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Metabolic engineering for valorization of macroalgae biomass

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ABSTRACT

Marine macroalgae have huge potential as feedstocks for production of a wide spectrum of chemicals used in biofuels, biomaterials, and bioactive compounds. Harnessing macroalgae in these ways could promote wellbeing for people while mitigating climate change and environmental destruction linked to use of fossil fuels. Microorganisms play pivotal roles in converting macroalgae into valuable products, and metabolic engineering technologies have been developed to extend their native capabilities. This review showcases current achievements in engineering the metabolisms of various microbial chassis to convert red, green, and brown macroalgae into bioproducts. Unique features of macroalgae, such as seasonal variation in carbohydrate content and salinity, provide the next challenges to advancing macroalgae-based biorefineries. Three emerging engineering strategies are discussed here: (1) designing dynamic control of metabolic pathways, (2) engineering strains of halophilic (salt-tolerant) microbes, and (3) developing microbial consortia for conversion. This review illuminates opportunities for future research communities by elucidating current approaches to engineering microbes so they can become cell factories for the utilization of macroalgae feedstocks.

1. Introduction

Commonly called seaweeds, macroalgae are multicellular marine organisms that float as free-living forms or are affixed to hard substrates such as rocks. "Macroalgae forests" have important roles in coastal ecosystems: they provide habitat for many species, shape coral-microbial ecology, protect organisms from storms, reduce deoxygenation and acidification, maintain biogeochemical cycling and storage, and contribute to fishery yields (Vega Thurber et al., 2012; Filbee-Dexter and Wernberg, 2018; Duffy et al., 2019). In addition, as a biomass resource, macroalgae can help achieve the United Nations' sustainable development goals (SDGs), mitigating CO_2 emissions but not competing with staple food crops. Surpassing the growth rate of terrestrial energy crops, natural and cultured brown macroalgae have been produced at up to ~113 MT dry weight ha^{-1} yr^{-1} and 131 MT dry weight ha^{-1} over 7 months, respectively, while sugar cane production reached only 29.4 MT dry weight ha^{-1} yr^{-1} (Kraan, 2013; Waclawovsky et al., 2010).

Macroalgae are grouped as red, green, or brown according to their photosynthetic pigments other than chlorophyll (Zhang et al., 2020). Red and green algae are in the kingdom *Plantae* (phyla *Rhodophyta* and *Chlorophyta*, respectively), while brown algae are in the kingdom *Chromista* (phylum *Ochrophyta*, class *Phaeophyceae*). The *Chlorophyta* phylum includes four classes: the predominant marine planktonic *Prasinophyceae*, the marine *Ulvophyceae*, and the freshwater or terrestrial *Trebouxiophyceae* and *Chlorophyceae* (Cocquyt et al., 2010). Pereira (2021) estimates that 6200 to 7000 different red macroalgae, 600 to 1800 green macroalgae, and 1500 to 2000 brown macroalgae species exist globally. Green algae usually grow in the upper littoral zone of the sea, while red and brown algae inhabit mainly the middle to lower littoral zone (Dave et al., 2019). Brown macroalgae are the largest; *Macrocystis pyrifera*, or giant kelp, can be up to 50 meters long (Makkar et al., 2016).

As biomass for producing bioenergy and bioproducts, macroalgae have both desirable and limiting characteristics. While the high cellulose and lignin content of lignocellulosic feedstocks such as agricultural and forest residues impede production processes, macroalgae contain little or no cellulose and lignin. However, macroalgae contain unique and diverse sugar components such as sulfated polysaccharides (e.g., fucoidans, agar, carrageenan, and ulvan) and carbohydrates (e.g., alginates, laminarin, and mannitol) that necessitate specific pre-treatment and

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fermentation technologies. In addition, high mineral content (Ross et al., 2008) and the presence of polyphenols (Cotas et al., 2020) in macroalgae can limit the utility of existing industrial microbes as production hosts. Chemical composition varies between species and growth phase, complicating microbial strain engineering and fermentation. Chemical composition also varies according to harvesting season, geographical location, habitat, environmental conditions, and extraction method (Kadam et al., 2015; Øverland et al., 2019), hindering efficient macroalgae utilization.

Variability in sugar content affects the biochemistry and physiology of host microbes because catabolism of sugars involves coordinated expression of diverse pathways and necessitates extensive metabolic engineering. In addition, redox balance is critical for maintaining metabolic and physiological homeostasis in cells (Chen et al., 2014). Perturbation of redox-neutral states leads to diverge metabolic fluxes for restoring balance, resulting in decreased metabolic capacity and fermentation performance (Liu et al., 2013). Moreover, extensive metabolic engineering often imposes metabolic load (also called metabolic burden or metabolic drain) on the host. The host must, therefore, re-allocate their native resources such as ribosomes, storage carbons, and cofactor molecules to maintain the host's viability (Glick, 1995). In summary, to advance macroalgae's use as feedstock, strategies that consider the unique features of each macroalgae input must be formulated.

This review article provides an overview of current efforts to tap the potential of macroalgae as feedstock for valuable bioproducts by discovering and enhancing the capabilities of microorganisms. Specifically, we review the chemical composition of macroalgae and state-of-the-art metabolic engineering approaches for the utilization of the unique sugars in macroalgae. We also discuss existing challenges and untapped potential and describe potential solutions that use synthetic biology and multi-omics techniques.

2. Macroalgae feedstocks

2.1. Global supply and demand

Global production of macroalgae was 35.8 million wet metric tons (MTs) (USD 14.9 billion) in 2019, most of which was produced by aquaculture in Eastern and Southeastern Asia (Cai et al., 2021b) (Table 1). Since 1950, total macroalgae cultivation has increased by ~1000 fold, from 34.6 thousand MT to 34.7 million MTs. Among these cultured macroalgae, brown macroalgae (47.3%) and red macroalgae (52.6%) were predominant (Cai et al., 2021a), with several species across five genera accounting for 95.5% of the total production of cultured brown and red macroalgae: *Laminaria/Saccharina* (35.4%), *Undaria* (7.4%), *Kappaphycus/Eucheuma* (33.6%), *Gracilaria* (10.5%), and *Porphyra/Pyropia* (8.6%). Green macroalgae accounts for a small proportion of total cultured macroalgae production (0.05%). However, the frequent occurrence of algal blooms (green algae) in recent years causes problems such as the need for proper disposal (Ghernaout et al., 2012). A large amount of vegetation could instead be excellent sources of biomass.

Around 83% of macroalgae is consumed as foods, food ingredients, or seasoning condiments (Loureiro et al., 2015). In particular, hydrocolloids such as agar and carrageenan are valued polysaccharides for their gelling features; agar preparations use red macroalgae species in the genera *Gracilaria*, *Gelidium*, *Pterocladia*, *Acanthopeltis*, and *Ahnfltia*, and carrageenan preparations use the red macroalgae species *Eucheuma cottonii*, *E. spinosum,* and *Chondrus crispus*. (Suganya et al., 2016). Agar is used in foods and for packaging alternatives to petrochemical-based plastics (Leandro et al., 2020). Carrageenan has extensive applications as an emulsifier, a stabilizer, and a thickener in the dairy industry (e.g., yogurt, ice cream, and jelly) and for meat products. Alginate, another hydrocolloid, is isolated from brown macroalgae. Species groups in *Laminaria hyperborea*, *L. japonica*, *Macrocystis pyrifera*, and *Ascophyllum nodosum* are major sources of alginate (Suganya et al., 2016). Like carrageenan, alginate is used in dairy products (e.g., cream and cheese), as a stabilizer and flavor enhancer (e.g., sauces, jam, and dressing), and as an emulsifier (Leandro et al., 2020).

Macroalgae also have a wide spectrum of applications in agriculture (as fertilizers and biostimulants), animal feeds, fuels and chemical compounds (as an alternative to petroleum), nutraceutical and pharmaceutical products, and cosmetics (Rathore et al., 2009; Torres et al., 2019). A species of macroalgae may therefore be selected for bioconversion to bio-based products based not only on its yield capacity and its nearness to accessible cultivation methods, but also on its nearness to existing markets for these products.

2.2. Chemical composition of macroalgae

Macroalgae are rich in carbohydrates, which compose up to 76% of macroalgae's dry weight (Kraan, 2012). This high carbohydrate content is critical for conversion into bio-based products. Some carbohydrate components are common among macroalgae and terrestrial crops, and other carbohydrate components are made up of monosaccharides found only in specific macroalgae species. In this section, carbohydrates unique to macroalgae are reviewed (Fig. 1A–E), and other macroalgae components of commercial value are discussed.

2.2.1. Carbohydrates in red macroalgae

Red macroalgae consist of sulfated galactans (e.g., carrageenan, agar, and porphyran) (Fig. 1A), structural polysaccharides (cellulose, mannans, and xylans), and storage carbohydrates (floridean starch and

Table 1
Global macroalgae production in 2019 (source data from Cai et al., 2021b).

	Production		Share in countries/regions	
Regions	**Total production (MTs)**	**Aquaculture (MTs)**	**Total production (%)**	**Aquaculture (%)**
Eastern Asia (4 countries)	23,133,367	22,883,407	64.7	66.0
China	20,296,592	20,122,142	56.8	58.0
South Korea[a]	1,821,475	1,812,765	5.1	5.2
North Korea[b]	603,000	603,000	1.7	1.7
Japan	412,300	345,500	1.2	1.0
Southeastern Asia (3 countries)	11,651,336	11,606,471	32.6	33.5
Indonesia	9,962,900	9,918,400	27.9	28.6
Philippines	1,500,326	1,499,961	4.2	4.3
Malaysia	188,110	188,110	0.5	0.5
Others[c] (4 regions)	935,755	165,912	2.6	0.5
World	35,762,504	34,679,134	100.0	100.0

[a] Republic of Korea.
[b] Democratic People's Republic of Korea.
[c] Others include Americas, Europe, Africa, and Oceania.

