
DISCOVERY, CHARACTERIZATION AND ENGINEERING OF ENZYMES INVOLVED IN POLYMER MODIFICATION FOR SUSTAINABLE BIOCATALYSIS AND BIOTRANSFORMATION

Carmen Ercolano

Dottorato in Biotecnologie – XXXVI ciclo

Università di Napoli Federico II



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Carmen Ercolano

Dottorando: Carmen Ercolano

Relatore: Prof. Marco Moracci

Co-tutor: Dr. Andrea Strazzulli

Coordinatore: Prof. Marco Moracci

Settore Scientifico Disciplinare

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*“Above all, don't fear difficult moments.
The best comes from them.”*

Rita Levi-Montalcini

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ABBREVIATIONS

Obscured due to 12-month embargo request

AA = Auxiliary Activity

Amp = Ampicillin

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BHET = Bis-2(hydroxyethyl) terephthalate

CE = Carbohydrate Esterases

Chf = Chloramphenicol

DMSO = Dimethyl Sulfoxide

EC number = Enzyme Commission number

EG = Ethylene Glycol

FACS = Fluorescence-Activated Cell Sorting

FCE = Free Cell Extract

GH = Glycoside Hydrolase

GT = Glycosyl Transferase

HMM = Hidden Markov Model

HPAEC-PAD = High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection

HPLC = High-Performance Liquid Chromatography

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IPTG = Isopropyl β -D-1-thiogalactopyranoside

LB = Lysogeny Broth

LCC = Leaf-branch Compost Cutinase

LPMO = Lytic polysaccharide monooxygenase

mDNA = Metagenomic DNA

MHET = Mono-(2-hydroxyethyl)terephthalic acid

MSA = Multiple Sequence Alignment

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PA = Polyamide

PE = polyethylene

PET = Poly(ethylene terephthalate)

PHE = PET Hydrolysing Enzyme

PL = Polysaccharide Lyase

PNA = Peptide Nucleic Acid

pNP = *para*-Nitrophenol

PP = polypropylene

PS = Polystyrene

PSP = Phenol red

PUR = Polyurethane ester-based

PVC = Polyvinylchloride

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Thc_Cut1 = Cutinase 1 from *Thermobifida cellulosilytica*

T_g = Glass Transition Temperature

TPA = Terephthalic acid

W/o droplets = Water/oil droplets

W/o/w droplets = Water/oil/water droplets

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X-substrates = 5-bromo-4-chloro-3-indolyl-substrates

RIASSUNTO

Gli uomini dominano il pianeta Terra, cambiandolo in modi che minacciano la loro sopravvivenza e quella di altri esseri viventi. La crescita della popolazione mondiale e il conseguente sfruttamento delle risorse, la trasformazione e la frammentazione degli habitat, la produzione e il consumo di energia e il cambiamento climatico sono meccanismi che causano una trasformazione dell'ecosistema su scala planetaria. Dati i rischi per la resilienza della Terra, l'ampia scala e la rapidità del fenomeno, ma soprattutto la plausibilità di un "punto di non ritorno", hanno portato a una maggiore consapevolezza da parte della popolazione e dei governi. Ciò ha innescato un aumento della richiesta di soluzioni di tipo biologico, su scala locale e globale.

Al fine di minimizzare la dipendenza da materie prime fossili, le industrie stanno cercando di introdurre alternative sempre più verdi e sostenibili, anche nell'ottica della realizzazione di una economia circolare. I concetti di bioeconomia ed economia circolare incoraggiano infatti uno sviluppo sostenibile, realizzando una sinergia tra società, economia e ambiente.

L'economia circolare ha vari target di interesse biotecnologico, tra cui i polimeri, sia naturali sia artificiali. Un insieme di biopolimeri dal grande potenziale è rappresentato dalla biomassa lignocellulosica, che può essere fonte di combustibili verdi come il bioetanolo, da utilizzare in alternativa a quelli fossili. A tal fine, la ricerca di enzimi che degradano la lignocellulosa capaci di resistere a condizioni industriali spinte è ancora oggi al centro dell'attenzione in ambito industriale, per sfruttare al meglio il potenziale di questa biomassa. Tra i polimeri artificiali, invece, il polietilene tereftalato (PET) ha richiamato su di sé una notevole attenzione negli ultimi anni. La sua refrattarietà alla degradazione naturale, così come l'assenza di metodi verdi efficaci per il suo riciclo o per la valorizzazione dei prodotti ottenuti, hanno infatti scatenato una crescente ricerca di biocatalizzatori utili a questi fini.

Scoperta di nuovi enzimi attivi sui carboidrati attraverso la metagenomica funzionale

La dipendenza dai combustibili fossili come fonte di energia ha provocato un aumento di anidride carbonica, uno dei gas che causano l'effetto serra, contribuendo ampiamente al

surriscaldamento globale. Questo problema ambientale, accompagnato dalla diminuzione della disponibilità di combustibili fossili, ha innescato la ricerca di una fonte alternativa di energia.

I biocarburanti da biomasse rinnovabili rappresentano una valida opzione, dal momento che riducono l'emissione globale di gas serra creando un circolo chiuso del carbonio. Una promettente fonte di biocarburanti (come il bioetanolo) è rappresentata dalla lignocellulosa, la più abbondante fonte di carboidrati in natura, sfruttata nelle bioraffinerie di seconda generazione. Inoltre, a differenza delle biomasse amidacee usate nelle bioraffinerie di prima generazione, la lignocellulosa non crea competizione con l'industria alimentare. Questa biomassa rappresenta quindi non solo una scelta virtualmente inesauribile, ma anche una alternativa più conveniente ed eticamente sostenibile.

Data la sua struttura complessa, la lignocellulosa è spesso recalcitrante all'idrolisi (saccarificazione). In particolare, la saccarificazione enzimatica rappresenta lo step limitante dell'intero bioprocesso nelle bioraffinerie di seconda generazione. Questo è principalmente dovuto ai loro costi, alla loro bassa efficienza catalitica e alla loro instabilità operativa in presenza di agenti denaturanti. A tal proposito, risulta necessaria la ricerca di nuovi ed efficienti *cocktails* enzimatici per l'idrolisi della biomassa lignocellulosica.

Grande attenzione è data agli enzimi da microorganismi (iper)termofili, in particolare agli enzimi attivi sui carboidrati (*Carbohydrate Active enZymes*, *CAZymes*) tra cui le glicosil idrolasi (*Glycoside Hydrolases*, *GHs*). Questi catalizzatori possono lavorare in condizioni in cui gli analoghi mesofilici denaturano rapidamente.

I microorganismi (iper)termofili, date le condizioni estreme in cui normalmente vivono, sono spesso difficili da isolare, coltivare e studiare in laboratorio. Per tale ragione, è necessario l'uso di tecniche non dipendenti dalla coltura. In questo frangente, la metagenomica funzionale rappresenta una valida alternativa per la ricerca di nuove GHs. Essa consiste nella costruzione e nello screening funzionale di librerie di vettori contenenti inserti di DNA metagenomico (mDNA).

Il DNA metagenomico utilizzato in questo studio è stato estratto da campioni raccolti in ambienti ipertermofili, specificamente da siti geotermali. L'obiettivo principale del progetto è stato sviluppare nuovi protocolli di analisi

metagenomica funzionale per lo screening di dataset metagenomici ipertermofili. A tal fine, sono stati utilizzati strumenti bioinformatici avanzati per l'annotazione di CAZymes, e di assegnazione tassonomica, al fine di selezionare in modo mirato i campioni più promettenti per la costruzione di genoteche fosmidiche. Queste genoteche sono state successivamente sottoposte a *screening* con l'intento di identificare geni codificanti per nuove GHs adatte a funzionare in condizioni estreme di temperatura. Per raggiungere questo scopo, i protocolli utilizzati, sia nei metodi convenzionali sia in quelli basati su tecnologie di microfluidica, sono stati appositamente adattati e ottimizzati per consentire l'identificazione di enzimi in condizioni ipertermofile. Sebbene l'esplorazione iniziale non abbia portato all'identificazione diretta di cloni positivi di GHs ipertermofile, questo lavoro ha permesso di sviluppare e raffinare tre nuovi protocolli di metagenomica funzionale aprendo nuove vie per la ricerca di enzimi capaci di operare efficacemente in contesti industriali che richiedono alte temperature.

Scoperta di enzimi attivi sul PET attraverso la metagenomica *in-silico* e ingegnerizzazione di enzimi già noti

L'uso massivo di plastica ha causato il suo accumulo nell'ambiente. Una delle plastiche sintetiche più utilizzate è il PET, un polimero ad alto peso molecolare composto da acido tereftalico (TPA) e glicole etilenico (EG) legati tramite legame estere. Le caratteristiche chimico-fisiche che rendono il PET un materiale vantaggioso, come versatilità e durezza, rappresentano allo stesso tempo il motivo per cui si accumula in natura, essendo recalcitrante alla degradazione naturale.

La degradazione biologica del PET consiste nell'idrolisi enzimatica, che converte la molecola nei suoi due monomeri ecocompatibili, TPA ed EG. Inoltre, poiché l'idrolisi del PET incrementa con il crescere della temperatura a causa dell'aumentata mobilità delle catene, la ricerca di nuovi enzimi che degradano il PET (*PET Hydrolysing Enzymes*, PHEs) ad alta temperatura è giustificata.

Date le problematiche già descritte, legate allo studio degli organismi (iper)termofili in laboratorio, sono necessari approcci indipendenti dalla coltura. La metagenomica basata sull'analisi computazionale del DNA metagenomico sequenziato con metodi massivi (*Next Generation Sequencing*), anche detta *in-silico*,

rappresenta una soluzione. Inoltre, per ottenere PHEs capaci di lavorare ad alte temperature, anche l'ingegnerizzazione enzimatica rappresenta una via utile. Adoperando la mutagenesi, sia random sia sito-diretta, è infatti possibile ottenere enzimi che degradano il PET che siano in grado di resistere ad alta temperatura.

Per quanto riguarda la scoperta di nuovi enzimi attraverso la metagenomica *in-silico*, le sequenze disponibili online di PHEs già caratterizzate hanno rappresentato il punto di partenza per la costruzione di un modello di Markov nascosto (*Hidden Markov Model*, HMM). Quest'ultimo è stato impiegato, in collaborazione con il gruppo del Prof. Wolfgang Streit dell'Università di Amburgo, curatore del *Plastics-Active Enzymes Database* (PAZy - <https://pazy.eu>), per la ricerca di sequenze interessanti di nuovi putativi PHEs in datasets metagenomici privati. Sono state così identificate due nuove putative PHEs, PP_EG e Sis, che sono state selezionate per l'espressione, purificazione e caratterizzazione. Sia Sis sia PP_EG hanno mostrato attività nei saggi cromogenici durante le analisi preliminari, ovvero su substrati esterei e su substrati plastici. Inoltre, l'enzima Sis ha mostrato attività su substrati plastici ed è stato caratterizzato in dettaglio.

Per quanto invece concerne l'ingegnerizzazione enzimatica, *Obscured due to 12-month embargo request*

SUMMARY

Humans dominate the Earth, changing it in ways that menace planet's resilience. For the plausibility of a “tipping point”, this situation enhances the demand for biological solving from the local to the global scale, in particular, concerning the discovery of biocatalysts. In the frame of a circular bioeconomy, the discovery of enzymes able to breakdown natural and artificial polymers is gaining much attention.

For the first objective of this PhD thesis, the scientific background concerns the dependence on fuels from fossil as energy source, contributing to global warming. Biofuels represent a valid alternative, and lignocellulose is a promising reservoir. As biocatalysts, great attention is given to (hyper)thermophilic glycoside hydrolases (GHs), which can operate under conditions in which the mesophilic ones quickly denature. To this aim, functional metagenomics can help find new GHs.

The project aimed to create new protocols for analysing (hyper)thermophilic metagenomic data, using advanced bioinformatics for enzyme annotation and sample selection for fosmid library construction. Despite not identifying hyperthermophilic GH clones initially, the project developed and refined three novel functional metagenomic protocols, enhancing the search for enzymes suitable for high-temperature industrial applications.

For the second objective, given the environmental accumulation of poly(ethylene terephthalate), the aim is to discover new PET Hydrolysing Enzymes (PHEs). Since the hydrolysis of PET increases with the rising temperature, screening for thermophilic enzymes may be helpful. *In-silico* metagenomics and protein engineering represent two approaches. For the enzyme discovery, two new PHEs, PP_EG and Sis, were identified, showing activity on ester and plastic substrates. Among them, Sis was biochemically characterized more in detail. For the engineering, *Obscured due to 12-month embargo request*

CHAPTER 1: INTRODUCTION

To date, humans dominate the Earth, changing it in ways that menace their and other animals' survival. The growth of the human population and the consequent resource exploitation, the transformation and the fragmentation of the habitat, the production and the consumption of energy and climate change are forcing mechanisms causing a planetary-scale transformation of the ecosystem of the planet [1], [2]. For its wide scale, its speed, and its menace to the resilience of the Earth, but mainly for the plausibility of a “tipping point”, this novel situation has led to an increasing awareness from both the population and the governments, enhancing the demand for biological solving from the local to the global scale [1].

