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Dissertation presented to the post-graduate program in Microbiology at the Institute of Biomedical Sciences at the University of São Paulo for the title of Master in Sciences

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A mi familia, mis padres Edgar y Miriam (mi ángel), mis grandes ejemplos de perseverancia y esfuerzo, a ustedes que me mostraron lo importante de la vida; a mis hermanos Andrea y Alex quienes me animan a seguir y luchar por mis sueños, gracias por contagiarme esas ganas de seguir y encontrar mi camino

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RESUMO

CHURASACARI, T. **Caracterização funcional de uma nova esterase de solo escuro da Amazônia com comportamento histérico**. 2020. 67 f. Dissertacao (Mestrado em Microbiologia) – Inituto de Ciencias Biomédicas, Universidade de São Paulo, SP, 2020.

No presente estudo, caracterizamos bioquimicamente uma nova enzima com atividade esterase membro da superfamília α/β-hidrolase obtida de uma biblioteca metagenômica de solo escuro da Amazônia. Essa enzima foi nomeada como Amazonian Dark Earth Esterase 1 (Ade1). Ade1 hidrolisa ligações éster de diferentes substratos como: tributirina (cadeia alifática com 3 carbonos), Tween 20 (cadeia alifática com 11 carbonos), *p*-nitrofenil butirato (cadeia alifática com 4 carbonos), e *p*-nitrofenil octanoato (cadeia alifática com 8 carbonos) e N-hexanoil-L-homoserina lactona (C6-HSL, cadeia alifática com 6 carbonos) onde Ade1 atua interrompendo o quórum sensing pela sua capacidade de hidrolisar o autoindutor N-hexanoyl homoserine lactone, processo conhecido como quórum quenching. Dessa forma, Ade1 mostra um grau de promiscuidade em sua atividade enzimática pela sua capacidade de hidrolisar ligações ésteres de diferentes moléculas cuja estereoquímica permita coloca-la/seu ingresso no bolsillo catalítico no não seja volumosa. Ade1 é um monómero que apresento um perfil sigmoidal evidente nos ensaios de cinética, conhecido como comportamento histerético com perfil transitório tipo "burst", o que já foi reportado para outras enzimas lipolíticas. Analise de dinâmica molecular mostra que Ade1 tem dois estados conformacionais: E1 e E2 cujo equilíbrio foi dependente da concentração do substrato. Alem, a atividade enzimática de Ade1 foi influenciada pela presencia de cobalto. O estado E2 possui uma maior velocidade de hidrólise de *p*-nitrofenil octanoato do que o estado E1. Ade1 pode ser uma enzima com interesse biotecnológico por poder modular "quorum quenching" em algumas bactérias que tem C6-HSL como autoindutor.

Palavras chaves: Caraterização bioquímica, cinética enzimática, quorum quenching.

ABSTRACT

CHURASACARI, T. **Functional characterization of a new dark soil esterase from Amazon with hysterical behavior**. 2020. 67 p. Master thesis (Microbiology) – Biochemical Science Institute, University of Sao Paulo, SP, 2020.

In the present study, we biochemically characterized a new enzyme with esterase activity that is a member of the α/β-hydrolase superfamily from a metagenomic library of dark soil in the Amazon. This enzyme was named as Amazonian Dark Earth Esterase 1 (Ade1). Ade1 hydrolyses ester bonds from different substrate such as: tributyrin (aliphatic chain with 3 carbons), Tween 20 (aliphatic chain with 11 carbons), *p*-nitrophenyl butyrate (aliphatic chain with 4 carbons), and *p*-nitrophenyl octanoate (aliphatic chain with 8 carbons) and N-hexanoyl-L-homoserine lactone (C6-AHL, aliphatic chain with 6 carbons) where Ade1 interrupts the quorum sensing by its ability to hydrolyze C6-HSL autoinducer. Thus, Ade1 shows a degree of promiscuity in its enzymatic activity due to its ability to hydrolyze ester bonds of different molecules whose stereochemistry is not bulky. Ade1 is a monomer that has a sigmoidal profile evident in kinetic test, known as hysteretic behavior with a "burst" profile, which has already been reported for other lipolytic enzymes. Molecular dynamics analysis shows that Ade1 has two conformational states: E1 and E2 whose equilibrium depend on the substrate concentration. In addition, the enzymatic activity of Ade1 was influenced by the presence of cobalt. The E2 state has a higher hydrolysis rate of *p*-nitrophenyl octanoate than the E1 state. Ade1 can be an enzyme with biotechnological interest because it can modulate "quorum quenching" in some bacteria that have C6-HSL as an autoinducer.

Key words: Biochemical characterization, enzymatic kinetics, quorum quenching

1 INTRODUCTION

1.1 Amazonian dark soil

The soil fertility is associated with the presence of great diversity of microorganisms (ZILLI; RUMJANEK; XAVIER; DA COSTA COUTINHO *et al.*, 2003). To illustrate the scale of this diversity, it is estimated that one gram of soil can contain over 10,000 species of bacteria, archaea, viruses, and eukaryotic microorganisms (TORSVIK; OVREAS, 2002). However, it is not yet been well characterized (WARD, 2002). A reason for that is the fact that, typically, only 1% of the total microbial population in a sample can be cultivated in laboratory conditions by traditional culture techniques (CURTIS; SLOAN, 2005). Nevertheless, the study of this type of environment has largely progressed from non-culture-based molecular techniques. The vast soil microbial diversity can be understood as a consequence of the multiple niches found within it: soil microorganisms are subjected to biotic stresses (as competition and parasitism) and abiotic stresses (temperature fluctuations, levels of humidity) that generate a dynamic ecosystem with different microbial functions and interactions (KAKIRDE; PARSLEY; LILES, 2010). How these multiple factors interact to the structure of the microorganism communities in the soil and how this impacts soil quality is of great interest but still a major challenge for microbiology and soil science.

In general, conventional soils from most of the Amazon rainforest is considered not very nutritious and with low fertility, and this would be related to a limited microbial diversity, however a particular type of soil called Amazon Dark Earth (ADE) is an exception. These soils are of anthropogenic origin from indigenous populations of the pre-Columbian era and present a higher content of organic matter (NAVARRETE; CANNAVAN; TAKETANI; TSAI, 2010). Specifically, ADE soils present a high concentration of carbon in the form of organic matter as result of accumulation of vegetable residues (leaves, seed shells, among others) and animal residues (bones, blood, fat, feces, shells, among others), in addition to a large amount of cinder and residues of bonfires (charcoal), and also phosphorus, calcium, magnesium, manganese, zinc, and other minor elements (DE AQUINO; MARQUES; CAMPOS; DE OLIVEIRA *et al.*, 2016). These factors may be contributing to microbial biomass. Previous studies by NAVARRETE; CANNAVAN; TAKETANI and TSAI (2010) and SILVA (2009) have explored the composition and diversity of the communities of microorganisms in ADE soils. The most abundant bacterial phyla in ADE soils were Proteobacteria (24%), Acidobacteria (10%), Actinobacteria (7%), Verrucomicrobia (8%), Firmicutes (3%), plus 36% of unclassified

bacteria (NAVARRETE; CANNAVAN; TAKETANI; TSAI, 2010), while the most abundant archaeal phyla were Crenarcheota (40%) and Euryarcheota (8%) (SILVA, 2009). Distinct environmental factors determine the metabolic activities essential for the survival of microorganisms that reside in these niches, and each species possess a great diversity of enzymes that have the potential to be used in industry. For example, the Amazon conditions ensure the presence of enzymes whose catalytic activity is highest at relatively high temperatures and acidic pH values, which can be useful for finding new enzymatic resources.

In a previous functional metagenomic study, seventy-five clones from a library of environmental DNA from Amazonian soil were found to present substantial lipolytic activity, which demonstrates the enzymatic potential of the microbial community in this region (WILLERDING; OLIVEIRA; MOREIRA; GERMANO *et al.*, 2011). In line with this approach, the genomes of uncultivated prokaryotes are known to code for a large pool of new enzymes with metabolic capabilities still completely unexplored (SIMON; DANIEL, 2011). In addition to that, in general, enzymes can present an extraordinary array of catalytic functions and substrate specificities which are often better than both synthetic and inorganic catalysts (SANGEETHA; ARULPANDI; GEETHA, 2011). Therefore, this evidences the large biotechnological potential literally hidden below ground in the Amazon, part of which we explore in-depth in this work.

