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MINI-REVIEW

Harnessing the sponge microbiome for industrial biocatalysts

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Abstract

Within the marine sphere, host-associated microbiomes are receiving growing attention as prolific sources of novel biocatalysts. Given the known biocatalytic potential of poriferan microbial inhabitants, this review focuses on enzymes from the sponge microbiome, with special attention on their relevant properties and the wide range of their potential biotechnological applications within various industries. Cultivable bacterial and filamentous fungal isolates account for the majority of the enzymatic sources. Hydrolases, mainly glycoside hydrolases and carboxylesterases, are the predominant reported group of enzymes, with varying degrees of tolerance to alkaline pH and growing salt concentrations being common. Prospective areas for the application of these microbial enzymes include biorefinery, detergent, food and effluent treatment industries. Finally, alternative strategies to identify novel biocatalysts from the sponge microbiome are addressed, with an emphasis on modern -omics-based approaches that are currently available in the enzyme research arena. By providing this current overview of the field, we hope to not only increase the appetite of researchers to instigate forthcoming studies but also to stress how basic and applied research can pave the way for new biocatalysts from these symbiotic microbial communities in a productive fashion.

Key points

- *The sponge microbiome is a burgeoning source of industrial biocatalysts.*
- *Sponge microbial enzymes have useful habitat-related traits for several industries.*
- *Strategies are provided for the future discovery of microbial enzymes from sponges.*

Keywords Bacteria · Biodiscovery · Enzymes · Industrial applications · Marine biotechnology · Sponge holobiont

Introduction

Aquatic ecosystems are one of the final frontiers for biodiscovery. Oceans and seas comprise more than 71% of the Earth's surface and 97% of its water content (Schmitt 1997; Costello et al. 2010). While fulfilling an important role in global biogeochemical cycling (Falkowski et al. 2008), they are also the largest and most ancient reservoirs of the planet's biodiversity (Costello and Chaudhary 2016). Human interest in the sustainable exploitation of these marine environments led to the birth of marine biotechnology: the use of marine bioresources as a target or source of biotechnological applications (Gov and Arga 2014). These include novel bioactive compounds, notably those with biopharmaceutical applications, and valuable processes based on marine-derived systems with multiple industrial uses, particularly in the energy, food, nutraceutical and biorefinery sectors (Greco and Cinquegrani 2016; Barcelos et al. 2018). The dawn of the 'blue biotechnology' era is particularly evident by the increased interest in the field of marine natural products in the last few decades (Hu et al. 2011; Choudhary et al. 2017) and by the growing business interests in the global marine biotechnology market, which is projected to rise from $4.8 billion in 2020 to $6.4 billion for 2026 (Smithers Group 2015).

By definition, biocatalysts include free enzymes or whole microbial cells involved in catalytic conversions, which can be naturally found or formulated in different formats (extra- or

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intracellular, single or multiple) (Woodley 2017; Sheldon and Brady 2018). Biocatalytic entities are amongst the most sought after commodities in blue biotechnology. It is beyond the scope of this review to discuss the entire array of applications resulting from marine-derived biocatalysts, details of which can be found in several other reviews (Zhang and Kim 2010; Trincone 2010, 2011, 2013, 2017; Wang et al. 2016; Bruno et al. 2019). Nonetheless, it is worth pointing out that the variety of different environmental conditions that microorganisms encounter within these ecosystems has resulted in the evolution and ultimate adaptation of their biochemistry to cope with these extremes. This has culminated in a remarkable diversity of enzymatic entities and systems. Halotolerance, pH stability, hyperthermostability, barophilic behaviour, cold adaptivity, and, in particular, chemo-, regio- and stereoselectivity stand out as the principal habitat-related properties in many of these biocatalysts (Arnosti et al. 2014; Trincone 2011, 2013). These traits favour the use of these enzymes in commonly used harsh industrial reaction conditions, resulting in lower energy requirement, waste reduction and less highly toxic byproducts and, consequently, providing more cost-effective and greener processes (Liszka et al. 2012; Chapman et al. 2018).

Marine microorganisms are considered the primary source of biocatalysts and bioprospecting efforts are heavily concentrated in this area (Trincone 2012; Wang et al. 2016). The isolation and characterisation of carbohydrate-active enzymes (CAZymes), a wide range of peptidases, esterases, lipases and halogenases, have been increasingly reported from marine microbiomes, with a significant number of these enzymes exhibiting the aforementioned marine adaptation-associated traits (Sana 2015; Beygmoradi and Homaei 2017). Together with extremophiles and their multifarious extremozymes (Dalmaso et al. 2015), symbiotic microorganisms have also followed the same path of resilience in their immediate and ever-changing habitats within their hosts, constituting the holobiont unit (Rosenberg and Zilber-Rosenberg 2018). The vast biocatalytic repertoire of these host-associated microbial communities is directly responsible for a number of metabolic lifestyles: from the bloom of deep-sea life through chemosynthesis (Cavanaugh et al. 2013) to the thriving heterotrophic establishment on the polysaccharide-enriched surface of the coastal macrofauna (Egan et al. 2013).

Sponges (Porifera), the oldest phylum in the metazoan lineage (Yin et al. 2015), are well-known to harbour complex, diverse and, generally, stable microbial communities from all the domains of life (Hentschel et al. 2012; Webster and Taylor 2012; Thomas et al. 2016). Mostly found in the sponge connective-like multicellular layer, the mesohyl, these associated microorganisms can comprise approximately 35% of the sponge biomass (Vacelet 1975). Up to now, more than 50 prokaryotic phyla have been detected in association with these invertebrates, with *Proteobacteria* (and its *Gamma-* and *Alpha-* classes), *Actinobacteria*, *Chloroflexi*, *Nitrospirae*, *Cyanobacteria* and *Poribacteria* being the most abundant bacterial phyla and *Thaumarcheota* the dominant archaeal phylum (Pita et al. 2018). Conversely, eukaryotic members of these symbiotic communities, most notably fungi, have received much less attention (Taylor et al. 2007; Suryanarayanan 2012). *Ascomycota* and, to a minor extent, *Basidiomycota* are the most reported divisions, with the ubiquitous presence of the genera *Aspergillus*, *Trichoderma*, *Penicillium* and *Cladosporium* (Gao et al. 2008; Li and Wang 2009; Nguyen and Thomas 2018).

Not so reliant on taxonomic affiliation, a 'core' and functionally equivalent microbiota is a crucial player in the homeostasis of the sponge holobiont (Fan et al. 2012). These functions can be mainly categorised as follows: (i) metabolic features, involving mainly carbon and nitrogen metabolism, including degradation of recalcitrant carbohydrates, photoautotrophic pathways, ammonia oxidation, nitrogen fixation, nitrification, denitrification and vitamin biosynthesis, and (ii) defensive features, ranging from the presence of restriction-modification and clustered regularly interspaced short palindromic repeat (CRISPR)–Cas systems, phages and other mobile genetic elements to the production of secondary metabolites (Webster and Thomas 2016; Moitinho-Silva et al. 2017a; Pita et al. 2018), with the latter probably being the driver by competition as a means of shaping the symbiotic microbial assemblages (Lurgi et al. 2019).

These sponge microbe-derived bioactive metabolites are an ongoing source of interest from a biotechnological standpoint but do not form the specific focus of this review. This review will cover enzymes that are produced from these sponge-inhabiting microorganisms with applications in various industrial sectors. Modular biosynthetic enzymes, such as polyketide synthases (PKS), non-ribosomal peptide synthetases (NRPS) and/or hybrid PKS/NRPS, implicated in the production of antimicrobial and other therapeutically relevant substances, the whole-cell biocatalysts (recently reviewed by Birolli et al. 2019), enzymes directly isolated from sponges (and not their associated microbial communities) and enzyme inhibitors (Pandey et al. 2014; Ruocco et al. 2017), will also not form part of this review.

Up until now, there have been few attempts to compile a report on industrial enzymes from the sponge microbiome. Wang (2006) was the first to review this topic, reporting primarily on hydrolases from cultivable marine sponge bacterial isolates. Eight years later, in an all-encompassing review on the biotechnological potential of the sponge-associated bacterial microbiota, Santos-Gandelman et al. (2014) reported on different enzyme classes, including some from sponge metagenomes as well. To the best of our knowledge, the latest summarisation was carried out by Karthik and Li et al. (2019) on a handbook chapter about the symbiotic microbial communities of reef ecosystems. Despite considerably updating the

number of reports, including non-bacterial enzymatic producers, enzymes from the coral microbiome and those related to the biosynthesis of natural products were also included, with the sponge microbiome not constituting the sole focus as an enzymatic reservoir.

The present review aims to summarise the biocatalytic capacity of the sponge microbiome and discuss the potential industrial application of these enzymes. A number of representative enzyme groups will be highlighted, including their biochemical characterisation following purification and their industrially relevant features, when available. Alternative approaches to isolate novel biocatalysts from the sponge microbiota will also be critically presented. In doing this, we hope to lay the foundations for the further establishment of sponge microbial enzymology as an emerging resource for enzyme discovery.

The sponge microbiome as a source of industrial enzymes: a bird's eye view

The biocatalytic potential of the marine sponge microbiomes is chronologically outlined in Supplementary Table 1. Figure 1 depicts the number of (non-redundant) publications in the years 1997–2020 when searching the accessible literature in PubMed and Web of Science databases (search filters used were the words 'sponge', 'microorganism', 'bacteria', 'enzyme' and/or 'biocatayst' in the Title/Abstract fields and the words 'sponge-associated micro*', 'biocatalyst*', 'industrial*' and 'biotechnol*' in the TextWord field, applying the asterisk wildcard to extend the term selection). Given the full 23-year interval, it is clear that there was a sudden rise in the number of studies between 2008 and 2013, with 2019 alone exceeding the number of reports from the beginning of the decade.

