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DAMARIS BATISTÃO MARTIM

DUAS NOVAS ACETIL ESTERASES DE *Pantoea dispersa*: EXPRESSÃO RECOMBINANTE, PURIFICAÇÃO E CARACTERIZAÇÃO

Maringá
2019

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Dissertação 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 Mestre em Ciências Biológicas.

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Dissertação apresentada como requisito parcial para obtenção do grau de Mestre em Ciências Biológicas do Programa de Pós-Graduação em Ciências Biológicas, da Universidade Estadual de Maringá, sob a apreciação da seguinte banca examinadora:

Aprovado em 28 de fevereiro de 2019.

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BIOGRAFIA

Damaris Batistão Martim nasceu em Japurá, Paraná, em 30 de Maio de 1994. Possui ensino médio pelo Colégio Santa Maria Goretti, Maringá, Paraná e bacharelado em Bioquímica pela Universidade Estadual de Maringá (UEM) – Maringá, Paraná (2013-2016). Em março de 2017 iniciou o curso de Mestrado em Ciências Biológicas - Biologia Celular e Molecular pelo programa de Pós-graduação em Ciências Biológicas (PBC-UEM). Tem experiência na área de Biologia Molecular e Bioquímica, atuando principalmente nas áreas de Microbiologia Aplicada, Expressão de Proteínas Recombinantes e Enzimologia.

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É melhor conseguir sabedoria do que o
ouro; é melhor ter conhecimento
do que a prata.

O que adquire entendimento ama a sua vida
e quem age com inteligência
encontra a felicidade.

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APRESENTAÇÃO

Esta dissertação é composta de um artigo científico que descreve a clonagem de dois genes que codificam acetil esterases da bactéria *Pantoea dispersa*. Em adição, estes genes foram expressos de forma recombinante em *Eschericha coli* e as enzimas produzidas foram purificadas e caracterizadas. Em consonância com as regras do Programa de Pós-graduação em Ciências Biológicas, esta dissertação foi redigida como um artigo científico que será enviado para análise quanto à publicação para a revista Enzyme and Microbial Technology.

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LISTA DE ABREVIAÇÕES

°C – Grau Celsius.

µL – Microlitro(s).

µmol – Micromol(s).

AN – GenBank Número de Acesso

bp – Pares de bases.

DNA – Ácido desoxirribonucléico.

EDTA – Ácido etilenodiamino tetra-acético

g – Grama(s).

g/L – Grama(s) por litro.

h – Hora(s)

kDa – KiloDalton(s).

K_M – Constante de Michaelis-Menten.

L – Litro(s).

M – Molar

M.M. – Marcador Molecular

m/v – Massa por volume.

mg – Miligrama(s).

mg/L – Miligrama(s) por litro.

min – Minuto(s)

mL – Mililitro(s).

mM – Milimolar.

ng – Nanograma(s)

nm – Nanômetro(s).

PAGE – Eletroforese em gel de poliacrilamida.

PCR – Reação em cadeia da polimerase.

pH – Potencial de hidrogênio.

PMSF – Fluoreto de fenilmetano sulfonila

p-NP – *p*-Nitrofenol.

p-NPA – *p*-Nitrofenol acetato.

p-NPB – *p*-Nitrofenol butirato.

p-NPD – *p*-Nitrofenol dodecanoato.

p-NPP – *p*-Nitrofenol palmitato.

rpm – Rotações por minuto.

SDS – Dodecil sulfato de sódio.

SDS-PAGE – Dodecil sulfato de sódio – Eletroforese em gel de poliacrilamida.

Tween 80 - Polioxietileno (80) sorbitan monooleate

U – Unidade Internacional.

UV – Ultravioleta.

v/v – Volume/volume.

V_{max} – Velocidade Máxima

w/v – Massa (*Weight*) por volume.

RESUMO GERAL

Introdução: Acetil esterases são enzimas capazes de hidrolisar ligações carboxílicas do éster acético. A maioria das acetil esterases apresenta estrutura similar às clássicas lipases e esterases, que consiste em uma dobra de α/β-hidrolase, normalmente composta por um domínio de oito folhas β circundadas por α-hélices. Além disso, seus sítios ativos apresentam a tríade catalítica Ser-Asp-His e uma sequência consenso Gly-x-Ser-x-Gly (onde x pode ser qualquer aminoácido) ao redor da serina do sítio ativo. Carboidrato acetil esterases são enzimas capazes de desacetilar carboidratos como pectina e xilano. A ação sinergética de pectina acetil esterases com pectinases pode aumentar a eficiência de filtração e clareamento de sucos de frutas. A ação de xilano acetil esterases permite a degradação do xilano por outras enzimas e a consequente completa degradação da matéria lignocelulósica. Anteriormente em nosso laboratório, uma cepa da bactéria *Pantoea dispersa* produtora de enzimas lipolíticas foi isolada de grãos de milho apresentando sintomas de podridão. **Objetivos:** Identificar prováveis genes de lipases e esterases da bactéria *P. dispersa*, clonar e expressar estes genes em *Escherichia coli*, purificar e caracterizar as enzimas expressas. **Métodos:** Inicialmente, seis genes de prováveis lipases e esterases foram encontrados no genoma sequeciado e anotado de *P. dispersa*. Iniciadores foram desenhados para amplificar os mesmos por PCR a partir do DNA genômico da cepa de *P. dispersa*. Todos os genes foram amplificados, clonados no vetor pCR2.1® e subclonados no vetor de expressão pET21a(+). Cada vetor construído foi transformado em *E. coli* cepa Bl21 Star^{TM}(DE3). Após cultura, as células da bactéria transformada foram sonicadas e o homogeneizado obtido foi centrifugado. A atividade enzimática de lipase e esterase foi testada no sobrenadante com os substratos *p*-nitrofenil acetato, *p*-nitrofenil butirato e *p*-nitrofenil palmitato. Os genes das enzimas que apresentaram atividade foram sequenciados e as sequências de aminoácidos obtidas pela tradução das sequências de nucleotídeos foram utilizadas para estudos filogenéticos e de modelagem por homologia. As enzimas expressas com atividade foram purificadas por meio de cromatografia de afinidade. Análises eletroforéricas foram realizadas para confirmar a homogeneidade, determinar a massa molecular das enzimas, identificar a presença de multímeros e realizar zimograma. Os dados cinéticos de pH e temperatura ótimos, estabilidade térmica e estabilidade em diferentes pHs e temperaturas foram obtidos com o ensaio enzimático realizado em diferentes condições com as enzimas purificadas e com o *p*-nitrofenil acetato como substrato. A especificidade das enzimas foi avaliada com substratos sintéticos, com triacetina e com os carboidratos acetilados pectina e xilano. **Resultados e discussão:** Apenas duas das enzimas expressas, denominadas Est-1 e Est-2, apresentaram atividade de esterase, sendo mais ativas contra o substrato *p*-nitrofenil acetato. A massa molecular encontrada para a Est-1 e Est-2 foi de 33 kDa and 37 kDa, respectivamente. Ambas as proteínas apresentaram estrutura modelada de homodímeros com monômeros com estrutura de α/β-hidrolase, com a tríade catalítica Ser-Asp-His presente no sítio ativo. As duas enzimas também apresentaram atividade contra triacetina e foram capazes de desacetilar pectina e, em menor proporção, xilano. Os valores de K_M e a $V_{máx}$ encontrados para a Est-1 foram de 1,52 mM e 9,0 μmol/min e para a Est-2 foram de 0,6 mM e 8,0 μmol/min, respectivamente. Ambas as enzimas apresentaram um pH ótimo de 7,0. A temperatura ótima da Est-1 foi de 40 °C e da Est-2 de 50 °C. A temperatura em que as enzimas Est-1 e Est-2 perderam metade da atividade (T_{50}) foi de 44,1 e 58,9 °C, respectivamente. As enzimas foram significativamente inibidas por SDS, EDTA e PMSF. **Conclusões:** Duas novas acetil esterases de *P. dispersa*, com baixa identidade na sequência de aminoácidos entre si, foram purificadas e caracterizadas. Ambas as enzimas mostraram atividades catalíticas similares, como a desacetilação de triacetina, pectina e xilano.

GENERAL ABSTRACT

Introduction: Acetylesterases are enzymes able to hydrolyze carboxylic bonds of the acetic ester. Most of the acetylesterases have a structure similar to the classical lipases and esterases, which consist of an α/β-hydrolase fold structure, normally composed by an eight β-strands domain surrounded by α-helices. In addition, their active sites have the conserved catalytic amino acid triad Ser-Asp-His and the consensus sequence Gly-x-Ser-x-Gly (where x can be any amino acid) around the serine of the active site. Pectin is one acetylated polysaccharide, which constitute the plant cell wall. Carbohydrate acetylesterases are enzymes capable to deacetylate carbohydrates as pectin and xylan. The synergic action of pectin acetylesterases with pectinases can increase the filtration efficiency and clarification of fruit juices. The xylan acetylesterases allow the xylan degradation by other enzymes and the consequent complete degradation of the lignocellulosic matter. Previously in our laboratory, a lipolytic enzymes producing strain of *Pantoea dispersa* was isolated from maize grains presenting rotten symptoms. **Objectives:** To identify putative genes of lipases and esterases from *P. dispersa*, to clone and express those genes in *Escherichia coli*, and to purify and characterize the expressed enzymes. **Methods:** Initially, six genes coding for putative lipases and esterases were found in the sequenced and annotated genome of *P. dispersa*. Primers were designed to amplify these genes by PCR from the genomic DNA of *P. dispersa*. All the genes were amplified, cloned into the pCR2.1® vector, and subcloned in the pET21a(+) expression vector. Each constructed vector was transformed into the *E. coli* Bl21 Star ™ (DE3). After culture, the transformed bacterial cells were sonicated and the obtained homogenized was centrifuged. The enzymatic activity of lipase and esterase was tested in the supernatant with the substrates *p*-nitrophenyl acetate, *p*-nitrophenyl butyrate, and *p*-nitrophenyl palmitate. The genes of the enzymes that presented activity were sequenced and the amino acid sequences were obtained by the nucleotide sequences translation. The obtained protein sequences were used for phylogenetic studies and homology modeling. The expressed enzymes presenting activity were purified by affinity chromatography. Electrophoretic analyses were performed to confirm the homogeneity, to determine the molecular mass of the enzymes, to identify the formation of polymers, and to perform a zymogram. The kinetic parameters of optimum pH and temperature, thermal stability, and stability at different pHs and temperatures were carried out with the purified enzymes and with modifications in the enzymatic assay conditions, using *p*-nitrophenyl acetate as substrate. The enzymes specificities were evaluated with synthetic substrates, with triacetin, and with the acetylated carbohydrates pectin and xylan. **Results and discussion:** Only two of the expressed enzymes, denominated Est-1 and Est-2, showed activity of esterase, being more active against *p*-nitrophenyl acetate. The determined molecular mass for Est-1 and Est-2 was of 33 kDa and 37 kDa, respectively. Both proteins presented a modelled structure of homodimers with monomers presenting the α/β-hydrolase fold, with the catalytic triad Ser-Asp-His present in the active site. The two purified enzymes also presented activity against triacetin and were able to deacetylate pectin and, in lower proportion, xylan. The K_M and V_{max} values found for Est-1 were of 1.52 mM and 9.0 μmol/min and for Est-2 were of 0.6 mM and 8.0 μmol/min, respectively. Both enzymes presented an optimum pH of 7.0. The optimum temperature for Est-1 was 40 °C and for Est-2 was 50 °C. The temperature in which the enzymes Est-1 and Est-2 lost half of its activity (T_{50}) was of 44.1 and 58.9 °C, respectively. The enzymes were significantly inhibited by SDS, EDTA, and PMSF. **Conclusions:** Two novel acetylesterases from *P. dispersa*, with low amino acid sequence identity among them, were purified and characterized. Both enzymes showed similar catalytic activities, such as the deacetylation of triacetin, pectin and xylan.

