* lipase synthesis inhibition by addition of Fe^{+2} and Ca^{+2} to the medium [26]. Contrarily, Ca^{+2} along with Mg^{+2} improved lipase secretion by *A. terreus* [25,27].

It was verified that the inoculum size had little impact on increasing esterase production by *A. westerkijkiae*, notwithstanding its importance for lipase production using solid-state fermentation by *Penicillium simplicissimum*, in which a maximum enzyme production was reached with a specific inoculum size [28]. Agitation was essential to the esterase production by *A. westerdijkiae*, but it was observed that a faster agitation of 100 rpm decreased the enzyme production (data not shown), probably because it disturbed the oil and mycelia contact. The aeration provided by agitation has been reported to improve lipase production by *Aspergillus* sp. [22]. Even though light may alter many aspects in fungi, including fatty acid metabolism [29], it did not affect esterase biosynthesis by *A. westerdijkiae*. The growth of *A. westerdijkiae* remained statistically the same regardless of agitation, light, or inoculum size.

Tween 80 has been reported as a good inducer of lipolytic activity production by *Trichosporon asahii* [30], due to its role in cell permeabilization and enzyme liberation. Which could explain, at least in part, the Tween 80 influence on the higher esterase production by *A. westerdijkiae*. Furthermore, Tween 80, without the addition of any oil, could support enzyme production and fungus growth, and this indicates that *A. westerdijkiae* can use it as a carbon

source, considering that Tween 80 has oleic acid in its composition. In fact, the lyophilized mycelium of *A. westerdijkiae* presented activity against Tween 80. The activity against long fatty acids esters in *A. westerdijkiae* mycelia is due to the presence of a true lipase [9].

The employed protocol of ammonium sulphate precipitation, dialysis, and size exclusion chromatography in Sephacryl S-200 HR was efficient at purifying the esterase from *A. westerdijkiae*. The monomeric purified esterase estimated molecular weight of 32 kDa falls into a range of various other fungal esterases [1,2].

The K_M of 638.11 μM for *p*-NPB of the *A. westerdijkiae* esterase is higher than the K_M of 164 μM for the carboxylesterase from *A. oryzae* [31], thus evidencing a lower affinity for *p*-NPB. The purified enzyme was more active in alkaline pHs. Esterases generally have acidic optimum pH [1], but alkaline esterases have also been reported in fungi and bacteria [1,2]. The thermal stability and optimum temperature found for the *A. westerdijkiae* esterase may prove to be advantageous, since many biocatalytic processes are carried out at temperatures below 40 °C to protect labile substrates.

The high specificity for *p*-NPB found for the purified esterase proves that this enzyme is a true esterase. The existence of an essential serine residue in its catalytic site and the participation of SH groups in catalysis or in the enzyme structure are supported by the strong inhibition by PMSF and Hg^{2+}, respectively. The esterase of *A. oryzae* was also strongly inhibited by Hg^{2+} [31]. The EDTA inhibition of the purified enzyme suggests that this esterase may need a divalent cation as cofactor. The enzyme activation by Na^+ and K^+ was probably due to a salting in effect.

The Ca^{2+} effect on the purified esterase has already been observed on other esterases [2]. Detergents can alter the tertiary conformation of enzymes, which probably caused the reduced esterase activity, whereas the slight activation by SDS was presumably a result of its capability to

prevent enzyme aggregation. Lipases and esterases ability to withstand water-restricted environments is crucial for their employment in organic synthesis, thus, the purified enzyme ability to hydrolyze *p*-NPB in the presence of organic solvents suggests its possible usage in this biotechnological realm.

Mass peaks differences among the purified protein in this work and the protein found in the sequenced genome of *A. westerdijkiae* could reflect strain specificity, which is common in microorganisms. The protein with the highest identity with the studied esterase (54.3%) is the one from *A. terreus*, which belongs to the *Aspergillus* genera Terrei section, phylogenetically closer with the *A. westerdijkiae* Circumdati section [32].

The obtained structure model of the *A. westerdijkiae* esterase awe06028 misses two αβ strands, a characteristic shared by the putative esterase YHR049W/FSH1 from *S. cerevisiae* [18]. The *A. westerdijkiae* esterase awe06028 has no secretion signal peptide and no *N*-glycosylation sites. Hence, this protein is probably secreted by a non-classical secretion pathway, different of the Endoplasmic Reticulum-Golgi apparatus secretion pathway, which is concordance with protein secretion in *S. cerevisiae*, where most of the proteins are secreted by the non-classical pathway [33]. Most of the fungi cell wall proteins are glycosylated and are secreted by the classical pathway [34]. Accordingly, Castro et al. [9] have found an esterase with a high activity against *p*-NPB in the *A. westerdijkiae* lyophilized mycelia, which could be ascribed to the esterase purified in this work in its secretion pathway.

The protein tree constructed with the *A. westerdijkiae* serine α/β-hydrolases and the esterases from *Aspergillus* spp. created seven distinct groups of proteins. Inside each group, the proteins had more than 40% of identity, but among the groups the identity was lower than 20%. No tree could be constructed when feruloil-esterases or carbohydrate esterases were used. All the

enzymes from the group containing the esterase here described (abH22) and the majority of the enzymes of two other groups had the sequence WGXG found by MS in this work. Those enzymes are putative noncytosolic or extracellular carboxylesterases (abH1) or lipases/esterases (abH7) without a signal peptide. Therefore, this sequence could be used as a marker for this kind of esterases. The other groups contained membrane segments (abh15), esterases with signal peptide (abH33), or lipases-like from the GGGX class (abH4).

