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Review Article

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Epiphytic Bacteria of Macroalgae of the Genus *Ulva* and their Potential in Producing Enzymes Having Biotechnological Interest

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Abstract

Marine environment represents broad potential for discovering novel enzymes having biochemical and functional properties with added value. Metagenomics approaches have promoted the search for and facilitated the discovery of genes encoding enzymes having particular characteristics. Such approaches have focused on exploring microbial diversity at the functional level, often starting with marine samples. Communities associated with living surfaces, particularly marine macroalgae from the genus *Ulva*, are known to harbor a great number of associated microorganisms having host-specific colonization patterns and are influenced by the macroalgae's characteristics, such as cell wall components and defense mechanisms. Monosaccharides, such as rhamnose, xylose, glucose, mannose and galactose, are part of algal polysaccharides constituting the cell wall and the storage material; these algal polysaccharides are a potential source of carbon and energy for numerous marine bacteria. Macroalgal production of these polysaccharides promotes the generation of enzymes synthesized by epiphytic bacteria that are able to degrade a great diversity of compounds. Bacteria associated with macroalgae from the genus *Ulva* thus represent a source of enzymes having biotechnological interest. Some of the most outstanding discoveries to date are discussed and described in this review.

Keywords

Epiphytic bacteria; Macroalgae; Enzymes; Marine bacteria

Introduction

Living surfaces in a marine environment represent potential sites for establishing microbial communities. Macroalgae from the genus *Ulva* represent one such site, being an important structural component of coastal intertidal areas and serving as substrate for establishing microbial communities characterized by remaining associated with living surfaces [1,2]. Spatial proximity leads to intercellular interactions in this kind of association and creates complex differentiated communities [3,4].

Macroalgae are known to harbor a great number of associated microorganisms which participate in relevant survival processes [5]. Macroalgae-bacterial relationships depend on macroalgae capacity to produce organic matter (food) and oxygen which are used by bacteria; moreover, associated bacteria provide CO_2 and minerals [6]. Some bacteria excrete specific regulatory factors which act similarly to cytokinins and auxins in higher plants enhancing cell division and *Ulva* thallus formation [7].

Another important characteristic is the metabolite or enzyme synthesis (in most cases) protects against opportunistic organisms and potential competitors colonizing macroalgal surface, or herbivores able to remove macroalgal biomass [8]. Microorganisms benefit from organic compounds produced by a macroalgal host and synthesize a variety of enzymes such as agarases, amylases, phosphatases, esterases, lipases, β-galactosidases, ureases and cellulases to be able to assimilate macroalgal compounds [9,10].

Nevertheless, despite their abundance and diversity, little interest has been shown in epiphytic bacteria from macroalgae compared to those associated with other hosts such as sponges and corals [11]. This review has thus been focused on the study of microorganisms associated with marine macroalgae from the genus *Ulva*. The search for and expression of enzymes synthesized by marine bacteria is discussed, also parameters influencing the relationship between bacteria and macroalgae is addressed, and finally the enzyme production by bacteria associated with macroalgae having a potential application in biotechnology and industry due to their functional characteristics is reviewed.

The search for and expression of enzymes synthesized by marine bacteria

Different methodologies have been used for assessing microbial diversity associated with living surfaces; one of them concerns culturing and isolating microbial species from environmental samples, such as sea water, soil and the human intestine. Nevertheless, despite recent advances, most microorganisms cannot be cultured and the method is unsuitable. Another broadly used method for assessing diversity is rRNA 16S gene amplification and sequencing which reflects evolutionary relationships between microorganisms due to its universal presence [12].

Despite such analysis have revealed a lot about microbial diversity distribution, it is difficult to draw inferences about functional attributes of non-cultivable microorganisms only based on taxonomic and phylogenetic analysis. More information about non-cultivable microorganisms' genomes is needed. Studies of the genomes of a complete microbial community (metagenomics studies) have thus revealed the metabolic and functional potential of non-cultivable microorganisms, the discovery of new bioactive compounds and enzymes being promoted through functional screening [13].

Metagenomics consists of assessing diversity and function based on the sequence of microbial genomes in an environmental sample (seawater, living surfaces, soils, etc.). These technologies have been applied on different scales from genes, metabolic pathway, to organisms and communities. To this end, sequence databases provide an important source of knowledge for this kind of study. Moreover, next generation sequencing technologies provide greater coverage compared to traditional sequencing methods, thereby enabling a large amount of sequences to be gathered for analysis [14].

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Metagenomics involves two approaches: constructing sequence-based and/or function-based libraries. The former implies designing primers from known conserved genes whilst the latter can be used for identifying new biomolecules. Multiple datasets can thus be compared taxonomically and functionally [13].