TWO NOVEL ACETYLESTERASES FROM *Pantoea dispersa*: RECOMBINANT EXPRESSION, PURIFICATION, AND CHARACTERIZATION

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ABSTRACT

Two novel acetylesterases from *Pantoea dispersa*, with low amino acid sequence identity among them, were expressed in *Escherichia coli* with a carboxyl-His_6 tail given by the expression plasmid, purified, and characterized. The purified proteins, named Est-1 and Est-2, had a molecular mass of 33 kDa and 37 kDa, respectively. Both proteins presented a modelled structure of homodimers with monomers presenting the α/β-hydrolase fold, with the catalytic triad Ser-Asp-His present in the active site. The K_M for *p*-nitrophenyl acetate and V_{max} values found for Est-1 were of 1.52 mM and 9.0 μmol/min and for Est-2 were of 0.6 mM and 8.0 μmol/min, respectively. Both enzymes presented an optimum pH of 7.0. The optimum temperature for Est-1 was 40 °C and for Est-2 was 50 °C. The temperature in which the enzymes Est-1 and Est-2 lost half of its activity (T_{50}) was of 44.1 and 58.9 °C, respectively. The enzymes were significantly inhibited by SDS, EDTA, and PMSF. The two purified enzymes also presented activity against triacetin and were able to deacetylate the carbohydrates pectin and xylan, with higher activity against pectin. Thus, they can be considered as carbohydrate esterases.

Key words: Esterases, *Pantoea dispersa*, Lipolytic enzymes, Hydrolases.

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1 INTRODUCTION

The industrial enzymes global market is expected to reach nearly 6.2 billion dollars by 2020 [1]. Almost 75% of all industrial enzymes are hydrolytic enzymes that accounts for more than 70% of all enzyme sales [2,3]. Microbial enzymes are preferred due to their stability and higher catalytic activity [3]. Besides, microorganisms grow abundantly on simple and low-cost substrates and their production is not affected by seasonal fluctuations [3]. Furthermore, by means of recombinant DNA technology, an enzyme gene can be cloned and expressed in a recombinant way to produce large quantities of a protein [1]. Currently, about 200 microbial enzymes are used commercially, but only about 20 enzymes are produced on an industrial scale [2]. Nearly 150 industrial processes use enzymes or whole microbial cell catalysts and over 500 industrial products are made by means of enzyme technology [4]. Thus, the finding of new enzymes is on demand.

After proteases and amylases, lipolytic enzymes, i.e. lipases and esterases, are the most commercialized enzymes [3]. Esterases hydrolyze short chain fatty acids esters, whereas lipases hydrolyze long chain fatty acids esters, mainly triacylglycerol [5,6]. These enzymes do not need cofactors and, in organic interfaces, they can also catalyze esters synthesis. Lipolytic enzymes can be obtained from microorganisms, plants, and animals [7,8]. Several bacteria genera are reported to produce lipases, and those from the genera *Burkholderia*, *Chromobacterium*, and *Pseudomonas* are commercially available [5]. The main bacterial esterase producers are from the genera *Bacillus*, *Pseudomonas*, *Vibrio fischeri*, and *Geobacillus* sp. [6,7]. Lipases are used in the detergent, paper, biodiesel, and food industries, and in effluent treatment, while esterases can be employed in the food, pharmaceuticals, detergents, textiles, leather, and cosmetics industries [5,6,7].

Carbohydrate acetylesterases are enzymes that are capable of removing the acetyl group of acetylated polysaccharides, such as pectin and xylan [7]. Pectin is a plant cell walls complex polysaccharide consisting mainly of D-galacturonic acid resides in an alpha-(1-4) chain, which can be methyl-esterified at C-6 and acetyl-esterified at C-2 and/or C-3. Pectin acetylesterase removes the acetyl-ester group from the pectin homogalacturonan region, allowing the action of depolymerization enzymes [10,11]. Pectin acetylesterases are interesting for industrial application because they can work together with pectinases to increase fruit juices filtration efficiency and clarification [10,11]. Xylan is another component of plant cell walls and constitutes up to 35% of the total dry weight of higher plants. It consists of a homopolymeric backbone chain of β-1,4-linked D-xylose units and short side chains including L-arabinofuranosyl, and *O*-acetyl-D-glucuronosyl or *O*-methyl-D-glucuronosyl residues. Xylan acetylesterases, together with other enzymes, play a role in the complete degradation of xylan and, consequently, of the lignocellulosic matter [10,11].

Most of acetylesterases has the same structure of classical lipases and esterases, which consist in the α/β-hydrolase fold. This structure is normally composed by a domain of eight segments in β-conformation surrounded by α-helices [8]. In addition, in most of their active sites there is the conserved catalytic amino acid triad Ser-Asp-His and the consensus sequence Gly-x-Ser-x-Gly (where x can be any amino acid) around serine in the active site [9,10,11].

Recently, a lipolytic enzyme producing strain of *Pantoea dispersa* was found in a bioprospection study on maize grains presenting rotten symptoms [12]. There was a report of esterase production by *P. dispersa* [13], but there is no information about carbohydrate acetyl esterase production by this species. In the present study, two carbohydrate acetylesterases from *P. dispersa* were expressed in *Escherichia coli*, purified, and characterized.

2 MATERIALS AND METHODS

2.1 Bacterial strains

The *P. dispersa* (formerly *Erwinia herbicola*) strain was obtained from maize grains presenting rotten symptoms and identified as lipolytic enzymes producer in Rhodamine B medium containing olive oil [12]. *P. dispersa* is a Gram-negative bacterium of the family Enterobacteriaceae, which ferment lactose, are motile, and form mucoid colonies. This species is commonly found in soils and plants, but has been also involved in infections in humans [14]. The strain of *E. coli* DH5α™ was used for cloning and *E. coli* BL21 Star™(DE3) was used for protein expression.

2.2 *P. dispersa* lipolytic enzymes production profile

The lipases and esterases production by *P. dispersa* was tested in Rhodamine B medium [2.0% (v/v) olive oil; 1.0% tween 80; 2 mg/L (w/v) Rhodamine B; 0.5% yeast extract; 0.3% peptone; 0.125% tryptone; 4 g/L NaCl; pH 7.0; 15 g/L agar] [15] and in 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 10 mm diameter dishes. The bacterium was transferred to the specific medium with a sterile loop from nutrient agar slopes. After growth for 48 h at 37 °C, lipase production was identified by the appearing of a pink fluorescent color under UV light (312 nm) in a transilluminator in the Rhodamine B agar and esterase production by the development of a clear halo around the colony in the Trybutirin agar.

The production of lipases and esterases by *P. dispersa* was also tested in a liquid medium developed based on bacterial media used for lipolytic enzymes production [7.0 g/L $NaNO_3$; 2.0 g/L K_2HPO_4; 1.0 g/L KH_2PO_4; 0.5 g/L $MgSO_4 \cdot 7H_2O$; 0.01 g/L $CaCl_2.2H_2O$;

1.0% olive oil; 1.0 g/L yeast extract; 0.1 g/L KCl; 0.012 g/L $FeSO_4.7H_2O$; pH 7.0] [16,17,18]. The bacterium was first streaked on nutrient agar medium in 10 cm diameter plates and incubated overnight at 37 °C. Afterwards, the obtained colonies were suspended in 5 mL of nutrient broth medium and 1 mL of this solution was transferred to three 125 mL flasks with 50 mL of the liquid medium. The inoculated flasks were incubated for 48 hours, 100 rpm, at 37 °C. To assess extracellular enzyme production, aliquots of 1.0 mL were removed with time and, after centrifugation (10,000*g*, 3 min) to remove cells, the enzymes were assayed in the supernatant. To search for cell-bound together with extracellular enzymes, aliquots of 1.0 mL were removed with time and cells were disrupted by sonication using a Fisher Scientific (USA) sonicator, with 48 cycles of 10 s (5 s on and 5 s off), with an amplitude of 30%. After a short centrifugation (10,000×*g*, 3 min), the enzymes assays were performed with the supernatant.

2.3 Enzymes assay

The lipase activity was assayed by the hydrolysis of *p*-nitrophenyl palmitate (*p*-NPP), following the methodology described by Winkler and Stuckmann [19], with modifications. For that, 100 μL of the sample 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 at 40 °C, for 10 min. The released *p*-nitrophenol (*p*-NP) was read at 410 nm. A calibration curve of *p*-NP was performed using 50 mM phosphate/citrate buffer, pH 5.0. One lipase unit was defined as the amount of enzyme that liberated 1 μmol/min of *p*-NP. A blank without enzyme was used to subtract the natural hydrolysis of the *p*-NP derivatives.

The esterase enzymatic activity was assayed by the hydrolysis of *p*-nitophenyl acetate (*p*-NPA) or *p*-nitophenyl butyrate (*p*-NPB) by the photometric assay described by Winkler and Stuckmann [19], with modifications. The reaction consisted of 100 µl of the sample, 100 µl of 0.1 M phosphate buffer, pH 7.0, and 900 µl of substrate solution. The substrate solution was prepared with 5 mL of isopropanol containing 1 mg of *p*-NPA or 1 µL of p-NPB mixed with 45 mL of 0.1% arabic gum and 0.4% Triton X-100, resulting in a final concentration of 113 µM of *p*-NPA or *p*-NPB. The reaction was carried out at 40°C, for 10 min. The released *p*-NP was read at 410 nm, unless otherwise stated. A calibration curve of *p*-NP was performed using 10 mM phosphate buffer, pH 7.0. A blank without enzyme was used to subtract the natural hydrolysis of the *p*-NP derivatives. One enzyme unit was defined as the amount of enzyme that liberated 1 µmol/min of *p*-NP.

