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Marine Microalgae

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Abstract Marine microalgae, the largest primary biomass, have been attracting attention as resources for new metabolites and biotechnologically useful genes. The diversified marine environment harbors a large variety of microalgae. In this paper, the biotechnological aspects and fundamental characteristics of marine microalgae are reviewed.

Keywords Marine microalgae · Useful material production · Genetic manipulation · Mass cultivation · Photoreactor

1 Introduction

The primary producers of oxygen in aquatic environments are algae, especially planktonic microalgae. These microorganisms are widely distributed in nature and have adapted to different environments with great diversity in size, morphology, life cycle, pigments, and metabolism. About one half of global photosynthesis and oxygen production is accomplished by marine microalgae. They play an important role in CO_2 recycling through photosynthesis, which is similar to higher plants in O_2-evolved systems (PSI and PSII).

Research in microalgae has been carried out not only on physiological aspects but also to develop production of useful biomaterials. The advantages of their utilization in production are (1) their ability to convert CO_2 to useful materials through photosynthesis and (2) their ability to grow in natural environments under inorganic conditions. For example, marine microalgae can be cultivated using seawater, CO_2, and sunlight. Recent developments in the biotechnology of microalgae have been focused on their production of useful materials applicable to the cosmetic and medical fields.

Genetic modification and molecular tools have been developed mainly in eubacterial microalgae, cyanobacteria (blue-green algae). In contrast, genetic modification has been only gradually applied to eukaryotic microalgae. Recently, whole genome sequences and EST analyses have been performed in marine strains. The elucidation of genomewide information may help in the development of new biotechnological applications using microalgae.

In this chapter, we review the useful applications of microalgae for genetic engineering, cultivation technologies, and CO_2 fixation as follows:

1. Production of useful chemicals by marine microalgae
2. Metabolic engineering of marine microalgae
3. Microalgal mass cultivation technologies
4. CO_2 fixation using microalgal cultures in industry

2 Production of Useful Chemicals by Marine Microalgae

2.1 Cyanobacteria

The cyanobacteria are oxygenic photosynthetic prokaryotes that show large diversity in their morphology, physiology, ecology, biochemistry, and other characteristics. Currently, more than 2000 species are recognized, which comprise two fifths of known bacterial species (5000 species). Such variation complicates estimation of species diversity. Cyanobacteria contain chlorophyll *a*,

phycobiliproteins, and characteristic glycosylated xanthophylls, such as myxoxanthophyll and oscillaxanthin [1–3]. The phycobiliproteins are water-soluble pigments consisting of red-colored phycoerythrin and blue-colored phycocyanin and allophycocyanin. They have a characteristic structural feature, the phycobilisome, that is used as a photosynthetic light-harvesting antenna. Cyanobacteria are unicellular, multicellular, colonial, and branched or unbranched filamentous forms. Specialized cells, heterocystous and akinates, are contained in some of the filamentous-form cells. Some cells produce extracellular matrix such as sheaths, capsules, and slimes that consist mainly of polysaccharides. Cyanobacteria are distributed widely not only in salt water but also freshwater, brackish water, polar regions, hot springs, and deserts. Some also exist as symbionts in sponges, ascidians, echiuroid worms, planktonic diatoms, and dinoflagellates in marine environments [4] and lichens and azollae in terrestrial environments. Cyanobacteria, especially marine pelagic *Synechococcus* and *Prochlorococcus*, contribute largely to global oxygen production.

Many commercial applications have been proposed for marine cyanobacteria, although no marine strain presently is commercially supplied. Recent proposals to evaluate potential commercial uses typically fall into three categories: bioactive chemical compounds [5–10], polysaccharides [11–14], and evaluation of new genes for recombinant expression. Cyanobacteria can produce a large variety of complex chemical compounds. Bioactive compounds isolated from marine cyanobacteria have recently been summarized by Burja et al. [5] and Takeyama and Matsunaga [15]. Novel plant growth regulators that promote redifferentiation, germination, and plantlet formation [16], tyrosinase inhibitors [17], UV-A absorbing compounds [18], sulfated polysaccharides showing anti-HIV activity, and novel antibiotics with light-regulated activity [19] are among the many compounds that have been studied. Matsunaga et al. [20] reported that several marine cyanobacterial strains, such as *Phormidium* sp. NKBG 041105 and *Oscillatoria* sp. NKBG 091600, showed high *cis*-palmitoleic acid content (54.5% and 54.4% of total fatty acid, respectively).

The discovery of biochemically active compounds from marine cyanobacteria, including enzyme inhibitors, herbicides, antimycotics, antifeedants, multi-drug-resistance reversers, and antimalarial and immunosupressive agents, has dramatically increased over the last few years. This has been due to the adaptation and use of current cyanobacterial collections and cyanobacteria-derived compounds for screening in new pharmaceutical and industrial assays.

The focus on the polysaccharides of marine cyanobacteria as well as freshwater strains also has greatly increased in relation to interest in their exploitation for various industrial applications [11–14]. Cyanobacteria produce three types of extracellular matrix consisting mainly of polysaccharides, which have unique bio- and physicochemical characteristics. Most of them are com-

posed of at least ten different monosaccharides and contain pentoses, which have not been observed in other prokaryotic polysaccharides. The anionic nature of these unique polysaccharides is due to the presence of acidic sugars and anionic organic and inorganic compounds. Little work has been devoted to potential applications of marine cyanobacterial polysaccharides. Extracellular polysaccharide production by cyanobacteria, as well as their possible applications, was reviewed by Philippis and Vincenzini [11] and Philippis et al. [13].

Marine cyanobacteria also are attractive as a resource for useful enzymes and genes [21–29]. Cyanobacteria commonly produce complex macromolecules that often possess biological activities such as cytotoxicity or microbial toxicity [5]. Recent genetic analyses have revealed that most of these macromolecules, as well as unusual small molecules, are coded for by large gene clusters representing nonribosomal peptide synthetase and polyketide synthetase [21–23, 25, 26, 28]. These gene clusters may be manipulated for the production of new chemicals. Many varieties of the gene clusters are present in most cyanobacteria [21, 24]. Therefore, cyanobacteria are attractive not only as producers of useful bioactive macromolecules and enzymes but also for production of complex macromolecules that may become important pharmaceuticals.