In the industrial field, the request for biological solutions concerns new biocatalysts. Enzymes are interesting for their vast range of applications, also being biodegradable and non-toxic, originating from renewable sources [3]. Enzymes involved in polymer modification play a pivotal role across diverse fields, spanning from biofuel production to plastic degradation. [4], [5]. In particular, most enzymes for industrial applications are hydrolases able to catalyse polymeric substrates breakdown into simpler molecules [6].

1.1. Metagenomics for enzyme discovery

1.1.1. Functional metagenomics

Enzyme discovery through functional metagenomics depends on the construction and functional screening of libraries of suitable vectors containing metagenomic DNA (mDNA) inserts to identify enzymes of interest [7], [8], [9]. Briefly, the first step consists of extracting mDNA and constructing the libraries, sometimes containing hundreds of thousands of clones. The fragments dimensions usually range from 2 to 200 kbp, depending on the cloning vector [8]. Then, the libraries can be functionally screened, exploiting different kinds of substrates and methodologies [8], [10], [11], [12].

Generally, the functional screening exploits chromogenic or fluorescent substrates to detect the presence of the enzymatic

activity of interest. For example, in the search for catalysts able to degrade polysaccharides, the insoluble azurine-cross-linked-polysaccharides (AZCL-polysaccharides) represent a solution because the presence of *endo*-hydrolase activity is indicated by the release of soluble azure products as AZCL-oligosaccharides (**Fig. 1a**) [8]. Resorufin-glycosides represent another suitable substrate for the functional screening since, by the exploitation of this class of molecules, it is possible to observe hydrolysis by the release of fluorescent resorufin aglycone (**Fig. 1b**) [11], [13].

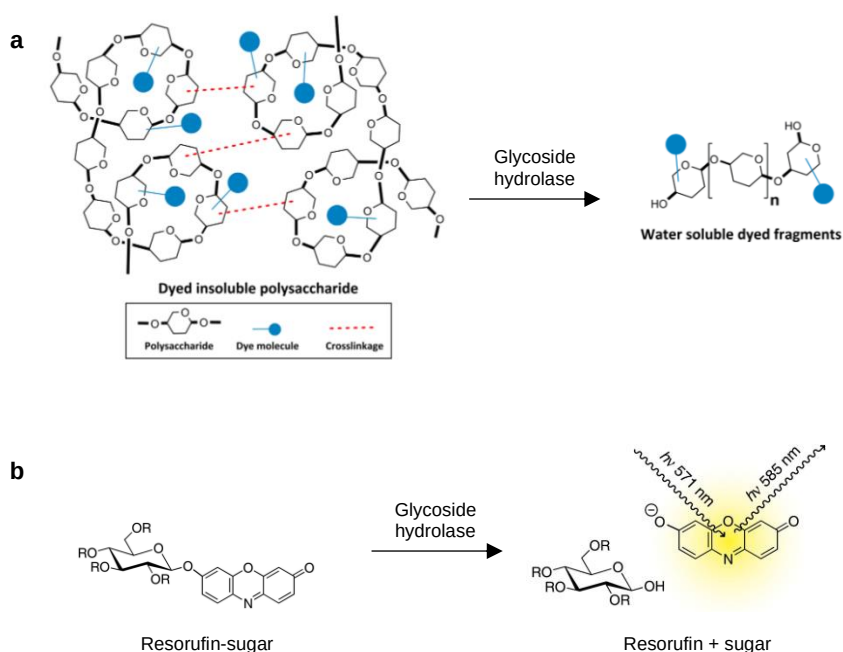


Figure 1. Schematic representation of the activity detection on (a) AZCL-substrates (adapted from <https://www.megazyme.com/shop-all-products/enzyme-substrates/insoluble-chromogenic-substrates>) and (b) resorufin-substrates (adapted from ref. [13]).

Concerning the methodologies of high-throughput screening for glycosidases, among the ones available, the most common ones exploit solid and liquid plates containing a cultivation medium and a chromogenic substrate. The most used substrates are represented by the AZCL-polysaccharides described above but also by Azo-polysaccharides and 5-bromo-4-chloro-3-indolyl-substrates (X-substrates) [8], [9], [10], [12]. Azo-polysaccharides are blue-coloured substrates, and the activity can be highlighted on a solid plate by forming a clear halo around the colony. X-

polysaccharides instead are non-coloured substrates, and the activity can be detected by the formation of a blue-coloured halo around the colony on a solid plate. Moreover, these substrates allow the differentiation between *endo*- and *exo*-acting enzymes during the screening since AZCL- and Azo-polysaccharides permit the identification of *endo*-activities, while X-substrates allow the detection of *exo*-activities [8]. As regards the screening methods, besides the conventional one described above, a valid alternative is represented by microfluidics-based high-throughput screening. More in detail, exploiting droplet-based systems, pipelines for the screening of new activities from mesophilic communities have been reported [11], [14]. This approach can exploit resorufin-substrates since the release of fluorescent groups can be detected downstream by Fluorescence-Activated Cell Sorting (FACS) [11]. Also, conventional and microfluidics-based methodologies can exploit minimal media supplemented with oligosaccharides as the sole carbon source, aiming to find clones that can degrade the target oligosaccharide, thus growing [8], [14].

Besides the discovery of glycosidases, functional screening has been extensively used in searching for esterase and lipase activities [15]. A generic pH indicator assay can be exploited to detect new activities by high-throughput screening. This method, in fact, represents a simple and generic way to detect activities targeting esters of interest [15], [16], [17], [18]. To this aim, the commonly used pH indicators are represented by *para*-nitrophenol (pNP), phenol red (PSP) and bromothymol blue [15]. For example, in the presence of activity, the expected colour change for bromothymol blue is from blue to yellow [15], [16], [17]. To identify activities of esterases and lipases, functional screening can utilize chromogenic substrates like α -naphthyl esters (for lipase/esterase activities), indoxyl esters (for lipase/esterase activities), tributyrin (specifically for lipase), and rhodamine (also for lipase). For instance, the successful activity detection on solid plates with α -naphthyl esters is indicated by a brown colour, whereas a blue colour signifies activity on indoxyl esters [18].

Once identified, the positive clones are usually processed in a secondary screening to confirm the activity, finally arriving at the sequencing step to identify the genes [8], [11]. For example, the

commonly used methods for the secondary processing of positive clones, which are putative glycosidases, are represented by another round of screening on AZCL-, Azo- or X-substrates, but also by *p*NP-mono/oligosaccharide and high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Regarding *p*NP-substrates, the yellow colour shows the release of *p*NP-OH as the leaving group in the reaction mixture. Regarding the HPAEC-PAD analysis, the activity validation involves chromatographically separating the reaction products and their identification using appropriate standards [8].

1.1.2. *In-silico* metagenomics

Besides the difficulties in constructing libraries (e.g. given by the low amount or the low quality of mDNA), a drawback in the functional metagenomics approach is represented by the considerable presence of false positives and false negatives [8], [11], [19], [20], [21]. Indeed, false positives often arise when non-specific activities or background noise are mistakenly identified as the target enzymatic activity [11], [19], [21], [22]. On the other hand, false negatives can occur when the actual enzymatic activity is present but goes undetected, possibly because the conditions used for the screening are not optimal for the enzyme's activity or the expression levels of the relevant genes are too low to be detected under the assay conditions [8], [11], [19], [22]. Moreover, given the difficulties in studying (hyper)thermophilic microorganisms and the aim to discover novel thermozymes, the screening temperature also represents a significant bottleneck [21].

In this framework, a complementary approach is given by sequence-based, or *in-silico*, metagenomics. The mDNA is extracted, sequenced, and assembled from environmental samples. This process allows for subsequent functional and taxonomic annotation without the biases associated with isolating novel microbial species, which often necessitate growth conditions that are challenging to replicate in the laboratory. In this way, it is possible to mine metagenomes, both from private and public datasets, searching for new enzymatic activities [23], [24] by exploiting suitable bioinformatic tools [25], [26]. One of the most efficient methods is the construction of Hidden Markov Models

(HMMs), statistical models built starting from the multiple sequence alignment of already reported enzymes with the activity of interest. Hence, Hidden Markov Models (HMMs) can be utilized to scan metagenomic datasets to discover new enzymes that are biotechnologically valuable [23], [24], [27].

1.2. Biotechnological applications of extremozymes

Even if some environments seem inhospitable for life (in terms of harsh conditions of temperature, pH, pressure, salinity, ionizing radiations, presence of heavy metals), these extreme habitats are populated by microorganisms from all three life domains: Eukarya, Archaea, and Bacteria [28]. The study of extremophilic microorganisms, known as extremophiles, has increased over time, given the biotechnological applicability of their enzymes (extremozymes) [28], [29], [30]. Extremophiles display ecological and biochemical adaptations that are atypical if compared to their mesophilic counterparts.

Defined the environmental parameters (e.g. pH, temperature, salinity), the extremophiles are classified in various categories, also with specifications of sub-categories to better arrange the organisms. Concerning the temperature, the extremophiles are classified as hyperthermophiles (optimal $T > 85$ °C), thermophiles (optimal T 45-85 °C) and psychrophiles (optimal growth under 15 °C) [28]. Even if the extremophiles are spread within the three domains of life, most belong to Archaea and Bacteria, and, in particular, hyperthermophiles are mainly Archaea [31], [32], [33].

The high stability of the enzymes from (hyper)thermophiles, namely thermozymes, can be exploited in a broad range of industrial applications, given the tolerance of these catalysts to temperature [28], [34]. Since they can catalyse reactions under conditions where the mesophilic counterparts are quickly denatured, the potential of thermozymes in biotechnological fields is therefore recognised [34]. These biocatalysts can be, in fact, used for cosmeceutical applications, waste management, food, and beverage, but also in biorefineries, e.g. for the pretreatment of lignocellulosic biomasses for biofuels production [3], [35], [36], [37], [38]. For example, concerning enzymes active on carbohydrates, xylanases can be exploited in pulp and paper bio-

bleaching since the process requires thermostable and alkalophilic enzymes [37]. Regarding the enzymes active on plastics, examples are represented by cutinases, which usually can degrade poly(ethylene terephthalate) (PET) at high temperatures [5], [39].

The commercialisation of enzymes started in 1873 with the production of pepsin preparations, aiming to treat digestive problems. This was possible thanks to Christian Hansen, an entrepreneur which opened the first enzyme factory, today known as the company Chr. Hansen [40]. The demand for enzymes has increased over time, given their advantageous properties such as selectivity and efficiency [36], [40]. For industrial enzymes, the global market was worth 2 billion dollars in 2004, 3.3 billion dollars in 2010 and 7 billion dollars in 2017, expecting to reach more than 10 billion dollars by 2024 [35], [36], [41], [42]. Since most commercial enzymes currently available have a mesophilic origin, the demand for thermozymes able to resist harsh industrial conditions is growing [36]. Thus, given the great potential of these biocatalysts in the biotechnological field, this represents another reason why their study keeps increasing over the years, aiming for a more complete exploitation for industrial applications [21], [36].

1.3. Carbohydrate-Active enZymes (CAZymes) and CAZy database

Carbohydrates can form many combinations in nature, given the different monosaccharides involved, the large variety of non-sugar substituents that can decorate the final chain, and the diverse regio- and stereospecificity of the glycoside bonds involved [43]. Complex carbohydrates diversity is regulated by enzymes that catalyse their assembly and their disruption [43], [44]. Collectively, these enzymes are indicated as Carbohydrate-Active enZymes (CAZymes), which are classified in families based on the sequence similarity for over 20 years [43], [44], [45]. The classification is available online in a daily updated database named CAZy (www.cazy.org) created in 1998, and it is possible to notice the division in enzymatic classes based on their activities, that are Glycoside Hydrolases (GHs), Glycosyl Transferases (GTs), Polysaccharide Lyases (PLs), Carbohydrate

Esterases (CEs) and Auxiliary Activities (AAs) [43], [44]. The classes are divided into families based on sequence similarity, and families can include enzymes with diverse substrate specificity. Moreover, when a high correlation between sequence and substrate specificity occurs, members of the same family can be sub-grouped into sub-families. Furthermore, families with the same 3D structure can be grouped into clans, indicating a common origin from an ancestral DNA sequence [43], [44]. The number of enzymes identified is continuously expanding [43], and to date (December 2023), the classification includes 187 GH families, 117 GT families, 43 PL families, 20 CE families and 17 AA families.

1.3.1. Glycoside Hydrolases (GHs)

GHs are enzymes that can act on complex sugars or glycosylated molecules, catalysing the hydrolysis of the glycosidic bonds that hold together these macromolecules. Given the central role of carbohydrates in the metabolism, GHs are widespread in all living organisms, freeing simple sugars processing larger carbohydrate molecules [46], [47].

GHs can be classified according to different criteria. First, they can be divided into *exo*- and *endo*-glycosidases based on the action mode on the long sugar chain. More in detail, *exo*-acting enzymes cleave the substrate at the end (most frequently the non-reducing end), and *endo*-acting enzymes cleave within the middle of the chain (**Fig. 2**) [48].