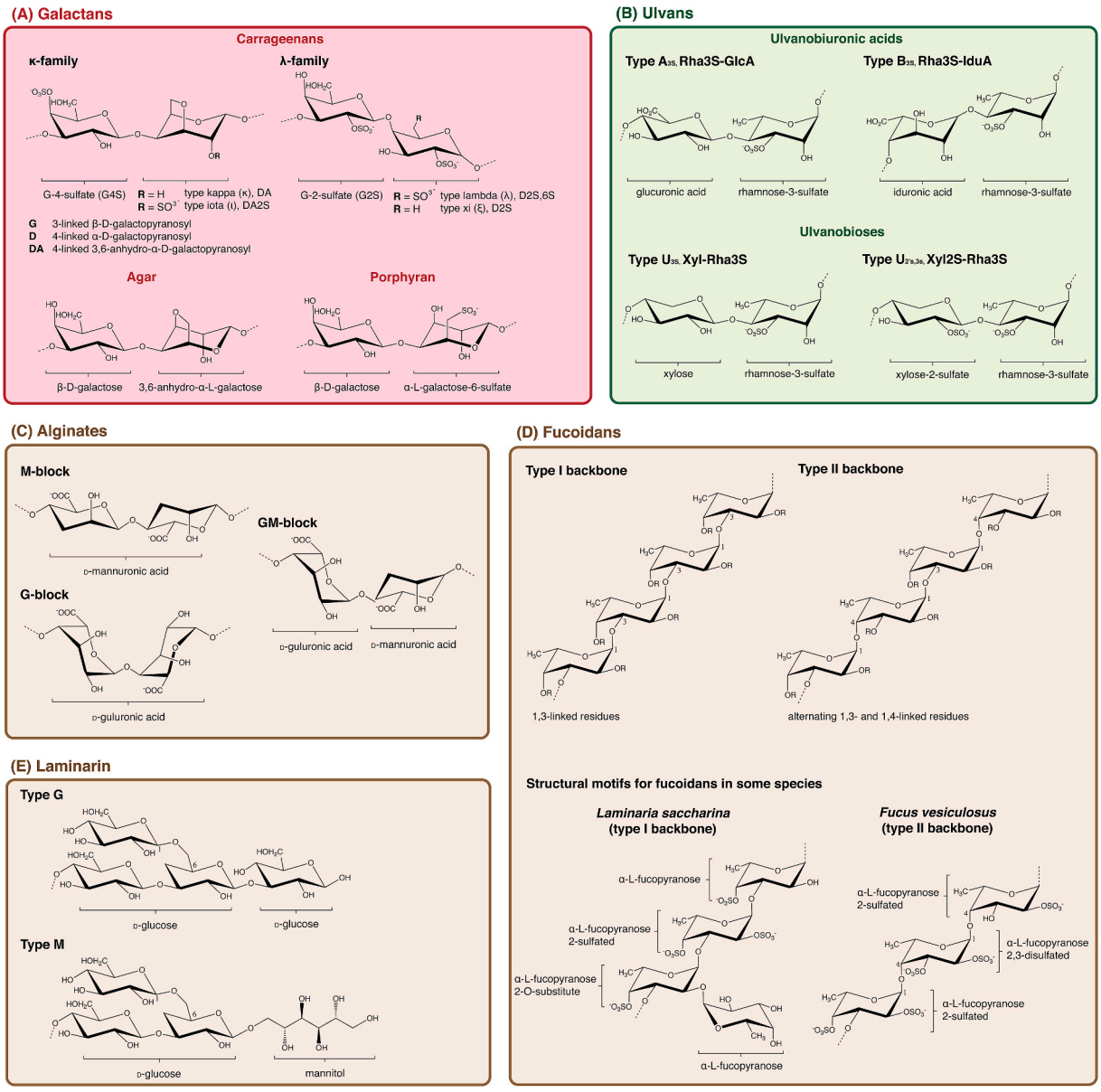

Fig. 1. Schematic representation of unique carbohydrates from macroalgae.
(A) Sulfated galactans such as carrageenan are the main structural carbohydrate components of red macroalgae. Carrageenan is classified into multiple families based on the constituents of its disaccharide units, which consist of β-D-galactopyranosyl residues and α-D-galactopyranosyl residues that are often found in cyclized form as 3,6-anhydro-derivatives. Representative families of carrageenans are illustrated. Agar and porphyran include β-D-galactopyranosyl residues as a common moiety, connected to 3,6-anhydro derivatives in agar and to sulfated α-L-galactose in porphyran. (B) Ulvans consist of two major disaccharides units: ulvanobiuronic acid and ulvanobioses. Sulfated rhamnose is commonly found in all disaccharide units, while another residue bound by β-1,4-glycosidic linkages to α-L-rhamnose-3-sulfate varies as glucuronic acid in type A_{3s}, iduronic acid in type B_{3s}, xylose in type U_{3s}, and xylose-2-sulfate in type $U_{2's,3s}$. (C) Alginates consist of residues of β-D-mannuronic acid (M) and/or α-L-guluronic acid (G) linked by 1,4-glycosidic bonds. Three types of polymeric forms are found as building blocks: homopolymeric G-residues (G-block), homopolymeric M-residues (M-block), and heteropolymeric alternating G- and M-residues (GM-block). (D) Fucoidans primarily consist of two backbones: 1,3-linked-α-L-fucopyranosyl residues (type I) and alternating 1,3- and 1,4-linked-α-L-fucopyranosyl residues (type II); structural motifs of fucoidans from *Laminaria saccharina* and *Fucus vesiculosus* are represented (Chevolot et al., 2001; Cumashi et al., 2007). (E) Laminarin consists of a linear chain of D-glucose residues with occasional branches of β-1,6-linked D-glucose. Modification patterns of the reducing end residue classify laminarin into M- or G-types.

α-1,4-glucan). Carrageenan, the main component of the sulfated galactans, consists of galactose and 3,6-anhydrogalactose with alternating α-1,3- and β-1,4-linkages. There are 15 types of carrageenans, categorized by the pattern of disaccharide units and the number and position of sulfate ester(s) (Usov, 2011). The carrageenan types vary among species (Jönsson et al., 2020).

Agar consists of galactans, mainly made up of D- and L-galactose and 3,6-anhydro-α-L-galactose (Fig. 1A). Agar is the most abundant cell wall polysaccharide in the *Gracilariaceae*, *Gelidiaceae*, *Pterocladiaceae*, and *Gelidiellaceae* families (Lee et al., 2017). Agarose, which constitutes approximately 70% of agar polymers, consists of repeated D-galactose and 3,6-anhydro-L-galactose units joined by β-1,3 and α-1,4 glycosidic linkages (Marinho-Soriano and Bourret, 2005; Lee et al., 2017).

Porphyran is a major polysaccharide component of *Porphyra* and *Pyropia* species. Porphyran chemical structure consists of 3-linked β-D-galactosyl units alternating with either 4-linked α-L-galactosyl 6-sulfate or 3,6-anhydro-α-L-galactosyl units (Fig. 1A). Rather than being used as a platform substrate, this polysaccharide has attracted much attention

because of its biological activities related to human health; for instance, porphyran is both antitumor and antioxidative.

Sulfated galactan polysaccharides found in red macroalgae also include hypneans from *Hypnea* spp., furcellaran from *Furcellaria lumbricalis*, dulsan from *Rhodymenia palmata*, and iridophycan from *Iridanea* or *Iridophycus* genera (Rinaudo, 2007).

2.2.2. Carbohydrates in green macroalgae

Marine green macroalgae, such as *Ulva* spp., contain from 14% to 40% carbohydrates dry weight (Kazir et al., 2019). These carbohydrates are made of sulfated and/or carboxylated polysaccharides and glucans, as well as floridean starch such as amylopectin. Green algae can be grouped into two major categories based on uronic acid content (either limited or rich), though different genera and species may have different amounts (Synytsya et al., 2015). Species in the genera *Codium*, *Bryopsis*, and *Caulerpa* are in the uronic-acid–limited group and primarily consist of sulfated galactans, arabinopyranans, and mannans (Jönsson et al., 2020). Species in the genera *Ulva*, *Gayralia*, *Acetabularia*, and *Monostroma* are in the uronic-acid–rich group and their primary cell wall component is ulvans (Jönsson et al., 2020).

Ulvans are polyanionic heteropolysaccharides. Accounting for 9%–36% of the dry weight of green algae. Ulvans are composed of α- and β-1,4-linked rhamnose (Rha) (5.0–92.2 mol%), uronic acids, glucuronic acid (GlcA) (2.6–52.0 mol%), iduronic acid (IduA) (0.6–15.3 mol%), and xylose (Xyl) (0–38.0 mol%), with sulfur modification at rhamnose and xylose residues (Lahaye and Robic, 2007). Structural types are defined by various numbers and placement of repeating disaccharide units: ulvanobiuronic acid types A (A_{3s}, Rha3S-GlcA) and B (B_{3s}, Rha3S-IduA), and, in lesser amounts, ulvanobioses type U (U_{3s}, Xyl-Rha3S and $U_{2's,3s}$, Xyl2S-Rha3S) (Kidgell et al., 2019) (Fig. 1B). The composition can fluctuate depending on species, habitats, and pre-treatment methods (Kidgell et al., 2019).

Potential biological activities of ulvans have attracted interest in diverse applications in biomaterial science, nutraceuticals, functional foods, and agriculture; details can be found in previous reviews (Alves et al., 2013; Kidgell et al., 2019).

2.2.3. Carbohydrates in brown macroalgae

Brown macroalgae are rich in carbohydrates. Their cell walls are made of cellulose, hemicellulose, matrix polysaccharides (alginates and fucoidans), and laminarin. After cellulose, alginates are the second-most abundant polysaccharide in the world (Leandro et al., 2020).

Alginates consist of the uronic acids, β-D-mannuronic acid (M) and/or its C-5 epimer, α-L-guluronic acid (G). Linked together with 1,4-glycosidic bonds, the monomers are arranged into three types of block-like polymeric forms: (1) homopolymeric M-residues (polyM, mannuronan), (2) homopolymeric G-residues (polyG, guluronan), and (3) heteropolymeric alternative residues (polyGM/MG) (Fig. 1C) (Synytsya et al., 2015). The G/M ratio varies in different parts of an alga and according to the harvest period and genetic differences between species. The structural arrangement of the monomers, the size of the formed alginate, and the presence or absence of ions (e.g., sodium, calcium, and magnesium) all affect the physicochemical properties of the alginate such as state (sol or gel) and viscosity. In particular, the selective binding of polyG with multivalent cations (e.g., Ca^{2+}) results in firm but brittle gels because of the "buckled" chain conformation, described as the "egg-box model" (Grant et al., 1973; Thiang Yian Wong et al., 2000; Draget and Taylor, 2011).

Fucoidans (or fucans) are a group of fucose-containing sulfated polysaccharides (FCSPs) found in the fibrillar cell walls or intracellular spaces of brown macroalgae. These fucoidans usually have a backbone of either 1,3-linked α-L-fucopyranose residues or alternating 1,3- and 1,4-linked α-L-fucopyranose residues; these residues are the primary fucoidan constituents (Fig. 1D). Additional constituents besides fucose include other sugars such as galactose, uronic acids, and xylose. The structure of fucoidans varies according to species, season, habitat, and maturity. For example, the fucoidan content in *F. serratus*, *F. vesiculosus*, and *Ascophyllum nodosum* is generally higher in the fall than in other seasons (Fletcher et al., 2017). In polymeric or oligomeric forms, fucoidans have exhibited therapeutic effects such as anti-tumor bioactivity (Kim et al., 2010).

Laminarin (also spelled laminaran) is a linear β-1,3-D-glucan with occasional β-1,6-linked side chains, with a degree of polymerization of 25 (Rioux et al., 2007). It is produced as a storage polysaccharide like the starch found in terrestrial plants. The ratio of β-1,3- and β-1,6-glycosidic bonds varies according to growth conditions and genetic background. For example, the bonds occur in laminarin in the fronds of *Laminaria* at a ratio of 3:1, but at a lower ratio in *Ascophyllum*, *Fucus*, and *Undaria* (Kraan, 2012). Laminarin chains can be classified as M or G depending on how the reducing end is modified (Kadam et al., 2015): M chains end with 1-O-substituted mannitol, while G chains end with glucose (Fig. 1E).

Mannitol, a reduced form of mannose, is a type of sugar alcohol, and is accumulated as a reserve polysaccharide, as are laminarins (Groisillier et al., 2014). Carbon storage is an important physiological process, since stored carbon protects the algae against osmotic stress (Conde et al., 2011). Mannitol content can increase up to 20%–30% of the dry weight of brown macroalgae in some species (Reed et al., 1985), although the content varies seasonally. Seasonal fluctuations in carbon stored in the form of laminarin and mannitol often occur (Adams et al., 2011b). For instance, in a 2-year observation of three *Laminariaceae* species (*Laminaria cloustoni*, *L. digitata*, and *L. saccharina*) in the Northern Hemisphere, Black (1950) reported that the content of laminarin dropped in the developmental period (spring) and greatly increased in the growth-restricted period (summer and fall). During the observation, mannitol ranged from 3% to 21% of dry weight and laminarin ranged from less than 1%–25% of dry weight (Black, 1950). Similar trends of seasonal variations in *Laminaria* have been reported in later studies performed in the UK (Adams et al., 2011a, 2011b) and in Scotland (Schiener et al., 2015). In contrast to the huge effect of season on brown macroalgae composition, the effects of season on green and red macroalgae are small (Robic et al., 2009; Kumar et al., 2013).

2.2.4. Other components in macroalgae

In addition to carbohydrates, macroalgae are sources of other compounds such as lipids, proteins, vitamins, minerals, and secondary metabolites. These byproducts may be important to offset the cost for the production of bioproducts from macroalgae sugars.

Protein content in macroalgae varies across groups and species. Red macroalgae have high protein levels, up to 47% (w/w), while green and brown macroalgae have low to moderate protein levels, from 9% to 26% (w/w) and 3%–15% (w/w) of dry weight, respectively (Harnedy and FitzGerald, 2011; Fleurence et al., 2018). The protein content of red macroalgae is comparable to that of soybeans (35% of dry weight), although the red macroalgae level can fluctuate and amino acids, and most macroalgae are especially rich in the acidic amino acids, aspartic acid and glutamic acid (Fleurence, 1999). The high proportion of glutamate contributes to the savory, or umami, taste imparted by macroalgae, especially kelp, when used as a condiment for flavor or nutrition. Enzymatic protein extract and peptides are excellent sources of natural antioxidants (Kazir et al., 2019). In addition, bioactive proteins such as lectin and phycobiliproteins are valuable in pharmaceutics and biotechnology (Pangestuti and Kim, 2015). Protein content in red and brown macroalgae increases from winter to early spring and decreases during summer and early fall, which is the opposite of the trend in storage carbohydrates (Banerjee et al., 2009; Schiener et al., 2015).