Moving to the enzyme class, hydrolases dominate the field, followed by oxidoreductases (Fig. 2a). Amongst the hydrolases, glycoside hydrolases (GHs) and carboxylesterases together constitute almost 63% of the studies reported, followed by peptidases and studies where several exoenzymatic activities were detected, mostly following screening on solid media (Fig. 2b). For most of the GHs that were analysed, the complex biopolymer (starch, carboxymethylcellulose, chitin) was tested as substrate, both for qualitative detection and biochemical characterisation, which does not rule out the concomitant participation of other CAZymes, such as polysaccharide lyases (PLs) and the oxidative enzymes within the auxiliary activities (AAs) class to fully degrade the polysaccharide. Around a third of the studies involved enzyme purification and/or confirmation of activity with crude extracts, which were often supernatants recovered from the selected production media in which the isolate was cultured (Fig. 2c).

With respect to the microbial sources, to date, biocatalytic research from the sponge microbiome has relied substantially on cultivable strains, which account for around 75% of the total, followed by either DNA libraries and metagenomes, all of which were screened by a functional-based approach (Fig. 2d). Considering the taxonomic affiliation of the microbial producers, the domain *Bacteria* is, unsurprisingly, the most investigated (66%), followed by the fungal eukaryotes (25%) and *Archaea* (9%) (Fig. 2e). The bacterial enzyme producers are predominantly from the phylum *Proteobacteria*, with most members from the *Gamma-* class, followed by *Actinobacteria*, with the majority belonging to the *Streptomyces* genus, and *Firmicutes*, all belonging to the family *Bacillaceae*. *Ascomycota* is the dominant fungal division, chiefly involving the genera *Aspergillus*, *Cladosporium*, *Cadophora* and *Penicillium*. For the five archaeal cases, one was assigned to the phylum *Thaumarchaeota* (Schleper et al. 1997) and the other four to *Euryarchaeota* (Malik and Furtado 2019a, 2019b; Gaonkar and Furtado 2018, 2020).

Finally, only two reports implicated non-demosponge specimens (Borchert et al. 2017a; Cretoiu et al. 2012), which is to be excepted as the *Demospongiae* class covers up to 82.7% of the 9320 accepted poriferan species (van Soest et al. 2020), and is also overrepresented in the Sponge Microbiome Project (Moitinho-Silva et al. 2017b). *Aplysinia*, *Dendrilla* and

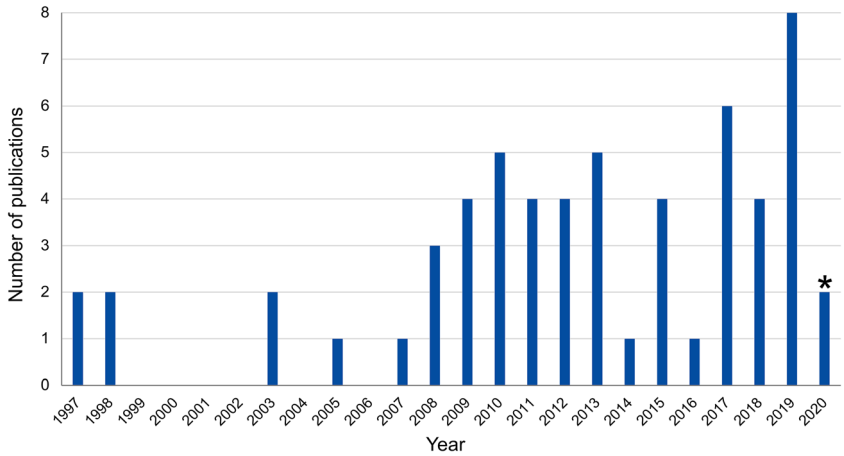

Fig. 1 Profile of the studies selected for the current review for the period 1997–2020. * = ongoing

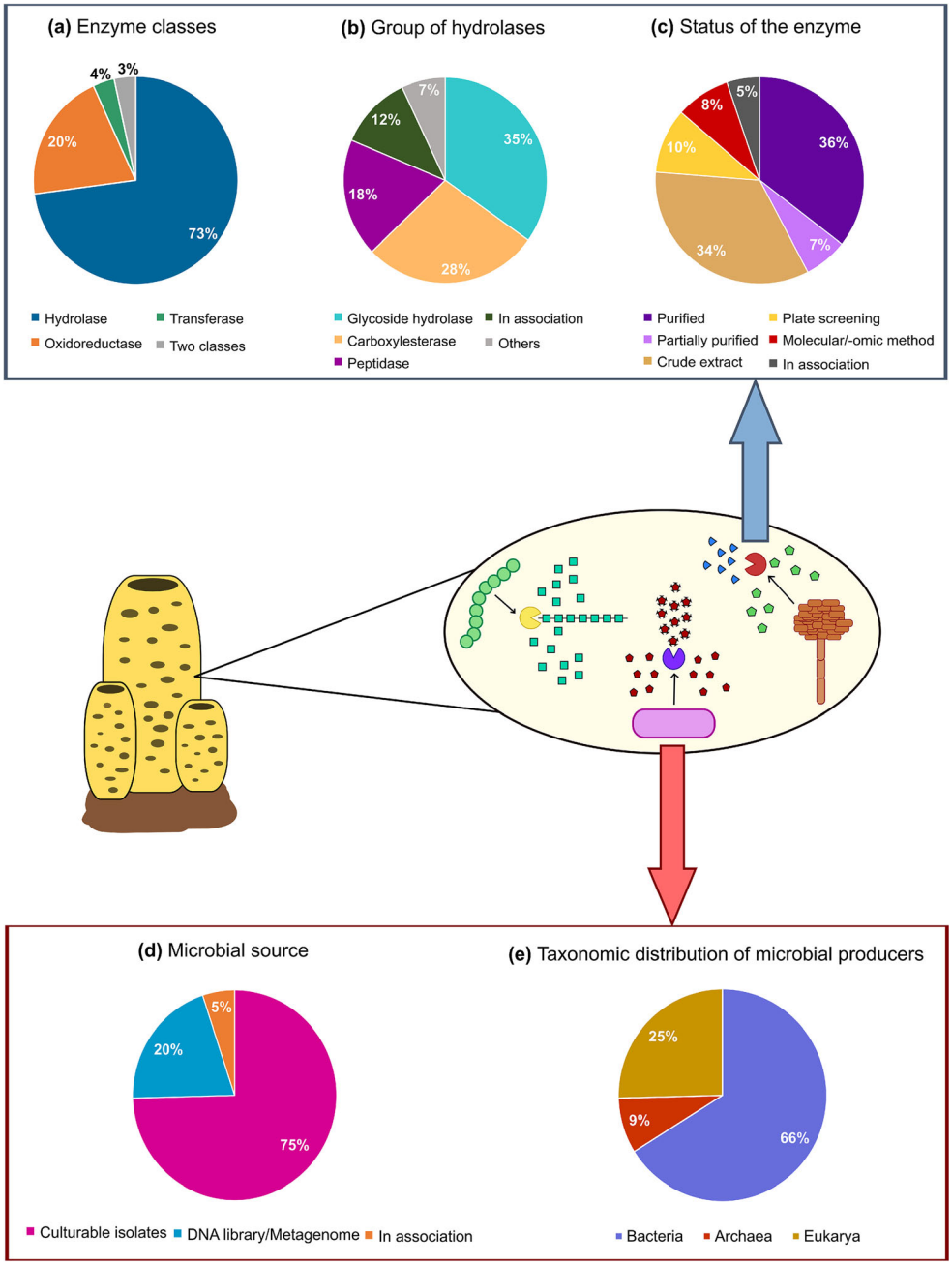

Fig. 2 A bird's eye view of the industrial enzymes reported from the sponge microbiome. **a** Enzyme classes. **b** Groups of hydrolases. **c** Status of the enzyme. **d** Microbial source. **e** Taxonomic distribution of microbial producers

Haliclona are the leading sponge genera from which enzyme-producing associated microorganisms have been isolated. Regarding the geographic origin of the source sponge specimens, the coastlines and sea zones of India, Brazil, Ireland and China are the most represented (Table S1).

Expanding on the hydrolytic CAZyme repertoire

As previously mentioned, most of the studies to date have focused on hydrolases, with almost 40% of these directed towards the activity of a GH and, in particular, those with broad polysaccharide-degrading capabilities. Following the first two reports (Mohapatra and Bapuji 1997; Schleper et al. 1997), a study was conducted to investigate a mild acid and salt-requiring glucoamylase-producing *Mucor* sp. strain associated with the intertidal sponge *Spirastrella* sp. The activity was estimated in crude extracts, with subsequent thin layer chromatography (TLC) analysis of the mono- and oligosaccharide hydrolysis products produced (Mohapatra et al. 1998).

A subsequent report involving a CAZyme from a sponge microbial symbiont occurred 5 years later. In a two-part parallel study, a *Streptomyces* sp. DA11 strain was isolated from

the South China Sea sponge *Craniella australiensis* and had its antifungal chitinase activity investigated by optimising enzyme production from the wild-type strain through Plackett–Burman and Box–Behnken designs (Han et al. 2008). A PCR-based cloning approach, followed by the heterologous expression, purification and biochemical characterisation of the enzyme, was then performed, with the recombinant chitinase showing an optimum pH 8.0 and temperature of 50 °C (Han et al. 2009). While rarely employed in other studies (Zhang et al. 2009; Selvin et al. 2012; dos Santos et al. 2016), even for other enzyme classes (Bonugli-Santos et al. 2010), this coupled strategy exemplifies the positive outcomes that can be achieved by applying statistical design on a large scale. In this case, chitinase production was 39.2-fold higher than the basic medium following optimisation, with a yield of 1559.2 U/g (36.43 U/mL) after 72 h of incubation (Han et al. 2008).