1.2 Metagenomic

The study of microorganisms has become very important because they are the cause of human and animal diseases, and they also have a fundamental role in some industrial processes. Microorganisms have a great variety of forms and consist of a great variety of metabolic enzymes that allow them to colonize different ecological niches, some of them with extreme conditions. 1g of soil may contain over 100 billion microbial cells representing several thousand to over a million distinct genomic species and, only 0.1 - 1 % of microbial species are cultured by standard microbiological methods (XU, 2006), for it, is important develop and use new tools. The metagenomic technique is a tool that facilitate studying and examining the diversity of microbial communities and molecules with industrial importance, using genetic technology and bioinformatics tools. Metagenomic technique is used to find new coding genes for enzymes as proteases, lipases, amylases, alcohol oxidoreductases, antibiotics, antibiotic resistance (VOGET; LEGGEWIE; UESBECK; RAASCH *et al.*, 2003) and drugs important to the industrial (CARVALHO; CALAFATTI; MARASSI; SILVA *et al.*, 2005).

The steps generally involved in this technique are shown in **Figure 1** (THOMAS; GILBERT; MEYER, 2012) . First, taking and processing of the sample, this step is relevant, total DNA extracted must have a high quality and be representative (it must include the greatest amount of diversity present in the sample). The DNA obtained is cloned into Fosmid plasmids and thereby generate gene libraries that can be used in functional screening for metabolic activity of metagenomic clones, or sequence and annotate sequences from which it is possible to predict conserved motifs (ESCHENFELDT; STOLS; ROSENBAUM; KHAMBATTA *et al.*, 2001). The sequencing of the clones of interest can be performed by the classical Sanger method (to generate complete genomes in samples with low diversity) or next generation sequencing (NGS) by 454/Roche or Illumina/Solexa. The assembly can be based on references, where the assembly is carried out with the help of related genomes as reference with differences such as: insertion, deletion or polymorphisms; or de novo assembly, it requires assembly tools based on Brujin graphics for a large amount of data, therefore it requires more computational resources. For the annotation it is necessary to identify the genes of interest and then, by prediction, assign functions of these putative genes and taxonomic neighbors (functional annotation) using different bioinformatic tools. After a statistical analysis, the genomic data are placed in public repositories where they can be made available to other researchers.

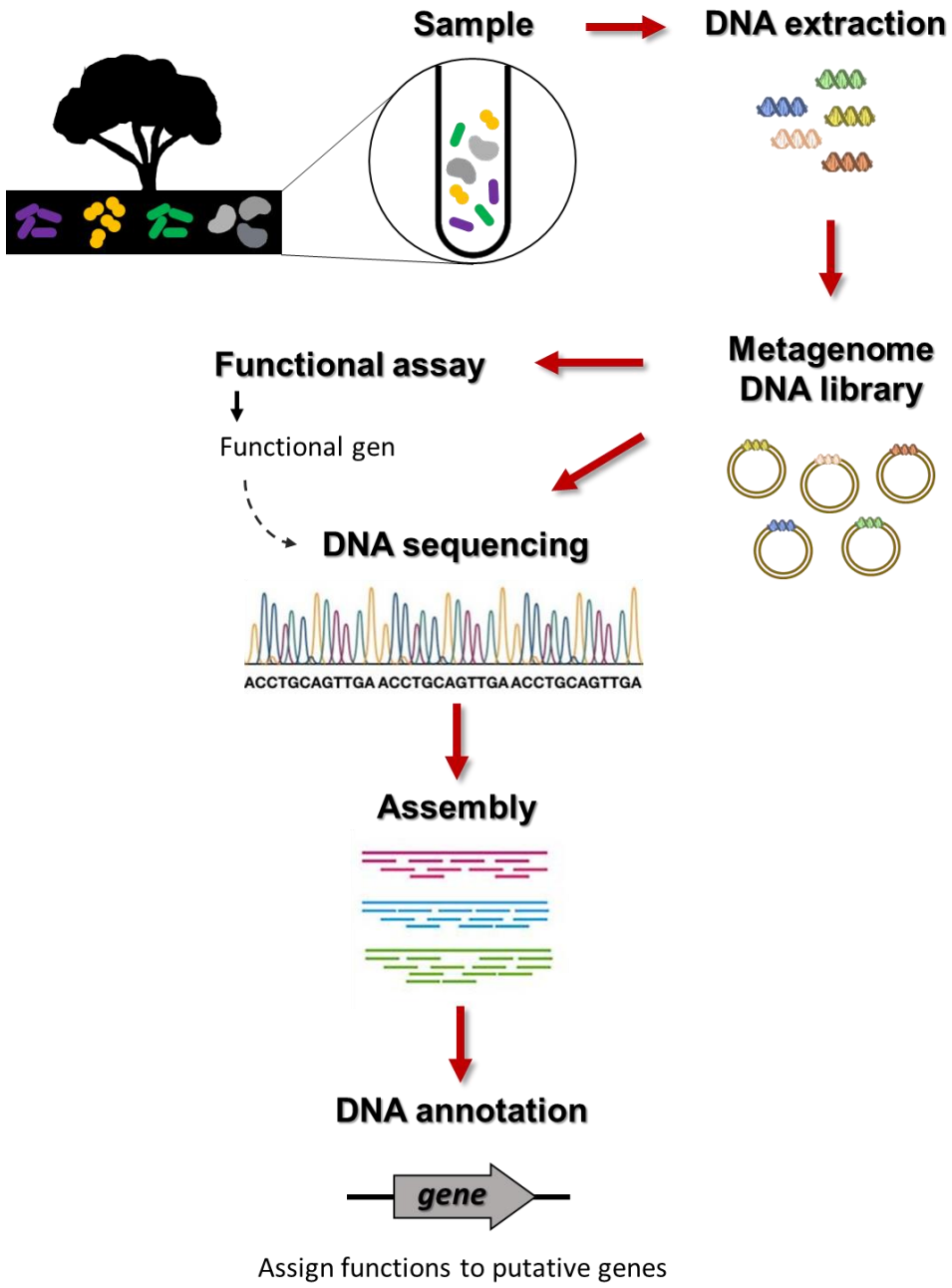

Figure 1. Metagenomic process. Sample processing, total DNA extraction and subcloning into fosmid plasmids to generate a metagenome DNA library. These clones can be used in functional screening or sequence processing and DNA assembly to annotate them and predict conserved motifs. The genomic data obtained is placed in public repositories.

1.3 Lipolytic enzyme

Lipolytic enzymes are part of the group of hydrolases, since catalysis is assisted by a water molecule (**Figure 2**). These enzymes hydrolyze ester groups of fatty acids and is composed of enzymes that have diverse amino acid sequences but related three-dimensional structure.

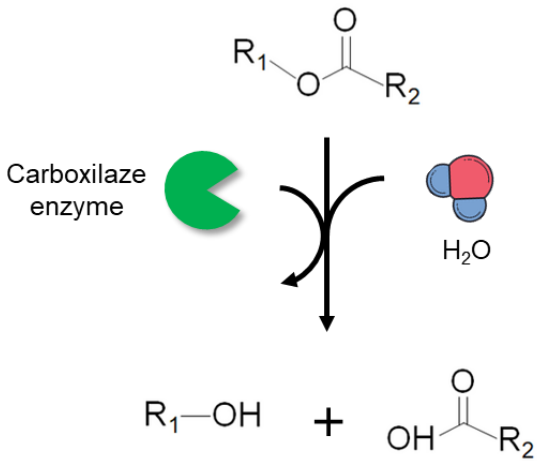

Figure 2. Carboxylase enzymes hydrolyze on carboxyl ester bond in lipids.