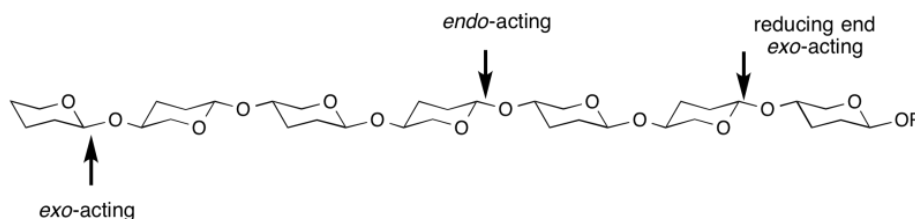


Figure 2. Schematic representation of *exo*- and *endo*-activities (https://www.cazypedia.org/index.php/Glycoside_hydrolases).

Furthermore, GHs can be divided into α - or β -glycosidases based on the stereo-specificity of the anomeric carbon involved in the glycosidic bond (**Fig. 3**) [49].

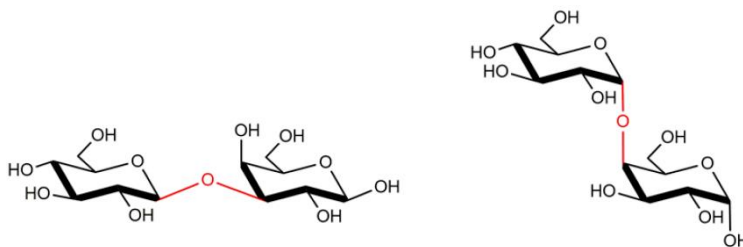


Figure 3. In red, examples of β -glycosidic bond on the left (in particular, β -1,3-glycosidic bond) and α -glycosidic bond on the right (in particular, α -1,4-glycosidic bond).

GHs can also be classified as *inverting* or *retaining*, based on the stereochemistry of the reaction product that is respectively inverted or maintained if compared to that of the substrate [49], [50]. More in detail, *inverting* GHs ($\alpha \rightarrow \beta$ and $\beta \rightarrow \alpha$) exploit a single-displacement mechanism that results in the inversion of the anomeric carbon in the product compared to the substrate. In this case, two carboxyl groups are in the active site, acting like an acid and a general base, respectively, with a typical distance of 7-11 Å. Since the acid protonates the glycosidic oxygen and the water is activated by the base, this situation leads to the inverted configuration of the anomeric carbon (**Fig. 4a**) [49]. Conversely, the *retaining* GHs ($\alpha \rightarrow \alpha$ and $\beta \rightarrow \beta$) follow a double-displacement mechanism, consisting of glycosylation and de-glycosylation steps with the formation of a covalent glycosyl-enzyme intermediate that is resolved to a product with a maintained anomeric configuration. Here, the two catalytic carboxyl groups are separated by a distance of about 5 Å, acting as a general acid/base and a nucleophile (**Fig. 4b**) [49]. Finally, concerning the classification of GHs, it is worth highlighting that some GH families do not follow the mechanisms described above [50]. Examples are represented by families GH4 and GH109, exploiting a mechanism that involves a NAD^+ molecule as cofactor [51].

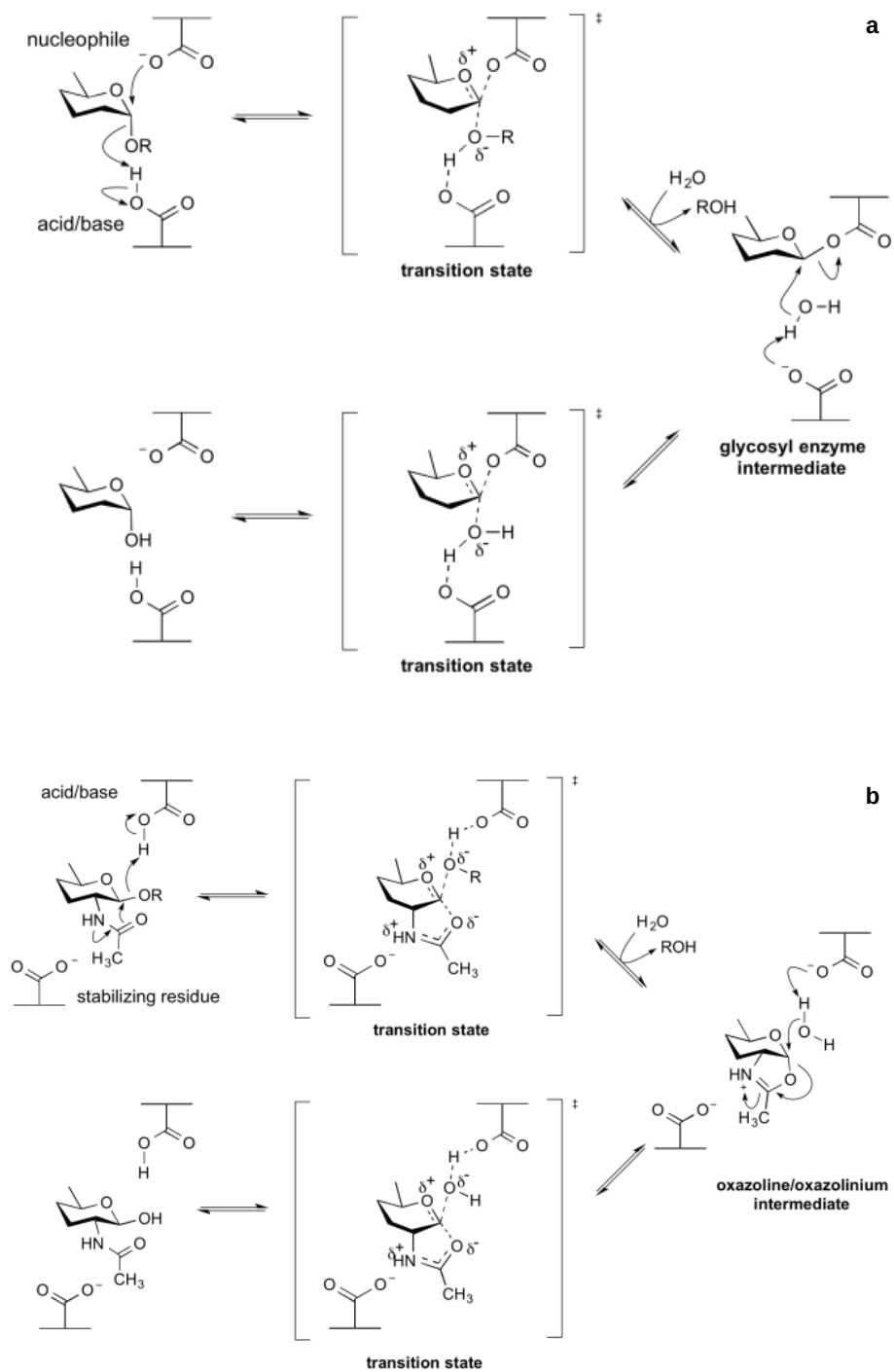


Figure 4. Mechanisms of reaction of (a) *inverting* GHs (here the mechanism of an *inverting* α -glycosidase is represented) and (b) *retaining* GHs (here the mechanism of a *retaining* β -glycosidase is represented) (https://www.cazypedia.org/index.php/Glycoside_hydrolases).

1.4. PET Hydrolysing Enzymes (PHEs) and PAZy database

Nowadays, plastic accumulation in nature represents a global challenge [52], [53]. These materials are hugely exploited in the economy, and around 350-400 million tons are produced every year at global scale. This prompted the request for biocatalysts, searching for enzymes able to degrade plastics without generating toxic substances, as in the case of chemical recycling processes [5], [52], [54], [55]. So far, only a limited selection of enzymes has been identified for the degradation of PET, polyurethane ester-based (PUR), and polyamide (PA). Moreover, no enzyme is known to degrade other hugely used fossil-fuels based plastics as polystyrene (PS), polyvinylchloride (PVC), and polypropylene (PP), and none has been characterized at the biochemical level for polymeric and crystalline polyethylene (PE) [54]. As CAZy for CAZymes, despite the considerable difference in the number of known enzymes given the lack of catalysts active on plastic polymers, thus requiring a massive work of discovery and annotation, Plastics-Active enZymes database (PAZy - www.pazy.eu) was developed in 2021 to collect the information about the enzymes described above. This database, in fact, collects activities, genes and protein data for verified enzymes acting on fossil fuel-based polymers and polymers from mainly renewable resources [54].

One of the most used synthetic plastics is PET, a high molecular weight polymer composed of ester bond-linked terephthalic acid (TPA) and ethylene glycol (EG) [5], [56]. PET's advantageous physical properties are excellent cheapness, versatility, and durability [5], [57]. Nevertheless, this plastic poses an environmental challenge due to its recalcitrance to natural degradation, thereby presenting a drawback for recycling and upcycling efforts. The leading techniques for plastic degradation consist of chemical and biological processes. However, chemical degradation processes require high temperature and pressure, consume much energy, and generate many toxic and polluting substances [5], [52], [53]. Conversely, biological processes of degradation are eco-friendly, and the degradation through PET Hydrolysing Enzymes (PHEs) could be a solution, converting the molecule into its building blocks [5], [52], [53], [58].

PET is a semicrystalline polymer consisting of an amorphous and a crystalline part. The latter strongly affects the enzymatic activity of PHEs since it is less accessible for the breakdown. This suggests that the enzymatic activity increases with the decreasing crystallinity [55], [59], [60]. Moreover, the enzymatic hydrolysis of PET increases with the rising temperature. This is possible because there is a higher probability for the surface chains to leave the polymer structure near its glass transition temperature (T_g around 75 °C). Thus, these chains could become targets fitting into the active site of PHEs [39]. In fact, as the temperature approaches the T_g , there is an increased chain mobility that results in a more rapid enzymatic hydrolysis. This underscores the critical importance of finding enzymes that are effective in these high-temperature conditions [2], [24], [52], [53]. Therefore, a significant number of these enzymes preferentially hydrolyse the amorphous regions of PET due to the less restricted chain mobility [39], [52], [55], [61].

1.4.1. PETases and MHETases

Among the PHEs, esterases named PET hydrolases (PETases) display the highest PET hydrolytic activity at ambient temperature. However, PETases are often heat-labile and an outstanding occurrence among the biochemically characterized PHEs is that they generally display high thermostability [2], [52]. In fact, concerning environmental remediation, in the past decade, several studies have identified enzymes from thermophilic organisms showing a detectable PET degrading activity [5], [62], [63]. For the biodegradation of PET, the pioneering researchers have mainly employed cutinases [64], [65], [66], enzymes that can degrade the plastic substrate up to 50 °C but are not able to perform efficiently the reaction at room temperature [5]. Therefore, PET degradation at this temperature represented a challenge, until Yoshida et al. in 2016 discovered a new bacterium, *Ideonella sakaiensis* 201-F6, able to grow on PET as the principal carbon source [58]. The degradation of PET is possible because the bacterium adheres to its surface, secreting a cutinase-like enzyme that can degrade it [52], [56], [57], [58]. The main products of this hydrolysis are mono-(2-hydroxyethyl)terephthalic acid (MHET), bis-2(hydroxyethyl) terephthalate (BHET), TPA, and EG. PETase

can further degrade BHET in MHET and EG (**Fig. 5a**). Meanwhile, a second enzyme of *I. sakaiensis*, the MHET-digesting enzyme (MHETase), is a tannase capable of degrading MHET to obtain TPA and EG (**Fig. 5b**).

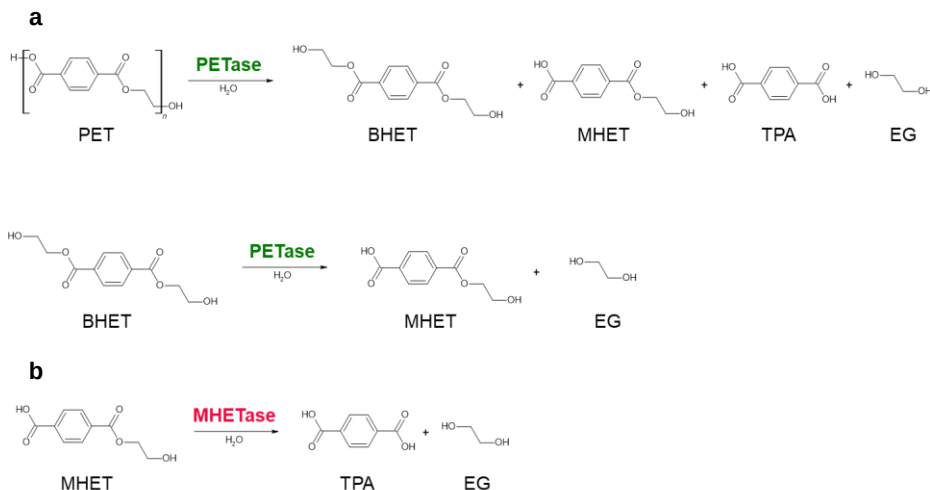


Figure 5. General reaction schemes of (a) PETases and (b) MHETases.