2.2 Rhodophyta

The rhodophytes, or red algae, contain chlorophyll *a*, carotenoids, and phycobiliproteins. Their red color is due to the presence of phycoerythrin in the outermost part of the phycobilisomes, while other regions of the algae look blue-green due to the absence of phycoerythrin. Rhodophytes are unicellular, or composed of simple or complex filamentous aggregates. Flagellated cells have not been observed. Red macroalgae commonly inhabit tropical and temperate zones near shores. About 600 genera and 5500 species have been recognized. Most of them (98%) are marine macroalgal species.

Red macroalgae are of economic significance [30]. *Porphyra* and a few other species are cultured for human food. The production of red algal polysaccharides such as agar, agarose, and carrageenans is also an important industry. These compounds are widely used for laboratory cell culture media, nucleic acid purification, or food processing, respectively.

Although rhodophyte microalgae have not been produced commercially as yet, their polysaccharides are considered to have commercial potential [31–35]. For example, in concentrated solutions of polysaccharides from the unicellular rhodophyte *Porphyridium* sp., viscosity is stable over a wide range of pH, temperature, and salinity. These properties indicate possible applications for use as a thickening agent in aqueous systems or as a stabilizer for emulsions and suspensions [31]. In addition to its potential application as

a viscosity stabilizer, the polysaccharide and the biomass of *Porphyridium* sp. have been used as functional food additives [32]. The colon and jejunum changed morphologically with hypertrophy in the muscularis layer in rats fed diets containing pelleted biomass or sulfated polysaccharides. In addition, it was shown in rats that the algal polysaccharide and biomass were potent hypocholesterolemic agents active at low concentrations in the diet. Moreover, the sulfated polysaccharide of *Porphyridium* sp. has shown promising antiviral activity against a variety of animal viruses including *Herpes simplex* viruses types 1 and 2 (HSV 1, 2) *Varicella zoster* virus (VZV), and HIV types 1 and 2. The compounds also showed significant inhibition of productive infection with retroviruses (murine leukenia virus, HIV-1, and HIV-2) and cell transformation by murine sarcoma virus in vitro [33–35]. Thus, red microalgae and their polysaccharides seem to be good candidates for the development of antiviral drugs.

2.3 Chlorophyta

Cells of chlorophytes are green due to chlorophyll *a* and *b*, the same predominant photosynthetic pigments as those of land plants. Some algae show yellowish-green or red-green colors due to the presence of a certain amount of carotenoids such as β-carotene, prasinoxanthin, siphonaxanthin, and astaxanthin. These chlorophyta form starch in the chloroplast as a storage product of photosynthesis. Chlorophytes are unicellular, multicellular, colonial, filamentous, siphonous, and thallus. The Chlorophyta consist of five classes, the Prasinophyceae, the Ulvophyceae, the Chlorophyceae, the Trebouxiophyceae, and the Charophyceae. The Treboxiophyceae recently were separated from the Chlorophyceae. The Chlorophyta are primarily freshwater algae with approximately 500 genera comprising 16 000 species. Only about 10% of these are marine species. The Ulvophyceae are primarily multicellular marine green algae. In addition, some species from the Prasinophyceae, Chlorophyceae, and Trebouxiophyceae families are found in the marine environment. Depending on the class, it has been estimated that there would be at least two to three times more species in this phylum.

A marine species of the Chlorophyceae, *Dunaliella*, has been cultivated commercially for food supplements and β-carotene production [36]. The microalgal biomass of some marine *Tetraselmis* and *Pyramimonas* strains in the Prasinophyceae family also are used for fish food additives [37]. Recently, anti-inflammatory and immunosupressive properties were discovered in the extracts [38] or extracted polysaccharides [39] of another marine species, *Chlorella stigmatophora*. Miura et al. [40] reported that *Chlorella* sp. NKG 042401 contains 10% γ-linolenic acid (C18 : 3), which is present in the cells mainly in the form of galactolipid.

2.4 Cryptophyta

Cryptophytes are unicellular, cryptomonad flagellates with 12 to 23 genera comprised of 200 species. A few species are colorless heterotrophs, but most possess various colored plastids with chlorophylls *a* and *c*, carotenoids, and phycobiliprotein. Alloxanthin is a xanthophyll that is unique to cryptomonads. Morphologically, cells have a flattened asymmetrical shape with two anterior flagella, slightly unequal in length. They are distributed widely both in freshwater and marine environments. Approximately half of the known species inhabit marine environments. It has been estimated that there would be about six times more species in this phylum.

Only a few strains such as *Rhodomonas minuta* and *Cryptomonas* sp. have been used for aquaculture feeds since they contain significant amounts of polyunsaturated fatty acids (PUFAs) [37]. No further application has been proposed.

2.5 Heterokontophyta

The phylum Heterokontophyta is the most diverse algal group with huge commercial and biotechnological potentials [30, 37]. They range in size from microscopic unicells to giant kelp averaging several meters. Cells of heterokontophytes contain chlorophyll *a* with chlorophyll *c* and carotenoids such as fucoxanthin or vaucheriaxanthin. They are characterized primarily by the similarities in their ultrastructural and biochemical characteristics.

Heterokontophyte microalgae are widely used as feed in mariculture/aquaculture [30, 37, 41]. Diatoms such as *Chaetoceros calcitrans*, *Chaetoceros gracilis*, *Chaetoceros muelleri*, *Skeletonema costatum*, and *Thalasiosira pseudodonana* are commonly used as live feeds for all growth stages of bivalve molluscs (e.g., oysters, scallops, clams, and mussels), for crustacean larvae, and for zooplankton used as feed for larvae. The genera *Navicula*, *Nitzschia*, *Cocconeis*, and *Amphora* also are used to feed juvenile abalone. Some Eustigmatophyceae species of the genus *Nannochloropsis* are commonly fed to Artemia or rotifers, which in turn are fed to crustacean and fish larvae.

The biotechnological potential of diatoms is also concerned with PUFA production. Most diatoms have a high content of eicosapentenoic acid (EPA) 20:5 (n-3). *Phaeodactylum tricornutum* and *Nitzschia laevis* especially have been investigated for EPA production. In addition, EPA production by diatoms has been reviewed recently by Lebeau and Robert [42, 43]. Recent advances in heterotrophic production of EPA by microalgae were also reviewed by Wen and Chen [44].