Minerals in macroalgae make up around 8%–40% of dry weight, including macro-minerals (e.g., Na, K, Ca, Mg, and P) and trace elements (e.g., Fe, Mn, Cu, Zn, and I); this content is higher than that of land plants and includes some elements that are not found in them (Rupérez, 2002). In brown macroalgae, the mineral content is more than 30%, much higher than the mineral content of other macroalgae, and the

iodine content is exceptionally high as well, reaching up to 1.2% of dry weight in *Laminaria/Saccharina* (Holdt and Kraan, 2011). Macroalgae also have a high capacity to accumulate heavy metals (e.g., As, Cd, Hg, Pb), which presents a potential threat to animals and humans if the macroalgae are consumed. The degree of accumulation depends on habitat and composition of polysaccharides in the macroalgae's cell walls. Therefore, it is imperative to determine toxic metal concentrations and respond appropriately, especially if macroalgal products are intended as food, feed, or fertilizer (Lähteenmäki-Uutela et al., 2021). Removing heavy metals during preprocessing reduces their concentrations downstream. Although this step increases production cost, the cost may be offset if value is added to the bioproducts by alleviating concerns about toxicity (Sadhukhan et al., 2019). In biotechnological applications, the ability of chassis microbes to tolerate these heavy metals is also an important factor to consider, because any macroalgae feedstocks can be utilized for production of bioproducts. In contrast to the drawbacks, macroalgae's high metal absorption capacity can be an asset that opens up other applications. For example, macroalgae has gained attention as an indicator of metal content in seawater (Chakraborty et al., 2014) and as a biosorbent that can be used to remove heavy metals (Ibrahim, 2011).

Lipid content is low in macroalgae, with lipids making up a maximum of 4% of dry matter except for some species (e.g., *Dictotales*, *Cladophorales*, and *Bryosidales*) (Gosch et al., 2012). Most lipids in macroalgae are polyunsaturated fatty acids (PUFAs) in the form of omega-3 (n-3) and omega-6 (n-6) lipids (MacArtain et al., 2007). Of the PUFAs, eicosapentaenoic acid (EPA; 20:5 n-3) and arachidonic acid (20:4 n-6) are predominant, particularly in red macroalgae such as *Palmaria palmata* (Ginneken et al., 2011), in which half of the fatty acids are EPA (Wells et al., 2017). Lipid saturation level and composition in macroalgae are influenced by water temperature.

The lipophilic vitamins A, D, and E are present in macroalgae, as well as the B-group representatives B_1 and B_{12}, ascorbic acid, riboflavin, niacin, pantothenic acid, and folic acid (Dhargalkar and Pereira, 2005).

Secondary metabolites of macroalgae represent a wide spectrum of biological activity and vary among different species and environments. They include pigments (e.g., β-carotene, astaxanthin, lutein), phenolic compounds (e.g., phenolic and cinnamic acids and flavonoids), mycosporin-like amino acids (MAAs), and complex phlorotannin polymers (Harrysson et al., 2018; Biris-Dorhoi et al., 2020).

3. Microbial conversion of macroalgae substrates into bioproducts

3.1. Bioconversion of red macroalgae

3.1.1. Decomposition of carrageenan and agar

Of the red macroalgae carbohydrates, carrageenan is the main component in carrageenophyte species such as *Eucheuma denticulatum*, and agar is the main component in agarophyte species such as *Gelidium amansii*. For example, the carbohydrate composition of *G. amansii* consists of agarose (~52 wt% of dry weight), including 3,6-anhydro-α-L-galactose (AHG; ~33 wt%) and D-galactose (~26 wt%), and cellulose (~15 wt%) (Park et al., 2012; Yun et al., 2015a).

Saccharification methods for agarose such as acid hydrolysis, enzymatic hydrolysis, and acid pre-hydrolysis with successive enzymatic hydrolysis allow the release of AHG and D-galactose for subsequent fermentation (Yun et al., 2015a). Specifically, agarose is depolymerized into agarooligosaccharides (degree of polymerization [DP] = 2–5) by chemical liquefaction by acetic acid, and these agarooligosaccharides are then enzymatically saccharified into monomer units of AHG and D-galactose by β-agarases types I and II, and α-neoagarobiose hydrolase (NABH) (Yun et al., 2015b).

Saccharification of carrageenan is difficult because acid treatments concomitantly generate inhibitory compounds such as furfural, 5-HMF, levulinic acid, and acetic acids (Monlau et al., 2014; Kumar et al., 2014). Also, a hydrolase that acts on the α-1,3-linkage of AHG and D-galactose (neocarrabiose), one of disaccharide units from carrageenan, has not been identified (Kawai and Murata, 2016). Since carrageenan saccharification is difficult, D-galactose and AHG from agarose are the most suitable target substrates for bioconversion of red macroalgae.

3.1.2. Bioconversion of D-galactose

D-Galactose is a C-4 epimer of glucose and is converted into glucose-6-phosphate in the Leloir pathway to enter glycolysis. In most organisms, this pathway involves five enzymes. In *S. cerevisiae,* β-D-galactose is imported to the cell through the galactose permease (Gal2) and enters the Leloir pathway (Sellick et al., 2008; Kawai and Murata, 2016) (Fig. 2). Mutarotase (Gal10) converts β-D-galactose into α-D-galactose, which is further phosphorylated into α-D-galactose-1-phosphate by galactokinase (Gal1). Galactose-1-phosphate uridyl transferase (Gal7) catalyzes an exchange between the phosphate group of α-D-galactose-1-phosphate and the uridine diphosphate (UDP) group of (UDP)-D-glucose to form UDP-galactose and α-D-glucose-1-phosphate. UDP-galactose is subsequently converted to (UDP)-D-glucose catalyzed by galactose-1-phosphate uridyl transferase (Gal7). The C-4 position of UDP-galactose is epimerized by UDP-glucose-4-epimerase (Gal10), and UDP-D-glucose is formed. After completion of the Leloir pathway, α-D-glucose-1-phosphate is converted to α-D-glucose-6-phosphate by phosphoglucomutase (Pgm2), after which glycolysis commences. Transcription of these GAL genes is tightly controlled by the availability and types of sugars (Gancedo, 1998). In the presence of galactose and glucose, yeasts sequentially catabolize these sugars with a diauxic lag-phase. Specifically, the Leloir-pathway-encoding genes are repressed by transcriptional repressor Mig1 and co-repressor complex Tup1-Cyc8 (Ssn6) (Papamichos-Chronakis et al., 2004), whereas in the presence of galactose and absence of glucose, these genes are activated by concerted actions of GAL1, GAL4, and GAL80 (Sellick et al., 2008).

Since red macroalgal hydrolysates typically contain both galactose (23%) and glucose (20%) (Wi et al., 2009), co-fermentation of these sugars is a promising strategy for bioproduction. However, in one study, the stringent repression system of GAL genes and two-stage use of these sugars resulted in a reduction of overall ethanol yield and productivity (Ostergaard et al., 2001). After transformation and screening of genome-wide fragmented libraries of *S. cerevisiae*, Lee et al. (2011) reported that overexpression of either a small nuclear RNA (SNR84), a truncated global transcriptional factor (tTUP1), or a rate-limiting gene in the galactose metabolic pathway (PGM2) improved the efficiency of separate fermentation of glucose and galactose into ethanol (ethanol fermentation), but simultaneous fermentation of these sugars was not achieved. In a successful effort to bypass the glucose repression, Ha et al. (2011a) designed a co-fermentation system of cellobiose (dimer of glucose) and galactose by intracellular hydrolysis of cellobiose. Specifically, they developed an *S. cerevisiae* D452-2BT strain harboring two plasmids encoded a cellodextrin transporter (cdt-1) and an intracellular β-glucosidase (gh1-1) from the cellulolytic fungi *Neurospora crassa*. This engineered yeast platform achieved simultaneous ethanol fermentation of galactose and cellobiose, with improved cell growth and all titer rate yield (TRY) metrics compared with single-sugar fermentation and diauxic fermentation (Ha et al., 2011b). Furthermore, the group adapted this approach for co-fermentation of xylose and cellobiose and developed *S. cerevisiae* DA24-16BT3, which harbors a xylose metabolic pathway in addition to the cellobiose assimilation system.

3.1.3. Bioconversion of AHG

Agar-assimilation capability, especially agarose activity, has been confirmed in several marine microorganisms (Yun et al., 2016), in particular, in *Saccharophagus degradans* 2–40^T (Ekborg et al., 2005), *Agarivorans albus* (Kurahasi and Yokota, 2004), *Alterococcus agarolyticus* (Shieh and Jean, 1998), *Pseudoalteromonas carrageenovora* (Barbeyron et al., 1994), and *Zobellia galactanivorans* (Jam et al., 2005). The catabolic pathway of AHG was identified in the agarolytic marine bacterium

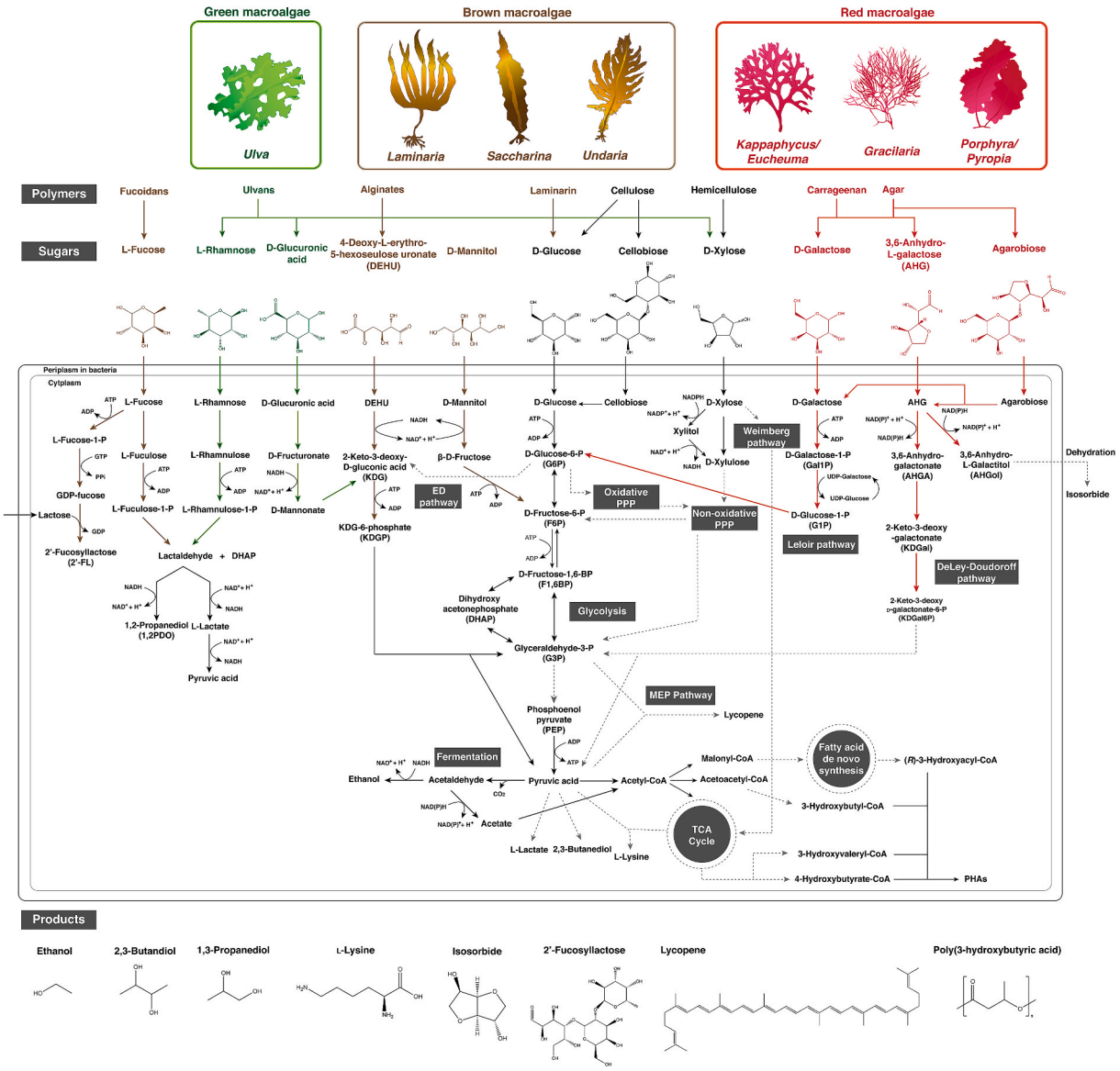

Fig. 2. Metabolic map of engineering strategies implemented for assimilation and conversion of sugars from macroalgae.
The chemical structure of each sugar discussed in this review is shown in the same color as the macroalgae type (green, brown, or red) that contains that sugar. Sugars shown in black are common to several types. Likewise, metabolic pathways for assimilating sugars unique to one macroalgae type are shown with arrows of the appropriate color. Black arrows indicate central fluxes and common metabolic pathways. Solid arrows indicate a single reaction, while dashed arrows indicate multiple-pathway reactions. Abbreviations of chemicals and pathways are as follows: P, phosphate; BP, bisphosphate; ED, Entner-Doudoroff; GDP, guanosine 5′-diphosphate; UDP, uridine diphosphate; PPP, pentose phosphate pathway; MEP, methyl erythritol 4-phosphate; TCA, tricarboxylic acid. Products from macroalgal sugars are also shown.