The enhancement of fermentation was also achieved by the incorporation of raw plant-derived biomass. In pursuit of cellulolytic enzymes produced by fungal strains isolated from the Egyptian sponge *Latrunuculia corticata*, a combination of solid-submerged fermentation (SSF) and saccharification with classical genetic engineering was employed (El-Bondkly and El-Gendy 2012). A hypercellulolytic mutant (Tahir 25) was generated by protoplast fusion of the three best producers, which were selected following a primary screen. Rice straw, wheat straw, corn straw, corncob and sugarcane bagasse were collected, processed and added to the fermentation media. A higher yield of all the enzymes from the cellulase complex was observed using the mutant strain, with sugarcane bagasse being the best inducer and giving stability up to 65 °C and pH 8.0. After a week of saccharification, the released reducing sugars were efficiently converted into ethanol by the *Saccharomyces cerevisiae* strain NRC2, and the enzymes were shown to be resistant to extraction with Tween 80. Thus, the combination of genetic engineering and ethanol production using cheap agricultural residues resulted in a reliable recombinant strain that could be used as a broad enzymatic cocktail, fulfilling some of the key needs for the biorefinery value chain (Cherubini 2010).

Batista-García et al. (2017) also incorporated agro-industrial lignocellulosic byproducts (maize stover and wheat straw) as substrates for SSF when characterising lignocellulosic enzymes from three deep-sea psychro- and halotolerant sponge-derived ascomycetes. As well as confirming the activities of carboxymethylcellulase (CMCase), xylanase, peroxidase and phenol oxidase, the authors also included a saccharification step with alkali-processed pure cotton fibres and the culture supernatants as the crude enzyme, adapted under improved cultivation conditions for each fungal strain. Beyond all relevant properties of broad pH tolerance for the xylanase and broader salt tolerance for the CMCase in each isolated case, the higher fibre saccharification was verified when the culture supernatant was supplemented with carboxymethylcellulose (CMC). Remarkably, the concentration of the produced reducing sugars released after treatment of the cotton fibres with the culture supernatants were 5–6 μmol, while commercial cellulolytic cocktails, such as Accellerase™ 1500 (Genencor International Corporation), produced 4.5 μmol of reducing sugars directly from CMC (Molina et al. 2014). This highlights the real potential of these lignocellulosic-degrading enzymes from sponge-associated ascomycetes to act directly on raw substrates, which would be particularly useful for biofuel production.

A similar approach was employed with two marine sponge-derived fungal isolates, confirming the efficient removal of lignin by pre-treatment of sugarcane bagasse, followed by their saccharification (Santos et al. 2017). Not conducted systematically in the same fashion as with the aforementioned ascomycetes (El-Bondkly and El-Gendy 2012; Batista-García et al. 2017), fungi isolated from an Antarctic sponge had their xylanases tested with wheat bran, wheat straw, oat bran (Del-Cid et al. 2014), sugarcane bagasse and rice straw (dos Santos et al. 2016). The xylanolytic enzymes displayed a mild acid behaviour and relatively wide thermostability, which was apparently dependent on the presence of low molecular weight solutes in the culture media. One xylanase, produced by a *Cladosporium* sp. strain, retained 30% of its activity at low temperatures, a clear habitat-adaptative feature (Del-Cid et al. 2014).

Cellulase and xylanases have been successfully used together for the production of biofuels and probiotics, food processing and in the bio-deinking of waste paper (Bajaj and Mahajan 2019). However, the synergistic applications of these two multi-component enzymes may not always be desirable. Active at higher temperatures and under alkaline conditions, cellulase-free xylanases are highly favoured for biobleaching and are involved in the selective degradation of the hemicellulose matrix, minimising the requirement for chlorine treatment, which results in a superior grade dissolving of pulp and increased paper brightness (Walia et al. 2017). After confirming a lack of CMCase or cellobiase activities on solid media, Malik and Furtado (2019b) tested the xylanase production in *Cinachyrella cavernosa*-associated haloarcheons in liquid media. The crude extract of one of the strains, *Halococcus thailandensis* GUMFAS7, produced a salt-dependent and relatively thermotolerant multimeric xylanase, with maximum activity at pH 5.0. The same research group reported a xylanase-free cellulase from another haloarchaeal isolate, *Haloferax sulfurifontis*, with activity at 5 M NaCl and throughout a broad range of pH and temperature (Malik and Furtado 2019a). Despite not being alkaliphilic, both endo-type enzymes have an extreme halophilic nature that would be of particular interest for xylanases applied in marine/salt food processing and waste treatment (Teo et al. 2019) and cellulases in the direct recovery of cellulose pre-treated with denaturing ionic liquids during biofuel production (An et al. 2015).

The focus on plant-derived or lignocellulosic biomass substrates for the reported GH examples reveals an aspect of the ongoing research that remains largely underexplored: a surprising disregard for marine polysaccharides, particularly the constituents of algae cell walls. Given the ecological interactions between sponge and macroalgae (Easson et al. 2014) and the constant flow of seaweed detritus as dissolved organic carbon (DOC) through the sponge loop (Pawlik and McMurray 2020), it is puzzling that little effort has to date focused on prospecting enzymes involved in the degradation of marine biomass by sponge-inhabiting microorganisms. They are likely to release GH and/or PLs to initially convert these recalcitrant polysaccharides, encountered through the sponge filtering activity, into simpler organic carbon sources that can subsequently be transported into the bacterial cell and assimilated via central metabolic pathways. In fact, some of the major bacterial members found in the sponge microbiome, mostly from the *Alpha-*, *Gammaproteobacteria* (*Proteobacteria*) and *Flavobacteria* (*Bacteroidetes*) classes, are recognised producers of biochemically characterised agarases (Jahromi and Barzkar 2018), carrageenases (Zhu et al. 2018), alginate-lyases (Zhu and Yin 2015), laminarinases (Labourel et al. 2015), fucosidases (Dong et al. 2017) and ulvan-lyases (Reisky et al. 2019).

To date, there have been just two reports of exoagarases from sponge-derived *Cytophaga* (Imhoff and Stöhr 2003) and *Bacillus* (Li et al. 2007) strains, both observed following screening on solid media. Putative gene sequences of β-agarases (family GH50), α- (families GH5 and GH36) and β-galactosidases (family GH2) and α-L-fucosidases (family GH95) have been detected in single-amplified genomes (SAGs) of the candidate phylum "*Poribacteria*", still regarded as a role model for true sponge symbionts (Kamke et al. 2013; Podell et al. 2019). Considering the variety of industries where marine carbohydrate-hydrolysing enzymes can be applied (Trincone 2018), particularly with respect to the anionic oligosaccharides with human health-related bioactivities that can be produced by these enzymes (Michel and Czjzek 2013; Imran and Ghadi 2019), the isolation, identification and subsequent biochemical characterisation of marine polysaccharide-depolymerising and modifying biocatalysts from the sponge microbial consortium should be receiving more attention.

In relation to discovering new CAZyme entities by using other saccharidic substrates, glycoconjugates have emerged as compelling alternatives. Sialic acid, a derivative of *N*-acetylneuraminic acid (Neu5Ac) and an example of this wide group of chemically modified sugars, has been identified in sponges (Garrone et al. 1971; Harrison and Cowden 1981), while sialyltransferases (STs) appear to be essential in cellular aggregation events in these invertebrates (Müller et al. 1977, 1978, 1979; Petit et al. 2015). Other sulphated and acidic glycans, such as chondroitin, also mediate cellular adhesion and recognition resulting from their interactions in marine sponges (Kamerling and de Souza 2011). Genomic data from certain sponge microbial symbionts have uncovered their potential capacity to degrade sialylated molecules and other glycosaminoglycan/proteoglycan components of the sponge extracellular matrix. This is believed to be achieved through the coordinated action of exosialidases (family GH33), other specific disaccharidases, galactosamine and uronic acid hydrolases, together with sulfatases and *N*-acetylneuraminate lyases (Kamke et al. 2013; Bayer et al. 2018; Podell et al. 2019). Catabolising these glycosylated ligands is one of the strategies used by these microorganisms to interact with their eukaryotic hosts, similar to what has been characterised in the human microbiome (Ndeh and Gilbert 2018) and bacterial pathogens, in the case of sialic acids (Haines-Menges et al. 2015). Chemoenzymatic synthesis of valuable sialoconjugates (including surface antigens, recombinant fusion proteins and oligosaccharides) and in-depth analysis of glycan structures are amongst some of the promising applications of marine bacterial sialidases, in particular STs (Fukano and Ito 1997; Yamamoto 2010; Kamimiya et al. 2013; Kang et al. 2015). Given the fact that sponge mesohyl is a selective microenvironment for microorganisms that are capable of degrading these glycoconjugates, it is clear that poriferan-associated microorganisms should be further explored as a source of enzymes involved in glycoconjugate metabolism.

Employing various -omics-based approaches to the discovery of novel GHs from the sponge microbiome is likely to be a useful strategy in the immediate future. This could range from an initial PCR-based detection of the genes encoding the enzymes being targeted (Sibero et al. 2019), to the application of functional and genetic screening of metagenomic libraries focusing on the biocatalyst-coding gene (Cretoiu et al. 2012) to ultimately using genome mining of cultivable bacterial isolates, coupled with the heterologous expression and characterisation of the industrially relevant enzyme (Borchert et al. 2017a). In this sense, lessons can be learned from recent reports, such as exploration of the genome, transcriptome and secretome of the laccase-producing *Peniophora* sp. CBMAI 1063 associated with the marine sponge *Amphimedon viridis*. The multi-omics strategy confirmed that this strain is a strong candidate for the production of a cocktail of lignocellulolytic enzymes, corroborated by testing the purified oxidoreductase on pre-treated sugarcane bagasse (Brenelli et al. 2019).

CAZymes comprised 3.7% of the metaproteome analysed from the Mediterranean sponge *Aplysinia aerophoba*. From the detected CAZyme families, those involved in the metabolism of chitin and *N*-acetylglucosamine, followed by enzymes responsible for the bioconversion of glycoproteins and glycolipids and the degradation of complex polysaccharides were the most predominant groups present (Chaib De Mares et al. 2018). Despite the low frequency of GH verified for the microbiome of marine sponges in relation to other

habitats (Berlemont and Martiny 2016), it should not be ignored the huge amount of information steadily accumulated from the -omics surveys. It is fundamental to increase efforts in biochemically characterising these putative carbohydrate hydrolases, adopting rational strategies similarly to those that have been employed recently (Schultz-Johansen et al. 2018; Helbert et al. 2019; Nguyen et al. 2019).