Both approaches involve cloning DNA obtained from samples and the further construction of clone libraries having small or big inserts (DNA fragments). The resulting clones are used to transform a host, most commonly *Escherichia coli* or species from the genera *Streptomyces* and *Thermus*. This step allows the heterologous production of cloned genes and therefore the phenotypic expression of the activity of interest. This ability depends on several factors, such as transcription, translation and post-translational modification, as well as the synthesized products compatibility with a particular host's biochemical properties [14,15].

Furthermore, depending on insert size, the libraries are constructed using plasmids (up to 15 kb), fosmids, cosmids (up to 40 kb), or artificial bacterial chromosomes (more than 40 kb) as vectors. Small-insert libraries in plasmid vectors (bearing less than 10 kb insert) are useful for isolating single genes or small operons encoding enzymes or small enzyme complexes; large-insert libraries are used for identifying gene groups encoding enzymes from a whole metabolic pathway [14].

Sequence-based analysis can involve complete sequencing of cloned DNA. An emerging and powerful direction for metagenomics analysis concerns functional anchor use, functional analogs of conventional phylogenetic anchors. Functional anchors define the functions that can be assessed rapidly in all of the clones in a library, whilst phylogenetic anchors indicate the probable taxonomic group and identify the source of the DNA fragment. Once a gene of interest has been identified, phylogenetic anchors can be sought in the flanking DNA to provide a phylogenetic link with a functional gene [16].

Functional-based screening involves selecting clones expressing desired traits from libraries and analyzing active clones' molecular and biochemical aspects. Currently, there are several screening methodologies; one of them is termed SIGEX and it has been developed for isolating novel catabolic genes from environmental metagenomes, particularly genes that are difficult to obtain using conventional gene cloning methods. This screening approach involves using an operon trap *gfp* expression vector in combination with fluorescence-activated cell sorting. Restriction enzyme-digested metagenome fragments are ligated into an operon-trap vector and a library is constructed and grown in a liquid culture by transforming a cloning host. The library is subjected to a substrate- dependent gene-induction assay and positive cells are selected by detecting the activity of a co-expressed marker encoded in the vector. However, this method is limited, as only genes homologous to known genes can be obtained and catabolic genes that are distant from a relevant transcriptional regulator cannot be identified [17].

Another type of screening is known as product-induced gene expression; it is a reporter assay-based screening method for enzymes which is used in libraries containing many clones. Enzyme activity is detected in this method by *gfp* expression which is triggered by product formation; the BenR activator is replaced upstream *gfp*. *E. coli* cells harboring the benR-gfp cassette which would fluoresce in the presence of a benzoate precursor compound if they expressed an enzyme capable of actively transforming the precursor into benzoate. This reporter assay system allows the identification of desired enzymatic activities by linking product formation to reporter gene expression. Using this system, amidases have been targeted which can convert benzamide to benzoate. The approach's strength is that it does not require the gene(s) of interest to be recognizable by sequence analysis, enabling entirely new classes of genes to be identified for new or known functions [16].

It should also be considered that, in metagenomics screening, the target gene represents a small proportion of the total nucleic acid fraction; pre-enriching a sample can thus enhance the screening hit rate. Culture enrichment on a selective medium favors the growth of target microorganisms. The inherent selection pressure can be based on nutritional, physical, or chemical criteria, although substrate use is the most common practice [15].

Using these tools has promoted the identification of several enzymes having various functional characteristics. Most enzymes used in different industries are microbial in origin and have specific characteristics, like stability, high specific activity, and facilitated mass transfer. High saline marine environments have proven to be a rich source of microorganisms harboring industrially important enzymes. Enzymes produced by marine microorganism can provide numerous advantages over traditional enzymes due to their habitat in wide range of environments. The quest for bacterial isolates producing enzymes with high efficacy and of commercial value is still going on to exploit enormous marine resources, one of these are marine macroalgae and bacteria associated to their surfaces [18,19].

Macroalgae contribute about half global primary production. The large amounts of polysaccharides synthesized by these algae are degraded and consumed by marine heterotrophic microbes that use carbohydrate-active enzymes (CAZymes) which digest the organic matter so produced into low molecular weight substrates. Marine microorganisms associated with macroalgae contain unexplored enzymes (glycoside hydrolases and polysaccharide lyases, agarases, etc) that participate in important metabolic pathways and whose characterization will provide new insights into marine enzymes having potential biotechnological use. Recent years have seen increasing interest in the analysis of CAZymes that degrade marine polysaccharides, mainly those from macroalgae. Such research is also providing biotechnological tools for producing oligosaccharides having therapeutic potential and generating the biocatalysts required for the efficient use of algal biomass as feedstock for bio-refinement [20]. Figure 1 shows a flowchart involving the different experimental strategies used for identifying enzymes synthesized by epiphytic bacteria from macroalgae of the genus *Ulva*.

