Chapter 5
Characterization and Engineering of Seaweed Degrading Enzymes for Biofuels and Biochemicals Production

Eva Garcia-Ruiz, Ahmet Badur, Christopher V. Rao and Huimin Zhao

Abstract Exploitation of natural sources and increasing concerns of environmental pollution are motivating a growing interest in renewable and sustainable feedstocks for biochemicals and biofuels. Marine macroalgae have many advantages over terrestrial plant biomass, including high carbohydrate content which converts seaweed in a cogent alternative feedstock. Algal carbohydrates show a diverse sugar composition, which implies that specialized enzymatic systems are required for their conversion into biofuels and chemicals. Discovery and characterization of degrading enzymes and assimilating the relevant pathways is a key step in the depolymerization of algal polysaccharides into fermentable sugars and their metabolism by fermenting microorganisms. Current advances in metabolic engineering have generated new microorganisms capable of efficiently metabolizing macroalgal carbohydrates while producing ethanol, the target product. However, more research is required to unlock the full potential of macroalgae biomass as a feedstock for biochemical and biofuels production. This book chapter provides an overview of seaweed polysaccharides properties, degrading enzymes, and their application in the bioconversion of macroalgae into biofuels and biochemicals.

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5.1 Introduction

5.1.1 Next Generation Feedstocks

Current trends in the production of biofuels and biochemicals utilize feedstocks from recalcitrant sources. So-called first generation feedstocks, including sugar cane and corn, were easily metabolized by fermentative organisms. However, these sources came with drawbacks; these drawbacks are sought to be answered by second generation feedstocks, including lignocellulosic- and algal-based biomasses.

There is much work being done to engineer lignocellulosic feedstocks for the production of biofuels and biochemicals. The perennial grass *Miscanthus* × *giganteus* has been reported as having high yields in moderate climates [1], an attractive feature for large-scale cultivation. Other feedstocks, such as *Opuntia ficus-indica* and *Agave tequilana,* have also been demonstrated as having high yields for biomass with advantageous climate and water requirements [2]. Regardless of the particular lignocellulosic feedstock, these biomasses contain polymers that cannot be directly utilized by most fermentative organisms. These polymers, cellulose, hemicellulose, and lignin, form a complex network which cannot be easily solubilized nor degraded [3, 4]. Additionally, these polymers often contain constituent monomers which cannot be natively metabolized by common fermentation strains [5]. Thus, before these lignocellulosic feedstocks can be utilized at industrial scales, much work has to be done both in engineering the pretreatment process to degrade lignocellulosic polymers and in engineering fermentative strains to metabolize non-native carbon sources.

5.1.2 Algal-Based Feedstocks

Algae are an exciting target for the production of biofuels and biochemicals. These feedstocks have many advantages over current terrestrial biomass. As algae are cultivated from marine environments, their use in a fermentative process would avoid the conflict between food and fuel [6]. Additionally, algae are photosynthetic organism, and therefore they convert sunlight into chemical energy which is used to efficiently fix atmospheric CO_2 into biomass. Algal photosynthetic efficiency (6–8 %) is threefold higher than that of terrestrial biomass (1.8–2.2 %), which makes algae one of the fastest growing organisms on the planet [7, 8]. This ensures high productivity for their cultivation. As an example, brown macroalgae from

Saccharina sp. can grow as much as 50 cm/day and reach lengths of more than 80 m [9]. Most importantly, algae contain low amounts of crystalline cellulose and lignin [10, 11], which avoid the need of expensive pretreatment processes for their removal. Thus, algae-based feedstocks have advantages over those of terrestrial origin.

Carbon flux through an ecosystem is a useful measure to indicate how amenable is the cultivation of carbon feedstocks. If an ecosystem has a large carbon flux compared to potential feedstock cultivation, this ecosystem is expected to be less affected by large cultivation than one in which carbon flux is similar to feedstock cultivation. The carbon flux through algal bed, algal reef, and estuary ecosystems has been estimated as 10^9 tons of carbon per year [12]. These systems make up two thirds of all ocean carbon biomass, indicating that marine grasses and algae in these ecosystems are an important component of global carbon flux. Additionally, these ecosystems are dense stores of carbon, further exemplifying that algal-based feedstocks are attractive targets for industrial cultivation.

Algae can be broadly divided into two groups: microalgae and macroalgae. Microalgae are unicellular, photosynthetic microorganisms, whereas macroalgae are leafy multicellular, photosynthetic plants. Microalgae have been targeted for production of biofuels since they produce large quantities of lipids, oils, and other storage polymers. Various microalgae have been shown to contain up to 40 % of their dry cell weight in lipids and oils [13]. Additionally, microalgae can have low nutritional needs compared to terrestrial feedstocks; however, special bioreactors must be constructed to maximize growth rates and light penetration [14]. Microalgae can also be engineered to produce heterologous biofuels and biochemicals. Work has been done to engineer the cyanobacterium *Synechococcus elongatus* to produce isobutyraldehyde and isobutanol [15]. Atsumi and coworkers were able to engineer this microalgae to produce more than 6 mg l^{-1} h^{-1} of isobutyraldehyde and 3 mg l^{-1} h^{-1} of isobutanol by introducing a ketoacid decarboxylase gene, *kivd*, and overexpressing other metabolic genes. Further work engineering *Synechocystis* sp. has yielded ethanol production greater than 8 mg l^{-1} h^{-1} by introducing pyruvate decarboxylase, *pdc*, and overexpressing other metabolic genes [16]. These works indicate that microalgae are an achievable platform for the production of biofuels and biochemicals.

5.1.3 Macroalgae-Based Feedstocks

As microalgae necessitate bioreactor design to maximize light penetration [17], economies of scale found in typical fermentative processes are not expected in microalgae-based biofuel and biochemical production. Macroalgae-based bioprocesses would provide a compromise, utilizing marine feedstock cultivation and current industrial fermentation techniques. Macroalgae are primarily comprised of polysaccharides and, as such, must be degraded to a monomeric form to allow for metabolism by fermentative organisms. During this degradation, pathways and

enzymes must be engineered to: (1) secrete polysaccharide degrading enzymes into the extracellular environment; (2) degrade the algal polysaccharides into oligomers; (3) transport the oligomers inside the cell; (4) degrade the oligomers into monomeric form; and (5) metabolize monomers into desired biofuel or biochemical product through metabolic engineering.

This chapter will discuss macroalgal polysaccharides properties and focus on biochemical and biophysical characterization and engineering of seaweed polysaccharides degrading enzymes and their applications for synthesis of biofuels and chemicals.

