Enzymes to unravel bioproducts architecture

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Research review paper

Enzymes to unravel bioproducts architecture

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ABSTRACT

Enzymes are essential and ubiquitous biocatalysts involved in various metabolic pathways and used in many industrial processes. Here, we reframe enzymes not just as biocatalysts transforming bioproducts but also as sensitive probes for exploring the structure and composition of complex bioproducts, like meat tissue, dairy products and plant materials, in both food and non-food bioprocesses. This review details the global strategy and presents the most recent investigations to prepare and use enzymes as relevant probes, with a focus on glycoside-hydrolases involved in plant deconstruction and proteases and lipases involved in food digestion. First, to expand the enzyme repertoire to fit bioproduct complexity, novel enzymes are mined from biodiversity and can be artificially engineered. Enzymes are further characterized by exploring sequence/structure/dynamics/function relationships together with the environmental factors influencing enzyme interactions with their substrates. Then, the most advanced experimental and theoretical approaches developed for exploring bioproducts at various scales (from nanometer to millimeter) using active and inactive enzymes as probes are illustrated. Overall, combining multimodal and multiscale approaches brings a better understanding of native-form or transformed bioproduct architecture and composition, and paves the way to mainstream the use of enzymes as probes.

1. Introduction

Enzymes, which can be naturally produced by microorganisms (bacteria, fungi, and yeasts), are widely used in the agrifood, chemical and pharmaceutical industries to catalyse specific steps in a range of biotechnology processes (Vogel and May, 2019). Enzyme catalytic properties are advantageously used to make paper and cardboard from plant lignocellulose, prepare fermented food products (wine, cheese, enzyme-modified milk, etc.), refine or structure oils and derivatives, produce detergents, and more. Industrial demand for process-adapted enzymes has evolved hugely for technical, environmental and economic reasons. In addition, the enzyme market has grown steadily in the last decade ("Industrial enzymes market analysis by product (carbohydrase, lipases, proteases, polymerases & nucleases and others), by application (textile, feed additive and food processing), by end-use (food & beverage, detergents, animal feed, textile, paper & pulp, nutraceutical,

Abbreviations: AA, auxiliary activity enzyme; AFM, atomic force microscopy; CAZyme, carbohydrate-active enzyme; CBM, carbohydrate-binding module; CE, carbohydrate esterase; CPD, computational protein design; EP, enzyme probe; FA, fatty acid; FCS, fluorescence correlation spectroscopy; FRAP, fluorescence recovery after photobleaching; FRET, fluorescence resonance energy transfer; FT-IR, Fourier-transform infrared spectroscopy; GH, glycoside hydrolase; LPMO, lytic polysaccharide monooxygenase; MALDI, matrix-assisted laser desorption/ionization; MD, molecular dynamics; MRI, magnetic resonance imaging; NP, nanoparticle; SIMS, secondary ion mass spectrometry; UnAA, unnatural amino acid

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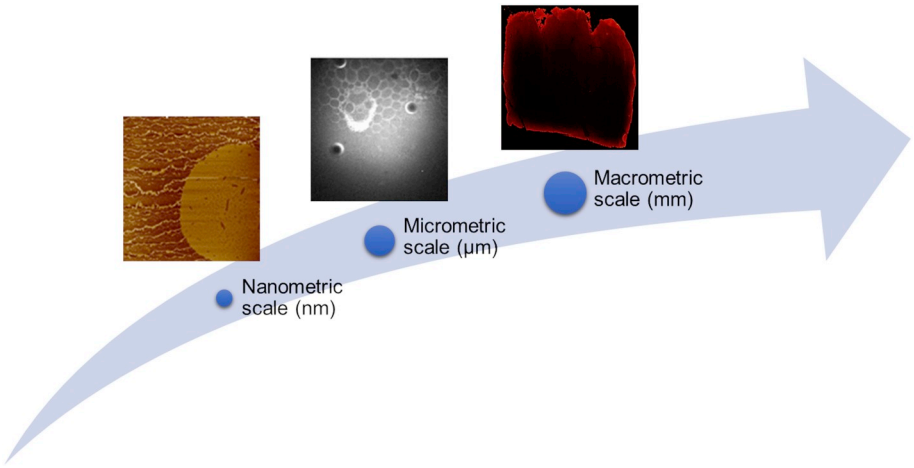

Fig. 1. Illustration of the concept of enzymes as probes for exploring the structure and chemical composition of complex bioproducts. Using enzymes as probes hinges on first knowing the physical-chemical properties, catalytic properties and specificity properties of the enzymes. Relevant data is then obtained by evaluating the effect of their catalysis, their interactions, and their dynamics in bioproducts as substrates, all from nanometric to macroscopic scale. Illustrations show adsorption of gastric lipase into milk fat globule at nanometric scale (Bourlieu et al., 2016), dynamics of cellulases in plant cell wall at micrometric scale (Chabbert et al., 2017) and diffusion of pepsin in meat at macrometric scale (Astruc et al., 2017).

personal care & cosmetics, and wastewater) and segment forecasts to 2024," 2016). Enzymes also serve as 'green' biocatalysts driving sustainable biomass transformation processes in conditions having low environmental impact (low temperature, no harsh chemicals or solvents).

There is already a continuous effort to gain deeper insight into enzyme structure-function relationships in order to create improved biocatalysts, but recently there has been increasing interest in using enzymes as analytical tools to better understand the bioproduct structure and composition. Using an enzyme as a probe (EP) exploits the specificity of each enzyme for a specific recognition site on its substrate, in much the same way as with antibodies for recognition of their epitopes. The development of real-time methods for monitoring enzyme activities in complex systems has further extended the scope for the applications of EPs.

One of the core classes of existing enzymes are hydrolases (EC 3), which can act as probes at different scales (nm/μm/mm), as shown in Fig. 1. These EPs can be used as characterization tools to define the chemical structure of polymers by profiling an enzymatic fingerprint (Quémener et al., 2015), to locate a polymer subunit in a complex multi-molecular structure by imaging the enzyme itself via microscopy techniques or by imaging its reaction products (Veličković et al., 2014). In research on plant tissues, enzymes are powerful tools for relating the mechanical properties of the cell walls to the composition, structure and organization of polysaccharides (Videcoq et al., 2017). Applying digestive enzymes on a complex substrate and then tracking both the enzymes and the biochemical and biophysical evolution of the substrate helps identify the structural parameters that impair substrate digestibility, including crystallinity, modulation of secondary or tertiary structure, or dispersion states. This kind of approach has been used on a wide variety of food products (Astruc, 2014; Bourlieu et al., 2016; Jamme et al., 2014).

The goal of this review is to describe the latest and most relevant ways to produce and characterize EPs and the way they can be applied to explore the structure and physico-chemical properties of bioproducts. We work to the definition that bioproducts are biological resources from plants and animals, ranging from small molecules to complex heteropolymer assemblies. First, we present the many sources of enzymes, with emphasis on their natural diversity and on the techniques used to mine natural sources, as well as computer-aided tools for designing optimized or even whole new enzymatic activities. We also briefly present the biochemical enzyme properties that need to be firmly mastered before using an enzyme as a probe. The following section overviews the theoretical basis and experimental applications of using EPs at nanometric to macrometric scales (Fig. 1). The review details the implications of using active or inactivated enzymes, along with examples of *in vitro* and *in silico* EP approaches. Promising developments in non-destructive quantitative and time-resolved *in situ* measurements of enzyme action on bioproducts are also presented.

2. Prerequisites to enzyme probes: diversity and characterization

2.1. Sources of recently investigated 'natural' enzymes

Microorganism diversity offers a great number of enzymes. Natural biodiversity has largely been explored via classical microbiological and biochemical approaches and by storming secretomes. This exploratory work has identified a huge diversity of enzyme functions. More recent breakthroughs in genome sequencing have expanded opportunities for discovering new enzyme functions. Furthermore, the unculturability of microorganisms is no longer a limitation in genomic data acquisition, as recently seen with the single-cell sequencing of several uncultured early-diverging filamentous fungi (Ahrendt et al., 2018). Large genome and metagenome sequencing programs such as the human microbiome project (Koppel and Balskus, 2016), 1000 Fungal Genome Project (Grigoriev et al., 2014) and global ocean project (Karsenti et al., 2011) have aimed to gain a global view of gene/protein/enzyme contents within each ecosystem. Extreme environments are increasingly being studied using metagenomic approaches to identify robust enzymes adapted to specific processes (Ferrer et al., 2015; Sarmah et al., 2018).

There are also a number of experimental strategies available for new enzyme discovery (Passerini et al., 2015; Vuillemin et al., 2016) that further expand the enzymatic diversity produced by microorganisms (Fig. 2). Some of these strategies, such as high-throughput functional (meta)genomics (Tasse et al., 2010), multi-omics analytical workflows (Miyauchi et al., 2016), CAZyChip technology (Abot et al., 2016) and carbohydrate microarrays (Salmeán et al., 2018; Vidal-Melgosa et al., 2015), aim to rapidly identify microbial strains with high biocatalytic potential. Putative enzyme functions/specificities can also be predicted using bioinformatics pipelines, and a huge diversity of sequence-based data has been gathered in expert databases dedicated to the display and analysis of genomic, structural and biochemical information related to specific enzymes acting on polysaccharides, proteins and lipids.