The Pinguiophyceae also have significant biotechnological potential for use in fish feed and for PUFA production [45]. Pinguiophyceae consist of five ma-

rine unicellular algal species, *Pinguiochrysis pyriformis*, *Phaeomonas parva*, *Pinguiococcus pyrenoidosus*, *Glossomastix chrysoplasta*, and *Polypodochrysis teissieri*. These algae have an unusually high percentage of PUFAs, especially EPA. The EPA content ranges from 23.5% to 56.0% of the total fatty acids in these five species. They also contain arachidonic acid (AA) 20:4 (n-6) and docoahexaenoic acid (DHA) 22:6 (n-3). *Pinguiococcus pyrenoidosus*, *Glossomastix chrysoplasta*, and *Polypodochrysis teissieri* have docosatetraenoic acid (DTA) 22:4 (n-3) ranging from 4.4% to 9.5% of total fatty acid content. This significant oil balance, as well as their lack of cell wall, indicates that they are good candidates as food/feed sources. Evaluations of the utility of Pinguiophyceae for food/feed supplements and optimization of growth conditions necessary for efficient production are being carried out.

Some marine colorless heterokontophytes also are being tested for the production of DHA [36, 46–48]. *Schizochytrium*, *Thraustochytrium*, and *Ulkenia* are representatives of the class Labyrinthulea. They produce substantial amounts of PUFAs, especially DHA [46–48]. The safety of biomass production and differences in oil content are intensively studied [49–53]. Utilization of fermented "okara" for DHA and/or EPA production by thraustochitrids also has been reported, while the yield was lower by growth in a glucose-yeast-extract medium than by fermentation [54].

2.6 Dinophyta

The Dinophyta include the dinoflagellates, most of which are unicellular, with two dissimilar flagella [1–3]. Flagellated cells show characteristic forward-spiraling swimming motions. Organisms in this phylum have remarkable morphological diversity including nonflagellate amoeboid, coccoid, palmelloid, or filamentous. Approximately 130 genera with about 2000 living and 2000 fossil species have been described in this group. Most are marine and only about 220 species are from freshwater. About half of the known species are colorless heterotrophs. Most of the plastid-containing phototrophic dinoflagellates contain chlorophylls *a* and c_2 and carotenoids such as β-carotene and peridinin, the unique accessory xanthophyll in this phylum. Dinoflagellates are also characterized by cell coverings consisting of a layer containing many closely adjacent, flattened amphiesmal (thecal) vesicles. In many species, each amphiesmal vesicle contains a thecal plate composed of cellulose. About 60 dinoflagellate species are known to produce cytolytic, hepatotoxic, or neurotoxic compounds. Some form harmful red tides. The majority of toxin-producing dinoflagellates are photosynthetic, estuarine, or coastal shallow-water forms that are capable of producing benthic resting cysts and that tend to form monospecific populations. Freshwater species are not known to produce toxins. The dinophytes comprise autotrophs, mixotrophs, osmotrophs, phagotrophs, and parasites. Dinoflag-

ellate endosymbionts known as zooxanthellae are essential for the existence of coral reef ecosystems. Symbiotic as well as parasitic forms also are present within the cells or tissues of fish, invertebrates, and filamentous algae.

Biotechnological applications for dinoflagellates have not been intensively performed, probably because most dinoflagellates can not be easily cultured. The only exception is the DHA produced by heterotrophically grown *Crypthecodinium cohnii* [36]. DHA produced by these cells is distributed widely as food supplements, such as for infant formula. Recently, significant biological activities have been attributed to the polysaccharide from *Gymnodinium* sp. [55–58]. For example, strong cytotoxicity against several human leukemic cell lines leading to apoptosis [55] and potent anticancer activity mediated by the inhibition of topoisomerase I and II [58]. Optimal growth conditions for *Gyrodinium impudicum* were reported to produce a sulfated polysaccharide that showed antiviral activity against encephalomyocarditis virus [59].

2.7 Haptophyta

The phylum Haptophyta (haptophytes or prymnesiophytes) is a group of unicellular flagellates characterized by the presence of a haptonema between two smooth flagella [1–3]. The role of haptonema is unclear, although it sometimes functions as a feed-capturing net, in avoidance reaction by coiling and recoiling, and as an attachment organ on surfaces. There also are haptonemaless flagellates or nonflagellate unicells or colonies. The cells of haptophytes are brownish or yellowish-green containing chlorophylls *a* and c_1/c_2 and carotenoids such as β-carotene, fucoxanthin, diadinoxanthin, and diatoxanthin. The cells are commonly covered with scales made mainly by carbohydrates or calcium bicarbonate. Many species known as coccolithophorids produce calcified scales called coccoliths. About 70 genera with 300 species have been recognized to date. Most are primarily marine species inhabiting tropical seawater. The group is distributed worldwide and is often an important source of food for aquatic communities. Some haptophytes, however, produce algal blooms and cause serious problems for fish and for fishermen by producing dimethyl sulfide (DMS), a noxious-smelling compound that affects fish migrations and alters their normal routes.

Microalgal biomass of haptophytes is commonly used as living feed in aquaculture [37]. *Isochrysis galbana* and *Pavlova lutheri*, especially, are used as living feeds for bivalve molluscs, crustacean larvae and zooplanktons that in turn are used for crustacean and fish larvae. Some cells can produce PUFAs such as DHA or EPA. In addition, the DHA content in *I. galbana* was shown to be enhanced by low temperature or incubation of the culture in the dark after reaching plateau phase growth [60]. Furthermore, it was shown that these algae are useful for DHA enrichment of feed such as rotifers for the larvae of several marine fish species [61].