5.2 Properties of Seaweeds

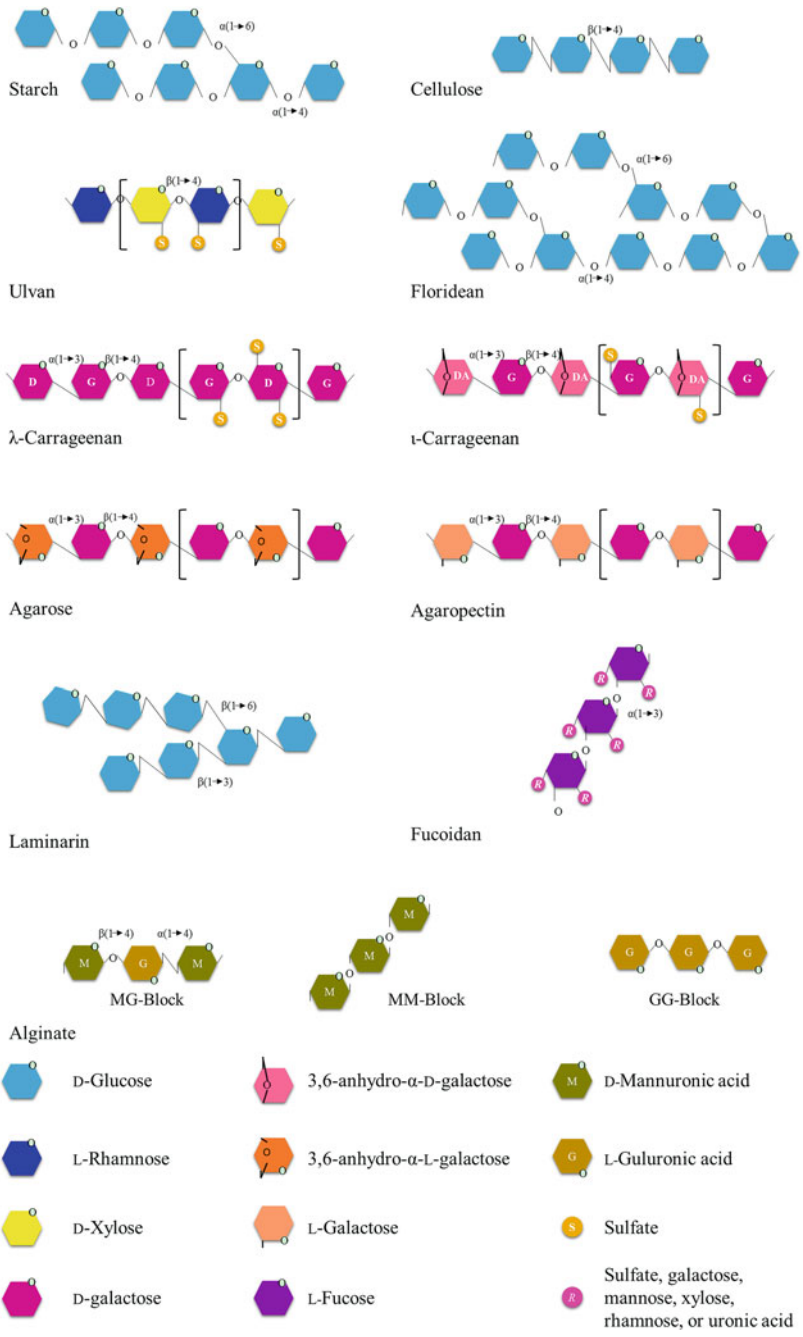
They are especially abundant in coastal waters; however, due to their diverse evolutionary origin, they can be found in a wide variety of habitats, show different growth, and have varied chemical composition [18–22].

Marine macroalgae, referred as seaweeds, include a variety of multicellular photosynthetic organisms with a diverse evolutionary origin. Traditionally, they are classified in three different groups based on the color of their natural pigment content: green macroalgae (Chlorophyceae), red macroalgae (Rhodophyceae), and brown macroalgae (Phaeophyceae). Typical green, red and brown macroalgae-species are *Ulva* sp. and *Codium* sp.; *Gelidium* sp. and *Eucheuma* sp.; and *Laminaria* sp. and *Sargassum* sp., respectively.

Macroalgae as a photosynthetic organism convert sunlight into chemical energy, which is used to efficiently fix inorganic carbon (in form of CO_2 and/or H_2CO_3) into carbohydrates, which can be exploited for biorefinery. However, to consider macroalgae as a feedstock for biofuels and chemical production, it is essential to understand the chemical composition of macroalgal biomass, and their available content. Therefore, understanding the composition of macroalgal biomass helps to determine the appropriate process and technology to produce the biochemical of interest.

In general terms, macroalgae have low content of lignin, lipids, and proteins [19, 23–26]. However, they are rich in carbohydrates, the cell wall being the primarily source. The carbohydrate content can account for more than 60 % of dry weight [18]. Thus, the conversion of these carbohydrates is expected to be the main source for biofuel and chemical production.

Carbohydrates in macroalgae show a diverse chemical composition, and some of them are restricted to a certain group of macroalgae. For example, green seaweeds have a sulfated polysaccharide named ulvan as major non-glucan carbohydrate. Ulvan is a water-soluble sulfated polymer consisting of repeating units of L-rhamnose, D-xylose, D-glucose, D-glucuronic acid, and L-iduronic acid with diverse pattern depending on the species (Fig. 5.1). This type of carbohydrate can be found in the cell wall representing up to 29 % of dry weight. Nevertheless, green macroalgae also contain other common glucans as starch and cellulose. Starch is a


Starch
Cellulose
Ulvan
Floridean
λ-Carrageenan
ι-Carrageenan
Agarose
Agaropectin
Laminarin
Fucoidan
MG-Block
MM-Block
GG-Block
Alginate
D-Glucose
L-Rhamnose
D-Xylose
D-galactose
3,6-anhydro-α-D-galactose
3,6-anhydro-α-L-galactose
L-Galactose
L-Fucose
D-Mannuronic acid
L-Guluronic acid
Sulfate
Sulfate, galactose, mannose, xylose, rhamnose, or uronic acid

◀ **Fig. 5.1** Scheme of the structure of macroalgae polysaccharides. Sugars and linkages between them are represented. Main repeating disaccharide units are indicated between brackets. In λ-carrageenan: D, denotes D-units (α-D-galactose); G, denotes G-units (β-D-galactose). In ι-carrageenan: DA, denotes DA-units (3,6-anhydro-α-D-galactose). In alginate: M, denotes M-residues (β-D-mannuronicacid); G, denotes G-residues (α-L-guluronicacid). Fucoidan polymer is widely heterogeneous and composition of monosaccharides, sulphate positions, linkages, and branching is highly variable, thus it is only represented a homopolymer of α-1,3-L-fucose where R may be any of the following: sulfate, galactose, mannose, xylose, rhamnose, or uronic acid. For more information refer to the text

linear molecule of α-1,4-D-glucose with α-1,6 branches which is produced as an energy store. Cellulose is a water-insoluble linear chain of β-1,4-D-glucose that can be found in the cell wall as a structural polysaccharide (Fig. 5.1) [27–29].

Red macroalgae contain a unique type of glucan, floridean starch, a highly branched polymer of α-1,4-glucosidic linked D-glucose with α-1,6-branches (Fig. 5.1), which green and brown algae do not have. Floridean starch can be found as carbon reserve starch granules in the cytosol of red algae and represent up to 80 % of the cell volume [30]. However, red seaweeds contain carrageenan and agar as major non-glucan polysaccharides constituents in the cell wall, which can account for more than 50 % of dry weight [19, 31].

Carrageenans are sulfated linear polysaccharides of D-galactose and 3,6-anhydro-D-galactose. These polysaccharides are traditionally divided into six basic forms: Iota (ι)-, Kappa (κ)-, Lambda (λ)-, Mu (μ)-, Nu (ν)- and Theta (θ)-carrageenan. The μ-, ν-, and λ-carrageenans consist of a repeating disaccharide formed by 3-linked β-D-galactose (G-units) and 4-linked α-D-galactose (D-units), and have one, two or three sulfate ester groups, respectively. They are the natural biological precursor of κ-, ι-, and θ-carrageenans that consist of a repeating disaccharide formed by 3-linked β-D-galactose (G-units) and 4-linked 3,6-anhydro-α-D-galactose (DA-units) (Fig. 5.1) [26, 32].

Agar consists of the combination of agarose and agaropectin. Agarose is a linear polymer comprising of the repeating unit of agarobiose, which is a disaccharide of 3-linked β-D-galactose and 4-linked 3,6-anhydro-α-L-galactose, whereas agaropectin contains 3-linked β-D-galactose and 4-linked α-L-galactose (Fig. 5.1) [19, 26, 33–35].

Brown macroalgae contain alginate, mannitol and laminarin as major carbohydrates. However, other carbohydrates such as fucoidan and cellulose may also be present. The alginate content in brown algae cell wall can represent up to 40 % of dry weight. Alginate is a water-soluble, linear, unbranched polymer containing two types of uronic acid units: 4-linked β-D-mannuronic acid (M-residues), and 4-linked α-L-guluronic acid (G-residues) (Fig. 5.1). These units can be found forming G-blocks (repeating G units), M-blocks (repeating M units), and MG-blocks (alternating M and G units) [36, 37].

Laminarin is a water-soluble glucan carbohydrate produced as an energy store. It is composed of 3-linked β-D-glucose with β-1,6-branches (Fig. 5.1), and represents up to 35 % of dry weight of algae. There are two different types of laminarin chains

depending on the terminal end: "M" laminarin contains a mannitol residue as a non-reducing end; "G" laminarin contains a glucose residue as a reducing end instead [19, 38]. Mannitol is the sugar alcohol of mannose, and together with laminarin, is the most readily accessible sugar in brown macroalgae.