Vibrio sp. strain EJY (Yun et al., 2015b). In this pathway, the AHG is oxidized into 3,6-anhydrogalactonate (AHGA) by $NADP^+$-preferred AHG dehydrogenase (*Vej*AHGD), and then AHGA is isomerized to 2-keto-3-deoxy-galactonate (KDGal) by AHGA cycloisomerase (*Vej*ACl). The KDGal is thought to be further metabolized through the DeLey-Doudoroff pathway (Wong and Yao, 1994). In addition, Yun et al. (2015b) designed and built the AHG catabolic pathway (Fig. 2) into the ethanologenic *E. coli* KO11 strain by introducing *Vejahgd* and *Vejaci* genes. Under modified M9 media with 1.2% (w/v) agarose hydrolysate including 3.2 g/L AHG and 4.1 g/L galactose, the engineered *E. coli* strain showed 2.0-fold higher AHG consumption and 1.2-fold higher ethanol production than the control strain (without these genes) after 52 h of fermentation (Yun et al., 2015b).

Physiochemical instability of AHG hinders its valorization as a biomass source. Kim et al. (2020) proposed and demonstrated an alternative strategy, in which AHG is converted into its sugar alcohol form, 3,6-anhydro-L-galactitol (AHGol), to form a new platform chemical that has the potential to be used in foods, cosmetics, and polymer industries. First, agarose is chemically decomposed into agarobiose (AB) and disaccharides consisting of D-galactose and L-AHG bound by β-1, 4-glycosidic linkages using phosphoric acid, which preferentially cleaves α-1,3-glycosidic linkages (Kim et al., 2018). The released AB is transported into an engineered yeast platform (*S. cerevisiae* D452-L124) through the lactose permease (*LAC12*). Then, the imported AB is intracellularly hydrolyzed into AHG and galactose by β-galactosidase (*LAC4*), and aldose reductase (AR, *GRE3*) converts the released AHG into AHGol (Fig. 2), while small amounts of agarobitol and galactitol are produced. In a fed-batch scheme, Kim et al. achieved 41.18 g/L AHGol with a yield of 0.46 g-AHGol/g-AB and demonstrated chemical conversion (using 1% [w/v] H_2SO_4 at 105 °C) of AHGol to isosorbide (Kim et al., 2020). Potential applications include using isosorbide monomers as building blocks for designing alternatives to petroleum-based products in diverse

industries, including functional materials, solvents, and pharmaceuticals (Rose and Palkovits, 2012). In addition to this engineering effort, the group has characterized a novel agarolytic pathway in marine bacterium *Vibrio* sp. strain EJY3, which opens further opportunities to advance agarose utilization (Yu et al., 2020).

3.2. Bioconversion of green macroalgae

Green macroalgae contain sulfated and/or carboxylated polysaccharides, glucans (cellulose and mannan), and floridean starch such as amylopectin. As mentioned earlier, the major categories are based on uronic acids content (rich and limited).

3.2.1. Decomposition of ulvans

The major carbohydrates of green macroalgae *Ulva* species are ulvans and glucans, in which median values for rhamnose and glucuronic acid are 45.0 mol% and 22.5 mol% of carbohydrate chains, respectively (Kidgell et al., 2019). Natural ulvan-utilizing microbes are *Alteromonas* spp., *Pseudoalteromonas* spp., and *Formosa* sp. In these species, ulvan-utilizing enzymes are found, as predicted by a polysaccharide utilization locus (PUL) in their genomes. Ulvan polysaccharide lyases produce ulvan oligosaccharides, including unsaturated uronic acid, at the non-reducing end of ulvan oligosaccharides via the β-elimination reaction in an endolytic mode of action. These ulvan oligosaccharide lyases are categorized into five families: PL24, PL25, PL28, PL37, and PL40 (Li et al., 2020). According to a model proposed for the ulvan-utilizing pathway of the marine flavobacterium *F. agariphila* KMM 3901^T (Salinas and French, 2017; Reisky et al., 2019), the formed ulvan oligosaccharides are further depolymerized by outer-membrane enzymes, then bound by a SusD-like protein and transported into the periplasm by a TonB-dependent receptor (TBDR) that has a linkage with a TonB-ExbBD complex. A hybrid two-component system protein (HTCS) detects the incorporation of the oligosaccharides and activates the PUL for periplasmic depolymerization and production of monomeric sugars via specific lyases such as unsaturated glucuronyl hydrolases (i.e., GH105), rhamnosidases, xylosidases, and sulfatases. The released monosaccharides (i.e., rhamnose, glucuronic acid, xylose, and unsaturated uronic acid) are transported into the cytoplasm through sugar permeases that belong to the major facilitator superfamily (MFS) and catabolized into pyruvate or GAP.

3.2.2. Bioconversion of D-glucuronic acid

D-Glucuronic acid in the form of glucuronoxylan is a hexuronic acid that, along with ulvans, makes up plant cell walls (Reis et al., 1994). Several types of D-glucuronate catabolic systems have been found in bacteria, fungi, and animals (Kuivanen et al., 2016). Some bacteria, including *E. coli*, possess the isomerase system (Fig. 2), in which D-glucuronate is metabolized to D-glyceraldehyde-3-P and pyruvate through five enzymatic reactions in which D-glucuronate isomerase converts D-glucuronate to D-fructuronate, which is then reduced to D-mannonate by an NADH-dependent reductase. D-Mannonate dehydratase then converts D-mannonate to 2-keto-3-deoxy-D-gluconate, and this is then phosphorylated to 2-keto-3-deoxy-D-gluconate-6-P by a kinase. Finally, an aldolase splits 2-keto-3-deoxy-D-gluconate-6-P into glyceraldehyde-3-P and pyruvate (Kuivanen et al., 2016). Ethanologenic *E. coli* strains have enabled the conversion of D-glucuronic acid into ethanol at yields of around 0.2 g/g (Lawford and Rousseau, 1997).

3.2.3. Bioconversion of L-rhamnose

L-Rhamnose is a deoxy hexose sugar (methyl-pentose) that exists widely as one of the components of pectin in bacteria and plants. Various bacteria, including *E. coli*, possess an L-rhamnose catabolic system known as the phosphorylated catabolic pathway (Fig. 2), which converts L-rhamnose into L-lactaldehyde and dihydroxyacetone phosphate (DHAP). The phosphorylated catabolic pathway comprises three enzymes: L-rhamnose isomerase, L-rhamnulose kinase, and L-rhamnulose-1-phosphate aldolase (Rodionova et al., 2013). DHAP can be fluxed into the glycolytic pathway, whereas L-lactaldehyde is converted into different products depending on oxygen availability; L-lactaldehyde is oxidized to L-lactate by NAD^+-dependent dehydrogenase under aerobic conditions, while under anaerobic conditions, NADH-dependent oxidoreductase reduces L-lactaldehyde to 1,2-propanediol (1,2-PDO) as a redox reservoir (Zhu and Lin, 1989). This pathway is common for metabolizing another methyl pentose, L-fucose, which is described in section 3.3. An alternative non-phosphorylated L-rhamnose catabolic pathway (or aldolase pathway) exists mainly in fungi and a few bacteria (e.g., *Azotobacter vinelandii*, *Sphingomonas* sp.). This alternative pathway converts L-rhamnose into pyruvate and L-lactaldehyde, and consists of four enzymes: L-rhamnose-1-dehydrogenase, L-rhamnono-γ-lactonase, L-rhamnonate dehydratase, and L-2-keto-3-deoxyrhamnonate aldolase (Watanabe and Makino, 2009; Rodionova et al., 2013).

3.2.4. Bioconversion of xylose

Because xylose is a major constituent among the hemicellulose components of lignocellulose, metabolic engineering of xylose catabolism has been extensively reviewed (Moysés et al., 2016; Kwak and Jin, 2017). Please see these articles for pathway engineering of xylose assimilation.

3.3. Bioconversion of brown macroalgae

Metabolic engineering strategies implemented for microbial conversion of sugars derived from brown macroalgae are represented in Table 2.

3.3.1. Decomposition of fucoidans

Fucoidan-degrading enzymes (i.e., fucoidanases) cleave linkages of bridged non-sulfated fucose residues from the non-reducing end of fucoidans. Fucoidanases are generally classified according to modes of action into endohydrolases and fucosidases. Fucoidanases have been found in marine organisms that have various catalytic and physicochemical properties; these organisms include bacteria (e.g., *Pseudoalteromonas* spp.), invertebrates such as sea cucumber (*Acaudina molpadioides*) and sea urchin (*Strongylocentrotus nudus*), and some fungi (e.g., *Fusarium* sp. LD8) (for a comprehensive review, see Kusaykin et al., 2015). Because fucoidans from different sources have complex chemical structures and different compositions, it is challenging to produce fucose in its monomeric form by enzymatic hydrolysis, and chemical hydrolysis may be a better alternative. In spite of the difficulties, the variations between fucoidans generate various bioactive functions that allow fucoidans to provide special benefits as nutraceutical and therapeutic agents. These benefits have drawn attention to the catalytic patterns of fucoidanases (Fitton et al., 2015).

3.3.2. Bioconversion of fucose

The monosaccharide L-fucose that is released from fucoidanases is a methyl-pentose that is also found in bacterial cell walls, plants, and animal cells. Some bacteria, such as *C. phytofermentans* (Petit et al., 2013), *E. coli* (Boronat and Aguilar, 1981), and *Salmonella typhimurium* (Badía et al., 1985) possess intrinsic metabolic capabilities (i.e., the propanediol pathway) for assimilating L-fucose and metabolizing it into two different products, 1,2-propanediol and lactate, depending on cellular redox states. The biosynthesis of 1,2-propanediol and lactate occurs through the L-rhamnose metabolic pathway, in which L-lactaldehyde is mediated as a branching metabolite (Fig. 2) (Baldomà and Aguilar, 1988).

Besides being converted into diols, L-fucose can also be used for 2'-fucosyllactose (2'-FL) production. 2'-FL is one of the most abundant of the human milk oligosaccharides (HMOs), and it therefore has important nutraceutical and pharmaceutical benefits (Reverri et al., 2018). Because infant formula made from non-human-animals' milk contains very few oligosaccharides (Vandenplas et al., 2018), the microbial

Table 2
Metabolic engineering strategies for brown macroalgae utilization.

