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Microalgae As Sources of High Added-Value Compounds—A Brief Review of Recent Work

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Microalgae have found commercial applications as natural sources of valuable macromolecules, including carotenoids, long-chain polyunsaturated fatty acids, and phycocolloids. As photoautotrophs, their simple growth requirements make them attractive for bioprocesses aimed at producing high added-value compounds that are in large demand by the pharmaceutical market. A few compounds synthesized by microalgae have indeed proven to possess anti-inflammatory, antiviral, antimicrobial, and antitumoral features; astaxanthin, a known antioxidant produced by Haematococcus pluvialis*, is an illustrative example with important anti-inflammatory and antitumoral roles. From a chemical standpoint, several such compounds are polysaccharides or long chain fatty acids, where the latter can be either saturated or unsaturated. Additionally, their chemical structures are often atypical, whereas their concentrations can exceed those found in many other natural sources. The productivity and biochemical composition of microalgae depend strongly on the mode of cultivation, medium composition, and nutrient profile. Consequently, numerous efforts aimed at elucidating the practical impacts of the aforementioned parameters have been developed. This review accordingly covers the knowledge produced in the last two decades on the uses of microalgae to obtain physiologically active compounds, and on the optimization of the underlying production and purification processes. It also identifies major gaps and opportunities in this field that should be addressed or exploited in the near future.* © 2011 American Institute of Chemical Engineers *Biotechnol. Prog.*, 27: 597–613, 2011
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Introduction

The marine environment constitutes two thirds of our planet, encompasses a considerable part of its biodiversity, and offers huge potential for citizens' well-being. The extensive resources provided thereby now constitute the basis of many economic activities; and looking ahead, the marine environment offers a wide array of applications of biotechnology. Indeed, according to the *Green Paper – Towards a Future Maritime Policy for the Union* and the indications set forth by the EU-US Task Force on Biotechnology Research, "blue biotechnology" has a predicted growth of 10% per year and an expected global market of 2,400 M€.

Microalgae (especially those from marine origin) remain to date largely unexplored, so they represent a unique opportunity to discover novel metabolites and produce known metabolites at lower costs. In fact, the rate of rediscovery, which entails finding metabolites that were already obtained from other biological sources, is expected to be far lower (i.e., <5%) in microalgae than in traditional, better-studied organisms (i.e., > 90%).[1] These microorganisms possess the extra advantage of a huge metabolic plasticity that leads to opposite results in bioactive screening programs depending on their physiological state (i.e., stressed vs. nonstressed). Similarly, their secondary metabolism can be easily triggered by most forms of externally applied stress (e.g., lack of a nitrogen source).

Despite the almost 18,500 new compounds isolated from marine sources between 1965 and 2006, one estimates that ca. 97% of all existing marine compounds have not yet been isolated, and thus they did not undergo chemical or biological characterization whatsoever.[2] Besides their peculiar structures, most marine compounds entail a wide diversity of molecular targets with a marked selectivity, thereby raising a pharmaceutical interest.[3] Considering only the products

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currently undergoing clinical testing, their specific therapeutical targets encompass ion channels, metabolic enzymes, microtubules, and DNA; and five of them actually belong to novel classes (e.g., the antitumoral Yondelis® and the analgesic Prialt®). On the other hand, the antitumoral Citarabina® and the antiviral Virabadina® are already in current use. However, commercial development has been hampered by the difficulties in assuring controlled and consistent production on large scales.[4]

In the USA, several Universities and private alga-biotech Companies (mostly located in Hawaii and California) have been engaged for more than a decade now in comprehensive screening for bioactive metabolites produced by marine organisms. Microalgae and cyanobacteria were the subjects of most of these efforts. Major contributions to bioactive compound libraries have accordingly been made, as well as assessments on their potential for cultivation at industrial scales. Thus far, however, the development of innovative and efficient bioprocesses remains an obstacle to economical commercialization at a large scale. Despite said obstacle, the commercial potential of microalgae is widely recognized. The production of astaxhantin-rich biomass from *Haematococcus*, for example, has been already pursued by Cyanotech (Hawaii), Mera Pharmaceutical (Hawaii), and Fuji Health Science (Japan). Likewise, β-carotene production from *Dunaliella* is the focus of a number of companies, including Betatene, Western Biotechnology, and AquaCarotene, all in Australia. Cyanotech (Hawaii) and Inner Mongolia Biological Engineering (China) also pursue β-carotene production.[5]

In this article, the most relevant features of microalgal biotechnology are reviewed, with a specific focus on antioxidant, anti-inflammatory, antimicrobial, antiviral, and antitumoral compounds. The optimization of their production and purification strategies is tackled, especially in the case of antioxidants, lipids, and polysaccharides. Finally, economic considerations and future perspectives in the field are discussed in brief.

Antioxidant compounds

Recent decades have witnessed a growing interest in compounds with antioxidant properties because such compounds can play favorable roles in human health. This expands the role of antioxidants from classically functioning as a food preservative to that of a therapeutic, which can be either ingested in native foods or as a part of formulated functional foods. The evidence gathered from a large number of studies has in fact confirmed the positive effects of antioxidants on the prevention and control of growth of certain tumors (see the Antitumoral section). Other studies demonstrate that the incidence and severity of cardiovascular and degenerative diseases are lessened, and other health conditions involving inflammatory states are ameliorated (see the Anti-inflammatory section). Furthermore, a positive effect on extending one's life span and associated delay in ageing has been reported. Such a conclusion was based on a decrease in the abundance of biomarkers, such as protein carbonyls produced by oxidative damage.

Carotenoids are potent biological antioxidants that are able to absorb the excitation energy of singlet oxygen radicals (SOR) into their complex ringed chain. The radical scavenging lessens tissue decay by reducing the damage to molecules such as DNA, proteins, and membrane lipids.[6] Furthermore, the processes brought about by SOR have also been linked to the ageing process and to the pathogenesis of several diseases, viz., cancer, cardiovascular diseases, atherosclerosis, rheumatoid arthritis, muscular dystrophy, cataracts, and several neurological disorders.[6,7] For instance, lutein has been recommended (and even prescribed) for the prevention of cancer and diseases related to retinal degeneration.[8,9] Other studies indicated that the decline of cognitive ability in Alzheimer's disease may be caused by the persistent oxidative stress in the brain.[10] By using transgenic mice fed with *Chlorella* sp. containing carotenoids such as β-carotene and lutein, Nakashima et al.[11] found that progression in cognitive impairment could be prevented to a significant extent.

Table 1. Selected Antioxidant Features of Microalgae

Microalga Source	Active Compound	Reference
Dunaliella salina	β-carotene	13,14
Haematococcus pluvialis	Astaxanthin, cantaxanthin, lutein	14,15
Chlorella vulgaris	Cantaxanthin, astaxanthin	14,16
Chlorella pyrenoidosa	Lutein, violaxanthin	14,17,18

Generally, microalgae are rich in carotenoids, some of which have been recognized to exhibit a strong antioxidant capacity (see Table 1). Therefore, production of these compounds in larger scale after bioprocess optimization is urged, even though photoautotrophic biotechnology is far from being fully mastered.[12]

Anti-inflammatory compounds

Oxidative stress and inflammation are implicated in several health conditions such as cardiovascular diseases and atherosclerosis. In a normal cell, transcription factors, such as nuclear factor κB (NF-κB), upregulate production at the cellular level of downstream inflammatory mediators; these include inducible nitric oxide synthase, matrix metalloproteinases, cyclooxygenase-2, tumor necrosis factor α, and interleukin-1β. During regular cell function, reactive oxygen species (ROS) lead to the synthesis of appropriate amounts of signalling molecules that operate along with the downstream products in the metabolic pathways; hence, a balanced environment encompassing ROS and reactive nitrogen species (RNS) arises, which will promote regular cell health. Natural enzymatic antioxidants (e.g., superoxide dismutase and glutathione peroxidase) scavenge or quench the excess lipid- and water-soluble ROS and RNS. This, then, allows signalling molecules and cellular pathways that upregulate the production of inflammatory mediators to operate normally toward homeostasis.

However, in response to commonly encountered inflammatory stimuli (e.g., cytokines or pathogens), the production of ROS and RNS increases dramatically and eventually overwhelms the capacity of normal cell antioxidants. In prolonged disease pathologies, a state of chronic oxidative stress and inflammation thus results, so depletion of natural (and even dietary) antioxidants ultimately leads to a persistently malevolent redox homeostasis. The aforementioned diseases are generated, in part, by overproduction of ROS and RNS, which activates transcriptional messengers (e.g., NF-κB) that contribute, in turn, to endothelial dysfunction and initiation and progression of atherosclerosis, as well as irreversible damage after ischemic reperfusion and even arrhythmia (e.g., atrial fibrillation[19]).