These combined efforts have produced a large and diverse set of enzymes that can be used as tools to investigate bioproduct structure and composition, and that needed to be databased. Carbohydrate-active enzymes (CAZymes; CAZy database; www.cazy.org) have been grouped based on comparisons of amino acid sequence, structure and catalytic mechanism. Proteases (EC 3.4; The MEROPS database; http://www.ebi.ac.uk/merops/) are another important class of enzymes involved in metabolism (protein trafficking, nutrient digestion, immune regulation, etc.) and employed in industrial processes. Lipases and esterases (E.C. 3.1.1.3; Lipase Engineering Database; http://www.led.uni-stuttgart.de) are other ubiquitous enzymes that play a central role in lipid

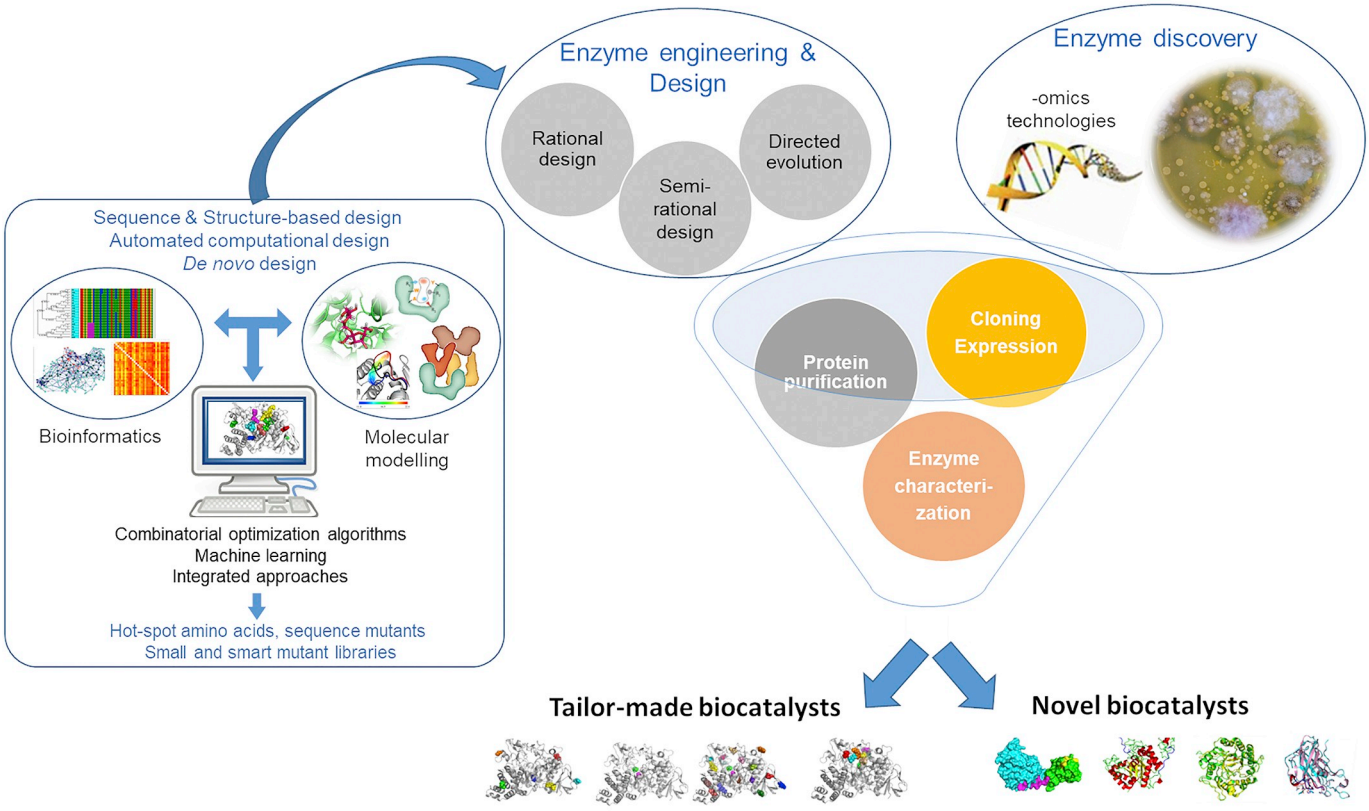

Fig. 2. General approaches to enzyme discovery and engineering. Omics technologies can rapidly identify new enzymes from microorganisms. Rational (site-directed mutagenesis of synthetic genes) or semi-rational (controlled randomization) design and directed evolution (random mutagenesis) are also powerful technologies for engineering novel enzymes with tailored properties and/or activities. Based on sequence-structure-function relationships, these rational or semi-rational engineering strategies mobilize a combination of bioinformatics methods (multiple sequence alignment, coevolutionary and phylogenic analysis, ancestral sequence reconstruction), molecular modelling (3D model building, docking, molecular dynamics, quantum mechanics, etc.) and various computational predictive tools (machine learning, combinatorial optimization algorithms) to preselect target amino acids and predict sequence mutants, with the aim of establishing small and smart mutant libraries (as illustrated in blue inset). These approaches, completed with enzyme expression, purification and characterization, are driving a rapid expansion in the repertoire of hydrolytic enzymes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

metabolism in a vast array of biological systems.

2.2. Expanding the enzyme repertoire through protein design and engineering

Enzyme engineering through directed evolution, rational or semi-rational design has become a powerful technology for generating new tailor-made enzymes (Fig. 2) (Badenhorst and Bornscheuer, 2018; Bornscheuer et al., 2012; Chowdhury and Maranas, 2020; Davids et al., 2013; Korendovych, 2018; Lutz and Lamurri, 2018; Sinha and Shukla, 2019; Zorn et al., 2016). Directed evolution is widely used as an approach for fine-tuning enzyme properties. It mimics Darwinian evolution using iterations of genetic variation by randomly recombining a set of related sequences (e.g. gene shuffling) or by introducing random changes in single protein sequences (e.g. error-prone PCR). Combining these genetic variations with high-throughput screening leads to mutants with improved properties. Such technology has been successfully used to increase enzyme performance under stern reaction conditions (including higher temperatures, high acidity or basicity and high concentrations of organic solvents), to expand the range of substrates that enzymes accept as well as to alter and optimize product selectivity (Arnold, 2018; Bradley et al., 2019; Chen and Arnold, 2020; Heater et al., 2019; Sanchez and Ting, 2019; Zeymer and Hilvert, 2018). Enzyme engineering strategies have also evolved towards more data-driven rational and semi-rational approaches that enable the construction of a limited number of mutants or small-size libraries where the diversity is focused on key regions encoding the desired property (Gao et al., 2019; Gordon et al., 2012; Li et al., 2018a, 2018b; Verges et al., 2015; Watanabe et al., 2018; Zeuner et al., 2018; Zhang et al., 2019; Zorn et al., 2018). Such data-driven engineering strategies include also the construction of chimeric enzymes (Chang et al., 2016; Saadat, 2017; Smith et al., 2012). The main advantage of rational and semi-rational approaches is they limit downstream high-throughput screening assays. The current trend is thus to develop, use and combine computational methods in hybrid approaches to guide enzyme engineering (Fig. 2). Advances in computational methodologies have notably made it possible to design *de novo* enzymes with new 'non-natural' activities (Kiss et al., 2013; Kries et al., 2013; Lewis et al., 2018; Vaissier Welborn and Head-Gordon, 2019). Further technologies that incorporate unnatural amino acids (UnAA) into proteins either by genetic means (Drienovska and Roelfes, 2020; Dumas et al., 2015; Young and Schultz, 2010; Zhang et al., 2013) or by chemical modification (Díaz-Rodríguez and Davis, 2011) provide novel opportunities to generate an artificial enzyme diversity offering novel catalytic capabilities.

In conclusion, a steady increase in the quantity and quality of biological data coupled with recent development of powerful mutagenesis and computational methods has accelerated and expanded the array of enzymes available to explore and transform bioproducts.

2.3. Enzyme characterization

The strategy of using an EP to characterize bioproducts hinges on exploiting specific enzyme catalytic properties to deduce certain properties of the bioproducts. This means that the structure–function

relationship, the specific mode of action of the enzyme, and the impact of the environment on the enzyme properties must all be known and controlled in order to effectively and efficiently use an enzyme as a probe.

Enzyme structure and dynamics is conditioned by the catalytic domain (the region of an enzyme that interacts with its substrate) but also by non-catalytic modules or domains such as carbohydrate-binding modules (CBMs) in CAZymes that drive substrate recognition. The binding specificity of CBMs means they can be used alone as probes for structural elucidation of complex polysaccharide matrices (Gilbert et al., 2013). Some CBMs recognize specific polysaccharides (chitin, β-1,3-glucans, xylan, mannan, galactan and starch), specific physical states of polysaccharides (crystalline *versus* non-crystalline cellulose) or present 'lectin-like' affinity since they are able to bind to a variety of glycans (Boraston et al., 2004).

Enzyme–substrate affinity can also be modulated by the presence of a cofactor, i.e. a compound such as a small organic molecule or certain metallic ions required for the enzyme activity. Proteases often require cofactors (López-Otín and Bond, 2008) whereas most lipases do not. An important biological exception is human triglyceride pancreatic lipase, the primary lipase that hydrolyses dietary fat, which is inhibited by bile salts and needs a small protein cofactor colipase (112 amino acids) binding to the non-catalytic C-terminal domain of lipase to restore its activity (van Tilbeurgh et al., 1999).

The ability of an enzyme to carry out its function depends on its ability to fold into a specific three-dimensional structure. This makes it essential to analyse enzyme 3D structure in order to decipher the molecular determinants responsible for enzyme activity and properties. For EP, there are several key aspects of enzyme 3D structure to consider: i) the active site topology, ii) the localization of catalytic residues in the 3D architecture, iii) the mode of interaction with a model substrate, and iv) the functional oligomerization state. The combination of *in vitro* and *in silico* techniques provides ways to examine these aspects and seems very promising although they should be further developed in future research projects (Aschauer et al., 2018; Brison et al., 2016; Calzado et al., 2016; Koliński et al., 2020; Kovaľová et al., 2019).