Versatile carboxylesterases

Carboxylesterases are conventionally classified as lipases (EC 3.1.1.3, triacylglycerol hydrolases), which are active on water-insoluble longer fatty acid chains and subject to interfacial inactivation, and esterases or non-specifically esterases (EC 3.1.1.1, carboxyl ester hydrolases) with short acyl chain ($>C_6$) as substrates (Chahinian and Sarda 2009). There has been a growing interest in marine-derived carboxylesterases, as they can be employed in food and feed preparation, in the detergent industry, in leather and textile processing, in pharmaceutical synthesis and in biodiesel production (Navvabi et al. 2018). Open and deep-sea waters, coastal sediments and hydrothermal vents are amongst the leading microbial sources of carboxylesterases (Patnala et al. 2016; Zhang et al. 2017). After the polysaccharide-degrading CAZymes, carboxylesterases are the second most common group of hydrolases that have been characterised from the sponge microbiome (Fig. 2b), and have mostly been identified by functional metagenomics-based approaches. Except for a defensive phospholipase A2 (PLA2) from the *Streptomyces* MSI051 strain and the extract of its original sponge (Selvin 2009), together with a report of antibacterial activity credited to lipase-encoding genes in the *Cymbastela concentrica* metagenome (Yung et al. 2011a), the other characterised sponge microbial lipases and esterases possess at least three or more habitat-adapted features, such as alkaliphily, halotolerance and stability to surfactant agents and organic solvents.

In addition to its alkaline and psychrophilic nature, the lipase purified from the *Dendrilla nigra*-associated *Pseudomonas* sp. MSI057 strain had its relative activity increased by approximately one third at low concentrations of detergents, including Triton-X and SDS, and was quite stable under acetone extraction (Kiran et al. 2008). A moderately alkaline lipase was also detected from a *Bacillus pumilus* B106 strain, a biont from the South China Sea sponge *Halichondria rugosa* (Zhang et al. 2009). In stark contrast to the *Pseudomonas* case, the enzyme had an optimum temperature of 50 °C and was remarkably stable at high salt concentrations, retaining 78% activity from 0 to 150% g/kg of KCl, and was inducible in the presence of 10% methanol.

Following the screening of a *Haliclona simulans* metagenomic library, Selvin et al. (2012) identified a novel halotolerant lipase. The enzyme exhibited a broad pH and thermotolerance associated with an extreme halotolerance (99% activity retained at up to 5 M NaCl). The activity was also positively influenced by detergents and organic solvents, revealing a biocatalyst with potential utility in various industrial scenarios, such as the manufacturing of marine products. Another functional metagenomics-based study resulted in the isolation and identification of a *lipA* gene, with carboxylesterase activity from the microbiome of the sponge *Ircina* sp., sampled in the South China Sea (Su et al. 2015). Biochemical characterisation of the heterologously expressed LipA protein determined the thermostability and alkaline nature of the Ca^{2+}-dependent lipase, whose activity was also enhanced by methanol, isopropanol and acetone, a particular advantage for biodiesel production and transesterification and ester synthesis by eliminating the need for solvent removal or solvent-free systems.

A mildly alkaline active acetylcholinesterase producing *Arthrobacter ilicis* was isolated from the marine sponge *Spirastrella* sp. (Mohapatra and Bapuji 1998), and two recombinant esterases, named EstB1 and EstB2, were isolated following the extensive screening of a genomic library from an *A. aerophoba*-associated *Bacillus* sp. strain (Karpushova et al. 2005). Both these short-chain fatty acid-hydrolysing esterases differed in their sequence-based classification, which was reflected in minor differences in their biochemical properties: EstB1 is slight thermophilic, while EstB2 exhibited more mesophilic characteristics. Despite their similar properties in terms of substrate affinity, inhibition by metal ions and stability in the presence of organic solvents (stability up to 50% of DMSO), EstB1 had a relatively higher NaCl and KCl tolerance than EstB2.

Another functional-directed screening of a metagenomic library of *Hyrtios erecta* using a substrate formulation of Tween 20 and $CaCl_2$ resulted in the isolation of an esterase-coding gene, *estHE1* (Okamura et al. 2010). The His-tagged EstH1 protein was found to have specificity towards $>C_6$ fatty acids (excluding triglycerides and tributyrin—C_4), and retained activity when kept between 25 and 55 °C. It also attained 55% of its activity at 40 °C after 12 h, a thermostability that would be convenient in industrial set-ups demanding longer storage times. What is particularly unique about this esterase is its interesting behaviour under rising concentrations of NaCl: with up to 1.9 M of the salt, enzyme activity decreased steadily; however, a subtle recovery took place when the NaCl concentration was adjusted to 3.8 M. A likely structural flexibility inherent to this esterase family was suggested as the true explanation behind this peculiar property (Okamura et al. 2010).

In another study, Borchert et al. (2017b) isolated a novel esterase, 7N9, from the metagenome of the deep-sea sponge *Stelletta normani*. The combined slight alkaliphily, salt tolerance, reactivity to metal ions and, mainly, psychrophily

highlighted its potentiality for low-temperature processes, such as in the manufacture of food, thermolabile pharmaceutical products and cold-wash detergents. Subsequent protein homology, phylogenetic analysis and molecular docking analyses indicated the potential relatedness of the 7N9 esterase with sequences from the not-yet cultured sponge bacterial symbiont *Candidatus* "Entotheonella", also hinting the role of this esterase in niche adaptation in harsh conditions.

An interesting esterolytic enzyme from a sponge-associated microorganism has recently published (Almeida et al. 2019). Underpinned by the discovery of a polyethylene terephthalate (PET)-hydrolytic strain, *Ideonella sakaiensis* (Yoshida et al. 2016), and subsequent structure elucidation of its PETase (*Is*PETase) (Liu et al. 2018), several research groups have attempted to identify PETase homologues from different ecosystems. Following an in silico-based screening to reveal potential PETase homologues from both terrestrial and marine *Streptomyces* isolates, Almeida et al. (2019) interrogated the genomes of 52 streptomycete strains. Heterologous expression of one candidate PETase-like gene, *sm14est*, which was derived from a *Haliclona simulans*-associated *Streptomyces* sp. SM14 strain, was carried out and the protein was shown to have polyester-degradation activity on polycaprolactone (PCL), a preliminary substrate used for the screening of plastic hydrolysis. It is likely that marine sponge-derived *Streptomyces* isolates may have had previous exposure to plastics and/or microplastics present in seawater, resulting from their association with the filter-feeding animal (Almeida et al. 2019). Enzyme promiscuity, a term which describes an enzyme with a broad substrate range and a preference for accepting bulkier substrates, has been reported for esterases and may provide an explanation for the PCL hydrolysis observed with SM14 (Martínez-Martínez et al. 2017b). It should also be noted that PCL is considered a biodegradable polymer, whereas PET has a complex structure, with properties such as aromaticity and crystallinity remaining a key challenge in enzymatic PET degradation (Goldberg 1995; Kawai et al. 2019). Upon comparison of the SM14 amino acid sequence and overall structure with *Is*PETase, both enzymes were shown to contain the conserved catalytic triad as well as the serine hydrolase motif (Almeida et al. 2019). This work opens up a new area of exploration for polyesterases from sponge-derived microbial strains, offering a supplementary microbial source to solve the crisis caused by the ever-increasing plastic pollution on aquatic environments (Amaral-Zettler et al. 2020).

Leveraging up the proteolytic reservoir

Despite being the most economically important group of marketed enzymes (Razzaq et al. 2019), proteases derived from sponge-associated microorganisms have been largely overlooked when compared with the aforementioned hydrolase groups. Most work to date on the purification and biochemical characterisation of marine sponge-derived proteolytic enzyme has centred on a virulence-related collagenase (Bhattacharya et al. 2018), with little focus on its potential industrial uses.

Thus, Webster et al. (2002) initially isolated this collagenolytic *Pseudoalteromonas agarivorans* NW4327 strain (Choudhury et al. 2015) from a necrotising Great Barrier Reef sponge, *Rhopaloeides odorabile*, fulfilling the Koch's postulates of its pathogenic potential by electron microscopy and an Azocoll-degrading assay. Next, this collagenase was purified, biochemically characterised and found to have a mildly acidic, mesophilic and high proteolytic profile. It demonstrated an ability to degrade gelatin, casein, bird feather and collagenous spongin obtained from different demosponge skeletons. Interestingly, collagenase production reached its peak when natural seawater and the specific host spongin were supplemented to the production media of the strain NW4327, reflecting the importance of the original symbiont microenvironments for expression of the enzyme (Mukherjee et al. 2009). Finally, the purification and characterisation of this collagenase were completed by integrating structural prediction and functional sequence analysis, identifying them within the U32 peptidase family (Bhattacharya et al. 2018, 2019). Additionally, four collagenase-producing bacterial isolates (*Vibrio* spp. and *Bacillus* sp.) have also been isolated from the demosponge *Cymbastela concentrica*, with one gene homologous to an annotated collagenase formerly found on the host metagenome which may be a potential colonisation/virulence factor (Yung et al. 2011b).

Apart from their ecological importance, microbial collagenases have a wide range of applications in the food and nutrition industries, particularly in the extraction of bioactive collagen-derived ingredients, in the preparation of collagen hydrolysate and in meat and seafood processing (Pal and Suresh 2016). Laboratory treatments for the detachment of tissue culture cells and the medical healing of burns, ulcers and diseases with an accumulation of fibrous plaques are amongst the commercially available uses for these enzymes. Marine environments are likely to be a good source of collagenolytic proteases since this polymer does not appear to accumulate in these habitats (Zhang et al. 2015; Bhagwat and Dandge 2018). Indeed, collagenases of some free-living *Pseudoalteromonas* strains have been successfully applied in meat tenderisation (Zhao et al. 2012) and for the production of antioxidant hydrolysates from seafood waste (Yang et al. 2017); it is, therefore, likely that novel biotechnologically relevant collagenases can be discovered in sponge-associated microbial strains.