1.3.1 α/β-hydrolase superfamily

Lipolytic enzymes belong to the α/β-hydrolase superfamily, which present different types of catalytic activity such as carboxylic ester hydrolase, lipid hydrolase, thioester hydrolase, peptide hydrolase, halo peroxidase, dehalogenase, epoxide hydrolase, and C-C bond breaking. The α/β-hydrolases present similar tertiary structures which consist of 8 central β-strands surrounded by a variable number of α-helices (**Figure 3a**), composing two domains. The core domain is described by an 8-stranded β-sheet region surrounded by a variable number of α-helices (**Figure 3b**) (BAUER; BUCHHOLZ; PLEISS, 2020; LENFANT; HOTELIER; BOURNE; MARCHOT *et al.*, 2013; MINDREBO; NARTEY; SETO; BURKARTL *et al.*, 2016). The cap domain is composed of a variable number of α-helices (**Figure 3b**). BAINS; KAUFMAN; FARNELL and BOULANGER (2011) suggest that the cap domain is important for dimerization of two monomers and define the architecture of the active site helping in the substrate selectivity of the enzyme (MINDREBO; NARTEY; SETO; BURKARTL *et al.*, 2016). The enzymatic activity is due to the presence of a catalytic triad composed of a nucleophilic residue, a histidine, and an acidic residue. Only the histidine is invariant within the superfamily members, while the nucleophilic residue can be a serine, a cysteine, or an asparagine, and the acidic residue can be either an aspartate or a glutamate (HOLMQUIST, 2000; LEE; KWON; PARK; KIM *et al.*, 2017; MINDREBO; NARTEY; SETO; BURKARTL *et al.*, 2016; SAYER; ISUPOV; BONCH-OSMOLOVSKAYA; LITTLECHILD, 2015). Although this group of enzymes shares similar catalytic triads, they hydrolyze many different substrates and act in diverse biological contexts. The main factor contributing for the substrate specificity of each enzyme of this family could be the orientation of the additional residues

located in the pocket of the active site (FOJAN; JONSON; PETERSEN; PETERSEN, 2000). Having the same core structure and similar residues in the catalytic pocket (HOLMQUIST, 2000; MINDREBO; NARTEY; SETO; BURKARTL *et al.*, 2016), only experimental assays can be used to identify the substrates and catalytic pathways of a novel α/β-hydrolase enzyme.

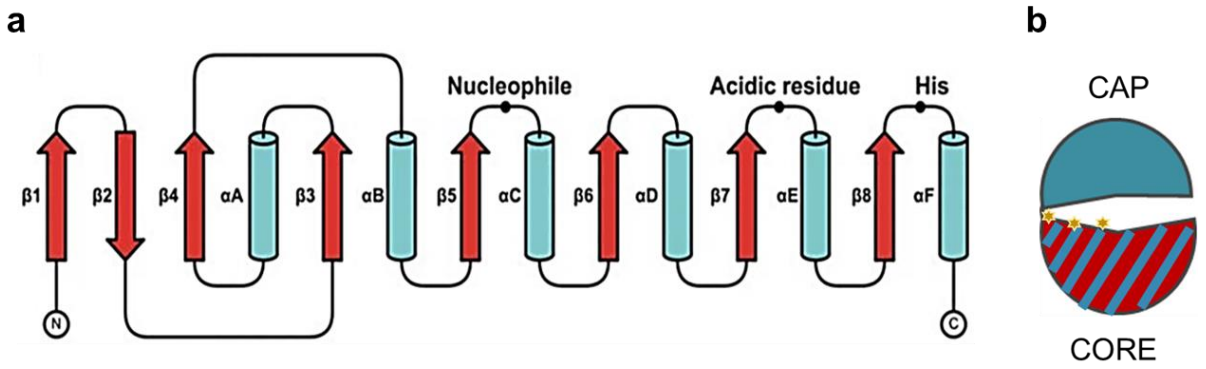

Figure 3. Schematic representation of the α/β-hydrolase fold. (a) β-sheets (1–8) are shown as red arrows and α-helices (A–F) are shown as blue cylinders. The positions of each amino acid that compose the catalytic triad is also indicated. Source: (LEVISSON; SUN; HENDRIKS; SWINKELS *et al.*, 2009). **(b)** Typical 3D representative scheme of α/β-hydrolase, signaling the CAP and CORE domains, the yellow stars, located in the catalytic pocket, represent the catalytic triad.

Lipolytic enzymes are ubiquitous (CHAHINIAN; SARDA, 2009), and can be classified in two groups: esterases (EC 3.1.1.1) and lipases (EC 3.1.1.3) (HOLMQUIST, 2000), **Figure 4**, both of which are capable of catalyzing the hydrolysis of esters into carboxylic acid and alcohol molecules, differing mainly in the size of the carbon chain of their preferred substrates (**Figure 5**). Based on their source, biochemical properties, protein fold, and the position of the catalytic triad these enzymes can be classified into one of the XXXV families (HITCH; CLAVEL, 2019).

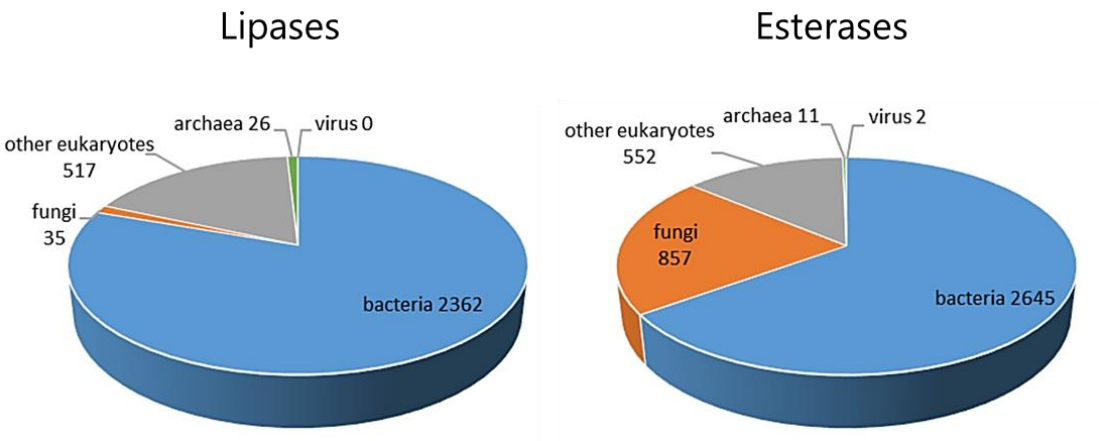

Figure 4. Lipolytic enzymes diversity. Taxonomic distribution of lipases and esterases using Brenda database (KOVACIC; BABIC; KRAUSS; JAEGER, 2019)

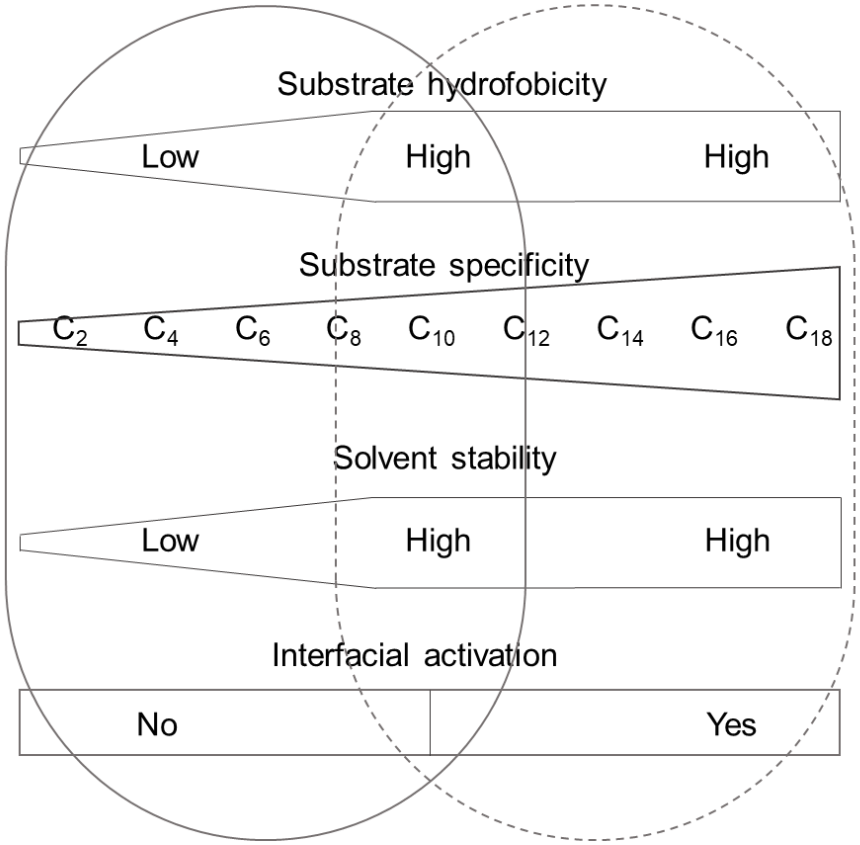

Figure 5. Lipase and esterases enzymes properties and features. (ELLEUCHE; SCHRÖDER; ANTRANIKIAN, 2016)