The monomers resulting from PET breakdown by PETase and MHETase are finally used for the metabolism of *I. sakaiensis* [52], [58], [67].

1.4.2. Engineering of PHEs

Newly discovered enzymes are often responsible for biochemical functions that can be interesting for industrial applications, but usually, they are not suitable for a complete biotechnological exploitation. For example, low resistance under harsh conditions or a low substrate specificity can hamper their use [2]. To overcome these hurdles, as well as to better understand the adaptation that can contribute to the improvement of the activity, protein engineering represents a solution [2], [55], [57].

Many efforts have been made to produce PETase variants, especially the one from *I. sakaiensis*, and the preferred host for the expression is *E. coli* [2], [57], [68], [69]. There is still the necessity of a robust and straightforward expression procedure concerning the large-scale production of these enzymes. To this aim, *Obscured due to 12-month embargo request*

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1.5. Enzymatic polymer modification for circular economy

In the 17th century, the invention of steam engines started the industrial revolution. At that time, energy and raw materials were considered unlimited, creating a linear economy with a flow summarised as: take - make - dispose [74]. This economic model generated 110 million tons of municipal solid waste per year in 1900, over 1 billion tons in 2000, and about 2 billion tons in 2016 [74], [75]. Due to the estimated scenarios concerning the increasing accumulation of waste in the future, a new economic model, called a recycling or reusing economy, was developed. The critical point of this model was to reuse a part of the materials and disposing of them at the end of their second life [74].

Given the amounts of raw materials used, the quantity of waste generated, and the risk of an ecological collapse, the linear and the recycling economy do not represent the ideal economic model for modern society [74], [76]. Thus, the circular economy concept was born to introduce a sustainable growth model, aiming to encourage a synergy between society, economy, and environment [76], [77]. This model emerged in the 1970s, prompting the adoption of sustainable strategies for industrial production and representing the key to sustainability [78], [79]. The goal is preserving natural resources, reducing or eliminating

waste, and using renewable resources in a biobased economy [79]. The global masterplan, in fact, considers the negative impact of the consumption of non-renewable resources on the environment and, thus, on the quality of life. Concerning the industrial field, the producers are lowering their dependence on unsustainable resources, applying the principles of a circular economy, and creating new employment [77].

1.5.1. GHs in the frame of circular economy

The human dependence on fossil fuels as energy source, led to a dramatic increase of the atmospheric concentration of CO₂, in a time span much reduced if compared to the formation of fossil carbon derivatives. Thus, the gases causing the greenhouse effect widely contributing to global warming, is a current problem extensively discussed nowadays. This environmental issue, accompanied by the diminishing supply of fossil fuels, prompted the search for an alternative energy source [80]. Biofuels represent a valid option since they reduce the global emission of greenhouse gases and they are renewable [80], [81], [82]. One of the most promising reservoirs of biofuels, as ethanol, is represented by lignocellulose, the most abundant carbohydrate source in nature [81], exploited in the second-generation biorefineries. The carbon present in plant lignocellulose results from its assimilation during photosynthesis, in which CO₂ is converted into organic compounds. Thus, the using of lignocellulosic biomasses as fuel sources allows a homogeneous emission of CO₂ and its re-assimilation during photosynthesis. This close circle of carbon, occurring in the same time frame, can lead to a decrease of greenhouse gases emission derived from fossil fuels. Furthermore, unlike the starchy biomasses currently used in first-generation biorefineries, lignocellulosic biomasses do not compete with the food industry, representing not only a choice of more straightforward procurement but also a more convenient and ethically sustainable alternative [78], [80], [81], [82].

Unfortunately, given its complex structure (typically composed of 40-80% cellulose, 15-30% hemicellulose and 10-25% lignin [83]) (**Fig. 7**), lignocellulose is generally recalcitrant to hydrolysis (saccharification) [82].

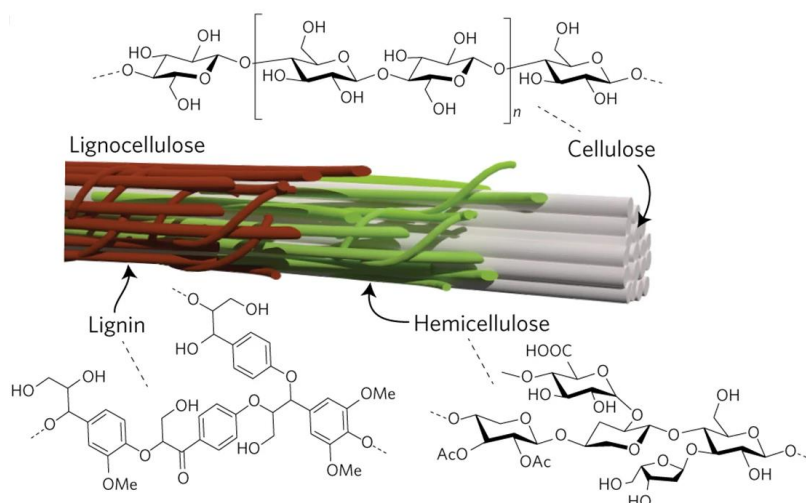


Figure 7. The structure of lignocellulose, existing as microfibrils in plant cell walls, consisting of cellulose bordered by hemicellulose and lignin [84].

The complete bioconversion of lignocellulosic biomasses involves the following basic steps: mechanical or chemical pre-treatment; saccharification by acid or enzymatic hydrolysis to obtain simple sugars (pentoses and hexoses); microbial fermentation of monosaccharides to obtain chemical building blocks; separation and concentration of the final bioproducts (**Fig. 8**) [4], [85]. Due to their cost, low catalytic efficiency and operational stability to denaturant agents, the enzymes for the saccharification represent the limiting step of the entire large-scale bioprocess in second-generation biorefineries. This issue makes it necessary to search for new efficient cocktails of enzymes for this hydrolysis [82].

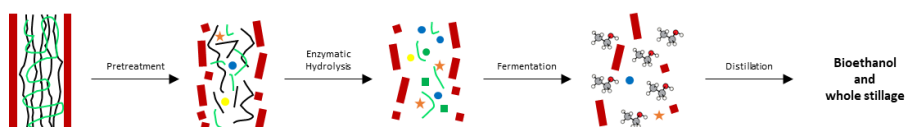


Figure 8. Bioprocess of conversion of lignocellulosic biomass into bioethanol and co-products.

To this aim, CAZymes play a significant role. Among them, lytic polysaccharide monooxygenases (LPMOs) can be found, being classified in CAZy database as AAs. LPMOs gained much attention in the last years, having an exceptional role in the polysaccharide degradation performing the oxidative cleavage of β -1,4-glycosidic bonds [86], [87], [88]. Furthermore, great

attention has been given to hydrolytic enzymes as the (hyper)thermophilic GHs and, in particular, to the (hemi)cellulases, able to degrade the (hemi)cellulosic fraction of lignocellulose. This interest is justified by their ability to operate under conditions where mesophilic GHs quickly denature [82]. Therefore, the research for new (hemi)cellulases as GHs from (hyper)thermophilic microorganisms, with new properties in terms of substrate specificity, stability to denaturant agents and improved conditions of reaction compared to those already known, plays an essential role in the field of biofuels [46], [48], [80], [81], [82].

1.5.2. PHEs in the frame of circular economy

In less than a century of manufacturing, plastics have become essential to modern society, determining its accumulation in nature with severe ecologically damaging effects [2], [5], [23], [54], [56], [89]. The production, in fact, is estimated to be almost 400 million tons/year, with applications in textile and electrical equipment. However, overall packaging (around 36% of the production) is the main constituent of plastic waste (around 47%) [55].

The problems concerning marine and terrestrial pollution and the negative impacts of microplastic exposure on human health have caused many worries [2]. The wasted plastic materials in the environments are exposed to oxygen and intense sunlight, causing plastics' partial deterioration and fragmentation, thus forming microplastics [62]. Both large debris of plastic and microplastics are thus disseminated worldwide in terrestrial and oceanic environments. The Great Pacific Garbage Patch, a collection of debris in the North Pacific Ocean, is probably the most famous instance of plastic accumulation. Furthermore, environmental accumulation of plastic negatively affects the health of humans and other animals [23], [89]. For instance, marine creatures such as fish often eat plastic debris, thus introducing plastics into the food chain [23]. Juvenile animals, for example, often get entangled in plastic fragments, which can lead to growth issues, both in terms of restriction of movements and difficulty in feeding and breathing [89].

Among plastics, PET is the most abundant since it is largely exploited for different applications such as beverage bottles and clothing [55]. PET waste is spread in the environment both during its production and after its use, and its accumulation keeps increasing over time [5], [54], [55]. Given its recalcitrance to natural degradation, concerning waste management, the most practised methods are currently disposal (landfill, open-air), incineration, recycling, and biodegradation.

Biodegradation, foreseeing the use of enzymes and microbes, is the most eco-friendly method to degrade PET. In the frame of circular bioeconomy, biodegradation, in fact, gives many advantages as relatively low energy input and no need for costly machines [55], [89]. The rising awareness about environmental issues thus prompts the search for novel bio-based strategies to mitigate the dependence on petroleum resources [55], [90]. Recycling of PET through enzymes has represented an attractive research topic for twenty years, and it is now gaining more attention [52], [55], [58].

PHEs are typically serine hydrolases with a Ser-His-Asp catalytic triad, mainly belonging to the superfamily of α/β hydrolase [91], [92]. Moreover, most of them are included in the carboxylic ester hydrolases class, with the Enzyme Commission (EC) number 3.1.1.-, as cutinases [90], lipases [93], and esterases [94]. Pioneeristic studies, in fact, involved cutinases (EC 3.1.1.74), a class composed of secreted catalysts able to degrade the cutin, an insoluble aliphatic polyester that is ejected from the cuticle of the plant [95]. This research allowed the identification of thermostable enzymes able to degrade PET [64], [92], [96]. Aiming to efficiently degrade PET, a high reaction temperature is, in fact, preferable, better if near to T_g [55], [97], [98]. Nevertheless, these catalysts could also be used to treat PET surface at lower temperatures but with slow responses [55]. Lipase activities have also been used for PET breakdown, but generally, they have low catalytic efficiency on this substrate [55], [99], [100]. Esterases, instead, usually display activity on esters with a short aliphatic chain, but only a few of them act on PET [55], [101]. More recently, given the discovery of PETase and MHETase from *I. sakaiensis* in 2016 [58], this research topic has gained more and more attention. Thus, from this perspective, the discovery and

engineering of PHEs appears to be necessary, given the lack of knowledge to scale-up this biodegradation technology [55]. Therefore, this represents an eco-friendly strategy within the frame of a circular bioeconomy. This approach is also a valid option from a biotechnological point of view since it includes biorecycling, bioremediation, and sustainable polymer modification. Since significant steps forward have been taken, even if there are still significant challenges to commercialise these technologies, a circular bioeconomy for PET still represents a global expectation [52], [55].

1.6. Discovery of (hyper)thermophilic enzymes

Enzymes are catalysts with a great potential to untie current industrial problems. Given the necessity to obtain concrete solutions starting from this potential, the demand for their discovery has been growing over the years [102]. With great processivity and specificity, enzymes catalyse chemical reactions increasing the quality and the yields of the products [6], [103], [104]. To this aim, it is possible both to identify novel enzymes and to engineer the already available ones [5], [82].

Historically, enzyme discovery has leaned on cultivating organisms and their screening. However, less than 1% of them can be accessible through cultivation-dependent approaches, neglecting the uncultivable majority [102], [105]. Moreover, (hyper)thermophilic microbes often are difficult to cultivate due to the harsh conditions in which they normally thrive [106], [107]. Currently, metagenomics represents a valid tool for studying these microorganisms to obtain access to the entire metagenome of the microbial communities populating extreme environments such as hot springs in geothermal areas. This approach allows obtaining information about the metabolic and enzymatic potential of the entire microbiota, including the uncultivable species [82], [106], [107].

Among the metagenomic approaches, a classical approach is given by sequence-based, or *in-silico*, metagenomics, where taxonomic annotation is performed by suitable bioinformatic tools. Instead, in a complementary approach, functional metagenomics represents a straightforward way to identify genes showing the desired functions, starting from genomic libraries built using the

extracted mDNA of mixed microbial populations from environmental metagenomes [14], [31].

1.7. Aims of the PhD thesis

Aiming to improve the current bioprocesses in the frame of a circular bioeconomy, the identification and engineering of enzymes useful for biotechnological purposes represent a valid option. The aim of this PhD thesis is thus to develop new functional metagenomic protocols for identifying GHs (glycoside hydrolases) from (hyper)thermophiles, as detailed in Chapter 2, and to discover new PHEs using a sequence-based metagenomic approach in Chapter 3. Specifically, Chapter 2 focuses on fine-tuning these new functional metagenomic methods for GH discovery, employing conventional and microfluidics-based screening techniques. Furthermore, since protein engineering represents another way to contribute to the exploitation of PHEs in biotechnological processes, another purpose is to improve the catalytic properties of already characterised catalysts, as reported in Chapter 3.