Another major example is skin ageing, which is a chronic low-level inflammation, triggered by environmental stressors

Table 2. Selected Anti-Inflammatory Features of Microalgae

Microalga Source	Active Compound	Mechanism of Action	Reference
Haematococcus pluvialis, *H. lacustris*, *C. zofingiensis*, *C. vulgaris*, *Chlorococcum* sp.	Astaxanthin	Inhibition of intracellular ROS accumulation, decrease of H_2O_2-induced NF-κB activation, expression of inducible NOS in primary macrophages, suppression of serum levels of NO	23
Porphyridium sp.	Sulphated polysaccharides	Inhibition of PMN migration toward chemoattracting molecules, partial blocking of adhesion to endothelial cells	24
Gyrodinium impudicum	p-KG03	Immunostimulatory effects	25,26
Chlorella stigmatophora	Hydrosoluble components, sterols	Immunosuppressant effects	27

such as ultraviolet and infrared radiation, as well as ozone and bacterial by-products.[20] Chemotaxis and adhesion of neutrophils to endothelial cells are the body response to the damage process, which is inhibited by sialyl lactose. Antioxidants such as astaxanthin provide a broad, "upstream" approach that quenches ROS/RNS or promotes free radical chain-breaking. Accordingly, antioxidants appear to be an appropriate therapeutic option, at least based on the epidemiologic, dietary, and in vivo animal model data already available.

Recent work by McNulty et al.[21] suggests that conformational differences brought about by specific antioxidants in the cell membrane and mitochondrial intermembrane space account for their anti-inflammatory activity. The precise transmembrane alignment in the lipid bilayer provides exposure of the polar (hydrophilic) ends of the antioxidant molecules to the internal cytoplasm and the aqueous outer environment of the cell (or the mitochondrial matrix and the intermembrane space of mitochondria), which facilitates electron transfer via the double bonds of the carbon scaffold of those compounds. This apparently prevents lipid-based oxidation, and constrains the ability of hydrophilic biophores to be exposed to and to interact with ROS and RNS in their microenvironment. Additionally, access is provided to cofactors (e.g., vitamin C) that serve as sink for the radical cations, thereby recharging the electron transfer capacity.[22] A number of recent examples of anti-inflammatory compounds originating from microalgae are listed in Table 2.

Unlike vitamin E and lutein, dietary astaxanthin has been proven to reduce low-density lipoprotein oxidation,[28] while being safe for human use.[29] Sulphated polysaccharides from *Porphyridium* sp., composed of 10 sugars (primarily xylose, glucose, and galactose) in addition to glycoproteins and inorganic sulphate, inhibited the spreading of immune cell recruitment toward inflammatory stimuli in vivo.[24] Moreover, a soluble fraction isolated from *Porphyridium* strain UTEX 637 inhibited the auto-oxidation of linoleic acid and other forms of oxidative damage in a dose-dependent manner. It has been suggested[30] that the antioxidant activity of sulfated polysaccharides in vivo protects microalgae against ROS produced under high solar irradiation, possibly by scavenging the free radicals produced in the cell under stress conditions and transporting them from the cell to the outer medium.

Further studies revealed an anti-inflammatory capability in crude extracts of *Chlorella stigmatophora* and *Phaeodactylum tricornutum*, which attained 25%–30% of that exhibited by the reference compound indomethacin.[27] In the former case, the active components were found to be water-soluble.[31] Guzmán et al.[32] purified such extracts to yields of 7.14% and 6.85%, respectively; they proved successful, both in vitro and in vivo, in terms of delay of hypersensitivity response and effect on phagocytic activity.

Antimicrobial compounds

Several pathogenic bacteria, e.g., multidrug-resistant *Staphylococcus aureus* (MRSA) strains, have caused an increased concern in healthcare institutions worldwide because they are not susceptible to most conventional antibiotics. Hence, the discovery of novel antibacterial compounds that follow distinct biochemical mechanisms of action, for eventual use in human patients, is crucial to overcome the resistance problem. Although marine microalgae are known to produce a few useful products of this type, the search for novel antibiotics is still ongoing; examples are tabulated in Table 3.

The activity of cell lysates of *P. tricornutum* against both Gram-positive and -negative bacteria (including MRSA), even at micromole levels, was attributed to eicosapentanoic acid (EPA), a compound synthesized de novo by diatoms.[46] This polyunsaturated fatty acid (PUFA) is found chiefly as a polar lipid species in structural cell components (e.g., membranes). It plays a role in microalgal defense due to it being toxic to grazers,[47] and it is also a precursor of aldehydes that have deleterious effects on consumers such as copepods.[48] However, the mechanism of its action remains unknown. EPA may act on multiple cellular targets, even though cell membranes are the most probable ones because membrane damage may lead to cell leakage and reduce nutrient uptake, as well as decrease cellular respiration. Conversely, Desbois et al.[33] claimed a peroxidative process involving H_2O_2. Compounds synthesized by *Scenedesmus costatum*, and partially purified from its organic extract, also showed antibacterial activity against aquaculture bacteria. The activity was associated with fatty acids longer than 10 carbon atoms in chain length, which apparently induce lysis of bacterial protoplasts.

The ability of the fatty acids to interfere with bacterial growth and survival has been known for several decades, but recent structure-function relationship studies have made it clearer that such antimicrobial activity depends on both the chain length and the degree of unsaturation. Compounds such as cholesterol can antagonize antimicrobial features,[49] so both the composition and concentration of free lipids should be taken into account.[50] Finally, the reported antialgal ability can be derived either from interference on chlorophyll and protein synthesis[42] (as in *Isochrysis galbana*) or from changes in membrane permeability, coupled with dissociation of phycobilin assemblages on the thylakoid membranes that lead to leakage across the cell wall.[40]

Antiviral compounds

A number of viral infectious diseases have emerged (and re-emerged) in recent years. For example, a new avian

Table 3. Selected Antimicrobial Features of Microalgae

Microalga Source	Active Compound	Type of Action	Target Microorganism	Reference
Phaeodactylum tricornutum	Eicosapentaenoic acid	Antimicrobial	MRSA, *Listonella anguillarum*, *Lactococcus garvieae*	33
Haematococcus pluvialis	Short chain fatty acids		–	34
Skeletonema costatum	Unsaturated, saturated long chain fatty acids	Antibacterial	*Vibrio* spp.	35
Euglena viridis	Organic extracts		*Pseudomonas*, *Aeromonas*, *Edwardsiella*, *Vibrio*, *Escherichia coli*	36
Askeletonema costatum	Extra-metabolites	Bacteriostatic	*Listeria monocytogenes*	37
Chlamydomonas reinhardtii, *Chlorella vulgaris*	Methanolic and hexanolic extracts	Antifungal, antibacterial	*Staphylococcus aureus*, *S. epidermidis*, *Bacillus subtilis*, *E. coli*, *S. typhi*, *Candida kefir*, *Aspergillus niger*	38
Amphidinium sp.	Karatungiols	Antifungal, antiprotozoan	*Aspergillus niger*, *Trichomonas foetus*	39
Peridinium bipes	Water-soluble extract		*Microcystis aeruginosa*	40,41
Isochrysis galbana	$C_{22}H_{38}O_7$ from cell-free filtrates of cultural medium at death phase	Antialgal	*Dunaliella salina*, *Platymonas elliptica*, *Chlorella vulgaris*, *Chaetoceros muelleri*, *Chlorella gracilis*, *Nitzschia closterium*, *P. tricornutum*	42
Nitzschia frustulum, *Scrippesiella trochoidea*, *Prorocentrum donghaiense*	–		–	
Goniodoma pseudogoniaulax	Goniodomin A	Antifungal	–	41,43
Gambierdiscus toxicus	Polyether compounds (gambieric acids A and B)			41,44
Prorocentrum lima *Dinophysis fortii*	Polyether compounds			41
Staurastrum gracile, *Pleurastrum terrestre*, *Dictyosphaerium pulchellum*, *Klebsormidium crenulatum*	Methanol extracts	Antibacterial	–	41
Chlorococcum sp.	Aqueous extract			41
Chlorococcum HS-101	α-Linolenic acid			45
Chlorokybus atmophyticus	Acetone extract			41

Table 4. Selected Antiviral Features of Microalgae

Microalga Source	Active Compound	Mechanism of Action	Target Virus	Reference
Navicula directa	Polysaccharide	Inhibition of hyaluronidase	HSV1, 2, influenza A virus	55
Gyrodinium impudicum	p-KG03 exopolysaccharide	Inhibition (or slowing down) of cytopathic effect	Encephalomyocarditis virus	25
Dunaliella primolecta	Pheophorbide a-, b-like compounds	Inhibition of cytopathic effect	HSV1	56
Chlorella autotrophica, *Ellipsoidon* sp.	Sulfated polysaccharides	Replication inhibition in vitro of *C. autotrophica*: range 47.4–67.4%; *Ellipsoidon* sp.: up to 44%	VHSV, ASFV	57
Cryptomonads	Allophycocyanin	Inhibition of cytopathic effect, delay in synthesis of viral RNA	Enterovirus 71	58

influenza virus that originated in Southeast Asia in 2005 has become a serious worldwide problem. New subtypes caused by an antigenic shift have spread to humans and periodically have increased the risk for a pandemic outbreak. Although several antiviral drugs have been specifically developed, drug-resistant mutations do constantly take place. Therefore, new antiviral active principles are required, especially from sources that do not constitute, or are directly exposed to viral pools. Microalgae have consequently received more attention as a potential source of antiviral agents;[51–54] a few selected examples are listed in Table 4.