Lipolytic enzymes share the same physicochemical nature of their catalytic triad and present many structural similarities. These enzymes can be broadly applied in biotechnological processes due to their promiscuity, high regioselectivity, and stereoselectivity to accommodate substrate for different reactions (HOLMQUIST, 2000). Due to the characteristics that these

enzymes present, they are the third largest group with industrial importance (ELLEUCHE; SCHRÖDER; ANTRANIKIAN, 2016). Because of this, lipolytic enzymes can be used in food modifications, detergent formulations, and chemical synthesis, as well as for drug metabolism and drug detoxifications in pharmacological and clinical contexts (**Figure 6**) (MURUGAYAH; GERTH, 2019)

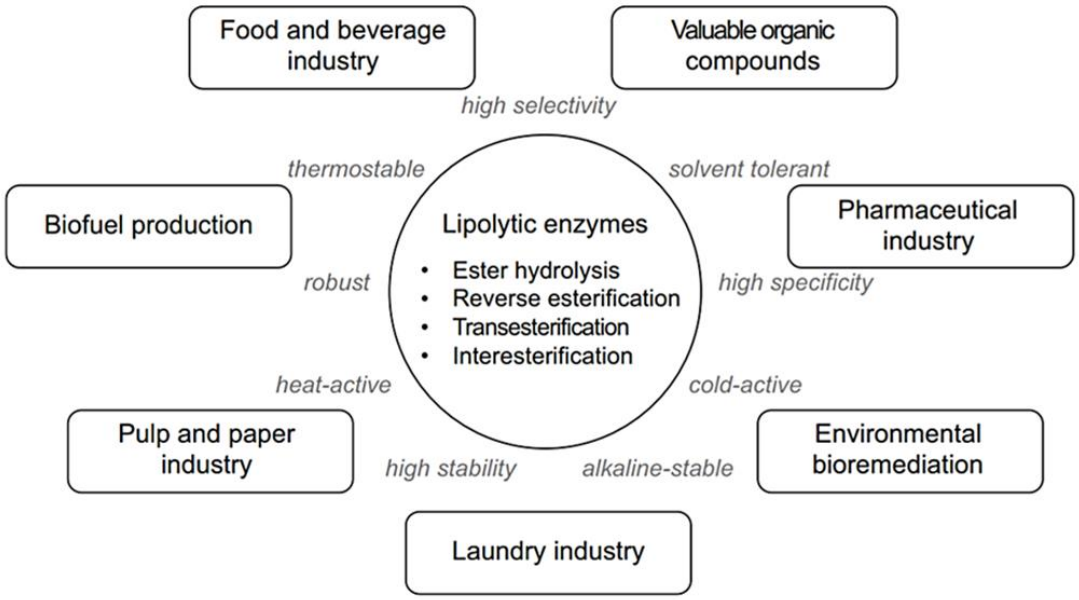

Figure 6. Industrial importance of lipolytic enzymes. Lipolytic enzyme catalyzed reactions, features and several applications (ELLEUCHE; SCHRÖDER; ANTRANIKIAN, 2016).

1.3.2 Lipases

Lipases can be distinguished from esterases by the phenomenon of interfacial activation in kinetics terms, this refer that the lipase activity increases considerably at the lipid-water interface (BRZOZOWSKI; DEREWENDA; DEREWENDA; DODSON *et al.*, 1991; VERGER, 1997). These enzymes can hydrolyze short and long chain acyl-glycerides (≥ 10 carbons) and exhibit high activity towards aggregated states of its substrates (JAEGER; STEINBUCHEL; JENDROSSEK, 1995).

1.3.3 *Esterases*

Esterases hydrolyze only short-chain esters (< 10 carbons) when its substrate is in a soluble state (ARPIGNY; JAEGER, 1999). This group of enzymes hydrolyzes ester linkages in compounds such as aryl-ester, acyl-glycerol, and carboxylic esters. Esterases are known to play important physiological roles in lipid metabolism and detoxification of xenobiotics agents in different organisms and thus are ubiquitous in nature (LEE; KWON; PARK; KIM *et al.*, 2017).

1.4 Quorum Sensing and Quorum Quenching

1.4.1 Quorum sensing

Quorum sensing (QS) is a process related to bacterial cell-to-cell communication related to bacterial cell-population density to coordinate the gene regulation and therefore the behavior to the community, changes in the cell-population density is perceived by the accumulation of signal molecule called the "autoinducer" (**Figure 7 a** and **b**) (MILLER, 2001). The autoinducer is a small signal molecule produced by bacteria that diffuses out of the bacterial cell and accumulates in the extracellular environment. QS-mediated regulation is activated by sensing high cell densities and synchronizes gene expression for coordinating inter-bacterial behavior (KALIA; PATEL; KANG; LEE, 2019; PIEWNGAM; CHIOU; CHATTERJEE; OTTO, 2020). It is also related to the regulation of biofilm formation, antibiotic resistance, bioluminescence, and bacterial virulence in different species (CHOO; RUKAYADI; HWANG, 2006; FETZNER, 2015; LIN; XU; HU; WANG *et al.*, 2003; TORRES; UROZ; SALTO; FAUCHERY *et al.*, 2017). Different types of signal molecules have been identified, but the most studied correspond to N-acyl homoserine lactone (AHL) analogs that differ in the length of their N-acyl chains (BAINS; KAUFMAN; FARNELL; BOULANGER, 2011). The side-chain length of AHL from different bacterial species is often in the range between 4 to 14 carbons connected by an ester linkage to the lactone ring. These distinct molecular structures influence the binding of the signal molecule to the receptor protein. In this manner, AHL communication systems are usually highly species-specific, but crosstalk which disturbs proper signaling may also happen (MARTINELLI; GROSSMANN; SEQUIN; BRANDL *et al.*, 2004). AHLs autoinducers regulate several functions in terrestrial and aquatic environments such as the production of exoenzymes in *Pectobacterium carotovun*, bioluminescence in *Aliivibrio fisheri*, activation of the conjugative transfer of plasmid Ti in *Agrobacterium tumefaciens*, motility in *Aeromonas hydrophyla* (TORRES; UROZ; SALTO; FAUCHERY *et al.*, 2017) and is also related with bacterial pathogenicity.

1.4.2 Quorum quenching

In nature, it has been reported the ability of different organisms (bacteria, fungi, plants, and animals) to inhibit and/or interfere with the bacterial QS, suggesting that host cells or competing organisms have developed QS-interference methods as evasion mechanisms to avoid infection or to obstruct the development of adversary microbes (TORRES; UROZ; SALTO;

FAUCHERY *et al.*, 2017). Quorum quenching (QQ) is any process involved in the disturbance of the QS through quorum sensing inhibitory molecules (QQ enzymes) or physical processes that affect the temperature and pH upon infection of animal or plant hosts, which inactivate the AHL QS signal. QQ was revealed to play a fundamental role in microbial competition (GRANDCLEMENT; TANNIERES; MORERA; DESSAUX *et al.*, 2016; MURUGAYAH; GERTH, 2019; WANG; DAI; ZHANG; HU *et al.*, 2007). One efficient way to avoid cell-to-cell communication is by the degradation or modification of autoinducers by different classes of enzymes, including lipolytic enzymes (**Table 1**). QQ enzymes have gained importance in the last years because they can be used as a strategy for the development of antibacterial, antiviral or compounds against diseases of important pathogens (GRANDCLEMENT; TANNIERES; MORERA; DESSAUX *et al.*, 2016). In this context, AHLs are one example of autoinducer well-characterized in Gram-negative bacteria that can be specifically targeted for interference by QQ (FETZNER, 2015; MURUGAYAH; GERTH, 2019). The lactonases are examples of QQ enzymes that show a high range of selectivity, from highly promiscuous, to considerably both stereo and regio-specific facilitating the correct accommodation of the substrate. Enol-lactone-hydrolases (ELH) are the more selective family, which convert the terminal cyclic structure 3-oxoadipate-enol-lactone (β-ketoadipate-enol-lactone) into β-ketoadipate as part of the β-ketoadipate pathway, where the aromatic metabolites - protocatechuate and catechol - are turned into intermediaries of the Krebs cycle (BAINS; KAUFMAN; FARNELL; BOULANGER, 2011). Another group of enzymes are Acyl homoserine lactone lactonases (AHL lactonases) that inactivate acyl homoserine lactone molecules by hydrolyzing the lactone ring (LIN; XU; HU; WANG *et al.*, 2003).