2.8
Euglenophyta

Euglenophytes are unicellular organisms with two pantonematic flagella arising from the bottom of a flask-shaped invagination called a "gullet." A few have stages with colonies or are enclosed within a mucilaginous capsule. There are about 40 genera comprised of 900 species, of which one third have green plastids. The chloroplast originating from green algae contains chlorophylls *a* and *b* and carotenoids such as diadinoxanthin, neoxanthin, and β-carotene. Two thirds of the genera are heterotrophic, some having colorless plastids and some lacking plastids altogether, living either saprotrophically or phagocytically. Cell walls are absent, but there is a characteristic cell-covering structure called a pellicle, composed of protein-rich spiral strips beneath the cell membrane. One to several flagella may be present, and nonflagellate cells can undergo a type of motion involving changes in cell shape. Although most species are found in highly eutrophic freshwater environments such as ponds and ditches, some euglenoids have important ecological roles in particular marine environments. Very few euglenoids have been grown in axenic culture, and euglenoid culture media are generally very nutrient rich. No direct economic significance has been associated with this phylum, probably because of the difficulties in culturing. Although euglenoids generally are harmless, toxin production has been demonstrated in some freshwater *Euglena* sp. [62]. On the other hand, *E. gracilis Z* is one of the few microorganisms that simultaneously produces antioxidant vitamins such as β-carotene and vitamins *C* and *E*. Efficient production of these vitamins consists of a two-step culture under photoheterotrophic/photoautotrophic conditions [63].

3
Metabolic Engineering of Marine Microalgae

3.1
Gene Transfer Methods for Marine Microalgae

Genetic studies on microalgae have been redirected mainly toward analysis of photosynthesis and metabolic pathways. A limited number of microalgae such as cyanobacteria have been used in biotechnological applications. Development of molecular techniques for physiological analysis and enhancement of biotechnological applications is necessary.

Many attempts at gene transfer have been made in eukaryotic and prokaryotic microalgae. Genetic manipulation in prokaryotic microalgae cyanobacteria have been studied extensively after several transformable unicellular

strains were discovered. At first, the freshwater cyanobacterium *Synechococcus* PCC7942 was reported to have an ability to take up DNA. Subsequently, several other naturally transformable freshwater strains have been found. Gene transfer has been developed mainly in freshwater strains, *Synechococcus*, *Synechocystis*, *Anabaeba*, and *Nostoc* [64]. Only a few marine cyanobacterial strains of the genus *Synechococcus* have been used for heterogeneous gene expression and other genetic applications [65, 66]. There are two commonly used gene transfer procedures: transformation using naturally occurring or artificially competent cells, e.g., conjugation with *Escherichia coli*, or physical methods for gene introduction, e.g., electroporation and particle bombardment.

In marine cyanobacteria, natural transformation has been reported for *Synechococcus* sp. PCC7002 [67]. Other strains have been transformed successfully by electroporation or conjugation. Further, plasmids harvested from several marine microalgal species have been used as vector DNA for gene transfer. Many cyanobacteria-harboring endogenous plasmids have been reported. Some functional genes were found to be coded on freshwater cyanobacterial plasmids [68]; however, most cyanobacterial plasmids are cryptic. Marine plasmids have been found in *Synechococcus* sp. NKBG042902, which has high phycocyanin content and a rapid growth rate. Extracts from this strain promote plant germination [16]. This strain contains more than three cryptic endogenous plasmids, pSY09 (> 10 kbp), pSY10 (2.6 kbp), and pSY11 (2.3 kbp). Plasmid pSY10 has the unique replication characteristic that their copy number increases under high salinity conditions [70]. To investigate the function and replication mechanism of these plasmids, the plasmids pAQ1 (4.8 kbp) of the marine strain *Synechococcus* PCC7002 and pSY10 of NKBG 042902 were entirely sequenced [71, 73], and a gene transfer system using pSY10 was established [69]. The complete sequence of *Synechococcus* pSY10 (2561 bp) includes seven potential open reading frames (ORFs). The longest ORF has homology with the replication region of plasmids from several bacteria [72]. pSY10 did not hybridize with other plasmids purified from *Synechococcus* sp. NKBG 042902.

Replication of these plasmids appears to be controlled by different mechanisms. Plasmids are maintained at high copy number in cyanobacteria, suggesting the possibility that they act as a shuttle vector between cyanobacteria and *E. coli*. In fact, a shuttle vector with *E. coli* has been constructed using pSY10.

Conjugative gene transfer has been reported mainly for the freshwater filamentous cyanobacterium *Anabaena* PCC7120, which is not a naturally transformable strain. Conjugation in *Anabaena* PCC7120 was carried out using conjugal plasmids such as RP4 (IncP), a helper plasmid carrying a mobilization gene, and a shuttle vector carrying cyanobacterial replicons [74]. The other filamentous freshwater cyanobacteria *Plectonema boryanum* [75] and *Fremyella diplosiphon* [76] also can be successfully transformed by conju-

gation. A conjugative plasmid vector in unicellular freshwater cyanobacteria has been constructed [77].

Conjugative gene transfer using a broad-host range vector pKT230 (IncQ, Km^r, and Sm^r) was successful for the marine cyanobacterium *Synechococcus* sp. NKBG 15041C [78]. It was demonstrated that this plasmid is stably maintained in cyanobacterial cells [79]. This introduced a new tool in cyanobacteria biotechnology since most transformations were carried out using the shuttle vector plasmid containing a cyanobacterial plasmid origin of replication. Random gene insertion into the genome using the transposon vector pSUP 1021 (which carries the RP4-specific mob site) and the transposon Tn5 was demonstrated in NKBG 15041C.

In marine cyanobacteria, besides the plasmid vector system, the construction of a phage vector system also is required to enable the cloning of large DNA fragments in specific cyanobacterial hosts. Since cyanophages were first reported by Safferman and Morris [80], various types of cyanophages have been found in seawater [81, 82] and have been characterized as to their genetic diversity and phylogenetic affiliations [83]. These vectors could be utilized for gene transfer in the near future.

The particle gun or microprojectile method has been developed for delivering DNA into plant cells and tissues. In prokaryotes, Shark et al. [84] reported the biolistic transformation of *Bacillus megaterium*. Both gold and tungsten particles have been used as DNA carriers in this system. However, small particles are required for prokaryotic transformation. DNA (pSUP1021) conjugated onto nano-sized bio-magnetic beads (50–100 nm in diameter), purified from the magnetic bacterium *Magnetospirillum* sp. AMB-1 [85], was used to successfully transform a marine cyanobacterium, *Synechococcus* sp. NKBG15041c [86].