Industrially relevant peptidases from sponge-associated prokaryotes for their part have been the focus of some research interest. Many of the proteases discovered from sponge

microbiomes display biochemical properties matching with the previously related industrial proteases, such as resistance to elevated temperatures and non-aqueous organic solvent-based media (Barzkar et al. 2018). Shanmughapriya et al. (2008) purified a thermotolerant and alkaline protease from a potential gelatinolytic *Roseobacter* strain, isolated from the marine sponge *Fasciospongia cavernosa*. The enzyme retained almost 92.5% and 89% of its activity, respectively, under pH 9.0 and at 50 °C.

Working with halophilic microbial strains isolated from *Haliclona* specimens sampled in the Indian seashore, Gaonkar and Furtado (2018) confirmed the proteolytic activity in the crude extract of the *Halococcus* sp. GUGFAWS-3 strain, following cultivation in a liquid media supplemented with 25% NaCl. This protease was subsequently characterised as an extremozyme (Gaonkar and Furtado 2020). The 65-kDa monomeric cysteine protease retained 85% of its activity at 5 M NaCl, had an optimum temperature of 70 °C and attained 82.4% of its activity at 80 °C. Moreover, this thermotolerant enzyme was unaffected by organic solvents, being stimulated in particular by acetone and sodium benzoate, and even remaining high levels of stability in the presence of detergents and surfactants, making it ideally suited in the detergent industry or elevated temperature-dependent processes. In addition, inhibitory/disruptive activities on the formation and maturation of *Staphylococcus aureus* biofilm were also confirmed for this haloarchaeal protease.

Marine sponge-derived bacteria have also been the source of proteolytic enzymes with biomedical applications, with fibrinolytic proteases being particularly important in this respect. A fibrinolytic protease was isolated from a highly caseinolytic *Streptomyces radiopugnans* strain, following the screening of proteolytic bacteria isolated from three different demosponges. Besides the ability to degrade casein plasminogen, this moderately alkaline and thermostable protease was shown to be able to release the red blood cells (RBCs) at a higher efficiency (91, 100 and 100% at 10, 20 and 30 min) than the drug streptokinase (Sigma-Aldrich) (87, 94 and 100% at 10, 20 and 30 min) in the clot lysis assay. Thus, it is clear that this enzyme could be useful as a thrombolytic agent for cardiovascular disorders, such as acute myocardial infarction and strokes (Dhamodharan et al. 2019). New bacterial fibrinolytic proteases have been reported for a number of bacterial genera, particularly *Bacillus* (Singh and Bajaj 2017), and, in truth, this Indian research group has been successful in finding potential antithrombotic actinoproteases in several marine- and sponge-derived *Streptomyces* strains (Jemimah Naine et al. 2016; Mohanasrinivasan et al. 2017; Verma et al. 2018), which prompted their screening of different sponge specimens (Dhamodharan and Chandrasekaran 2018, Dhamodharan et al. 2019).

The action of L-asparaginases reduces one of the main nutrients required by cancer cells, L-asparagine, making them important antitumor agents, specifically in the treatment of acute lymphoblastic leukaemia (ALL). Marine microorganisms have been shown to be prolific producers of these hydrolases, which to date have mostly been identified from the *Actinobacteria* and *Ascomycete* phyla (Izadpanah Qeshmi et al. 2018). One such example is an L-asparaginase which has been characterised from *Streptomyces noursei* MTCC 10469, isolated from *Callyspongia diffusa*. The optimal activity of the enzyme was shown to be 50 °C and pH 8 (Dharmaraj 2011). Two distinct L-asparaginases were characterised from an *Aspergillus* sp. strain ALAA-2000 associated with the marine soft sponge *Aplysinia fulva*. One of the purified L-asparaginases, AYA-1 (25 kDa), was thermostable (67 °C) and had an optimal alkaline pH (pH 10.0), while AYA-2 (31 kDa) was optimally active at more neutral pH values (pH 6.0) and was less thermostable (47 °C). Neither activities were affected by EDTA, indicating that both AYA-1 and AYA-2 were not metalloproteases (Ahmed et al. 2015). In this way, it is clear that sponge-associated microbes are potentially good reservoirs of chemotherapeutical enzyme-based drugs, such as L-asparaginases.

Other hydrolases

Ethyl carbamate (urethane) is a highly toxic byproduct released from urea during the alcoholic fermentation of several beverages and food, generally under low pH conditions (Weber and Sharypov 2009). Acidic and alcohol-tolerant ureases and urethane hydrolases (EC 3.5.1.75) are thereby valuable in helping to reduce or eliminate the levels of these harmful carcinogens (Zhang et al. 2016). One of the first reports of a marine-derived urethanase came from a *Spirastrella* spp.-derived *Micrococcus* sp. strain (Mohapatra and Bapuji 1997). The enzyme was partially purified and shown to be optimally active at pH 5.0 and 45 °C, as well as being unaffected by the presence of 10% ethanol, and retained around 82% of its activity at levels of 20% alcohol.

Unfortunately, no urease has to date been purified and biochemically characterised from a sponge-associated microbial strain or from a sponge metagenome. Beyond the sole confirmation of producers by solid media screening (Feby and Nair 2010; Gaonkar and Furtado 2018), Su et al. (2013) assayed urea utilisation by the sponge *Xestospongia testudinaria* and performed a functional microbial diversity analysis, by constructing genomic and cDNA libraries of the *ureC* gene, which encodes the alpha and largest urease subunit. The sponge slurry was positive for urea elimination, which coincided with an ureolytic *Marinobacter litoralis* strain being isolated from the same host species. The common *ureC*-positive operational taxonomic units (OTUs) to both libraries matched with the sequences of the same gene in *Proteobacteria*, also found as predominant in the microbial

community, affirming the active transcription of the urease genes. It is believed that urea degradation is a widespread trait in sponge-associated microorganisms (Moitinho-Silva et al. 2017a), and urease expression has been employed as a marker to track the effects of ocean acidification in sponge-associated microbial communities (Botté et al. 2019). However, a plethora of biotechnological applications known for this enzyme has not yet been fully recognised in this holobiont, such as biocementation (Sarayu et al. 2014), diagnostic determination of urea levels in organic fluids (Qin and Cabral 2002), removal of heavy metal (Li et al. 2013) and insecticides (Kappaun et al. 2018).

Non-hydrolase universe

Lignin is a polyaromatic component of plant cell walls and its degradation is achieved through the concerted action of three sets of ligninolytic enzymes, namely lignin peroxidases (LiP, EC 1.11.1.14), manganese peroxidases (MnP, EC 1.11.1.13) and multicopper oxidases, in particular laccases (EC 1.10.3.2) (Janusz et al. 2020). These enzymes are mostly employed in biorefinery, food and textile and in paper industry applications; notwithstanding, their other biotechnological uses include enzyme-based diagnostics, nanotherapeutic biofuel cells and the generation of personal-care cosmetics and in the preparation of paint resins and furniture drying (Mate and Alcalde 2016).

A diversity of crude ligninolytic activities have been reported from a number of basidiomycetes strains isolated from the sponges *Amphimedon viridis* and *Dragmacidon reticulata* (Bonugli-Santos et al. 2010). Further screening involving Remazol Brilliant Blue R (RBBR), a dye commonly discharged by the textile industry, confirmed both MnP and LiP activities by these demosponge-derived fungi (Bonugli-Santos et al. 2012). One of these, the strain *Tinctoporellus* sp. CBMAI 1061, had the RBBR degradation products isolated and characterised by spectroscopic analysis, confirming the potential of the fungal ligninolytic arsenal in the metabolisation of this synthetic dye (Rodriguez et al. 2015).

Subsequent RT-PCR of laccase gene expression in the fungi isolated from *D. reticulata* resulted in the identification of a basidiomycete strain, namely *Peniophora* sp. CBMAI 1063, which displayed good levels of laccase expression (Passarini et al. 2015). Increased MnP and laccase expression in this strain were accompanied by decolorisation of the azo dye Reactive Blue 5 (RB5) (Bonugli-Santos et al. 2016). Marine sponge-derived fungal strains have also been reported to be able to remove polycyclic aromatic hydrocarbon (PAH) under saline and alkaline conditions. For example, the strains *Tolypocladium* sp. strain CBMAI 1346 and *Xylaria* sp. CBMAI 1464 were shown to remove pyrene and benzo-a-pyrene, with the former degrading 95% of the pyrene present following 7 days of incubation, highlighting the potential of marine sponge-associated fungi to degrade environmental pollutants in saline environments (Vasconcelos et al. 2019). Following further transcriptome (Otero et al. 2017), fermentative scaleup (Mainardi et al. 2018), genome and secretome studies (Brenelli et al. 2019) on the aforementioned *Peniophora* sp. CBMAI 1063 strain, a laccase was purified and biochemically characterised from this marine basidiomycete which displayed a very good ability to degrade sugarcane lignin. Hence, these outcomes indicate the utility of this laccase as an efficient biocatalyst for the bioconversion of industrial waste streams containing lignin and, especially, those generated by the feedstock-processing biorefineries (Brenelli et al. 2019).

Due to the presence of a halogen group, naturally occurring organohalogens exhibit a diversity of pharmaceutical-related bioactivities, including important antimicrobial, anticancer, immunomodulating, hormonal and halogen-harbouring agents currently on the market (Gribble 2004; Wagner et al. 2009). Generally classified in agreement with their required coenzymes or chemical cofactors, halogenases are responsible for the incorporation of these halides into organic molecules, often as tailoring reactions in the final steps of biosynthetic pathways. Nowadays, these biocatalysts are being targeted due to their potential use as environmentally friendly alternatives for selective chemical synthesis (Schnepel and Sewald 2017; Gkotsi et al. 2018). Marine sponges with other sessile organisms, such as corals, seaweeds and hemichordates, have been majorly prospected as sources of these biologically active halogenated molecules (Gribble 2015), where they are believed to play a defensive role (Atashgahi et al. 2018). Several different classes of organohalogens, many of which are brominated, have thus far been characterised in sponges and their associated microbiota (Li and Shi 2020).