A common organism used to investigate QS and QQ in laboratory studies (also used in this work) is *Chromobacterium violaceum*, a Gram-negative bacterium which resides in the tropical and subtropical areas. This bacterium synthesizes an easily measured violet pigment – called violacein – as a result of biofilm formation by quorum sensing signaling. The autoinducer is a N-hexanoyl homoserine lactone (C6-HSL). C6-HSL binds to the transcriptional regulator (CviR) and activates the expression of the violacein biosynthetic genes (Fig. 3b) as well as other virulence factors (CHOO; RUKAYADI; HWANG, 2006). Quorum-quenching by degradation of homoserine lactones is observed in different classes of enzymes (such as lactonases, decarboxylases, acylases, and deaminases) that cleaves the molecule at different sites (**Figure 7c**) (CZAJKOWSKI; JAFRA, 2009; DONG; ZHANG, 2005).

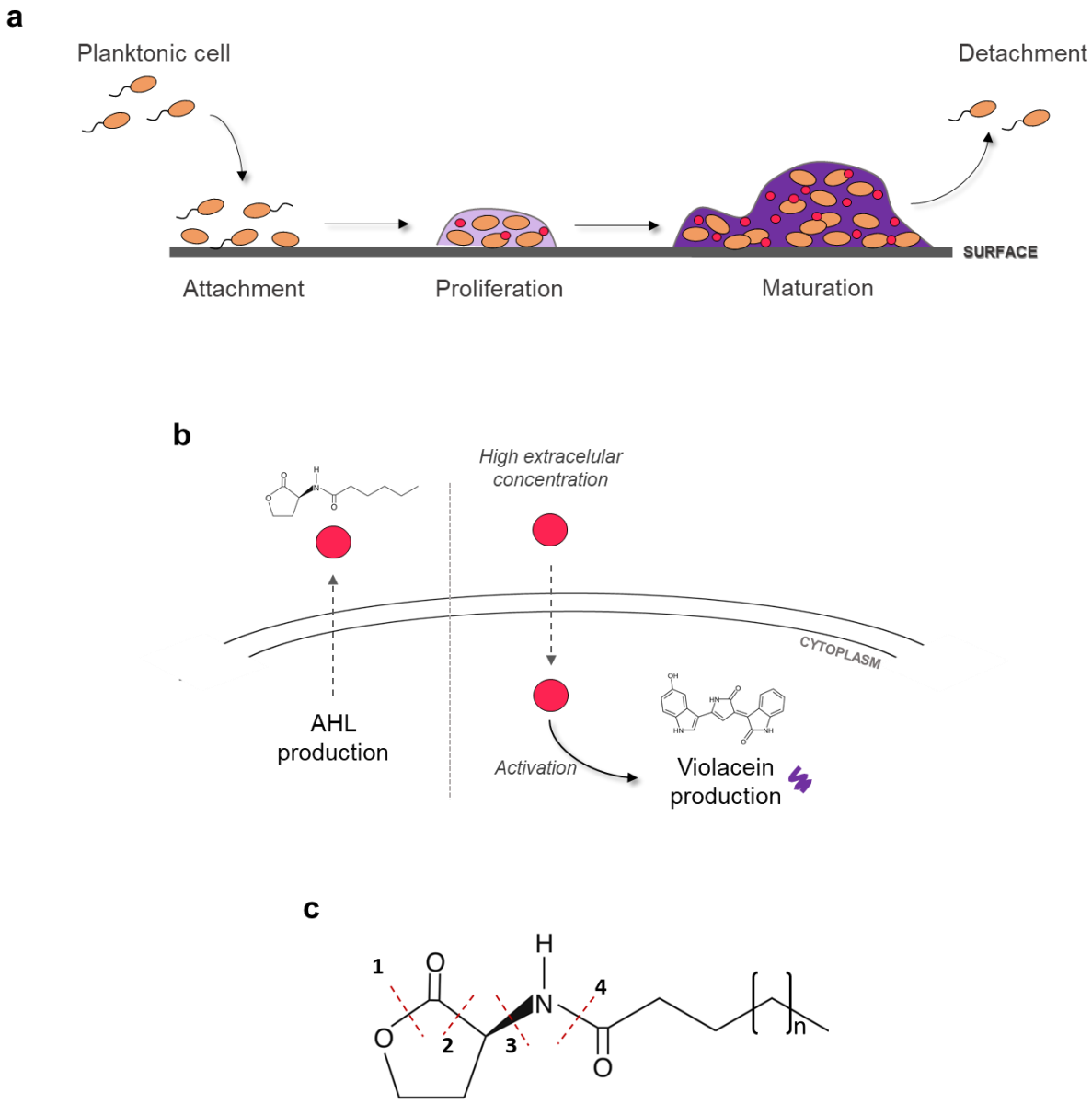

Figure 7. Quorum sensing and biofilm formation in *Chromobacterium violaceum*. (**a**), Biofilm formation, **Attachment**, the planktonic cells attach to a surface, the interaction with the surface is by protein-protein interaction; **Proliferation**, these cells divide and increase their population density, and form microcolonies; **Maturation** or colonization, the microcolony growth, bacterial cells secret extracellular matrix and virulence factors regulated by quorum sensing (QS), until some cells detach from the matrix and return to a planktonic state (JOO; OTTO, 2012). (**b**), Regulation of violacein production mediated by the autoinducer acyl homoserine lactone (AHL). AHL (red ball) is secreted out of the bacteria upon adhesion to a surface, when AHL is in high concentrations, enter into bacteria by diffusion and, activates the transcription of violacein (purple pigment). (**c**) Possible ways of enzymatic degradation of AHLs, broken lines mark the position of possible sites cleaved by the following enzymes: 1, lactonase; 2, decarboxylase; 3, deaminase and 4, acylase (CZAJKOWSKI; JAFRA, 2009).

Table 1. Quorum-quenching activity of different α/β-hydrolase members. The table has information about the enzyme, organism, biological function and if there is the presence or absence of metal during the enzymatic catalysis. There are proteins that have quorum-quenching activity.

Name	Organism	Function	Reference	metal/no metal
3-hydroxy-palmitic acid methyl ester hydrolases	metagenomic library	esterase	(LEE; KHAN; TAO; CHOI *et al.*, 2018)	no metal
XB7, XB122 and XB102	*Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*	esterase	(ACHARI; RAMESH, 2018)	Cu^{2+} and Ca^{2+} enhanced activity of quorum quenching enzymes
Est816 esterase	Turban Basin metagenomic library	esterase	(LIU; CAO; FAN; LIU *et al.*, 2016)	no metal
Porcine kidney acylase (PKA), Porcine liver esterase (PLE) and horse liver esterase (HLE)	Porcine and Horse	acylase and esterase	(KISCH; UTPATEL; HILTERHAUS; STREIT *et al.*, 2014)	no metal
Est816 esterase	Turban Basin metagenomic library	esterase	(FAN; LIU; LIU, 2012)	Ca^{2+}, activity increases slightly
QsdH	*Pseudoalteromonas byunsanensis*	esterase	(HUANG; LIN; YI; LIU *et al.*, 2012)	Zn^{2+}, Ni^{2+}, Cu^{2+}, Ba^{2+}, Mg^{2+}, Sr^{2+}, Ca^{2+} and Mg^{2+}
beta-hydroxypalmitate methyl ester hydrolase	*Ideonella* sp. 0-0013	esterase	(SHINOHARA; NAKAJIMA; UEHARA, 2007)	Fe^{2+} and Sr^{2+} inhibit the enzyme activity. Na^{+} and K^{+} promoted enzymatic activity. Zn^{2+} and Mg^{2+} do not inhibit