Substrate	Target product	Base host	Reported TRY metrics	Fermentation type	Genome and metabolic engineering approaches and features	Reference
Fucose and lactose	2'-fucosyllactose (2-FL)	*E. coli* BL21star(DE3)	0.52 g g^{-1} lactose 1.06 g g^{-1} fucose 0.39 g L^{-1} h^{-1} productivity	Fed batch	Overexpression of the salvage pathway genes *Bacteroides fragilis* fkp and *Helicobacter pylori* fucT2 Deletion of lacZ and fucI-fucK gene cluster	Chin et al. (2016)
Fucose and lactose	2-FL	*S. cerevisiae* D452-2 (MATα, leu2, his3, ura3, and can1)	0.43 g g^{-1} lactose 1.87 g g^{-1} fucose	Fed batch	Overexpression of the salvage pathway genes *B. fragilis* fkp, codon-optimized *H. pylori* fucT2, and *Kluyveromyces lactics* LAC12	Yu et al. (2018)
Alginate	Ethanol	*Sphingomonas* sp. A1	0.26 g g^{-1} alginate	Fed batch	Overexpression of the homoethanol pathway genes *Zymomonas mobilis* pdc and adhB Improved expression of adhB and pdc by use of an endogenous promoter and increments of copy-number Deletion of ldh	Takeda et al. (2011)
Saccharina japonica including alginate, mannitol, glucan (without any pre-treatment)	Ethanol	*E. coli* EPI300	0.28 g g^{-1} kelp powder (yields of over 80% of the theoretical maximum)	Fed batch	Overexpression of *Pseudoalteromonas* sp. SM0524 alginate lyase and the scattered alginate assimilation system of *Vibrio splendidus* 12B01 Overexpression of the homoethanol pathway genes *Z. mobilis* pdc and adhB Deletion of pflB-focA, frdABCD, and ldhA	Wargacki et al. (2012)
Alginate, mannitol, and glucose mixture at a ratio of 5:8:1	Ethanol	*E. coli* ATCC8739	Improved titer and productivity ~330% and ~1200%, respectively, over its plasmid counterpart	Batch	Chromosomal integration of the alginate-utilizing pathway (35.3 kb in total) used in Wargacki et al. (2012), through recombinase-assisted genome engineering technology (Nicole Santos and Yoshikuni, 2014)	Santos et al. (2013)
Alginate monomer (DEHU) and mannitol mixture at a ratio of 1:2	Ethanol	*S. cerevisiae* SEY6210	0.22 g g^{-1} sugars (yields up to 83% of the theoretical maximum from consumed sugars)	Batch	Identification and overexpression of alginate monomer (DEHU) transporter DHT1 from *Asteromyces cruciatus* Overexpression of DEHU catabolic pathway genes with codon optimization: *Vibrio harveyi* dehR, *E. coli* kdgK, and *V. splendidus* 12B01 kdgpA Overexpression of endogenous mannitol pathway genes: YNR073C, YNR072W, and YNR071C Adaptive laboratory evolution (ALE) in media with DEHU as the sole carbon source over 400 generations Acclimation under anaerobic conditions on agar plates with DEHU and mannitol as the carbon sources	Enquist-Newman et al. (2014)
Alginate and mannitol mixture at a ratio of 1:2	Ethanol	*S. cerevisiae* SEY6210	0.15 g g^{-1} sugars (yields up to 32% of the theoretical maximum from consumed sugars)	Batch	Immobilization of endo- and exo-alginate lyases, *Saccharophagus degradans* Alg7A and Alg7K, on the yeast cell surface (Takagi et al., 2015) Overexpression of DEHU catabolic pathway genes (Enquist-Newman et al., 2014) ALE and acclimation were performed following the previous procedure (Enquist-Newman et al., 2014), using alginate polymer as the carbon source instead	Takagi et al. (2017)
Kelp powder (without any enzymatic pre-treatment, hydrolysis)	Ethanol	*Vibrio* sp. dhg	0.35 g g^{-1} kelp powder (yields to 63% of the theoretical maximum) and 0.8 g L^{-1} h^{-1} productivity	Fed batch	Overexpression of the homoethanol pathway genes *Z. mobilis* pdc and adhB, under control of the synthetic promoter (P_{VP15}) and a synthetic 5'-UTR Deletion of ldhA, frdABCD, and pflB genes	Lim et al. (2019)
Alginate and mannitol mixture at a ratio of 1:2	2,3-butanediol (BDO) + acetoin		0.40 g g^{-1} sugars (yields to 81% of the theoretical maximum) and 1.3 g L^{-1} h^{-1} productivity	Fed batch	Overexpression of *Enterobacter aerogenes* budABC operon genes, under control of the synthetic promoter (P_{VP15}) and a synthetic 5'UTR Deletion of ldhA, frdABCD, and pflB genes	
	Lycopene		0.62 mg g^{-1} sugars	Batch	Overexpression of *Lamprocystis purpurea* crtEBI genes and *E. coli* dxs, idi, and ispA genes, under control of the synthetic	

(*continued on next page*)

Table 2 (*continued*)

Substrate	Target product	Base host	Reported TRY metrics	Fermentation type	Genome and metabolic engineering approaches and features	Reference
					promoter (P_{VP13}) and synthetic 5'UTRs Additional overexpression of idi, ispA, and dxs genes	
Alginate	β-carotene	*Vibrio* sp. SP1	0.26–0.34 mg g^{-1} alginate	Batch	Overexpression of L. *purpurea* crtEBI genes and *Pantoea ananatis* crtY gene, under control of P_{tac} and synthetic 5'-UTRs	Park et al. (2021)
	Lycopene		0.18–0.25 mg g^{-1} alginate	Batch	Overexpression of L. *purpurea* crtEBI genes, under control of P_{tac} and synthetic	
Sargassum fusiforme (without any pre-treatment)			0.017–0.024 mg g^{-1} algal powder	Fed batch	5'-UTRs	
Laminaria japonica hydrolysate including 30.5% mannitol and 6.98% glucose	Ethanol	*E.coli* KO11	0.4 g g^{-1} sugars	Batch	*E. coli* KO11 harbors homoethanol pathway genes *Z. mobilis* pdc and adhB	Kim et al. (2011)
L. japonica hydrolysate primarily including glucose and mannitol	BDO + acetoin	*E. coli* MG1655	0.43 g g^{-1} hydrolysate	Fed batch	Overexpression of *E. aerogenes* budABC operon genes Deletion of pta, adhE, frdA, and ldhA genes	Mazumdar et al. (2013)
L. japonica hydrolysate including primarily glucose and mannitol	L-Lactate	*E. coli* MG1655	0.8 g g^{-1} hydrolysate (yields to 80% of the theoretical maximum)	Fed batch	Overexpression of *Streptococcus bovis/equinus* ldh Deletion of pta, adhE, frdA, ldhA, mgsA, and lldD	Mazumdar et al. (2014)
Mannitol	L-Lysine	*Corynebacterium glutamicum* LYS-12	0.19 g g^{-1} mannitol	Batch	Overexpression of *E. coli* K12-MG1655 mak and *Streptococcus mutans* UA159 gapN Deletion of mtlR Identification of metabolic bottlenecks by flux balance analysis	Hoffmann et al. (2018)
L. digitata hydrolysate	L-Lysine	*Corynebacterium glutamicum* SEA-2B	0.27 C-mol $C\text{-mol}^{-1}$	Batch	Overexpression of *E. coli* K12-MG1655 pntAB and codon-optimized *S. mutans*	Hoffmann et al. (2021)
Durvillaea antarctica hydrolysate	L-Lysine		0.40 C-mol $C\text{-mol}^{-1}$	Batch	UA159 gapN Flux balance analysis-driven evaluation of individual metabolic engineering	
Laminarin	Ethanol	*S. cerevisiae* BY4741/Δsed1	0.26 g g^{-1} laminarin	Batch	Immobilization of endoglucanase or β-glucosidase, *S.degradans* Gly5M or *Aspergillus aculeatus* BGI, on the yeast cell surface Optimization of inoculation ratio in the co-culture of endoglucanase- or β-glucosidase-displaying yeast strains	Motone et al. (2016)

biosynthesis of 2'-FL has garnered much attention (Ni et al., 2020). The *salvage* pathway, which exists exclusively in eukaryotes (with the exception of *Bacteroides fragilis* 9343 [Coyne et al., 2005]), can metabolize L-fucose into guanosine 5'-diphosphate (GDP)-L-fucose, and α-1, 2-fucosyltransferase transfers a fucosyl residue of GDP-L-fucose into lactose (Yu et al., 2018). So far, microbial platforms of engineered *E. coli* (Chin et al., 2016) and *S. cerevisiae* (Yu et al., 2018) have been developed for 2'-FL production using L-fucose and lactose as the substrates. Although 2'-FL can be produced from other substrates such as glycerol using the *de novo* pathway (Ni et al., 2020), this review focuses on an L-fucose-oriented biosynthetic scheme as an example of a downstream application of fucoidans.

3.3.3. Decomposition of alginate

Alginate-degrading capabilities have been characterized in various organisms, including bacteria (*Pseudomonas* spp. and *Azotobacter* spp), fungi, and algae, all isolated from both marine and soil environments. These organisms possess a class of alginate lyase (Aly) that endolytically depolymerizes alginate (DP > 100) into unsaturated oligosaccharides (2- to 5-mers) via the β-elimination reaction. The unsaturated oligomers are further decomposed into unsaturated monomers (i.e., 4,5-unsaturated mannuronate or 4,5-unsaturated guluronate) by exolytic catalysis of oligoalginate lyase (Oal) (Wargacki et al., 2012). This class of alginate lyases consists of two endo-types, polyM-lyase (1, 4-β-D-mannuronan lyase; EC 4.2.2.3) and poly-glyase (1,4-α-L-guluronan lyase; EC 4.2.2.11), as well as one exo-type, poly-MG/GM-lyase (EC 4.2.2.-). According to the carbohydrate-active enzyme (CAZy) database (http://www.cazy.org), these lyases are classified into 12 polysaccharide lyase families (PLs; EC 4.2.2.-) (Thiang Yian Wong et al., 2000; Lombard et al., 2010; Meng et al., 2021). There are more endolytic lyases than exolytic lyases, and they have been characterized in various species such as *Sphingomonas* sp., *Stenotrophomas* sp., *Pseudomonas* sp., *Chlorella virus*, *Agrobacterium* sp., *Saccharophagus degradans* 2–40, and *Pseudoalteromonas* sp. (Zhu and Yin, 2015).

3.3.4. Bioconversion of alginate monomers

As alginate-utilizing systems in natural bacteria, three models have been proposed (for illustration and review, see Zhang et al., 2021): (1) the polysaccharide utilization loci (PUL) system, which is typical machinery in some *Bacteroidetes* and *Gamma-proteobacteria* species (this model is called the PUL-like system); (2) the scattered system, found in *Vibrio* spp.; and (3) the pit transport system, identified in *Alpha-proteobacteria* and *Sphingomonas* spp. The monomer produced by these systems is rearranged into 4-deoxy-L-erythro-5-hexoseulose uronate (DEHU). DEHU is reduced into 2-keto-3-deoxy-gluconate (KDG) by DEHU reductase (DehR), the metabolite of which, in bacteria, enters into the Entner-Doudoroff (ED) pathway. Metabolic enzymes KDG kinase (KdgK) and KDG-6-phosphate aldolase (KdgpA) convert KDG into the glycolytic

intermediates pyruvate and glyceraldehyde-3-phosphate (G3P) (Fig. 2).

About a decade ago, these strings of enzymatic processes for alginate assimilation were functionally characterized in the bacterium *Sphingomonas* sp. A1 through the engineering of a homoethanol pathway (Takeda et al., 2011). Subsequently, Wargacki et al. (2012) developed an *E. coli* platform, BAL1611, that achieved direct ethanol production from brown macroalgae biomass containing alginate, mannitol, and glucan with a yield of 0.28 g-ethanol/g-dry macroalgae, more than 80% of the maximum theoretical yield. In this platform, the alginate degradation, periplasm and cytoplasm transports, and metabolic capabilities were harnessed by introducing extracellular production of alginate lyase from *Pseudoalteromonas* sp. SM0524 and the scattered alginate assimilation system from *V. splendidus* 12B01. Furthermore, genes (*pdc* and *adhB*) for a homoethanol pathway consisting of pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase B (*adhB*) from *Zymomonas mobilis* were chromosomally introduced for ethanol production, in addition to endogenous gene deletion for blocking byproduct formation (Wargacki et al., 2012).

Synthetic *E. coli* platforms have been further engineered for stable expression of lengthy heterologous pathways using Recombinase-Assisted Genome Engineering (RAGE). This technology enables chromosomal integration of heterologous genetic clusters through construction of genetic modules on a bacterial artificial chromosome or a single-copy plasmid by site-specific recombinases (Santos et al., 2013; Nicole Santos and Yoshikuni, 2014). Using RAGE, the consecutive alginate-utilizing pathway (35.3 kb in total) was integrated into the chromosomes of *E. coli* strains. After selection of the engineered strains, the final strain (BAL1611) showed efficient ethanol fermentation capability that was ~40% higher titer than that of the plasmid-based strain at the initial generation. After 50 generations, titer and productivity were substantially improved (by ~330% and ~1200%, respectively, over the plasmid control) (Santos et al., 2013).

In the research that followed this work, the Yoshikuni group sought an alginate-utilizing system in the ethanologenic yeast *S. cerevisiae*. In 2014, they reported the development of a synthetic yeast platform (BAL3215) that enabled co-fermentation of alginate monomer and mannitol into 0.22 g-ethanol/g-DEHU and mannitol equivalent to ~83% of the maximum theoretical yield (Enquist-Newman et al., 2014). In eukaryotes, DEHU is transported into the cytoplasm by the DEHU transporter. To synthetically build the DEHU catabolic pathway in yeasts, the Yoshikuni group initially explored a DEHU transporter (DHT1) from the marine alginolytic yeast *Asteromyces cruciatus* and confirmed function in *S. cerevisiae*. Then, codon-optimized genes for the DEHU metabolic pathway were chromosomally integrated. These genes were dehR from *Vibrio harveyi*, kdgK from *E. coli*, and kdgpA from *V. splendidus* 12B01. An additional copy of mannitol pathway genes that are cryptic in the native host (YNR073C, YNR072W, and YNR071C) were chromosomally introduced, which imparted a redox control capability by adjusting the molar ratio of input substrates. To synchronize the heterologous system with the native yeast metabolic network, the group employed adaptive laboratory evolution (ALE) in media with DEHU as the only carbon source over 400 generations. With further acclimation under anoxic conditions, the final strain (BAL3215) was generated, possessing anaerobic growth and fermentation capabilities when grown on both DEHU and mannitol.