CHAPTER 2: DEVELOPMENT OF NEW PROTOCOLS FOR THE DISCOVERY OF GLYCOSIDE HYDROLASES THROUGH FUNCTIONAL METAGENOMICS FROM GEOTHERMAL SITES

2.1. Aim of the Chapter

The aim of this Chapter was the development of novel functional metagenomics protocols for the discovery of (hyper)thermophilic GHs. The aim was pursued by exploiting both conventional and droplet-based microfluidics approaches, using chromogenic and fluorescent substrates, as well as natural oligosaccharides, overcoming the limits of the existing protocols used for screening mesophilic hydrolases.

This research activity was performed in my laboratory in Naples and during my 6-months-long period abroad in Toulouse, France, at the National Institute of Applied Sciences of Toulouse, INSA - Toulouse Biotechnology Institute, TBI. In particular, the libraries were constructed in Naples and functionally screened in Toulouse. It was possible to perform the functional screening thanks to the platform PICT-ICEO "Engineering and Screening for Original Enzymes" (<https://www.toulouse-biotechnology-institute.fr/en/plateformes-plateaux/pict-iceo/>), certified ISO 9001:2016 and NFX 50-900. This is a platform led by Dr. Sophie Bozonnet, open to international academic and industrial scientific communities, providing services for high-throughput enzymatic screening, characterisation of the reaction products and purification and biophysical characterisation of proteins in analysis. Platform PICT-ICEO is a partner of the European/French infrastructure IBISBA "Industrial Biotechnology Innovation and Synthetic Biology Accelerator" (<https://www.ibisba.eu/>).

2.2. Results and Discussion

2.2.1. Bioinformatic analysis of metagenomic DNA

The first aim was to identify interesting mDNA candidates for the construction of libraries to be screened by exploiting the new protocols of functional metagenomics. The mDNAs were extracted from samples from geothermal sites (in collaboration with Prof. Donato Giovannelli's group, University of Naples "Federico II", Naples, Italy) and, provided as contigs, they were bioinformatically analysed. Among the available mDNAs, the investigation was done for nine of them due to the interesting extreme condition of the environments from which they come (metadata in **Tab. 1**).

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2.2.2. Construction of fosmid libraries from mDNA

Concerning the type of libraries, the choice fell on the fosmid ones, systems that exploit a DNA vector based on the bacterial plasmid F and that allowed the discovery of many GHs [11], [12], [118]. In detail, the fosmid library kit required at least 2.5 µg of mDNA concentrated at approximately 500 ng/µL. Furthermore, the kit prefers a dimension in the range of 30-40 kbp to avoid undesired chimeras since the vector can accommodate these large inserts. These restrictions thus limited the usage of all the mDNAs extracted (**Tab. 1**) since having a huge amount of high-weight DNA highly concentrated is uncommon [7]. Therefore, each mDNA sample was analysed by electrophoresis on an agarose gel to evaluate the dimensions of DNA fragments, avoiding the ones that appeared as a long smear. Among all the samples from Argentina, the one with the highest amount and concentration and the most adequate dimensions was sample *IN* with a concentration of 566 ng/µL. The sample *Pool1* from Pisciarelli showed instead a concentration of 829 ng/µL, and, also in this case, the dimension of DNA fragments adequate for constructing the library. Thus, starting from the mDNAs of these two samples, two fosmidic libraries have been constructed. The reactions resulted in two small libraries of about 300 clones for *IN* and 2,300 clones for *Pool1*.

The dimension of the libraries, cloning a metagenome, usually ranges from 10,000 to 100,000 clones [20]. However, the size of the libraries from environmental samples can be compromised by the presence of humic substances, interfering with the library construction. The DNA extraction from soil in fact always results in co-extraction of these substances, that are difficult to remove, compromising the DNA purity [20], [119], [120]. Given the small dimension in the number of clones in the *IN* library, the subsequent microfluidics-based screening analysis was performed only on the one derived from *Pool1*. Instead, conventional screening was performed for both libraries. It should be noted that the selected fosmid kit ceased production between

2021 and 2022, making it impossible to procure additional units. Moreover, no alternative product with comparable characteristics could be identified.

2.2.3. Development of a novel conventional screening protocol exploiting chromogenic substrates for (hyper)thermophilic GHs discovery

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2.2.4. Modification of droplet-based microfluidics screening for thermophilic GHs and FACS analysis

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2.3. Conclusion

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2.4. Materials and Methods

2.4.1. Metagenomic DNA extraction and bioinformatic analysis

The mDNA samples were purified from 10 g of sediments collected in each site. The extraction protocol consisted of modifying a protocol already reported [119]. The sediments were resuspended in a ratio 1:1 = w:v in the extraction buffer (100 mM TRIS-HCl pH 8.0, 100 mM sodium phosphate pH 8.0, 1.5 M NaCl, 1% CTAB, 100 mM EDTA pH 8.0). After the addition of 10 mg of proteinase K, samples were incubated at 37 °C for 30 min. SDS was added with a final concentration of 20%.

Samples were frozen/thawed three times (10 min in dry ice and 10 min in a thermal bath at 65 °C) and centrifugated at 6,000 × *g* for 10 min at room T, recovering the supernatant. Then, before wasting the derived pellets, this protocol was repeated three times: they were resuspended in 4.5 mL of extraction buffer, adding SDS with a final concentration of 20%, vortexed, put at 65 °C for 10 min, centrifugated at room T for 10 min at 6,000 × *g* and the derived supernatants were recovered.

A solution of chloroform:isoamyl alcohol (24:1) was added to the samples in a 1:1 (v:v) ratio that were centrifugated at 6,000 × *g* for 10 min at RT. The aqueous fractions (above) were recovered, adding then isopropanol in a ratio of 0.6:1.0 (isopropanol:sample) for the precipitation phase. After 1 h of incubation at room temperature, samples were centrifugated at 11,000 × *g* for 20 min at room T. The supernatants were discarded, and their residues were left evaporating. The obtained pellets were resuspended in 100 µL of sterile water and transferred in sterile 1.5 tubes.

To concentrate the DNA, samples were incubated in a concentrator (Eppendorf Concentrator 5301) to altogether remove water. Finally, the pellets were resuspended in 10 µL of sterile water. Each sample was analysed by electrophoresis on 1% agarose gel (10 cm length, 100 V, 30 min) to evaluate the DNA fragments dimension.

2.4.2. Fosmidic libraries construction

Starting from the mDNAs of the samples IN from Argentina at the concentration of 566 ng/ μ L and Pool1 from Pisciarelli Solfatara at the concentration of 829 ng/ μ L, two fosmid libraries in *E. coli* were constructed (CopyControl HTP Fosmid Library Production Kit with pCC2FOS Vector, Lucigen), following the instructions of the manufacturer.

Briefly, the high-molecular-weight DNAs were end-repaired, generating blunt-ended, 5'-phosphorilated DNA, and then they were ligated into the fosmid vector. Subsequently, packaging and titration of the CopyControl fosmid clones occurred. The last step was represented by plating and selection, spreading the library on LB medium + Chf 12.5 ng/ μ L. Libraries were stored both in multiwell-plates (for conventional screening) and tubes (for microfluidics-based screening) with 20% glycerol at -80 °C.

2.4.3. High-throughput primary and secondary screening on chromogenic substrates

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2.4.4. Water/oil and water/oil/water droplets production with fluorescent substrates or natural oligosaccharide

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2.4.5 FACS analysis and de-emulsification

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CHAPTER 3: *IN-SILICO* METAGENOMICS FOR THE DISCOVERY OF NEW PET HYDROLISING ENZYMES FROM METAGENOMIC DATASETS FROM GEOTHERMAL ENVIRONMENTS AND THEIR ENGINEERING

3.1. Aim of the Chapter

This Chapter aimed to identify novel (hyper)thermophilic PHEs through an *in-silico* metagenomics approach through the screening of private databases. Moreover, considering the necessity for more resilient enzymatic systems for biotechnological applications [69], another purpose was to exploit enzyme engineering.

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This objective is part of the REPLAY Project (Progetti di ricerca di Rilevante Interesse Nazionale, PRIN - funded under the call PRIN 2020). More in detail, this Italian national project involves three universities (University of Milano “Bicocca”, University of Bari “Aldo Moro” and University on Naples “Federico II”), aiming to contribute to the upcycling of the PET (<https://indbiotechlab.btbs.unimib.it/replay/>).

3.2. Results and Discussion

3.2.1. Identification and structural analysis of two new putative PHEs: Sis and PP_EG

To identify novel PHEs, collaboration was established with Prof. Wolfgang Streit's team from the University of Hamburg, Germany. Within this framework, exploration of private metagenomics datasets was conducted, utilizing a Hidden Markov

Model (HMM) developed with PHE sequences sourced from the PAZy database [54], as previously described [23], [24]. Based on the percentages of identity and on the 3D structure prediction (**Fig. 32**), Sis and PP_EG (**Tab. 7**) were selected from geothermal assembled metagenomic sequences provided by Prof. Donato Giovannelli (University of Naples “Federico II”, Naples, Italy).

Both the putative PHEs displayed an overall 45% identity percentage *versus* PETase from *I. sakaiensis* [58] (*IsPETase* in **Fig. 32**). For the structural analysis, the 3D models of Sis and PP_EG were predicted using AlphaFold2 [126]. The models highlight a higher presence of α -helix structures in both Sis and PP_EG (**W-Z** in **Fig. 32**): four regions (**W-Z** in **Fig. 32**) are present in Sis and three regions (**X-Z** in **Fig. 32**) are present in PP_EG.



Figure 32. AlphaFold2 models of Sis (on the left, in green) and PP_EG (on the right, in yellow) vs *IsPETase* 3D structure (purple, PDB: 6QGC). Note: the signal peptide regions were removed for all the models of the enzymes. (**W-Z**) α -helix regions present in Sis and PP_EG but not in *IsPETase*.

Internal ID	HMM score	NR reference	% Identity vs NR	Query coverage for HSP	Reference scientific names	% Identity vs PAZy	PAZy reference
Sis	279.3	MBP7342671.1	83.7	99.6	<i>Deltaproteobacteria bacterium Syntrophaceae bacterium</i>	56	<i>Thermobifida cellulosilytica</i> ADV92526.1
PP_EG	366.1	GBD22443.1	85	99.7	<i>bacterium HR29</i>	85	BhrPETase from <i>HR29 bacterium</i>

Table 7. Data collection for Sis and PP_EG.

Based on the aminoacidic sequence alignments, the sequences encoding for the leader peptide sequences were

removed obtaining Sis and PP_EG shortened of the residues 1-24 and residues 1-34, respectively (**Fig. 33**). The alignments also allowed the identification of the conserved residues of the catalytic triad, oxyanion hole, aromatic clamp, and disulfide bond. Furthermore, the common trait of α/β serine hydrolases, which is represented by a GX SXG motif, was identified (**Fig. 33**) [23], [24], [91], [127].

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Figure 33. Protein alignments *versus* IsPETase of Sis and PP_EG.

The new putative PHE genes were produced in outsourcing as synthetic genes in the pET21b(+) vector, allowing obtaining His-tagged proteins. Thus, the 267 aa sequence of Sis resulted in a 28.4 kDa molecular weight, and the 268 aa sequence of PP_EG gave a 28.95 kDa weight. IsPETase was considered as a control for this study (31.4 kDa molecular weight, including signal peptide). To ensure a standardised comparison, the wild-type IsPETase was specifically expressed in recombinant form and subsequently purified.

The proteins were produced in *E. coli* BL21(DE3) RIL strain (**Fig. 34**), identifying the best conditions of expression (0.6 A_{600 nm}, 1 mM IPTG with 18 °C/16 h induction for IsPETase and 37 °C/2 h induction for Sis and PP_EG). All enzymes were purified by Ni-

affinity chromatography (His-Trap) (**Fig. 35**). Preliminary assays on *p*NP-acetate on His-Trap fractions allowed to identify both for Sis and for PP_EG the elution peaks containing esterase activity (data not shown).

Molecular weight was determined for every step through SDS-PAGE electrophoresis, exploiting 10% polyacrylamide gel.