In conclusion, the production of an esterase by *A. westerdijkiae* has been optimized and the produced enzyme was purified and characterized as a potential biotechnological catalyzer.

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CONFLICTS OF INTEREST

The authors indicate no conflicts of interest.

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Table 1. Two-step purification of the esterase from *A. westerdijkiae*

Sample	Volume (mL)	Proteins (mg)	Total activity (U)	Specific activity (U/mg protein)	Recovery (%)
Filtrate	230.0	10.5	1619.8	153.9	100.0%
Precipitation and dialysis	3.5	2.8	933.2	338.8	57.6%
Sephacryl S-200 HR	4.0	0.2	279.1	1468.7	17.2%

Table 2. Impact of activators, inhibitors, and organic solvents on the esterase activity.

Substance	Concentration	Relative activity (%)[a]	Concentration	Relative activity (%)[a]
Control	-	100	-	100
$HgCl_2$	5 mM	0	10 mM	0
$MgSO_4 \cdot 7H_2O$	5 mM	98	10 mM	62
NaCl	5 mM	124	10 mM	95
KCl	5 mM	121	10 mM	101
$NiCl_2 \cdot 6H_2O$ [b]	5 mM	32	10 mM	26
$ZnSO_4 \cdot 7H_2O$[b]	5 mM	12	10 mM	8
$CaCl_2 \cdot 2H_2O$[b]	5 mM	101	10 mM	62
EDTA	5 mM	48	10 mM	15
SDS	5 mM	112	10 mM	81
PMSF	5 mM	0	10 mM	0
Tween 20	1%	60	2%	29
Tween 80	1%	43	2%	23
Triton X–100	1%	46	2%	22
Ethanol	25%	34		
Methanol	25%	39		
Isopropanol	25%	27		
Acetone	25%	54		
Acetonitrile	25%	20		

[a]The results are the average of duplicates.

[b]The enzyme assay contained 50 mM Tris buffer, pH 7.0, in substitution to the phosphate buffer, to avoid precipitation of the reaction.

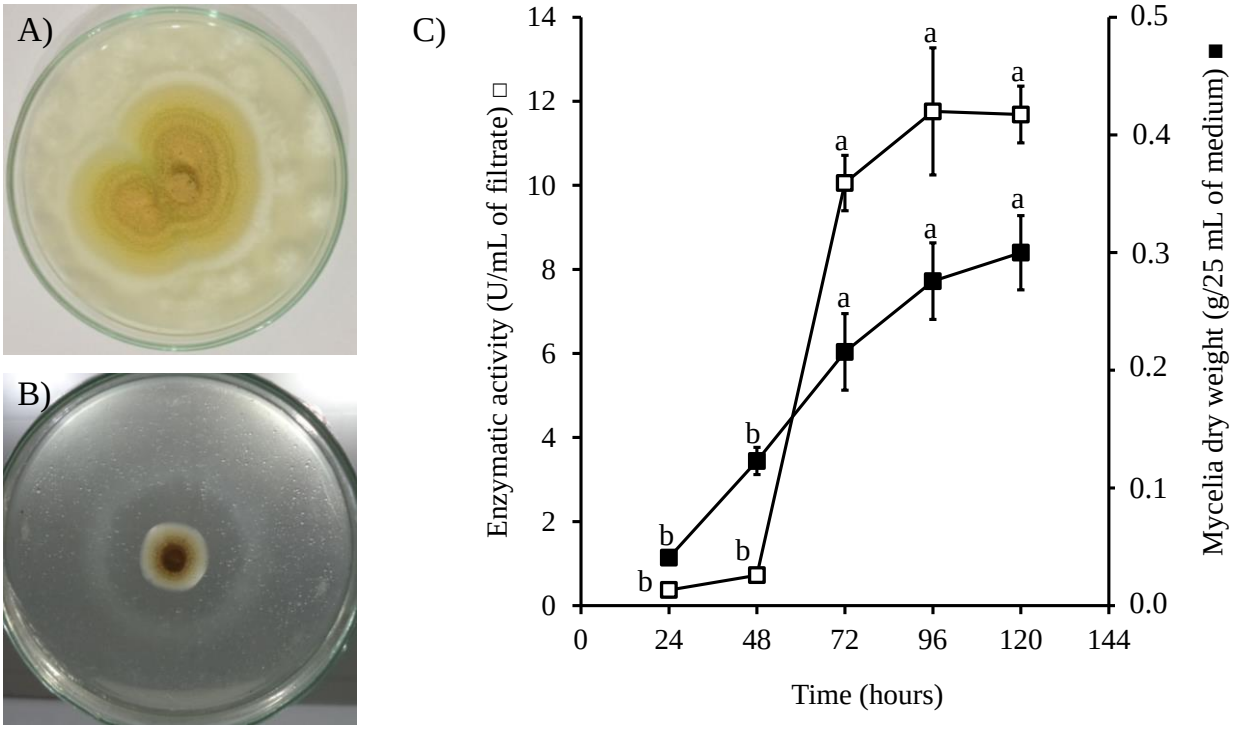

FIGURE 1. *A. westerdijkiae* esterase production. A) *A. westerdijkiae* culture in PDA. B) *A. westerdijkiae* culture in tributyrin Agar, showing the hydrolysis halo. C) *A. westerdijkiae* growth and enzyme production curve. CV for enzymatic activity was 12% and for mycelium dry weight was 11.9%.