2.4 *P. dispersa* lipolytic enzymes genes search

Initially, lipases and esterases genes and/or proteins were searched in the sequenced genome of *P. dispersa*, by name and by BLAST analysis using other bacteria lipolytic enzymes amino acid sequences. Two lipases, one carboxylesterase, and three α/β-hydrolases were found in the sequenced and annotated genomes of two *P. dispersa* strains. First, the obtained protein sequences were analyzed in the program Signal IP (http://www.cbs.dtu.dk/services/SignalP/) to search for secretion signal peptides. Then, PCR primer pairs were designed to amplify the truncated genes, coding only for the mature enzymes without the signal peptides (Table 1).

2.5 DNA extraction, cloning, subcloning, and expression analysis of lipolytic enzymes genes

For the DNA extraction, the bacterium was inoculated in 5 mL of Luria Bertani (LB) medium [10 g/L tryptone; 5 g/L NaCl; 5 g/L yeast extract] and incubated for 18 h, at 37 °C, under orbital stirring at 100 rpm. Cells from 1.5 mL of this culture were harvested by centrifugation and washed 3 times with 500 μL of TE buffer [10 mM Tris; 1 mM Na_2EDTA; pH 8.0]. Then, cells were resuspended in 200 μL of TE buffer and boiled for 10 minutes. The obtained lysate was centrifuged for 1 min (10,000×*g*) and the supernatant obtained was stored at -20 °C until its use as the DNA source.

The PCR amplification reactions were performed in a thermocycler Techne TC-312 (England) in PCR tubes containing 25 μL of following reaction mixture: 1 X enzyme buffer; 50 mM $MgSO_4$; 1.0 U of Platinum®*Taq* DNA Polymerase High Fidelity (Thermo Scientific, USA); 0.2 mM of each dNTP; 25 pmol of each primer (forward and reverse); and 2 μL of the DNA solution. The PCR reaction consisted of a 5 min initial incubation at 94 °C and 25 cycles of 1 min at 94 °C for denaturation, 1 min at 50 °C or 60 °C for primers annealing, and 1 min and 30 s at 68 °C for DNA synthesis. After the cycles, the samples were incubated for 10 min at 68 °C for the complete extension of the fragments and frozen at 4 °C until use.

The amplified DNA fragments were cloned into the TA Cloning vector pCR®2.1 (Life Technologies, USA), following the manufacturer's instructions. The obtained recombinant plasmids were transformed in *E.coli* DH5α™ according to Chung et al. [20]. The plasmids were recovered from *E. coli* by alkaline lyses, according to Sambrook and Russel [21], and analyzed by restriction analysis.

The cloned genes were then transferred from pCR®2.1 vector to the expression vector pET21a(+), using the well stablished digestion and ligation technology [21]. The recombinant

plasmids were transformed in *E. coli* DH5α^{TM} [20], recovered by alkaline lysis [21], and analyzed by restriction digestion.

The pET21a(+) recombinant vectors containing the genes (40-60 ng) were used to transform *E. coli* BL21 Star™(DE3), as described by Chung et al. [20]. The empty pET21a(+) vector was used as a control. The transformed bacteria were inoculated in 5 mL of LB medium-ampicillin (50 μg/mL) and the cultures were grown overnight at 37 °C, with agitation (100 rpm). Five hundred microliters of each culture were used to inoculate 10 mL of LB medium containing ampicillin (50 μg/mL). After 4 h at 37 ^{o}C, 100 rpm, 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the media, to induce protein expression, and the temperature was reduced to 20 ^{o}C. After incubation for 18 h, the obtained cell were collected by centrifugation (6.000*g*, 5 min, 4 °C) and the pellets were resuspended in 2 mL lysis buffer (50 mM Tris, 50 mM NaCl, pH 7.0). The bacteria were disrupted by sonication as described above. The sonicated samples were centrifuged (9.000×*g*, 5 min, 4 °C) and the pellet was separated from the supernatant. The supernatants were used in the enzyme assays to screen for soluble and active enzyme expression. The obtained homogenates, pellets, and supernatants proteins were analyzed by SDS-PAGE to evaluate protein overexpression.

2.6 Gene sequencing and analysis

The genes which coded protein presented enzyme activity were sequenced at the Studies Center of the Human Genome (CEGH) at the University of São Paulo, using the M13 universal primers (forward and reverse) and primers internal to the gene, which were designed based on the *P. dispersa* genes sequences found in the sequenced genomes. The sequencing files were opened in the Chromas Lite 2.01. Consensus sequences were generated in the BioEdit program [22] and the genes sequences were deposited in GenBank.

2.7 Protein *in silico* analysis

The proteins amino acids sequences were obtained by translating the sequenced genes in the Translate program at ExPASy (https://www.expasy.org/). The proteins structural models were generated using the Modeller program [23] or the Swiss-Model program at ExPASy. The obtained PDB were analyzed in VADAR version 1.8 (http://vadar.wishartlab.com/) to determine the percentage of secondary structure. The modeled PDB structures were visualized and colored using the PyMOL program [24]. The proteins were also analyzed for structural domains using the databanks Conserved Protein Domain Database (CDD) from NCBI (https://www.ncbi.nlm.nih.gov) and Pfam from EMBL-EBI (https://pfam.xfam.org/). Molecular size and pI were calculated using the Compute pI/Mw program from ExPASy.

The obtained amino acid sequences were also analyzed in platforms used to study and classify lipases and esterases, such as ESTHER (ESTerases and alpha/beta-Hydrolase Enzymes and Relatives) database (http://bioweb.ensam.inra.fr/esther), Lipase Engineering Database (LED; http://www.led.uni-stuttgart.de/), and Carbohydrate-Active enZYmes Database (CAZY; http://www.cazy.org/),.

Blast searches were carried out using the protein sequences in order to retrieve other similar bacterial proteins from GenBank. The proteins full-length sequences, without their signal peptides, were aligned and the alignment was used to build a phylogenetic tree by employing the neighbor-joining method [25] of MEGA 7.0 program [26]. Bootstrap analyses were conducted to assess the confidence limits of the branching with 1000 heuristic replicates [27]. Values higher than 70% in the bootstrap test of phylogenetic accuracy indicated reliable grouping among proteins. The evolutionary distances were computed by the maximum composite likelihood method [28]. The grouping was also performed by other methods, such as maximum parsimony, minimum evolution, and UPGMA, with similar results.

2.8. Protein expression and purification

The recombinant vectors, containing genes coding for soluble and active enzymes, were transformed in *E. coli* BL21 Star™(DE3) [20]. The transformation reactions were inoculated in 5 mL of LB-ampicillin (50 μg/mL), which were cultured overnight at 37 °C with agitation (100 rpm). Five hundred microliters of each culture were used to inoculate 250 mL flasks containing 50 mL of LB-ampicillin (50 μg/mL) medium. After 4 h at 37 ^{o}C, 100 rpm, 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the media and the temperature was reduced to 20 ^{o}C. After incubation for 18 h, the obtained cell were collected by centrifugation (6.000 *g*, 5 min, 4 °C) and the pellets were resuspended in 10 mL of lysis buffer (50 mM Tris, 50 mM NaCl, pH 7.0). The bacteria were disrupted by sonication, as described above. After centrifugation (9.000×g, 5 min, 4 °C) of the obtained homogenized, the expressed proteins on the supernatants were purified by means of immobilized metal affinity chromatography (IMAC). For that, the supernatant was applied to a HisTrap™ HP column (GE Healthcare Life Sciences, USA) equilibrated with washing buffer (0.1 M Tris, pH 7.0, 0.1 M NaCl, 20 mM imidazole) at a rate of 1 mL/min. Afterward, the column was washed with 20 mL of washing buffer, and the recombinant proteins were eluted with an 150-450 mM imidazole gradient, in 100 mM phosphate buffer, pH 7.0, containing 100 mM NaCl. Protein elution was followed by absorbance at 280 nm. Fractions containing the enzyme were pooled and dialyzed twice against 0.1 M Tris buffer, pH 7.0. After dialysis, the enzyme was supplemented with glycerol 20% (v/v) and kept frozen at −20 °C until use.

2.9 Protein concentration determination and electrophoresis analysis

Protein concentration was determined according to the method of Bradford [29] using bovine serum albumin as a standard. The purified proteins molecular weight and purity were

evaluated by SDS-PAGE. For that, aliquots of the purified proteins (10 μg) were diluted (1:1) 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 boiled for 10 min. Then, samples were loaded in a discontinuous denaturing 10% gel, pH 8.9, with a 4.5% stacking gel, pH 6.8, which was run in the Tris-glycine buffer system [30]. After the run, the gel was stained with Coomassie Brilliant Blue. Broad range molecular marker (BioRad, USA) was used to determine the approximate size of the enzymes.

A non-denaturing and discontinuous PAGE was also performed to test for the proteins multimeric forms. Aliquots of the purified proteins (10 μg) were diluted (1:1) in the sample dilution buffer[20% glycerol, 0.03 mg/ml bromophenol blue, 125 mM Tris-base, pH 6.8] containing or not 0.72 mM β-mercaptoethanol. The samples that were resuspended in the buffer containing β-mercaptoethanol were also boiled for 10 min. Then, samples were loaded in a 7.5% gel, pH 8.9, with a 4.5% stacking gel, pH 6.8, which was run in the Tris-glycine buffer system, without SDS. After the run, the gel was stained with Coomassie Brilliant Blue.

For zymogram analysis, a PAGE gel containing the purified proteins was loaded in the top of a Petri dish containing a substrate solid medium (2% triacetin, 10 mM phosphate buffer, pH 7.5, 0.004 % bromothymol blue). The dishes were incubated for 2 h at 40 °C before analyzes.

2.10 Enzyme characterization

The purified proteins optimal temperature was determined by changing the assay temperature from 20 to 60 °C. The thermal stability was determined by 30 min pre-incubations of the purified enzymes at different temperatures, and then measuring the residual activity in the standard enzymatic assay. The temperature in which the enzyme lost 50% of its activity (T_{50}) was estimated through the second order equation obtained from the polynomial

regression curves. To analyze the thermal stability over time, the enzymes were also incubated in their optimal temperatures in 0.1 M phosphate buffer, pH 7.0, for up to 48 h, and aliquots were used to assay the activity at intervals.

The optimal pH was estimated in the standard enzymatic assay using the Britton and Robinson buffer (0.1 M boric acid, 0.1 M acetic acid, 0.1 M phosphoric acid) in several pH values. The released *p*-NP was read at its isosbestic point, 348 nm. A 48 h pH stability time course was performed at pHs 6.0, 7.0, and 8.0, in 0.1 M phosphate buffer, at 4 °C. Aliquots of the incubated enzymes were removed at time intervals and the enzyme assay was accomplished at pH 7.0. The released *p*-NP was also measured at 348 nm.