Fucoidan is a sulfated polysaccharide composed chiefly of α-1,3-L-fucose alternating with other monosaccharides as galactose, mannose, xylose, rhamnose, and uronic acid (Fig. 5.1). Fucoidan structure is widely heterogeneous as the composition of monosaccharides, sulphate positions, linkages, and branching is highly variable [39–41].

The breakdown of macroalgae carbohydrate composition shows that the glucan content is low (around 20–25 % dry weight) compared to that of terrestrial biomass (30–45 % dry weight) [42], and hence use of these carbohydrates as a feedstock is not enough to achieve high productivities by their conversion into a biochemical of interest. Accordingly, the conversion of other macroalgae carbohydrates is needed to attain industrial productivities. Whereas glucans are easily fermentable carbohydrates, the uniqueness of non-glucan macroalgae carbohydrates makes them difficult to use.

A number of microorganisms have developed specialized enzymatic systems for the efficient utilization of these non-glucan carbohydrates as a carbon source. These carbohydrate-degrading enzymes, which will be discussed below, can be implemented in different biotechnological processes to make the macroalgae sugar content accessible to fermentative microorganism that eventually can be engineered to synthesize a biochemical of interest.

5.3 Enzymes Involved in Macroalgae Degradation

As the feedstocks found in macroalgae are polymers, degradation of these polymers is necessary for their utilization. The enzymes which accomplish this degradation can either be endolytic (acting within the fragment) or exolytic (acting from the outside of a fragment). The resulting degradation products must then be transported into the cell for metabolism. Here we present an overview of the proteins and enzymes necessary for an organism to metabolize a polysaccharide from macroalgae.

5.3.1 Alginate Lyases

5.3.1.1 Background and Families

Alginate lyases (EC 4.2.2.3 and 4.2.2.11) are enzymes that cleave the glycosidic bonds of alginate. These enzymes accomplish the cleavage of alginate through β-elimination. The cleavage of the glycosidic bonds occurs through a sequence of

three reaction steps. First, the negative charge of the carboxyl anion is neutralized via the amino acid residues arginine, histidine, glutamine, glutamic acid, or tyrosine or a divalent cation. Second, the C-5 proton is abstracted from the sugar ring by either an aspartic acid, cysteine, glutamic acid, histidine, or lysine residue. Last, a tyrosine or arginine residue is used to accept the electrons from the C-4 and C-5 bond. The net result of these steps is the formation of a double bond between C-4 and C-5 in the cleaved guluronate or mannuronate [43, 44].

As alginate lyases are defined for their proclivity to degrade the bonds within alginate, these enzymes can have different 3-D structures, indicating that alginate lyases have evolved many times. Each of these structures can be classified into polysaccharide lyases (PL) families. These families show a wide variety in their structures, including β-helices and α/α barrels. There exist seven PL families typically associated with alginate lyases [44, 45], including PL5, PL6, PL7, PL14, PL15, PL17, and PL18. The families PL5 and PL16 contain an $(\alpha/\alpha)_6$ barrel structure, wherein the α barrels form a tube-like structure [46–48]. The families PL7, PL14, and PL18 contain a β-jelly roll with β-sheets in antiparallel, adjacent configuration [49, 50]. The β-sheets are arranged to form a cleft shape. The PL6 family contains a parallel β-helix structure with a long repeated series of β-strands forming a long tube [51]. The PL17 family has a structure with many α-helices forming a barrel structure [52].

5.3.1.2 Enzymatic Mechanism of Alginate Lyase Degradation

Alginate lyases cleave the glycosidic bonds within alginate using β-elimination. β-elimination is a reaction process wherein glycosidic bonds are cleaved by the formation of a reducing end on one end and an unsaturated ring on the other end of the broken bond [43]. This reaction mechanism differs from that of hydrolysis in that water is not used to break the glycosidic bond. Most broadly, an alginate lyase acts on the glycosidic bond at the C-4 position by first neutralizing the negative charge on the carboxylic group on C-5. This neutralization is accomplished by the action of a positively charged cation or amino acid residue. Once the neutralization occurs, the C-4 proton is abstracted and an electron transfer occurs which cleaves the glycosidic bond and forms a double bond between C-4 and C-5.

There are two general mechanisms for the neutralization of the positive charge by an alginate lyase. The first mechanism utilizes a divalent cation (such as Ca^{2+}) to neutralize the negative charge on the C-5 carboxylic group. A lysine group is then used to abstract the C-4 proton, while a lysine or arginine group accepts the required electrons. This metal-assisted mechanism is found in the PL6 family of alginate lyases [53].

The second catalytic mechanism employed by alginate lyases does not utilize a divalent cation for neutralization, but rather uses an asparagine, arginine, glutamic acid, or glutamine residue for neutralization. Independent of the amino acid residues used for carboxyl-group neutralization, the members of PL5 [54], PL7 [55], and PL15 [56] families of lyases utilize a histidine residue to abstract the proton from

C-4 and a tyrosine residue to accept the electrons which cleave the glycosidic bond. Interestingly, recent work has shown that the PL18 [57] family of alginate lyases utilizes a tyrosine residue to both abstract the proton from C-4 and then donate the same proton to the linked C-1 to cleave the glycosidic bond. Arginine and lysine are used to stabilize the carboxyl group. A similar mechanism has been proposed for a PL17 alginate lyase [52]. Two different tyrosine residues deprotonate the hydrogen from C-4 and accept the electrons to cleave the glycosidic bond. Asparagine and histidine residues were proposed to stabilize the carboxylic group.

The enzymatic mechanism for glycosidic cleavage of the PL14 family alginate lyases has not been fully elucidated. A representative of the PL14 family has been crystallized [50]. It was found that in this alginate lyase, mutagenesis of all the amino acids within the catalytic pocket reduced enzymatic activity. In addition, mutagenesis of K197A and S219A abolished enzymatic activity. These residues were conserved from other alginate lyases. This suggests that these amino acid residues might play a role in the cleavage of alginate.

(1) Oligoalginate Lyases

As alginate can contain up to 70 monomers within its structure, its degradation is expected to occur in several steps. First, the full alginate polymer is degraded into smaller fragments, called oligoalginate. These oligoalginate fragments are then degraded into the constitutive monomers by oligoalginate lyases which can act exolytically. The PL15 and PL17 families of alginate lyases are associated with the degradation of oligomers of alginate. A PL15 alginate lyase, Atu3025, from *Agrobacterium tumefaciens* has been crystallized [56] to understand how PL15 lyases act exolytically. Ochiai and coworkers found that this lyase contains a pocket-like structure which can recognize the terminal monomer within alginate [56]. Only when this terminal monomer is situated in the pocket, Atu3025 will adopt a "closed form" wherein β-elimination can occur. The enzyme then adopts an "open form" that releases the degraded monomer and a new alginate fragment can approach the enzyme.

Recent work has sought to characterize the oligoalginate degrading enzymes of *Vibrio splendidus* 12B01 [58]. This bacterium has three PL15 oligoalginate lyases (OalA, OalB, and OalC). OalA was found to have high catalytic efficiency (k_{cat}/K_m) on an alginate trisaccharide and less than 10 % catalytic efficiency on larger alginate fragments. It was confirmed that OalA acts on alginate exolytically since only monomers were released upon degradation of alginate. Other PL15 and PL17 oligoalginate lyases have also been expressed and characterized [52, 59].

(2) Specificity of Alginate Lyase Degradation

Since alginate contains different sequence combinations of L-guluronate and D-mannuronate, alginate lyases have evolved the ability to target specific regions of alginate. Alginate lyases targeting polyM [60], polyG [61, 62], and polyMG [63] regions within alginate have been identified. The specificity of an alginate lyase can be determined through multiple experimental techniques. Partial degradations of alginate can be chemically generated [64, 65], which results in the formation of polyG- and polyM-rich alginate substrates. The enzymatic activity towards these substrates can be then compared to that of the full alginate polymer. Another means

by which substrate specificity can be determined is through NMR analysis of the enzymatically degraded alginate [66, 67]. In this procedure, the degraded products are analyzed for the formation and destruction of NMR peaks which correspond to specific linkages in alginate. Since alginate degradation is expected to target specific dyads within the structure, the dyad specificity can then be determined. This analysis has successfully identified the dyad specificity of four alginate lyases within a single bacterium [68].