The relationship between bacteria and macroalgae from the genus *Ulva*

Marine ecosystem hosts harbor a great diversity of microbial communities, most of them forming biofilms or other consortia consisting of cells embedded in an extracellular matrix. Epibiosis is a direct consequence of such narrow associations; it occurs when a group of organisms survive on an individual living host. The hosting organism is called a basibiont, the organisms growing on the surface are called epibionts (epizoos if the substrate is an animal and epiphytes if the substrate is a plant) and the relationship is called a holobiont [21].

Macroalgae, as well as other marine surfaces, play an important role as basibionts, being the most important surface colonized

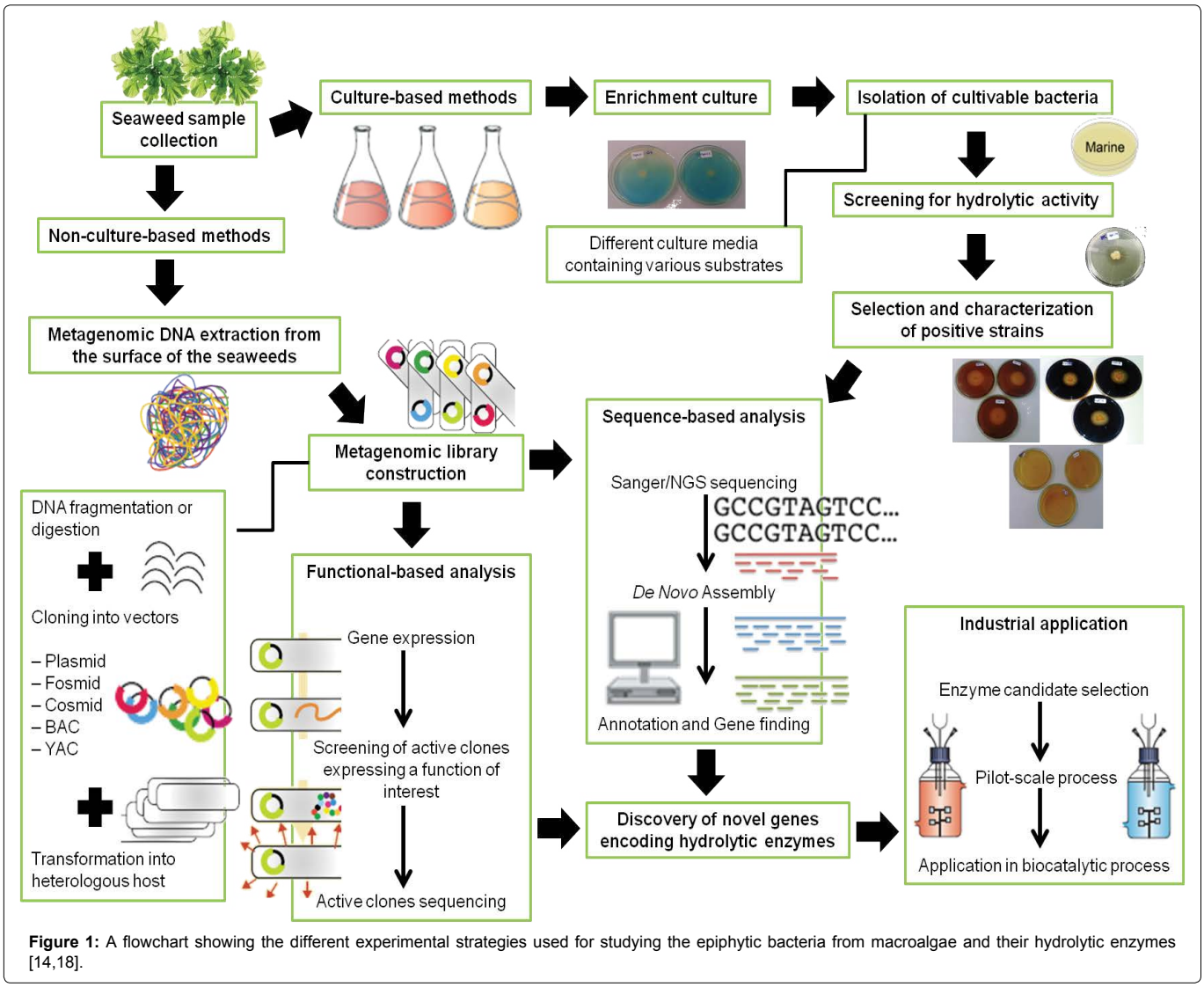

Figure 1: A flowchart showing the different experimental strategies used for studying the epiphytic bacteria from macroalgae and their hydrolytic enzymes [14,18].