On top of this platform, Takagi et al. (2017) harnessed a capability for polymeric alginate degradation by producing *S. degradans* Aly (Alg7A) and Oal (Alg7K) on the yeast cell surface. The developed strain (AM1) achieved an 8.8 g-ethanol/g-alginate and mannitol mix with ~32% of the maximum theoretical yield.

In addition to *Sphingomonas* sp. A1 and industrial microbes, other naturally occurring alginate-utilizing microorganisms have been characterized and demonstrated for production. These include *Defluviitalea phaphyphila* Alg1 (Ji et al., 2016a, 2016b), *Vibrio algivorus* sp. (Doi et al., 2017), *Hydrogenophaga* sp. strain UMI-18 (Yamaguchi et al., 2019), *Vibrio* sp. dhg (Lim et al., 2019), *Clostridium phytofermentans* (Dharshini et al., 2020), and *Vibrio* sp. SP1 (Park et al., 2021). Of these, the alginolytic bacteria *Vibrio* sp. dhg had beneficial properties as a platform microorganism; for instance, it was fast-growing (the growth and sugar uptake rates were higher than those for *E. coli*), and it had high tolerance for salts (10% [w/v] sodium chloride), ethanol, and other major industrial products such as organic acids and solvents (Lim et al., 2019). *Vibrio* sp. dhg possesses the scattered-type alginolytic system, including four Alys, three Oals, and transports (OMP, KdgM, IMP, and Toa) (Zhang et al., 2021).

For ethanol fermentation, *Z. mobils* pyruvate decarboxylase (*pdc*) and aldehyde dehydrogenase (*aldB*) genes were expressed in addition to endogenous genes. For 2,3-butanediol production, *Enterobacter aerogenes* operonic genes (*budABC*) encoding acetolactate decarboxylase (*budA*), acetolactate synthase (*budB*), and acetoin reductase (*budC*) were adopted. For lycopene production, six heterologous genes were used; these were *Lamprocystis purpurea* operonic genes (*crtEBI*) encoding geranylgeranyl pyrophosphate (GGPP) synthase (*crtE*), phytoene synthase (*crtB*), phytoene desaturase (*crtI*), *E. coli* 1-deoxy-D-xylulose-5-phosphate (DXP) synthase (*dxs*), isopentenyl diphosphate (IPP) isomerase (*idi*), and farnesyl diphosphate (FPP) synthase (*ispA*) genes. By engineering promoters and optimizing metabolic fluxes to target products by controlling precursor balances and consumption in competing pathways, Lim et al. (2019) established a microbial brown macroalgae conversion platform using *Vibrio* sp. dhg for biofuel and biochemical production. The group achieved superior TRY: (1) 0.35 g ethanol fermentation per g raw kelp powder (without enzymatic pre-treatment or hydrolysis), (2) 0.40 g 2, 3-butanediol production per g alginate-mannitol mixture (1:2 ratio), equivalent to 81% of maximum theoretical yield, and (3) 0.62 mg lycopene per g alginate-mannitol mixture (1:2 ratio).

3.3.5. Bioconversion of mannitol

Many bacteria, fungi, and yeasts can catabolize mannitol. There are three known pathways for organisms to convert mannitol to fructose-6-phosphate (F6P) for assimilation (Enquist-Newman et al., 2014). (1) Some bacteria use a phosphoenolpyruvate-dependent mannitol phosphotransferase system (PTS) that catalyzes phosphorylation of mannitol and produces mannitol-1-phosphate (M1P). Then, M1P-dehydrogenase (M1PDH) oxidates M1P to fructose-6-phosphate (F6P). (2) Other bacteria and fungi use mannitol-2-dehydrogenase (M2DH), which directly converts mannitol into fructose (Fig. 2). [The efficiency of the forward (mannitol to fructose) reaction is high in fungi, whereas that of the reverse reaction is high in bacteria.] The fructose is then phosphorylated into F6P via hexokinase. (3) Higher plants, including algae, use machinery similar to that used by fungi, by which mannitol-1-dehydrogenase (M1DH) catalyzes the oxidation of mannitol and produces mannose. Mannose is phosphorylated to mannose-6-phosphate (M6P) by hexokinase. Then, M6P is isomerized to F6P by M6P-isomerase (Iwamoto and Shiraiwa, 2005). All three types of mannitol assimilation pathways involve a redox reaction in which NAD(P)$^+$ is reduced to NAD(P)H, resulting in an accumulation of excess NAD(P)H under anaerobic conditions due to absence of an electron shunt (Loescher et al., 1992).

The innate mannitol catabolic pathways of several bacteria have been harnessed for mannitol utilization. For example, *Zymobacter palmae* (Horn et al., 2000a, 2000b) produced 0.38 g-ethanol/g-mannitol in a synthetic mannitol medium under oxygen-limiting conditions. *E. coli* also has an intrinsic mannitol assimilation mechanism, and Wargacki et al. (2012) built an alginate assimilation pathway (depolymerization and DEHU catabolism) for co-utilization. Some yeast strains also have an intrinsic mannitol assimilation pathway. For example, *Pichia angophorae* (Horn et al., 2000a) was able to co-ferment mannitol and laminarin in *Laminaria hyperborean* extracts to 0.43 g-ethanol/g-substrate. *S. paradoxus* NBRC 0259, can metabolize mannitol (Ota et al., 2013). In *S. cerevisiae,* mannitol assimilation is regulated by a cryptic mechanism; ALE allows ~50% of *S. cerevisiae* strains to activate the mannitol pathway. Enquist-Newman et al. (2014) also demonstrated that

overexpression of the cryptic genes (YNR072W and YNR073C or YNR071C) responsible for mannitol assimilation enabled an *S. cerevisiae* strain (SEY6210) to grow on mannitol.

Other biofuels besides ethanol have also been produced from mannitol. For example, Mazumdar et al. (2013) developed 2,3-butanediol (BDO)-producing *E. coli* by harnessing a BDO pathway derived from *E. aerogenes*. They designed the metabolic flux to pool pyruvate, which is a starting substrate for the BDO pathway, and to block sub-product formation through disruption of phosphoacetyltransferase (*pta*), alcohol/acetaldehyde dehydrogenase (*adhE*), fumarate reductase (*frdA*), and lactate dehydrogenase (*ldhA*) for metabolic flux optimization.

Another value-added chemical, lactic acid (LA, 2-hydroxy-propanoic acid), has been produced from *L. japonica* hydrolysates (primarily glucose produced hydrolytically from laminarin and mannitol) with engineered *E. coli* (Mazumdar et al., 2014).

An industrial L-lysine-producing workhorse, *C. glutamicum*, was metabolically engineered to utilize mannitol for L-lysine production (Hoffmann et al., 2018). Although *C. glutamicum* possesses a mannitol catabolic system, the system is highly regulated through repression of the mannitol transporter (*mtlT*) and NAD-dependent M2DH (*mtlD*) controlled by the repressor MtlR (*sucR/mtlR*) (Peng et al., 2011). Deletion of the *mtlR* gene releases the repression of *mtlT* and *mtlD*, and consequently mannitol can be incorporated and oxidated into fructose in cells. The fructose is initially exported by a transporter, after which it undergoes reuptake by a PTS-dependent fructose uptake system, and then F1P kinase (*pfkB*) catalyzes the fructose-1-phosphate (F1P) into fructose-1,6-bisphosphate (FBP), which is fluxed into the Embden-Meyerhof-Parnas (EMP) pathway or the pentose phosphate pathway (PPP). Hoffmann et al. (2021) also further extended the engineering platform for valorization of two commercially relevant macroalgae, *L. digitata* and *Durvillaea antarctica*.

Naturally occurring mannitol-assimilating microbes have been characterized such as *Burkholderia* sp. AIU M5M02 (Yamada et al., 2018) and *Vibrio* sp. dhg (Lim et al., 2019). In the latter, mannitol is imported into the cell by PTS (MtlA) and then converted into F6P by M1PDH (MtlD). F6P is then fluxed into the EMP pathway for glycolysis.

3.3.6. Utilization of laminarin

Laminarinases decompose the laminarin polymer into glucose. These enzymes are classified into β-1,3-endoglucanases (EC 3.2.1.6 and EC 3.2.1.29) and β-1,3-exoglucanases (EC 3.2.1.58). Endo-type laminarinases belong to multiple glycoside hydrolase families (GH16, GH17, GH55, GH64, and GH81), whereas exo-type laminarinases belong to the GH3 family (Becker et al., 2017). The endo-acting glucanases break down the laminarin polysaccharide into laminarin oligosaccharides (β-1,3-linked oligomers with a length of 2–4 units) and gentiobiose, a β-1,6-linked biose. Specific exo-acting glucanases then decompose the oligosaccharides into glucose, which is readily utilized for glycolysis. Since β-glucanases are widely found in natural organisms (Martin et al., 2007) and commercialized, laminarin is a relatively easy-to-use compound from brown macroalgae.

3.4. Bioconversion of macroalgae hydrolysates

Various fermentation procedures for macroalgae have been explored. Separate hydrolysis and fermentation (SHF) is a traditional approach in which biomass is saccharified and fermented separately. Enzymatic hydrolysis is typically performed at 45 °C–55 °C, whereas typical fermentation temperatures are 28 °C–40 °C (Offei et al., 2018). This approach allows researchers to select the ideal condition for each step. However, fermentation inhibitors (e.g., phenolics and salts) are built up during hydrolysis. For example, in butanol production from sulfuric-acid–treated hydrolysates of *Ulva lactuca*, productivity fell by 75% if excess solids from the hydrolysate were not removed prior to fermentation (Potts et al., 2012).

In contrast, the simultaneous saccharification and fermentation (SSF) approach loads both the saccharification enzymes and the fermentation microbe concurrently into a single reactor. Benefits of SSF include low capital costs, low contamination risk, and little buildup of fermentation inhibitors; lower inhibitor content would presumably improve overall fermentation yield (Jang et al., 2012). For example, Lee et al. (2013) further treated an mild acid pre-treated *Saccharina japonica* with cellulase and β-glucosidase. Ethanol fermentation using thermotolerant *S. cerevisiae* DK 410362 was simultaneously performed at 43 °C, which resulted in 6.65 g/L ethanol production, or an SSF yield of 67.41%. Although this approach is potentially cost-effective, identifying optimal conditions for both saccharification and fermentation is still challenging. For example, in a study by Huang et al. (2013), the recombinant Alg2A from *Flavobacterium* sp. S20 showed optimal catalytic activity at a pH of 8.5 and a temperature of 45 °C, conditions too harsh for yeast fermentation.

Approaches such as gene mining and protein engineering the hydrolyzing enzymes and using thermotolerant microorganisms will expand the applications of SSF. Additionally, multiple parameters such as substrate types, availability, and inhibition; product inhibition; cell loading; and cell growth and death need to be optimized (Das Neves et al., 2007). For example, Hargreaves et al. (2013) performed ethanol fermentation of acid-pretreated hydrolysates of the red macroalgae *Kappaphycus alvarezii*. Specifically, two streams, a galactose-containing liquid fraction processed from carrageenan and a cellulose-containing solid fraction, were generated. Ethanol fermentation by *S. cerevisiae* CBS1782 using the liquid and solid fractions resulted in 38 g/L and 53 g/L ethanol production, respectively. However, co-fermentation of the mixed liquid and solid fractions yielded 65 g/L ethanol. Although yeasts generally repress catabolism of sugars such as galactose in the presence of glucose because of the yeasts' catabolite repression system, the result of Hargreaves et al.'s work suggests that increased inoculum size helped reduce inhibition of the Leloir pathway.

Consolidated bioprocessing (CBP) integrates all processes required for saccharification and fermentation in a single pot. Fermentation microbes are engineered to secrete hydrolysis enzymes and simultaneously perform saccharification and fermentation of substrates (Parisutham et al., 2014). CBP is more cost-effective than SSF because the cost of producing the hydrolysis enzyme is lower (Olson et al., 2012; Parisutham et al., 2014). Several strategies for designing microbes for CBP have been explored. These include the native strategy (which leverages an innate capability of a hydrolytic microbe such as cellulolytic *Clostridium* sp., *Bacillus subtilis*, or *Trichoderma reesei*), metabolic engineering, and the microbial consortia approach (for a comprehensive review, see Jouzani and Taherzadeh [2015]). One example of work on these strategies is cell-surface display systems. These have allowed several CBP processes to be carried out in *E. coli* (Wargacki et al., 2012) and in the yeast chasses *S. cerevisiae* (Ueda and Tanaka, 2000), *Pichia pastoris* (Tanino et al., 2006), and *Yarrowia lipolytica* (Yuzbasheva et al., 2011). Some studies have shown that co-display of endo- and exo-glycosyl hydrolases on yeast cell surfaces could enhance saccharification efficiency (Fujita et al., 2004; Katahira et al., 2004; Sasaki et al., 2017; Ishii et al., 2016; Motone et al., 2016), and Takagi et al. (2015) demonstrated that co-display of endo- and exo-alginate lyases on yeast cell surfaces enhanced the saccharification efficiency of alginate.

