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Innovative Natural Functional Ingredients from Microalgae

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Nowadays, a wide variety of compounds such as polyphenols, polyunsaturated fatty acids (PUFA), or phytosterols obtained, for example, from wine, fish byproducts, or plants are employed to prepare new functional foods. However, unexplored natural sources of bioactive ingredients are gaining much attention since they can lead to the discovery of new compounds or bioactivities. Microalgae have been proposed as an interesting, almost unlimited, natural source in the search for novel natural functional ingredients, and several works have shown the possibility to find bioactive compounds in these organisms. Some advantages can be associated with the study of microalgae such as their huge diversity, the possibility of being used as natural reactors at controlled conditions, and their ability to produce active secondary metabolites to defend themselves from adverse or extreme conditions. In this contribution, an exhaustive revision is presented involving the research for innovative functional food ingredients from microalgae. The most interesting results in this promising field are discussed including new species composition and bioactivity and new processing and extraction methods. Moreover, the future research trends are critically commented.

KEYWORDS: Microalgae; functional foods; extraction; antioxidant

INTRODUCTION

In the last years, there has been a growing interest in functional food development because of the beneficial health effects that it can promote (*1*). The rising demand on such foods can be typically explained by the increasing costs of healthcare, the steady enhancement in life expectancy, and the desire of older people to improve their health quality (*2*).

Functional food was born as a new concept in Japan at the beginning of the 1980s, as a means to protect the health of the consumers and to reduce the high health costs derived from a high population with high life expectancies (*3*). In 1993, the Ministry of Health and Welfare established a policy for "Foods for Specified Health Uses" (FOSHU) by which health claims of some selected functional foods were legally permitted and regulated (*4*). In the United States, the Food and Drug Administration (FDA) has accepted a correlation between some nutrients in the diet and the possibility to prevent several diseases when "the totality of publicly available scientific evidence, and where there is substantial agreement among qualified experts that the claims were supported by the evidence" (*5*). On the other hand, in Europe, a working group coordinated by the European Section of the International Life Science Institute (ILSI) and supported by the European Commission was created in the second half of the 1990s to promote the action FUFOSE (Functional Food Science in Europe, under the IV Framework Program) (*6*). This particular action's aim was to stimulate the scientific study on functional food.

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Besides, from this project, a standard definition for functional food was agreed. Namely, a food can be regarded as "functional" if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or a reduction of the risk of disease (*7*−*10*). Moreover, the novel functional food must remain the same as the original food (not in the form of pills or capsules, for instance), and it has to be demonstrated that it can cause its effects in amounts that can be normally expected to be consumed in a regular diet (*5*). In this sense, from the research carried out in the past decade, the European Regulation (CE) 1924/2006 about functional foods (approved in December 2006 by the European Union: regulation of the European Parliament and of the Council, of December 20, 2006, relative to the nutritional declarations and of healthy properties in the food) was derived. In this regulation, the nutritional allegations and/or healthy properties of the new products are regulated, including their presentation, labeling, and promotion.

From a food science point of view, a particular action of a functional food is derived from one or more functional ingredients. The functional food can be classified depending on the type of food or depending on the type of the functional compound employed. This type of classification is shown in **Table 1**. As can be observed, there are a wide range of compounds and beneficial activities that have already been described to develop new functional products. Therefore, because the demand on functional food is continuously increasing, there is a need to find new adequate functional ingredients to be employed by the food

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Table 1. Examples of Functional Ingredients That Are Commonly Consumed in Functional Foods and Their Possible Effect on Human Health