5.3.1.3 Alginate Transport and Metabolism

Since alginate is a large molecule, a microorganism must utilize a means of transporting alginate or oligoalginate into the cell. The bacterium *Sphingomonas* sp. strain A1 contains a periplasmic alginate protein-dependent ATP binding cassette (ABC) transporter [69]. The overall transport mechanism contains three components: a pit on the cell surface which transports the full alginate polymer into the cell periplasm, proteins within the periplasm which bind alginate, and the aforementioned ABC transporter which is found in the inner-membrane. *Sphingomonas* sp. strain A1 then uses alginate lyases contained within the cytoplasm to degrade alginate into the constituent monomers.

Another mechanism by which degraded alginate is effectively transported into the cell can be found in the bacterium *V. splendidus* 12B01. This bacterium contains an outer-membrane porin KdgMN [70]. Long chain oligoalginate fragments diffuse into the periplasm of 12B01 through KdgMN. These long chains are then degraded into chains two to four units long within the periplasm by alginate lyases. The two- to four-mers are then transported into the cytoplasm by the symporter ToaABC. These short fragments are then degraded into monomeric form by oligoalginate lyases. The two transport mechanisms described illustrate how alginate must be carefully transported, either through assisted transport of the full polymer or initial degradation outside the cell followed by diffusion of the degraded oligoalginate.

Once alginate is degraded and transported into the cell, an organism must metabolize the alginate monomers. An alginate lyase will cleave alginate using β-elimination which results in an L-guluronate or a D-mannuronate and a non-reducing end. The generated non-reducing end is 4-deoxy-L-erythro-hex-4-enepyranosyluronate (DEH) [64]. L-Guluronate and D-mannuronate can also be converted to DEH non-enzymatically, so DEH serves as an important step in alginate metabolism. Once inside the cell, DEH is converted to 2-keto-3-deoxy-D-gluconic acid (KDG) by the NADPH-dependent enzyme DEH reductase [71]. KDG is then converted by the enzyme KDG kinase [70] to 2-keto-3-deoxy-6-phosphogluconate (KDPG). KDPG enters the Entner–Doudoroff pathway to be converted into pyruvate and glyceraldehyde-3-phosphate by the enzyme KDG-6-phosphate aldolase [70]. The final product of alginate metabolism is ATP and NADH.

5.3.1.4 Alginate Lyase Engineering

Many different alginate lyases from diverse organisms have been characterized, leading to a wealth of information on the enzyme kinetics and substrate binding of alginate lyases to alginate. However, attempts at protein engineering to improve alginate lyase enzymatic activity are less frequent. The alginate lyase, AlyVI, from *Vibrio* sp. QY101 was altered to improve enzymatic function [72]. Cho and coworkers built a homology model of this enzyme and modeled the binding of a repeating α-L-guluronic acid, trimer GGG, to the enzyme. Based on this model, they then mutated the amino acid residues within AlyVI predicted to be involved in enzymatic function; mutation of these residues abolished activity, which confirms their role in β-elimination. The authors also found that the double mutation L224V/D226G in AlyVI increased catalytic activity almost twofold without changing the binding affinity of GGG. Computational modeling of this double mutant indicated that the β-sheets of this PL7 lyase were rearranged allowing for increase in the enzymatic activity.

Another study unveiled the catalytic mechanism of the PL5 lyase Smlt1473 from *Stenotrophomonas maltophilia* k279a [73] to degrade polysaccharides. This lyase can degrade the polysaccharide hyaluronic acid along with polyM and polyG. Smlt1473 has optimal activity towards polyM at pH 9, while it has optimal activity towards polyG at pH 7. The authors sought to understand how this lyase degrades polyM and polyG preferentially by mutating non-catalytic residues within the catalytic cleft of the enzyme. They identified two single mutations, H221F and Y225F, which increased activity toward polyM twofold, and two other single mutations, Y115F and R312L, which increased activity toward polyG twofold. As polyM and polyG have different conformational structures, the authors concluded that these mutations resulted in subtle changes in Smlt1473 which increase activity towards either polyM or polyG.

These two mutational studies demonstrate that rational engineering of alginate lyases can lead to significant changes in enzymatic activity and specificity without sacrificing function. Other attempts at mutagenesis [74] have resulted in increased activity at the expense of weaker alginate binding. Specificity appears to be a parameter easily modified by rational design. However, random mutagenesis has been also used to modify specificity [75], albeit coming at the expense of enzymatic activity.

5.3.1.5 Multiplicity of Alginate Lyases

Alginate-degrading bacteria usually contain multiple putative lyases, thus some studies have sought to understand the purpose of these seemingly redundant enzymes. The marine bacterium *V. splendidus* 12B01 contains three oligoalginate lyases and four alginate lyases. Each of these enzymes was characterized. The three oligoalginate lyases, OalA, OalB, and OalC, were found to have optimal enzymatic activity between 16 and 35 °C and pH 6.5 and 7.5 [58]. Additionally, OalA was

found to have both polyM and polyG specificity, while OalB and OalC were found to have either polyMG or polyM specificity, respectively. These enzymes act exolytically, cleaving the terminal moiety from alginate or oligoalginate. The four alginate lyases, AlyA, AlyB, AlyD, and AlyE were found to be optimally active between 20 and 25 °C and pH 7.5 and 8.5 [68]. Detailed NMR analysis identified that AlyA and AlyB act on the G-M dyad, corresponding to polyMG specificity, while AlyD and AlyE act on the G-G dyad, corresponding to polyG specificity. AlyA, AlyB, AlyD, and AlyE were found to act endolytically, cleaving alginate to an average degree of polymerization from 4 and 21. These alginate lyases also degraded alginate much slower than the oligoalginate lyases OalA, OalB, and OalC. Taking these two studies together, a picture of alginate degradation of *V. splendidus* 12B01 can be formed. AlyA, AlyB, AlyD, and AlyE degrade alginate to oligomers of average length between 4 and 21. These fragments are then transported into the cell and are degraded into monomeric form by OalA, OalB, and OalC with faster kinetics than the alginate lyases. Since the 12B01 enzymes have polyM, polyG, or polyMG specificity, these enzymes are expected to fully degrade alginate, since all portions of the polymer can be targeted.

Another study characterized two of the seven alginate lyases from the marine bacterium *Zobellia galactanivorans* [49]. One of these alginate lyases, $AlyA1_{PL7}$, was determined to act endolytically with a G-G dyad specificity. Conversely, AlyA5 was found to act exolytically on DEH, G, or M-terminal ends. These findings suggest that pairing of endolytic and exolytic alginate-degrading enzymes is advantageous for heterologous alginate degradation outside of its native host. Additionally, the pairing of enzymes with differing dyad specificities is expected to allow full alginate degradation.

In summary, enzyme multiplicity in alginate-degrading organisms aims to produce a set of enzymes with a wide spectrum of biochemical properties to fully degrade the alginate polymer.

5.3.2 Laminarinases

5.3.2.1 Background

Laminarinases (EC 3.2.1.6) are the enzymes capable of breaking the β-1,3- and β-1,6-glycosidic linkages of laminarin. These enzymes belong to the wider class of enzymes called glycoside hydrolyases (GHs) [76]. These enzymes are fundamentally different than the previously discussed alginate lyases, in that, they cleave the glycosidic bonds using water. More specifically, laminarinases cleave the bonds of laminarin in either a one-step or two-step process.

The one-step process is an inversion mechanism [76]. In this mechanism, the glycosidic linkage is simultaneously attacked by two amino acid residues, one acting as an acid while the other acts as a base. Through transfer of electrons from the basic amino acid residue to a water molecule, the water molecule attacks the

anomeric center of the glucose residue. Simultaneously, the acidic amino acid residue accepts electrons from the glycosidic oxygen. The net result of this sequence is the cleavage of the glycosidic bond via hydrolysis. Since the newly reacted hydroxyl group from the water attack exists in the opposite configuration of the original β-glycosidic bond, the cleaved hemiacetal contains a 6C hydroxyl group in an α-configuration, hence the inversion mechanism.