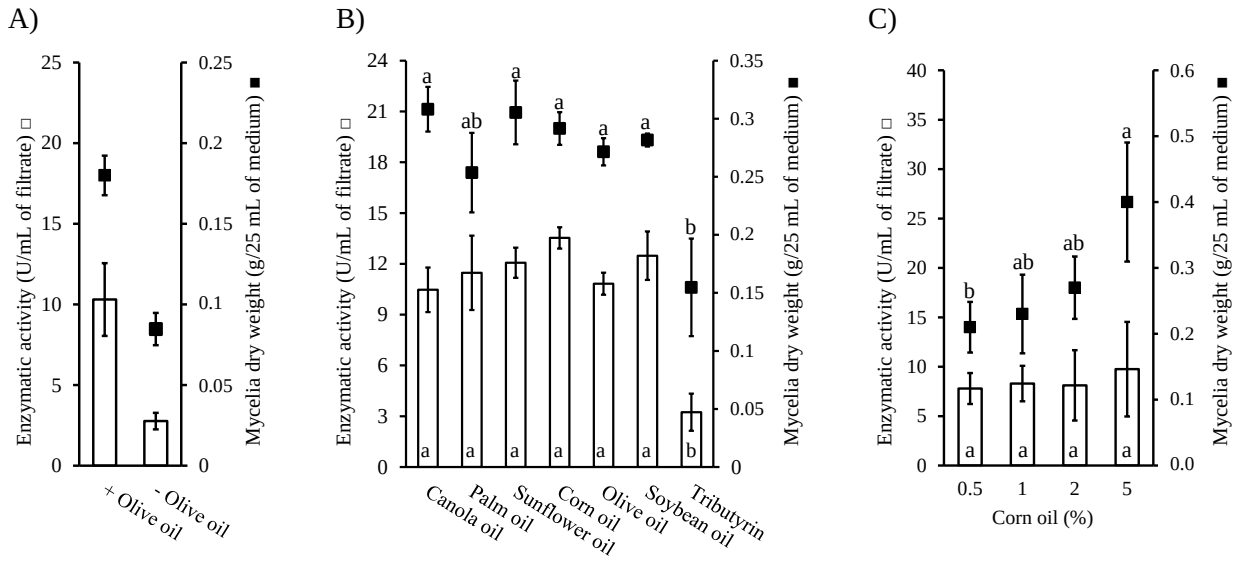

FIGURE 2. The carbon source effect on the *A. westerdijkiae* esterase production. A) The culture was performed with and without vegetal oil. B) Culture was executed with different vegetal oil (1%, v/v). C) Several concentrations of corn oil were used to induce the enzyme production. In (B) CV for enzymatic activity was 12% and for mycelium dry weight was 11.2%. In (C) CV for enzymatic activity was 43% and for mycelium dry weight was 22.8%.