In eukaryotic microalgae, some unicellular and multicellular algae have been successfully transformed, although most of them are freshwater strains of diatom and chlorella. Marine strains, such as diatom *Phaeodactylum tricornutum*, *Thalassiosira weissflogii*, and green algae *Dunaliella salina*, have been reported to be transformed as well [87–90]. Particle bombardment and electroporation are used mostly for these microalgae. Stable transformation for the purpose of enhancing the production of useful materials or analyzing gene expression has been carried out. However, the level of protein production by transformants varied due to multiple insertion of the target gene into the genome and to variation in transcriptional efficiency caused by random integration.

3.2 Metabolic Engineering of Marine Microalgae for Producing Valuable Metabolites

Enhanced production of valuable primary or secondary metabolites in microalgae can be rendered possible by high density cultivation and/or application of genetic manipulation. Recent pharmaceutical interest in unsaturated fatty acids has triggered the search for sources of these valuable compounds. Several eukaryotic microalgae are known to produce highly unsaturated fatty acids such as EPA and DHA, which are valuable dietary components [44, 61]. Genetic engineering has been applied to produce EPA in the marine cyanobacterium *Synechococcus* sp. [66]. Cyanobacteria do not have the biosynthetic pathway to produce them. The EPA synthesis gene cluster (ca. 38 kbp) isolated from a marine bacterium *Shewanella putrefaciens* SCRC-2738 was cloned to the marine cyanobacterium using a broad-host cosmid vector, pJRD215 (10.2 kbp, Sm^r Km^r). The cyanobacterial transconjugants grown at 29 °C produced EPA only at 0.12 mg/g dry cell, whereas those grown at 23 °C produced EPA at 0.56 mg/g dry cell. The content of EPA grown at 23 °C increased to 0.64 mg/g dry cell after 24 h incubation at 17 °C. Furthermore, EPA production was improved by partial deletion of the EPA gene cluster to stabilize its expression and maintenance in host cyanobacterial cells [91].

Most diatoms do not have the capacity to grow on exogenous glucose in the absence of light. The transformable marine diatom *Phaeodactylum tricornutum* exhibited heterotrophic growth after the introduction of a single gene for glucose transporters *glut1* or *hup1* [92]. For this purpose, plasmid (pPha-T1; *glut1-gfp*) was introduced into *P. tricornutum* by using a biolistic procedure, and transformants were selected for zeocin resistance in the light. Exogenous glucose entering the transformants can be metabolized at a high rate of flux, allowing the cells to thrive in the absence of light. The trophic conversion of microalgae, such as diatoms, is a critical first step in engineering algae for successful large-scale cultivation.

The genetic engineering of microalgae for industrial purposes also has been performed in freshwater cyanobacteria where the ketocarotenoid astaxanthine, an extremely efficient antioxidant, was synthesized by introduction of the beta-c-4-oxygenase gene (*crt*O) from the green alga *Haematococcus* [93]. Ethylene production also was demonstrated in the cyanobacterium *Synechococcus elongates* PCC7942 by chromosomal insertion of an ethylene-forming enzyme [94]. However, the reaction catalyzed by the ethylene-forming enzyme induced metabolic stress that was detrimental to the host cell.

3.3
Whole genome analyses in marine microalgae

Sequencing of microbial genomes has become a routine procedure for gene discovery. The most abundant population in marine cyanobacteria is *Prochlorococcus*, which are the smallest phytoplankton known, with a diameter of about 0.6 μm. Now the complete genomes of three strains of *Prochlorococcus* [95, 96] and one strain of *Synechococcus* [97] have been sequenced and analyzed. The information obtained from genome sequences and subsequently by comparative genome analyses takes on importance as the 2000 genes of these minimal life units are sufficient to generate the most abundant global biomass from solar energy and inorganic compounds. The genome database for cyanobacteria is available at (http://www.kazusa.or.jp/cyano/cyano.html).

In eukaryotic microalgae genomics, the genome composition of the genetically transformable diatom strain *Phaeodactylum tricornutum* was analyzed by the generation of approximately 1000 express sequence tags (ESTs) [98]. Interestingly, many sequences were shown to have more similarity with animal genes than with their plant counterparts.

4
Microalgal Mass Cultivation Technologies

Photosynthetic microorganisms play an important role in the conversion of solar energy into chemical energy. Photosynthetic conversion is an efficient and alternative process used in several industrial fields. Attempts to develop large-scale methodologies for the cultivation of microalgae have been performed using many different kinds of cultivation systems for providing alternatives to fermentation and agriculture products [99]. Algal biomass has historically served as fertilizer [100] and a food source for both humans and animals [101, 102] for secondary waste water treatment [103] and bioremediation [104, 105]. This use of algal biomass is an important consideration for industrial applications of microalgal cultures. With advances in processing technology, algal biomass has come to be seen as a possible source of fuels, fine chemicals, and pharmaceuticals [106]. Several species of microalgae, which produce useful chemicals such as amino acids, vitamins, carotenoids (β-carotene), fatty acids (DHA, EPA, γ-LA 18:3 (n-6)), polysaccharides, and antibiotics have been reported. Many microalgal products have already been commercialized [36, 107–109]. Further, microalgal production of energy resources has been extensively investigated. Development of processes that utilize the majority of the resulting microalgal biomass as energy sources would be preferred. Such processes may allow the recycling of evolved

CO_2 from human energy consumption rather than direct emission, as is the present case for fossil fuels. The following six products for use as fuels can be produced from microalgal biomass: hydrogen (through biophotolysis), methane (through anaerobic digestion), ethanol (through yeast or other alcohol fermentation), triglycerides (through extraction of lipids), methyl ester fuels (through transesterification of lipids), and liquid hydrocarbons (from *Botryococcus braunii*).

The development of efficient culture systems is necessary for algal mass production and the industrial applications of microalgae. The growth rate and maximum biomass yield of microalgal strains are affected by culture parameters (light, temperature, and pH) and nutritional status (CO_2, nitrogen, and phosphate concentration). On the other hand, increasing the density of cultures decreases photon availability to individual cells. Light penetration of microalgal cultures is poor, especially at high cell densities, and such poor photon availability decreases specific growth rates. Higher biomass yields can be expected if sufficient photons are provided in high density cultures of microalgae.

Large-scale culture systems have been constructed (classified as open and closed systems) with the greatest attention directed to the light supply (Fig. 1) [110]. Strains such as *Chlorella*, *Scenedesmus*, *Dunaliella*, *Spirulina*, *Porphyridium*, and *Haematococcus* have been cultured using photobioreactors to obtain several useful materials.