functional food	functional ingredients	possible health effect	refs
prebiotics	oligosaccharides (inulin, fructo-oligosaccharide, etc.)	play important role in obesity control	*11*, *12*
functional drinks	(n-3) fatty acids	reduce risk of certain heart diseases	*13*
	vitamin A and lutein	reduce risk of certain eyes diseases.	*14*
	calcium	increase bone health	*15*
functional cereals	β-glucans	into low-fat dairy products can make their sensory properties resemble those of full-fat products	*16*
functional meats	(n-3) fatty acids	reduce risk of certain heart diseases	*17*
	probiotics (lactic acid bacteria and bifidobacteria)	anticholesterol activity, improved lactose utilization, and anticarcinogenic activity	*18*
functional eggs	(n-3) fatty acids	reduce risk of certain heart diseases	*19*
fermented dairy products	probiotics (lactic acid bacteria and bifidobacteria)	anticholesterol activity, improved lactose utilization, and anticarcinogenic activity	*18*
	antihypertensive peptides	reduce risk of certain heart diseases	*20*
bakery products	vitamin B_{12}, folic acid, vitamin C, and prebiotic fiber	reduce risk of coronary heart diseases	*21*
	phytosterol esters, α-tocopherol, and β-carotene	reduce risk of certain heart diseases	*22*

industry. In this regard, special attention has to be paid to the origin of the functional ingredients considering that their natural origin will always be preferred by consumers as compared to the synthetic one (*23*). These characteristics have led to an increase in the research of new natural sources for this kind of compound. At this point, some plants have already been pointed out as rich sources of important compounds (*23*).

More recently, the field of available natural sources has been further increased by also including some algae (*24*) and, even more interestingly, microalgae. These microorganisms are a potentially great source of natural compounds that could be used as functional ingredients. The chemical composition of microalgae can present great variations and is mainly related to environmental factors such as water temperature, salinity, light, and nutrients available. Most of the environmental parameters vary according to season, and the changes in ecological conditions can stimulate or inhibit the biosynthesis of several nutrients (*25*). Microalgal biomass has already been found as a natural source of a number of biologically active compounds, such as carotenoids, phycobilins, fatty acids, polysaccharides, vitamins, and sterols (*26*). Nevertheless, not only the presence of a particular compound makes these microorganisms interesting but also their huge diversity and the possibility of not only harvesting them but also growing them at different conditions, which means using them as natural reactors, leading to an enrichment of some bioactives (*27*).

Additionally, with the aim to obtain new functional ingredients to be used by the food industry, efficient, selective, and environmentally friendly extraction techniques are required. In this sense, supercritical fluid extraction (SFE) or pressurized liquid extraction (PLE) have gained importance in the last years. These techniques have been selected, even considering that other interesting procedures have also been applied to isolate and purify interesting compounds from natural matrices, such as countercurrent chromatography or expanded bed adsorption. References to some key works dealing with these techniques have also been included in the review.

In this work, an exhaustive revision is presented involving research for innovative functional food ingredients from microalgae. The most interesting results in this field are presented and commented, paying attention to the particular species of microalgae, the activity of the compounds obtained, and the type of extraction mechanisms used. Lastly, the future research trends and research needs for the attainment of bioactives from microalgae are critically commented.

FUNCTIONAL INGREDIENTS FROM MICROALGAE

A relatively high number of microalgae species have been used up to now, with the aim of obtaining different functional ingredients. To describe the work carried out in this research area, this section has been divided into different subsections attending to the particular microalgae species studied. This way, the published results concerning each microalga are grouped so that is possible to have a general idea of the potential and global chemical composition of a given species. Besides, the particular extraction methods as well as the bioactivity studies carried out are thoroughly described. A summary of the information described below is shown in **Table 2**.

Spirulina platensis. *S. platensis* is a microalga belonging to the group of cyanobacteria (or blue-green algae) and has been traditionally used as food for different cultures. At present, this alga has attracted special attention due to its importance as a nutritious supplement and its demonstrated (in vitro and in vivo) functional properties, being one of the most studied species.

This microalga presents a high protein content, up to 70% dry weight (*52*). Its amino acid composition has a great interest, not only because *S. platensis* possesses all of the essential amino acids but also because these amino acids have a great bioavailability (*52*). The carbohydrates of *Spirulina* constitute approximately 15% of the dry matter. The major carbohydrates are polysaccharides. Among the monomeric forms, glucose, galactose, ribose, and mannose are preferentially found (*52*). On the other hand, its lipid fraction accounts for about 5% of its dry weight (*52*).