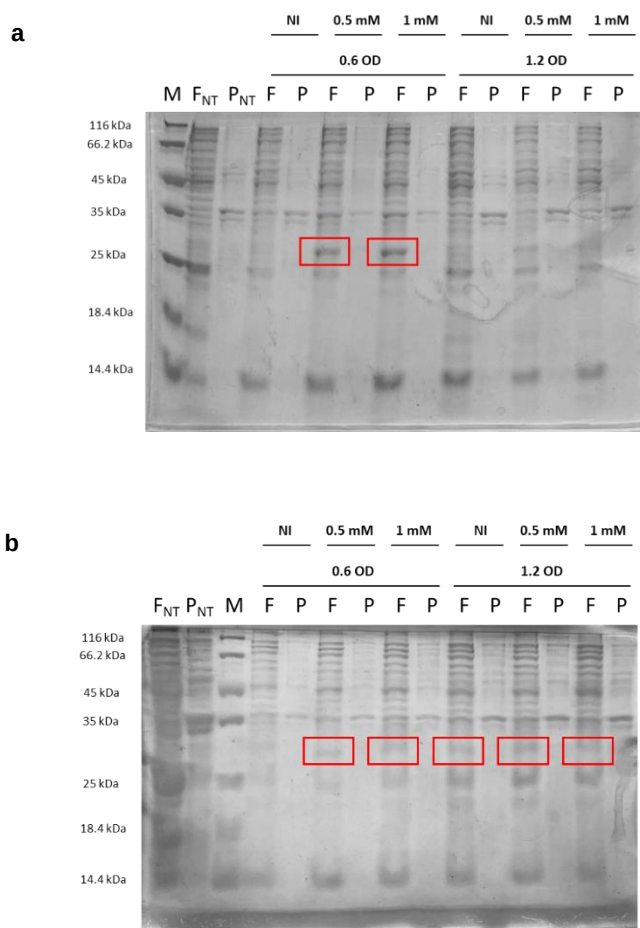


Figure 34. Expression in *E. coli* BL21(DE3) RIL for **(a)** Sis and **(b)** PP_EG in different conditions of IPTG concentration and at different $A_{600 \text{ nm}}$ of induction. Growth time after induction: 2 h. Temperature of growth after induction: 37 h. Red boxes highlight the detection of the proteins of interest, among which the best expression conditions were chosen. F = Free cell extract; P = pellet resulted from the free cell extract obtaining; F_{NT} = F not-transformed; P_{NT} = P not-transformed; M = Marker Thermo Scientific™ Pierce™ Unstained Protein MW Marker (Catalog number: 26610); NI = not-induced expression.

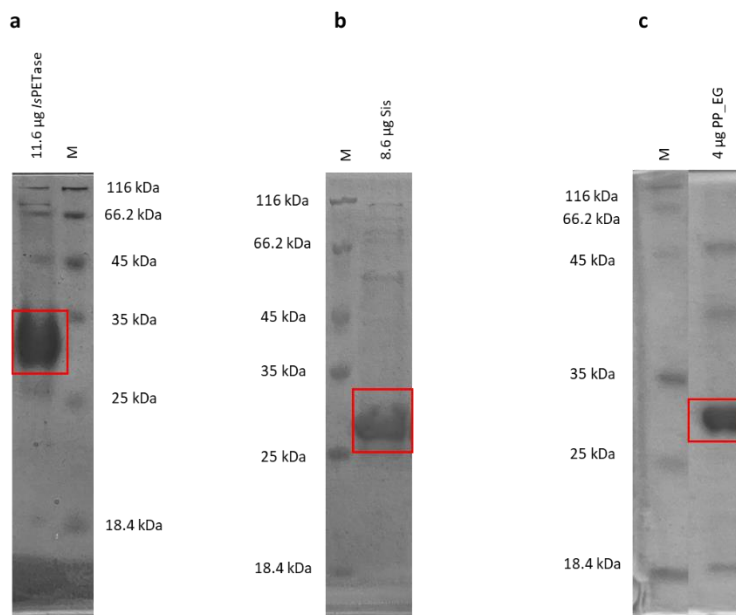


Figure 35. Purified (a) *IsPETase*, (b) *Sis* and (c) *PP_EG* after His-Trap chromatography and dialysis, highlighted by a red box. M = Marker Thermo Scientific™ Pierce™ Unstained Protein MW Marker (Catalog number: 26610).

Given the lower identity percentage of *Sis* against PAZy database reference over *PP_EG*, priority for the subsequent analyses was given to *Sis*.

To confirm the correct folding of *Sis* and to structurally characterize it, a far-UV circular dichroism (CD) analysis was performed in collaboration with Prof. Filomena Sica (University of Naples “Federico II”, Italy). The analysis showed a higher presence of α -helix structures in *Sis* than *IsPETase* (**Fig. 36**), as suggested by the 3D model (**Fig. 32**).

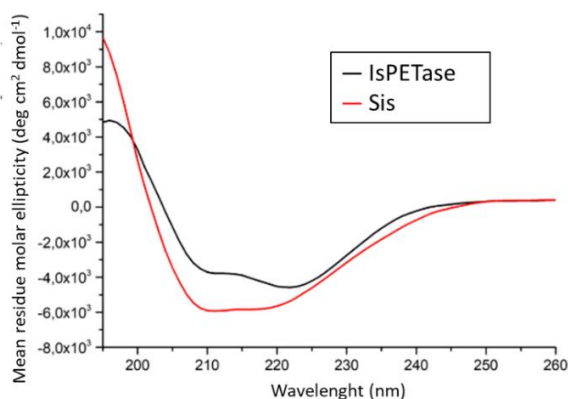


Figure 36. CD spectra of *IsPETase* (in black) and *Sis* (in red).

3.2.2. Biochemical characterization of Sis on *p*NP-butyrate

The initial biochemical characterisation of Sis was performed on *p*NP-butyrate, a commonly used *p*NP-aliphatic ester substrate mimicking the ester bond of PET [58]. The aim was to establish the optimum pH and temperature to conduct subsequent reactions on plastic substrates under the most favourable conditions. On *p*NP-butyrate, Sis revealed a broad pH range of activity with a pH_{opt} in the range 5.0-7.0 (**Fig. 37a**) and T_{opt} of 70 °C (**Fig. 37b**). Furthermore, the structural stability of Sis was assessed using Differential Scanning Fluorimetry, which revealed a melting temperature (T_m) of 82 °C. (**Fig. 38**). Since a rapid spontaneous degradation of the substrate with the rising temperature was observed, the study of the effect of temperature on Sis activity was conducted up to 70 °C (**Fig. 37b**). Sis was thus kinetically characterized on *p*NP-butyrate at pH_{opt} and T_{opt} , revealing that it efficiently hydrolysed the substrate with a k_{cat} value of $447 \pm 24 \text{ s}^{-1}$, a K_M of $0.08 \pm 0.01 \text{ mM}$ and a k_{cat}/K_M ratio of $5,587 \text{ s}^{-1} \cdot \text{mM}^{-1}$. Compared to *IsPETase* activity on the same substrate under its optimal conditions [128], Sis exhibited a specificity constant 1.3-fold greater than *IsPETase* ($4,298 \text{ s}^{-1} \cdot \text{mM}^{-1}$ and $5,587 \text{ s}^{-1} \cdot \text{mM}^{-1}$ for *IsPETase* and Sis, respectively). When compared to its PAZy reference (**Tab. 7**), the thermophilic Cutinase 1 from *Thermobifida cellulosilytica* (Thc_Cut1), on the same substrate under optimal conditions, Sis displays a specificity constant that is 13.1-fold greater ($425 \text{ s}^{-1} \cdot \text{mM}^{-1}$ and $5,587 \text{ s}^{-1} \cdot \text{mM}^{-1}$ for Thc_Cut1 and Sis, respectively) [129]. Moreover, if compared to the k_{cat}/K_M ratio of another thermophilic PETase, that is the Leaf-branch Compost Cutinase (LCC), on the same substrate under optimal conditions, Sis shows a specificity constant that is 3.4-fold bigger ($1630 \text{ s}^{-1} \cdot \text{mM}^{-1}$ and $5,587 \text{ s}^{-1} \cdot \text{mM}^{-1}$ for LCC and Sis, respectively) [130].

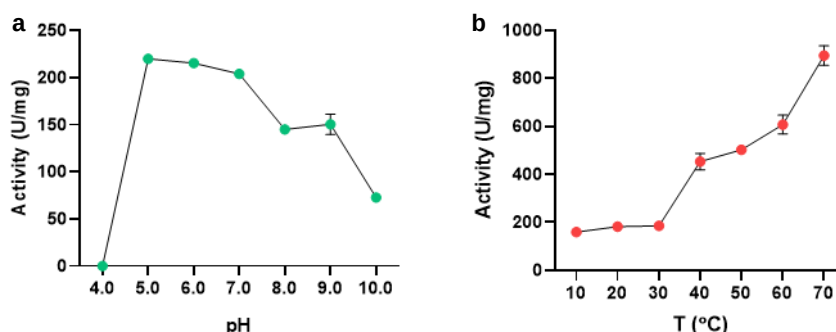


Figure 37. Determination of optimal (a) pH and (b) temperature on *p*NP-butyrate for Sis. All the assays were performed in triplicate.

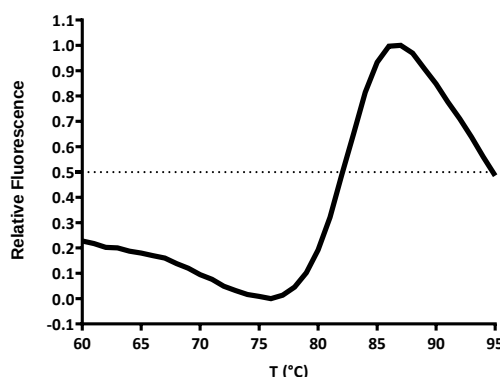


Figure 38. DSF analysis revealed a T_m of 82 °C for Sis.

Concerning the determination of functional thermostability, Sis was assayed in optimal conditions (0.1 M sodium-phosphate buffer pH 7.0, 70 °C) on *p*NP-butyrate after incubation at two different temperatures (50 °C and 70 °C) up to 96 h (**Fig. 39**). The enzyme lost almost 40% of its activity after only 30 min of incubation, which is in contrast with the DSF experiment where Sis showed a T_m of 82 °C once incubated with a thermal gradient of +1 °C/min, in the range from 20 °C to 95 °C. Possibly, the lowered amount of NaCl that was present in the incubation buffer used for the DSF stabilized the enzyme (**Fig. 38**). Nevertheless, Sis kept around 50% of its activity after 24 h of incubation under both temperatures tested. After 4 days, Sis kept around 30% of its activity when incubated at 50 °C, instead losing around 80% of activity at 70 °C (**Fig. 39**).

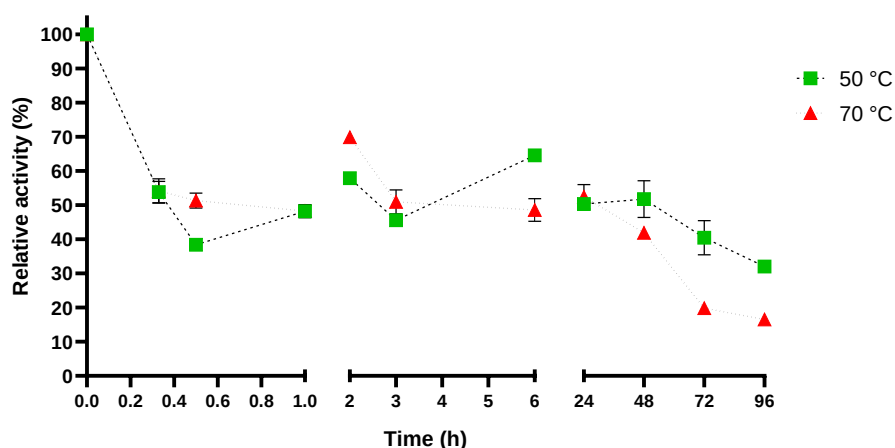


Figure 39. Functional thermostability of Sis at 50 °C and 70 °C on *p*NP-butyrate under optimal conditions. 100% of activity is equal to $1,199.87 \pm 11.74$ U/mg.

In addition, the hydrolytic activity of Sis on *p*NP-butyrate was evaluated, in quadruplicate, at 30 °C in the presence of two different concentrations of organic solvents or detergents (1% and 10%) (**Fig. 40**). Sis displayed a remarkable tolerance to a significant part of the additives tested. The presence of 1% ethanol increased the enzymatic activity by 50%. Conversely, Triton X-100 lowered the enzymatic activity by more than 50% at both concentrations of detergent.

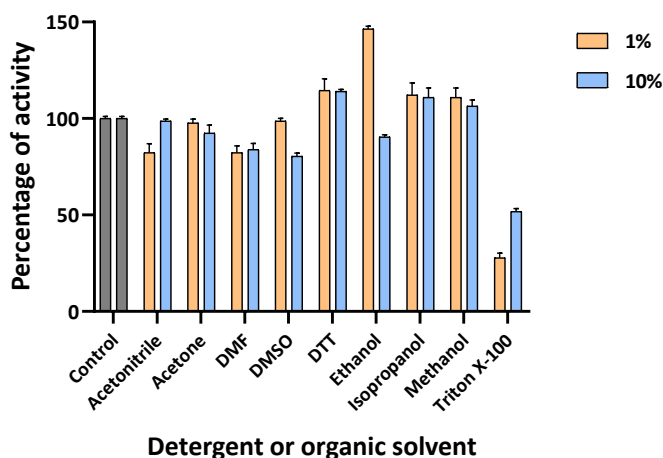


Figure 40. Effect of different detergents and organic solvents on Sis' activity on *p*NP-butyrate at 30 °C, pH 7.0. All the assays were performed in quadruplicate compared to a control without adding detergents and organic solvents. The standard error ($n = 4$) represents the percentual error.