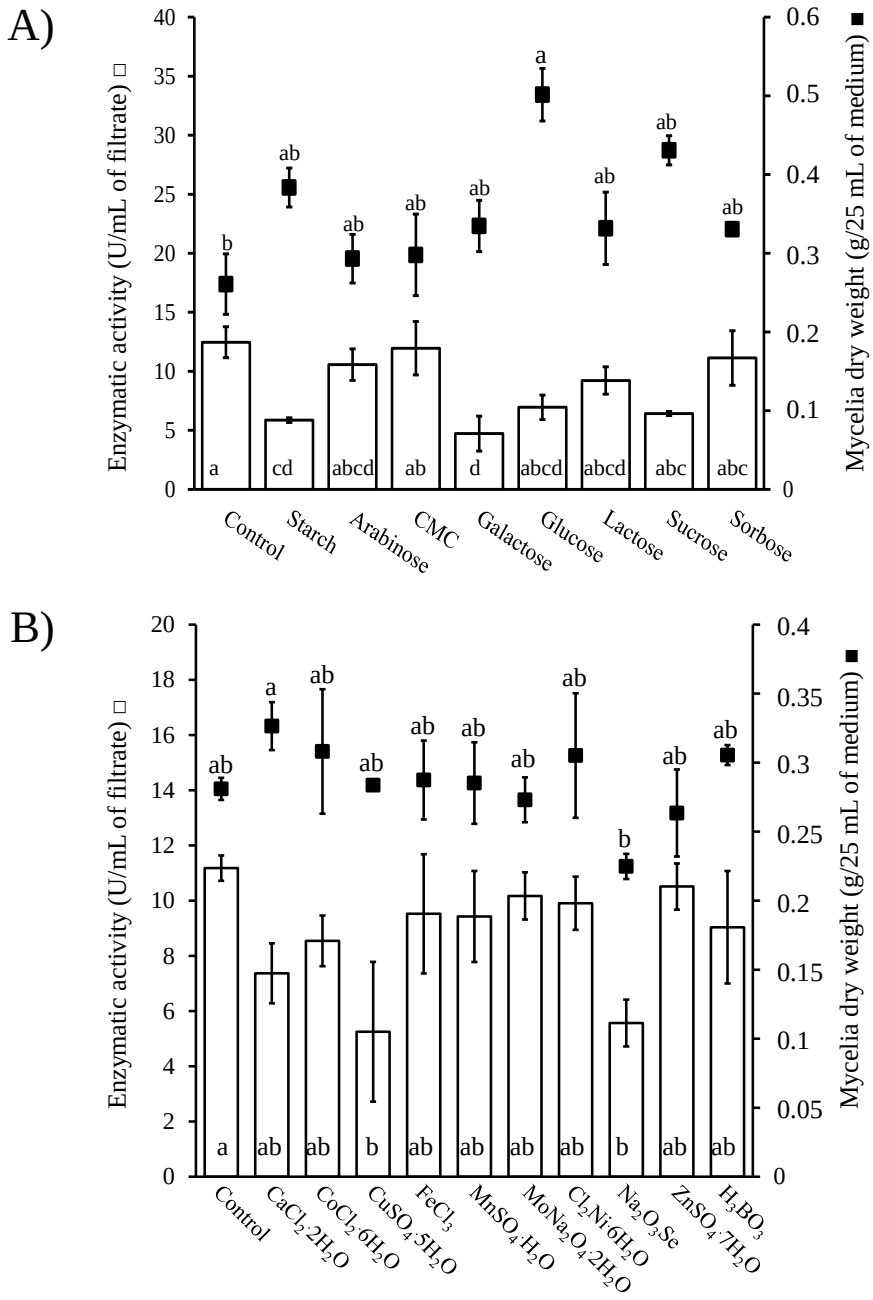

FIGURE 3. Effect of sugars and ions addition to the culture medium on the *A. westerdijkiae* esterase production. A) Different sugars were added to the culture medium at concentration of 1%. Control had no sugar added. B) Different salts were added to the medium at concentration of 20 μM. Control had no salt added. In (A) CV for enzymatic activity was 18% and for mycelium dry weight was 14%. In (B) CV for enzymatic activity was 16.9% and for mycelium dry weight was 8.3%.

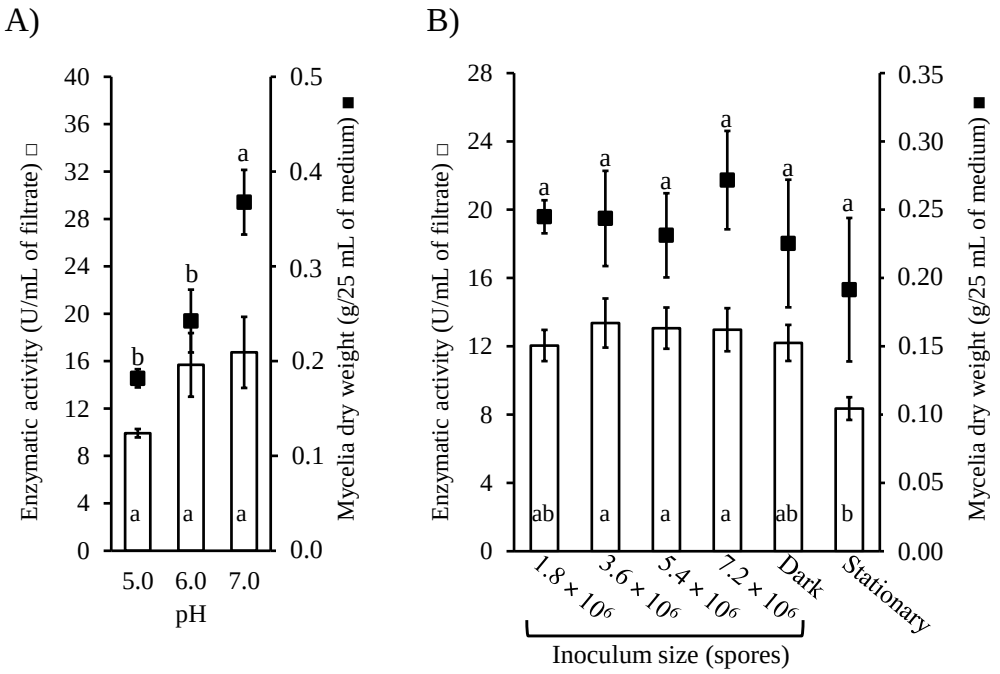

FIGURE 4. Effect of the medium initial pH, inoculum size, light, and agitation on the *A. westerdijkiae* esterase production. A) Effect of the culture medium initial pH. B) Effect of the inoculum size, light, and agitation. In (A) CV for enzymatic activity was 21% and for mycelium dry weight was 9.3%. In (B) CV for enzymatic activity was 9% and for mycelium dry weight was 15.1%.