One of the major compounds in *S. platensis* is phycobiliprotein [namely, allophycocyanin (APC) and c-phycocyanin (CPC)] (*53*, *54*), a group of proteins involved in photosynthesis. Purified phycobiliproteins can have several uses such as cosmetics, colorants in food, and fluorescent labels in different analytical techniques (*55*, *56*). These proteins are characterized by having a tetrapirrolic pigment, called phycobilin, covalently attached to their structure. Important medical and pharmacological properties such as hepatoprotective, antiinflammatory, and antioxidant properties (*57*−*59*) have been described and are thought to be basically related to the presence of phycobilin. Besides, phycobiliproteins might have an important role in different photodynamic therapies of various cancerous tumors and leukemia treatment (*60*). Different works have been aimed to the selective extraction and analysis of the phycobiliproteins from this organism (*41*, *61*). It has been demonstrated how by means of PLE using water as an extraction solvent, it was possible to extract both phycobiliproteins from the microalgae. Later on, it was possible to perfectly identify the two subunits of each protein, namely, APC-α, APC-β, CPC-α, and CPC-β,

Table 2. Potential Functional Ingredients Found in Different Microalgae, Bioactivity and Extraction, and Analytical Procedures Employed for Their Attainment

functional ingredients	possible health effect	extraction method[a]	analytical techniques	microalgae	refs
carotenoids					
β-carotene	antioxidant activity	SFE	LC-UV/vis	*Dunaliella salina*	28
		SFE	LC-UV/vis	*Haematococcus pluvialis*	29
astaxanthin	antioxidant, immunomodulation, and cancer prevention	SFE	LC-UV/vis	*H. pluvialis*	29
		SFE	HPLC	*Chlorella vulgaris*	30
cantaxanthin	antioxidant, immunomodulation, and cancer prevention	SFE	HPLC	*C. vulgaris*	30
		SFE	LC-UV/vis	*H. pluvialis*	29
lutein	antioxidant activity	SFE	LC-DAD	*Chlorella pyrenoidosa*	31
		SFE	LC-UV/vis	*H. pluvialis*	29
violaxanthin	antioxidant activity	L-L	LC-MS	*Chlorella ellipsoidea*	32
fatty acids					
EPA fatty acid	reduce risk of certain heart diseases	L-L	GC	*Phaeodactylum tricornutum*	33
		L-L	GC	*Monodus subterraneus*	34
		L-L	GC-FID	*Porphyridium cruentum*	35
oleic acid	antioxidant activity	L-L	GC	*H. pluvialis*	36
		SFE	GC-FID	*C. vulgaris*	30
		PLE	GC-MS	*D. salina*	37
		SFE	GC-FID	*Spirulina platensis*	38
linolenic acid	antimicrobial activity	PLE	GC-MS	*D. salina*	37
		L-L	LC-UV/vis	*S. platensis*	39
palmitic acid	antimicrobial activity	PLE	GC-MS	*D. salina*	37
palmitoleic acid	reduce risk of certain heart diseases	SFE	GC-FID	*S. platensis*	38
DHA fatty acid	reduce risk of certain heart diseases	L-L	GC-FID	*S. platensis*	40
proteins					
phycobiliproteins	immunomodulation activity, anticancer activity, and hepatoprotective, anti-inflammatory, and antioxidant properties	PLE	CE-TOF-MS	*S. platensis*	41, 42
		L-L	spectrophotometry	*Porphyridium* spp.	43
polysaccharides					
sulfated polysaccharide	antiviral, antitumor, antihyperlipidemia, and anticoagulant	ultrasounds	GC	*C. pyrenoidosa*	44
		ultrasounds	GC-FID	*Porphyridium* spp.	45
insoluble fiber	reduce total and LDL cholesterol	L-L	assay for glucose consumption	*C. vulgaris*	46
vitamins					
tocopherols (vitamin E)	antioxidant activity	ultrasounds	HPLC-FD	*Porphyridium* spp.	47
		SFE	LC-DAD	*S. platensis*	48
phenolic compounds					
benzoic acid derivatives, hydroxybenzaldehydes, and cinnamic acid derivatives	antioxidant activity	SPE/SFE	HPLC-MS	*S. platensis*	49
volatile compounds					
neophytadiene, phytol, etc.	antimicrobial activity	PLE; SFE	GC-MS	*S. platensis*	38, 50
		PLE	GC-MS	*D. salina*	37
		PLE	GC-MS	*Phormidium*	51

[a] L-L, liquid−liquid extraction.

in the water extracts from *S. platensis* microalgae, (*41*, *42*) by using a novel capillary electrophoresis-mass spectrometry (CE-MS) method. Expanded bed adsorption has also been used to purify these proteins (*62*). The main advantage claimed by authors was the high yield achieved using this method in the steps of product extraction (crushing cells by osmotic shock)

and adsorption, being able to reduce both processing time and cost (*62*).