The substrate concentration effect was evaluated by varying the *p*-NPA concentration from 0.113 to 2.486 mM and using a constant amount of the purified enzymes in the enzyme assay. The kinetic constants K_M and V_{max} were determined by linear regression from a double-reciprocal plot. The constant k_{cat} was calculated using the obtained V_{max} divided by the used enzyme concentration and the obtained value was used to calculate k_{cat}/K_M.

The effect of potential inhibitors and activators in the enzymes activity were evaluated by addition of metal ions, EDTA, SDS, PMSF (5 and 10 mM), and detergents (1%) to the standard enzyme assay. The ability to promote *p*-NPA hydrolysis in a water-restricted environment was estimated by adding 25% of different organic solvent in the enzyme assay. The relative activity was defined as the percentage of activity compared with the control without any addition.

2.11 Substrate specificity

The substrate specificity was verified in the enzyme assay, by measuring the enzyme activity against *p*-nitrophenyl acetate (*p*-NPA), *p*-nitrophenyl butyrate (*p*-NPB) and *p*-

nitrophenyl formate (*p*-NPF) at 113 μM, and *p*-nitrophenyl palmitate (*p*-NPP) and *p*-nitrophenyl dodecanoate (*p*-NPD) at 0.8 mM.

The activity against triacetin was measured spectrophotometrically using the bromothymol blue to evaluate pH change. The reaction consisted of 2 mL of 2% (v/v) triacetin in a 10 mM phosphate buffer, pH 7.5, 0.004% (w/v) bromothymol blue, and 100 μL of the purified enzymes (0.29 mg). A control reaction was prepared in the same way, but without the enzyme. The increase of absorbance at 430 nm was recorded at 15 s or 30 min intervals. The reaction initial rate was determined from the linear portion of the curve where one unit of activity was defined as a change in absorbance of 0.001 per min of reaction [31]. Each test was carried out in triplicate.

The activity against pectin and xylan was measured by titration of the acetic acid released, according to the methodology described by Talboys and Busch [32], with modifications. The pectin deacetylation reactional mixture was prepared in 125 mL Erlenmeyer flask, in triplicate, with 1% (w/v) citrus pectin in 10 mM phosphate buffer, pH 7.0, and 0.29 mg of the purified enzymes, in a total volume of 10 mL. This mixture was incubated for 60 min, at 100 rpm, at 40 °C. The xylan deacetylation reactional mixture was prepared in the same way, with 1% (w/v) oat spelt xylan in 10 mM phosphate buffer, pH 7.0 (made soluble by boiling in a water bath for 20 min and stirring overnight), with 0.725 mg of the purified enzymes, in a total volume of 10 mL. This mixture was incubated for 90 min at 100 rpm, at 40 °C. Afterwards, the reactions were stopped by the addition of 40 ml of 99.5% ethyl alcohol and 5 drops of 1% phenolphthalein were added to the reaction. Titration was done with 10 mM NaOH. The control reactions consisted of the same mixtures but with the enzyme inactivate by boiling in a water bath for 10 min. Pectin or xylan acetylesterase activity were calculated by using the following formula [33]:

$$\text{Acetylesterase (U)} = \frac{(Vs - Vb) \times [\text{NaOH}]}{VT}$$

Where , *Vs* - Volume of NaOH used to titrate sample (mL), *Vb* - Volume of NaOH used to titrate blank (mL), [NaOH] – NaOH concentration in mM, *V* - Volume of the enzyme solution used in the reaction (mL), *T* - Reaction time (min). One enzyme unit was defined as the amount of enzyme that released 1.0 milequivalent/min of acid from pectin or xylan.

3 RESULTS AND DISCUSSION

3.1 *P. dispersa* lipolytic enzymes production profile

The Rhodamine B test agar has confirmed the lipolytic enzymes production by *P. dispersa* (Fig. 1A and B). The tributyrin agar test did not present a clear halo around the colony. The lipolytic enzymes production was also examined in liquid culture. The enzymes peak production occurred at 24 h of culture, except for the total and extracellular butyrilesterase production, which peaked at 30 and 6 h of culture, respectively (Fig, 1C, D, and E). Lipase was produced in higher amount by *P. dispersa*, both total (294 U/mL) and extracellular (164 U/mL). Species of the *Pseudomonas* genus are among the best bacterial lipase producers and the *P. dispersa* yield of lipase production was close to the ones obtained by species of this genus, such as the production of 156 U/mL by *Pseudomonas gessardii* and 222 U/mL by *Pseudomonas aeruginosa* [34, 35]. Butyril esterase was the second enzyme most produced, both total (89 U/mL) and extracellular (16.6 U/mL). The butyril esterase production by *P. dispersa* in liquid culture contrasts with the negative results in tributyrin agar and this may be due to differences in the used substrate in each test. Total (36.8 U/mL)

and extracellular (9.2 U/mL) acetylesterase was also produced by *P. dispersa*, but in lower amount. In contrast, *Bacillus subtilis* was shown to produce approximately 450 U of extracellular and 4 U of cell-associated carboxylesterase after 48 h of culture [16]. However, a rumen bacterium was also shown to produce a lower amount (0.5 U/mL of culture medium) of an acetylesterase after 48 h of culture [18].

3.2 Genes amplification, cloning, and expression analysis

Although the extracellular expression of lipolytic enzymes by *P. dispersa* was reasonable high for purification and characterization, the recombinant expression offers some important features such as higher yields; introduction of a tag for affinity chromatography purification; and work with well stablished laboratory bacterial systems. All six targeted genes were successfully amplified, cloned, and subcloned into pET21a(+) plasmid. In the expression analysis, there was protein overexpression for all cloned genes, as seen in the SDS-PAGE analysis (not shown). However, not all proteins were completely soluble. In addition, there was no lipase activity among the expressed proteins, because none could release *p*-NP from *p*-NPP, and no expressed protein was very active with *p*-NPB. However, two of the proteins, named Est-1 and Est-2, presented a strong acetylesterase activity, releasing *p*-NP from *p*-NPA.

3.3 Analysis of the esterases genes and protein sequences

The two cloned esterases truncated genes sequences, *Est-1* and *Est-2*, were deposited in GenBank with accession numbers MK503264 and MK503265, respectively. The truncated 855 bp *Est-1* gene codes for the 285 amino acid residues Est-1 enzyme. The truncated 941 bp *Est-2* gene codes for the 317 amino acid residues Est-2 enzyme. The 20 and 19 *N*-terminal amino acid sequences, which codes for the secretion signal peptides were missing in the

cloned *Est-1* and *Est-2* genes, respectively. An extra *C*-terminal 13 amino acid sequence, which includes a 6-His tag, given by the plasmid, was incorporated in both expressed proteins.

The Est-1 and Est-2 deduced amino acid sequences were 99% identical to the amino acid sequences of the proteins derived from the *P. dispersa* genes, which sequences were used for primer design (GenBank AN WP_058775601.1 and WP_021509014.1, respectively). Est-1 and Est-2 are only 21.4% identity with each other. Est-1 has 18% and Est-2 19% of identity with another *P. dispersa* esterase previously described in the literature [13].

The deduced amino acid sequences of Est-1 and Est-2 were used for BLAST analysis to search for similar proteins. The characterized protein with higher sequence identity with Est-1 (43%) was the PaeX pectin acetylesterase of *Erwinia chrysanthemi* (GenBank CAD45188.1) [36] (Fig. 2A) and with Est-2 (66.6%) was the acetylesterase EstK from *Pseudomonas putida* (GenBank Q88GB2) with activity for deacetylation of xylan and poly(vinylacetate) [37] (Fig. 2B). The alignment among these proteins shows the characteristic penta peptide for most lipases and serine proteases (Gly-x-Ser-x-Gly) and the conserved catalytic triad Ser-Asp-His in both *P. dispersa* Est-1 and Est-2 esterases (Fig. 2A and B).

The evolutionary history of Est-1 and Est-2 with putative or characterized esterases retrieved in BLAST searches is shown in Fig. 3. The retrieved proteins were chosen because they have sequence identity above 40% with Est-1 or with Est-2. The used protein sequences are all from the Phylum Proteobacteria and Family Enterobacteriaceae, except one that is from Family Pseudomonadaceaea. The constructed phylogenetic tree had two distinct protein branches, each one containing one of the characterized *P. dispersa* esterase. The proteins identity among branches was below 19% and inside branches was above 41% for the Est-1 branch and 68% for the Est-2 branch.

The structural models of *P. dispersa* Est-1 esterase was built by comparison with a esterase of an uncultured archaeon (PDB 2c7b, GenBank AAW62260.1) [38], with 22% of sequence identity and 88% of coverage, and the and Est-2 esterase with a *P. putida* esterase structure (PDB 4ob8, UniProt L7PYQ2) [39], with 51% of sequence identity and 98% of coverage (Fig. 4A and B). The structural models obtained for Est-1 and Est-2 revealed that both enzymes are composed of eight segments in β-conformation (only the second β strand is antiparallel) surrounded on both sides by α-helices, which is a characteristic of the α/β-hydrolase domain [9]. The predicted structures for Est-1 and Est-2 had, respectively, 41% and 44% of α-helices, 21% and 22% of segments in β-conformation, 37% and 33% of coil, and 22% and 21% of turns. Positions of the triad in the mature enzymes are consistent with their expected position in the α/β-hydrolase fold family (Fig. 2A and B; Fig. 4A and B). The active site serine is located between strand β5 and helix αC [9]. A long loop, which is located after the strand β7, carries the acid member (Asp). The third member of triad, the His residue, is located in a loop between strand β8 and helix αF.

In silico analysis revealed that both Est-1 and Est-2 belong to the AB_hydrolase (CL0028) protein domain clan. Est-1 has a peptidase_S9 (PF00326) or an acetyl esterase/lipase – Aes family domain, with an e-value $3.1 \times e^{-15}$ and $4.63 \times e^{-28}$, respectively. Est-2 has a Abhydrolase_3 family (PF07859) domain with an e-value of $3.1 \times e^{-66}$ (Fig. 4A and B). Est-1 and Est-2 proteins aligned with proteins of the abH04 - Moraxella lipase-2 like proteins family from the GGGX class of α/β hydrolases in LED database and with Hormone-sensitive_lipase_like proteins family in ESTHER database.

The enzymes Est-1 and Est-2 showed homology with homo-dimeric structure esterases in a Swiss-Model analysis. Est-1 was modeled into a homo-dimer using as template the esterase of *Lactobacillus plantarum* (PBD 3bxp.1.B) [40], with 23.7% of sequence identity,

and Est-2 using as template the esterase of *Thalassospira* sp. (PBD 4v2i) [41], which has 50.6% of sequence identity (Fig. 4C and D).