While there have been reports on the detection of halogenases from sponge microbial symbionts, coupled with the characterisation of the microbial diversity associated with the host (Öztürk et al. 2013; Rua et al. 2018; Gutleben et al. 2019), there are limited reports on the isolation and biochemical characterisation of halogenases in these holobionts. One such report (Smith et al. 2017) involves a gene cluster encoding a putative flavin-dependent halogenase from the metagenome of a specific chemotype of the marine sponge *Theonella swinhoei*, the alleged source of cytotoxic keramamides (Wakimoto et al. 2015). Whereas the authors speculate that a specific microbial symbiont is the likely source of the gene cluster encoding this singular halogenase, no microbial analysis was performed on *T. swinhoei*, precluding the assignment of the enzyme to a microbial source (Smith et al. 2017).

Using functional metagenomics and single-cell genomics, Bayer et al. (2013) discovered several $FADH_2$-dependent halogenases from the microbial consortia of several

Mediterranean and Caribbean sponges. The sponge microbial-derived halogenase sequences segregated into four unique clusters, that were phylogenetically unrelated to previously known halogenases. Additionally, they were assigned to bacterial symbionts from various bacterial phyla and were suggested to have different functions than the expected halogenation of defensive secondary metabolites. Tryptophan halogenase sequences were identified in the metagenome of *Crambe crambe* specimens and in one cultivable *Psychrobacter* isolate, despite this genus not being abundant amongst the bacterial communities within the sponge microbiome. The identities of the predicted halogenases also varied considerably with those deposited in the databases, indicating some degree of novelty in these biocatalysts and that they might be engaged in the biogenesis of yet uncharacterised active halogenated compounds (Öztürk et al. 2013).

Employing metagenomics sequencing, Rua et al. (2018) have determined the distribution pattern of halogenases and the associated microbiomes in the Brazilian demosponges *Agelas* spp. and *Tedania* spp., from which several bioactive bromopyrrole alkaloids had previously been identified. They observed a higher abundance of flavin-dependent halogenases, mostly from rare members of the sponge microbial communities. Phage genes were found to be enriched in one species, namely *Tedania brasiliensis*, supporting the author's hypothesis that the presence of these halogenated compounds in this sponge could be mediated by viral transduction.

Subsequently, four distinct clades of tryptophan halogenases were detected by pyrosequencing in six Mediterranean and Caribbean *Aplysinia* specimens, most of which were distantly related to hitherto described microbial halogenases (Guttleben et al. 2019). Further correlation with 16S rRNA gene-based diversity data demonstrated some specific sponge microbial-specific taxons, such as *Chloroflexi*, as the main sources of these putative halogenases. Interestingly, the dominant tryptophan halogenase sequences in the Mediterranean *A. aerophoba* were closely related to the above-mentioned *C. crambe*-derived *Psychrobacter* halogenase (Öztürk et al. 2013).

While halogenases are generally regarded as having key physiological roles in the biosynthesis of natural products, dehalogenases for their part are often associated with respiratory processes and have in particular been shown to be involved in the degradation of anthropogenic-derived toxic compounds (Agarwal et al. 2017). Considering the pressure imposed by the high contents of natural and man-made halogenated compounds in marine ecosystems and their accumulation in sponges due to their filter-feeding activity, it would seem likely that certain members of the sponge microbiota would be capable of metabolising these organohalides.

After a long-term enrichment culture regime from the brominate-containing *A. aerophoba* sponge, Ahn et al. (2003) isolated an anaerobic deltaproteobacterium that could degrade several halophenols. This isolate was subsequently described as a new species, namely *Desulfoluna spongiiphila* AA1^T, which was exclusively found in sponges and was characterised as a sulfidogenic non-obligate organohalide-respiring bacterium, capable of using various brominated compounds, but not other halogens as electron acceptors (Ahn et al. 2009). Subsequent '-omics'-based approaches (Liu et al. 2017, 2020; Peng et al. 2020) uncovered the genetic organisation and metabolic requirements for the synthesis and action of this diverse array of reductive dehalogenases (RDases). Regrettably, none of these *D. spongiiphila*-derived RDases have as of yet been biochemically characterised or studied for their potential use in bioremediation strategies.

Irrespective of this, there are reports on the purification and biochemical characterisation of dehalogenases from other sponge bacterial cultivable isolates. With an adapted enrichment medium, Huang et al. (2011a) initially isolated potential bacterial dehalogenase producers from the sponge *Hymeniacidon perlevis*, sampled in a highly polluted area containing chlorinated industrial waste. Firstly, a *Paracoccus* sp. DEH99 strain showed a 2-haloacid dehalogenase activity, which was characterised from the crude cell extracts (Huang et al. 2011b). The enzyme showed a broad substrate specificity, including iodoacetic acid and 2-chloropropionic acid (CPA), which was selectively active on the *S* enantiomer of 2-CPA. A putative group 2 dehalogenase gene was subsequently PCR-amplified from the *Paracoccus* sp. DEH99 genome. In addition, another *H. perlevis*-associated strain, *Pseudomonas stutzeri* DEH130, was reported to contain two dehalogenases in its cellular supernatant, both with different stereospecificities and with traits that were completely unrelated to bacterial dehalogenases which had been described up until then. One of these, dehalogenase II, was subsequently purified and was shown to be a dimer, with an optimum pH 10 and temperature at 40 °C and with a high substrate specificity for L-2-CPA (Zhang et al. 2013).

There have only been a few reports of the characterisation of sponge microbial transferases. While focused on elucidating the symbiont physiology, Schleper et al. (1997) characterised a non-thermostable single strand-specific DNA polymerase from a fosmid library of the psychrophilic strain *Cenarchaeum symbiosum*, an archaeon that was firstly discovered in the Californian *Axinella mexicana* sponge (Preston et al. 1996), which later helped with the establishment of the phylum *Thaumarchaeota* (Brochier-Armanet et al. 2008). Recently, Gavin et al. (2019) have identified a potential transaminase in the genome of a sponge-associated *Pseudovibrio* sp. aiming at overcoming the challenging stereospecific resolution of active pharmaceutical ingredients (API) and other drugs. The purified alkaline ω-transaminase displayed strict enantioselectivity for the remote stereocentre of various aminated substrates, including intermediates in the synthesis

of sertraline, one of the most commonly prescribed antidepressant agents. Thereby, this transferase appears to be a good candidate for a 'first in class' biocatalyst to be ameliorated in future rational design and directed evolution.

Where should we strive to succeed?

Building on the successes to date in prospecting the marine sponge microbiome as an important source of biocatalysts, it is important to focus on the approaches that may lead to breakthroughs in this field. We have addressed this from two different perspectives (Fig. 3). Firstly, from the perspective of the biocatalyst (Fig. 3a), several strategies will be proposed to enhance the efforts in the discovery, optimisation and practical use of these biocatalysts. A plurality of these approaches are cornerstones of contemporary applied enzymology and have already been employed and in some instances already described in this review (e.g. the use of crop residues for optimising enzyme production in fermentation experiments). The second perspective will focus on surveying the biology of the sponge–microbe partnership to help in the unravelling of a wealthy enzymatic toolbox. In that sense, we will pinpoint the use of classical microbial bioprospecting techniques, the exploitation of the rapidly growing data generated by the modern -omics approaches and the search in as yet poorly assessed environments.

As previously discussed regarding the use of marine polysaccharides and acidic complex sugars in uncovering new CAZymes, the incorporation of non-conventional substrates may prove to be a valuable resource in the identification of other enzyme groups and classes from the sponge microbiome (Fig. 3a). For example, the use of protein components of the sponge mesohyl, as verified for the *P. agarivorans*-collagenase active on spongin (Mukherjee et al. 2009) and type IV collagen (Bhattacharya et al. 2018), could be important in testing the specificity of peptidases, with potential utility in the healthcare or cosmetic sectors. Amorphous mineral elements present in the mesohyl matrix and external sponge body layers could also be employed to identify other biocatalysts, such as calcium-precipitating enzymes, that have been reported in sponge bacterial symbionts (Uriz et al. 2012; Garate et al. 2017), and silicases, naturally produced by sponge specialised cells to control the skeletal formation (Schröder et al. 2003; Wang et al. 2012). Silica-degrading proteins are interesting for nanobiotechnological applications, with potential uses mainly in the preparation and encapsulation for the modification of medical biomaterials (Schröder et al. 2007) and even the removal of electronic waste (Spangle 2018). Amongst the silicase patents, one (US 8,822,188 B2) was