Another critical point to consider is that enzymes are not static entities but rather dynamic systems that host a large range of internal motions. Enzymes change their conformation several times during the catalytic process, and the extent and timescale of these changes can cover several orders of magnitude, from bond vibrations (femtosecond timescale) through to loop motions (microsecond to millisecond) and up to large-scale domain rearrangements (few seconds or longer). The question of how enzyme dynamics relates to chemical events and their associated reaction remains open to debate (Kohen, 2015; Warshel and Bora, 2016) but numerous examples have demonstrated that some motions are crucial for the ligand access and binding to active sites, for product release, and even for priming the active site for chemical catalysis. Approaches combining robotics algorithms with molecular modelling have been applied to a 'thumb-like' structure in xylanases to investigate its role during catalysis, and predictions of the importance of the amino-acid type at the tip of the thumb were validated by *in vitro* site-directed mutagenesis experiments (Paës et al., 2012b). Similarly, structural investigations on lipases have revealed that most lipases feature a mobile subdomain lid or flap composed of an amphiphilic peptide loop that covers the active site of the enzyme in its inactive state. This lid undergoes a conformational change that makes the active site accessible to substrate in the presence of a hydrophobic interface (Barbe et al., 2009, 2011). This conformation change is called 'interfacial activation' (Khan et al., 2017). The lid domain involves specific interactions with substrate and controls the inactive/active-form enzyme equilibrium, making it a hotspot for lipase engineering.

In complex bioproducts, enzymatic reaction kinetics can be limited by enzyme diffusion through pores and entanglements within the matrix (Tanaka et al., 1988). Enzyme size is thus another key structural property to consider. It can be determined from a geometrical or hydrodynamic standpoint, which corresponds to the gyration radius, R_G, and hydrodynamic radius, R_H (He and Niemeyer, 2003). The R_G of a protein is the radius of a sphere in which protein perfectly fits, whereas R_H is the radius of a sphere that has the same diffusion as the studied protein. In the ideal case of perfectly spherical protein, $R_G = R_H$. Actually, R_G and R_H are rarely identical when enzymes are made of different domains, their measurement provides indications on the sphericity of enzymes.

Enzyme specificity, i.e. the "ability to distinguish a substrate of well-defined structure and composition", is what gives them specificity as probes and distinguishes them from chemical catalysts. However, this notion of specificity varies across different hydrolase classes. For GHs, substrate specificity is related to the type of glycosidic bonds between sugar residues and to the presence and distribution of sugar and non-sugar substituents along the polysaccharide chain. It also depends on the chain length of the substrate, as enzyme activity is often different depending on degree of oligo/polysaccharide polymerization. Also, the presence of non-catalytic domains can favour interactions with substrate and promote transglycosylation vs hydrolysis (Light et al., 2017). Proteases, which are involved in multiple biological processes (digestion, hemostasis, apoptosis, signal transduction, and more), gather enzymes of either broad specificity (e.g. digestive proteases cleaving after hydrophobic residues) or very narrow specificity (recognition of a five-residue cleavage site in a precise location and environment) (López-Otín and Bond, 2008; Schauperl et al., 2015). Protease specificity is determined by the size and topology of the substrate binding site, or 'active site cleft', *i.e.* where there are few residues adjacent to the catalytic residues. Lipases and esterases show several types of fairly loose specificities. They can target a specific fatty acid (FA) or group of FAs (typoselectivity), but they can also distinguish the two external positions of the triacylglycerol backbone (positional specificity or regioselectivity) or the *sn*-1 and *sn*-3 positions of the triacylglycerol molecule (stereospecificity) (Villeneuve et al., 2000).

Enzyme specificity is also modulated by the substrate properties (3D structure, conformation and flexibility of biopolymers, presence of interfaces or defect) and physical state. For proteases for instance, one striking example is that the activity of pepsin on β-lactoglobulin, the main protein of bovine milk whey, can vary between 0 and 100% depending on its structural sate (native globular from vs heat-induced gels, respectively) (Macierzanka et al., 2012). Regarding CAZymes, for instance, polysaccharides have no single 3D structure and their monomers may adopt a wide range of ring conformations. One of the factors responsible for the high catalytic efficiency of GHs is the change in conformation of a sugar unit. Similarly, the physical state of lipids (crystallinity, liquid condensed phase) affects lipase and phospholipase binding. Several studies have found that phospholipases and lipases preferentially adsorb at the edge of defects, where less tight molecular spacing and the increase of curvature favour their adsorption (Noll et al., 2000). Gastric and pancreatic lipases get inserted in the liquid expanded domain of a triacylglycerols interface but do not get inserted in more rigid and packed liquid condensed parts of a hydrophobic interface (Bourlieu et al., 2016; Chu et al., 2010). This specific modulation can obviously be exploited to characterize unknown substrates but has not yet been implemented in *in silico* docking approaches.

In addition to specificity, processivity is another critical property of enzymes that affects their usability as probes. Processivity refers to the ability of enzymes to catalyse successive reactions on a unique molecule without releasing its substrate. Processive enzymes are particularly relevant for use as probes as their binding time onto the substrate is longer than for an enzyme with a random mode of action (Nakamura et al., 2018). For GHs, processivity is governed by multiple interactions with consecutive monomer units along the polymer chain. It has been extensively studied for cellulose-degrading enzymes, and particularly GH6- and GH7-family cellobiohydrolases. The processivity concept was also introduced for digestive lipases having long a residence time at hydrophobic interface. Processivity was posited to explain the extensive

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hydrolysis of a lipid droplet before the lipase attacks another droplet (Mansbach II et al., 2001).

2.4. Enzyme synergy and environmental conditions

In nature, enzymes are most often exposed to substrates that we can qualify as complex substrates, i.e. of variable structure and composition (proteins, polysaccharides, lipids) and that make up supramolecular structures. The high specificity of enzymes means that it takes many different types of enzymes to hydrolyse a complex biostructure. Indeed, bio-products are complex structures made of polymer assemblies. The recognition site of the substrate must remain accessible to the enzyme and if it is masked by other polymers that are not the substrate of a given specific enzyme, it must be exposed by the action of another specific enzyme so that the first enzyme can reach it. These enzymes act in synergy when the result of their activity is higher than the sum of their individual activities. Hence, there is increasing research into combining enzyme activities to deconstruct complex bioproducts. As explained in the review by Bouws et al. (2008), microorganisms have evolutionarily learned to use lignocellulose as carbon source. Extensive analysis of particular secretomes, such as that of *Trichoderma*, highlighted different sets of associated enzymes allowing some synergistic effects. Complex polysaccharides such as hemicelluloses require a synergistic action of several GHs in order to be turned into monosaccharides (Scheller and Ulvskov, 2010). It was recently demonstrated that lignocellulose degradation involves not just numerous GHs but also oxidative enzymes such as lytic polysaccharide monooxygenases (LPMOs) (Vaaje-Kolstad et al., 2010). In contrast, anaerobic cellulolytic bacteria produce multienzyme complexes, called 'cellulosomes', allowing spatial proximity of the different catalytic domains, which enhances their synergistic capacity (Artzi et al., 2017). Animal digestive enzymes also act synergistically. Preduodenal lipases plus phospholipases (A1 and A2), carboxyl ester hydrolase, pancreatic triglyceride-related lipases 1 and 2 and pancreatic triglyceride lipase all contribute to extensive hydrolysis of dietary fat.

In order to optimally use enzymes, the conditions in which they best act need to be assayed for pH, temperature, ionic strength, water activity, and of course the presence of inhibitors/inactivators. The impact of such external factors on the physical-chemical state of complex substrates should also be mastered before EPs are used. For instance, all enzymes present a range of optimal activity that covers a given range of temperature and pH. Thermophilic enzymes have been targeted for effective processing, but there is evidence that they have lower catalytic power at a given temperature than the corresponding mesophilic enzymes, as the thermophilic enzymes are less flexible (Roca et al., 2007). pH is an equally critical factor since it directly affects the state of acid-base amino acid residues involved in catalysis. Very few lipases are acidophilic, it is more common to find acidophilic proteases and CAZymes. For example, in the case of GH11 xylanases, the optimal pH range can span one up to several pH units depending on the enzyme (Paës et al., 2012a). Water availability is also a critical factor for hydrolases because water is not only one of the reactants involved in catalysis, but also critical for the structural conformation of the enzyme. In addition, in multiphasic environment the thermodynamic activity of the water controls water transfer between phases and local concentration gradients, and thus, strongly impacts reaction equilibria (Halling, 1984; Nadim et al., 1992). When analysing solid matrices using enzymes, it may be found that the local dry matter content decreases the availability of water for the enzymatic reaction. However, the analysis is carried out in an aqueous system and thus, contrary to what can be observed in water-organic two-phase systems, the solubility of enzyme and products is not altered and the active conformation of the enzyme is maintained. It was shown in family GH1 that highly conserved water molecules are organized in chains extending from the surface of the protein to the catalytic residues, inducing the formation of 'water channels' involved in enzyme function (Teze et al., 2013). It was also shown that introducing hydrophobic amino acids in a positive subsite can reduce water diffusion and improves the transglycosylation activity of GHs (Durand et al., 2016). Acyltransferase lipases have been identified that catalyze acyl transfer faster than hydrolysis in high-water-activity media such as CpLIP2 from *Candida parapsilosis* (Subileau et al., 2017).