by microorganisms in marine environments and a considerable percentage regarding submerged intertidal marine surfaces [1]. The macroalgal of the genus *Ulva* stands out because of the high diversity of associated microorganisms; abundance greater than 1.1×10^8 microorganisms (including yeast, fungi, protozoans, microalgae and bacteria) on one square centimeter have been reported [10]. Some studies have demonstrated that clades of bacteria such as *Proteobacteria* and *Bacterioidetes* are more likely to be associated with green algae than other phylotypes; these bacteria are also functionally equipped to be associated with these algae and, presumably, are considered to be microbial weed species [22,23].

The variability of established groups on algae surfaces indicates the colonizing microbiota functional diversity. A metagenomic analysis of the microbial community associated with macroalgae from the specie *U. australis* has supported the hypothesis that the algal surface provides a niche for microorganisms having gene products performing particular functions, beyond the functions found in one particular organism. Such community functions are generally related to detecting host movement, biofilm adhesion and formation in response to environmental stimuli, an ability to degrade complex polysaccharides, to stave off competition, and processes related to defense or horizontal gene transfer [22]. In most cases, bacteria and algae define the survival characteristics of each other in such environment through specific interactions (mutualism, commensalism or parasitism) [24]. Phylogenetic and functional analysis has also revealed that functions are not restricted to a particular taxonomic group, thereby indicating that the different taxa are responsible for such functions present in the *U. australis*-associated bacterial community [22].

Ulva species have been studied extensively, including *U. lactuca, U. linza, U. pertusa, U. fasciata,* and *U. mutabilis*. Morphogenesis-inducing extracts have been obtained from various isolated bacteria and a bacteria from the genus *Cytophaga* has been identified; it excretes the morphogenesis-inducing substance thallusin that partially promotes the formation of distromatic thalli of *U. pertusa* and other *Ulva* species, highlighting the potentially important role of thallusin for green macroalgae's normal development [25,26]. Similarly, *Ulva mutabilis* morphogenesis regulation is mediated by marine bacteria through compounds excreted into the environment. Examples are species from the genera *Roseobacter, Sulfitobacter* and *Halomonas* present in macroalgae which produce molecules similar to cytokines [7].

Likewise, auxin has been shown to promote algal growth or rhizoid formation in seaweeds from the genus *Ulva* in culture conditions. Seaweeds develop into a callus-like morphotype without appropriate specific bacteria; this is sometimes described as an atypical but viable pincushion-like morphology composed of uniseriate branching filaments. These abnormal algal colonies can be partly or completely recovered to their typical thallus by inoculation with appropriate marine bacteria or partly purified morphogenetic compounds (morphogens) [26]. Also, *Marinomonas* sp. and *Bacillus* sp. bacteria have been shown to be involved in the differentiation and growth of members from the genus *Ulva* [27]. These examples illustrate how microorganisms act in conjunction, using chemical communication conditioning macroalgal morphology [7].

On the other hand, several studies have pointed out that at least a part of the *Ulva* sp. lifecycle depends on the microbial community associated with the macroalgal surface [28]. It is known that spore settlement on a substrate is mediated by the recognition of N-Acyl homoserine lactones (quorum sensing molecules), due to macroalgal spore recognition using a chemokinetic mechanism in response to the presence of bacteria [29]. In species like *Bacillus flexus*, polymeric extracellular substance production facilitates *U. lactuca* spore settlement on a particular substrate in which macroalgal colonization will take place [30-32]. However, direct cell-cell contact is not necessary for the bacteria-induced morphogenesis of *Ulva*; some studies have already highlighted the fact that interactions between macroalgae and bacteria depend strongly on positive and negative chemical stimuli [7,33]

Moreover, macroalgae surfaces are free of epibiotic macroorganisms. Since these organisms generally lack mechanical defenses, the observation of reduced macrofouling on their surfaces is indicative of natural antifouling strategies based on chemical modes of action. Allelopathic activity of secondary metabolites synthesized by epiphytic bacteria and macroalgae provide an effective means of controlling surface colonization [34]. Bacteria from the genera *Bacillus, Pseudoalteromonas, Pseudomonas* and *Streptomyces* are characterized by producing antimicrobial compounds which contribute to defense against colonization by opportunistic organisms [35,36].