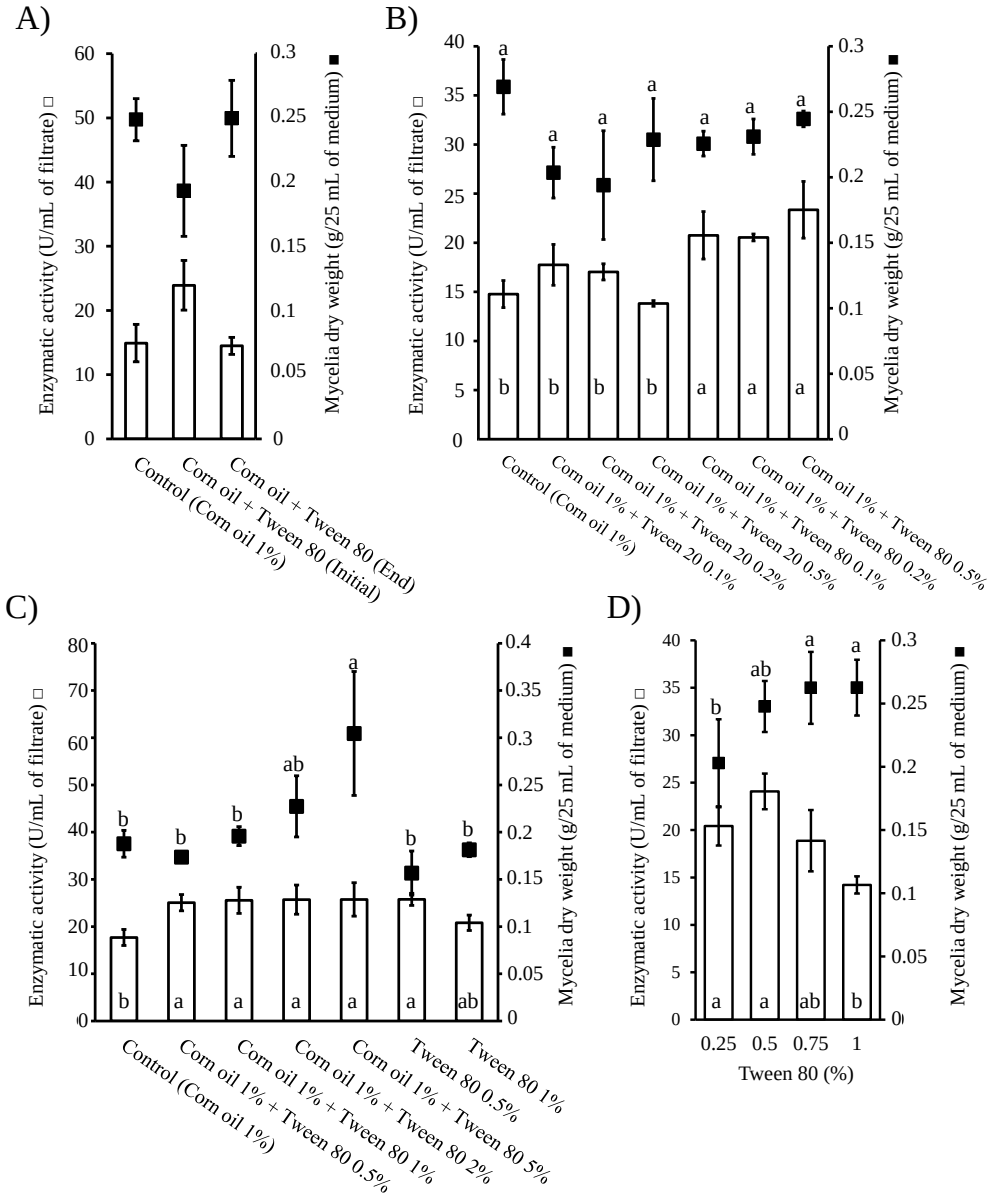

FIGURE 5. Effect of Tween 80 addition to the medium on the *A. westerdijkiae* esterase production. A) Tween 80 was added to the culture medium containing 1% corn oil, at the concentration of 0.2%, at the beginning of the culture or in the last 30min of the culture. B) Different concentrations of Tween 80 and Tween 20 were added to the culture medium containing 1% corn oil. Different letters indicates differences among averages in the esterase production data ($\alpha = 0.05$). C) Higher concentrations of Tween 80 with and without 1% corn oil were added to the culture medium. D) The effect of Tween 80 alone. In (B) CV for enzymatic activity was 9% and for mycelium dry weight was 10.9%. In (C) CV for enzymatic activity was 7.3% and for mycelium dry weight was 14.4%. In D) CV for enzymatic activity was 9% and for mycelium dry weight was 7.8%.