The presence of enzyme inhibitors/activators is another key point to consider when using EPs. Seed, for instance, contains high contents of protease inhibitors (Kunitz inhibitors that inhibit trypsin, Bowman-Birk inhibitors that inhibit both trypsin and chymotrypsin). They are generally partially eliminated by heat treatments, which improves plant protein digestibility by restoring trypsin/chymotrypsin activity. Similarly, cereal grains contain a number of GH-inhibitors specific to pectinases, amylases and xylanases. These are peptides or proteins expressed as part of the defence response. Examples are inhibitors of polygalacturonases (PGIP), pectin methylesterases (PMEI), xylanases (XIP, TAXI) and amylases (BASI) (Juge, 2006).

3. Multiscale use of enzymes to probe bioproducts

Determining bioproduct composition is just one step towards process optimization. Equally important is to understand bioproduct architecture at different scales. Comparing results from the action of EPs on bioproducts before and after transformation provides unique information on the way a transformation step can affect the structure and organization of the bioproducts.

Also, as described in Sections 2.3 and 2.4, the information gained will only be relevant if it also integrates the intrinsic properties of EPs (catalytic activity and specificity, size, etc.) and their environment (pH, water content, etc.). This leads to two complementary strategies:

- Inactive EPs used with no catalytic activity: i) inactive as they are far from their optimal pH or temperature conditions or lack a co-factor but avoiding stern conditions that could affect enzyme tertiary structure or ii) inactivated using rational mutagenesis of catalytic amino acids or inhibitors. The enzymes thus diffuse as nanoparticles (NPs), without impact on bioproduct structure, revealing structural and topochemical information through their diffusion and interaction properties.
- Active EPs used under optimal conditions: bioproducts are hydrolyzed so that the released products and the enzyme-modified structure of the bioproducts can be analyzed to indirectly yield chemical and structural information on the bioproducts.

These two strategies can be advantageously applied to elucidate the bioproduct architecture modifications induced by transformation processes. However before designing an EP and choosing one of the two strategies, the pros and cons should be balanced: when using inactive EPs, their size and surface charge distribution are key factors controlling the bioproduct-enzyme interactions. In addition, the fact that enzyme is inactive makes tracking easier but gives a rather static picture of the bioproduct structure. Conversely when active EPs are used, the specificity of the enzyme is the key factor controlling the interaction. For a complex bioproduct, several EPs acting in synergy or the enzyme processivity will help giving a detailed and dynamic description of the bioproduct structure.

Below, we describe the complementary experimental (biochemical, spectral and microscopy techniques) and theoretical approaches to investigate bioproduct structure at different scales through the use of inactive or active EPs.

3.1. Experimental and theoretical approaches

Information collected using EPs is length-scale-dependent and thus requires complementary techniques including microscopy and imaging modalities (Fig. 3). The action of the enzyme inside the bioproduct can

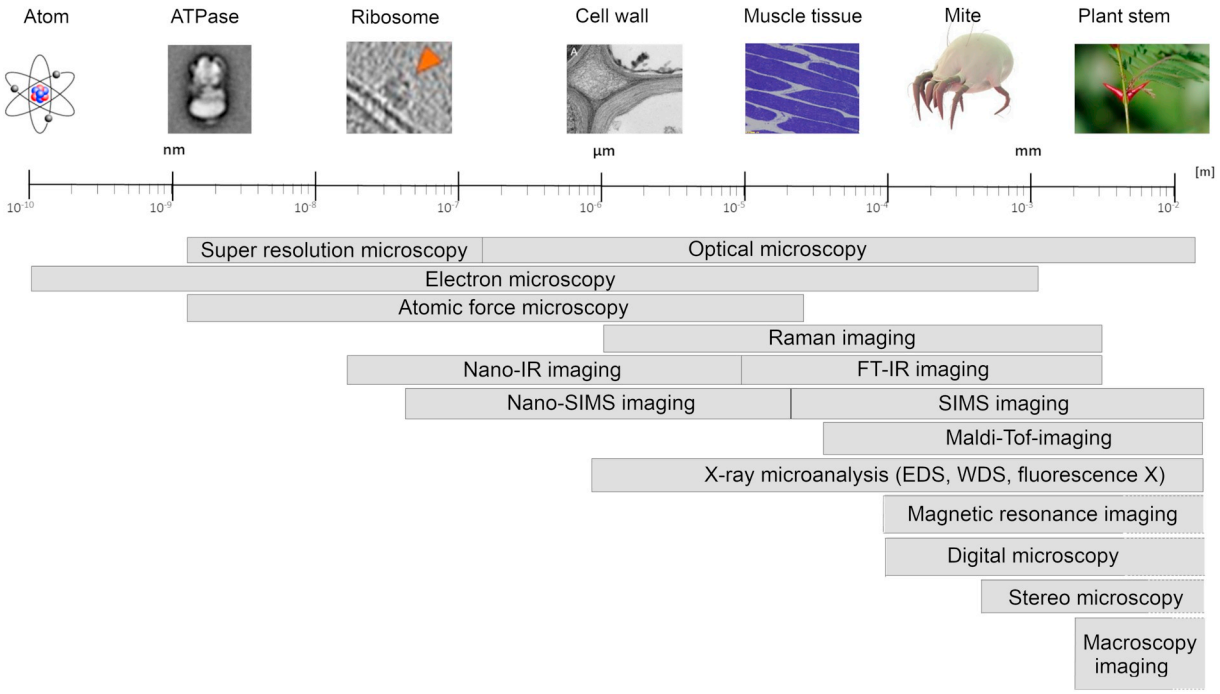

Fig. 3. Most common microscopy and microscopy–spectroscopy techniques geared to exploring bioproduct structure and composition. They span length-scales from nm to mm, representing six orders of magnitude, many of them can address several length-scales.

Table 1
Main experimental methods applied to using enzymes as probes at nanometric and micrometric scales.

Scale	Retrieved information	Methodology	References
Angstrom to nanometer	Chemical composition of the substrate	Analysis of products released under the action of specific enzymes, using chromatography and spectral techniques (mass spectrometry, infrared spectroscopy, etc.).	Böcker et al. (2017), Güler et al. (2011), Huang et al. (2011), Poulsen et al. (2016), Quémener et al. (2015)
Angstrom to nanometer	Information on the chemical composition of the substrate	Measurements of interactions between EPs and bioproducts using spectroscopy, acoustics or AFM	Arredondo et al. (2018), Bourlieu et al. (2016), Carvalho and Santos (2012), Lambert et al. (2019)
Few nanometers	Enzyme localization within the bioproduct	Direct enzyme imaging using immunocytochemistry (with labeled antibody) coupled to super resolution fluorescence microscopy (with fluorescent dye) or electron microscopy (using gold particles); AFM.	Astruc (2014), Berge et al. (2001), Bourlieu et al. (2016, 2020), Neumann et al. (2010), Poreba et al. (2019)
Few nanometers	Perspective of enzyme localization within the bioproduct and its interaction with structural matrix components at a given time	Nano-stable isotope probing, i.e. detection of enzyme enriched in stable isotope (e.g. ^{12}C-^{13}C, ^{14}N-^{15}N, etc.) within a matrix using a nano-secondary ion mass spectrometry (nanoSIMS) tool	Agüi-Gonzalez et al. (2019), Jiang et al. (2014, 2016), Nuñez et al. (2017), Proetto et al. (2018)
10–100 nanometers	Nanoporosity of the bioproduct and its evolution under enzyme action	AFM, assessing evolving bioproduct topology; FRAP, measuring the diffusion of molecular probes, typically using a set of fluorescent EPs of various known hydrodynamic radii R_H; fluorescence correlation spectroscopy (FCS) which measures diffusion in much lower volumes and thus detects lower concentrations of EPs than FRAP	Igarashi et al. (2011), Lambert et al. (2019), Luo et al. (2017), Thévenot et al. (2017), Videcoq et al. (2013)
0.2–100 micrometers	Dynamical distribution of enzyme within the bioproduct and evolution of bioproduct structure	Confocal microscopy observation of dye-labeled enzyme; deep-UV fluorescence imaging (synchrotron sources) coupled to bright field transmission microscopy; FRET interactions between EPs and polymers	Astruc (2014), Beaugrand et al. (2005), Bonnin et al. (2019), Bourlieu et al. (2015), Chabbert et al. (2017), Devaux et al. (2018), Floury et al. (2018), Jamme et al. (2014), Poreba et al. (2019), Tawil et al. (2011), Yoshida et al. (2006)
10–100 micrometers	Distribution of enzyme-induced modifications in macromolecular structure	FT-IR, Raman, fluorescence microspectroscopy	Chagnot et al. (2015), Day et al. (2010), Gierlinger et al. (2012), Yang et al. (2016)
10–100 micrometers	Highlight of molecules distribution, substrates, enzymes and reaction products	Mass spectrometry imaging (MALDI-ToF; ToF-SIMS) on histological sections previously subjected to partial enzymatic hydrolysis followed by sample fixation	Caprioli et al. (1997), Cillero-Pastor and Heeren (2014), Gessel et al. (2014), Porta et al. (2015), Théron et al. (2014, 2016, 2019)

generally be characterized by morphological and chemical imaging methods. Morphological imaging methods make it possible to map the shape of objects at scales from a few mm (macroscopy) down to a few nm (electron microscope and AFM). Coupling these morphological imaging modalities to spectrometers (FT-IR, Raman, fluorescence, mass, X-rays …) enables spectral acquisitions on histological sections of biological matrices or, less frequently, on whole samples. Chemical imaging methods can acquire information on matrix composition and

on molecular structure in an area of interest at spatial resolutions ranging from 50 μm to 50 nm depending on spectral modality and equipment used. Information is mainly gathered at nanometric and micrometric scales, using methods detailed below and summarized in Table 1.