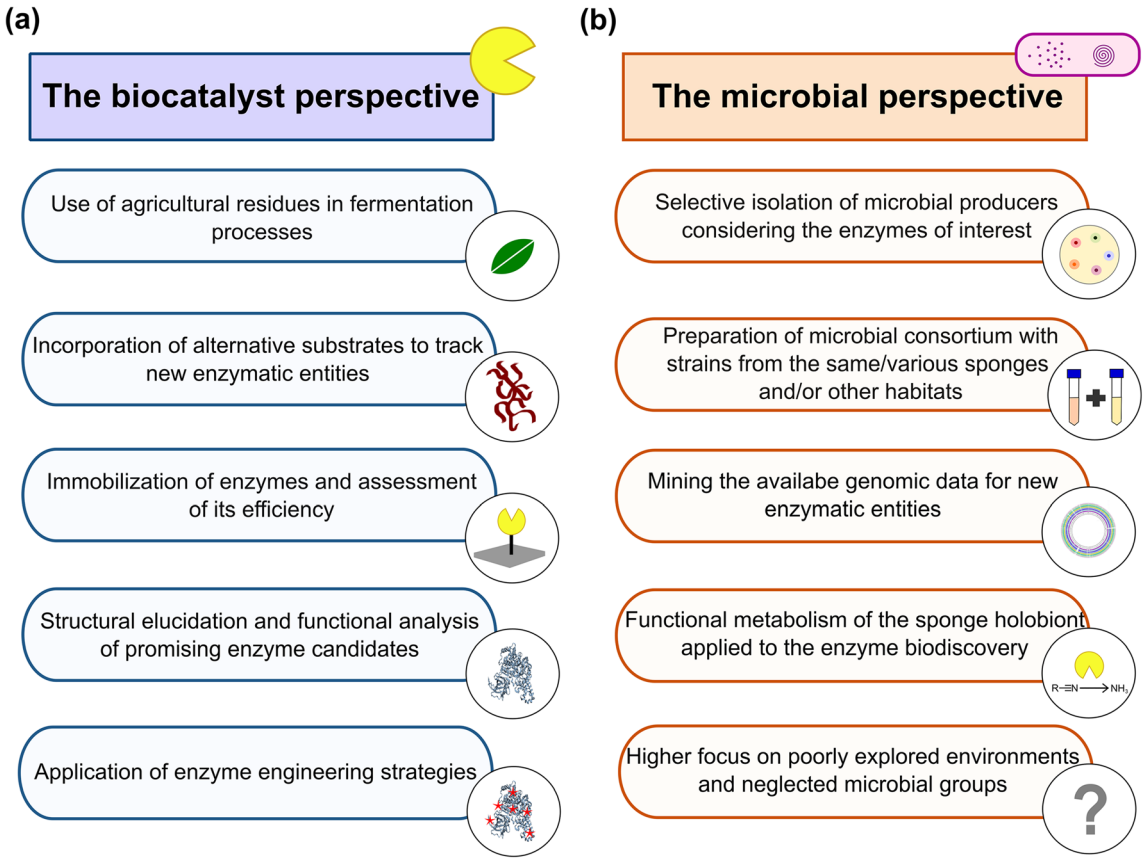

Fig. 3 Future strategies for the sponge microbial enzymology: **a** the biocatalyst and **b** microbial perspectives

derived from *Bacillus plakortidis* (Toender and Borchert 2009), an alkaline-tolerant strain first described from the marine sponge *Plakortis simplex* (Borchert et al. 2007) and later reclassified by genome-based taxonomy to *Bacillus oshimensis* (Liu et al. 2019). This silicase displayed carbonic anhydrase-like activity on silica-based materials, such as aerosil (fumed silica) and Sipernat® 22S (hydrated silica), sand and glass wool. This is an excellent example of how potential products from sponge-derived microorganisms, not confined to antimicrobial secondary metabolites, can transcend the academic sphere and lead to the generation of intellectual property.

Despite the examples outlined here, where enzyme production has been optimised by simpler one-parameter-at-time or using various complex statistical design strategies, there are no reports in the literature about the utilisation of immobilisation for any of these sponge-derived microbial biocatalysts. Enzyme immobilisation offers several benefits, notably reuse of the biocatalyst and enhancement of stability and efficiency in the presence of organic solvents and under extremes of pH and temperature, together with minimisation of enzyme contamination on its products and low or no allergenicity (Sheldon and van Pelt 2013). Utilisation of different types of enzyme immobilisation for marine microbial enzymes has increased in recent years, including α-amylases (Chakraborty et al. 2014), alginate-lyases (Li et al. 2019), β-agarases (Xiao et al. 2018), chitinases (Beygmoradi et al. 2019), lipases (Hassan et al. 2018) and esterases (Rahman et al. 2016), to name a few examples.

In this regard, immobilisation has been acknowledged to yield superior catalytic features and recycling of lipases, especially in solvent mixtures (Thangaraj and Solomon 2019). Given the tolerance or complete resistance of the majority of the hitherto described sponge microbial-derived lipases and esterases to various organic solvents and harsh chemical agents, the adoption of an immobilised biocatalytic system is likely to prove extremely worthwhile (Fig. 3a). Recently, agro-industrial residues have been suggested as useful support carriers for virtually all enzyme immobilisation methods. After their use as carriers, they can be redirected as substrates, rendering cheaper, more sustainable and efficient biocatalytic processes (Girelli et al. 2019). In this respect, plant waste material could be applied as immobilisation supports for the lignocellulosic-degrading GHs and copper oxidases produced by sponge-associated microorganisms.

Information on the structure of a protein is paramount in defining the potential heterologous host and conditions for expression of a recombinant enzyme; nonetheless, this knowledge can be also taken to delineate adequate enzyme engineering strategies (Ali et al. 2020). In this respect, the *P. agarivorans* NW4327 U32 collagenase is a good example of such a strategy. Initial characterisation of the enzyme structure and function was determined by a series of complementary computational tools, inferring some of the main traits of this peptidase, which were later confirmed following biochemical characterisation of the purified enzyme (Bhattacharya et al. 2018, 2019). Modelling the protein based on its primary structure can provide clues regarding the potential catalytic mechanism involved which can then be further altered to enhance or specifically engineer the enzyme's performance (Fig. 3a). The *Pseudovibrio* sp. WM33-ω-transaminase (Gavin et al. 2019) is another example for such approach. The selective activity of this enzyme with respect to the remote stereocentres, which is novel when compared to other transaminases, could potentially be adapted by directed evolution approaches, focusing on the already identified key catalytic residues. This could permit an increase in the range of possible substrates for the enzyme and other pharmaceutically active aminated products. The aforementioned PETase-like SM14est enzyme (Almeida et al. 2019) could also be a good target for protein engineering in order to allow the recognition of other polyesters, including PET, thereby enhancing its degradation ability, in a similar fashion to approaches that have been successfully employed with the *Is*PETase (Austin et al. 2018).

The study of the cultivable fraction of microbes associated with sponges has to date largely hinged upon the use of broad, rich-based media or a myriad of different culture media, with their compositions varying in line with the physiological and metabolic requirements of the target group of microorganisms in a culturomics-like approach (Laport 2018). Selective isolation of environmental microbes who are producers of enzymes is one of the most traditional methods employed in the discovery of industrially relevant biocatalysts (Steele and Stowers 1991). This saves a considerable amount of time when examining large numbers of strains in primary and secondary screening rounds (Schafer and Borchet 2004). Selective media have been purposely used for the isolation of specific enzyme microbial producers from sponge specimens. However, their use is still incipient and the growing adoption of selective isolation media may prove to be useful, particularly for enzymatic activities adapted to extreme conditions (Fig. 3b).

In this context, a particular good example was the addition of 25% crude salt/NaCl in the basal medium to isolate bacterial and archaeal bionts from sponges collected along the Indian seashore (Malik and Furtado 2019a; Gaonkar and Furtado 2018). These strains, and notably the archaea, were subsequently shown to possess extremely halophilic cellulase (Malik and Furtado 2019a), xylanase (Malik and Furtado 2019b), lipase (Gaonkar and Furtado 2018) and protease activities (Gaonkar and Furtado 2018, 2020). Coupling a qualitative pH indicator method and applying the chlorinated substrate, 2-CPA, as the sole carbon source in enrichment cultures resulted in the successful isolation of specific dehalogenase-producing bacterial strains (Huang et al. 2011a), from which

enzymes were subsequently purified (Huang et al. 2011b; Zhang et al. 2013). In addition, a quantitative high-performance liquid chromatography (HPLC) method was adopted to follow the 2-CPA degradation. In fact, detecting chlorine (Cl^-) in seawater is a challenge during the isolation of dehalogenating microorganisms from marine sources; by quantifying the degradation of the substrate, the group additionally circumvented this obstacle (Huang et al. 2011a). The sole use of starch casein agar (SCA), a classic actinobacterial isolation medium, by Dhamodharan et al. (2019) to retrieve the fibrinolytic *Streptomyces* sp. reflects another good example of selective isolation in aiming at a specific microbial taxon, considering the former knowledge of the huge potential of these thrombolytic actinoproteases (Dhamodharan et al. 2015).

By shifting away from the culturing and screening of pure isolates, an appealing alternative would be the use of microbial consortia, which is the natural state of these microbial communities in their sponge hosts. Strategies involving consortia containing different microbial strains have successfully been employed for the production of antimicrobial molecules; metabolism of biopolymers; production of biodiesel, organic acids, pigments and enzymes; and also importantly, environmental elimination of dyes, oil spills and other organic pollutants (Bhatia et al. 2018). Co-culture between sponge-associated bacteria originated from the same sponge (Dashti et al. 2014; Matobole et al. 2017; El-Hawary et al. 2018) or with other strains (Abdel-Wahab et al. 2019; Frank et al. 2019; Yu et al. 2019) has proven fruitful in inducing the expression of the so-called silent or cryptic gene clusters encoding pharmacologically active secondary metabolites. The association of microbial isolates—derived from the same, various sponge specimens, or other unrelated strains—for the degradation of complex polymeric substrates or the multi-step co-utilisation of two chemically different substrates would be relevant to assess their biocatalytic potentialities for some applications to which these microbial consortia are particularly suitable (Fig. 3b), such as for biorefinery and industrial effluent treatment.

While the classic concept of 'genome mining' had proven useful in the natural products discovery field (Ziemert et al. 2016), the exploitation of the huge and exponentially increasing amount of genomic data has also been successfully directed to reveal new industrial enzymes with singular properties. This 'mining' approach is a particularly powerful when rationally used, and when employing the broad arsenal of bioinformatics resources which are currently available to interrogate the genome sequences deposited in several databases (Zaparucha et al. 2018). This in silico initially guided strategy has been effectively applied in some cases which have previously been highlighted in this review, with the *Pseudovibrio*-ω-transaminase being a fine example (Gavin et al. 2019). Their strategy was different than the one adopted to recover the cold-adapted glucosidase from a *Pseudoalteromonas* sp. EB27 (Borchert et al. 2017a), where the authors followed the classic route from the isolation of the sponge-associated microorganisms to the purification of the biocatalyst following the genome screening of a number of strains. Gavin et al. (2019) focused on the genome sequence of culturable deep-sea sponge bacteria in the databases, searching specifically for aminotransferase genes, which resulted in the identification and subsequent characterisation of a promising enzyme with an extraordinary substrate specificity. Hence, if resources are employed in examining the hundreds of available sponge-associated microbial genomic sequences for functional enzymes, it is likely that we uncover more 'first in class' biocatalysts in a faster and more cost-effective fashion (Fig. 3b).