However, in the search for new functional compounds from *S. platensis*, new compounds, other than phycobiliproteins, have been described. In this sense, several antioxidants have been extracted using PLE. By combining these extraction techniques with a multistep chemical analysis including preparative thin-layer chromatography (TLC), DPPH staining to test the antioxidant activity of the separated bands, and high-performance liquid chromatography−diode array detection (HPLC-DAD) to identify the separated compounds in each band, it was possible to determine different chlorophylls and carotenoids in the *S. platensis* PLE extracts, among them, β- and α-carotene, β-cryptoxanthin, asthaxanthin, zeaxanthin, lutein, echinenone, oscillaxanthin, and myxoxanthophyll (*63*). In this case, different solvents were tested to maximize the attainment of antioxidant compounds. Ethanol was found to be the most appropriate solvent considering the high yields and good antioxidant activities produced and its GRAS (generally recognized as safe) nature, which was of great importance in the context of the food industry. Other kinds of antioxidants were extracted by Klejdus et al. (*49*). These researchers proposed a novel extraction technique based on the combination of solid phase and solid-phase extraction (SPE/SFE) with subsequent analysis by reversed-phase HPLC. By using this methodology, these authors could identify different simple phenolics such as benzoic acid derivatives (*p*-hydroxybenzoic, protocatechuic, vanillic, and syringic acids), hydroxybenzaldehydes (4-hydroxybenzaldehyde and 3,4-dihydroxybenzaldehyde), and cinnamic acid (caffeic and chlorogenic acid) derivatives in this microalga (*49*).

Other compounds with demonstrated antimicrobial activity were also identified in this organism. In fact, the antimicrobial activity of different *S. platensis* PLE and SFE extracts was tested (*38*, *50*) against a panel of microorganisms that included a Gram positive bacteria (*Staphylococcus aureus*), a Gram negative bacteria (*Escherichia coli*), a yeast (*Candida albicans*), and a fungus (*Aspergillus niger*) (*50*). The antimicrobial activity was quantitatively assessed by the determination of the minimum inhibitory concentration (MIC) and minimal fungicidal and bactericidal concentrations (MBC). Derived from these experiments, it could be observed that both kinds of extracts presented certain antimicrobial activities. However, the supercritical CO_2 extracts showed better values of antimicrobial activity than those obtained by means of PLE using more polar solvents, such as ethanol (*38*). Therefore, it was suggested that the antimicrobial activity would be related to nonpolar compounds present in the microalga composition. Several authors have associated the antimicrobial activity in different cyanobacteria to the presence of different compounds (*64*−*66*), such as γ-linolenic acid ($C_{18:3}$, n-3), an antibiotically active agent that has been found in relatively great extent in this microalga (*39*) that was detected in *S. plantesis* (*67*) as well as other species of *Spirulina* (*28*). However, the fatty acid profiling of the supercritical extracts did not reveal the presence of significant amounts of this fatty acid; instead, other active fatty acids were determined in greater amounts, particularly lauric, palmitoleic, and oleic acids (*38*), which have been previously pointed out to have also some antimicrobial activity (*68*−*70*). Besides, the antibacterial capacity of *S. platensis* has been correlated to its volatile composition (*66*), determined by gas chromatography−mass spectrometry (GC-MS), resulting in the identification of 15 compounds, which constituted 96% of the total compounds. The main volatile components of *S. platensis* consisted of heptadecane (40%) and tetradecane (35%) (*66*).