Recall that viral growth is generally divided into three stages: Stage I, adsorption and invasion of cells; Stage II, eclipse phase; and Stage III, maturity and release of virus particles. For instance, the anti-HSV activity of the routinely applied antiviral compound acyclovir® is expressed at Stage II, but the anti-HSV factor from *Dunaliella* sp. extracts inactivates the initial viral function right after Stage I.[59,60] Sulphated exopolysaccharides from marine microalgae have been claimed to interfere with Stage I of some enveloped viruses;[61] they offer unique advantages, due to their antiviral spectrum against, e.g., HSV and HIV-1 viruses.[62] Their inhibitory effect is attributed to interaction with the positive charges on the virus or on the cell surface, thereby preventing penetration of the former into the host cells.[63,64] They may also selectively inhibit reverse transcriptase in the case of HIV, thus hampering production of new viral particles after infection.[65] However, the exact step during viral replication when they act remains to be fully elucidated. Highly sulfated polysaccharides from several species of red microalgae that bear antiviral features consist mainly of xylose, glucose, and galactose;[55,66] they are unusually stable to extreme pH and temperature conditions.[67]

Another important antiviral action attributed to sulfated polysaccharides is against two enveloped rhabdoviruses with significant economic importance: the viral hemorrhagic

Table 5. Selected Antitumoral Features of Microalgae

Microalga Source	Active Compound	Mechanism of Action	Target cells	Reference
Haematococcus pluvialis	Astaxanthin	Expression decrease of cyclin D1, increase of p53 and some cyclin kinase inhibitors (p21WAF-1/CIP-1, p27)	Colon cancer cell lines	80
Peridinium bipes	Diadinochrome A, B, diatoxanthin/cynthiaxanthin	Cytotoxic effect	HeLa cells	81
Gymnodinium sp.	Extracellular polysaccharide GA3P, D-galactan sulfate associated with L-(+)-lactic acid	Catalytic inhibition of DNA topoisomerase (topo I, topo II)	Human myeloid leukemia K562 cells	82

septicemia virus (VHSV) of salmonid fish and the African swine fever virus (ASFV) (Table 4). Extracts from marine microalgae can find prophylactic usefulness in hatcheries against VHSV and perhaps also against other fish-infecting enveloped viruses, besides against mammalian viral diseases.[57]

Hence, application of sulfated polysaccharides appears to be a particularly attractive option for the viral therapy because their pleiotropic mode of action is less likely to promote the development of resistant mutants when compared with other compounds that attack only one target throughout the viral life cycle. To gain some insights, NMR and MS analyses were performed on the antiviral compounds synthesized by *Dunaliella primolecta*[56] and unfolded structures that had not been found before in nature. The pheophorbide-like substances found and structurally characterized had the proton at position 21 replaced by a hydroxyl group, which might be the key for the unique antiviral activity observed.

A homopolysaccharide of galactose with uronic acid (3.0%, w/w) and sulfate groups (10.3%, w/w) from *Gyrodinium impudicum* strain KG03[25] exhibited an impressive activity in vitro (EC_{50} = 26.9 μg/mL) against swine encephalomyocarditis virus, which is widespread at the subclinical level. Its acute form leads to sudden death in piglets and reproductive failure in adult animals,[68] and has consequently caused major economic losses in Belgium,[69] Greece, Cyprus, and Italy.[70]

Despite their successful performance, there is still limited information on the metabolic pathways that lead to sulphated polysaccharides. Their secretion by unicellular red algae was originally characterized via radiolabeling, which showed biosynthesis of the carbon chain and sulphation of the resulting polysaccharide in the Golgi apparatus.[71] These findings were confirmed in *Porphyridium* sp.[72] and other red microalgae.[73] More recently, Keidan et al.[74] used ^{14}C pulse-chase experiments coupled with ultrastructural microscopy to conclude that brefeldin A (a membrane-traffic inhibitor of the Golgi apparatus) decreases the contents of both bound and soluble polysaccharides. It also inhibits formation of the cell-wall bound polysaccharide to a greater extent than its soluble counterpart in either actively growing or resting cells.

Finally, note that the discovery of small molecules that can specifically disrupt a particular protein–protein interface is a challenging endeavor. However, such a discovery is of keen interest in virology because the antiviral drugs currently available target only viral proteins.

Antitumoral compounds

More than eight million men in Europe and North America undergo proliferation of prostate cells. Also, each year ca. one million new cases are diagnosed, of which 360,000 correspond to cancer, thus making its incidence particularly high (second only to skin cancer). Unfortunately, this incidence has doubled in the latest decade, which is attributed to, in part, an extended life expectation. Current therapies are not very selective, so they prompt several side effects such as erectile disfunction, cleft-communicating bladder and rectum, and inflammation. Preventive properties have been assigned to antioxidant micronutrients, including those belonging to the carotenoid group.

Recent clinical trials have demonstrated that phytomedicine is effective to treat pathologies related to vascularization and cell proliferation in prostate hyperplasia.[75,76] Several marine microalgae can in particular synthesize bioactive compounds with antitumoral performance. For example, campesterol, a sterol found in *Tetraselmis suecica*,[77] has been reported to be antiangiogenic.[78] Extracts of *Chlorella vulgaris* have also been found to be active against liver cancer in vitro and in vivo, where the inhibition of proliferation and increased apoptosis were reported.[79] Additional examples of antitumoral compounds synthesized by microalgae are provided in Table 5.

There is an well-established evidence confirming that microalgal carotenoids possess potent cancer chemopreventive features.[83,84] Their activity is thought to rely on the stabilization of DNA-topo cleavable complexes, which are intermediates in the catalytic cycle of those enzymes,[82] to eventually produce apoptosis. Recall that topos are nuclear enzymes that regulate DNA topology, which encompass two classes (topo I and topo II) that differ in their functions and mechanisms of action. Topo I acts by making a transient break in one DNA strand that allows the DNA helix to swivel and release torsional strain and, thus, changes the linking number by steps of one. Topo II makes transient breaks in both strands of one DNA molecule, thus permitting the passage of another DNA duplex through the gap, and accordingly changes the linking number by steps of two. Both enzymes are crucial for cellular genetic processes, viz. DNA replication, transcription, recombination, and chromosome segregation during mitosis.[85]

The red ketocarotenoid astaxanthin (3,30-dihydroxy-β,β-carotene-4,40-dione) produced by microalgae has attracted considerable interest because of its outstanding antitumoral activity, which is clearly different from and much more potent than that of β-carotene and other carotenoids.[80,86] Dietary astaxanthin displays antitumoral effects in the post-initiation phase of carcinogen-induced colon and oral cancer models.[87,88] Suppressive effects thereof have also been reported in transplantable tumor cells, including methylcholanthrene-induced fibrosarcoma[80] and murine mammary tumor cells.[89,90] It has been further suggested that astaxanthin attenuates liver metastasis induced by stress in mice, thus promoting the immune response via inhibition of lipid peroxidation.[91] Kang et al.[92] reported that astaxanthin protects rat liver from damage induced by carbon tetrachloride, via inhibiting lipid peroxidation and stimulating its cell antioxidant