3.4 The *P. dispersa* esterases expression and purification

Both *Est-1* and *Est-2* truncated genes cloned in pET21a(+) were successfully expressed in *E. coli* Bl21 Star^{TM}(DE3) and Ni-column purified. The yield of purified Est-1 after dialysis was 935 U and 1.62 mg of protein per 50 mL of culture, with a specific activity of 530 U/mg, and for Est-2 was 736 U and 1.74 of protein per 50 mL of culture, with a specific activity of 455 U/mg. Similarly, a recombinant acetyl xylan esterase from *Anoxybacillus flavithermus* was purified in concentrations in the range of 0.015–1.2 mg/ml of protein from 200 ml culture, in an analogous expression system [42]. In addition, Kakugawa et al. [43] obtained 4 mg of a purified thermostable carboxylesterase from the hyperthermophilic bacterium *Thermotoga maritima*, with a specific activity of 13 U/mg from 400 ml of culture, in a comparable expression system.

According with the SDS-PAGE analysis, the purified enzymes were homogeneous and the denatured Est-1 ran as a single protein of 32.6 kDa and Est-2 as a single protein of 37.4 kDa (Fig. 5A and B), what is similar with the predicted molecular mass for these proteins of 33 kDa for Est-1 and 37 kDa for Est-2. Other characterized acetyl esterases have similar molecular masses, such as the ones from *Nocardia mediterranei* and *Pseudomonas mandelii*, which have 37 kDa and of 33 kDa, respectively [44,45].

The PAGE analysis showed the enzymes Est-1 and Est-2 in their denatured form (boiled in the presence of β-mercaptoethanol), which run faster than the native form (Fig. 5C). The predicted isoelectric point (pI) for Est-1 and Est-2 were 9.03 and 6.11, respectively. Because the probable higher pI of Est-1, it did not enter in the gel in its native form. However, it is possible to conclude that these proteins have a higher molecular weight in their native

form when compared with their denatured form. The zymogram for Est-2 shows a yellow circle in the triacetin medium (Fig. 5D), which indicates pH change and acetylesterase activity, only in the native Est-2 lane of the gel. These results corroborate the structural modeling analysis results, where a homodimer structure was obtained for both enzymes.

3.5 Biochemical properties of the recombinant acetylesterases

The optimum temperatures for the Est-1 and Est-2 activity were 40 °C and 50 °C, respectively (Fig. 6A). Most esterases optimum temperature positions in the range of 40 to 60 °C [6,7]. In accordance with our results, the acetylesterases from a rumen bacteria and from *B. subtilis* have optimum temperature at 40 °C and 50 °C, respectively [18,46]. Regarding *P. dispersa* recombinant acetylesterases thermal stability after a 30 min incubation, the temperature in which 50% of the enzyme activity is lost (T_{50}) was 44.1 °C for Est-1 and 58.9 °C for Est-2 (Fig. 6B). Concerning thermal stability with time in their optimal temperature, the enzyme Est-1 remained 79% active after 48 h incubation at 40 °C, but the enzyme Est-2 was only 36% active after 48 h incubation at 50°C (Fig. 6C). Although the thermal stability of Est-1 and Est-2 stands in the range of most esterases [6,7], enzymes with higher thermal stability have been described. For example, the *T. maritima* carboxylesterase stayed 100% stable when incubated up to 90 h in its optimum temperature of 60 °C [43] and the *Thermoanaerobacterium* sp. acetyl xylan esterase remained almost 100% active after 4 h incubation at 88 °C [47].

Both purified *P. dispersa* Est-1 and Est-2 were most active at pH 7.0 (Fig. 6D). This maximum enzymatic activity against *p*-NPA in the neutral range is comparable to the values reported for the *Streptomyces* sp. acetylesterase [48]. Nevertheless, the *P. mandelii* acetylesterase has a more alkaline optimum pH of 8.5 [49]. In the pH stability time course at 4 °C, the enzyme Est-1 was slightly more stable at pH 8.0 and the enzyme Est-2 at pH 7.0 (Fig.

6E). This difference may be related to the predicted pI of those enzymes, since Est-1 a more basic protein.

The Lineweaver-Burk plots with different concentrations of *p*-NPA for the recombinants Est-1 and Est-2 are shown in Fig. 6F. A K_M of 1.5 mM and 0.6 mM for *p*-NPA was found for Est-1 and Est-2, respectively. Accordingly, a K_M value of 1.15 mM was found for the recombinant acetylesterase of *T. maritima* [43]. However, a greater K_M value of 119 mM was reported for the recombinant acetylesterase of *B. subtilis* [46]. Considering the diversity among esterases, this variation could be expected.

A V_{max} of 9.0 μmol/min and 8.0 μmol/min of released *p*-NP was found for Est-1 and Est-2, respectively. The obtained values for the turnover number (k_{cat}), considering the dimeric mass of the proteins, and the catalytic efficiency constant (k_{cat}/K_M) were 42,477 s^{-1} and 2.7 × 10^7 $M^{-1}s^{-1}$ for Est-1 and 41,986 s^{-1} and 7.0 × 10^7 $M^{-1}s^{-1}$ for Est-2, respectively. These values were considerably higher than those observed for the *Thalassospira* sp. esterase, which has a k_{cat} of 47.7 s^{-1} and a k_{cat}/K_M of 50.6 × 10^3 M^{-1} s^{-1} [41], and for the *T. maritima* acetylesterase, which has a k_{cat} of 57.5 s^{-1} and a k_{cat}/K_M of 0.31 × 10^3 $mM^{-1}s^{-1}$ [43]. This indicates that Est-1 and Est-2 are reasonably efficient enzymes.

The results of potential inhibitors and activators on the Est-1 and Est-2 activity are summarized on Table 2. The ions Zn^{2+}, Ni^{2+}, and Co^{2+} inhibited the enzymes at 5 and 10 mM. The inhibitory effect of Zn^{2+} and Ni^{2+} on the *T. maritima* carboxylesterase was described as well as the inhibitory effect of Zn^{2+}, Ni^{2+}, and Co^{2+} on the *P. aeruginosa* carboxylesterase [43,51]. However, the ion Co^{2+} acted as an activator of the *Thalassospira* sp. acetylesterase [41]. PMSF strongly inhibited both Est-1 and Est-2, in both used concentrations. The PMSF inhibition confirmed that a serine residue is catalytically important for both purified enzymes, as predicted from the sequence alignment analysis (Fig. 2). The carboxylesterases from *T. maritima*, *Sulfolobus shibatae*, and *P. aeruginosa* PMSF inhibition was also reported

[43,50,51]. EDTA inhibited *P. dispersa* Est-1 in a great extension than Est-2. In agreement, EDTA inhibited the *Kluyveromyces marxianus* esterase [52], but had no effect on the *P. aeruginosa* carboxylesterase [51].

The anionic detergent SDS and the neutral detergents Tween 80, Tween 20, and Triton X–100 also inhibited *P. dispersa* Est-1 and Est-2 (Table 2). Detergents can alter enzymes tertiary conformation, which probably caused the Est-1 and Est-2 reduction in activity. The *K. marxianus* carboxylesterase was also inhibited by SDS [52].

The *P. dispersa* acetylesterases Est-1 and Est-2 were moderately stable in 25% water-miscible organic solvents (Table 2). The maximum residual activity for Est-1 was obtained in the presence of acetone (29%) and for Est-2 in the presence of methanol (23%). The *T. maritima* carboxylesterase and *K. marxianus* esterase were also tolerant to organic solvents (up to 15%), with increased residual activities when compared with the purified *P. dispersa* Est-1 and Est-2 [43,52]. Esterases ability to withstand water-restricted environments is crucial for their employment in organic synthesis, thus, the purified enzyme ability to hydrolyze *p*-NPA in organic solvents presence suggests its possible usage in this biotechnological dominion.

3.6 Substrate specificity of Est-1 and Est-2

The purified *P. dispersa* Est-1 and Est-2 exhibited high specificity for *p*-NPA (C2) (100%) and very low activity for *p*-NPB (relative activity of 5% for Est-1 and 2% for Est-2, when compared with the activity against *p*-NPA). However, no activity was detected with *p*-NPF (C1), *p*-NPD (C12), and *p*-NPP (C16). These results endorse the activity of acetylesterases of the purified enzymes.

To confirm the acetylesterase activity of Est-1 and Est-2, a time course curve of triacetin degradation based in the pH indicator bromothymol blue color change was

performed (Fig. 7). Est-2 was able to completely change the reaction color in 10 min. However, it took 180 min for the enzyme Est-1 to do the same. Nonetheless, the results confirm both enzymes as acetylesterases, having Est-2 a greater affinity for triacetin (163.4 ± 14.8 U/mg of protein) in comparison with Est-1 (4.3 ± 1.2 U/mg of protein). The *K. marxianus* esterase, *Thermoanaerobacterium* sp. acetyl xylan esterases, and *E. chrysanthemi* acetylesterase were also active with triacetin [36,47,52].

Both *P. dispersa* purified Est-1 (25.5 ± 2.6 U/mg of protein) and Est-2 (27.4 ± 6.9 U/mg of protein) were active in removing acetyl groups from citrus pectin, in the titration assay. Nevertheless, these activities are lower than the activity found for *p*-NPA and triacetin (above). This may be explained by the low acetyl group content in citrus pectin [53]. In addition, the hydrolysis of polymeric substrates can be more complicated because not all esterified substituents are chemically equal and because of the effect of steric hindrance. Furthermore, the polymer decreased solubility disturbs the enzymes action [53].

The *P. dispersa* purified Est-1and Est-2 had a small activity toward oat spelt xylan, in the titration assay, 1.3 ± 0.1 and 1.2 ± 0.1 U/mg of protein, respectively. The used xylan was not artificially acetylated and this may have caused this low level of enzyme activity. A rhamnogalacturonan acetylesterase from *B. subtilis* is also reported to have xylan acetylesterase activity [54].

According with the Carbohydrate-Active enZYmes Database (CAZY), there are 16 families of carbohydrate esterases (CE) [10]. Pectin acetylesterases are present in families CE12 and CE13. Xylan acetyl esterases are present in eight families, CE1-7 and CE12. Considering the dual carbohydrate acetylesterase action of the purified enzymes on pectin and xylan and the predicted structures, it can be proposed that they both could be classified as proteins from CAZY CE12 family.

4 CONCLUSIONS

In this study, two acetylesterases from *P. dispersa* displaying carbohydrate deacetylation activity were purified and characterized for the first time. Although, both enzymes have low sequence identity between them, both presented similar catalytic activities, such as triacetin, pectin, and xylan deacetylation. This remarkable capacity to remove acetyl groups from carbohydrates and the low sequence identity with other esterases suggest that both characterized enzymes are two novel carbohydrate acetylesterases.