This microalga is also a natural source of docosahexaenoic fatty acid (DHA) ($C_{22:6}$, n-3), which can account for up to 9.1% of the total fatty acids content (*71*). This compound has shown several interesting functional properties (*72*, *73*). Moreover, this microorganism also contains tocopherols in a significant amount (*70*, *74*) that can be selectively enriched by using SFE (*48*).

Dunaliella salina. *D. salina* is a green unicellular marine microalga well-known for being one of the main natural sources of β-carotene. Under particular conditions, *D. salina* is able to produce β-carotene up to 14% of its dry weight (*75*). For this reason, and given the importance of this product, it is undoubtedly one of most studied microalgae (*76*−*79*). In fact, the particular growing conditions able to maximize production of β-carotene at an industrial scale have been investigated (*77*, *80*−*84*). Because β-carotene has important applications in food, pharmaceutical, and cosmetics industries (*82*), different procedures have been studied, not only for the production of this compound but also for its extraction and isolation (*28*, *77*, *85*−*88*). The most widely employed technique has probably been SFE. The low polarity characteristics of the supercritical CO_2 make this solvent appropriate for the β-carotene extraction from this microalga. Mendes et al. (*28*) studied the different solubility in supercritical CO_2 of both synthetic and natural β-carotene. Besides, they could observe that the extraction of the *cis* isomer was more favorable than that of the *all-trans* isomer. The separation and identification of the different β-carotene isomers extracted have also been the aim of other researches (*86*, *88*, *89*), considering that there is no absolute certainty of the similar antioxidant activity of the different isomers. Jaime et al. (*86*) reported the strong influence of the supercritical extraction conditions in both the β-carotene isomer composition of the extracts and the antioxidant activity. In particular, a statistically significant relationship between antioxidant activity of the extracts and their β-carotene isomeric ratio, 9-*cis*/*all-trans*, was observed (*86*).

Also attending to the selective extraction of carotenoids, supercritical CO_2 was demonstrated to be more selective and effective than ultrasound-assisted extraction, providing a higher carotenoids/chlorophylls ratio (*88*). Other extraction techniques have also been studied, based on the use of pressurized solvents (*90*). In this study, ethanol was selected as the most suitable solvent considering the yield and the antioxidant activity provided by the different extracts produced. Besides, the extracts were analyzed to determine the relative amounts of carotenoids. *all-trans*-β-Carotene and 9-*cis*-β-carotene were the main carotenoids, whereas other carotenoids (α-carotene, 13-*cis*-β-carotene, 15-*cis*-β-carotene, and 11-*cis*-β-carotene) were determined in smaller amounts (*90*).

Additionally, it has been observed that under irradiance stress, *D. salina* can accumulate significantly high amounts of xanthophylls, in particular zeaxanthin (*91*). This carotenoid has raised attention since it has been demonstrated to possess interesting functional properties helpful for preventing several diseases (*92*). Thus, it could be possible to make use of this characteristic to selectively obtain different interesting pigments other than β-carotene.

Nevertheless, this microalga has not only been studied as a source of β-carotene and other antioxidants. Recently, the possibility to obtain antimicrobial compounds from *D. salina* has been evaluated (*37*). These authors reported PLE extracts using ethanol and hexane as solvents that presented certain antimicrobial activity, especially against bacteria. Later on, the extracts were characterized by GC-MS according to their volatile compounds and fatty acid profiles with the aim to determine the compounds responsible for the antimicrobial activity detected. β-Cyclocitral, α- and β-ionone, neophytadiene, and phytol were identified in the extracts among other compounds. Besides, palmitic, linolenic, and oleic acids accounted for more than

85% of the total fatty acids content. All of these compounds had previously been described as antimicrobial agents (*37*).

Other components of interest are proteins, which account for about 50% of dry weight. Its amino acid composition shows a low content in cysteine, tyrosine, methionine, and tryptophan (*93*). Carbohydrates make up about 30−40% of the dry weight of *D. salina,* including mono- and disaccharides, galactose, glucose, mannose, xylose, ribose, rhamnose, and polysaccharides, especially α-1,4-glucosan. Nevertheless, as it has been stated before, depending on the particular growing conditions, the different ratios among the diverse components can vary significantly (*93*).