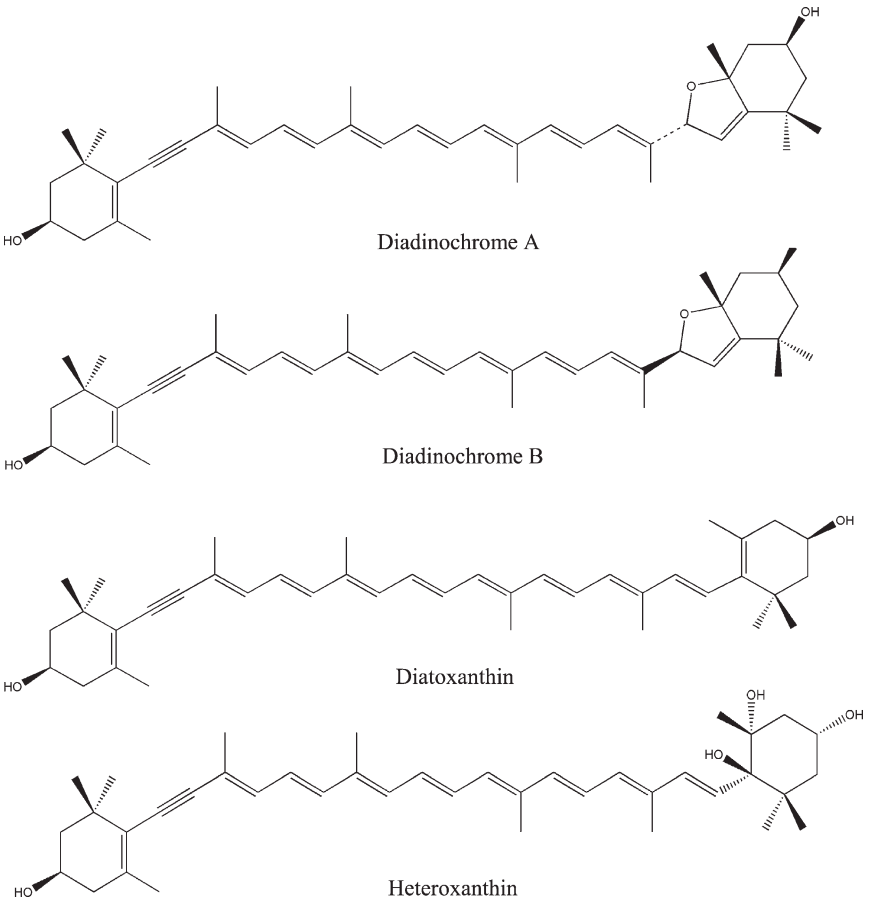

Figure 1. Selected acetylenic carotenoids from microalgae.

system. Additionally, the effects of astaxanthin on human breast cancer cells have been studied,[93] in which case astaxanthin inhibited the proliferation of an MCF-7 cell line although less effectively than β-carotene and lycopene.

Several mechanisms have been proposed to explain the putative role of astaxanthin in modulating cell growth, including its ability to induce xenotoxic-metabolizing enzymes in the liver,[94] to modulate the immune function[95] and gap functional communication,[96,97] and to regulate the intracellular redox status.[98,99] Because of the prohibitive price of synthetic astaxanthin, several efforts resorting to *H. pluvialis*,[100] *Haematococcus lacustris*,[101] *Chlorococcum* sp.,[102] and *C. vulgaris*[103] as natural sources of that compound have meanwhile met with success. Despite *H. pluvialis* being the most studied microbial source of astaxanthin, the associated production capacity is constrained by its relatively slow growth, poor cell yield, ease of contamination, susceptibility to adverse conditions, and requirement of extremely high irradiance.

In addition to astaxanthin, more than 1,000 natural acetylenic carotenoids have been isolated from a wide variety of sources, including microalgal species. Several acetylenic carotenoids exhibited cytotoxic activities against human neoplasm cells and other antitumoral properties.[81,104,105] Oono et al.[106] studied 51 acetylenic carotenoids belonging to diverse structural classes with cytotoxic activity against Raji cells (human neoplasm), three of which, viz. diadinochromes A and B and diatoxanthin/cynthiaxanthin (see structures in Figure 1), were isolated from *Peridinium bipes* (Dinophyceae). Diadinochrome A proved cytotoxic to HeLa cells, whereas the latter two exhibited unspecific anticarcinogenic activity.[107,108] Quantitative carotenoid analysis of *Euglena viridis* revealed the presence of more than 86% acetylenic carotenoids, including monoacetylenic diatoxanthin (61%), diadinoxanthin (12% of which rearranged to diadinochrome), heteroxanthin (1%, see Figure 1), and the diacetylenic 3,4,7,8,3′,4′,7′,8′-octadehydro-β,β-carotene (6%). However, other microalgae such as *Tribonema aequala*, *Gonyostomum semen*, *Vacuolaria virescens* (Raphidophyceae), and *Pleurochloris meiringensis* (Xanthophyceae) appeared to also have significant amounts of this constitutive compounds.[106]

Production in photobioreactors

Development of microalga-mediated biotechnology has been constrained by their relatively low volumetric productivities in industrial photobioreactors. Hence, most microalgal industrial production nowadays occurs in outdoor open ponds that are relatively inexpensive, but where processing conditions are far from being optimal. It is indeed difficult to control temperature, light distribution, and CO_2 concentration therein and to prevent microbial contamination that would reduce the throughput rate and compromise the quality of the final product(s).[107,108] Therefore, the next generation of microalgal bioreactors for the manufacture of high added-value products will eventually resort to closed bioreactors in which the product yields will be optimized by the informed manipulation of culture conditions that are carefully selected for each individual microalgal species according to its unique physiological and growth characteristics.[109]

Two processing factors require particular attention: light supply and temperature,[110,111] although heat and mass transfer should also be fine tuned for proper operation.[112,113]

A variety of closed photobioreactor configurations have accordingly been proposed to suit the particular characteristics of distinct microalgal strains. Their designs include horizontal tubular systems,[114,115] helical tubular reactors,[116] cascade reactors,[117] alveolar flat panels,[118] vertical flat panels,[119] or bubble columns.[120] The designs that can be more easily scaled up correspond to horizontal or helical tubular systems, as well as combinations of vertical flat panels and bubble columns; these types of photobioreactors have to date received most attention. Comprehensive and integrated characterization of sunlight supply, fluid dynamics, and mass transfer rates in both tubular and bubble column photobioreactors are available,[120–122] which is in contrast to the current situation for flat panel photobioreactors. However, the flat panel design has important advantages for the bulk production of photoautotrophic microorganisms and may likely become one of the standard types of system for industrial production in the future.

Particularly, Samon and Leduy[119] used vertically translucent flat plates illuminated on both sides and stirred by aeration. Tredici and Materassi[123] built on this idea by using rigid alveolar panels, whereas Pulz et al.[107,108] used flat panels with inner walls arranged to promote an ordered horizontal culture flow driven by a mechanical pump. The most innovative aspect of this configuration was that parallel plates were packed together and close enough to attain up to 6 m^3 of culture volume on a 100 m^2-implantation surface area (corresponding to ca. 500 m^2 of total illuminated culture surface area).[112,124,125]. A type of flat plate reactor made of glass sheets glued together with silicon rubber to obtain flat vessels was proposed as well, thus providing a rather robust and inexpensive construction technology for any light path. Recently, a new design of vertical flat panel photobioreactor was suggested that consists of a plastic bag located between two iron frames;[126] this design substantially reduced the capital investment.

Optimization of metabolite synthesis

Allelopathic compounds excreted by microalgae may account for several bioactive principles—so experimentation aimed at simplifying their recovery from the culture medium is in order. However, most such compounds are expected to be intracellular, which requires further steps of cell-wall disruption and separation downstream that will be discussed in the next section. Herein, the processing conditions leading to the maximum yields and productivities for the compounds of interest will be reviewed at some length. Selected examples are accordingly listed in Table 6, with explicit indication of their bioactivities, relevant metabolites, source, cultivation medium, major operating conditions (encompassing temperature, pH, aeration, stirring, and irradiance), and reactor configuration, along with the associated productivity. More detailed considerations on each type of compound family follows in specific subsections.

Inspection of Table 6 reveals that not only the type of nutrients but also the operating conditions are critical for driving the synthesis of specific bioactive products. However, a large number of combinations of processing levels may be required for comprehensive testing. One way to overcome this constraint is to use rapid optimization based on high-throughput methodologies. A recent example used a hydrophobic foil that was treated by plasma technology to generate hydrophilic spots, and with sufficient mass transfer rates of CO_2 and O_2 in opposing directions.[127] This device can be tuned, especially to minimize water evaporation during regular operation.

Therefore, microalgae possess different productivities and exhibit distinct biochemical compositions, depending on the mode of cultivation and the nutrient profile supplied via the medium. On the other hand, one of the major reasons why they are not yet drug producer candidates in the pharmaceutical industry is their relatively small yield (usually 1–50 g $kg^{-1}_{biomass}$); hence, this realization demands specific attention to be paid to medium compositions and underlying processing conditions.