3.1.1. Nanometric scale (0.1 nm–100 nm)

The techniques operating at this scale can capture diverse chemical information about the bioproducts. The most evident information retrieved from the use of EPs on bioproducts is the chemical composition of the substrates, which is either directly derived from analysis of the products released under the action of specific enzymes or deduced from measurements of interactions between EPs and bioproducts. Interaction analysis is generally done with simple isolated substrates or polymers or assemblies of polymers (considered as simplified models of bioproducts), but much less with complex bioproducts.

Direct imaging of EPs in bioproducts is now possible, using a panel of method illustrated in Fig. 3. Some of the techniques also give pieces of information on the effect of EPs on bioproduct structure.

In silico methods have been developed which can overcome the limitations of experimental nanometric methods for locating EPs. Numerical approaches based on 3D modelling and simulations can be used to investigate the behaviour, structure and dynamics of complex bioproducts and their interactions with enzymes.

The numerical approaches presented here work by i) modelling the (macro)molecular system of interest defined as a large set of interacting atoms or grains, and ii) simulating the equilibrium or non-equilibrium (in the case of enzymes acting on their substrate) behaviour of these particle sets. These *in silico* experiments serve to address a number of structural, dynamical and mechanical properties of the system, which can be seen as the numerical counterpart of the measurements performed in real experiments.

There are two main scales of description: atomistic models and coarse-grain models. While atomic-scale models describe details of the atomic composition and connectivity of the molecules, coarse-grain models map groups of atoms onto single interaction sites, therefore giving access to a larger space and timescale but at the expense of the smaller-scale details. In all-atom models, the interactions between particles rely on force-fields defined for carbohydrates, lipids or proteins. In coarse-grain models, the force fields can be derived from atomic-scale models of a specific system of interest (Harmandaris et al., 2006; Muller-Plathe, 2002) or generic potentials can be used.

Whether using atomic-scale or coarse-grain models, both enable the collective behaviour of assemblies of (macro)molecules to be computed using large-scale simulations executed on massively parallel supercomputers. This approach is able to simulate large and complex molecular systems. The structural properties of the simulated systems can be obtained from Monte Carlo simulations, while MD simulations simultaneously give access to structural, dynamical and mechanical properties of the modelled systems (Allen and Tildesley, 1987; Frenkel and Smit, 2001).

In atomic-scale modelling, MD simulations of complex biostructures such as the carbohydrate assemblies representative of plant cell wall give insight into the structural (e.g. the R_G of polymers) and some dynamical (e.g. mean-square displacements) properties of these systems (Beckham et al., 2011; Charlier and Mazeau, 2012; Oehme et al., 2015; Payne et al., 2011). It is also possible to simulate the effect of linkage type in the macromolecular backbone (Berglund et al., 2016) or of distinct intramolecular motifs on the conformation of and interactions with biopolymers (Martinez-Abad et al., 2017). MD simulations have already been used to investigate recalcitrance to hydrolysis in a mixed cellulose-lignin substrate (Lindner et al., 2013). A step further consists in simulating the interactions between enzymes and a complex substrate, which was done on the cellulose-lignin substrate model (Lindner et al., 2013) and led to a better mechanistic understanding of how lignin impedes cellulase-to-cellulose binding and thus hinders biomass hydrolysis (Vermaas et al., 2015).

At larger scales, coarse-grain models coupled with MD simulations give insight into the collective structural and dynamical properties of the simulated systems. This kind of approach helps to decipher the general mechanisms at work in a class of systems and is widely used in the study of the generic properties of liquid polymers, gels or glasses (Binder, 1995) or polymer–nanoparticle systems (Kalathi et al., 2014; Sorichetti et al., 2018). In the case of enzymes acting on their substrate, which can be generically described as reactive polymer–nanoparticle systems and correspond to out-of-equilibrium situations, the simulations give access to the simultaneous evolutions of i) substrate structure (made of a large number of coarse-grain polymers) and ii) the action/diffusion of the enzymes, and the approach has managed to establish the main mechanisms at work (Hugouvieux and Kob, 2017).

3.1.2. Micrometric scale (0.1 μm–100 μm)

Technically speaking, imaging is generally easier at micrometric scale than nanometer scale, largely because the animal and plant tissue samples are easier to prepare. The main tool used for micrometric-scale studies is optical microscopy, as it allows static imaging of fixed samples (bright field and fluorescence modes, chemical imaging) and to a lesser extent, dynamic imaging of unfixed samples (confocal microscopy, FRET, FRAP, synchrotron deep UV microspectroscopy). At this scale, observation of EPs within bioproducts can be (Fig. 4):

- Indirect, using techniques to gain access to the structure of the bioproduct to image the effect of EPs;
- Direct, using techniques to visualize the enzyme itself (label-free or after labelling).

Indirect observations at these scales (10–100 μm, Table 1) are well geared to revealing structural changes in the biological matrix under the action of an enzyme. Imaging is either static (MALDI-ToF; ToF-SIMS) or dynamic (FT-IR, Raman, fluorescence microspectroscopy). Direct detection of EPs is also achievable with static or dynamic methods. The most common static method used is immunohistochemistry, although it does pose challenges as EPs can get partially lost during the incubation or washing steps. Chemical imaging, using MALDI-selected reaction monitoring mode for instance (Gessel et al., 2014; Porta et al., 2015), is another static method that gives access to the location and local concentration of a specific ion within a tissue (see Veličković et al., 2014 in Fig. 4 for an example).

Dynamic tracking brings complementary information by continuously visualizing the enzyme as it diffuses inside the solid matrix. The sample is generally incubated in EP solution, and videos or images are recorded using an inverted confocal microscope with a digital camera. As a rule, the EP is labelled with a fluorescent tracer dye that has to fit its optimal physical-chemical properties (pH, temperature, etc.) while being compatible with the fluorescence lasers and filters available on the microscope. Dye-labelling the EP increases the enzyme's steric hindrance and can modify its catalytic activity, which makes it necessary to control the activity of the enzyme after labelling. These labelling issues can be circumvented using direct imaging. With intense synchrotron light sources, deep-UV fluorescence imaging directly reveals the autofluorescence of in-protein aromatic amino acids (mainly tryptophan; (Jamme et al., 2013)). In a protein-free matrix (like seed compartments), any fluorescence from aromatic amino acids necessarily comes from the enzyme. This autofluorescence property makes it possible to follow diffusion of the enzyme in the matrix in real time by fluorescence microscopy, while the bright-field mode makes it possible to characterize hydrolysis-induced changes in bioproduct structure (Bonnin et al., 2019; Chabbert et al., 2017; Devaux et al., 2018).

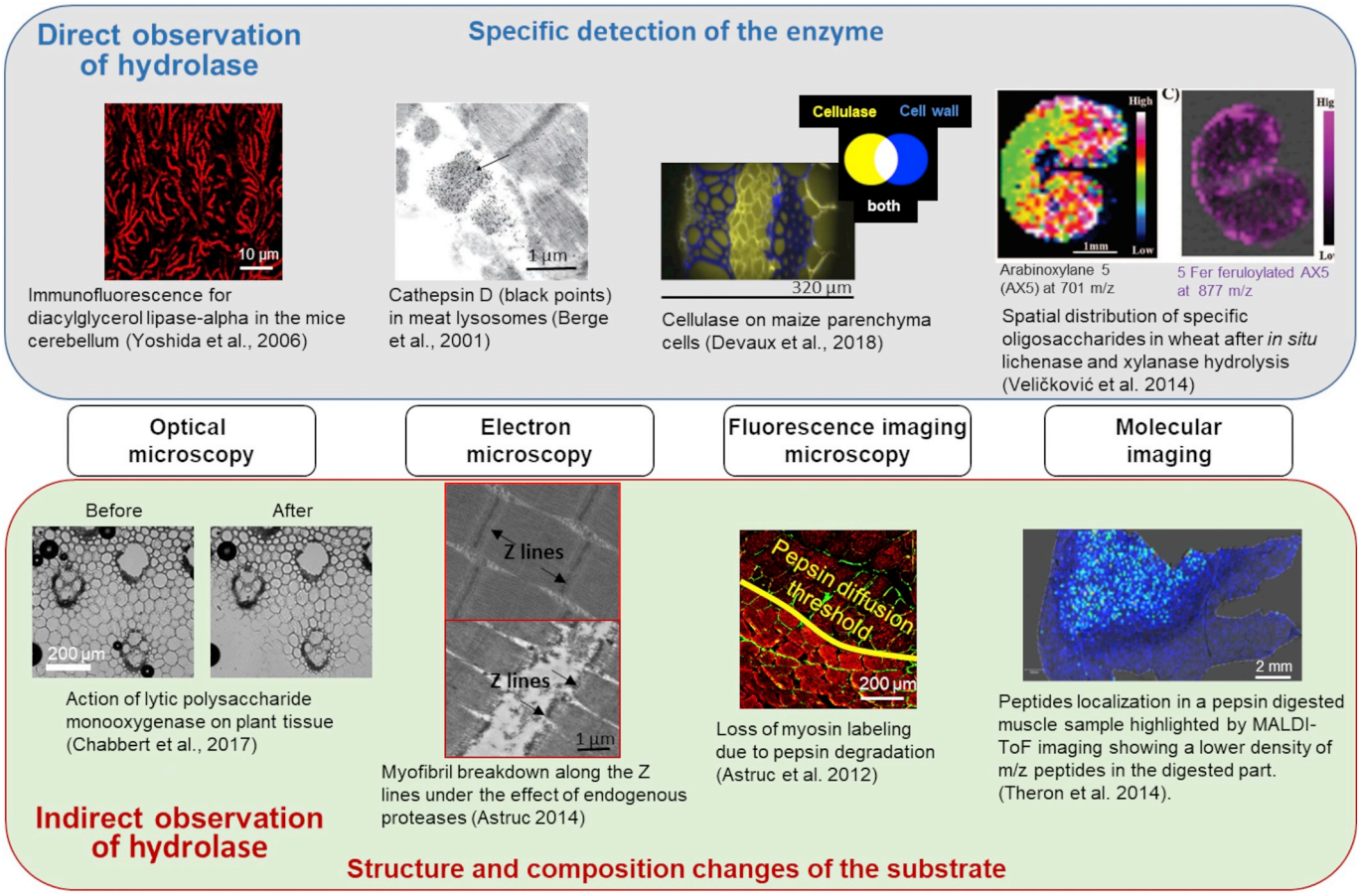

Fig. 4. Complementary approaches for using enzyme probes. Direct observation of hydrolases (upper panel) provides spatial information related to localization, interactions and accessibility. Indirect observation of hydrolases (bottom panel) points to their effects and modifications in structure and composition of bioproducts.