***Chlorella* spp.** *Chlorella* is a genus of single-celled green algae, belonging to the Chlorophyta, and therefore containing the green photosynthetic pigments chlorophyll *a* and *b* in its chloroplast. The unicellular algae *Chlorella vulgaris* contain many bioactive substances with medical properties. Experimental studies carried out under *Chlorella* have demonstrated its antitumor effect (*94*), hepatoprotective properties (*95*), antioxidant properties (*96*), antibacterial effects (*97*), or even the immunostimulant activity of enzymatic protein hydrolysates from this microalgae (*46*). In this later work, the authors specifically proposed these hydrolysates to be used for developing functional foods with immunopotentiating activity, since they could demonstrate their activity in vivo (*46*). Moreover, the hypocholesterolemic mechanism of *Chlorella* powder has also been investigated by Shibata et al. (*98*). This study suggests that *Chlorella* powder enhances the hepatic degradation of cholesterol by up-regulating the expression of cholesterol 7α-hydroxylase in rats with or without diet-induced hypercholesterolemia. The active components in *Chlorella* responsible for the up-regulation of cholesterol 7α-hydroxylase expression might be part of the indigestible fraction.

Chlorella contains many dietary antioxidants, which could eventually be responsible for some of its functional activities. In fact, lutein, α-carotene, β-carotene, ascorbic acid, and α-tocopherol, compounds with the capacity to scavenge free radicals, have been found (*97*). According to Wu et al. (*31*), *Chlorella* contained 2−4 mg/g dry cell weight of lutein. Lutein is not only an important natural food dye and additive but also a strong antioxidant that may be useful in reducing the incidence of cancer (*99*) and preventing macular degeneration (*100*). For instance, the antiproliferative activity of carotenoids extracted from *Chlorella ellipsoidea* and *C. vulgaris* on human colon cancer cells has been investigated by Cha et al. (*32*). It has been reported that the main carotenoids from *C. ellipsoidea* were violaxanthin together with other two minor xanthophylls, antheraxanthin and zeaxanthin, whereas the main carotenoid from *C. vulgaris* was lutein. Both extracts of *C. ellipsoidea* and *C. vulgaris* inhibited HCT116 cell growth, yielding IC_{50} values of 40.73 ± 3.71 and 40.31 ± 4.43 μg/mL, respectively. Besides, the *C. ellipsoidea* extract produced an apoptosis-inducing effect almost 2.5 times stronger than that of the *C. vulgaris* extract on these cells, indicating that bioactive xanthophylls of *C. ellipsoidea* could be useful functional compounds in the prevention of human cancers (*32*). High-speed counter-current chromatography (HSCCC) was applied to the isolation and purification of lutein from *C. vulgaris*. In a first step, this liquid−liquid partition chromatography was optimized at an analytical scale to select the most appropriate solvents, and later, preparative HSCCC was applied obtaining lutein at 98% purity in a one-step separation (*101*).

As for other carotenoids, when carotenogenesis is induced by nutrient starvation and NaCl addition at high luminosity, *Chlorella* accumulates canthaxanthin and astaxanthin (*26*, *30*). Canthaxanthin and astaxanthin as well as other nonprovitamin A carotenoids enhance the immune response (*102*). Moreover, both carotenoids have been used as pigments in feeds to color salmonid fish, trout, and egg yolk (*30*). Besides carotenoids, other antioxidant components have been described in *C. vulgaris* (*103*). In this study, the authors demonstrated that purified peptides from *C. vulgaris* had significant protective effects on DNA and prevented cellular damage caused by hydroxyl radicals. These results suggested that peptides from *C. vulgaris* protein waste had the potential to be a good dietary supplement for the prevention of oxidative stress-related diseases, such as atherosclerosis, coronary heart disease, and cancer (*103*).

Concerning its lipidic composition, according to Mendes et al. (*30*), oleic, palmitic, and linolenic acids were the main constituents of the glycerides and fatty acids fraction, accounting for 41, 22, and 9% of the total amount, respectively. These results agreed with other published studies (*104*).