3.2.3. Assessment of the PETase activity of Sis and PP_EG

Preliminary tests for Sis and PP_EG on plastic substrates were conducted using BHET and PET nanoparticles (nanoPET, diameter < 0.22 μm) as substrates. Through a preliminary PSP-based assay [16], [17] at 30 °C and 50 °C, activity on nanoPET and BHET was detected for both Sis and PP_EG. In particular, Sis and PP_EG exhibited higher activity at 50 °C on both substrates in comparison to *IsPETase* (**Fig. 41**).

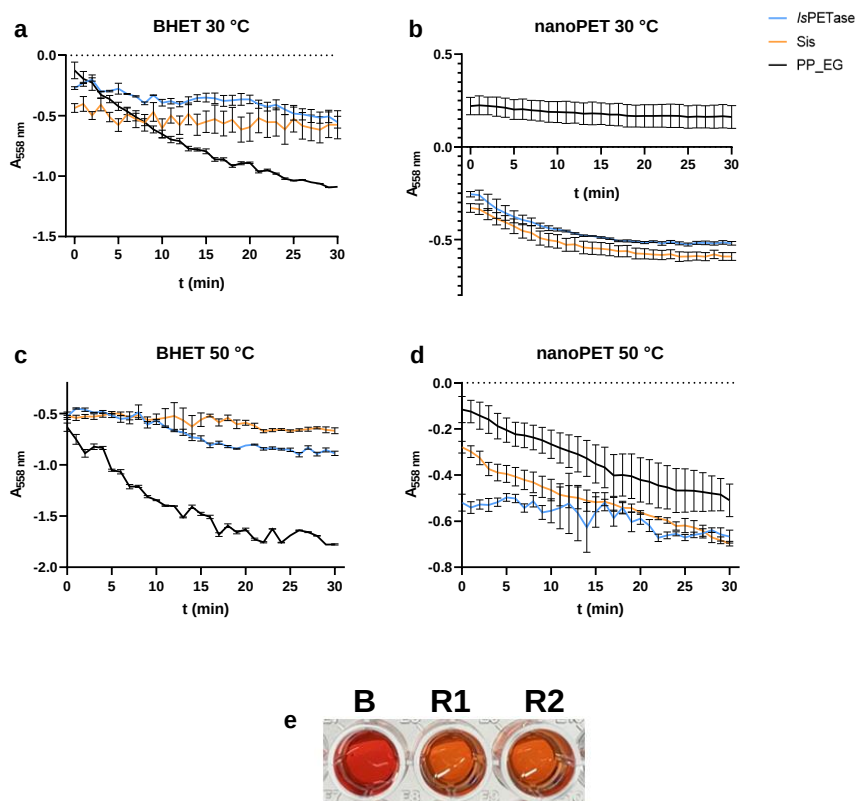


Figure 41. PSP of Sis, PP_EG and *IsPETase* on BHET and nanoPET. Highlighted in the graphs: decrease of absorbance signal at 558 nm in 30 min (a) on BHET at 30 °C, (b) on nanoPET at 30 °C, (c) on BHET at 50 °C and (d) on nanoPET at 50 °C. (e) Example of visive change of reaction colour produced by Sis on nanoPET after the 30 min incubation at 50 °C, performed in duplicate (R1 and R2). A shift from red to orange is observed if comparing B (Blank with no enzyme), R1 and R2.

Based on the previous observations, further assays of Sis on BHET and nanoPET substrates were carried out in triplicate at pH 7.0 at 30 °C, 50 °C, and 70 °C and analysed by HPLC. The same analysis was performed on *IsPETase* (**Fig. 42 and 43**) as a

comparison. Even if the analysis on BHET revealed a 5-fold higher release of MHET by *IsPETase* over *Sis* at 30 °C, it also showed a higher MHET production by *Sis* over *IsPETase* at 50 °C and 70 °C (**Fig. 42**). In these cases, the decrease in MHET concentration can be attributed to a combined effect of the enzymes' reduced thermal resistance (as evident with *IsPETase* at 70 °C after 40 min, in **Fig. 42**) and the spontaneous degradation of BHET at high temperatures.

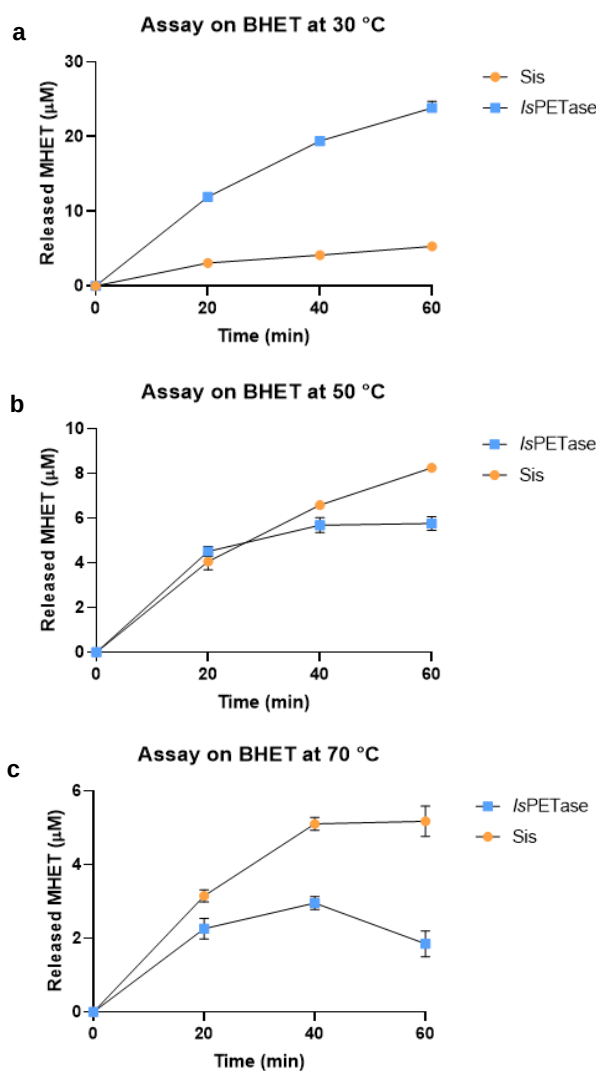
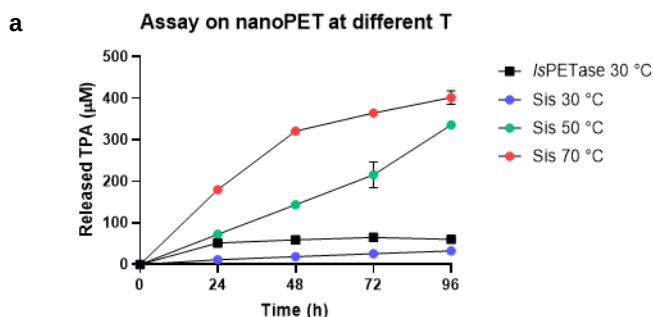


Figure 42. HPLC analysis of released MHET from the reaction of BHET of *IsPETase* and *Sis* at (a) 30 °C, (b) 50 °C and (c) 70 °C.

The release of TPA, MHET, and BHET by Sis and *IsPETase* assayed on nanoPET was also measured by HPLC (**Fig. 43**). The TPA concentration over time revealed that Sis, already after 24 h, produces 3.5- and 1.5-fold more product, at 70 °C and 50 °C respectively, than *IsPETase* incubated at its optimal conditions. After 96 h of incubation, the difference in terms of TPA concentration further increases 6.5-fold at 70 °C and 5.5-fold at 50 °C (**Fig. 43a**). After 48 h of incubation, we observed a decrease of BHET and MHET concentrations during the time with the rising temperature is visible in all the conditions tested (**Fig. 43b and 43c**), particularly for MHET at 70 °C (**Fig. 43b**). The decrease of BHET concentration can be explained both by the spontaneous degradation with the rising temperature and by the enzyme activity, considering that BHET is itself a substrate of PETases (**Fig. 43c**). Instead, the decrease of MHET can be ascribed to the loss of stability of the product with the rising temperature (**Fig. 43b**). Nevertheless, the increase of the total released products after 96 h at 50 °C, if compared to the concentration obtained at 70 °C, cannot be explained only by the spontaneous degradation of MHET and BHET (**Fig. 43d**). The absence of a rise in the concentration of total released products at 30 °C and 70 °C may be due to the thermophilicity and the biochemical thermal stability of the enzyme (**Fig. 37b** and **Fig. 39**, respectively). These observations validate Sis' PETase activity and its thermophilic nature, demonstrating its ability to hydrolyse nanoPET at 50 °C and to maintain its activity for up to 4 days.



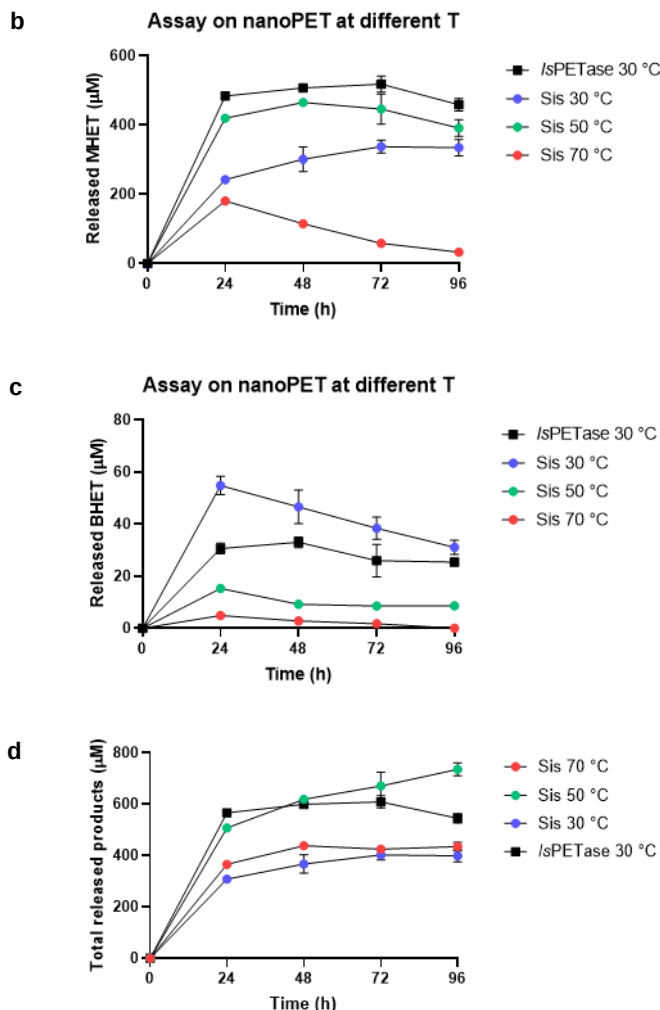


Figure 43. HPLC analysis of released (a) TPA, (b) MHET, (c) BHET and (d) total released products from the reaction of BHET of /sPETase at 30 °C and for Sis at 30 °C, 50 °C and 70 °C.

In light of these results, Sis clearly represents a thermophilic and thermostable PHE, thus being a good candidate for PET degradation. Thus, the discovery of the two new PHEs, Sis and PP_EG, aims to contribute to a better degradation of plastics through protein engineering and the construction of stronger HMMs for the enzyme discovery.

3.2.4. Expression on *Escherichia coli* outer membrane

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3.2.5. Preliminary activity assays of whole cells on *p*NP-esters

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3.3. Conclusion

The discovery of PHEs is essential for waste plastic bioconversion, as a green approach in the frame of a circular economy [52], [55]. In this frame, this chapter described a valid strategy to identify new PHEs through an *in-silico* metagenomics approach. The identification of two novel PHEs, and, in particular, the deep characterisation of one of them, could represent a step forward to constructing more reliable Hidden Markov Models for enzyme discovery.

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3.4. Materials and Methods

3.4.1. Identification and structural prediction of new putative PET Hydrolysing Enzymes

The in-silico identification of novel PHEs was performed as previously reported in the literature [24]. ChimeraX v. 1.4 AlphaFold tool was used to predict the 3D structure [126]. PyMOL v. 2.4.1 was used to detect the structural features and to visualise the superposition of 3D models.

3.4.2. Construction of expression vectors

The gene coding for the new putative PHEs Sis and PP_EG were codon-optimised for the expression in *E. coli*, exploiting the GenSmart™ Codon Optimization online tool by GenScript (<https://www.genscript.com/gensmart-free-gene-codon-optimization.html>, GenScript Biotech Corporation, New Jersey, US). The DNA sequence corresponding to the signal peptide was removed. The obtained DNA for Sis and PP_EG was synthesised in pET21b(+) plasmid by GenScript. Conversely, the gene coding for *IsPETase*, containing the nucleotide sequence corresponding to the signal peptide, was obtained by AddGene (Massachusetts, USA) in pET21b(+) plasmid.