The two-step reaction mechanism is a retaining mechanism. First, in a glycosylation step, one of the catalytic residues acts as a nucleophilic center by attacking the anomeric center of the linked glucose residue. Simultaneously, the other catalytic residue acts as an acid by protonating the glycosidic oxygen. The net result of these steps is the cleavage of the glycosidic bond [77]. Following the glycosylation step of laminarin cleavage, a complex is formed between one of the catalytic amino acid residues and the cleaved laminarin molecule. In order to complete the overall cleavage of the laminarin glycosidic bond, a second step must be performed: deglycosylation. In this step, a molecule of water is used to attack the complex, which frees the laminarinase catalytic residue from the cleaved laminarin [78]. Since the newly added hydroxyl group exists in the same β-configuration as the original β-glycosidic bond, this mechanism is a retaining mechanism. In laminarinases, the catalytic residues used by either the retaining or inverting mechanism are typically glutamic and aspartic acids [79, 80].

5.3.2.2 Laminarinase Characterization

Laminarinases from diverse organisms have been characterized. A laminarinase from the archaeon *Pyrococcus furiosus* has been overexpressed and purified [81]. This enzyme was shown to be optimally active between pH 6.0 and 6.5 and at 100 °C. As the glycosidic linkages found in laminarin are similar to other storage glucans, Gueguen and coworkers sought to determine if this laminarinase would degrade other glucans [81]. The authors found that the *P. furiosus* laminarinase had maximal activity on laminarin and approximately 90 % less activity on lichenan and barley β-glucan (both have β-1,3-1,4 glucose linkages). The laminarinase had no activity on glucans with only β-1,4 glucose linkage.

Another laminarinase from the actinobacterium *Streptomyces sioyaensis* was also characterized [82]. This laminarinase had optimal activity at pH 5.5 and 75 °C. The laminarinase was found to be most active on the β-1,3-linked glucans curdlan, laminarin, and pachyman, while the enzyme had fourfold less activity on lichenan (a β-1,3-1,4 glucan). A fungal laminarinase from *Phanerochaete chrysosporium* was purified from the secreted proteins and characterized [83]. This enzyme was tested to degrade various glucans and found to degrade laminarin and lichenan, β-1,3-1,6- and β-1,3-1,4-linkages, respectively. The laminarinase had no activity on cellulose (β-1,4 linkage).

5.3.3 *Fucoidanases, Agarases, and Carrageenanases*

Alginate and laminarin are the primary constituents of macroalgae; however, other polysaccharides are found within macroalgae. Fucoidanases (EC 3.2.1.44) are enzymes that degrade the glycosidic bonds within fucoidan. These enzymes have been purified from bacterial species and have shown optimal activity between pH 5 and 8 and between 30 and 45 °C [84, 85]. Fucoidan degrading enzymes are also found in marine invertebrates; an α-L-fucosidase from the marine mollusk *Pecten maximus* has been purified [86]. This enzyme was shown to have large enzymatic activity and high thermal stability. Additionally, the α-L-fucosidase was shown to have low fucoidan linkage specificity.

Enzymes have evolved to degrade the polysaccharides carrageen and agar. An agarase (EC 3.2.1.81) from *Pseudomonas* sp. was characterized and found to have optimal activity at pH 9 and 35 °C [87]. The agarase also had exolytic degradation. An agarase from the bacterium *Rhodococcus* sp. Q5 [88] was characterized and found to be optimally active at pH 6 and 40 °C.

Enzymes which degrade κ-, ι-, and λ-carrageen (EC 3.2.1.83, 3.2.1.157, and 3.2.1.162, respectively) have also been identified and purified. An ι-carrageenase was found to cleave ι-carrageen with a one-step inversion mechanism [89], in contrast to κ-carrageenases, which employ a retaining mechanism [90]. This indicates that enzymes have evolved to accommodate the specific degree of sulfation of carrageen. A λ-carrageenase has also been purified and characterized [91]. This enzyme was found to degrade only λ-carrageen and not κ- and ι-carrageen, again indicating that different enzymes have evolved to target the different forms of carrageen.

5.4 Gene Expression and Regulation

As polysaccharide degrading enzymes are expected to be expressed by their hosts in response to the presence of their corresponding substrates, a macroalgae degrading organism must be able to detect the presence of polysaccharides in the extracellular medium. A previous study has sought to understand how the hyperthermophilic bacterium *Thermotoga maritime* utilizes diverse carbon sources [92]. This bacterium is capable of metabolizing many simple sugars along with complex polysaccharides including β-1,3/1,4-glucan from barley, laminarin, and carboxymethylcellulose (CMC). The authors demonstrated that upon growth on different carbon sources, clusters of genes are overexpressed. Upon growth on laminarin, a laminarinase (Lam16), an exoglycosidase (Cel3 which cleaves exolytically), and a XlyR-related transcriptional regulator were overexpressed compared to the other growth conditions [92]. Similarly, upon growth on β-1,3/1,4-glucan from barley (β-1,3-1,4-linked glucose), β-1,4- and β-1,3-glucanases and Lam16 were overexpressed compared to other growth conditions. Conversely, when *T. maritime* was grown on CMC

(β-1,4-linked glucose), intra- and extracellular endoglucanases were exclusively overexpressed. These findings indicate that *T. maritime* was able to detect the precise inter linkages of polysaccharides in the extracellular environment and then expressed the corresponding enzymes for degradation. Since a transcriptional regulator was identified upon growth on laminarin, a regulatory target exists for future genetic manipulation.

The alginate regulatory mechanism has also been investigated in the marine bacterium *V. splendidus* 12B01 [68]. This bacterium contains four alginate lyases and the gene expression of each of these enzymes was investigated. Each of the lyases was found to be conditionally expressed upon growth on alginate with higher gene expression at higher alginate loadings. Between 0.1 and 1.0 % alginate in the culture medium, the gene expression of three alginate lyases was found to increase by more than twofold. These findings indicate that polysaccharide degrading enzyme gene expression is often tied to detection of specific polysaccharides in the culture medium. Subsequently, these enzymes will degrade the carbon sources allowing the organism to metabolize the constituent monomers.

5.5 Carbohydrate Binding Modules

A common domain found within polysaccharide degrading enzymes is the carbohydrate binding modules (CBMs). These modules are implicated with binding to both monosaccharides and polysaccharides. A laminarinase, Lam16A, from *Thermotoga neapolitana* was found to contain two CBMs surrounding the catalytic domain [93]. By overexpressing and purifying the CBMs independently and with the catalytic domain, Zverlov and coworkers demonstrated that each CBM was bound to different glucans with different affinities [93]. One of the CBMs was bound to laminarin and barley glucan (β-1,3/1,4-linked glucose), while the other CBM was bound to curdlan (1,3-β-glucose), pustulan (1,6-β-glucose), and insoluble yeast cell wall glucan. When the catalytic domain was incubated with the tested glucans, no binding was found indicating that the CBM is essential for binding a catalytic domain to the substrate for eventual lytic action.

A further investigation of a CBM-containing β-1,3-glucanase found similar results [82]. This enzyme was cloned from *S. sioyaensis* and characterized. The authors found that the CBM was not essential for enzyme activity toward laminarin, but dramatically decreased activity toward curdlan and pachyman. They also found that the CBM was directly bound to these glucans. Curdlan and pachyman are insoluble glucans, so these findings indicate that the CBM of the *S. sioyaensis* glucanase is used to bind insoluble glucans, which facilitates their degradation.

The CtCBM11 CBM from the *Clostridium thermocellum* Lic26A-Cel5E glucanase was found to bind both β-1,4- and β-1,3-1,4-linked glucans [94]. The CBM was found to bind to the tested glucans with an association constant between 4.4×10^4 and 30.1×10^4 M^{-1}. Additionally, through crystallization of the CBM, the authors were able to identify the putative amino acid residues responsible

for binding. By mutating several of these residues, three amino acid residues were determined to independently abolish binding of the CBM to the tested glucans. Results of investigations into CBM binding indicated that CBMs are critical for the enzymatic activity of polysaccharide degrading enzymes, particularly toward insoluble polymers. Interestingly, CBMs are not found in all polysaccharide degrading enzymes.