4.1 Open Culture Systems

Several different types of open culture systems have been proposed (Fig. 1a-d). These open culture systems are the simplest method of algal cultivation and offer advantages in low construction cost and ease of operation [111]. The open culture systems require large surface areas and shallow depth (ca. 12–15 cm) to improve light penetration. Furthermore, agitation of the culture prevents the cells from sinking to the bottom and facilitates efficient cell growth with sunlight. The raceway pound has been developed into various types, where those employing a paddle wheel for agitation have been used most frequently for outdoor production of microalgae [112, 113]. The raceway pound for commercial production of microalgae requires an area of 1000 to 5000 m^2.

Contamination by different algal species and other organisms is the biggest problem in open culture systems, and therefore *Chlorella*, *Dunaliella*, and *Spirulina*, which are tolerant to extreme conditions (high nutrient concentrations, high salinity, and high pH), are especially desirable strains for open culture systems. Vonshak et al. [114] demonstrated that contamination by *Chlorella* in outdoor *Spirulina* cultures was prevented by maintaining the culture medium at high bicarbonate concentration (0.2 M). Grazers sometimes

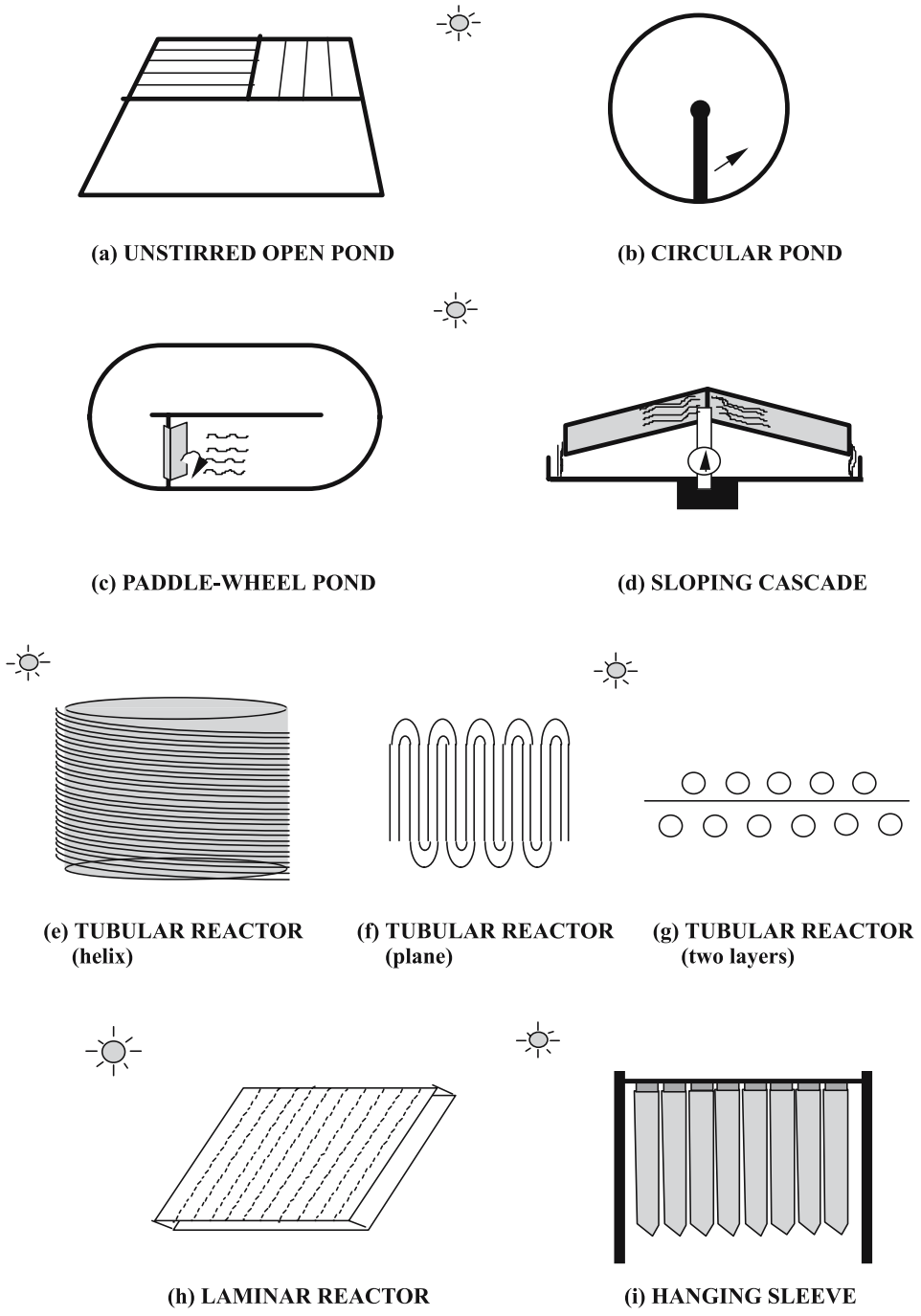

Fig. 1 Illustration of algal mass culture systems

found contaminants in *Spirulina* cultures could be arrested by addition of ammonia (2 mM). The open culture system is easily affected by weather conditions. For example, rain dilutes salinity, causing contamination. Outdoor open culture systems are chosen mainly for production of food sources in aquaculture [43, 115, 116]. However, several algae producing useful chemicals require more restricted conditions for efficient growth and for metabolite production.

4.2 Photobioreactor (Closed Culture Systems)

Closed systems have been expected to overcome the disadvantages of open culture systems, and several types of photobioreactors have been devised. EPA-producing microalgae diatoms have been cultured at various scales in photobioreactors [43]. The closed system is required here because the species used have no selective advantages like those of *Dunaliella* and *Spirulina*. Figure 1e–i shows an example of large-scale closed photobioreactors. These photobioreactors offer several advantages: (1) facilitate maintenance of monoalgal cultures by protecting them from contamination; (2) reduce water loss and the subsequent increase of salinity in the culture medium; (3) result in higher productivity with greater cell densities, reducing harvesting costs; and (4) are applicable to various microalgal species under favorable culture conditions. However, for an efficient and reliable large-scale culture system, several criteria need to be considered [117] such as light utilization efficiency, homogeneous mixing (turbulence), low shear environment, temperature control, and efficient gas transfer.