Antioxidants

Lutein. The most significant factors known to affect lutein content in microalgae are irradiance, pH, temperature, nitrogen availability and source, salinity (i.e., ionic strength), and presence of oxidizing substances (i.e., redox potential). However, the specific growth rate also plays a crucial role.

High temperature favors the accumulation of lutein, as is the case for other carotenoids (e.g., β-carotene) in *Dunaliella* sp.[112] Such temperatures are close to the edge of causing environmental stress; hence, the operational window is narrow because further temperature increases would be harmful and eventually cause decreases in biomass productivity.

A high irradiance level appears beneficial, but its effect depends on the culturing mode (indoor or outdoor). Furthermore, it is hard to reproduce in vitro all parameters that characterize outdoor operation, including solar cycle and temperature oscillation. Cultures of *Murielopsis* sp. and *Scenedesmus almeriensis* produced contradictory results, thus raising the question of whether there is an interaction between irradiance and temperature. Thus, it might be more useful to study these factors in combination rather than separately.[8,128–130] Moreover, the O_2 concentration outdoors cannot be manipulated, but it may also interact with illumination and temperature levels.

Similarly, the effect of pH is not consistent between batch and continuous cultivation. In the former case, lutein content increased at the extreme pH values experimented with, whereas the best results under continuous operation were obtained at the optimum pH for growth rate. Recall that pH is particularly relevant in microalgal cultures because it also determines CO_2 availability owing to the interconversion between CO_2, H_2CO_3, HCO_3^-, and CO_3^{2-}. These different outcomes raise a distinction between tests in which CO_2 is supplied continuously as a fraction of the aeration stream and others that use pH-controlled injection. In either case, however, the maximum productivity of lutein is attained at the optimum pH for biomass productivity, thus overriding putative differences in lutein specific content.

The concentration of nitrogen in the culture medium, supplied in the form of nitrate, does not apparently cause any significant effect on the lutein content of the biomass. However, nitrogen limitation decreases biomass productivity, thus leading to low lutein synthesis. Consequently, nitrate should be supplied to a moderate excess so that growth rate is not hampered, while avoiding saline stress caused by nutrient excess that may severely affect the culture performance.[8]

A slight, but positive effect on lutein synthesis occurs when chemicals such as H_2O_2 and NaClO are added to

Table 6. Optimal Conditions for Production of Selected Metabolites (a) with and (b) without Antioxidant Features by Microalgae

Feature	Active Compound	Microalga Source	Cultivation Medium	Processing Conditions	Reactor Configuration	Productivity	Reference
			(a) Optimal conditions for production of selected metabolites without antioxidant features by microalgae				
Antiviral, antitumural	p-KG03 exopolysaccharide	*Gyrodinium impudicum*	MKG03	**T:** 22.5°C; **pH**: 8; **LI**: 150 μE m^{-2} s^{-1}; **LDC**: 16:8 h; **AF**: 50 ml min^{-1} (1% CO_2)	Airlift balloon	Biomass:134.7 ± 5.9 g_{cells} mL^{-1} p-KG03:123.077 ± 1.597 mg L^{-1}	25
Antitumural, anti-inflammatory	Asthaxanthin	*Chlorella zofingiensis*	Bristol's supplemented glucose 50 g L^{-1}; *C/N* = 180	**T**: 30°C; **pH**: 6.5; **LI**: absence of light; **SR**: 130 rpm; **MM**: heterotrophic	Batch (250 mL)	Astaxanthin:10.3 mg L^{-1}	86
		Haematococcus pluvialis	Medium[1] (specific nitrate input: 0.5 mmol g^{-1} d^{-1})	**LI**: daylight cycle; **MM**: chemostat	Continuous tubular (50 L)	Biomass: 0.7 g $L^{-1}d^{-1}$ Astaxanthin: 8.0 mg $L^{-1}d^{-1}$	109
			BAR medium (1 g L^{-1} sodium acetate)	**T:** 28°C; **LI**: 345 μmol $_{photon}$ m^{-2} s^{-1}	Batch (1 L)	Astaxanthin: 98 mg $g^{-1}_{biomass}$	110
			Modified Bolds basal medium	**T**: 15–25°C; $\mathbf{LI}_{max}$: 2000 μmol_{photon} m^{-2} s^{-1}	Enclosed outdoor (25,000 L)	Biomass: 90 g m^{-2}; Astaxanthin: 13 g m^{-2} d^{-1}	111
Antimicrobial	Eicosapentanoic acid	*Phaeodactylum tricornutum*	Mixothophic growth acetate (0.01 M urea)	**LI**: 165 μE m^{-2} s^{-1}	Batch, fed-batch modes (1 L)	Biomass: 1.52 g L^{-1} d^{-1}; EPA: 43.13 mg L^{-1} d^{-1}	112
			Mann & Myer's medium (nutrient 3×)	**pH**: 7.7; **T:** 20 ± 2°C; **LI**: 200–1000 μE m^{-2} s^{-1}	Continuous tubular system (diameter 0.03 m)	EPA: 50 mg L^{-1} d^{-1}	117
			(b) Optimal conditions for production of selected metabolites with antioxidant features by microalgae				
Antioxidant	β-carotene	*Dunaliella salina*	Medium described by Shaish et al. (1992)	**T**: 25°C; **pH**: 7.5 ± 0.5; **LI**: 281 ± 89 μmol $_{photon}$ m^{-2} s^{-1}; **FR**: 38 cms^{-1} s^{-1}; **R**: 0.7 g MJ^{-1}	Semi-continuous outdoor closed ubular (55 L)	Biomass: 2 g m^{-2} d^{-1} Total carotenoids: 102.5 ± 33.1 mg m^{-2} d^{-1} (β-carotene: 10% of biomass)	112
	Lutein	*Muriellopsis* sp.	Medium described by Arnon et al. (1972), supplemented with 20 mM $NaNO_3$ and 35 mM NaCl	**T**: 28 °C; **pH** 6.5; **LI**: 460 μmol $_{photon}$ m^{-2} s^{-1}	Batch (0.2 L, 4–7d)	Lutein content: 5.5 mg g^{-1} $L^{-1}d^{-1}$; Lutein: 1.4–0.8 mg L^{-1} d^{-1}	113
			Medium described by Arnon et al. 1972, modified to contain 4 mM K_2HPO_4 and 20 mM $NaNO_3$	**T**: 28°C; **pH**: 7; **LI**: continuous 200 μmol_{photon} m^{-2} s^{-1}; **AF**: 50–100 L^{-1} h^{-1} (1% ,v/v CO_2)	Continuous outdoor tubular (55 L)	Biomass: 7.2 mg L^{-1} d^{-1}; Lutein: 5.5 mg g^{-1} L^{-1} d^{-1}	113
			Medium described by Arnon et al. (1972), modified to contain 4 mM K_2HPO_4 and 20 mM $NaNO_3$	–	Semicontinuous outdoors, open tank (100 L)	Biomass: 100 mg m^{-2} d^{-1}; Lutein: 100 mg g^{-1} $L^{-1}d^{-1}$	114
		Scenedesmus almeriensis	Mann and Myer's medium (5.0 g/L NaCl)	**T**: 30°C; **pH**: 8.0; $\mathbf{LI}_{max}$: 1700 μE m^{-2} s^{-1}; **AF**: 0.5 (v/v) min^{-2} s^{-1}; **LDC**: solar cycle	Continuous (2 L)	Lutein: 4.9 mg L^{-1} d^{-1}	8
			Mann and Myer's medium	**T**: 35°C; **LI**: 1900 μE m^{-2} s^{-1}	Continuous outdoor tubular system	Lutein: 5.31 mg m^{-2} d^{-1}	114
		Chlorella protothecoides	Modified basal medium with further addition of 0.1 mmol L^{-1} H_2O_2, 0.01 mmol L^{-1} NaClO and 0.5 mmol·L^{-1} Fe^{2+}	**T**: 28 C; **pH**: 6.5; **LI**: absence of light; **MM**: heterotrophic	Batch (16 L)	Lutein: 10 mg L^{-1} d^{-1}	116
		C. zofingiensis, Chlorococcum citriforme, Neospongiococcus gelatinosum	Medium described by Arnon et al. (1972), modified to contain 4 mM K_2HPO_4 and 20 mM $NaNO_3$	**T**: 28°C; **pH** 7; **LI**: continuous 200 μmol_{photon} m^{-2} s^{-1}; **AF**: 50–100 L^{-1} h^{-1} (1%, v/v CO_2)	Batch (0.2 L)	Lutein: 3.4 mg L^{-1} d^{-1} Lutein: 0.70 mg L^{-1} h^{-1}	113