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Table 1. Primers used in this work to amplify lipolytic enzymes from *P. dispersa*

Enzyme (GenBank Accession Number)	**Primers***
Lipase 1 (AN WP_058771629.1)	FW 5´-TTCATATGGCAAATAATTCTCTGGTTACTTCC RV 5´-TTAAGCTTGACATTTTCCATTACCTCT
Lipase 2 (AN KTS88797.1)	FW 5´-TTCATATGTGGGATAACCTGACGGTTTTCG RV 5´-TTAAGCTTAAATGTCGCGTTCAGGCCA
Carboxylesterase 1 (AN ERH66909.1)	FW 5´-TTCATATGAAAAATGAACGACGTCTGAGG RV 5´-TTAAGCTTAAGTTTAACGTGGCTGCGCATC
Esterase 1 (AN WP_058775601.1)	FW 5´-TTCATATGAATAAGGAAATTCCGATCTGG RV 5´-TTAAGCTTCCGCTTCACCAGGCTGCTTTG
Esterase 2 (AN WP_021509014.1)	FW 5´-TTCATATGTTTGCTGACGCACCACAACCT RV 5´-TTAAGCTTCTGCAGATGGGTTTTCAGCTC
Esterase 3 (AN WP_031279766.1)	FW 5´-TTCATATGCGGGATGACATTATTCCGCTC RV 5´-TTAAGCTTACCCAGCTTATCCAGCCAGCG

* All forward primers contained a site for the restriction enzyme *Nde*I that provided the first ATG. The reverse primers had a site for the restriction enzyme *Hind*III and they had not the stop codon. The restriction sites (underlined) were protected by two additional T residues.

Table 2. Impact of activators, inhibitors, and organic solvents on the purified acetylesterases activities.

Substance	**Concentration**	**Residual activity (%)**		**Concentration**	**Residual activity (%)**	
		Est-1	Est-2		Est-1	Est-2
Control	-	100 ± 4.0	100 ± 5.9	-	100 ± 5.2	100 ± 5.4
$MgCl_2$	5 mM	87 ± 1.0	96 ± 3.2	10 mM	103 ± 3.4	96 ± 0.7
KCl	5 mM	100 ± 1.8	98 ± 1.5	10 mM	97 ± 3.4	100 ± 2.9
NaCl	5 mM	98 ± 0.9	100 ± 1.6	10 mM	102 ± 4.4	95 ± 1.0
$CoCl_2.6H_2O$	5 mM	49 ± 5.8	73 ± 1.3	10 mM	53 ± 0.6	75 ± 0.8
$CaCl_2.2H_2O$	5 mM	90 ± 3.5	98 ± 0.6	10 mM	99 ± 2.4	92 ± 4.9
$ZnSO_4.7H_2O$	5 mM	39 ± 6.8	58 ± 2.0	10 mM	16 ± 0.6	44 ± 2.5
EDTA	5 mM	14 ± 3.4	73 ± 0.5	10 mM	0	7 ± 2.0
PMSF	5 mM	16 ± 1.8	9 ± 0.9	10 mM	0	0
SDS	5 mM	11 ± 0.6	12 ± 2.9	10 mM	7 ± 1.2	3.4 ± 2.0
Triton X-100	1%	44 ± 3.6	60 ± 0.9			
Tween 20	1%	56 ± 4.8	66 ± 4.0			
Tween 80	1%	50 ± 4.9	65 ± 7.0			
Methanol	25%	25 ± 1.7	23 ± 0.7			
Ethanol	25%	26 ± 0.6	7 ± 0.5			
Isopropanol	25%	3 ± 0.4	0.9 ± 0.5			
Acetone	25%	29 ± 3.6	12 ± 0.7			

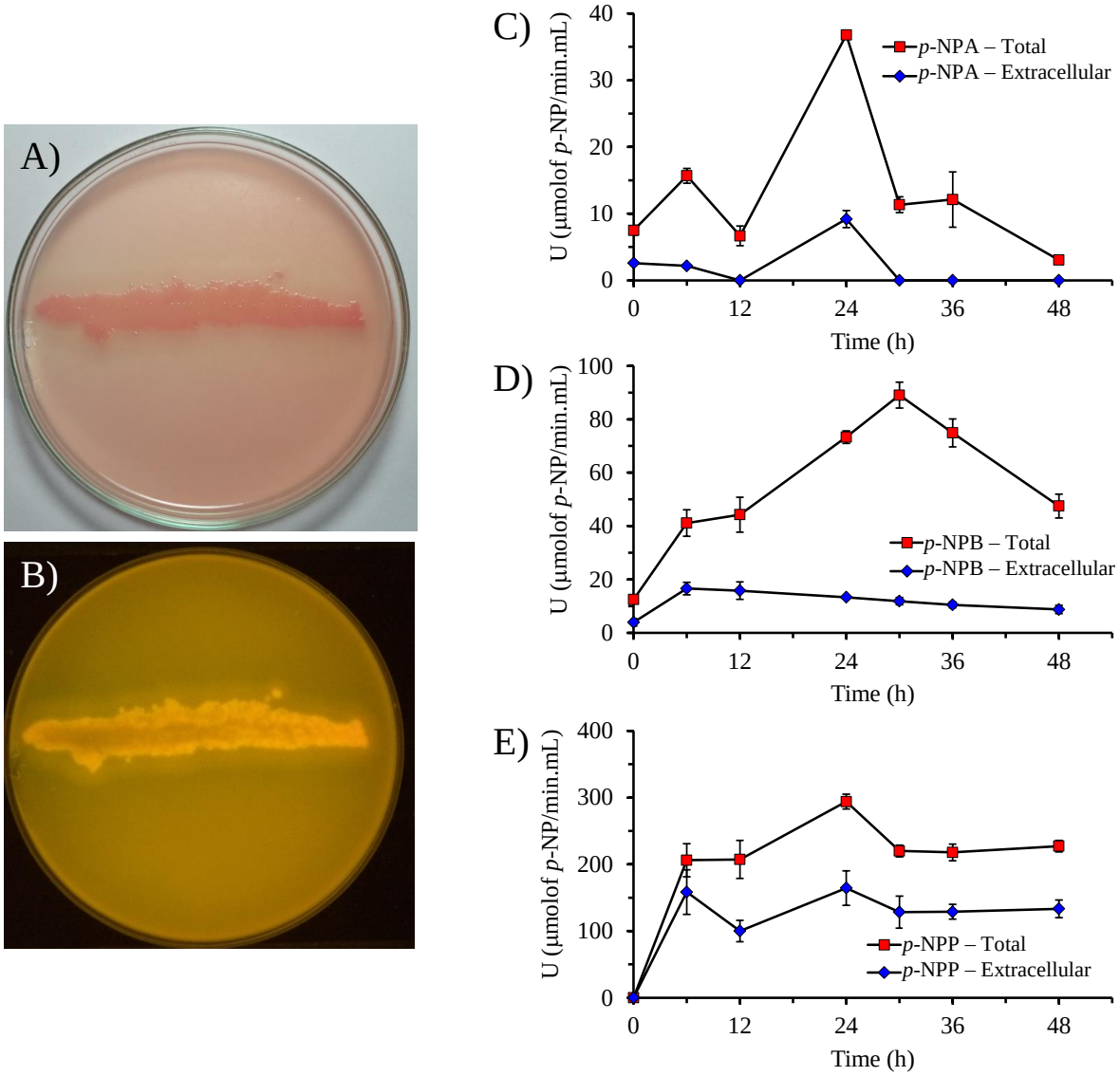

Fig. 1. Expression profile of lipolytic enzymes by *P. dispersa*. A) A Petri dish containing *P. dispersa* cultured in Rhodamine B medium (front). B) The same culture of *P. dispersa* in Rhodamine B medium photographed under UV light (reverse). Total (cell-bound + extracellular) and extracellular expression profile of acetylesterase (C), butyrilesterase (D), and lipase (E), assayed with *p*-NPA, *p*-NPB, and *p*-NPP, respectively, in a 10 min enzyme reaction. The data represents the average and standard deviation of the results obtained in three culture flasks.

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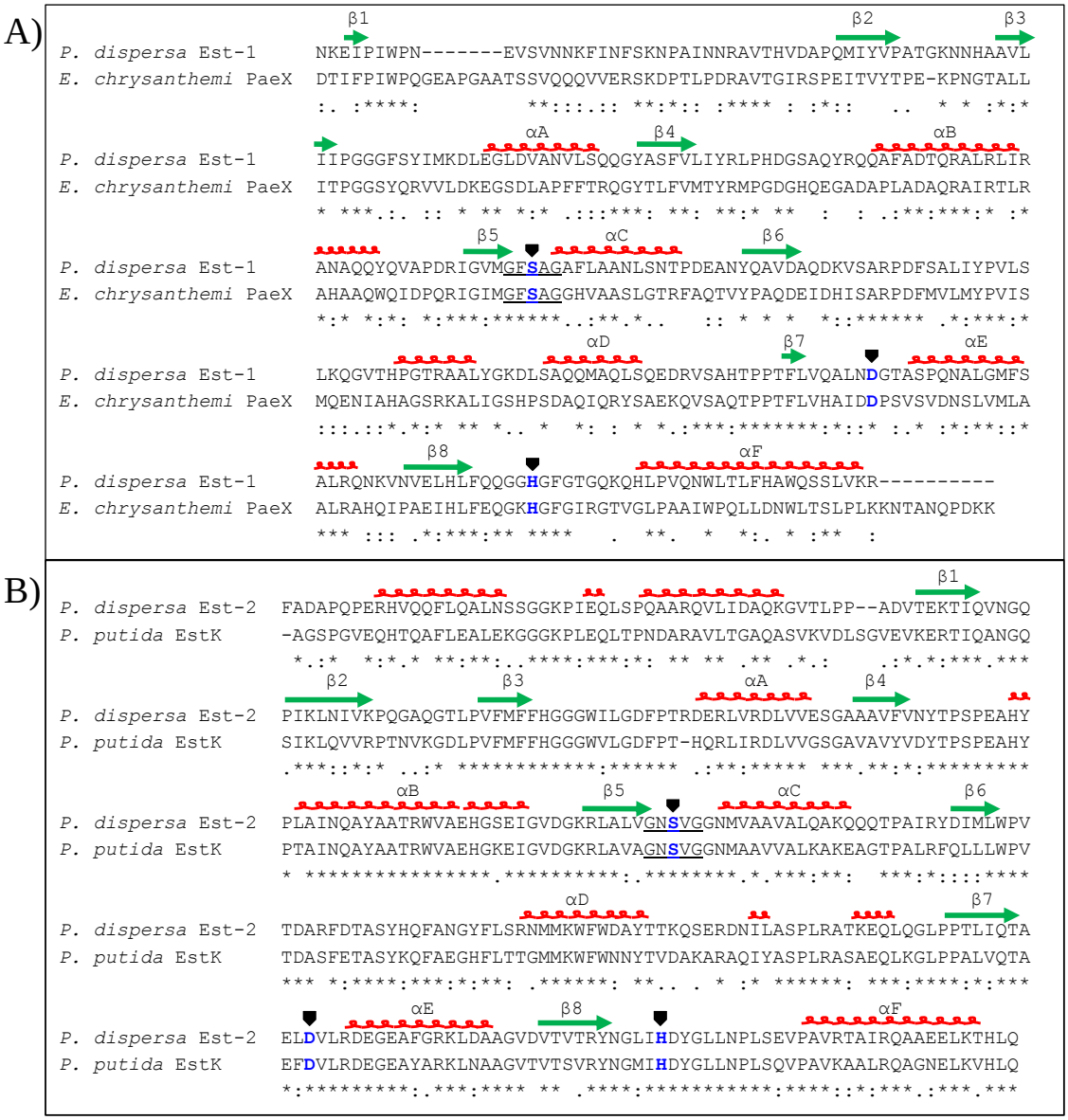