Polysaccharides have also been identified in *Chlorella pyrenoidosa* (*44*); purified algal polysaccharides, such as agar and carragenan, are extensively used in the industry (*105*). The biological activities of some species of algae (antioxidant, antitumor, antihyperlipidemia, and anticoagulant) have been associated with polysaccharides, which have been purified and developed as a new generation of drugs (*81*, *106*−*108*). Extracellular carbohydrates in *C. pyrenoidosa* cultures were represented mainly by water-soluble polysaccharides containing galactose, mannose, arabinose, xylose, ribose, fucose, and rhamnose (*109*). In general, it is clear that for *Chlorella*, as in general for all microalgae, it is mandatory to control the particular growing conditions, since those factors can significantly determine the whole and final chemical composition of the microalga.

Haematococcus pluvialis. *H. pluvialis* (Chlorophyceae) is a unicellular green alga common in small, transient, freshwater bodies. *Haematococcus* produces chlorophylls *a* and *b* and primary carotenoids, namely, β-carotene, lutein, violaxanthin, neoxanthin, and zeaxanthin, while it has the ability to accumulate, under stress conditions, large quantities of astaxanthin, up to 2−3% on a dry weight basis (*110*). During this carotenogenesis process, it undergoes different changes in cell physiology and morphology, giving as a result large red palmelloid cells (*111*, *112*). Astaxanthin is present in lipid globules outside the chloroplast. Their functions in the cell include protection against light-related damage by reducing the amount of light available to the light-harvesting pigmented protein complexes. These pigments possess powerful biological activities, including antioxidant capacity (*36*), ulcer prevention (*113*), and immunomodulation and cancer prevention (*114*). In fact, the extraction of astaxanthin has been thoroughly investigated. Different methods have been tested, including neat supercritical CO_2 (*115*) or supercritical CO_2 with different cosolvents (*29*, *116*, *117*), PLE (*85*), microwave-assisted extraction (*118*), direct extraction with vegetable oils (*111*) or solvents (*119*), or even treating cells with various solvents and organic acids at 70 °C before acetone extraction, with the aim to facilitate the astaxanthin extraction from the thick cell wall without affecting the original astaxanthin esters profile (*120*).

The carotenoid content in *H. pluvialis* has been determined by Nobre et al. (*29*). This study revealed that the main carotenoid was the esterified astaxanthin (about 73%). Other carotenoids present were lutein, astaxanthin (in its free form), β-carotene, and canthaxanthin (*29*). The antioxidant activity presented by *H. pluvialis* has also been measured and related to their carotenoid content (*36*). Besides, the particular growing conditions were considered. Interestingly, it was observed that the astaxanthin diesters possessed 60% higher antioxidant capacity than the astaxanthin monoester fraction and twice that of free astaxanthin. Therefore, a relationship between the fatty acids bound to the astaxanthin structure and the antioxidant activity was

observed (*36*). Authors concluded that controlling the growing conditions, astaxanthin and oleic acid-rich cells could be obtained, which exhibited the maximum antioxidant activity that would make them adequate for human consumption from a functional point of view (*36*). Different analytical methods have been developed to analyze specifically the astaxanthin esters. These methods were based on the HPLC separation coupled to atmospheric pressure chemical ionization−mass spectrometry detection (*121*, *122*).

Nevertheless, this microalga has not only been studied as a source of astaxanthin and other carotenoids. Recently, the possibility to obtain other antioxidant compounds from *H. pluvialis* has been evaluated (*123*, *124*). In these works, extraction of antioxidant compounds combining PLE and using hexane, ethanol (*124*), and water (*123*) as extraction solvents has been studied. The effect of the extraction temperature (50, 100, 150, and 200 °C) and the polarity of the solvent has been estimated in terms of in vitro antioxidant activity. Results in both works demonstrated that the extraction temperature had a positive influence in the extraction yield. Its effect in the antioxidant activity was negative, lowering the activity of the extracts with an increase of the extraction temperature, in ethanol and hexane extracts, while in water extracts, the extraction temperature had a positive influence on the antioxidant activity. In the water extracts, a possible correlation was found between the antioxidant activity and vitamin E, simple phenols, caramelization products, and possible Maillard reaction products obtained during the extraction at high temperatures. Nevertheless, the compounds responsible for this activity in the ethanol and hexane extracts were carotenoids (astaxanthin, lutein, etc.).