The potential of some already well-characterised metabolic aspects of sponge-associated microorganisms has recently been reported in numerous (meta)-omics–based approaches (Moitinho-Silva et al. 2017a; Chaib De Mares et al. 2018). While being crucial in furthering our comprehension of the nature of the host–microbe interactions, some of these features could also be employed and exploited from a biotechnological standpoint (Fig. 3b). As an example, more insights into the nitrification mediated by sponge-associated *Thaumarchaeota* have been recently gained (Feng et al. 2016; Moeller et al. 2019). Given that nitrilases are one of the most abundant putatively predicted cluster of orthologous genes (COGs) in a survey of enzymes with biotechnological potential from environmental microbiome samples (Parages et al. 2016), coupled with the fact that nitrile-converting catalysts lie at the core of many green chemistry processes (Nigam et al. 2017), attempts to isolate and characterise industrial interesting nitrifying enzymes from these sponge microbial symbionts could prove worthy, particularly from a functional metagenomics-based perspective. The contributing role of the RDases from *D. spongiiphila* AA1^T in the organohalide cycle involved in the sponge host/environment interface has also been well substantiated by recent genomic and transcriptomic surveys (Liu et al. 2017, 2020). Notwithstanding, their characterisation for any biotechnological prospect still remains an open question. Key enzymes in other biogeochemical cycles driven by these host-associated microbial communities could also be targeted accordingly.

Biotechnological innovation is accelerated primarily through novelty in microbial diversity, commonly brought to light by 'tapping into' and exploiting unusual environments, in other words, environments that are poorly explored, taxonomically distant from the human-associated microbiome and which are exposed to extremophilic conditions (Tanner et al. 2017). In this respect, freshwater sponges have recently been shown to harbour richer bacterial communities when compared to their marine counterparts (Laport et al. 2019). However, there are only a few reports on the microbiota of

Table 1 Examples of biochemically characterised enzymes from the sponge microbiome with industrial-related features comparable to commercially available enzymes

Enzyme (microbial producer)	T_{opt} (°C)	T range (°C)	pH_{opt}	pH range	Salt tolerance/ dependence	Comparable commercial enzyme	Potential applications of sponge microbial enzyme	References
Chitinase (*Streptomyces* sp. DA11)	50	30–60	8.0	6.0–9.0	45 g‰ psu	Chitinase from *Streptomyces griseus*	Phytopathogen control	Han et al. (2008, 2009)
Laccase Pnh_Lac1 (*Peniophora* sp. CBMAI 1063)	30	30–60	5.0	4.0–6.0	Dependence on ASW for production	MetZyme® LignO™	Treatment of lignocellulosic waste; biorefinery	Mainardi et al. (2018), Brenelli et al. (2019)
Lipase (*Pseudomonas* sp. MSI057)	37	20–50	9.0	5.0–12.0	Highest enzyme production at 1.5 M NaCl	CalB lipase	Food, detergent, pharmaceutical, chemical synthesis industries	Kiran et al. (2008)
Lipase Lpc53E1 (not identified—*Bacteria*)	40	4–60	7.0	3.0–8.0	Increased with 5 M NaCl	CalB lipase	Production of marine goods	Selvin et al. (2012)
Lipase LipA (not identified—*Bacteria*)	40	30–55	9.0	7.0–12.0	NA	CalB lipase	Detergent industry and enzyme-mediated organic synthesis	Su et al. (2015)
Protease (*Halococcus* sp. GUGFAWS-3)	70	20–80	7.0	3.0–13.0	Optimum activity at 3 M NaCl	Alcalase	Detergent/pharmaceutical industries	Gaonkar and Furtado (2020)
Protease (*Roseobacter* sp. MMD040)	50	27–50	8.0	6.0–9.0	Highest enzyme production at 3 M NaCl	Alcalase	Aquaculture	Shanmughapriya et al. (2009)
L-Asparaginase AYA-1 (*Aspergillus* sp. ALAA 2000)	47	20–70	6.0	3.0–12.0	NA	PEG-asparaginase	Pharmaceutical industry	Ahmed et al. (2015)
L-Asparaginase AYA-2 (*Aspergillus* sp. ALAA 2000)	67	20–70	10.0	3.0–12.0	NA	PEG-asparaginase	Pharmaceutical industry	Ahmed et al. (2015)

ASW, artificial seawater; *NA*, not available. pH_{opt}, optimum pH; *psu*, practical salinity unit; *T range*, temperature range; T_{opt}, optimum temperature

these sponges as potential enzymatic reservoirs. Two of these studies have focused on the detection of chitinase genes (*chiA*) in distinctive gammaproteobacterial symbionts from the freshwater sponge *Ephydatia fluvialis* (Cretoiu et al. 2012) and the amylolytic fungal strains isolated from mangrove sponges (Sibero et al. 2019). Thus, the microbiome of sponges inhabiting inland waters may provide a profitable source of novel enzymes in the future.

Marine phages, the largest biological entity in the oceans and the Earth (Breitbart et al. 2018), have been credited as valuable repositories of putative endolysins with broad activity against both Gram-positive and Gram-negative strains (Fernández-Ruiz et al. 2018) and polysaccharidases active on exopolyssacaride (EPS) (Lelchat et al. 2019). Lately, sponge viromes have proven to constitute a completely new universe thriving in this holobiont, with each sponge having a unique, host-specific and functionable pleomorphic viral fingerprint (Jahn et al. 2019). The yet largely underexplored virosphere coupled with the fact that a phage likely mediated the incorporation of a halogenase gene between two phylogenetically distant sponges (Rua et al. 2018) expose the hidden biocatalytic potential of these sponge-associated viral communities with respect to another potential source of novel enzymes.

Conclusions

The implementation of biotechnology in industrial sectors is estimated to rise by 40% in 2030. This will lead to an inherent increase in the annual demand for new enzymes, which has been calculated to grow at an annual rate of approximately 10% by 2030 (Martínez-Martínez et al. 2017a), with the industrial enzyme market expected to reach global $7.0 billion by 2023 (BCC Research 2018). Marine environments are an exciting emerging alternative source for enzyme biodiscovery, particularly due to their ecosystem-driven properties, which make them useful for application in multiple industrial settings (Trincone 2017). The marine microbial realm represents the most propitious enzymatic source (Ferrer et al. 2019), and amongst the infinity of habitats found therein, the sponge microbiome remains as yet a largely underexploited source of novel biocatalysts.

From the current scientific literature, it is difficult to precisely quantify the significance of the sponge microbiome as a reservoir of enzymes when compared to other marine-associated sources, which are receiving increasing interest in the last few years. This is reflected in major strategic and collaborative initiatives for the specific discovery of novel biocatalytic entities in oceanic habitats, such as the recently

concluded INMARE (Industrial Applications of Marine Enzymes) project (Ferrer et al. 2019) and the recently launched MARIKAT (https://bluebioeconomy.eu/marikat-new-catalytic-enzymes-and-enzymatic-processes-from-the-marine-microbiome-for-refining-marine-seaweed-biomass/), both funded by the European Union's Horizon 2020 Research and Innovation Programme. The inclusion of sponge specimens amongst the samples for bioprospecting in projects such as those just mentioned will expand the number of new catalytic hits originating from their microbial symbionts and raise interest both from the academy and industry.

In this context, sponge microbial enzymes have surely not been well surveyed. Most research efforts to date have focused on the heterotrophic role of the sponge-associated microbial communities in consuming organic matter and their released enzymes involved in aiding the energetics of the sponge host (Wang 2006; Santos-Gandelman et al. 2014). What has been largely overlooked is the multitude of microbial metabolic lifestyles flourishing in this holobiont and, consequently, the biocatalytic reservoir available therein for a wide range of biotechnological purposes. From this premise, we have endeavoured to highlight the biocatalytic reservoir of the sponge microbiome, by drawing attention to the most relevant industrial traits of these enzymes, which have to date being isolated and characterised, together with describing potential important and suitable approaches, that could further help in accelerating applied research in this arena.

Some of the enzymes reported here have biochemical features comparable to those found in currently available commercial enzymes (Table 1). For instance, lipases discovered either from a cultivable sponge-associated strain (Kiran et al. 2008) or by functional metagenome screening of sponge microbiota (Selvin et al. 2012; Su et al. 2015) have close optima pH and temperature to the commonly industrially employed CalB lipase, which has similar activity in the alkaline pH range. The biochemical profile of two thermotolerant and alkaline proteases from bacteria isolated from sponge specimens are also equivalent from an activity perspective to Alcalase, the sole accessible thermophilic peptidase currently on the market (Barzkar et al. 2018). The *Peniophora* laccase meets all the needs for a lignocellulose-degrading catalyst, similar to those found in the recently launched MetZyme® LignO™ (MetGen), a genetically engineered thermotolerant alkaline bacterial laccase particularly designed to efficiently optimise pulp/paper processing and lignin valorisation (Hämäläinen et al. 2018). Additional kinetics assays with the same substrates tested for these benchmark enzymes, in particular for the ones with broad substrate specificity (lipases, esterases and proteases), and safety/cytotoxicity tests, specifically for those applicable in pharmaceutical and food industries (e.g. asparaginase), would be indispensable to truly access this equivalence or even potential superiority with respect to the performance of the sponge microbial enzymes in comparison to their equivalent industrial counterparts.

Undoubtedly, halotolerance is the most common habitat-related trait in the majority of these biochemically characterised enzymes derived from sponge microbial symbionts. In many cases, salt is also required by the producing strain for enzyme production. Enzymes with salt tolerance are also known to possess other interesting properties such as increased thermo-, pH or solvent stability, which significantly broadens the biotechnological potential of these enzyme beyond their use at high salt concentrations (Oren 2010). Thus, further exploitation of the representative microbial enzymes mentioned throughout this review is warranted given that their salt tolerance may also result in them having some of the aforementioned properties that may make them useful in other industrial applications.

In summary, the poriferan microbiome is a burgeoning source of industrial biocatalysts. Future research involving screening, isolation, purification and complete characterisation of novel enzymes from this microbiome is therefore clearly warranted. Research in this area is likely to increase our repertoire of sponge microbial-derived biocatalysts, with many of these new enzymatic entities likely to have a multiplicity of potential biotechnological applications.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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