5.6 Applications of Algal-Polysaccharide Degrading Enzymes

Macroalgae have high potential as a feedstock due to their high carbohydrate content among other advantages as mentioned above. As discussed in Sect. 5.2, because macroalgae contain various types of glucans they can be easily hydrolyzed to glucose for bioconversion into value added chemicals. However, due to the relatively low glucan content in macroalgae compared to that of terrestrial biomass, exploitation of glucan carbohydrates from macroalgae is not competitive enough for its implementation in biorefinery. On the other hand, the high content of non-glucan carbohydrates present in macroalgae makes the exploitation of seaweed feedstock feasible for biofuel and chemical production.

Despite the potential shown by macroalgal carbohydrates for the industrial sector, bioconversion of non-glucan polysaccharides into high value compounds is still challenging, and it hampers the development of this field. Along these lines, recent studies focused on harnessing specialized enzymatic pathways from those microorganisms that naturally metabolize algal-carbohydrates to transform them into energy metabolites and other biocompounds. For this purpose, different fermentation microorganisms have been engineered to produce macroalgal polysaccharides degrading enzymes. The action of these enzymes releases oligosaccharides and/or monosaccharides that could be directly used by the central carbon metabolism. However, due to the peculiarity of algal carbohydrates, the released oligosaccharides and/or monosaccharides were occasionally found not to be metabolized. So, further metabolic engineering of the appropriate microorganism is necessary.

In the following section, recent advances in implementation of polysaccharide-degrading enzymes and specialized metabolic pathways for biofuel and biochemical production from macroalgae are discussed.

5.6.1 Biofuel Production

Currently there are a number of technologies for conversion of macroalgal biomass into biofuels. Bioconversion, hydrothermal liquefaction (HTL), and pyrolysis have

been developed for the production of biogas, bioethanol, biodiesel and bio-oil from macroalgal feedstock. HTL and pyrolysis are thermochemical conversion processes that require a high thermal treatment to decompose the biomass. These technologies do not require the use of seaweed polysaccharide-degrading enzymes, and thus they will not be discussed here. Readers are referred to various reviews on the subject [18–21, 95].

On the other hand, bioconversion harnesses the microbial enzymatic machinery to depolymerize macroalgal carbohydrates into sugars for production of liquid biofuels by fermentative microorganisms. Consequently, the choice of appropriate microorganisms for assimilation of these monomers is pivotal for successful fermentation. For example, green and red macroalgae contain relatively high levels of accessible fermenting sugars that can be fermented by common tractable microorganisms such as *Saccharomyces cerevisiae*. Conversely, biofuel production from brown macroalgae (with alginate as the main carbohydrate) is limited by the availability of microorganisms that can metabolize alginate.

Prior to fermentation by an appropriate ethanologenic microorganism, *S. cerevisiae*, for example, the carbohydrate fraction of macroalgae is usually released by physical and chemical treatments. The most widely adopted pretreatment is hydrothermal treatment with acid or alkali. These treatments release the polymers from the algal cell wall. However, these methods usually lead to the formation of fermentation inhibitors [96]. To reduce the toxicity of these treatments and also favor the availability of sugars to the fermentative microorganism, some studies have combined mild pre-treatments with enzymatic saccharification leading to improved ethanol productivities. Adams and coworkers combined enzymatic saccharification of extracts derived from the brown macroalgae, *Saccharina latissimi,* with fermentation by the ethanologenic *S. cerevisiae*. A laminarinase was also used to hydrolyze laminarin into glucose which was fermented to ethanol by the yeast. Ethanol yield obtained was 0.45 % (v/v) [97]. However, mannitol present in *S. latissimi* extracts was not consumed by *S. cerevisiae*. A microorganism with a more versatile sugar metabolism would allow more efficient conversion of the mixed carbohydrates in seaweed extracts. Thereby, simultaneous fermentation of mannitol and laminarin was performed by the yeast *Pichia angophorae* CBS 5830 in extracts of the brown algae *Laminaria hyperborean*. The *Pichia* yeast is able to convert mannitol and glucose into ethanol as well as hydrolyzing laminarin as it produces laminarinase. In this study, the maximum ethanol yield obtained was 0.43 g ethanol/g substrate [98]. In another study, extracts of *Laminaria digitata* were fermented by *P. angophorae* as in the above example. However, the addition of commercial laminarinase enzyme in the extract expedited the hydrolysis of laminarin and hence led to increase the ethanol yield to 0.89 % (v/v) [99].

Another microorganism capable of metabolizing mannitol and glucose is *Escherichia coli*. An engineered ethanologenic *E. coli* KO11 (ATCC55124) that contained the *Zymomonas mobilis* ethanol production genes [100] was used to produce ethanol from brown macroalgae. The acid lysates of *Saccharina japonica* (*Laminaria japonica*) treated with enzymes released high concentrations of glucose

and mannitol, and thus simultaneous saccharification and fermentation (SSF) resulted in a final ethanol yield of up to 29 g/L [101].

In these studies, the main fermentation sugars were mannitol and glucose derived from laminarin, but in brown macroalgae the major carbohydrate content is alginate, which is underused due to the lack of an efficient alginate saccharification method. Jang and coworkers tried to increase the use of the macroalgae sugar content by employing *Bacillus* sp. JS-1 for saccharification, a marine bacterium isolated from seawater capable of degrading different seaweed polysaccharides such as alginate, fucoidan and laminaran. In this study, extracts from *S. japonica* treated with thermal acid hydrolysis were inoculated with *Bacillus* sp. JS-1 and *P. angophorae* KCTC 17574. The combination of thermal acid hydrolysis with saccharification by *Bacillus* sp. JS-1 increased the release of reducing sugars from *S. japonica* by threefold compared to treatment of thermal acid hydrolysis alone. Whereas, combination of thermal acid hydrolysis with enzymatic treatment using a commercial α-amylase (Termamyl 120 L) gave a twofold increase of sugar release. The maximum ethanol concentration obtained by SSF with *Bacillus* sp. JS-1 and *P. angophorae* KCTC 17574 was 9.8 mL/L [∼1 %, (v/v)] [102].

These studies revealed that the customization of saccharification methods depending on the mixed carbohydrates from macroalgae together with the selection of an appropriate fermentative microorganism would increase bioethanol production. Pursuant to this goal, the most promising SSF approach is engineering the fermentative microorganism to combine saccharification enzyme production, carbohydrate degradation and monosaccharides metabolism, and ethanol formation in a single-process step.

Along these lines, *Sphingomonas* sp. A1 is an interesting candidate for metabolic engineering. *Sphingomonas* sp. A1 is a Gram-negative alginate-assimilating bacterium that directly incorporates alginate into the cytoplasm through a periplasmic alginate-binding protein-dependent ATP-binding cassette transporter. In the cytoplasm, alginate is degraded to monosaccharides by endo- and exotype alginate lyases. The released monosaccharides are then metabolized to pyruvate. By introduction of an ethanologenic pathway, pyruvate from alginate degradation can be redirected to produce ethanol. Takeda and coworkers engineered an ethanologenic *Sphingomonas* sp. A1 by introducing the pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase B (*adhB*) genes from *Zymomonas mobilis* under the control of a strong constitutive promoter isolated from *Sphingomonas* sp. A1 [103]. Also, the lactate dehydrogenase gene (*ldh*) was deleted to redirect the carbon flux to production of ethanol. The combination of these modifications led to a maximum of 13 g/L of ethanol after 3 days of incubation [103].

One of the most remarkable breakthroughs in the field was the development of consolidated bioprocessing (CBP) for production of biofuels from macroalgal biomass by Wargacki and coworkers. In this work, an *E. coli* platform that can degrade, uptake, and metabolize alginate to produce ethanol was constructed [70].