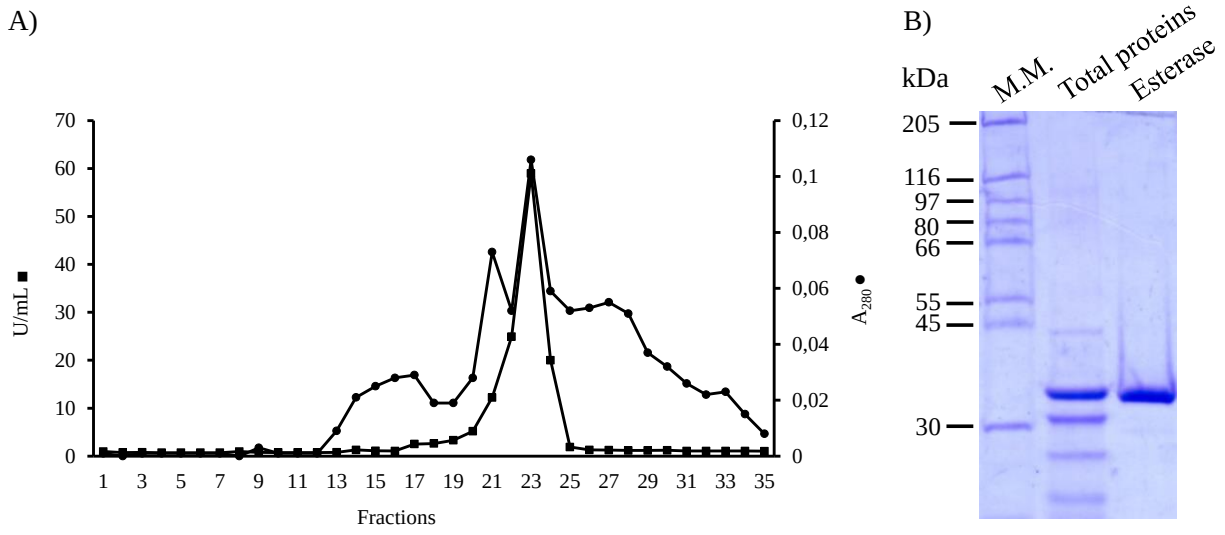

FIGURE 6. Purification of the esterase of *A. westerdijikiae*. A) Elution profile of the Sephacryl S-200 HR column, which was equilibrated and eluted with 50mM phosphate buffer, pH 7.0. The flow rate and fraction volume were 0.6 ml/min and 4 ml, respectively. B) Electrophoresis analysis. Proteins were developed in a 10% discontinuous SDS-PAGE. Total proteins are the proteins obtained after ammonium sulphate precipitation and dialysis (30 μg). Esterase is the purified enzyme (15 μg). The positions of the M.M. are indicated. The gel was stained with Coomassie blue.

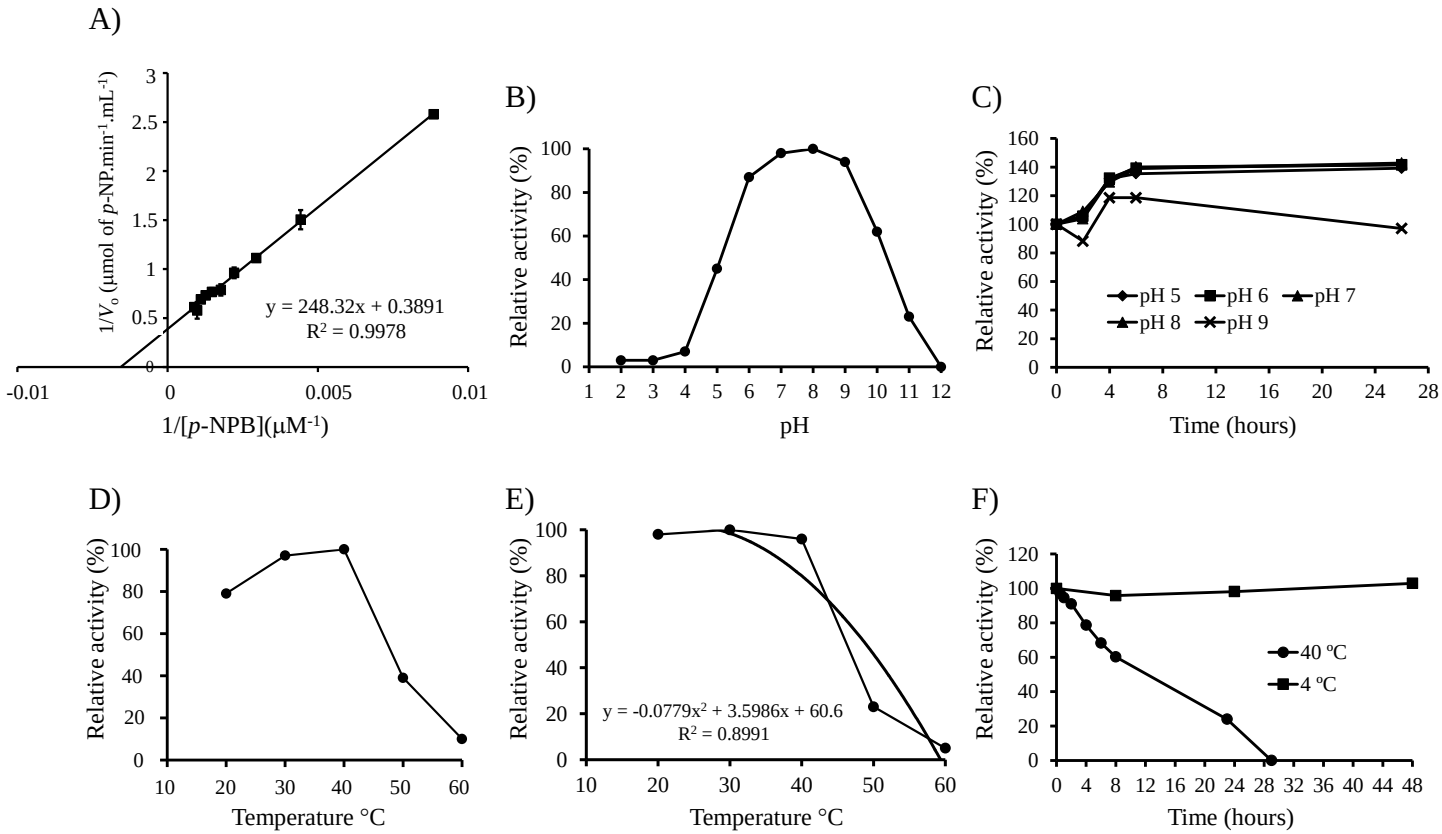

FIGURE 7. Kinetic analysis of the purified esterase from *A. westerdijkiae*. A) Lineweaver-Burk plot, varying the *p*-NPB concentration from 113 to 1130 µM. The data represents the average and the SD of three experimental data. B) Optimum pH. C) pH stability over time. D) Optimum temperature. E) Thermal stability. F) Thermal stability over time. The data represent the average of duplicates.