3.4.3. Heterologous expression and purification

The constructs were used to transform *E. coli* BL21(DE3)-Codon Plus RIL strain. To find the best expression conditions for Sis and PP_EG, cells were cultured at 37 °C in LB medium supplemented with 100 ng/μL ampicillin and 30 ng/μL chloramphenicol. Reached $A_{600\text{ nm}} = 0.6$ or 1.2, the induction of protein expression was performed by adding 0.5 or 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), incubating the flasks for 2 h or overnight at 18 °C or 37 °C. Non-induced and non-transformed were also assayed. Concerning *IsPETase*, cells were cultured at 37 °C in LB medium supplemented with 100 ng/μL ampicillin and 30 ng/μL chloramphenicol until $A_{600\text{ nm}}$ reached a value of 0.6, inducing the expression with 1 mM IPTG overnight at 18 °C as already reported [58]. Cells were withdrawn by centrifugation ($5,000 \times g$, 20 min, 4 °C) and resuspended in lysis buffer (150 mM NaCl, 20 mM NaH₂PO₄ pH 7.3, Triton X-100 1%) in a ratio w:v = 1:4. Then, 25 mg/L lysozyme and 5 mg/L DNase I were added, followed by a freeze/thaw lysis. The FCEs were clarified by centrifugation ($17,000 \times g$, 20 min, 4 °C) and filtered through a 0.45 μm PVDF membrane. The lysates were applied to a 1 mL His-Trap HP column exploiting an ÄKTApure chromatography system (Cytiva Life Sciences). The protein elution was performed by exploiting a discontinuous gradient of an imidazole-containing buffer (150 mM NaCl, 50 mM NaH₂PO₄ pH 7.3, 500 mM imidazole). 20 μL of every fraction derived from His-Trap purification was assayed on *p*NP-acetate in a 1 mL final volume in 45 mM Na₂HPO₄ at pH 7.0, 90 mM NaCl and 10% (v/v)

DMSO [58]. The fractions containing the enzyme of interest were pooled, followed by dialysis (cutoff 12 kDa) overnight against 150 mM NaCl, 20 mM NaH₂PO₄ pH 7.3. The concentrations of the purified proteins were determined by Bradford assay, using Bio-Rad Protein Assay Dye Reagent Concentrate by Bio-Rad (California, USA).

3.4.4. Circular Dichroism analysis

Circular dichroism (CD) spectra were recorded with a Jasco J-715 spectropolarimeter equipped with a Peltier temperature controller. Far-UV measurements were carried out at a protein concentration of 0.1 mg·ml⁻¹ in 10 mM NaH₂PO₄ and 50 mM NaCl pH 7.3 at 10 °C, using a cell with an optical path length of 0.1 cm. Spectra, registered with 50 nm·min⁻¹ scanning speed, 2 s response time, 1 nm data pitch, and 2.0 nm bandwidth, were obtained averaging three scans. Data were expressed as mean residue molar ellipticity in deg·cm²·dmol⁻¹.

3.4.5. Differential Scanning Fluorimetry

Differential Scanning Fluorimetry (DSF) analysis aimed to evaluate the thermal stability of Sis by determining the T_m value. A 0.3 mg/mL concentration of purified enzyme was mixed with 0.2 M NaH₂PO₄ pH 8.5 and with 5X SyproTM Orange Protein Gel Stain by Invitrogen (Thermo Fisher Scientific, Massachusetts, USA) to a final volume of 50 µL. The mix was prepared in triplicate and loaded into a transparent sealed PCR 96-wells plate in the StepOnePlus Real-Time PCR System by Applied Biosystems (Thermo Fisher Scientific, Massachusetts, USA). Samples were incubated at 20 °C for 10 min, then a melting scan from 20 °C to 95° C at 1 °C/min was recorded, with a final incubation at 95 °C for 10 min as previously reported [24]. Protein unfolding was monitored by detecting changes in SYPROTM Orange fluorescence, extracting thermal stability parameters by non-linear curve fitting.

3.4.6. Preliminary assays and biochemical characterisation on pNP-esters

Preliminary analyses on pNP-esters were conducted to determine optimums of pH and T for Sis. In detail, the enzyme

(0.05 μM) was incubated at 30 °C with 1 mM *p*NP-C4 (*p*NP-butyrate, dissolved in isopropanol) in 100 mM buffer citrate (pH 4.0 – 5.0), phosphate (pH 6.0 – 8.0) or borate (pH 9.0 – 10.0). The release of *p*NP-OH was monitored continuously at 405 nm in triplicate, exploiting a BioTek Synergy HTX plate reader (Agilent Technologies, California, USA).

To evaluate the thermophilicity of Sis, the enzyme (0.01 μM) was incubated with 1 mM *p*NP-C4 in 100 mM NaH_2PO_4 buffer pH 7.0 at different temperatures, ranging from 10 °C to 70 °C. The absorbance change was recorded in triplicate with a Cary 3500 Multicell UV-Vis Spectrophotometer equipped with a Peltier temperature controller (Agilent Technologies, California, USA).

The effects of different detergents and organic solvents were evaluated by testing Sis (0.05 μM) at pH 7.0 and at 30 °C on 1 mM *p*NP-butyrate in quadruplicate. For each detergent/solvent, the final concentration of 1% and 10% in the reaction mixture was tested.

To assess the thermal stability of Sis, the enzyme (0.05 μM) was incubated in 100 mM NaH_2PO_4 at pH 7.0, at 50 °C and 70 °C for up to 4 days. The residual activity was measured in triplicate after 20 min, 30 min, 1 h, 2 h, 3 h, 6 h and then every 24 hours, up to 96 h, on 1 mM *p*NP-C4 under the optimal reaction conditions.

3.4.7. NanoPET production

NanoPET was prepared as previously described [16], [17], [131]. Briefly, PET microplastics (maximum particle size 300 μm , crystallinity 40%, natural colour) by Goodfellow (Bad Nauheim, Germany) were dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol, with a ratio of 1 g of PET every 100 mL of water-soluble solvent. The solution was put drop by drop, under stirring, in a 10-fold larger volume of ddH_2O . Since the boiling point for hexafluoro-2-propanol ($T = 58.2$ °C) is lower than that of H_2O , the solvent was removed by heating the mixture during the stirring. Removed the bigger particles by filtration (cutoff 0.22 μm), the concentration was determined by weighing the nanoPET after the water evaporation exploiting Concentrator 5301 by Eppendorf.

3.4.8. Preliminary enzymatic degradation of nanoPET through PSP assay

Preliminary degradation analyses of nanoPET were performed using the colorimetric assay based on PSP dye [16], [17], [18]. Briefly, the $A_{558\text{ nm}}$ signal was detected continuously for 30 min at 30 °C and 50 °C exploiting a BioTek Synergy HTX plate reader (Agilent Technologies, California, USA). The enzyme (final concentration 0.5 μM) was added to 1 mM BHET (TCI, Tokyo Chemical Industry) or 0.2 mg/mL nanoPET and 0.2 mM PSP, up to a final volume of 200 μL by adding 1 mM NaH_2PO_4 buffer at pH 8.0. All the assays were performed in duplicate, compared to an enzyme-free control.

3.4.9. HPLC analysis of enzymatic degradation of BHET and nanoPET

Sis and IsPETase were assayed by using 0.05 μM of enzyme on 150 μM BHET up to 1 h, and on 50 $\mu\text{g/mL}$ nanoPET by using 0.01 μM of enzyme up to 5 days, at 30 °C, 50 °C and 70 °C in 0.1 M sodium-phosphate buffer pH 7.0. For the time-course, 50 μL of reaction were withdrawn and put on ice in a 150 μL final volume containing 15% acetonitrile and 0.1% trifluoroacetic acid (TFA). After centrifugation ($15,000 \times g$, 5 min, 4 °C), the supernatant was injected in a 20 μL loop and analysed by HPLC (LC-4000 Series System by Jasco) equipped with a C18 column Reversed-phase, 4.6 x 250 mm, 5 μM particle size (Hamilton Company, Nevada, USA). The $A_{240\text{ nm}}$ signal was monitored during the isocratic elution by using a solution 15:85 = acetonitrile: ddH₂O (ratio 15:85), acidified with 0.1% TPA. BHET, MHET, and TPA were used as standards to evaluate the retention time and quantify the products obtained. Every assay was performed in triplicate.

3.4.10. Heterologous expression for the exposure on *E. coli* outer membrane

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3.4.11. Assays of whole cells on *p*NP-esters

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CHAPTER 4: CLOSING REMARKS

The planetary-scale transformation of the planet triggered the enhancing request for biological remediation on a local and global scale. Concerning the industrial field, the demand for new biocatalysts as enzymes has increased in recent years, given their specificity and processivity. Among the available ones, the focus is on thermozymes, able to resist harsh temperatures over their mesophilic counterparts. To this aim, enzyme discovery and engineering represent valid strategies to contribute to the realisation of a circular bioeconomy.

This thesis work aims to expand the knowledge about the industrial applications of enzymes that can degrade (hemi)cellulose and PET. To this aim, Chapter 2 unveils the possibility of using high-throughput screening, particularly microfluidics-based, to screen for thermophilic CAZymes. Moreover, the discovery of novel PHEs in Chapter 3 contributes to the knowledge of this class of catalysts and the growth of PAZy database entries. These results also reinforce the bioinformatic models, which are helpful to screen for new activities. Finally, Chapter 3 also laid the foundations for a novel methodology to screen for novel PETase and MHETase activities and obtain an improved degradation of PET.

5. APPENDIX

5.1. Publications

Manuscript in preparation

Title: "Discovery and Characterization of Sis: A Novel Thermophilic PETase Identified through Sequence-Based Metagenomics".

5.2. Communication

- 10th International Congress on Biocatalysis
University of Technology (TUHH) - Am Schwarzenberg-Campus, 1, 21073, Hamburg, Germany.
Presenting author (poster session and lightning talk), **awarded with a travel grant**. *Discovery of novel thermophilic PET hydrolyzing enzymes by sequence-based metagenomics*. **Carmen Ercolano**, Andrea Strazzulli, Roberta Iacono and Marco Moracci.
- XVI FISV Congress (Federazione Italiana Scienze della Vita)
Reggia di Portici - Via Università, 100, 80055, Portici, Naples, Italy
 - Contributing author (poster session and short oral presentation). *Characterization and engineering of thermophilic α -glucosidase MalA from the hyperthermophilic crenarchaeota Saccharolobus solfataricus*. Francesca Maria Pia Paragliola, **Carmen Ercolano**, Andrea Strazzulli, Roberta Iacono and Marco.
 - Presenting author (poster session and short oral presentation). *Functional metagenomics approach for the identification of new (hyper)thermophilic carbohydrate active enzymes of biotechnological interest*. **Carmen Ercolano**, Andrea Strazzulli, Roberta Iacono, Donato Giovannelli and Marco Moracci.
- Meeting Sezione SIB Campania 2022

Complesso di Biotecnologie dell'Università degli Studi di Napoli Federico II - Via Tommaso De Amicis, 95, 80131, Naples, Italy

Contributing author (poster session). *Engineering of thermophilic α -glucosidase MalA from the hyperthermophilic crenarchaeota Saccharolobus solfataricus to change the substrate specificity*. Francesca Maria Pia Paragliola, **Carmen Ercolano**, Andrea Strazzulli, Roberta Iacono and Marco Moracci.

- CIMEs Team Seminar (Catalysis and Enzyme Molecular Engineering Research Team)

La Grange de Holle, Route de Boucharo, 65120, Gavarnie, France.

Presenting author (shared talk). *EAD1's advances in droplet-microfluidics based screening of enzymatic and microbial functions*. Sophie Lajus, Simon Ladèveze, **Carmen Ercolano** and Jean-Luc Parrou.

- 62° SIB Congress 2023 (Italian Society of Biochemistry and Molecular Biology)

Polo Didattico Morgagni, Università di Firenze - Viale Giovanni Battista Morgagni, 40, 50134, Florence, Italy. Presenting author (poster session). *Engineering PET-hydrolysing enzymes for display on E. coli outer membrane: a promising approach for plastic waste management*. **Carmen Ercolano**, Andrea Strazzulli, Roberta Iacono, Rosa Merlo, Rosanna Culurciello, Elio Pizzo, Giuseppe Perugino and Marco Moracci.

- 4th IBBR Memorial Workshop Maria Ciaramella

Institute of Biosciences and Bioresources of CNR - Via Pietro Castellino, 111, 80131, Naples, Italy.

Discovery, characterization and engineering of PET-hydrolysing enzymes for sustainable plastic degradation. **Carmen Ercolano**, Andrea Strazzulli, Roberta Iacono, Rosa Merlo, Golo Feuerriegel, Rosanna Culurciello, Donato Giovannelli, Elio Pizzo, Giuseppe Perugino, Wolfgang R. Streit and Marco Moracci.

5.3. Period spent abroad

From October 4th, 2022, to march 31st, 2023, under the supervision of Dr. Gabrielle Potocki-Veronese, at Institut National des Sciences Appliquées de Toulouse, INSA - Toulouse Biotechnology Institute, TBI - Avenue de Rangueil, 135, 31077, Toulouse CEDEX 4, France.

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