Although CBP is an ideal design, the metabolic burden created by the excessive expression of pathway genes may also cause undesirable physiological changes and reduce fermentation efficiency (Wu et al., 2016; Lu et al., 2019). Use of multiple strains or species playing synergistic roles in one pot may offer a way to bypass this limitation; this approach is further discussed in section 4.3.

Lastly, all the above fermentation methods may be performed continuously. For example, Park et al. (2012a) evaluated ethanol fermentation of a hydrolysate using batch or continuous fermentation procedures. The group used a modified yeast, *Brettanomyces custersii* KCTC 18154P, to ferment hydrolysate made from the red macroalgae

Gelidium amansii. In the batch mode, this yeast produced 11.8 g/L ethanol ($Y_{p/s} = 0.13$), whereas the continuous procedure produced 27.6 g/L ethanol ($Y_{p/s} = 0.38$). This difference might be explained by the concentrations of remnant inhibitory compounds (formic acid, levulinic acid, and 5-HMF) in the hydrolysate.

4. Advancing macroalgae-based bioproduction

As previously mentioned, the chemical composition of macroalgae varies widely, depending on species and harvesting season. For example, sugar content is greatly reduced in winter, while mineral content increases, requiring host microbes to tolerate high salt concentrations. The ratio of sugar types discussed in section 2 also changes dramatically, and host microbes must be metabolically flexible. Diverse metabolic and microbial engineering strategies are needed to deal with these variations. In this section, we discuss (1) dynamic metabolic engineering of pathways controlled by genetic circuits, (2) marine halophile hosts, and (3) microbial consortia (Fig. 3).

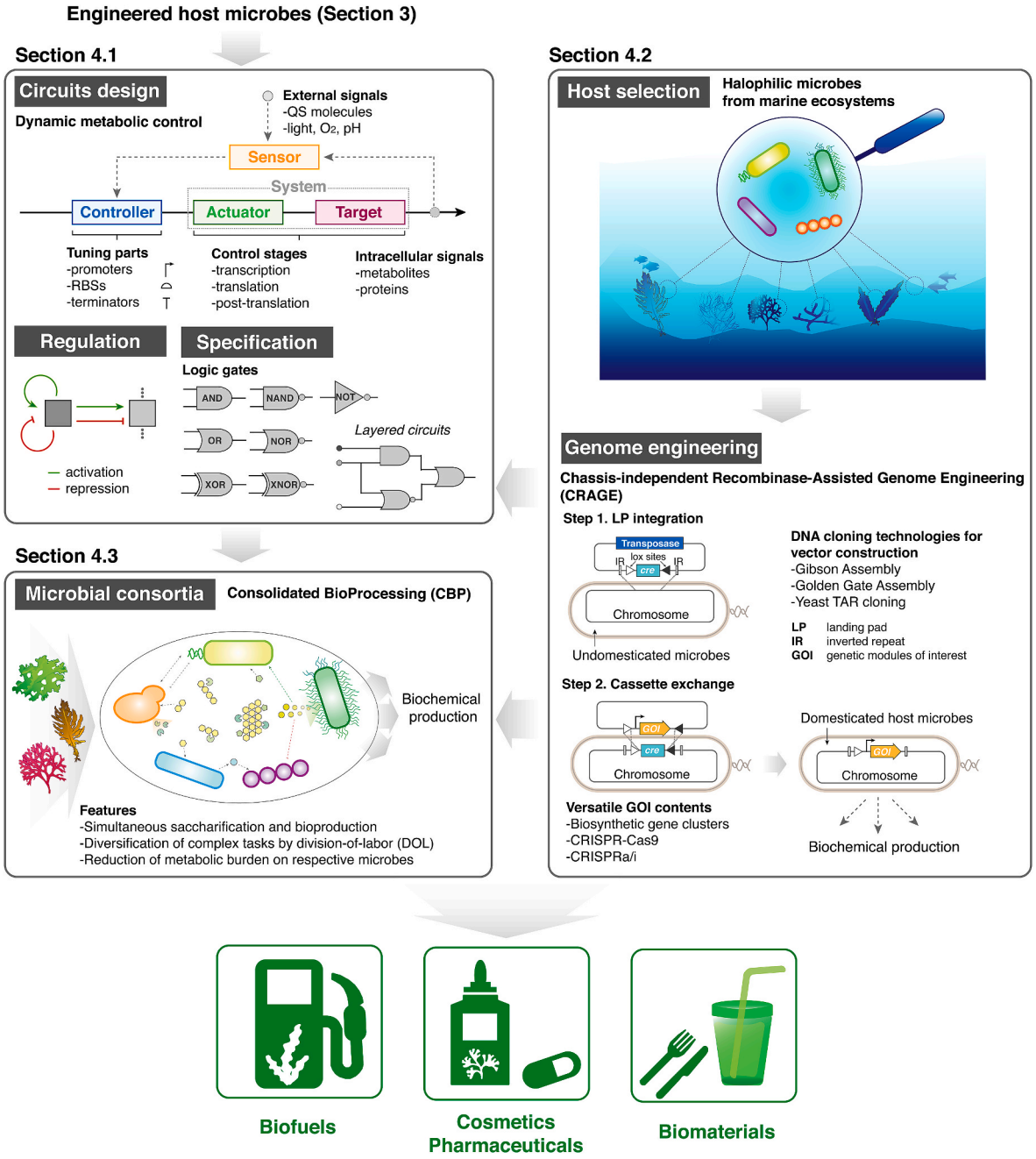

Fig. 3. Overview of metabolic and microbial engineering strategies.
Dynamic metabolic control allows cells to recognize extracellular and/or intracellular signals, transduce the information to cognate coctroller parts, and invoke actuation accordingly. Engineering of the circuit with extensive techniques such as open- and closed-loop regulations and logic gates enables in-situ tuning of cellular metabolic networks (section 4.1). Emerging genome engineering technologies can domesticate natural isolates and tailor them as bioconversion hosts (section 4.2). Microbial consortia, comprising canonical or newly domesticated microbial host cells, can extend dynamic metabolic control for intracellular population-level control, helping to streamline complex bioprocessing and bioconversion processes in a division-of-labor manner (section 4.3).

4.1. Dynamic metabolic control

Conventional metabolic engineering strategies rely on open-loop (feed-forward) regulation (Chubukov et al., 2014; Nielsen and Keasling, 2016). Pathways responsible for target metabolite production are often overexpressed, or competing pathways are deleted or repressed, using transcriptional, translational, and post-translational methods. Transcriptional regulations are controlled by different promoter types (constitutive, inducible, and repressive) and strengths, and transcript stability can also be controlled by untranslated regions and terminators. Translational regulations are controlled by ribosome binding sites, the translation initiation site of each coding sequence, and the organism's codon usage (Bhandari et al., 2021). Post-translational regulations are controlled by addition of tags (e.g., signal peptides), maturation of co-factors, and introduction or elimination of allosteric effects (Jones et al., 2015).

These conventional strategies have contributed to improving TRY metrics by increasing the activity of bottleneck pathways, eliminating byproduct-formation pathways, balancing redox states, and rewiring metabolic fluxes. However, these strategies often focus on specific biochemical reactions or pathways under defined conditions. They do not usually consider that cell physiology and pathway efficiency can change appreciably according to growth conditions such as media composition and conditions such as levels of nutrients, salts, or metals; temperature; osmotic pressure; pH; and oxygen levels. Because the pathways are not flexible or robust enough to adapt to these changes, target metabolites are often produced at sub-optimal levels. To overcome this limitation, many researchers have explored strategies for dynamic metabolic control.

Dynamic metabolic control uses closed-loop regulation, which involves response to feedback. While microbes often use this strategy to increase their fitness under diverse environmental conditions (Chubukov et al., 2014), its use has been limited in metabolic engineering because the design of closed-loop regulation is so complex. However, the development of systems and synthetic biology is allowing increasing use of dynamic control in metabolic engineering. We believe dynamic control will become an effective way to maintain high production yields from macroalgae feedstock.

Dynamic metabolic controls typically include sensors (or analytes), transducers, and actuators. Sensors can be transcription factors (TFs), riboswitches, or other designed peptides or proteins that can detect a broad spectrum of chemical inducers, environmental stimuli, and cellular factors. Upon detection of signals, these sensors can form transducers that access their corresponding actuators. Microbes can be engineered to sense diverse environmental cues (e.g., amounts of substrates and products) and to use them as inputs to control the activity of metabolic pathways. The genetic parts composing sensor-transducer units define the duration, dynamic range, and turnover rate of actuators. A dynamic control system can be made from layers of basic regulatory motifs (Alon, 2007). These regulatory motifs underlie diverse biological networks such as toggle switches (Gardner et al., 2000) and gene oscillators (Elowitz and Leibler, 2000; Stricker et al., 2008). The wiring of individual units abides by the rules of Boolean logic gates, enabling researchers to build synthetic gene circuits and networks analogous to basic electrical circuits (Khalil and Collins, 2010; Moon et al., 2012). The regulatory system's design plasticity allows researchers to program various regulatory patterns (Brophy and Voigt, 2014; Roquet et al., 2016).

Dynamic control systems that are sugar-responsive have been among the most widely studied. For example, the *HXT* system (Özcan and Johnston, 1999), the *GAL* system (Johnston and Davis, 1984), the *SUC* system (Williams et al., 2015), and the P_{BAD} system (Khlebnikov et al., 2000) can control gene expression depending on the concentration of glucose, galactose, sucrose, or arabinose, respectively. The discovery of microbial genetic circuits relevant to the catabolism of unique sugars in macroalgae feedstock can enable dynamic control of macroalgae sugar metabolism in the engineered cells. For example, mannitol sensor-actuator systems (e.g., MtlR) in prokaryotes have been discovered (Tan et al., 2009; Hoffmann and Altenbuchner, 2015; Byer et al., 2017). These systems can sense mannitol or other polyols and control gene expression. Additionally, dynamic control can change cellular physiology based on fermentation conditions via extrinsic and intrinsic signals. For example, redox reactions catalyzed by mannitol dehydrogenase and DEHU reductase need to be controlled by the amount of mannitol and alginate available in the fermenters and by the redox state of the hosts, which will allow macroalgae feedstocks with various compositions to be utilized.

Dynamic metabolic control strategies can also be used to produce targeted metabolites while minimizing metabolic burden to host cells. For example, changes in cellular physiology due to host-microbe interactions can trigger activation of dynamic control. Quorum-sensing (QS) molecules such as homoserine lactones (HLs) are widely used for intercellular communication among bacteria (Mukherjee and Bassler, 2019). Various QS systems have been applied for cell-density-dependent metabolic control. For example, in the lux system, luxR, which activates its cognate promoter (P_{lux}), was used to produce isopropanol (Soma and Hanai, 2015). EsaRI70V, which controls inhibition or activation under the cognate promoter P_{esaS} in response to the HL levels, was used to switch metabolic fluxes between glycolysis and aromatic amino acids synthesis (Gupta et al., 2017). Dinh and Prather (2019) further expanded the tunability of QS circuits by two independent QS systems, *luxR* and *esaR* sensors, with their cognitive promoters P_{lux} and $P_{esaR\text{-}H}$ (respectively). Using this dynamic control system, Dinh and Prather improved naringenin and salicylic acid production.

Cell-growth-dependent control also enables down-regulation of essential pathways so that metabolic resources can be re-allocated to targeted pathways (Doong et al., 2018). In this strategy, key metabolites can be used as signals for sensors. For example, FadR is a transcription repressor sensing acyl-CoA (Zhang et al., 2012). Other metabolites such as farnesyl pyrophosphate (Dahl et al., 2013), malonyl-CoA (Xu et al., 2014), L-tryptophane (Fang et al., 2016), and *myo*-inositol (Doong et al., 2018) have been used as signals for cognitive actuators. Farmer and Liao (2000) designed a controller that can monitor the overflow of acetyl phosphate in the glycolytic pathway by an Ntr regulation system controlled by the *glnAp2* promoter. The system activated the expression of pathway genes (*pps* and *idi*) upon high acetyl phosphate levels in lycopene biosynthesis. Notably, the system built by Dahl et al. (2013) can sense toxic intermediates and use them to negatively regulate their accumulation. Similarly, Ceroni et al. (2018) used a CRISPR-based actuator to sense and mitigate the burden associated with accumulated intermediates.