A)

```
A.aculeatus - XP_020060660.1    ----------------------------------------MYLDTPLQTPRKAKILMLHGTGQSAHTFQS
A. versicolor - OJI99616.1      MNRPKSSVPSFDHPMLEMASIRSYFEPFAPGNG-------GHT---PSTKGPTRILMLHGHGQSGQFFYH
A. sydowii - OJJ60284.1         MNRAKSSVPSFEHPLLEMASIRSYFEPFEPGNN-------GRKSPASSSKRPTRILMLHGHGQSGRFFYH
A. westerdijkiae - BK010323     ----------------------MNYVADYVPSDLLPIPPISPETSLPELVDKPIKILMIHGHGSSSSRLFY
A. terreus - XP_001210885.1     ---------------------------------MGVTGNIVPSASAASSANKPVKILMMHGNGSSGSRLDY
                                                                                :***:** *.*.  :

A.aculeatus - XP_020060660.1    KMRYLHEPIARALSATSMQHEILYPGGVEFFYLDGPIPASSIDACDETYMKPNRLPDSWLWGYGDPETS-
A. versicolor - OJI99616.1      KPTRLIETLHEIALDR---DTKRCSDRIELFYVNGPLLAGE-----------GPESDIWTWGQGDFEAET
A. sydowii - OJJ60284.1         KTTRLVEALHEIALDR---DAKRCSERIELFYVNGPLSAGQ-----------GPESDVWTWGQGDFEAER
A. westerdijkiae - BK010323     KYRALQPSTR----HE---ILHALHRDVEFYFPNAPILP-A-----------GFEPGMWTWGLGDYRVD-
A. terreus - XP_001210885.1     KTRHLQPLIR----DA---IKQRTNQNVEFFFPNAPFLP-T-----------GFDEDSFTWGLGDYRMS-
                                *   *                     :*::: :.*:              .   . : ** ** . .
                                                          Ser131
A.aculeatus - XP_020060660.1    ELKGLEHTWKRLANVLTEHGPFVGVIGFSAGAAWSVILTALLEQNRKN------PYFQVNHPPMHFCIAF
A. versicolor - OJI99616.1      HIWGLDASINKIMDVLAKHGPFDGVIGFSTGASIAAIITSLLEGNRRTRLDINQVFLQTRHPPFRFAVCF
A. sydowii - OJJ60284.1         RIWGLDASINKIMDILAKHGPFDGIIGFSTGATVAAIITSLLEGNRKARLDIKQ----IRHPPFRFAVCF
A. westerdijkiae - BK010323     RVPRLQESITYLVRYMEEHGPFDGIIGSSAGASVAVLVGSLLEGGVL-REGCEEVKMVTTHPPLKFILSY
A. terreus - XP_001210885.1     RVPGLDKSVAFLLSYLEEHGPFDGIIGSSAGCCVAVALASLLEN----PDRCAEFSVKTSHPRLRFILAY
                                .:  *: :   :   : :**** *:** *:*.  :. : :***                ** ::* :.:
                                                                  Asp188                  His225
A.aculeatus - XP_020060660.1    SAFMFEHRTYEWIYARRIQTPVLHFIAALDTWVSERQSLKLVGRCANAQVEYFQGSHYIPRGPQIRELVT
A. versicolor - OJI99616.1      SGFMLQHPDYRSFYQPQIKTPVMHFIAEYDTMIPEGLTRQLAAACSQREVQKFKGTHYVPRQREEVVHVS