Fig. 2. Alignment of the *P. dispersa* Est-1 and Est-2 amino acid sequence with the sequence of other esterases. A) Amino acid sequence alignment of the *P. dispersa* Est-1 with the *E. chrysanthemi* pectin acetylesterase PaeX [36]. B) Amino acid sequence alignment of the *P. dispersa* Est-2 with the *P. putida* acetylesterase Est-K [37]. The conserved catalytic triad Ser, Asp, and His residues are shown in bold blue and have an arrow above them. The consensus G-X-S-X-G motif of serine hydrolases is underlined. Signal peptide sequences were removed. The predicted secondary structures of Est-1 and Est-2 are indicated by green arrows (β-conformation) and red helices (α-helices) above the sequences.

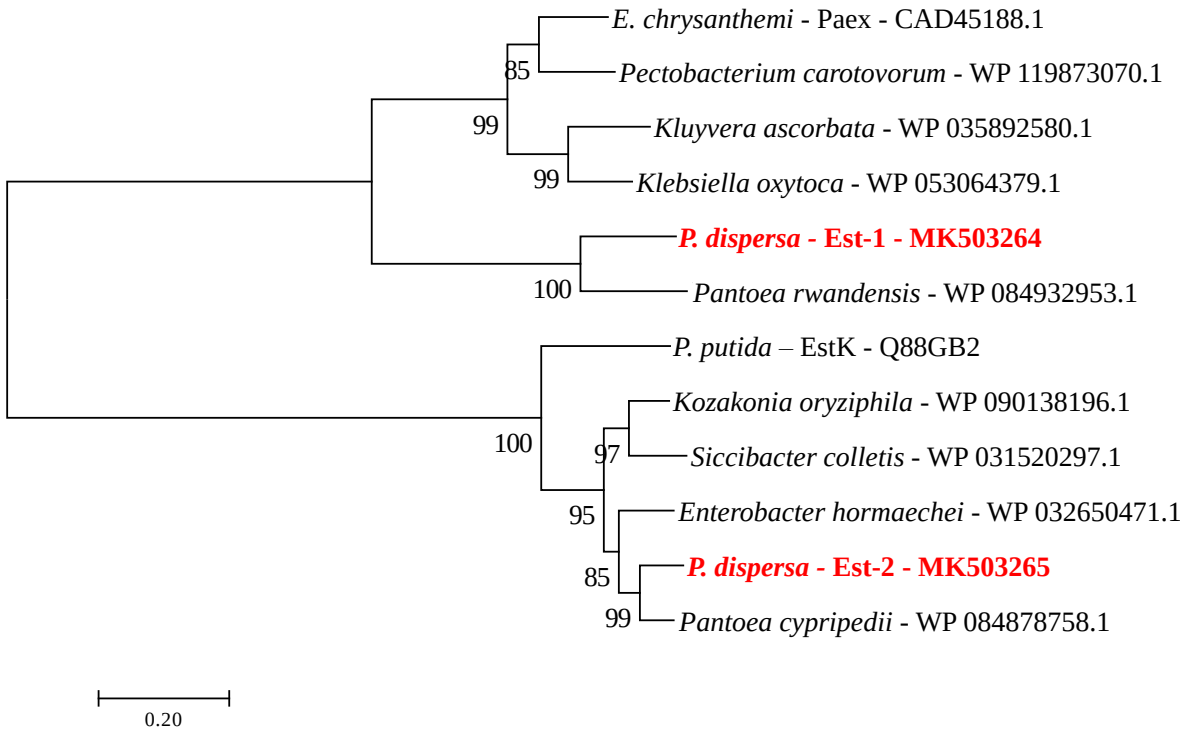

Fig. 3. The evolutionary relationships of Est-1 and Est-2 protein sequences. The evolutionary history was inferred using the Neighbor-Joining method [25]. The optimal tree with the sum of branch length = 3.54318161 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown below the branches [27]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved 12 amino acid sequences. All ambiguous positions were removed for each sequence pair. There were a total of 333 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [26].

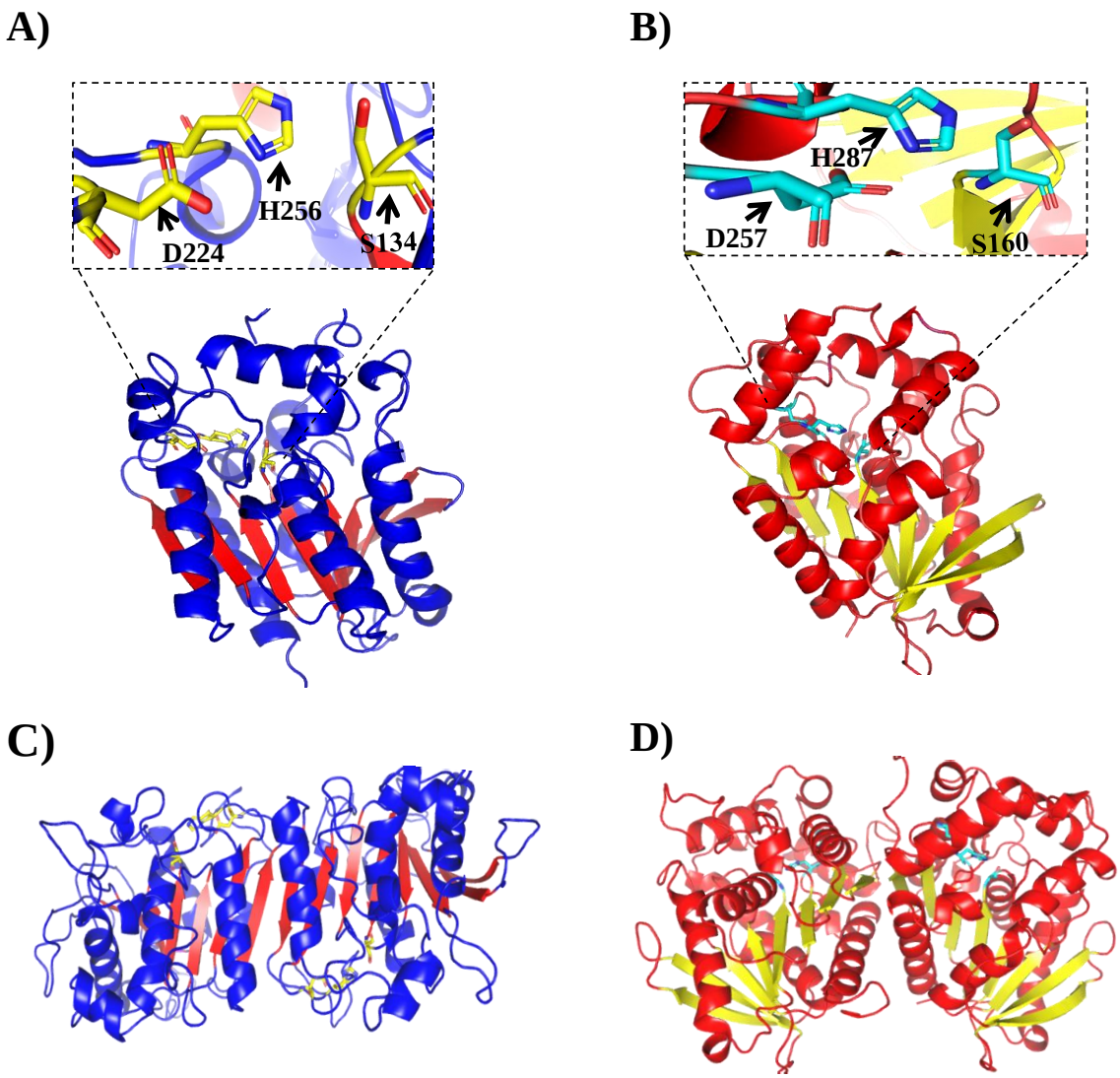

Fig. 4. Representative three-dimensional structures of the acetylesterases Est-1 and Est-2. A) The predicted Est-1 structure modelled using an uncultured archaeon esterase structure (PDB 2c7b) [38] as template. B) The predicted Est-2 structure modelled using the *P. putida* esterase structure (PDB 4ob8) [39] as template. The predicted active sites for Est-1 (A) and Est-2 (B) are zoomed inside the boxes and show the catalytic residues of Ser, Asp, and His as sticks. C) The dimeric structure of Est-1 modelled using the *L. plantarum* carboxylesterase structure (PDB 3bxp.1.B) [40] as template. D) The dimeric structure of Est-2 modelled using the marine bacterium *Thalassospira sp.* esterase structure (PDB 4v2i) [41] as template.