Initially, an alginate lyase (Aly) secretion system was created. A truncated Aly from *Pseudo-alteromonas* sp. SM0524 (tSM0524 Aly) was fused to an engineered portion of the antigen 43 (Ag43) which allowed the extracellular release of the

functional tSM0524 Aly. This step enabled the degradation of alginate into oligoalginate without the need of thermal and chemical pretreatment or enzymatic saccharification before fermentation. The next step was the identification and isolation of a putative alginate-metabolizing gene cluster from the marine bacterium *V. splendidus* 12B01 that enabled oligoalginate transport and conversion into pyruvate and glyceraldehyde-3-phosphate. The metabolic pathway was further optimized by expression of auxiliary genes. The putative alginate-metabolizing pathway allowed oligoalginates to pass through the outer membrane to the periplasmic space via porin (KdgM-KdgN). Then, oligoalginates were further degraded into smaller oligomers by alginate lyases (AlyABCD). The symporter (ToaABC) transported the oligomers to the cytoplasm where oligoalginate lyases (OalABC) degraded oligomers into monomers that were spontaneously transformed into DEH. DEH reductase (DehR) reduced DEH into KDG which is phosphorylated by KDG kinase (KdgK) into KDPG. Finally, KDG-6-phosphate aldolase (Eda) cleaved KDPG into pyruvate and glyceraldehyde-3-phosphate (Fig. 5.2) [70]. The alginolytic *E. coli* platform was further engineered to efficiently produce ethanol. To this end, the pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase B (*adhB*) genes from *Zymomonas mobilis* were integrated into the genome. Additionally, *pflB-focA*, *frdABCD*, and *ldhA* genes were deleted to avoid the synthesis of fermentation by-products [70]. The *E. coli* strain and the copy number of the alginate-assimilating cluster and the ethanologenic pathway were optimized to enhance the microbial platform performance. *E. coli* ATCC8739 exhibited faster growth rates in pre-degraded alginate media than other strains evaluated. Also, single copy of each

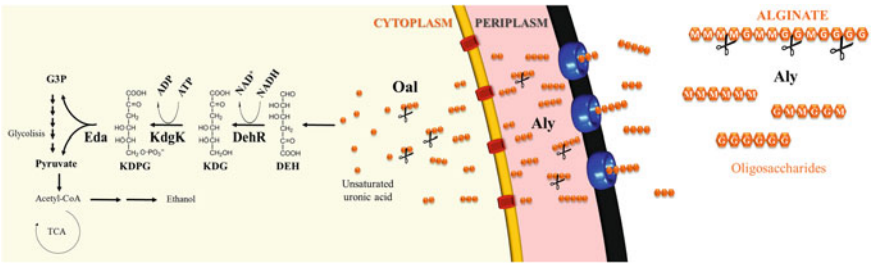


Fig. 5.2 Metabolic pathway for alginate degradation and ethanol production in an engineered *E. coli*. Alginate lyase (Aly, endo-type) degrades the alginate polymer into oligoalginate. These oligomers pass through the outer membrane (in *black*) to the periplasmic space via porin (KdgM-KdgN) (in *blue*). Then, oligoalginates are further degraded into smaller oligomers by alginate lyases. The symporter (ToaABC) (in *red*) transports the oligomers to the cytoplasm where oligoalginate lyases (Oal, exo-type) degrade oligomers into monomers of uronic acid that are spontaneously transformed into DEH (4-deoxy-L-erythro-5-hexoseulose uronic acid). DEH reductase (DehR) reduces DEH into KDG (2-keto-3-deoxy-D-gluconate) which is phosphorylated by KDG kinase (KdgK) into KDPG (2-keto-3-deoxy-6-P-gluconate). Finally, KDPG aldolase (Eda) cleaves KDPG into pyruvate and G3P (glyceraldehyde-3-phosphate). G3P is converted into pyruvate through the glycolysis metabolic pathway. Pyruvate can be redirected to be converted into ethanol by the introduction of an ethanologenic pathway. G, guluronic acid; M, mannuronic acid

pathway (alginate-metabolizing and ethanologenic) was found to produce higher ethanol titers [104]. The resulting strain (BAL1611) was evaluated for its ability to ferment *S. japonica* extracts. BAL1611 produced an average of 37 g/L of ethanol from dry milled brown macroalgae, which represented a final titer of ~4.7 % v/v of ethanol [70, 104].

Similarly, Enquist-Newman and coworkers developed a yeast platform that can simultaneously metabolize DEH and mannitol to produce ethanol [105]. Due to the lack of the metabolic machinery for alginate and/or DEH assimilation, the yeast *S. cerevisiae* cannot utilize either alginate or DEH. Therefore, several approaches were needed to achieve an ethanologenic DEH-assimilating strain. To this end, the DEH transporter AcDHT1 was identified from the alginolytic eukaryote *Asteromyces cruciatus*. This transporter was highly induced when *A. cruciatus* was grown on alginate. Also, yeast cells containing this transporter showed improved growth on DEH-containing media. Another important development was the identification of essential mannitol catabolism genes. The expression of an NAD^+-dependent mannitol-2-dehydrogenase (M2DH) (encoded by *yel070w*/*ynr073c*) and a mannitol transporter (encoded by *hxt13*/*hxt17*) were critical for mannitol assimilation in yeast. Finally, the encoding genes for DEH reductase, KDG kinase and KDPG aldolase, involved in DEH assimilation from different bacteria were studied. Those with maximum specific activity were overexpressed in the engineered yeast. Since the growth rate in DEH medium was low, the authors implemented an adaptive evolution strategy for several months to enhance the efficiency of DEH metabolism in aerobic and microaerobic conditions. BAL3215, the adapted strain containing the *Vibrio harveyi* DEH reductase (VhDehR), which uses both NADH and NADPH efficiently, stood out as the strain with improved ability to produce ethanol from mannitol and DEH in microaerobic conditions. Ethanol titers of 4.6 % (v/v) were obtained from fermentation of DEHU:mannitol 1:2 molar ratio, representing 83 % of the maximum theoretical yields [105]. This study also revealed the relevance of the cofactor balance for efficient ethanol production from macroalgae derived sugars. Contador and coworkers developed a genome-scale metabolic model that simulated the cellular behavior of the engineered yeast platform under macroalgal sugar fermentation to analyze the redox balance. The model pointed out the NADH-NADPH bias of DehR as the main determinant factor for efficient ethanol production. The model also detected other metabolic limitations that would be difficult to determine by other means. The authors suggested that this sort of metabolic model would help to improve the performance of the ethanol fermentation from alginate and mannitol [106].

Recently, the metabolic pathway for assimilation of 3,6-anhydro-L-galactose (AHG) has been elucidated. Red macroalgae contain high amounts of agar which is a combination of agarose and agaropectin. The enzymatic treatment of agarose with β-agarases and neoagarobiose hydrolase released β-D-galactose and AHG [107]. Galactose can be easily fermented by tractable microorganism, whereas AHG is an unusual sugar that is not fermented. To harness the full potential of red macroalgae for biofuel production, it was essential to discover the key enzymes that allow the catabolism of AHG. Yun and coworkers identified two essential enzymes for AHG

assimilation from *Vibrio* sp. strain EJY3. A $NADP^+$-dependent AHG dehydrogenase (*Vej*AHGD) oxidized AHG to 3,6-anhydrogalactonate (AHGA) which is subsequently isomerized to 2-keto-3-deoxy-galactonate (KDGal) by a AHG Acycloisomerase (*Vej*ACI). Then, KDGal can be converted to 2-keto-3-deoxy-6-phosphogalactonate (KDPG), which is a key metabolite in the galactonate metabolic pathway. The authors reconstructed the AHG assimilation pathway in an ethanologenic *E. coli* KO11. This engineered strain was able to produce ethanol from a lysate of agarose (containing galactose and AHG), and showed a 24 % increased ethanol production than the control with an empty vector. Further optimization of the metabolic pathway will increase the ethanol production from red macroalgal carbohydrates [108].