				the enzymatic activity.
3-hydroxy-2-methyl-4(1H)-quinolone 2,4-dioxygenase (Hod)	*Paenarthrobacter nitroguajacolicus*	oxidoreductase	(PUSTELNY; ALBERS; BULDT-KARENTZOPOULOS; PARSCHAT *et al.*, 2009)	no metal
AiiA	*Bacillus* sp. strain DMS133	esterase	(MAHMOUDI; NADERI; VENTURI, 2013)	no metal
AiiSSl – 5 EstSl – 5	*Altererythrobacter* sp. S1-5	esterase	(WANG; GUAN; PAIN; KAKSONEN *et al.*, 2019)	no metal
AHL-lactonase	*Rhodococcus* sp. BH4	esterase	(RYU; LEE; MIKOLAITYTE; KIM *et al.*, 2020)	no metal
BpiB05	metagenome-derived hydrolase	esterase	(BIJTENHOORN; SCHIPPER; HORNUNG; QUITSCHAU *et al.*, 2011)	Ca^{2+} for carrying out the catalysis
AqdC	*M. abscessus* subsp. *abscessus*	esterase	(BIRMES; SARING; HAUKE; RITZMANN *et al.*, 2019)	no metal
3-Phenyllactic acid (PLA) inhibitor	Lactobacillus species	-	(CHATTERJEE; D'MORRIS; PAUL; WARRIER *et al.*, 2017)	no metal
AiiAQSI-1	*Bacillus* sp. strain QSI-1	esterase	(ZHANG; ZHUANG; GUO; MCLEAN *et al.*,	no metal

			2019)	
EstDL30	Alluvial soil metagenomic library	esterase	(TAO; LEE; WU; KIM *et al.*, 2011)	no metal
EST816 esterase	Metagenomic library construction and AHL-lactonase screening	esterase	(LIE; MEYER; PEDERSEN, 2014)	no metal
AHL-lactonase	Human, mouse and fish	esterase	(YANG; WANG; WANG; DONG *et al.*, 2005)	Ca^{2+}
AHL lactonase	*Bacillus thuringiensis*	esterase	(LIU; MOMB; THOMAS; MOULIN *et al.*, 2008)	Zn^{2+}
Phosphotriesterase-Like Lactonases (PLLs) family	*Vulcanisaeta moutnovskia*	esterase	(HIBLOT; BZDRENGA; CHAMPION; CHABRIERE *et al.*, 2015)	Co^{2+}
N-acyl-homoserine lactone acylase	*Ralstonia solanacearum* GMI1000	acylase	(CHEN; CHEN; LIAO; LEE, 2009)	no metal
no enzymatic characterization	*Acinetobacter lactucae* strain QL-1	no enzymatic characterization	(YE; ZHOU; FAN; BHATT *et al.*, 2019)	no metal
no enzymatic characterization	*Acinetobacter* sp. XN-10	no enzymatic characterization	(ZHANG; LUO; ZHANG; FAN *et al.*, 2020)	no metal

AidF	*Ochrobactrum intermedium* D-2	esterase	(FAN; YE; LI; BHATT *et al.*, 2020)	no metal
FadY	*Acinetobacter lactucae* strain QL-1	acyl-CoA synthetase	(YE; ZHOU; XU; ZHANG *et al.*, 2020)	no metal
no enzymatic characterization	no microrganism was characterized	acylase	(BAO; HOSOE; HOSOMI; TERADA, 2020)	no metal
GcL	*Geobacillus caldoxylosilyticus*	esterase	(BERGONZI; SCHWAB; ELIAS, 2016)	Co^{2+}
PFE esterase	*Burkholderia anthina* HN-8	esterase	(YE; ZHANG; FENG; FAN *et al.*, 2020)	no metal
PLL (PTE-like lactonase)	*Mycobacterium avium* subsp. *paratuberculosis* K-10	esterase	(CHOW; WU; YEW, 2009)	no metal
QsdA	*Rhodococcus erythropolis*	phosphodiesterase	(UROZ; OGER; CHAPELLE; ADELINE *et al.*, 2008)	no metal

1.5 Hysteretic behavior

An enzymatic reaction has two important components, enzyme and substrate, which will form a complex and after catalysis a product will be generated. Esterases metabolize ester groups in alcohol and a carboxylic acid, and kinetic data tend to fit the Michaelis-Menten model (ADLER; KISTIAKOWSKY, 1962; HOFFSTEE, 1952; KHUSHAIRI; SAMAD; RAHMAN; YUSSOF *et al.*, 2020; SHENOUDA; GREEN; SULTATOS, 2009; VERPOORTE; MEHTA; EDSALL, 1967; ZAINOL; ISMAIL, 2019). The Michaelis-Menten model assumes that the enzymatic activity is one enzyme, one substrate and one product. Along the enzymatic turnover, the enzyme structure conformation is restored for recognizing the substrate and performs a new catalytic cycle (QIAN; ELSON, 2002).

The cooperative activity profile is a feature of some enzymes capable of forming an oligomeric state or having multiple substrate binding sites (VIVOLI; PANG; HARMER, 2017). Interestingly, some monomeric enzymes with one substrate site, show enzymatic activity with a profile of cooperativity (sigmoidal curve) and this characteristic is due to a slow conformational change in response to the environment, substrate or product, also known as hysteresis (**Figure 8**). The conformational changes of a protein can occur from very short periods of time (nano or picoseconds) to hours (BEHZADI; HATLESKOG; RUOFF, 1999; FRIEDEN, 1979). Behzadi and collaborators determined the influence of pH on the activity of an alkaline phosphatase, they observed that the change of activity was generated by a change in the conformation of the enzyme (conformational adaptation to pH), being that when increasing the pH the enzyme presented less activity (BEHZADI; HATLESKOG; RUOFF, 1999). The hysterical behavior of certain enzymes is due to the mechanism to control the activation or inhibition of a biological pathway using only one enzyme, **Figure 9** (FRIEDEN, 1979). One example of that is the modulation of the enzymatic activity based on the oscillation of a metabolic pathway benefiting the cell (CORNISH-BOWDEN; CÁRDENAS, 1987; JIANG; LI; MORROW; POTHUKUCHY *et al.*, 2019). The hysteresis can be observed by the transient kinetics as a burst or lag in substrate utilization, most of them belong to a metabolic (some detailed examples in **Table 2**). Burst is described as a change from an initially higher rate of activity to a lower steady state rate. Lag is described as a change from a lower rate to a higher rate of activity. There are two ways that cause enzymatic hysteresis behavior: Mnemonic model and Ligand-induced slow transitions model, described below (AINSLIE; NEET; SHILL, 1972; PORTER; MILLER, 2012).

1.5.1 Mnemonic model

In mnemonic model, the enzyme can keep or "recalls" a structural memory substrate-induced by a short time after releasing the product. In this model the enzyme present two conformational state, one of low affinity state and other of high affinity state. In absence of substrate, the enzyme maintains only one conformational state. However, the presence of substrate cause a second conformational state of the enzyme (with high or low affinity) and, the two conformational states with different initial velocities are present in equilibrium in the reaction (PORTER; MILLER, 2012).

1.5.2 Ligand-induced slow transitions model

The ligand-induced slow transitions (LIST) the enzyme visits two structural conformations in absence or presence of substrate. In contrast, the presence of substrate changes the equilibrium constant of the structural conformation states. In addition, the LIST behavior can be caused not only by the substrate but also by the solvent nature or pH (FOLOGEA; KRUEGER; MAZUR; STITH *et al.*, 2011; KE; KLIBANOV, 1998; STAN; BHATT; DE CAMARGO, 2020).