On the other hand, some water soluble monosaccharides, such as rhamnose, xylose, glucose, mannose and galactose, are part of algal polysaccharides constituting the cell wall and the storage material [6]. These algal polysaccharides are a potential source of carbon and energy for numerous marine bacteria [37]. Regarding the genus *Ulva*, its total biomass consists of proteins (27%), lipids (0.3%) and carbohydrates (62%); carbohydrates are mainly represented by ulvan (a compound formed by sulfate, rhamnose, xylose, iduronic acid and glucuronic acid) which, together with cellulose, constitutes the macroalgal cell wall [32]. These polysaccharides production by the macroalgae, promotes the generation of enzymes synthesized by epiphytic bacteria which can degrade a great diversity of compounds available on macroalgal surface [38-40].

Enzymes synthesized by epiphytic bacteria

Marine enzymes accelerate, or catalyze, many biochemical reactions having several biotechnological applications. Microorganisms in the sea are exposed to changing pressure, temperature and salinity, thereby synthesizing enzymes having particular characteristics enabling them to constantly adapt [40]. Some of these enzymes are released to the environment to degrade complex macromolecules whilst others are intracellular and participate in molecular pathways for producing energy [41,42].

Marine environments are considered one of the most important reservoirs of novel enzymatic biocatalysts [43]. Therefore, the search for enzymes has mainly been centered on planktonic and extremophiles microorganisms and, to a lesser degree, on microorganisms associated with sediments, muds, sandy soils and living surfaces (basibionts) [44]. Although, the importance of such extreme environments is known, the search has been restricted due to difficult access and the high investment involved regarding time and resources [45]. The successful search for new biocatalysts in epibionts associated with living surfaces like marine macroalgae has thus attracted significant attention. Proof of this lies in several patents having been granted between 1973 and 2007 for obtaining marine enzymes in applications related to pharmacological, medical and food industry, textile production, and make-up fields and others like environmental biotechnology, biofuel and bioenergy resource production [46].

Nutrient exchange has been considered to be one type of interactions between macroalgae and bacteria [47]. The major structural components of the cell walls of most marine macroalgae are polyanionic homo- and hetero-polysaccharides; moreover, compounds such as starch and laminaran are contained in green macroalgae as storage carbohydrates. Polysaccharides are degraded by enzymes secreted by microorganisms associated with macroalgae; these enzymes include various depolymerases acting on glycoside bonds [48]. Identifying the microbial enzyme systems responsible for polysaccharides degradation is important for unlocking the potential of using green algae as feedstock [20].

The following enzymes are synthesized by bacteria associated to macroalgae: amylases, glycosidases, pullulanases, agarases, lyases, galactosaminidases, galactosidases, cellulases, xylanases, glucanases, quitinases, laccases, cresolases, proteases, lipases and hydrogenases [20,40,48,49].

Some of the enzymes of importance regarding their application in biotechnology and industry are described below. The first section provides promising evidence for finding new enzymes from bacteria associated with macroalgae from the genus *Ulva*; the second section provides information regarding other enzymes which have been not discussed in the context of *Ulva* but are synthesized by epiphytic bacteria from macroalgae.

Enzymes synthesized by bacteria associated to *Ulva* sp.

Cellulases: Cellulose is the most abundant carbohydrate in nature, mainly forming the primary structural cell wall component in lower and higher plants. Macroalgae mainly contain α-cellulose without much complex lignin, thus differentiating them from those of terrestrial plants and making them a preferential source of cellulose [50].

Microorganisms having cellulolytic activity can provide unique opportunities for cellulose biodegradation, converting it into energetic molecules [51]. Several marine bacteria produce cellulases, especially those which are epiphytes growing on plants and green macroalgae, i.e. the genera *Cytophaga, Cellulomonas, Vibrio, Clostridium, Nocardia, Pseudoalteromonas* and *Streptomyces* [52].

Bacteria associated with degraded macroalgae *Ulva lactuca* (rich in cellulosic content as its cell wall component) were screened for extracellular cellulase enzymes, one strain from the specie *Bacillus flexus* having enzymatic activity; this enzyme was found to tolerate the presence of various solvents having alkaline and saline stability [51]. Furthermore, amongst marine bacteria isolated from

deteriorated *Ulva* macroalgae and investigated for solvent tolerance, a bacterium from the specie *Bacillus aquimaris* showed potential for producing an extracellular alkaline cellulase having functional stability at extremities of pH, solvents and ionic and non-ionic detergents. Increased enzyme activity when pre-incubated with benzene, 1-ethyl-3-methylimidazolium methanesulfonate and 1-ethyl-3-methylimidazolium bromide suggested prospects for the enzyme regarding industrial processes involving biphasic organic-aqueous fermentation and hydrocarbon-saturated environment bioremediation [53].