AF: air flow; IL:inoculum level; LDC: light dark cycle; LI: light irradiance; MM: metabolic mode; SR: stirring rate; T: temperature

[1] 12 mM $NaNO_3$, 0.17 mM $Ca(NO_3)_2{\cdot}4H_2O$, 0.37 mM KH_2PO_4, 0.2 mM $MgSO_4{\cdot}7H_2O$, 12.3 mM EDTA-Fe-Na, 12.1 mM DTA-$Na_2{\cdot}2H_2O$, 0.16 mM H_3BO_3, 14.1 mM $MnCl_2{\cdot}4H_2O$, 8 mM $CaCl_2{\cdot}2H_2O$, essential micronutrients (0.3 mM $ZnCl_2$, 0.17 mM $CoCl_2{\cdot}6H_2O$, 15.5 nM $(NH_4)_6Mo_7O_{24}$, 0.16 mM $CuSO_4{\cdot}5H_2O$), vitamins (0.2 mg L^{-1} Biotin, 1 mg L^{-1} Vitamin 1, 0.05 mg L^{-1} Vitamin B12)

induce stress. In the presence of Fe^{2+}, they affect the redox state and generate stress-inducing chemical species. This induction of oxidative stress is crucial (and logical) because lutein provides a protective antioxidant role. Inducing stress is particularly relevant in heterotrophic cultures, for which spontaneous oxidative stress is essentially absent, and unlike the situation arising in phototrophic cultures.[131]

Finally, an effect of specific growth rate was also observed in both continuous and semicontinuous cultures. Lutein tends to accumulate at low dilution rates but not to levels that can balance the decrease in biomass productivity under these circumstances. Therefore, the maximum lutein productivity was again attained at the dilution rate that is optimal for biomass production.[131]

Asthaxanthin. Cyanotech and Aquasearch have developed commercial processes for producing astaxanthin by *Haematococcus* sp. that are based on a two-stage strategy. The first stage produces green biomass under optimal growth conditions, and it is referred to as the "green" stage. The second stage then exposes the microalga to adverse environmental conditions that induce the accumulation of astaxanthin, and this is referred to as the "red" stage.[132] In large-scale facilities, the two-stage system yields astaxanthin productivities[133] of 2.2 mg L^{-1}, whereas maximum astaxanthin productivities of 11.5 mg L^{-1} d^{-1} can be attained at the laboratory scale under continuous illumination.[134]

A single-step, continuous manufacture process has meanwhile been proposed by Micro Gaia, under moderate nitrogen limitation.[135,136] This single-step process leads to productivities of biomass and astaxanthin of 8.0 and 0.7 mg L^{-1} d^{-1}, respectively.[137] The feasibility of the latter approach for continuous production of astaxanthin by *H. pluvialis* was tested outdoors by García-Malea et al.[138] The Aquasearch Growth Module (AGM) used was a 25,000 L-enclosed, computerized outdoor photobioreactor. Three AGMs were used to produce larger amounts of clean, fast growing *H. pluvialis* that was transferred daily to a pond culture system where carotenogenesis and astaxanthin accumulation were then induced. After a 5-day synthesis period, cells were harvested by gravitational settling to obtain an average of 2.5% (w/w_{DW}) astaxanthin. A high pressure homogenizer was used to rupture the cell walls, and the disrupted biomass was then dried to less than 5% (w/w) moisture using a proprietary drying technology. The dried product was finally ready for packaging as per customer specifications. The underlying photobioreactor research program almost doubled the performance of AGM in the first 9 months of operation, during which the biomass concentration increased from 50 to 90 g m^{-2} and the productivity increased from 9 to 13 g m^{-2} d^{-1}.[133]

However, the production capacity of *H. pluvialis* at large is hindered by its intrinsic slow growth, low cell yield, ease of contamination by bacteria and protozoa, and susceptibility to adverse environmental conditions.[139] Moreover, *H. pluvialis* cannot grow efficiently in the dark heterotrophic culture mode. Although production of astaxanthin might in principle adopt the photosynthetic mode, its optimum would require an extremely high irradiance (e.g., 950 μmol m^{-2} s^{-1}), which is well beyond the limit of economic justification. Because of its ease of culture and high tolerance to environmental fluctuations, *Chlorella zofingiensis* (another green microalga) has been proposed as an alternative for astaxanthin production: it grows very fast (ca. three times faster than *H. pluvialis*) and accumulates significant amounts of secondary carotenoids in the dark, thus facilitating large-scale cultivation in denser cultures.[86]

Oxidative stress by intense light illumination has indeed been shown to play an important role in inducing the synthesis of astaxanthin.[140] Apparently, the ROS generated by the excess photooxidation driven by high light irradiance [e.g., singlet oxygen ($^1O_2$), superoxide anion (O_2^-), and hydroxyl radicals ($^\bullet OH$)] trigger the synthesis of carotenoids that can protect against oxidative damage.[86] Continuous illumination rather than light/dark illumination cycles has been demonstrated to favor astaxanthin accumulation in *H. pluvialis*, as additional stress may be provided.[141] Furthermore, light quantity appears more important than light intensity.[142]

The effect of irradiance depends also on other operating variables—viz. culture density, cell maturity (flagellates are much more sensitive than palmelloids), medium nutrient profile, and light path.[143] The predominant roles of light stress and nitrogen deprivation in the induction and enhancement of biosynthesis in the aplanospores of *H. pluvialis* were originally suggested in the 1950s.[144] Astaxanthin accumulation is associated with cessation of growth, as in almost all other cases of microalgal stress.[143,145] Imamoglu et al.[137] compared the effect of various stress media on astaxanthin accumulation under high light intensities. For a photon flux of 546 $\mu mol_{photons}$ m^{-2} s^{-1}, they concluded that the addition of CO_2 in an N-free medium was the best condition for astaxanthin accumulation, and yields up to 30 mg g^{-1} were in fact reported.

Astaxanthin may thus be efficiently produced outdoors in continuous mode if accurate dosage of the nitrate input is assured.[138] Besides nitrogen, however, trace elements also play an important role. Iron, for example, is one of the most essential elements for the metabolism of microalgae, as it takes part in assimilation of nitrate and nitrite, deoxidation of sulfate, fixation of nitrogen, and synthesis of chlorophyll, among other biological synthesis and degradation reactions.[146–148] Iron deficiency was demonstrated to limit growth of microalgae even in the presence of rich nutrient environments,[149] and its addition was found to promote astaxanthin formation.[78,150–152] Furthermore, Cai et al.[153] studied the effects of different iron electrovalencies and the nature of its counter ion on cell growth and accumulation of astaxanthin. It was found that 18 μmol L^{-1} Fe^{2+}-EDTA stimulated synthesis of astaxanthin more effectively, leading to a maximum content of 30.70 mg g^{-1}. Despite the lower cell density attained (2.3×10^5 cell/mL), a higher concentration (36 μmol L^{-1}) of $FeC_6H_5O_7$ yielded cell density and astaxanthin production levels that were 2- and 7-fold, respectively, greater than those of its iron-limited counterpart.

In the "red stage" of growth, *Haematococcus* cells require only carbon as major nutrient source, which is usually supplied via direct injection of CO_2 into the photobioreactor during daylight time.[145] Furthermore, high light irradiance provides more energy for photosynthetic fixation of carbon, thereby resulting in a higher rate of astaxanthin synthesis;[154] the rate is further enhanced by high C/N ratios.[155]

Recently, Chen et al.[156] studied for the first time heterotrophic conditions based on pyruvate, citrate, and malate as substrates to produce astaxanthin by *C. zofingiensis* in the dark. Presence of either of those substrates above 10 mM in the culture medium stimulated biosynthesis of astaxanthin and other secondary carotenoids; ca. 100 mM pyruvate permitted yields of astaxanthin to be obtained that ranged from 8.4 to 10.7 mg L^{-1}, which represent an increase of 28%.