The production yield of algal biomass depends on the light path length to each cell, and therefore the surface-to-volume ratio is an important factor for efficient light utilization in photobioreactors. The productivity of photobioreactors is determined by the light regime inside the bioreactors. In addition to the light regime, oxygen accumulation and shear stress limit productivity in certain designs [118]. Tubular reactors have been refined, and the diameter of a tubular reactor is now less than 40 cm. Richmond et al. demonstrated that reduction in the tube diameter from 5.0 cm to 2.8 cm enhanced the biomass yield 1.8 times [119]. Narrower tube diameters may increase efficient light utilization as well as promote a faster flow rate, enhancing the algal productivity and reducing fouling on the inside wall of the tubes. In tubular reactors, flow rates of 30–50 cm/s generally are used and airlift is the most effective circulation method of the culture rather than using centrifugal, rotary positive displacement, and peristaltic pumps. The main advantages of airlift systems are their low shear and relative simplicity of construction. Several modifications in tube arrangement have been carried out for optimizing light penetration. Until recently, most of the tubular reactor was laid on the ground such that the lower part of the tubes received less light than the upper part. Torzillo et al. constructed a two-plane tubular bioreactor for optimizing light availability, where each tube in the lower plane is placed in the vacant space between two tubes in the upper plane [120]. They showed that this two-plane tubular bioreactor (145 L of culture volume) has an effective surface-to-volume ratio (49 L/m) resulting in a net volumetric productivity of 1.5 g dry wt/L/d using *Spirulina platensis*.

The helical tubular reactor shown in Fig. 1e consists of a vertical tower coiled up within a lone tube, increasing the land use efficiency. When biomass

productivity of various tubular reactors was compared on a footprint basis, the values were in the range of 15 to 30 g dry wt/m/d for all reactor types.

The effect of temperature on biomass yield is significant in algal culture. The culture in the tubular reactor often is maintained at higher temperatures than that in the open raceway. *Spirulina* cultured in the tubular reactor could be warmed faster than in the open raceway up to 35–37 °C, the optimal range for growth [119]. The closed reactors are sometimes overheated and thus are more suitable for the thermophilic or thermotolerant strains. Temperature control using a heat exchanger and/or evaporative cooling by spraying water onto the surface is sometimes required for the cultivation of general strains [120–122]. The effect of hydrodynamic stress on two different microalgae strains, *Dunaliella tertiolecta* and *D. salina*, also was investigated [121]. The data demonstrated that bubble rising and bubble bursting were not responsible for cell death. Regarding nozzle diameter, small nozzles were more detrimental to cells. Bubble formation at the sparger was the main cause of cell death.

A problem in the closed system is photooxidative damage to the cells caused by accumulation of dissolved oxygen produced by photosynthesis during the light period. In the open system, evolved oxygen is diffused easily to the atmosphere. By contrast, because oxygen cannot escape from closed reactors, degasser systems sometimes are required.

The culture part of closed photobioreactors has been constructed with several materials such as glass, methyl-polymethacrylate, polyethylene, polypropylene, vinyl-polychlorine, silicone, and stainless steel. These photobioreactors have been designed for optimal utilization of external illumination like sunlight. Optical fibers also have been employed as internal light sources. Photobioreactors employing optical fibers have the advantage of controlling illumination and light period duration and have a high surface-area-to-volume ratio [123]. The efficiency of light utilization of microalgae also was studied under light/dark cycles encountered in airlift photobioreactors using *D. tertiolecta* [124]. Optimization of cultivation parameter growth kinetics has increased productivity in photobioreactors. The Acceleration-stat (A-stat) cultivation method has been proposed to determine the culture steady state where the dilution rate is increased at a constant rate under light-limiting conditions in a photobioreactor [125].

Open pond systems have lower productivity of algal biomass, require larger land areas, and involve higher land costs. By contrast, closed culture systems can achieve high-density culture and the overall volume of algal culture can be reduced, resulting in decreased land costs. However, a certain amount of land area is still required for collecting a sufficient amount of solar energy, and therefore operating costs are higher than for open systems. Moreover, solar radiation, temperature, and other factors regulating algal productivity are significantly affected by location. Suitable culture systems

should be chosen according to the target products and available environmental conditions.

5 CO_2 Fixation Using Microalgal Cultures in Industry

The possible use of biological CO_2 fixation to reduce anthropogenic CO_2 emission has been investigated. However, the questions related to CO_2 reduction on the basis of global net amount are debated because biomass must be decomposed and CO_2 is released into the atmosphere as a result.

For development of onsite CO_2 fixation systems using microalgae, efficient photobioreactors and strains that can fix large quantities of CO_2 are required. Several applied studies have been conducted that consider the direct biological utilization of CO_2 in emission gases from coal-fired power plants and the steel and cement plants that produce large quantities of CO_2, NO_x, and SO_x (inhibitory gases for photosynthesis). Therefore, microalgae that can grow under such extreme conditions will be required for direct CO_2 fixation. Many microalgal strains capable of rapid growth in water sparged with emitted gases and under other extreme conditions such as high pH have been screened, and a marine alga *Chlorococcum littorale* showing high CO_2 tolerance and high growth rate in the linear growth phase was obtained [126].

In the report of the IEA Greenhouse Gas R&D Program, a system for the reduction and recycling of CO_2 emission from coal-fired power plants was designed, where CO_2 fixed products generated by microalgal culture are used as biomass fuels, which will substitute eventually for fossil fuels [110]. Costs of microalgae CO_2 mitigation using the designed systems have been estimated based on several design specifications such as (1) plant size, (2) gas condition, (3) conditions for CO_2 biofixation, (4) plant operation, (5) CO_2 production rate, and (6) algal strain (*Nannochloropsis* sp). Analyses of the designed system showed that CO_2 mitigation costs closely depend on productivity of algae and solar radiation. In spite of these recent advances, microalgal strains that can achieve higher photosynthetic efficiency at higher solar radiation are necessary. In addition, a photobioreactor, in which microalgae converts CO_2 to biomass at high photosynthetic efficiency, also is required.