Because of their usefulness in several biotechnological and industrial processes and extremely rapid development of high-throughput sequencing techniques, 180 open reading frames (ORF) have been characterized for cellulolytic enzymes in marine bacteria. Most have been expressed and characterized using a heterologous host, such as *Escherichia coli* [41]. Due to their functional characteristics, cellulases have a broad industrial application spectrum for many reactions related to processing bio-textiles, cotton, spinning, in the biofuel industry, alcohol production, flavors, detergents and treating residual water and hydrocarbon-saturated environments [51].

Lipases: Lipolytic enzymes have tremendous catalytic versatility regarding lipid hydrolysis, esterification, transesterification and interesterification, as well as fat and oil aminolysis and alcoholysis [54]. The construction of metagenomic libraries and the lipolytic gene expression in heterologous hosts during the last few years has led to recombinant enzymes being produced from samples obtained from soils and environments polluted by oils and other difficult to degrade polymers [55-58]. Metagenomic studies have led to lipolytic enzymes being recovered from sediments near hydrothermal basins which have multiple thermophilic and halotolerant properties [59,60]. There has been a considerable increase in prospecting studies searching for lipases in marine environments during the last decade. Some recombinant enzymes have been produced and new bacteria-synthesized enzyme families have been described [61]. More than 100 clones having lipolytic activity have been identified since 2009 in samples from several sources, most of them marine [62].

A functional metagenomic screening of microbial communities associated with a temperate marine sponge and a green alga from the genus *Ulva* identified three novel hydrolytic enzymes having antibacterial activities; these enzymes are lipases which act on lipids to release different chain length fatty acids which are known to have a broad spectrum of antibacterial activity. The mode of action is thought to be related to the detergent properties of these acids, which allow them to create pores or, at high concentrations, to cause cell lysis through cell wall degradation. This study's results suggest that uncultured alpha- and gamma-proteobacteria contain new classes of proteins that may be a source of antibacterial agents from host-associated microorganisms. This, in turn, may prevent the colonization or growth of certain bacteria and hence may have an impact on the community composition of a host's microbiota [63].

In another study, a metagenomic survey of alga-associated biofilms has revealed that genes involved in B vitamin biosynthesis and those for lipases and esterases are abundant and functional, suggesting their key roles in macroalgae-associated bacteria [64].

Lipases are considered to be one of the most important biological catalysts in biotechnological applications, such as the food, pharmaceutical and cosmetic industries, and also agrochemical, biofuel and detergent production [65,66]. These enzymes are also used in bioremediation and some of them have antimicrobial and antifouling activity [67,68]. There is great interest regarding all these applications in the search for lipases synthesized by marine bacteria, and isolating lipases from macroalgae opens up novel routes for industrial production [64].

Amylases: Amylases participate in the hydrolysis of starch into various products, including dextrin and small glucose units. Although amylases can be isolated from different organisms including animals, microorganisms and plants, microorganism-derived enzymes are of special interest for several industrial applications due to their functional properties. Alpha-amylases are one of the most important groups; they are used in textile production, paper, detergents and the food industry. Amylases have completely replaced the use of chemical products for hydrolyzing starch during the last few years. The search for new amylolytic enzymes thus poses a great challenge at the moment. Amylases synthesized by bacteria isolated from marine environments have been cloned and expressed in heterologous species, showing high thermal stability and tolerance to alkaline conditions [69].

Likewise, amylases having tolerance to salinity and different pH ranges have been isolated from marine bacteria and identified in metagenomics libraries obtained from marine samples [70]. Moreover, bacteria living in very low temperature regions and synthesizing enzymes having amylolytic activity have been studied, showing promise for industrial applications which imply fluctuations in salt concentration and which are undertaken at very low temperatures [71].

There is also evidence regarding an amylase isolated from the bacterial strain *Cellulosimicrobium* sp. which was obtained from the green alga *U. rigida*. It is worth stressing that this amylase's highest activity was obtained in the presence of 3% sodium chloride (NaCl); moreover, there was no amylase production in the absence of NaCl, clearly indicating the strain's halophilic nature. These enzymes with polymer degrading ability involving low water activity are of interest for many harsh industrial processes where concentrated salt solutions would inhibit many enzymatic conversions. Furthermore, most halobacterial enzymes are known to be thermotolerant and remain stable at room temperature for a long time [72].