Ethanol is not the only biofuel that has been produced by macroalgal carbohydrate fermentation. The so-called ABE fermentation produces acetone, butanol and ethanol by anaerobic fermentation of sugars. Bearing in mind the high content of sugars that macroalgae contain, it is presumed that the ABE fermentation of algal carbohydrates will produce acetone, butanol and ethanol. Thermal lysates of the green macroalgae *Ulva lactuca* treated with cellulases released glucose, rhamnose and xylose that were fermented by *Clostridium acetobutylicum* and *Clostridium beijerinckii* producing 0.35 g ABE/g sugars. The authors also found that 1,2-propanediol was produced. A study in more detail unveiled that *C. beijerinckii* produced 1,2-propanediol from rhamnose. The authors suggested that rhamnose-rich seaweeds can be used as feedstock for 1,2-propanediol production [109].

Anaerobic digestion (AD) is another approach that has been used to obtain biofuels from macroalgae. AD comprises a bacterial consortium that performs a number of sequential biological processes (hydrolysis, acidogenesis, acetogenesis and methanogenesis) that convert a feedstock into methane and carbon dioxide. In the first step, hydrolytic bacteria release sugars and amino acids. These are converted into volatile fatty acids (VFA) as carboxylic acids and alcohol by acidogenic bacteria. Then, acetic acid, hydrogen and CO_2 are produced from VFA, and subsequently converted in methane and CO_2 by methanogenic bacteria [95]. The biomethane yields reported from different macroalgae range between 0.12 to 0.48 m^3 CH_4/kg volatile solids (VL) [21]. However, little is known about the bacterial communities involved in the anaerobic degradation of macroalgal biomass. Recently, Kita and coworkers unveiled the bacterial consortium structure and alginate-degrading pathway in an alginate-assimilating microbial community obtained from sand from Hiroshima Gulf in Japan [110]. The metagenome study revealed that almost 98 % of the bacterial species were related to *Clostridium*, *Citrobacter* and *Dysgonomonas* genera, and two species related to Clostridiaceae bacterium SK082 and *Dysgonomonas capnocytophagoides* dominated the consortium followed by a third one related to *Citrobacter freundii*. The authors also determined the presence of all the genes involved in the metabolic pathway of alginate. They found 10 putative alginate lyase genes, 9 putative KDG reductase genes, 6 putative KDG kinase genes, and 6 putative KDPG aldolase genes which are the essential genes involved in the degradation of alginate to glyceraldehyde-3-phosphate and pyruvate. Further

elucidation of the role of each strain in the degradation and metabolism of alginate was made. The *Clostridium* species seemed to contain alginate lyases, KDG reductases, KDG kinases, and KDPG aldolases. *Citrobacter* species contained KDG kinases and KDPG aldolases, so it was concluded that they were not involved directly in the degradation of alginate. The contribution of *D. capnocytophagoides* to alginate degradation was unclear as only KDG reductase genes were confirmed, which barely justify why it was a dominant strain in the consortium [110].

More studies of the metagenomic kind will uncover microorganisms and their algal-carbohydrate-metabolizing pathways generating a wealthy source of options for metabolic engineering of a tractable microorganism for efficient assimilation of macroalgal carbohydrates, and eventually their conversion not only into ethanol but also into value added chemicals.

5.6.2 Other Chemicals

Macroalgae biomass has great potential in the production of added value biochemical other than biofuels. In a recent study, 2,3 butanediol and acetoin (BA) were produced by an engineered *E. coli* from macroalgal biomass. *S. japonica* lysates treated with a cocktail of commercial enzymes were fermented with an *E. coli* strain containing a synthetic 2,3 butanediol pathway from *Enterobacter aerogenes* KCTC 2190. The final yield of the fermentation was 0.43 g/g of BA [111].

The production of L-lactate from macroalgal biomass has been also reported. In this case, the L-lactate dehydrogenase from *Streptococcus bovis/equinus* was introduced in an *E. coli* strain where synthesis of the competing by-product was blocked. *S. japonica* lysates treated with a cocktail of commercial enzymes were fermented with the engineered *E. coli* to produce 37.7 g/L of L-lactate with 80 % of the maximum theoretical yield [112]. Similarly, pyruvate was produced from alginate by an engineered *Sphingomonas* sp. strain A where the D-lactate dehydrogenase gene (*ldh*) was deleted. The concentration of pyruvate was 4.6 g/L, representing 18.6 % of theoretical yield [113].

Alginate lyases and oligoalginate lyases have been used to produce DEH that can be converted into 2,5-furandicarboxylic acid (FDCA). FDCA has generated great interest as it can be used as a precursor for the synthesis of polyethylene terephthalate (PET), nylons, jet fuels, and otherdiol-, diamine-, or dialdehyde-based chemicals [114].

On the other hand, algal carbohydrate degrading enzymes can be used to release oligosaccharides. Some of these oligosaccharides have shown bio-activity with applications in medicine and cosmetics. In this case, the use of enzymes is even more relevant due to their high specificity, and also the mild reaction conditions preserve the integrity of the native chemical structure (maintaining the bioactivity of the oligosaccharide). Further, physicochemical treatments used to release oligosaccharides lead to the formation of toxic by-products [96] that must be removed prior to use in medicine or cosmetics. Thus, by employing enzymatic

treatments, expensive and tedious purification steps to eliminate these toxins can be avoided [40].

Fucoidanases or α-L-fucosidases can modify the algal polysaccharide fucoidan. These enzymes preserve the sulfation pattern, and thus the bioactivity of fucoidans is retained. It has been reported that fucoidan oligosaccharides obtained by enzymatic digestion of *Cladosiphon novae-caledoniaekylin* inhibited invasion and angiogenesis of tumor cells, showing a potential antitumor activity [115]. Also, fucoidan oligosaccharides can generate plant immunity against virus as described by Klarzynski and coworkers. The authors showed that fucoidan oligosaccharides prepared from enzymatic digestion of *Pelvetia canaliculata* stimulated defense against the tobacco mosaic virus (TMV) in *Nicotiana tabacum* plant [116]. Similarly, κ-carrageenases have been used to produce κ-carrageenans oligosaccharides that showed *in vivo* and *in vitro* anti-tumor and anti-angiogenic activity [117–119].

The application of algal-carbohydrate degrading enzymes to produce oligosaccharides is still in the early stages. Many enzymes are not presently commercially available thus making their implementation on an industrial scale not competitive enough, in part due to the high enzyme cost.

5.7 Conclusions and Perspective

Marine macroalgae are attracting great attention as a promising feedstock for production of both biofuels and chemicals, showing several advantages over terrestrial plant biomass feedstocks. Their potential in biorefinery lies in their high content of carbohydrates. These carbohydrates are generally complex and show diverse sugar composition, some of them exclusive to seaweed biomass. Thus, the depolymerization of these carbohydrates needs specific enzymes capable of releasing the uncommon sugars. Then, the transformation of these sugars into biofuels and chemicals by a fermenting microorganism requires specialized metabolic pathways that convert sugars into cellular metabolites. These specialized enzymes and metabolic pathways are not evenly distributed in nature. Actually, tractable microorganisms lack some of these assimilating mechanisms. Recent studies elucidated the alginate and agarose metabolism of a few microorganisms. However, more efforts are needed to discover the metabolism of agaropectin and carrageenan to exploit the full potential of seaweed polysaccharides.

Metabolic engineering approaches allowed the development of fermenting microorganisms that convert algal carbohydrates into biofuels. However, the limited number of known microbial mechanisms hinders further development. Therefore, the discovery, isolation and characterization of new degrading enzymes and pathways are key steps for the development of improved fermenting microorganisms capable of converting efficiently macroalgal carbohydrates into biofuels and biochemicals.

The exploitation of seaweeds, and hence their carbohydrates in biorefinery not only depends on improved enzymes and engineered microorganisms, but requires

improved seaweed farming and harvesting technologies. The biochemical composition, carbohydrate content and growth rate of macroalgae vary depending on the species, the period of harvesting and growth temperature. Thus, the farming technology should consider these features to obtain maximal mass-cultivated productivities to sufficiently supply feedstocks for industry [19].

Despite the challenges to be overcome before industrial exploitation, current developments mitigate key bottlenecks that hindered the practical implementation of macroalgal carbohydrates-degrading enzymes and assimilating pathways. It is envisaged that future efforts will make feasible the cost-effective exploitation of these systems for biofuel and value added chemical production.

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