4.2. Domesticating non-model marine microbes as chassis for bioconversion of macroalgae feedstock

Model microbes such as *E. coli* and *S. cerevisiae* have played pivotal roles in metabolic engineering, but they are suboptimal for bioconversion of macroalgae feedstock because they cannot tolerate the high salt content in macroalgae feedstock. Marine microbes, on the other hand, are salt-tolerant (halophilic) and have gained increased attention as potential hosts (Rotter et al., 2021). In addition to their salt tolerance, many marine microbes already possess pathways responsible for decomposition and assimilation of sugar polymers unique to macroalgae (see section 3.3 for metabolic engineering efforts that have leveraged these abilities). Some of these microbes can also tolerate high concentrations of heavy metals, a unique trait associated with macroalgae feedstock. Isolating microbes that can assimilate macroalgal sugar polymers on the basis of their growth phenotype is straightforward because they use those sugar polymers as their sole source of carbon and energy (Takeda et al., 2011; Ji et al., 2016a; Lim et al., 2019; Yamaguchi et al., 2019; Yu et al., 2020; Park et al., 2021). Additionally, recent advances in sequencing technologies and the expansion of public

sequence databases make it easier to identify gene clusters responsible for the metabolism of these sugars.

Metabolic engineering applications for marine microbes are still limited. However, the recent advent of genome engineering has made it more feasible to use marine microbes as chassis for bioconversion of macroalgae feedstock. For example, the Yoshikuni group has recently developed a technology called "chassis-independent recombinase-assisted genome engineering" (CRAGE). CRAGE enables single-step integration of large complex biological constructs (~60 kbp) directly into the chromosome of recipient bacteria with high accuracy. Using CRAGE, the group has already domesticated ~60 species of bacteria among α, β, and γ-proteobacteria, actinobacteria, and firmicutes (Santos et al., 2013; Wang et al., 2019a, 2019b). The Yoshikuni group has also demonstrated that CRAGE is fully compatible with modern DNA cloning technologies such as Gibson Assembly, Golden Gate Assembly, and yeast TAR cloning, as well as with new genome engineering tools such as CRISPR-Cas9, CRISPRa, and CRISPRi (Wang et al., 2019a, 2019b; Ke et al., 2021). The speed, accuracy, flexibility, and efficiency of CRAGE make it invaluable for developing marine microbial strains that can bioconvert macroalgae feedstocks to produce value-added chemicals.

In addition to their useful sugar metabolism, many marine microbes can natively produce diverse biopolymers with different physicochemical properties, such as hetero/homo-polysaccharides (e.g., alginates, cellulose, xanthan, and hyaluronate), polyamides (e.g., cyanophycin, γ-PGA, ε-PL), polyesters (e.g., polyhydroxyalkanoates, PHAs), polyphosphates (polyP), and others (e.g., extracellular DNA and polypeptides) (Moradali and Rehm, 2020). These biopolymers have numerous applications, including specialty materials for medical applications, food ingredients, cosmetic supplies, and alternatives to petrochemical-based plastics, with their associated environmental concerns.

Among them, bio-based plastics are increasingly getting attention. By 2019, global plastics production had grown to around 370 million MT/yr (Plastics Europe, 2020). Extrapolating from current growth, it is estimated that plastics production will grow to over 1 billion MT/yr by 2050, and that these materials will account for 20% of total oil consumption (World Economic Forum, 2016). As raw materials for biodegradable plastics, PHAs are a family of biopolymers of increasing interest. PHAs occur in over 150 chiral (*R*)-hydroxyalkanoic acid (HA) variations (Chen and Wu, 2005). Several types have been commercially produced, such as poly(3-hydroxybutyrate) (PHB) and poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) (Chen, 2009).

In marine environments, bacteria that can produce PHAs are spread widely across multiple phyla including *Proteobacteria* (e.g., *Alteromonas* spp., *Halomonas* spp., and *Pseudomonas* spp.), *Firmicutes* (e.g., *Bacillus* spp.), *Actinobacteria* (e.g., *Brevibacterium casei* MSI04), and *Cyanobacteria* (e.g., *Spirulina subsalsa*) (Suzuki et al., 2020). Several halophilic microbes have been investigated as hosts for the production of PHAs because of their valuable properties (Oren, 2008; Quillaguamán et al., 2009). For example, a moderate halophile, *Halomonas* TD01, accumulates PHB at up to 80% of cell dry weight (CDW) (Tan et al., 2011). Further strain engineering has improved metabolic fluxes and co-factor balances by deleting pathways competing for the precursors and overexpressing pyridine nucleotide transhydrogenase, which resulted in accumulation of PHB at up to 92% of the CDW, with ~42% glucose conversion efficiency (Fu et al., 2014). Development of genetic engineering tools such as CRISPRi in *Halomonas* spp. (Tao et al., 2017) will expand opportunities for PHA production with the benefits of halophile-specific properties (e.g., lower contamination risks).

Macroalgae carbohydrates have also been utilized to produce PHAs (Fig. 2). For example, two newly isolated marine bacteria, *Burkholderia* sp. AIU M5M02 and *Hydrogenophaga* sp. strain UMI-18, utilize mannitol and alginate as carbon sources and accumulate PHB at around 58% and 40% of the CDW, respectively (Yamada et al., 2018; Yamaguchi et al., 2019). Brown macroalgal hydrolysates prepared from *Sargassum* sp. have been used to produce PHB by the hydrogen-oxidizing bacterium *Cupriavidus necator* PTCC 1615 with 8 g/L NaCl stimulation, resulting in accumulation of PHB at 74.4% of the CDW (Azizi et al., 2017). Red macroalgal hydrolysates prepared from *Gelidium amansii* have also been utilized for PHA production by *Bacillus megaterium* KCTC2194 and co-culture of *S. degradans-B. cereus* pairs, yielding PHA at up to 54% and 35% of the CDW, respectively (Alkotaini et al., 2016; Sawant et al., 2018). In addition, PHAs have been produced from green macroalgal hydrolysates from *Ulva* sp. with the halophilic archaeon *Haloferax mediterranei*, with a PHA content of up to 58% of the CDW (Ghosh et al., 2019). Intriguingly, native properties of *H. mediterranei*, such as accumulation of PHBV and high salt tolerance, distinguish this archaeon as a production host.

4.3. Microbial consortia-based metabolic control

Besides the strategies involving unicellular metabolic control discussed in section 4.1 and 4.2, the consortia-based strategy (also called co-culture) has gained attention because it can perform complex fermentation. This strategy has been used extensively in diverse industrial processes including solid waste and wastewater treatments (e.g., anaerobic digesters). Consortia-based metabolic control uses a division-of-labor (DOL) approach. DOL allows complex tasks to be split into specific subtasks that can be distributed across diverse microbial strains, each specialized for a particular subtask (Tsoi et al., 2018). Thus, DOL can simplify engineering of each strain and potentially mitigate the burden associated with excessive metabolic engineering (Johns et al., 2016). Therefore, the consortia-based strategy may be a viable way to efficiently convert macroalgae feedstocks comprising complex mixtures of unique sugars into diverse bioproducts.

Successful DOL applications have established synthetic consortia for various applications in metabolic engineering: production of commodity chemicals in *E. coli–E. coli* pairs (Zhang et al., 2015), natural products by *E. coli–S. cerevisiae* pairs (Zhou et al., 2015), and biofuels from sugar cane bagasse hydrolysates by *E. coli–S. cerevisiae* pairs (Wang et al., 2019a, 2019b). The advantages of the consortia-based strategy may be more pronounced when it is applied to CBP. Microbial-consortia–based CBP could be an efficient and cost-effective way to use macroalgae as feedstock (Parisutham et al., 2014). Large quantities of carbohydrate-degrading enzymes (CAZymes) are needed for CBP, and their production is generally resource-intensive. In the microbial-consortia–based CBP strategy, therefore, one strain is dedicated for CAZyme production, while other strains are engineered to convert monomeric sugars into bioproducts. For CBP-based macroalgae conversion, a synthetic yeast consortium has attained direct ethanol fermentation from the brown macroalgae *Ecklonia kurome* (Sasaki et al., 2018). In this consortium, two different *S. cerevisiae* strains were used; one was engineered to assimilate alginate and mannitol, and the other to depolymerize glucans via surface-displaying CAZymes. This consortium produced 2.1 g/L ethanol from the raw macroalgae biomass. Another consortium built of *S. cerevisiae* KCTC 1126 and *Pichia angophorae* KCTC 17574 enabled researchers to convert hydrolysates prepared from waste red, brown, and green macroalgae into ethanol (Y_{EtOH} of 0.45 with ~94% substrate conversion yield). *P. angophorae* contains various CAZymes responsible for degrading macroalgae sugars (Sunwoo et al., 2017).

The microbial-consortia-driven CBP approach has also been used for other fermentations. For example, Potts et al. (2012) reported 4 g/L butanol production via acetone-butanol-ethanol (ABE) fermentation of the green macroalgae *Ulva lactuca* (av. 15.2 g/L of reducing sugars) using a bacterial consortium comprising *Clostridium beijerinckii* and *C. saccharoperbutylacetonicum*. Dark fermentation using a consortium of anaerobes (e.g., *Clostridia*) and facultative anaerobes (e.g., *E. coli*, *Enterobacter*) isolated from an anaerobic digester for wastewater treatment was evaluated for hydrogen production from red (*G. amansii*, *P. tennera*, and *G. verrucosa*), green (*C. fragile*), and brown (*L. japonica*, *U. pinnatifida*, *H. fusiforme*, and *E. stolonifera*) macroalgae (Ntaikou et al.,

2010; Jung et al., 2011). Among these macroalgae, *L. japonica* was confirmed to be a preferable feedstock with 67 mL-H_2/g total solid (TS), suggesting that the relatively high carbohydrate content (~60%) in brown *L. japonica* may contribute to hydrogen production.

While microbial-consortia–based control is promising for converting complex sugars in macroalgae feedstocks into bioproducts, several challenges remain. Microbial consortia are generally heterogeneous, making it difficult to control community stability, resilience, and robustness in the face of diverse perturbations, since population dynamics fluctuate according to fermentation conditions such as media composition, pH, and temperature. Additionally, the physiology of each community member needs to be precisely controlled. Heterogeneity often causes large batch-to-batch variation and affects the reproducibility of experiments (Jawed et al., 2019). Integrative multi-omics and systems biology could be used to improve understanding of factors that control the population dynamics and metabolic and energy states of each member of microbial consortia and full consortia. Knowledge gained could be incorporated into the design-build-test-learn (DBTL) cycle in microbiome engineering (Lawson et al., 2019).

5. Conclusions

The world is facing ever-increasing demand for sustainable sources of energy, foods, and materials. Macroalgae may be an ideal feedstock for processes designed to meet these demands. Macroalgae production does not compete with production of land-based food crops, as it does not require arable land, fresh water, or fertilizer. Furthermore, macroalgae can be produced abundantly at low cost, and they have high sugar content, desirable because sugars can be used to produce bioproducts. This review article therefore has focused on introducing recent advances in metabolic engineering efforts and remaining challenges to efficiently utilizing macroalgae sugars for production of biofuels and bioproducts.

Macroalgae comprise sets of unique sugars that are considerably different from land crop sugars. However, metabolic pathways for most macroalgae sugars have been identified in natural microorganisms, and some of them have been successfully engineered in various host chassis. Because macroalgae sugar composition varies widely depending on species and harvest season, several unique problems are associated with the utilization of macroalgae biomass. To efficiently utilize various compositions of sugars, microbes must be engineered to dynamically control sugar metabolisms in response to external sugar concentrations. Additionally, macroalgae, unlike terrestrial crops, contain sugars with different oxidative states. While these sugar variations may be beneficial for producing various chemicals, both the energy and the redox states of the microbes need to be well balanced in order to maintain bioconversion efficiency. Furthermore, the high salt content of macroalgae makes model microbes such as *E. coli* and *S. cerevisiae* less attractive for use in bioconversion of macroalgae than for bioconversion of terrestrial biomass.

Non-model microbes, in particular marine (halophilic) microbes, may prove to be more suitable chassis, and efficient genome technologies such as CRAGE can expand the repertoire of chassis from marine ecosystems. To accomplish bioconversion of complex mixtures of macroalgae sugars, microbial consortia comprising multiple species or strains might also be useful. In this strategy, each member of a consortium is specialized for decomposition and catabolism of different sugar components, but the carbon is funneled into a single final product. Microbial consortia can mitigate the metabolic burden associated with excessive metabolic engineering. Further developing models that design genetic circuits for monoculture-based and/or consortia-based dynamic metabolic controls will help predict viable ways to unleash the potential of macroalgal feedstock for bioprocesses.

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