Ulvan lyases: Ulvan is the main component of the genus *Ulva* cell wall; this compound is formed by complex polysaccharides, such as sulfated rhamnose, glucuronic acid, iduronic acid and xylose. Ulvans have promising potential with regard to their original chemical structure and physicochemical properties; however, green algal polysaccharides have been less investigated to date compared to the cell wall polysaccharides found in brown algae (alginates) or red algae (agars), both of which are widely-used in industrial applications for their gelling and thickening properties. Nevertheless, ulvan's unique chemical and physicochemical properties have many promising applications in the food, pharmaceutical, and chemical industries, as well as for aquaculture and agriculture [73].

Microorganisms able to completely degrade green algal biomass represent a good source of ulvanolytic enzymes for biotechnological applications. The first ulvanolytic enzyme was isolated from a macroalgal bloom in Great Britain and called ulvan lyase [74]. Moreover, ulvan lyases have been isolated from an uncharacterized Gram-negative marine bacteria found in decomposing algae, in a marine Bacterioidetes *Nonlabens ulvanivorans*, isolated from the feces of *Aplysia punctata* and a Proteobacteria species *Ochrobactrum*

tritici found in soil [73]. The ulvan lyase gene has been identified in *N. ulvanivorans*, expressed in *Escherichia coli* and subsequently characterized; nevertheless, no known similarity was found when its sequence was compared to other enzymes reported in databases, thus remaining an unclassified polysaccharide lyase [75,76]. A recent study identified a new bacterial ulvan-lyase producer having an interesting enzymatic cleaving activity on ulvan. Based on its 16S ribosomal sequence, it has been identified as belonging to the genus *Alteromonas* [77].

Green algae like *Ulva* sp. are also well-known because of their proliferation in eutrophicated coastal waters (also called green tides). Due to the environmental impact of these macroalgae, a broader knowledge base of this biomass is therefore required for better managing and using this resource. Further work is needed regarding the identification of ulvan-degrading microorganisms and the corresponding enzymes due to ulvan's potential in different applications for developing protocols for green algal biomass bioconversion [73].

Enzymes synthesized by epiphytic bacteria from other macroalgae

Proteases: These enzymes are distributed throughout microorganisms from terrestrial and marine environments and are very important, because they participate in cell growth and differentiation by catalyzing protein reactions and peptide hydrolysis into smaller molecules. This group of enzymes is considered to be one of the most in demand, representing 60% of industrial enzyme sales. They are used in processes related to detergent production and in the leather, pharmaceutical and food industries. Moreover, proteolytic enzymes are used in environmental strategies regarding decontamination and bioremediation [78].

Due to their numerous applications, there is great interest in the search for new proteolytic enzymes which are stable in different alkalinity and temperature conditions. Some of them which are stable in the presence of the organic solvents and surfactants used in industrial processes have thus been isolated during the last few years [43]. Some proteases have been identified which are synthesized by halotolerant bacteria, surviving in different salinity ranges [79].

Moreover, bacteria from the genus *Pseudoalteromonas* have been the object of intensive studies because they play an important role in marine environments due to their abundance and high metabolic activity. Bacteria from this genus have been isolated from the thallus of the brown alga *Fucus evanescens* collected in the Kurile Islands (Pacific Ocean). These halophilic bacteria have bacteriolytic, proteolytic and haemolytic activities and degraded algal polysaccharides, synthesizing a number of glycoside hydrolases [80]. Another study evaluated the enzyme profiles of three species of *Pseudoalteromonas* sp. Forty-one bacteria isolated from several invertebrates, macroalgae, sea grass and the surrounding water had different hydrolytic enzyme activity patterns, measured as the hydrolysis of either native biopolymers or fluorogenic substrates. Enzymatic activity occurrence varied over a broad range, depending not only on a strain's taxonomic affiliation but also on the source of its isolation; thereby suggesting species' specialization for different types of polymeric substrates. The incidence of certain enzymes, such as fucoidan hydrolases, alginate lyases, agarases, proteases and galactosidases, might be strain-specific and reflect their particular ecological habitat [81].

Furthermore, a metalloprotease synthesized by bacteria from the genus *Bacillus* associated with the genera *Sargassum* sp. and *Padina* sp. has been isolated recently; this new enzyme is a metalloprotease and, as it contains zinc, it is potentially useful in the pharmaceutical industry [82].

Agarases: Agarases are hydrolytic enzymes which are broadly distributed amongst marine organisms. They are divided into two classes: alpha-agarases and beta-agarases; these can hydrolyze alpha-1,3 and beta-1,4 linkages, respectively, in agar chains and participate in complex sugar depolymerization [43].