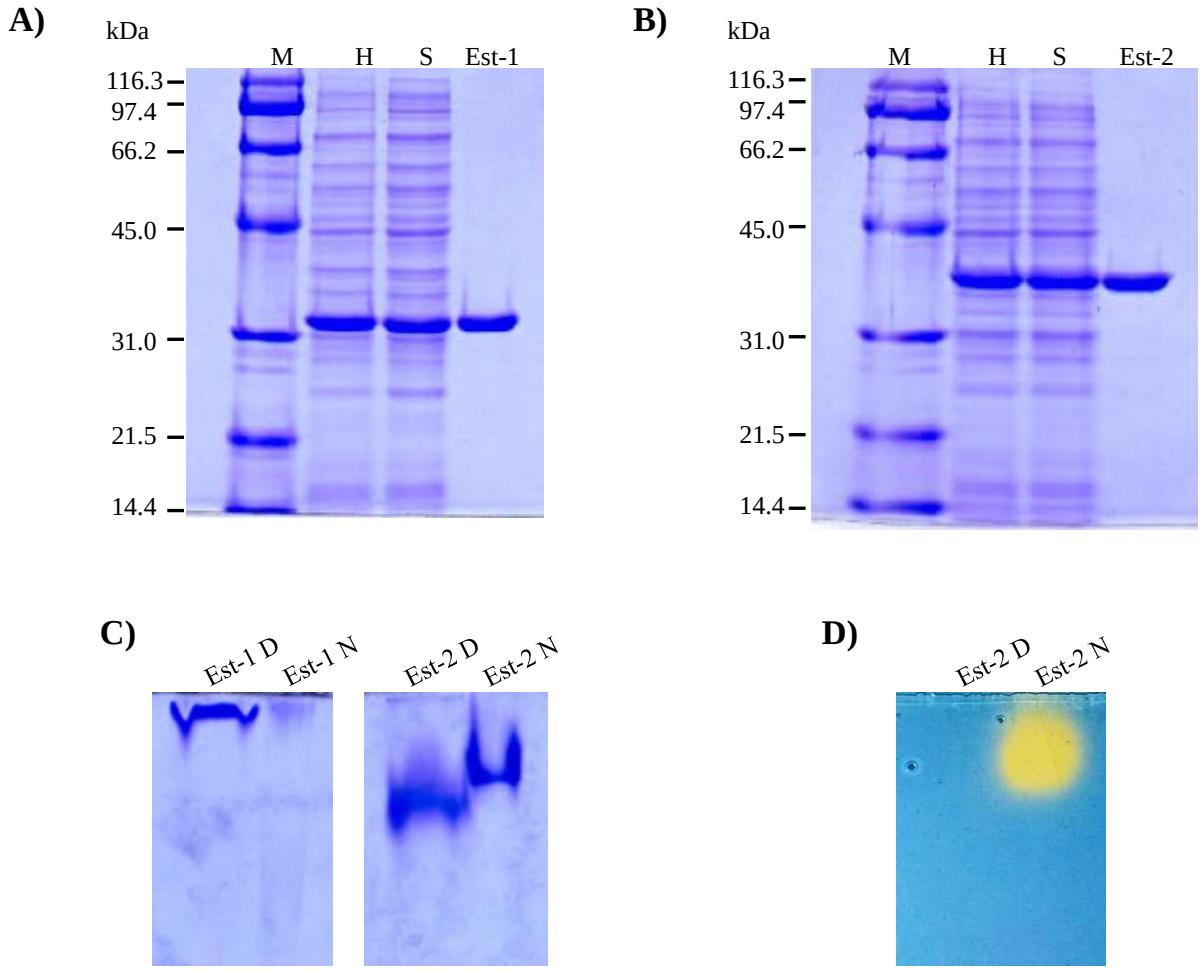

Fig. 5. Electrophoretic analysis. SDS-PAGE gels of purified *P. dispersa* recombinant Est-1 (A) and Est-2 (B). M is the Broad range molecular marker (BioRad, USA). H is the homogenized, S is the supernatant. Est-1 and Est-2 are the purified enzymes. In A), B), and C) the gels were stained with Coomassie blue. C) PAGE analysis. Est-1 D and Est-2 D are the denatured enzymes (monomeric). Est-1 N and Est-2 N are the native proteins. D) Zymogram showing the native Est-2 activity against triacetin, using bromothymol blue as pH indicator.

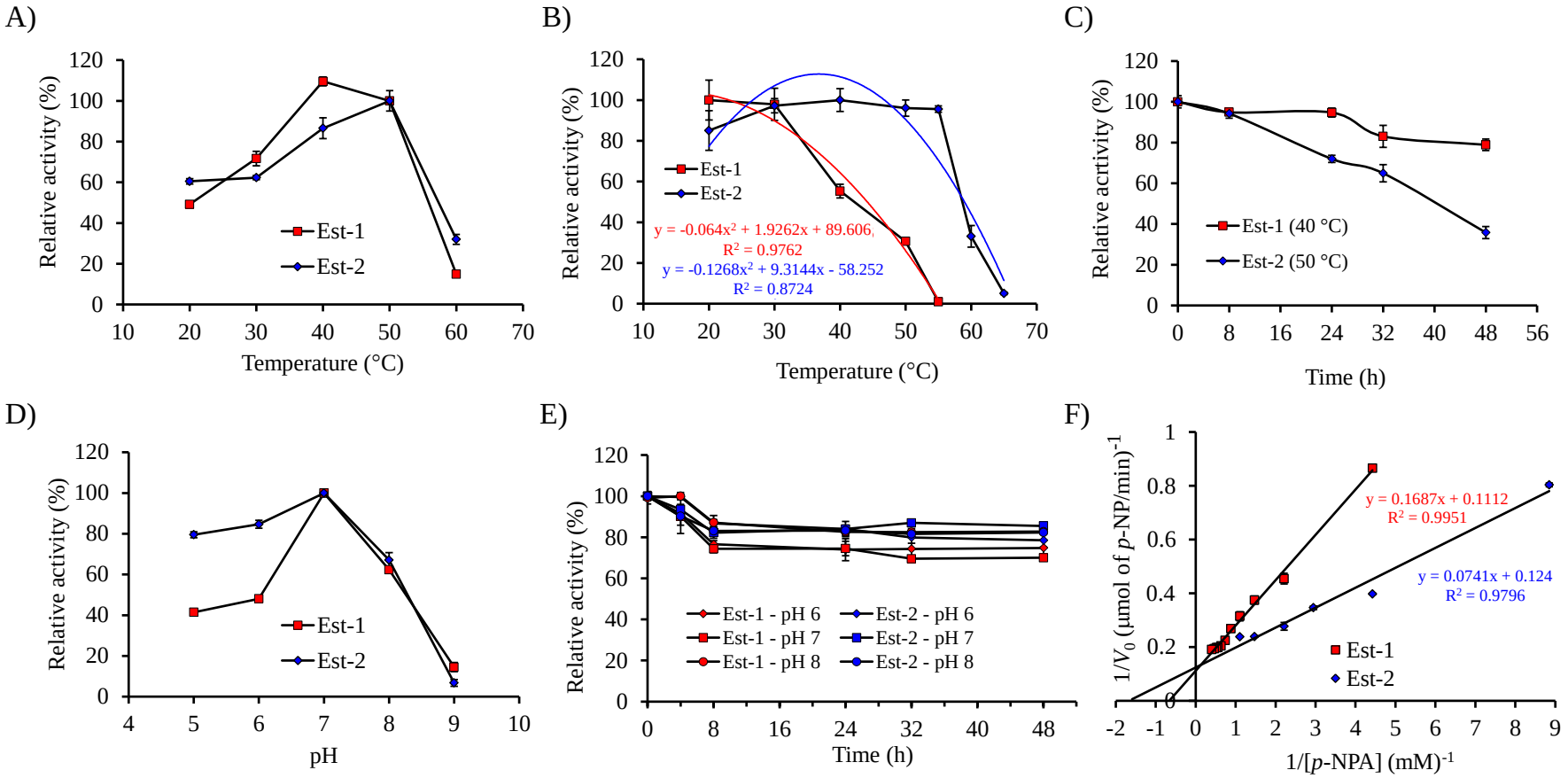

Fig. 6. Biochemical parameters of Est-1 and Est-2. A) Optimal temperatures. B) Thermal stabilities. The second order polynomial regression equations are indicated in colors. C) Temperature stabilities time course. D) Optimal pHs. E) pH stabilities time course at 4 °C. F) Lineweaver-Burk plots. The line equations are under the respective line. The data represents the average and standard deviation of three experimental sets.

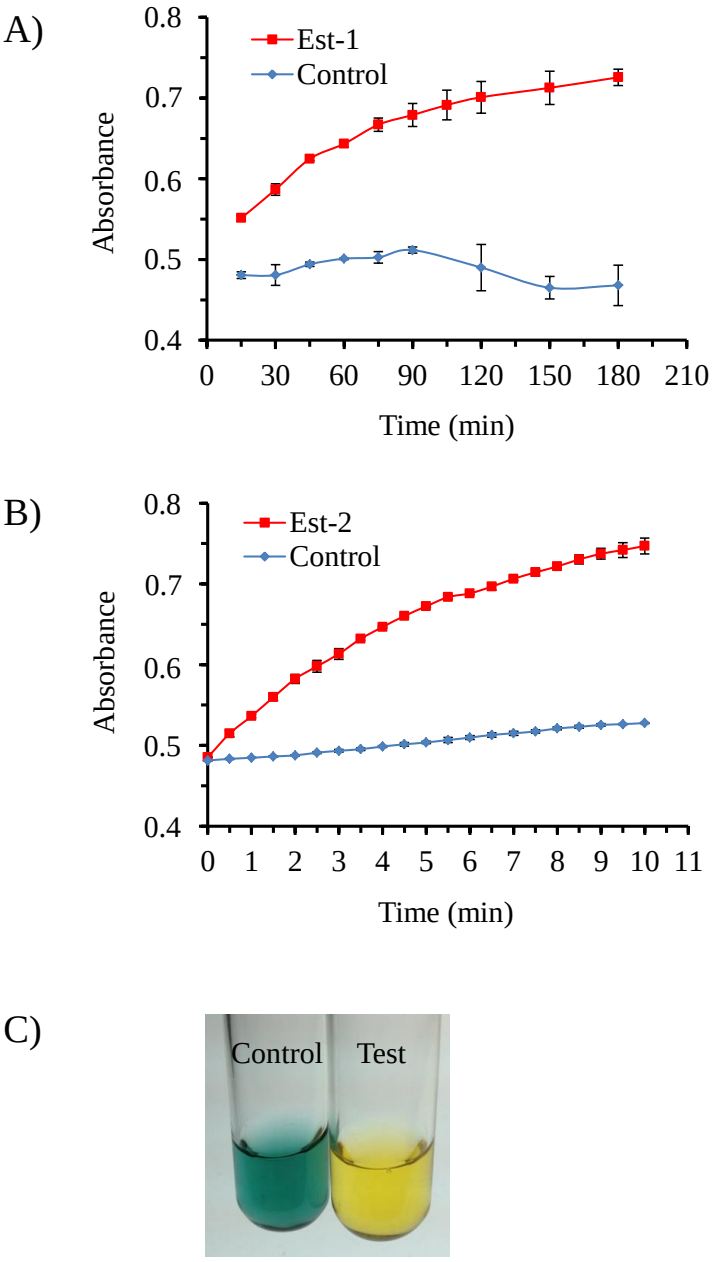

Fig. 7. Triacetin activity time course. A) Est-1 activity. B) Est-2 activity. C) A reaction illustration. Control is the tube with no enzyme added at the end of the reaction, with no color change from the beginning of the reaction. Test is the tube in which the enzyme was added at the end of the reaction, indicating the bromothymol blue color change with acidification by the enzymatic acetic acid release from triacetin. The results represent the average and the standard deviation of triplicates.