β-Carotene. Semicontinuous cultivation of *D. salina* at 25°C may produce 80 g m^{-3} d^{-1} of biomass,[128] from which 1.25 mg L^{-1} of β-carotene can be recovered;[157] however, 2.45 mg m^{-3} d^{-1} could be attained in continuous biphasic bioreactors.[158] The maximum levels of β-carotene and vitamin C in *Dunaliella tertiolecta* were obtained using urea as nitrogen source, but this simultaneously led to a minimum content of vitamin E.[145] A maximum cellular density was achieved equal to 12.5 × 10^6 cells mL^{-1} for 6.51 L_{air} min^{-1} L^{-1}, but only 7 × 10^6 cells mL^{-1} was attained without aerating and supplying CO_2 even at the optimum pH.[145] Under photoheterotrophic cultivation, a significant increase of cellular β-carotene content was observed, with a maximum of 70 pg $cell^{-1}$ in a culture enriched with 67.5 mM acetate and 450 μM $FeSO_4$.[159]

As happened with astaxanthin, Fe^{2+} ions play an important role in β-carotene accumulation in *D. salina* by inducing oxidative stress, especially in the presence of a carbon source that stimulates synthesis. The UV-A radiation (320–400 nm) added to the known photosynthetically active radiation (PAR, 400–700 nm) is an another efficient stress factor that fosters growth and carotenoid accumulation by *Dunaliella bardawil*, as demonstrated by using an air-fluidized bed photobioreactor. Compared with cultures exposed to only PAR, the addition of 8.7 W m^{-2} UV-A radiation to 250 W m^{-2} PAR stimulated long-term growth of *D. bardawil*, coupled with a 2-fold enhancement in β-carotene accumulation by 24 days.[160]

Lipids

Eicosapentanoic acid (EPA) production by *P. tricornutum* was studied elsewhere[128] under mixotrophic growth, using several substrates at distinct concentrations in both the batch and fed-batch modes. The results reported in Table 5 pertain to flask cultures of a 1 L-working volume and under low irradiance, yet similar data were generated for photoautotrophic growth on glycerol in outdoor tubular photobioreactors at the pilot scale (with 3–6 cm of diameter and 50–200 L of working volume). Hence, the possibility exists to use mixotrophic mass production of microalgae.

The contents of EPA and other PUFAs in *P. tricornutum* were found to be higher at lower temperature, in the range 10–25°C, with an increase of ca. 85% in the former case within that temperature interval.[161] On the other hand, a decrease in the fatty acid content with increasing irradiance is observed when photoinhibition becomes relevant; the percents of saturated and monounsaturated fatty acids decrease concomitantly, especially in the case of EPA. By taking into account the relationship between pigment and EPA content as a function of light irradiance, the variation in EPA productivity over the year can be simulated as a function of the average (or surface) irradiance. During the winter, the biomass productivity is limited by the low availability of light, despite the EPA content being maximum; conversely, the biomass productivity in summer is higher at the expense of a lower EPA specific content, as a consequence of photoinhibition (as expected, the higher the dilution rate the lower the productivity). Because the conditions that increase biomass productivity decrease PUFA content simultaneously, the optimum PUFA productivity is reached by operating under photolimitation and high growth rate.[162]

Polysaccharides

Concerning *P. cruentum*, patents exist that cover production in enriched seawater media using large initial inocula that are maximized for the productivity of polysaccharides. Product recovery then requires strong alkalinization and thermal treatment, as well as a stage that separates the solid and liquid phases after the precipitation of polysaccharides.[163] Precipitation is driven by adding a water-miscible organic solvent (e.g., ethanol), and overall yields can be obtained[164] that are as high as 4.5 g L^{-1}.

On the other hand, concentration of the sterol (24S)-24-methylcholesta-5,22E-dien-3-betaol was reported to vary between 29.1 under a renewal rate of 10%, and 20 fg $cell^{-1}$ under 50% of renewal in the case of *P. tricornutum*.[77] Furthermore, Singh et al.[165] optimized the production of sulphated polysaccharides when the process was conducted in the fed-batch mode. They reported that 1.32 g L^{-1} of product was attained for a light-path of 1.3 cm and 4.15 g m^{-2} d^{-1} for a light path of 20 cm, at a cell density of 1.4 × 10^{11} cell L^{-1}.

Processing for metabolite recovery

The production of specific metabolites by microalgae requires not only culturing for biomass build-up followed by secondary metabolism, but also entails recovery of the biomass and further downstream processing aimed at obtaining the desired metabolites in pure form. Unfortunately, the downstream processing is often more expensive than the bioreaction itself.

The relatively low biomass concentration classically obtained (viz., 1–5 g L^{-1}), because of the limit of light penetration coupled with the small cell size (viz., 2–20 μm in diameter), makes harvesting a costly process. This is so chiefly because of the large volumes to be handled per unit of final biomass, as a 50- to 200-fold concentration is expected; this operation usually accounts for 20%–30% of the total processing costs.[166] Centrifugation has often been used, because large volumes can be rapidly processed and the biomass remains fully contained. However, the efficiency of recovery depends on the intrinsic settling depth and other features of the cells, as well as on the residence time of the cell slurry. Heasman et al.[167] experimented with nine different microalgae and reported a harvesting efficiency above 95% only at 13,000*g*. The efficiency declined to 60% at 6000*g* and 40% at 1300*g*, whereas the cell viability depended significantly on the species and the mode of centrifugation used. However, no universal harvesting method exists, so each one has to be adapted to the conditions and objectives prevailing in each situation.

Specific postharvest processing depends strongly on the desired products and the market specifications. The recovery of microalgal metabolites usually involves mechanical crushing[168] to release the cell contents, followed by fluid extraction.[144] Most cell disruption methods previously developed for nonphotosynthetic microorganisms can be applied to microalgae.[169] For example, high-pressure homogenisers have been widely used to disrupt *Haematococcus* sp. cells for formulation of fish feed, whereas agitation of microalgal biomass in the presence of 0.5 mm-glass ceramic beads in mills has been successfully used to disrupt cells of *Scenedesmus obliquus*, *Scenedesmus platensis*, and *Monodus subterraneous*.[170] Bubrick[171] used cryogenic (−170°C) grinding of dried *Haematococcus* sp. biomass before the liquid extraction of astaxanthin. However, this method is not realistic for large-scale commercial use. Although treatment with alkali is effective in cell lysis,[172] it is not generally suitable for sensitive products, but it is appropriate for products such as PUFA.

Organic solvents are regularly used to extract metabolites from microalgal biomass, e.g., astaxanthin, β-carotene, and essential fatty acids from *P. cruentum*, *P. tricornutum*, *I. galbana*, or *M. subterraneous*. Hexane, ethanol, chloroform, and diethyl ether can indeed easily extract EPA, docosahexaenoic acid (DHA), and arachidonic acid from various microalgae.[173,174] Extraction with aqueous buffers has in turn been used to obtain phycobiliproteins from *P. cruentum*[175] and lutein from *C. vulgaris*.[176]

Supercritical fluid extraction and pressurized liquid extraction have meanwhile been proposed as useful green techniques. The former is suitable for industrial applications, because it enables the solvent to readily penetrate the solid biomass matrix, while being selective for specific solute families.[177] In the case of astaxanthin, its large molecular size requires cosolvents (e.g., ethanol or vegetable oils) to increase the extraction yield.[178,179] The latter was found appropriate for extracting valuable compounds from *H. pluvialis*,[180] in the absence of oxygen and light; although more prone to coextraction of impurities,[34,181] it used subcritical water extraction to recover antioxidant and antimicrobial compounds and achieved yields of ca. 30% (w/w_{DW}) at 200°C. On the other hand, such exopolysaccharides as p-KG03 from *G. impudicum* and others from *N. directa* can be obtained and purified via repeated precipitation in ethanol followed by gel filtration.[25,55]

Crude extracts are generally filtered and used to recover astaxanthin or PUFA.[93] Other methods have been used to successfully recover pure fatty acids (or their esters), which include reverse phase, silica gel-based adsorption, and argentated chromatographies.[174,182] High-value proteins are usually purified via ion exchange chromatography.[175]

Integrated production of biomass and extraction of metabolites have also been attempted; *n*-dodecane enhanced *Crypthecodinium cohnii* growth and DHA production,[183] whereas the growth of *Scenedesmus pleiomorphus* was essentially equivalent in conventional and dodecane-containing biphasic systems. A related possibility is "milking" microalgae, a technological concept announced and patented recently by OriginOil (USA) for the production of biodiesel in up to 200-gallon tanks. This process encompasses stimulating the cells through specific electrical modulation, which increases their membrane and cell-wall permeability and, consequently, allows oil droplets to be excreted without compromising cell viability. Those tanks were operated in a fed-batch mode to permit removal of waste toxins. The underlying principle may easily be extended to in situ production and recovery of specialty metabolites.

Economics of microalga-based processes

The major underlying problem with high added-value compounds from microalgae is how to produce them via processes that are cheap enough to entice market interest; it should be realised that most such compounds may eventually be synthesized via chemical routes, using microalgal libraries solely for identification purposes. Although screening is valuable, at present the manufacture of these products is limited by nonavailability of production processes, so a great deal of effort has been (and should continue to be) invested into design and operation of novel photobioreactor configurations.