One of the applications for $CaCO_3$ recycling also was demonstrated [127, 128]. Coccolithophorids are unicellular planktonic marine algae that produce elaborate structures called coccoliths comprised of scales or plates of $CaCO_3$. In the oceans, huge blooms of coccolithophorid algae occur that have been recognized as contributing to ocean floor sediment formation. Therefore, algae play an important role in the global carbon cycle by $CaCO_3$ recycling.

CO_2 fixation by artificial weathering of waste concrete and coccolithophorid algae cultures has been proposed (Fig. 2) [129]. During artificial

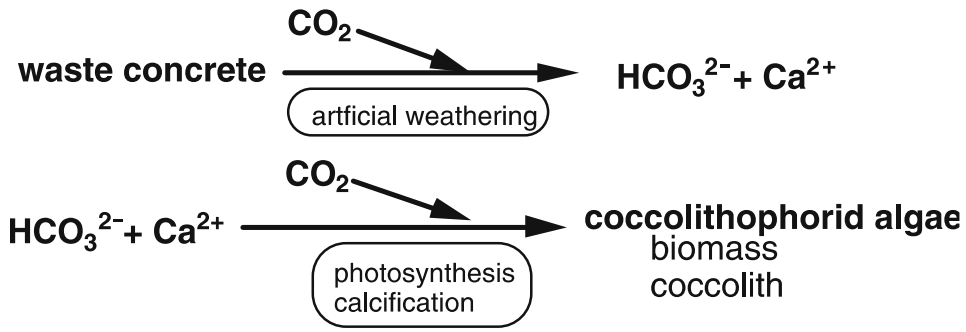

Fig. 2 Design of CO_2 fixation by artificial weathering of waste concrete and culture of coccolithophorid algae

weathering of waste concrete suspended in seawater, atmospheric CO_2 can be absorbed and dissolved as bicarbonate ions, which are a major source of coccolith particles. Coccolithophorid algae can use bicarbonate ions to form $CaCO_3$ particles. Consequently, CO_2 absorbed by artificial weathering can be mineralized and fixed permanently. Artificial weathering of waste concrete also is a useful method to supply bicarbonate ions to cells of the coccolithophorid alga *Emiliania huxleyi*.

CO_2 fixation by artificial weathering of waste concrete and coccolithophorid algae cultures can be applied to the reduction of CO_2 emission from cement plants (Fig. 3). Coccoliths can be used as an alternative to limestone, which is a carbonate source used for cement production. In the cement industry, CO_2 is produced mainly by decomposition of limestone during the burning of cement clinker. If $CaCO_3$ recycling can be achieved by artificial weathering of waste concrete and coccolithophorid culture, CO_2 emissions by the cement industry might be reduced. It has been estimated that the amount of CO_2 absorbed by the weathering of waste concrete is greater than that of CO_2 emitted during a cement production when CO_2 reduction and recycle systems using microalgae are implemented. Moreover, CO_2 is absorbed by the coccolithophorid alga cultures themselves [128]. Glucose oxidase and uricase have been immobilized onto purified ultrafine coccolith particles to illustrate their potential as a support material for biotechnological application [130]. If microalgal biomass can be stored in concrete without the decomposition of the biomass back to CO_2, removal of anthropogenic CO_2 may be achieved.

Extensive studies of biological CO_2 fixation using microalgal cultures have been pursued. A primary goal is the complete removal of CO_2 in discharged gas emitted by such an onsite system. Because of the land area requirements and a CO_2 mitigation cost of $264 per ton as carbon, it is difficult at present to apply microalgal cultures for CO_2 removal. Most nations are seriously concerned about the increase of atmospheric CO_2 concentration, and intensive efforts to reduce the anthropogenic CO_2 emissions are being made. Microalgae culture may be one of the important processes facilitating such efforts [131]. Increasing attention is being paid to resource sustainability in all

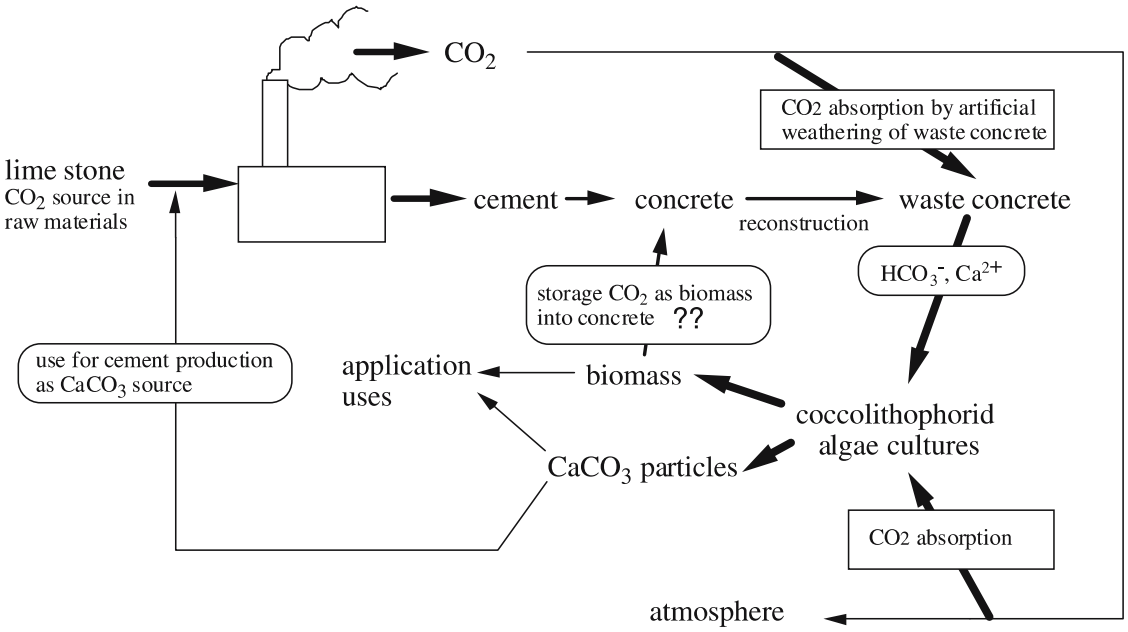

Fig. 3 Design of CO_2 fixation by artificial weathering of waste concrete and culture of coccolithophorid algae

industries, and developing new technologies for microalgal culture will help to provide sustainable resources.

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