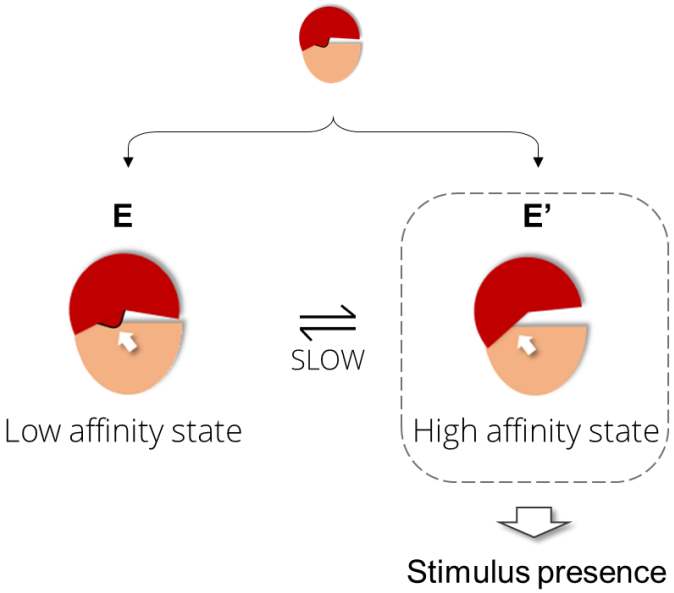

Figure 8. Hysteresis or Hysteretic behavior. When an enzyme presents two conformational states in response to a stimulus (presence or concentration of, substrate or product). Here, both states, E and E ', will be in equilibrium, being that the process of change of state, from E' to E, is slow; furthermore, the presence of E 'is conditional on the stimulus

Table 2. Different enzymes with hysteresis behavior. The table has information about the enzyme, transient behavior, organism, biological function and if there is the presence or absence of metal during the enzymatic catalysis and/or optimal pH.

Enzyme	Organism	Description	Transient behavior	pH and/or metal	Reference
Pyruvate kinase	*Bacillus licheniformis*	ATP obtention (metabolism)	Lag	Activation by Mg^{2+} (7.0 to 7.4)	(TUOMINEN; BERNLOHR, 1971)
Glutamine phosphoribosylpyro-phosphate amidotransferase	Pigeon liver	Purine biosynthesis (metabolism)	Lag	Activation by Mg^{2+} (8.0)	(ROWE; COLEMAN; WYNGAARDEN, 1970)
D-Lactate dehydrogenase	*Escherichia coli*	Fermentation – anaerobic metabolic pathway (metabolism)	Lag	(6.4 – 7.5)	(TARMY; KAPLAN, 1968)
Hexokinase B	*Saccharomyces cerevisiae*	Regulation of carbon catabolism (Metabolism)	Burst	(7.0 - 8.0)	(SHILL; NEET, 1971)
StEH1	*Solanum tuberosum*	Epoxide hydrolase	Burst	Activation by temperature change (8.0 to 9.0)	(LINDBERG; DE LA FUENTE REVENGA; WIDERSTEN, 2010)
BChE	Human	Butyrylcholinesterase (metabolism)	Burst	Activation by hydrostatic pressure, temperature, salts and pH	(MASSON; SCHOPFER; FROMENT; DEBOUZY *et al.*, 2005)
Nitrate reductase	*Cucurbita maxima*	Nitrate assimilation (metabolism)	Lag	Activation by phosphorylation state (7.5)	(LILLO; RUOFF, 1992)

Alkaline phosphatase (AP)	Calf intestine	metabolic process	?	(10.0 to 10.8)	(BEHZADI; HATLESKOG; RUOFF, 1999)
Trehalase	*Artemia salina*	Dormancy embryos	?	Intracellular pH dependent	(HAND; CARPENTER, 1986)
ProT (Protrombin)	Mamals	Blood coagulation	Lag	Activation by VWbp (Von Willebrand factor-binding protein, a glycoprotein) (7.0)	(KROH; PANIZZI; BOCK, 2009)
PFK (Phosphofructokinase)	Rat myocardium	Glycolysis (metabolism)	Lag	Alkalinization of the myocardium muscle	(HAND; CARPENTER, 1986)
ATP synthase	*Polytomella sp.*	Energy transduction in mitochondria (Oxidative phosphorylation metabolism)	Lag	Temperature (8.0)	(VILLAVICENCIO-QUEIJEIRO; PARDO; GONZÁLEZ-HALPHEN, 2015)
Oxidized fructose 1,6-bisphosphatase	Chloroplast	Glycolysis	Lag	fructose 2,6-bisphosphate (substrate analog), magnesium (7.5)	(SOULIE; RIVIERE; RICARD, 1988)
α-Acetyl-galactosaminidase	Bovine	Glycoside hydrolase	Lag	(4.7 - 5.0)	(WEISSMANN; WANG, 1971)
Homoserine dehydrogenase	*Escherichia coli*	Synthesis of L-homoserine	Burst	K^+ (6.9)	(BARBER; BRIGHT, 1968)

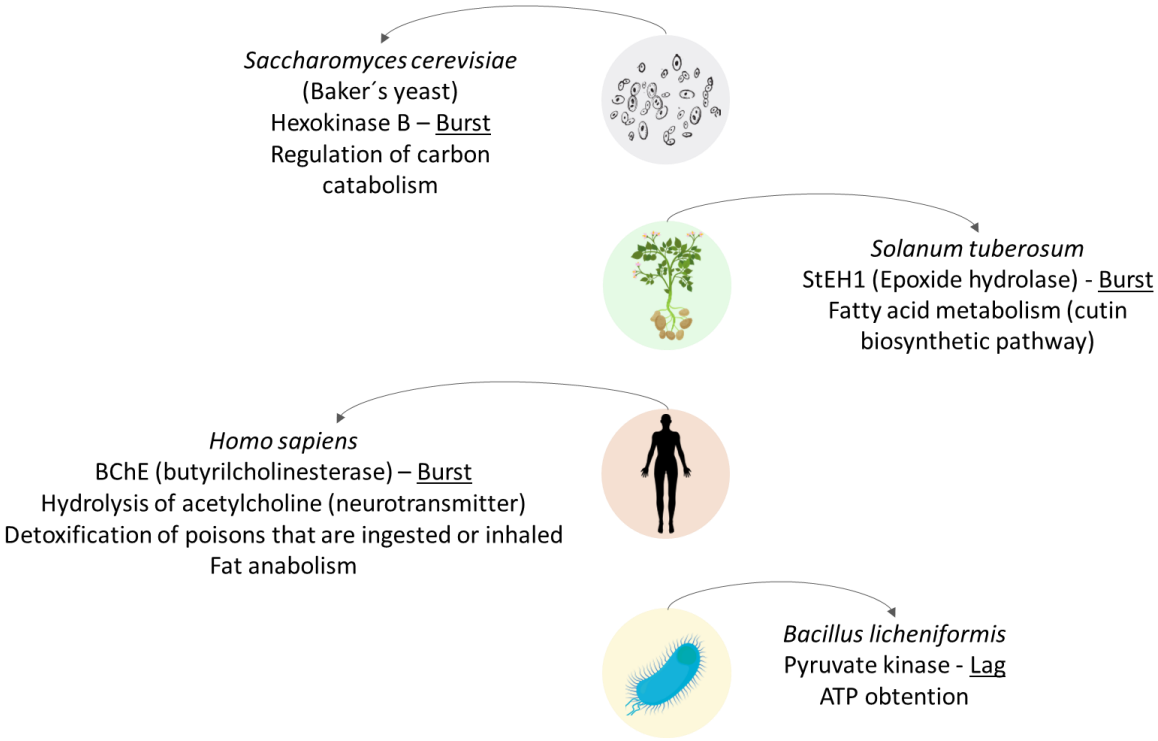

Figure 9. Examples enzymes with hysteretic behavior. Have been reported in different taxonomic groups, these enzymes participate in the regulation of different metabolic pathways in the metabolism of each organism**.**

1.6 Structural analysis of Ade1 (Amazonian Dark Earth esterase 1)

A novel lipolytic enzyme from a metagenomic library of Amazonian Dark Earth soils from the Amazonian Rainforest in Brazil, Ade1 (Amazonian Dark Earth Esterase 1), was characterized by CARVALHO (2015). Initially, the best four clones from the metagenomic library that showed triacyl-hydrolase activity were used to generate sub-libraries to identify the gene responsible for the expression of lipolytic enzymes. Using DNA sequencing with the "genome walking" technique, was identified an open reading frame (ORF) with a gene of around 804 pb, which codifies a protein of 268 residues (without a signal peptide) and theoretical molecular weight (MW) of 29.3 kDa. Ade1 was expressed, purified, and structurally characterized. Ade1 crystals diffracted at a resolution of 2.3 Å, the asymmetric unit contained four monomer units (**Figure 10 a** and **b**), and the special group was $P2_12_12_1$. The structural comparison of the Cα from structures of chains A, B, C, and D show a RMSD of 0.26 Å. This difference is located in the loop between α5 and α6 located at the beginning of the CAP domain of Ade1 (**Figure 10c**). It is an unstructured region in chain A (residues 138-140) and in chain C (residues 138 - 143).