Markets for a few microalga-derived products already exist and are steadily expanding, yet their substantial growth is hampered by the manufacture technology used. The cost of microalgal products is still high, and often their quality is substandard. Consequently, microalgal products are at a competitive disadvantage with similar compounds obtained from alternative sources and/or processes.

Several problems in producing microalgae at large scale exist at present. First, a large amount of biomass is needed, preferably in a compact installation because the land cost for industrial plants is increasing. However, to date, most photobioreactor configurations are too inefficient, thus requiring further development. Second, the microalgal biomass should contain high concentrations of the desired product(s), otherwise the separation and purification costs downstream will be high. This could be achieved via a better understanding of the underlying metabolic pathways and subsequent metabolic engineering.

The bioproduction costs per unit mass of product decrease as the installed capacity increases, in a manner that resembles the classical heuristic rule of the six-tenths power. However, separation costs vary with the degree of purity in a logarithmic fashion known as Sherwood rule, and often dominate over production costs as happens for other microbial processes, e.g., ca. 60% of the final cost of EPA arises from the recovery rather than the synthesis steps.[184] However, cases of economic success have been reported pertaining to microalgal metabolites, and lutein is an illustrative example. Lutein was originally obtained from the petals of marigold, thus presenting the drawbacks of being a labor-intensive and land-demanding process, as well as requiring several purification and concentration steps afterwards. Hence, the microalgal process can economically compete with both the flower-based and even the chemical synthetic route. Finally, microalgae can be fast growers and thus exhibit a high primary productivity, but many desired chemicals are secondary metabolites that are synthesized under conditions not compatible with a fast growth. On the other hand, once a chemical compound is discovered and characterized, it may be produced via chemical synthesis; and even a biochemical pathway leading thereto may be transferred to a more easily cultivable microorganism, as is often the case with bacteria. Hence, it seems that the future of microalgae in manufacturing will be limited to complex chemicals that cannot be easily synthesized via chemical processes or to unique chemicals derived from biochemical pathways that cannot be efficiently transferred to other microorganisms.[1]

The case of synthetic astaxanthin merits discussion at some length, because it exhibits a different ratio of stereoisomers than astaxanthin produced by *Haematococcus* sp. The world market price for astaxanthin is ca. US$ 2,000 kg^{-1}, but the actual production cost of synthetic astaxanthin is estimated to lie in the vicinity of US$ 1,000 kg^{-1}. To be able to earn a similar rate of return, and assuming a 3% (w/w) astaxanthin content, *Haematococcus* biomass should be produced at less than US$ 30 kg^{-1}. However, cell harvesting and breaking as done at present does not yet permit that threshold to be met. On the other hand, nutraceutical-grade astaxanthin sells for US$100,000 kg^{-1} in the dedicated market,[133] which thus fully justifies using microalgae to produce it, with the further advantage of its healthier image associated with a natural product label.

The overall competitiveness of microalgae-based bioprocesses will consequently demand new improved strains with faster growth and higher compound yield, via classical selection or targeted genetic manipulation. Bioreactor productivity should also be raised to at least 40–60 g m^{-2} d^{-1}.

Future prospects

From the biotechnology perspective, microalgae are far from being a well-studied group. In fact, from the (at least) 10,000 species that are believed to exist, only a few thousand strains are kept in formal collections. Moreover, only a mere few hundred have been investigated for chemical content, and just a handful are currently cultivated in industrial quantities (i.e., at the level of ton per year).[1] However, microalgae are rich sources of (novel) bioactive compounds that may be exploited for the benefit of mankind. To date, several microalgal secondary metabolites have been shown to possess promising pharmaceutical potential. As presented in this review, some of the metabolites offer anti-inflammatory, antimicrobial, antiviral, and antitumoral activities. However, many others exist that should be comprehensively screened in extended biomedical research programs, because successful drug discovery is one of the most promising aspects of microalgal biotechnology.[185]

The development of microalga-based biotechnology has been constrained by their limited growth rates in industrial photobioreactors. Indeed, the majority of microalgal production still takes place in outdoor open ponds, where processing conditions are not optimal. Closed-system commercialization began with *Haematococcus* sp. in Japan and Israel, and with *Chlorella* sp. in Germany. Developments afterwards have been comprehensively reviewed by Carvalho et al.[186] In general, existing production systems need further improvements to become more competitive and thus economically feasible.

Attempts to mass produce selected microalgal strains containing the desired high levels of bioactive metabolites will require use of photobioreactors capable of maintaining defined growth conditions. Thus, the real challenge is to design a new class of bioreactors from relatively inexpensive materials, which will provide economic feasibility while also assuring high productivities and accurate control of key parameters for biomass yield and composition. This improvement should, particularly, take into account the degree of mixing, the rate of gas exchange, the depth of light penetration, the shear forces, and the pH. Among the several photobioreactor configurations reported to date,[186] the most successful takes advantage of airlift-driven intermixing combined with static mixers. These offer efficient distribution of light with a low input of mechanical energy, coupled with low shear forces imposed on microalgal cells.[187] The configurations of flat panels, or arrays of transparent plastic or glass tubing that are exposed to direct or indirect sunlight merit further exploration and development.

In scale-up efforts, radiant energy for photosynthesis and mechanical energy for mixing are major issues.[188] However, the high cell densities therein preclude effective penetration of daylight, and so constrain the actual rate of photosynthesis. The consequent relatively low volumetric productivities may be overcome only if light is actually conducted into the tubing, which can be achieved by using optical fibers in standard form,[189] or specifically transformed for light dispersion in predefined regions of their path via either pattern nanotexturization or molecular coating.[190]

Photosynthesis by microalgae generates O_2 that may accumulate up to 10 g m^{-3} min^{-1} and eventually cause inhibition or even photooxidative damage.[191] Accumulated oxygen cannot be removed within solid tubing, whereas CO_2 depletion by photosynthesis will cause unwanted pH increases;[192] hence, the maximum length of a continuous tube run is rather limited, and the culture should periodically be returned to a degassing zone for stripping-out generated O_2 and feeding-in fresh CO_2. These drawbacks can be overcome via use of tiny, microporous hollow fibers. The original concept[193] has meanwhile been improved up to a 10-fold enhancement in effectiveness of mass transfer relative to bubbling,[194] but a few practical limitations are still to be overcome in attempts to assure sustained high mass transfer coefficients and large specific surface areas throughout the whole reactor length.

Furthermore, recovery of specific metabolites synthesized in advance by microalgae entails recovery of biomass and further downstream processing. No universal harvesting method exists, so one has to adapt to each particular situation. Alternative approaches based on sedimentation and membrane filtration remain to be fully optimized using novel separator designs, as well as induced flocculation and new concentration methods via e.g., drum-, spray-, fluidized bed-, and freeze-drying. Because most metabolites are located intracellularly, the disruption of cells is required; hence, methods such as autoclaving, bead-meating, microwaving, ultrasonication, or exposure to high concentrations of salts, acids, alkalis, or even enzymes have to be assessed as alternatives to cell disruption. Combinations of extraction solvents also merit investigation, along with the possibility of in situ extraction during active metabolism via addition of immiscible solvents or induction of cell permeability toward excretion of immiscible metabolites.

Heterotrophic and mixotrophic cultivation are also promising possibilities. Furthermore, genetic improvement of microalga strains holds a bright future despite being a challenge at present. The use of transgenic microalgae for commercial applications has indeed been hardly reported and has mainly been restricted to fresh water species (e.g., *Chlamydomonas reinhardtii*). Modified strains may overproduce traditional or newly discovered compounds that originated in microalgae themselves or elsewhere. Such strains could also be used to express specific genes that cannot be effectively expressed in yeasts.

Microalgae also share many attributes with higher plants, including patterns of protein glycosylation, and low risks of contamination by the viruses or prions that typically infect animals. However, microalgae have advantages over plants such as faster growth and higher cell densities that can be attained under optimized operating conditions. The relative ease and low cost of large-scale culturing will make this group of photosynthetic eukaryotes particularly attractive for use in bioreactors; yet, commercial competitiveness vs. bacterial and yeast bioreactors (and even chemical synthesis) still requires the volumetric productivity to increase by ca. 1 order of magnitude. Bridging this gap entails improving the performance of both the microalgal cell via genetic engineering and the microalgal bioreactor via process engineering; hence, fundamental and applied research in the microalgal field should focus on the aforementioned two issues in the coming future.

Overall, a two-step path for building microalgal technology can be foreseen encompassing biocatalyst engineering and bioprocess engineering, where the second will build on the first as well as require its own innovations. Use of microalgae as sources of nutraceutical ingredients for functional food and feed may soon reach the stage of commercial production. The application to pharmaceuticals appears to follow ahead in the future, with the start represented by several promising candidates that are now being tested at advanced stages.

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