3.2. Inactive EPs to explore bioproduct properties

Inactive EPs are thought to keep their interaction properties without impacting surrounding polymers, which means their behaviour can be assessed in the more general framework of NP diffusion in soft materials. Diffusion, or Brownian motion, is the mechanism by which molecules move in the absence of external forces. It is the most essential form of molecular transport, and tends to reduce concentration, temperature or pressure gradients. It is also a prerequisite for all (bio) chemical reactions, as reactants need to come into contact for a reaction to proceed. Mathematically, molecular diffusion of spherical particles that are infinitely diluted can be described by the following relation (1):

$$D = \frac{k\ T}{f} \tag{1}$$

where D is the diffusion coefficient, k is the Boltzmann constant, T is temperature, and f is the friction coefficient. In the case of a spherical particle of hydrodynamic radius R_H in a liquid of viscosity η, it results into the Stokes-Einstein equation (2):

$$D = \frac{k\ T}{6\ \pi\ \eta\ R_H} \tag{2}$$

However, in real systems that are far more complex than a viscous liquid, particle diffusion is influenced by the presence of compositional and/or structural heterogeneities (e.g. density, viscosity) that hinder the diffusion process. Hence, diffusion can be used to draw information from the surrounding environment, to probe a structure (density, shape, size, orientation of the obstacles), or to assess physical-chemical interactions (specific or non-specific) between the diffusing object and its surroundings (Fig. 5).

3.2.1. Inactive EPs to reveal structural properties

Because enzymes are globular proteins typically a few nanometers in size, they can generally be considered as spherical NPs. Their diffusion is strongly influenced by the length scales in the material through which they diffuse, and especially the ratio between the size of the enzyme and the characteristic length scales of the matrix. We can confidently posit that particles cannot penetrate substructures smaller or similar to their own size but can freely diffuse into channels larger than their own size.

A deeper understanding of the diffusion of inactive enzymes in biopolymer-based materials can be built upon the literature dealing with diffusion of NPs in liquid, gel or solid polymers (Masaro and Zhu, 1999). In this kind of systems, NP diffusion is affected by a number of length scales, such as monomer size, mesh size or chain size, independently of any physical-chemical interactions between NPs and polymers. In the case of generic liquid polymers and non-sticky NPs (no interactions between NPs, and no NPs adsorbing onto the polymers), it has been shown theoretically that different diffusion regimes can be predicted as a function of the size of the NPs with respect to the mesh size of the polymer network (Cai et al., 2011). Investigations led on NPs with a size of the same order as the mesh size in polymer networks and gels have evidenced a mechanism called 'hopping diffusion' (Cai et al., 2015). For much larger particles, hopping is no longer possible in solid polymers, and so the particles are trapped, whereas in liquid polymers, NPs can still move as the polymers can still rearrange. MD simulations of generic polymer–NP systems can also give insight into the diffusion regimes of NPs as a function of their size with respect to the typical mesh size of the polymer melts (Kalathi et al., 2014) or into the evolution of the diffusion coefficient of NPs when NP size or volume fraction are made to vary (Sorichetti et al., 2018).

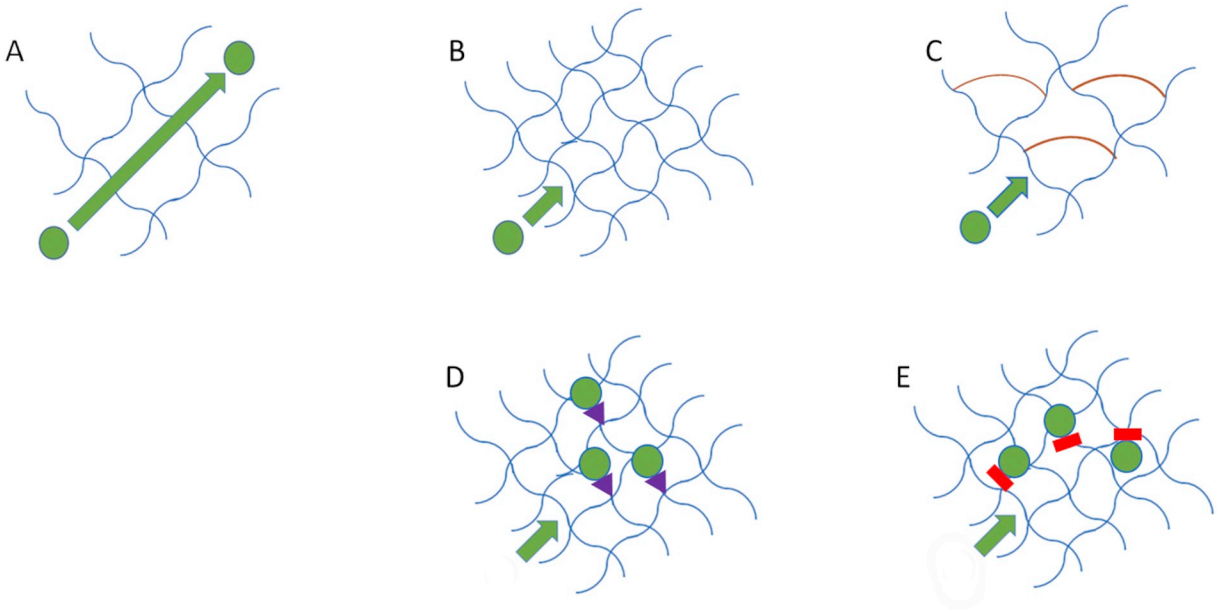

Fig. 5. Features impacting the diffusion of enzyme probes. Direct measurements of enzyme probe diffusion provide information on the structure and composition of bioproducts, since diffusion is directly related to pore size/entanglements of the bioproduct and to specific/non-specific interactions between EPs and polymers making the bioproduct. Free diffusion (A), diffusion slowing down due to: substrate porosity made by intra- (B) and inter-polymers (C), specific (D) or non-specific (E) interactions of EPs with the polymers.

Turning to the use of EPs to explore bioproducts, structural information can be obtained using techniques described in Section 3.1. EPs are perfectly complementary to NPs since they have biochemical (hydrophobicity, surface) and structural (size, shape, domain distribution) properties that are compatible with bioproducts. In plant cell walls, nanoporosity has been evaluated by confocal microscopy with fluorescent NPs by direct imaging (Donaldson et al., 2015) and FRAP analysis (Herbaut et al., 2018; Paës et al., 2017), which revealed entanglements which limited diffusion, even though pore size was three times higher than NP size. Bioinspired models of plant cell walls were used in a FRAP study on polymer network accessibility to measure the impact of relative polymer content and concentration (Paës and Chabbert, 2012) together with water concentration (Paës et al., 2013) and even to propose a diffusion model. A study comparing the localization of an GH11 xylanase inactivated by directed mutagenesis against its fully active parent in wheat tissue revealed that active EP had much higher penetration, thus demonstrating the role of hydrolysis in opening up the polymer network and facilitating diffusion (Beaugrand et al., 2005).

Similar approaches have led to comparable findings in the field of food science. Just recently, fluorescence techniques gave the first measurements of inactive pepsin diffusion in dairy gels (Luo et al., 2017; Thévenot et al., 2017), and the results overlapped with previous findings on the diffusion of NPs in dairy gel systems, showing a high dependency on both protein concentration and size ratio between the diffusing particle and the network mesh (Le Feunteun and Mariette, 2007, 2008a, 2008b).

3.2.2. *Inactive EPs to reveal chemical properties*

In addition to structural parameters, chemical properties can also be deduced from interactions between EPs and the polymers in the bioproduct. For instance, the diffusion of inactive GH45 cellulases as EPs (at far from optimal temperature) was measured by FRAP in bioinspired polymer assemblies to assay the impact of lignin on non-specific interactions (Fong et al., 2016). Variations in the modular structure of the cellulases demonstrated that the presence or not of one or several appended CBMs had a strong impact on the interactions between the EP and lignin.

In addition, several simulation studies have shown that the diffusion behavior of spherical NPs in polymers is generally strongly affected by the strength of polymer–NP interactions, and that NP diffusion deviates from the Stokes-Einstein equation due to monomer–NP attraction in unentangled and entangled polymers (Yamamoto and Schweizer, 2011). In this case, the diffusion coefficient of NPs can be estimated using effective R_H, which accounts for the polymer adsorption on the NPs, instead of using the simple NP radius in the Stokes-Einstein equation (Liu et al., 2008; Patti, 2014). These simulation approaches serve to explore the main parameters (size, charge, chemical surface properties) influencing EP diffusion, and pave the way to *in vitro* experiments to select the most appropriate EPs.