A. sydowii - OJJ60284.1         SGFMLQHPDYRAFYHPKIRTPVMHCIAEYDTMIPEALTRQLATACAQREVQKFKGTHYVPRQREEVVNIS
A. westerdijkiae - BK010323     SGFKMSHVCYRAIYRPKIQTPVMHFIGNLDTYIPGPLTLQLARRCINHKVVYFHGCHYIPRLGGTTMAAA
A. terreus - XP_001210885.1     SGCVMENPCYSSLYSPKVQTPAMFFIGELDSFIPPDLTMRLADCCSNSAVVTFWGTHYIPRFHETNSAAV
                                *.  :.:  *  :*  :::**.:. *.  *: :    : :*.  * :  *  * * **:**

A.aculeatus - XP_020060660.1    RFIRRTGGGVGEVDRGCIGV-----------------------------------------------
A. versicolor - OJI99616.1      EFILRHLGWLADEAGYITCSSDG--------------------------------------------
A. sydowii - OJJ60284.1         NFILRHLDWPEDETGYSTCSSNGR-------------------------------------------
A. westerdijkiae - BK010323     GFMCDCLGGGKRVVGKGEGNNRDRHRNSQGGDVEEVDGEKQENDDDGEWEWVEDEKIGDWVAKLL
A. terreus - XP_001210885.1     DFVCRALAGGREEDANNW---------------------------------VDI-----------
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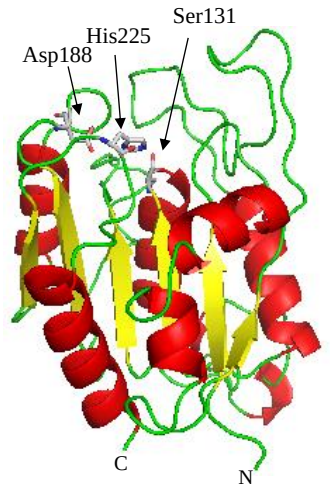
B)



C)

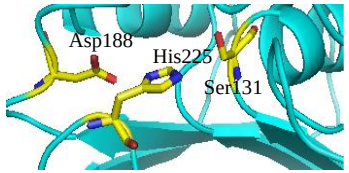


FIGURE 8. Analysis of the *A. westerdijkiae* awe06028 esterase sequence and structure. A) Clustal Omega alignment of the *A. westerdijkiae* awe06028 (BK010323) esterase sequence with other *Aspergillus* genera esterases sequences. In red and underlined are the MS obtained sequences. In blue are the active site catalytic triades Ser-Asp-His. In green are the serine-esterases common consensus sequence Gly-X-Ser-X-Gly, around the active site. B) The structural model of the esterase awe06028. C) Details of the predicted active site.

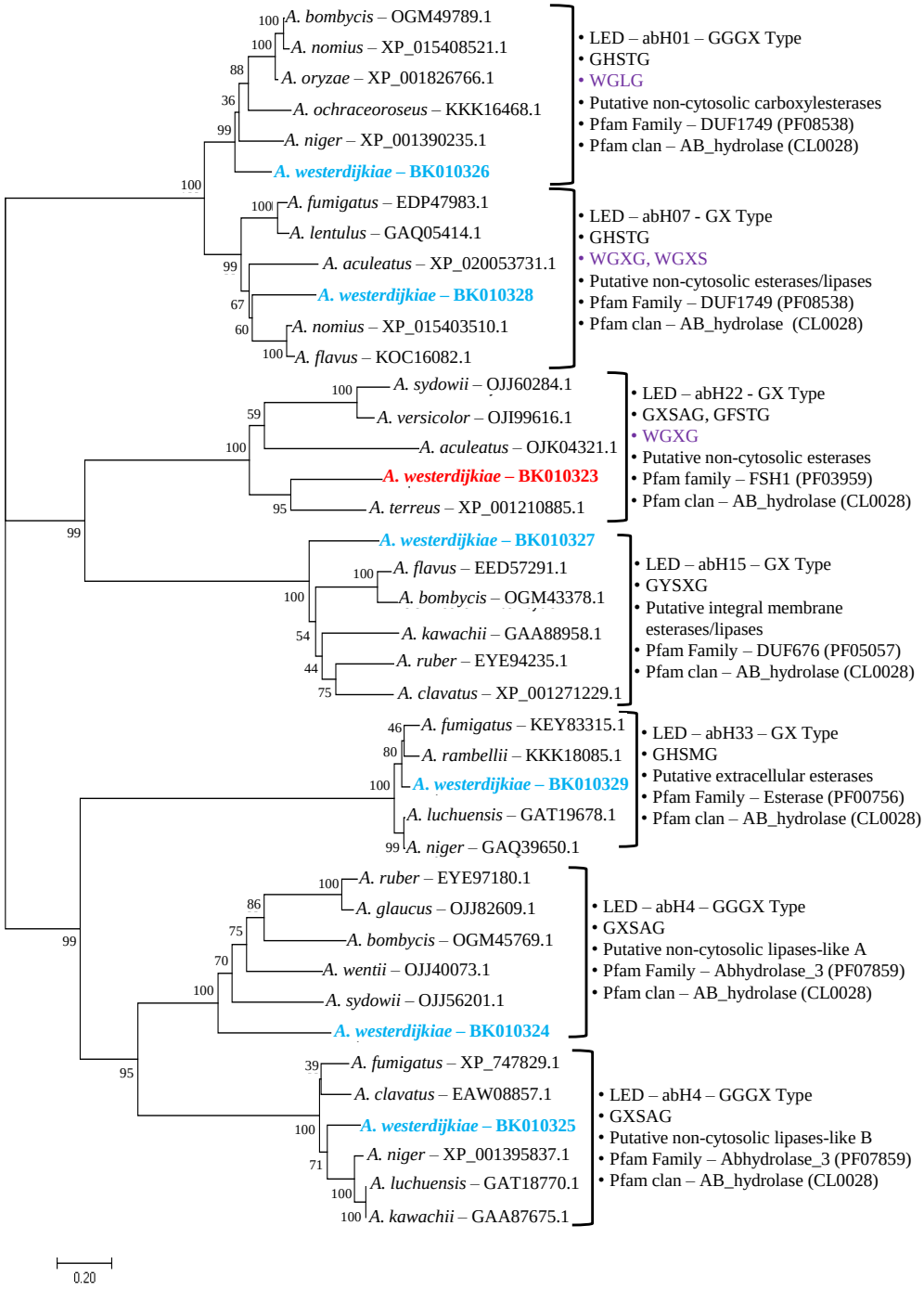


FIGURE 9. *Aspergillus* spp. serine-hydrolases amino acid sequence analysis. The tree was inferred using the Neighbor-Joining method using MEGA 7.0. Bootstrap values are indicated at 1000 replicates. Distances were computed using the Poisson method and are in the number of amino acid substitutions per site units. Ambiguous positions were removed. *A. westerdijkiae* sequences are in blue or red (protein from this work). The G-X-S-X-G consensus sequences around the active site and the WGXG (in purpur) consensus sequence are indicated. The alpha-beta hydrolases (abH) families found in the LED database and Pfam families and clans are also indicated.