Bacterial isolates have been associated with algal samples from the Rhodophytes species *Gelidium crinale* and *Pterocladiella capillacea* which can be exploited for agar and agarose production. Bacteria have been identified at genus level, such as *Alcaligenes* sp., *Bordetella* sp., *Acinetobacter* sp., *Pseudomonas* sp., *Flavobacterium* sp., *Vibrio* sp., *Vigribacillus* sp., *Marinobacterium* sp., *Bacillus* sp., *Pseudoalteromonas* sp., *Alteromonas* sp., *Streptomyces* sp., *Cytophaga* sp., *Aeromonas* sp., *Saccharophagus* sp. and *Streptomyces* sp. These bacteria can produce multiple agarases and thus grow rapidly on agars, agarose being the dominant carbon source [83,84].

Agarases are of interest in biotechnological and industrial processes related to degrading algal polysaccharides, agar and agarose gel liquefaction, biofilm removal in bioreactors, the production of simple sugars like neoagarobiose, neoagarotetraose and neoagarohexaose, as well as in the food industry for producing beverages, bread and low-calorie-content products [43]. Given the interest in these types of enzyme, several beta-agarases have been cloned from bacteria isolated from sea water and sea sediments [37,41].

Bacteria associated with macroalgae-degrading algal cell walls have been seen to successfully secrete hydrolytic enzymes. An agar-degrading bacterium from the genus *Pseudomonas* sp. has been isolated from a red alga used in aquaculture. The agarase gene from this strain was cloned in *E. coli* and some biochemical properties of the recombinant agarase were characterized. The results of biochemical study revealed that this enzyme's agarolytic activity was increased by adding NaCI because this compound could be involved in enzyme activation [85].

Laccases: Laccases are metalloenzymes (containing a copper nucleus) from the oxidoreductase class; laccasses are able to oxidize phenolic, polyphenolic substrate, aromatic amines, polyamines, aryl-amines, lignans, and other non-phenolic compounds. These enzymes catalyze an electron's transfer reaction, resulting in free radical production [86].

Laccases are also related to the degradation of organic compounds such as lignin because the free radicals completely cleave chemical bonds lignin molecules [87]. Laccases are used in processes related to the degradation of complex organic compounds, treating wastewater and polluted soils (bioremediation), but also in chemically modifying polymers used in the textile, paper and food industries [87,88]. Laccases synthesized by microorganisms such as fungi and yeast have been identified in a marine environment; nevertheless, genes encoding this type of enzyme have been discovered recently in the metagenome of bacteria isolated from marine sources [88].

Several of the bacterial laccases reported to date have been found to possess distinctive properties such as excellent activity and stability in alkaline conditions and having high halide tolerance. Recombinant expression has led to bacteria-derived laccases being produced within a short time at lower cost; hence, bacterial laccases may be

an alternative for specific industrial applications [89]. Bacteria from the species *Marinomonas mediterranea* isolated from the surface of the seagrass *Posidonia oceanica* has recently proven to be an excellent source of oxidative enzymes, including a tyrosinase responsible for pigmentation, a novel lysine oxidase and a multi-copper oxidase with laccase activity [90].

Polyphenol oxidase from the marine bacterium *M. mediterranea* is a 695-residue-long, blue, membrane-bound, multi-copper laccase; it has peculiar properties distinguishing it from known laccases, such as broad substrate specificity and high redox potential. The full-length enzyme has been overexpressed in *E. coli* cells to promote this laccase's biotechnological application. This revealed higher kinetic properties on catechol than for known laccases, very high thermal stability and strong resistance to NaCl, dimethyl sulfoxide (DMSO) and Tween-80, all being properties required for specific industrial applications [91].

Conclusion

Marine environment represents broad potential for the discovery of novel enzymes having biochemical and functional properties and added value. Metagenomics approaches have promoted the discovery of genes encoding enzymes having functional properties of interest regarding several industrial processes. It is known that around 3×10^3 enzymes in each bacterial species remain uncharacterized, most of them presumably functional in acid or alkaline conditions, in organic solvents, detergents or alcohols, as well as other compounds commonly used in industrially-related reactions [92].

Metagenomics analysis has focused on exploring microbial diversity at functional level, often starting from marine samples. Bacteria associated with macroalgae from the genus *Ulva* represent an important source of enzymes and bioactive compounds. Some epiphyte bacteria metabolize macroalgae-synthesized compounds through enzymes having very specific characteristics, such as ulvan lyases which are important enzymes in the ulvan degradation pathway. A better understanding of the mechanisms underlying macroalgae/bacteria interactions will facilitate the development of more efficient biotechnological processes, such as the fabrication of novel oligosaccharides and the production of enzymes synthesized by epiphytic bacteria having functional characteristics in specific environmental conditions.

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