3.3. *Active EPs to explore bioproduct structure: reciprocal influence of enzymes and substrate in time and space*

When EPs are active, they modify the bioproduct in which they catalyse a reaction. The reciprocal influence of enzyme(s) and substrate (s) in time and space is a challenge to address. The catalytic activity and diffusion of EPs trigger changes in the structural and chemical properties of the bioproduct, which in turn modifies the behaviour (diffusion, interactions, activity) of active enzymes. This is a good example of continuous reworking. For instance, a fully catalytically-active GH11 xylanase showed greater diffusion than its inactive counterpart, demonstrating that catalysis is critical to maximize EP diffusion into a plant tissue (Beaugrand et al., 2005). This is coherent with the fact that substrate degradation by the enzyme opens new paths for diffusion. Regarding the catalytic mechanism itself, recent studies have concluded that catalysis could enhance enzyme diffusion by up to 50% (Jee et al., 2018; Riedel et al., 2015). The enzyme-catalysed reaction appears to boost the motion of the focal enzyme for a few microseconds, thus enhancing its effective diffusion over longer timescales in the presence of substrate. This was reported to give rise to antichemotaxis, *i.e.* formation of an inverse enzyme concentration gradient to substrate gradient (Jee et al., 2018). Antichemotaxis could therefore hinder the overall process in applications where EPs are added to bioproducts for deconstruction purposes. More research is now needed to verify whether this phenomenon is common to all enzymes, and to determine its

precise influence on large-scale observations.

The strong interconnections between several parameters influencing EP diffusion create the challenging need to couple several types of imaging and spectral techniques together. Output of experimental and theoretical approaches developed in Section 3.1 are highlighted below through various examples.

Histological studies are a very common way to explore the impact of EPs. The diffusion of a pepsin solution in meat during enzymatic digestion was shown to be inhomogeneous: the part penetrated by the digestive juice showed a lower staining density than the undigested part (Astruc, 2014). Immunofluorescence staining of laminin in digested muscle became ineffective after exposure to pepsin which had hydrolysed the laminin antibody recognition site (Astruc et al., 2012a). Similarly, gastric lipase was localized on milk fat globules using indirect immunolabelling with purified polyclonal Ac anti-Human gastric lipase and Ac II anti-IgG (H + L) tagged with Alexa Fluo 488 (Bourlieu et al., 2015).

Confocal fluorescence microscopy provides useful information in space and time, shedding light on the impact of processing on bioproduct structure and its interactions with EPs. This technique was employed to show that cellulase hydrolysis of pretreated biomass samples led to very slight structural changes and equated to a hollowing mechanism. These results were used to propose a modelling approach to predict available surface areas (Luterbacher et al., 2013). Gastric lipase and pepsin applied to minimally-transformed (i.e. thermized) milk fat globules or further-transformed (i.e. homogenized or homogenized/pasteurized) milk emulsions gave important information on emulsion structure and composition throughout the kinetics of hydrolysis and on the nature of the products released (enhanced medium/long-chain FA and casein hydrolysis) in the transformed milk emulsions (Bourlieu et al., 2015).

In confocal microscopy, several techniques (FRAP, FRET, FCS) presented in Section 3.1 are particularly valuable for following dynamic processes. In the context of enzyme-mediated gel formation, a combination of FRAP, FCS and macroscopic biochemical analyses was used to assess diffusion in different pectin systems (solution, gel under formation, preformed gel) of two variants of the same pectin methylesterase enzyme with different processivity. This approach revealed that diffusion and processivity of the enzymes influenced the structuring of the pectins, and in return, the progressive structuration of the pectins had a reciprocal influence on behaviour of the enzymes (Videcoq et al., 2013). FRET has also been successfully used to measure interactions between cellulose fibers and cellulase (Wang et al., 2010). Recent developments have shown that the autofluorescence of complex bioproducts such as plant cell wall is no longer a limitation to quantitative measurements (Terryn et al., 2018).

Classic direct observation of enzymes with standard lab equipment requires labelling or antibody strategies. The emergence of synchrotron deep-UV beamlines has now made it possible to visualize label-free active EP. Elegant research has found that starch granules can be degraded in a very different pathway by various sources of amylases (Jamme et al., 2014; Tawil et al., 2011). One amylase showed very rapid diffusion towards the central part of the granule, following a pathway known for its low level of structural organization, before completing digestion from inside-out. In the same substrate, another amylase showed an erosion-like mechanism, with progressive disintegration from the external domains towards the interior of the granule. These highly contrasted behaviours could be related to the specificity of the enzymes' binding domains in combination with their catalytic core and preferential adsorption sites on the starch granule. These observations evidenced that both substrate structure and the enzyme specificities can be the main influencing factor.

The same technique has recently been adopted to monitor the disintegration of other bioproducts (Deuscher et al., 2018). In one example, Floury et al. (2018) studied the peptic digestion of two differently-structured dairy protein gels and found a highly contrasted sensitivity of the gel's inner structure to pepsin action. Another example investigated the degradation of maize stem by a cellulase: the study showed no evidence of enzyme binding on recalcitrant cell walls yet dense enzyme binding on degraded cell walls, with an unexpected variability in cell wall biochemical composition within a given cell type (Devaux et al., 2018). Combining LPMOs with cellulases improved the progression of the cellulases in some specific miscanthus tissues, thus validating not only the catalytic but also the spatial synergy between these enzymes (Chabbert et al., 2017). However, this kind of imaging approach which involves excitation in the deep UV remains difficult to implement, since it requires the use of microscopes coupled to a synchrotron radiation source.

Other microspectroscopy and chemical imaging techniques have been used in recent years to track hydrolases 'label-free', i.e. avoiding any labelling, in complex matrices. The enzyme itself is not necessarily visualized, rather the effects of hydrolysis can be followed. For example, MALDI imaging applied to blocks of muscle tissue incubated in pepsin solution revealed the presence of certain peptides in the infused area at the periphery of the block but not in its central part that is not reached by the digestion solution (Théron et al., 2014) (Fig. 4). It was concluded that the peptides in the infused part of the sample resulted from proteolysis of a larger protein. Combining SIMS with complementary analytical and imaging techniques can help identify and characterize subcellular structures highlighted in elemental distribution patterns.

FT-IR microspectroscopy is also a first-line method for mapping changes in macromolecular protein structure (such as α helices, β sheets, β turns) on a tissue section (Astruc et al., 2012b; Motoyama et al., 2018). The combination of electrophoresis (SDS-PAGE) and FT-IR microspectroscopy revealed that in soybean protein isolates, β-conformations were prone to be preferentially hydrolysed by pepsin and transformed to unordered structure during *in vitro* digestion, followed by digestion of α-helix and unordered structure (Yang et al., 2016).

Like FT-IR, Raman spectroscopy is a vibrational spectroscopy that can inform on macromolecular structure and composition. Raman microspectroscopy has been used to map the lipolysis of glyceryl trioleate emulsion droplets by porcine pancreatic lipase. This approach allowed to distinguish the undigested oil and the crystalline lipolytic products, and helped to understand the structural interaction between liposoluble crystals and lipolytic products (Day et al., 2010). Raman imaging of plant cell wall has also been developed to provide insights into chemical composition and structure at the micrometre level during enzymatic hydrolysis (Gierlinger et al., 2012).

AFM has been used to study the adsorption of phospholipases/lipases in homogeneous supported monolayers or bilayers, and yielded evidence that these enzymes generally adsorb at the edge of defects, where less tight molecular spacing and more curvature favour adsorption. AFM combined with ellipsometry, tensiometry and electrostatic enzyme surface modelling managed to elucidate the mechanisms of gastric lipase adsorption within a model membrane mimicking the milk fat globule membrane complete with a coexistent liquid–liquid phase (Bourlieu et al., 2016). AFM showed that gastric lipase partitions towards the liquid-expanded phase and at phase boundaries. Gastric lipase got adsorbed at three levels of insertion, suggesting a molecular cooperation that favoured insertion and strongly impacted the lateral lipid phase organization. Addition of negative charges using phosphatidylserine led to reinforced adsorption. Further investigation through surface potential modelling showed that besides hydrophobic interactions, gastric lipase adsorption was also favoured by electrostatic interactions, again indicating that subtle local changes in substrate structure can modulate EP activity and that, in return, EP can signal subtle changes in bioproducts.

High-speed AFM has been used to follow the motion of cellulases on cellulose nanofibers and the impact of catalytic activity on the substrate (Igarashi et al., 2011). Even though the focal substrate was pretty simple, this approach nevertheless represents a major technical

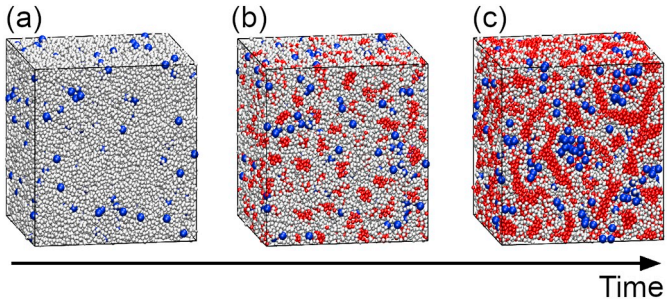

Fig. 6. Snapshots from coarse-grain MD simulations of enzymes acting on polymers modelled as chains of monomers. The enzymes (blue spheres) are able to convert initially repulsive monomers (white spheres) into attractive monomers (red spheres). The initially homogeneous system (a) forms clusters of attractive monomers at an intermediate stage (b) and eventually turns into a gel (c). The simulation box contains 400 polymers of 100 monomers and 500 enzymes, corresponding to a total volume fraction of 30%. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

breakthrough for studying enzymes: it reveals how crowding enzymes onto a single polymer could limit their processivity and thus the resulting hydrolysis rate. Similarly, a protocol developed to dynamically follow the enzymatic deconstruction of plant cell wall-bioinspired biopolymer over several hours provided structural data that was directly related to biochemical hydrolysis (Lambert et al., 2019). The recalcitrance of such films was modulated by varying the lignin concentration, thus demonstrating the influence of lignin at nanoscale.