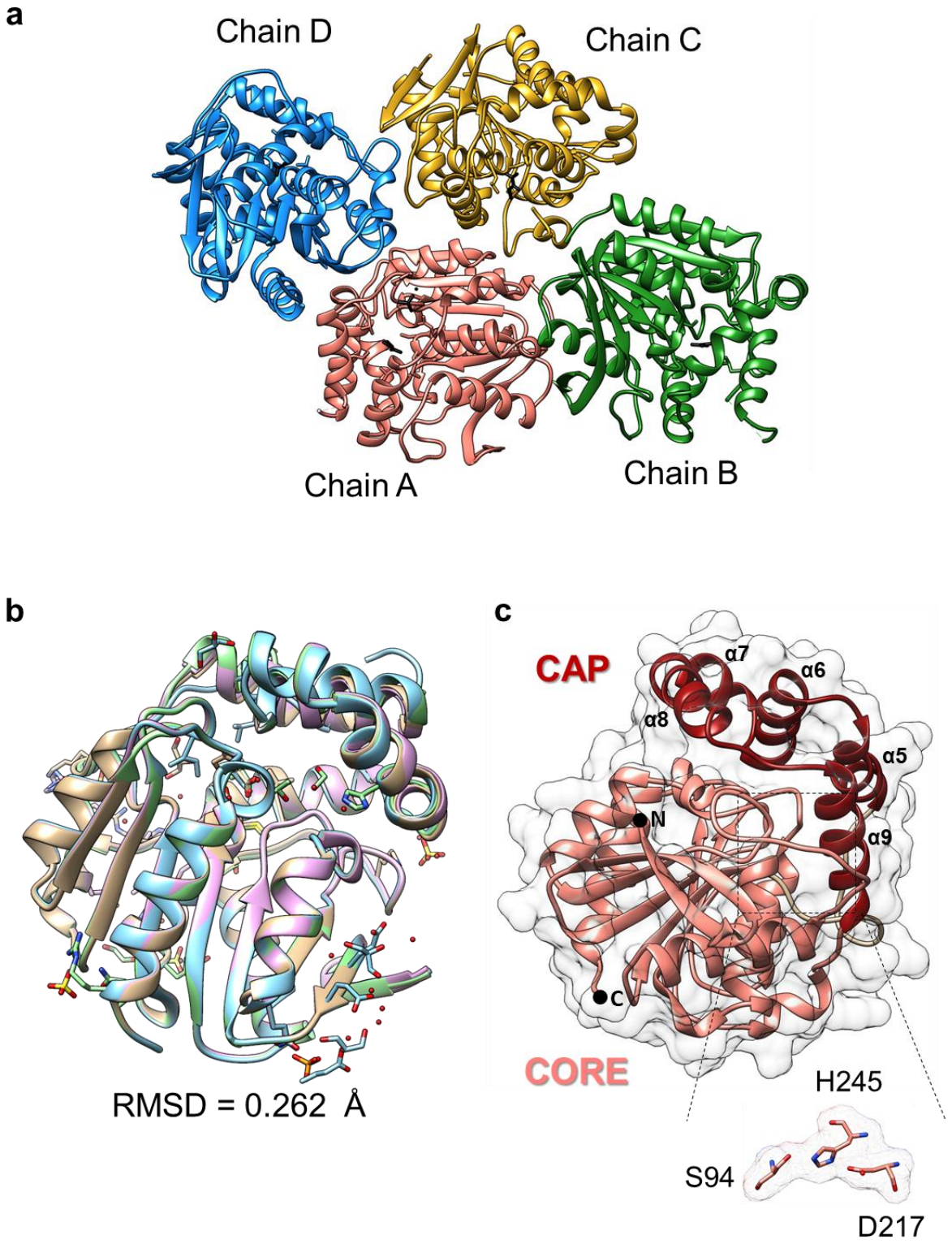

Figure 10. Ade1 structural characterization. (a) Show in ribbon four molecules contained in the asymmetric unit, chain A, B C and D, no interaction was observed between monomers. **(b)** Structural comparison of the C-alpha indicates that high similarity between the chains. **(c)** Ade1 monomer, results in dark red the Cap domain (α5 – α9), and in salmon the Core domain, highlight the catalytic triad (Ser94, His245 and D217) in the interface between these two domains, the black dots indicate the N- and C-terminal.

It was observed that Ade1 show a typical structure of an α/β-hydrolase (**Figure 10c**) with a conserved catalytic triad (Ser94, Asp217, and His245) in the core domain, while only Ser94 and His245 are exposed in the catalytic pocket. The Ser residue was found in the conserved pentapeptide motif G-X-**S**-X-G ("X" is any residue) and is important to start the catalytic cycle. Previous data classified Ade1 as a carboxylase, and two substrates were identified: tributyrin and *p*-nitrophenyl octanoate (*p*-NP C8). No influence of divalent metals ($CaCl_2$, $CoCl_2$, $MgCl_2$, $NiCl_2$) was observed in the enzymatic activity. However, when testing Zn, the activity decreased by 50%.

Two mutants were generated, S94A was used to abolish activity, and S94C was created to determine if the enzyme could acquire a cysteine hydrolase activity (dienelactone activity) (SCHLÖMANN; SCHMIDT; KNACKMUSS, 1990). None of the mutants presented any activity. Circular dichroism assays showed that in all constructions the structural conformation was preserved. Furthermore, it was observed that the Cys118 residue from the native polypeptide chain was at a distance of 2.88 Å from Cys94 in the Ade1 structure and was proposed that both could be interacting by forming a disulfide bond.

2 CONCLUSIONS

Based on our results we conclude the following:

- Ade1 is a monomeric esterase.
- Ade1 is an esterase with enzymatic affinity for esters with aliphatic groups with less than 12 carbons and more than 4, such as: tributyrin (aliphatic chain with 4 carbons), Tween 20 (aliphatic chain with 12 carbons), N-hexanoyl-L-homoserine lactone (aliphatic chain with 6 carbons), activity *p*-nitrophenyl butyrate and *p*-nitrophenyl octanoate showing a degree of promiscuity in its enzymatic.
- Ade1 shows quorum-quenching activity *in vivo* against *Chromobacterium violaceum*.
- Ade1 is possibly a moonlighting protein because it could perform more than one biological function.
- Cobalt enhances Ade1 enzymatic activity while other divalent cation doesn't affect the enzymatic activity.
- We show that Ade1 has hysteresis behavior due to a presence of two conformational states, E1 and E2. Molecular dynamics simulations revealed that the E1 state has an open cap conformation (inactive catalytic conformation) while the E2 state has a closed cap domain (active catalytic conformation).
- Dynamics simulation between $Ade1_{WT}$ and $Ade1_{S94C}$, shows that the cysteine mutant favors the E1 state resulting in abolishment the experimental catalytic activity by keeping the enzyme in the open cap state (inactive catalytic conformation).

In summary, esterase are enzymes widely used in the food industry, pharmaceutical industries, and agriculture. They may also be applied in the degradation of industrial pollutants, plastics, and other toxic chemicals. Furthermore, they are also used in the synthesis of optically pure compounds, antioxidants, and perfumes (PANDA; GOWRISHANKAR, 2005). Understanding of the molecular bases, catalytic and structural mechanisms of Ade1 may be applied to other esterases of biotechnological, food, and/or pharmaceutical interest. In addition, our study enlarges the knowledge of the molecular mechanism of monomeric esterase with hysteresis behavior. Moreover, our data also suggest that Ade1 belongs to a bacterial metabolic pathway of a proteobacteria phylum bacterium

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