Coarse-grain MD simulations were used to investigate the time and space evolution of a solution of polymers containing active EP (Hugouvieux and Kob, 2017). Starting from a solution of polymers, the action of enzymes converting the initially repulsive monomers into attractive (sticky) ones was shown to lead to the formation of a gel (where crosslinks were due to the attraction between the converted monomers) or to a macroscopic phase separation of the polymers depending on the conditions (Fig. 6). Under certain conditions, the enzymes can get trapped in the polymer gel, thus preventing further enzyme-driven conversion of the monomers.

4. Perspectives and outlook for exploring bioproducts with enzyme probes

Enzymes can become crucial tools for exploring complex biostructures in an effort to optimize their deconstruction. As demonstrated throughout this review, EPs carry several advantages for exploring biostructures: they offer a broad spectrum of substrate specificity and activity, are relatively easy to produce, amenable to engineering based on the large body of structure-function knowledge available, and have a protein-like nature that makes them chemically compatible with bioproducts (in contrast with inorganic molecules). EPs can also be used in two complementary modalities, *i.e.* as active or inactive catalysts. However, there are still some challenges to resolve before using them at their full potential.

Enzymes need to be further discovered and characterized. The enzyme repertoire is continuing to expand as novel enzymes, notably from microorganisms, continue to get identified. Furthermore, protein engineering can effectively design new enzymes with novel properties and/or activities. Nonetheless, even though the literature features a huge number of well-described enzymes, most of them annotated, few of them have validated functions. And yet a critical prerequisite to using an enzyme as a probe is to have sharp in-depth knowledge of its catalytic, biochemical and structural properties. Thus, there is still huge work needed to determine these properties for all unknown enzymes. The recent example of the discovery of LPMOs (Couturier et al., 2018) shows that there are still some key enzymatic activities to uncover.

The activity screening strategy remains a bottleneck. Substrates used to characterize an enzyme are most of the time pure isolated molecules or simple polymer assemblies only partly mimicking some chemical and structural features of bioproducts. These model substrates remain far from real-world complex bioproduct composition and architecture, and this substrate oversimplification is a barrier to the discovery of new activities. To overcome this lack of purpose-adapted substrates, it could be helpful to use artificial ones, either to create new linkages or to detect enzymes potentially active on various substrates in one shot by synthesizing the smallest common chemical part of several substrates. This approach—by engineering appropriate artificial substrates—has to be combined with an easy and rapid detection and screening system, based on chromogenic moieties for instance (Kračun et al., 2015).

Right now, we are unable to reproduce complex enzymatic systems and synergies between enzymes. In natural bioprocesses (food digestion, organic matter degradation in soil), bioproducts are deconstructed by several types of enzymes acting in synergy. Synergy should be considered not only between enzymes belonging to the same family (synergy between GHs, for instance) but also from different families (synergy between proteases and lipases). For instance, intramuscular connective tissue includes adipocytes that contain fat, which is known to stop the diffusion of small water-soluble molecules (Lebert and Daudin, 2014). We can thus expect these fat frames to also slow and/or disrupt the diffusion of water-soluble proteases. The concomitant use of lipases and proteases could reveal the characteristics of the fat frames (size, density, etc.) through the facilitated diffusion of proteases into the tissue.

Research should make more use of direct acquisition techniques for investigating the action of enzyme probes. First, direct imaging is set to evolve, as recently developed engineering methods that can incorporate UnAA into enzymes, including with fluorescent features, will likely facilitate direct EP observation (Ravikumar et al., 2015). Nevertheless, any such developments need to preserve the EPs' native properties, which remains a challenge. Furthermore, the behaviour of enzymes used as probes in complex polymer media is still poorly understood. The use of synchrotron light and the FRAP technique for example have brought valuable information on enzyme diffusion towards their substrate in complex systems, but there are still no physical-chemical models proposed to explain anomalous diffusion patterns and the subsequent enzyme distributions. A way forward is likely to come from numerical investigations on the reciprocal influence of i) the structure and dynamics of complex polymer materials and ii) enzymes acting on these materials.

There are promising recent developments for bioproduct characterization. FT-IR spectroscopy can highlight the consequences of proteolysis on bovine serum albumin films in muscle extracts (Böcker et al., 2017), plasma proteins (Poulsen et al., 2016) and milk proteins (Güler et al., 2011) in solution. These approaches could be transposed to FT-IR imaging in a near future. The MALDI-selected reaction monitoring modality should theoretically allow to identify specific enzymes (for example pepsin) in the bioproduct, but to our knowledge, no such study has yet been performed to localize pepsin inside digested food. The nano-stable-isotope probing (nanoSIP) approach would be particularly well suited to studying hydrolase diffusion and molecular interactions in complex matrices. The step involving enzyme enrichment in stable isotope is a major constraint, as is the cost of using nanoSIMS. This approach is already used in cancer research to locate and characterize the effect of drugs in the vicinity of cancerous tumours at ultrastructural scale (Legin et al., 2014). Among microscopy techniques, super-resolution techniques are filling the gap between standard photon and electron microscopy modalities, and could thus lead to a finer understanding of processual changes in bioproduct structure (Paës et al., 2018).

One important development expected soon is the routine acquisition of images in three spatial dimensions (3D) completed by one temporal

dimension (4D) and one spectral dimension (fluorescence, Raman, etc.) to reach 5D/6D datasets. The acquisition of this kind of data has become possible with X-ray tomography in synchrotron facilities and with instruments available in labs enabling the observation of mm-to cm-scale samples. However, dealing with bioproducts in water-buffered systems is still a challenge. Other photon microscopy techniques can handle such systems, but are generally suitable to only observe thin samples—the ability to work with thick samples would represent a significant technological leap forward (Susaki and Ueda, 2016). Macroscope tools ('stereomicroscopes') and digital microscopes that allow macroscopic observations can help to acquire knowledge on the macroscopic structure of biological tissues. The samples can be observed without preparation, and the large depth of field of the instruments allows dynamic observation of several-centimetre samples in 3D. In some cases, multispectral imaging systems can specifically contrast certain tissue structures or components based on their autofluorescence characteristics (Skjervold et al., 2003). The development of fluorescence macroscopes equipped with powerful digital zoom now makes it possible to acquire fluorescence images in a micro-/macroscopic range. MRI is also a powerful method for tracking enzyme diffusion and interaction with components of the bioproduct matrix, in order to derive information on the 3D organization of key tissue components (connective tissue, lipids, blood vessels, etc.). Unlike stereomicroscopes which can only produce surface images, MRI makes it possible to non-invasively acquire virtual images inside the sample (Damez and Clerjon, 2013). Recent developments in MRI contrast agents using a platform technology approach have made it possible to engineer new categories of agents with a core structure and ligands that can be easily modified to detect new enzymes (Hingorani et al., 2014). These newly developed technologies hold great promise as powerful ways to detect and monitor (in 4D) a variety of enzyme activities in biological matrices.

The overarching challenge is not only the acquisition but also the quantification of image parameters (structural properties, fluorescence intensity). Inspiration could come from recent developments done in biomedical fields and in plant morphogenesis science (Willis et al., 2016) and from machine learning to extract relevant information (Yuan et al., 2012). Simulations can also be run using structural data determined by microscope imaging. With a numerical description of the topology of tissues and the features of their different compartments, tissue behaviour can be simulated as a route towards predicting their properties. This approach has already been applied in research on the mechanical behaviour of wood tissue (Perré et al., 2016). Finally, management of multimodal and multiscale datasets requires new algorithms to be implemented in bioproduct models, including key chemical, structural and spectral information to predict reactivity to enzyme action.

Finally, EP science needs to look at the bigger picture so that synergies can be exploited to better investigate the structure of complex bioproducts and generate results that are transferable to industrial processes without overly expensive protein purification. In this context, it is essential to consider all the steps of the process before and after the catalytic step involving enzymes, in order to optimize the expected product yields and minimize the release of unwanted co-products (including inhibitors/inactivators). This strategy should help drive the design of more sustainable processes that are softer to bioassemblies (as needed for food applications) or that efficiently release target intermediate chemicals and synthons (as needed for biomass valorisation in green chemistry).

5. Conclusions

We have shown that there are several viable approaches for investigating the structure of complex bioproducts using enzymes as probes. Inactive or active EPs can be used on bioproducts as probes to get complementary information while capturing the impact of environmental factors on the physical-chemical state of the EP and its substrate. We learned that EP approaches gain power from working at different scales with dynamic techniques to follow bioproduct kinetics during hydrolysis (for active enzymes) and during other non-catalytic processes (using inactive enzymes). When using active EP, the EP/substrate interactions present continual changes that can be advantageously monitored using time-lapse high-throughput imaging systems. This information can be valuably completed using *in silico* techniques.

Recent breakthroughs in spectral and imaging techniques, data management, computational science and artificial intelligence set the scene for repositioning enzymes as essential probes to optimize bioproduct transformation.

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