The same extraction conditions were tested to obtain antimicrobial extracts from *H. pluvialis* (*123*, *125*). It was effectively observed that these extracts possessed certain antimicrobial activities. Short chain fatty acids turned out to be responsible for the antimicrobial activity presented by these extracts.

***Porphyridium* spp.** *Porphyridium* spp. is a unicellular red microalgae that belongs to Rhodophyta, and it is gaining importance as a source of valuable products such as phycobiliproteins, sulfated exopolysaccharides, and polyunsaturated fatty acids (PUFAs) with potential applications in the food industry (*126*). The phycobiliproteins in *Porphyridium* spp., like in *S. platensis*, have a photosynthetic function in the organism and are found aggregated in the cell in the form of phycobilisomes, which are attached to the thylakoid membrane of the chloroplast (*127*). Among them, the main protein of this type in *Porphyridium* is phycoerythrin, a red-colored pigment (*128*, *129*). These proteins are water-soluble and are generally employed as food dyes in different products (e.g., chewing gums, dairy products, and jellies, among others) (*126*), besides in the cosmetic and pharmaceutical industries (*130*). More importantly, several therapeutic bioactivities have been described related to phycoerythrin, namely, immunomodulating activities and anticancer properties (*131*). This protein has been recovered and purified from this microalgae by expanded bed adsorption chromatography (*132*, *133*). To obtain this interesting compound, several steps were performed, including extraction of the product from the cells, separation of phycoerithrin by expanded bed adsorption chromatography, and packed bed elution obtaining a concentrated product, and finally purification by packed bed ion-exchange chromatography. This strategy was also used to purify phycoerithrin from other samples (*134*, *135*).

In general terms, the *Porphyridium cruentum* biomass content is characterized by proteins (up to 34% of dry weight), carbohydrates (32% of dry weight), and low amounts of lipids (6.5% dry weight) (*136*). Interestingly, although these algae show a relatively low lipid content, several major interesting fatty acids can be found in significant amounts, namely, palmitic acid, arachidonic acid, eicosapentaenoic acid (EPA), and linoleic acid. This high proportion in PUFAs, and particularly EPA, has been reported to have beneficial effects such as reducing coronary heart disease risk and blood cholesterol, thus reducing the risk of arteriosclerosis, inflammation, and several carcinomas (*137*). *P. cruentum* also presents high levels of tocopherols as demonstrated by Durmaz et al. (*47*); for example, the contents of α-tocopherol and γ-tocopherol were 55.2 and 51.3 μg/g dry weight, respectively. These tocopherols (vitamin E) are lipid-soluble antioxidants that are considered essential nutrients because of their ability to protect membrane lipids from oxidative damage (*138*). Vitamin E has effects in the prevention of many diseases, such as atherosclerosis, heart disease, and also neurodegenerative diseases such as multiple sclerosis (*139*, *140*), thus also making it a very interesting functional compound.

Phorphiridium spp. cells are encapsulated within a sulfated polysaccharide, which has a wide range of promising industrial and medicinal applications (*45*). It is composed of about 10 different sugars, the main ones being xylose, glucose, and galactose, and the minor ones being mannose, methylated galactose, and pentose. The polysaccharide is negatively charged, due to the presence of glucuronic acid and half-sulfate ester groups (*141*). According to Huheihel et al. (*142*), the cell wall-sulfated polysaccharide exhibited impressive antiviral activity against herpes simplex virus types 1 and 2 both in vitro (cell culture) and in vivo (rats and rabbits). Moreover, the in vitro anti-inflammatory and antiproliferative activity of a sulfoglycolipidic fraction isolated from *P. cruentum* has also been studied (*143*). In this work, it was demonstrated that a sulfoquinovosylacylglycerols fraction exhibited in vitro antioxidant, anti-inflammatory, and antiproliferative effects and even might have a chemopreventive potential. The higher antiproliferative effect was observed on colon adenocarcinoma DLD-1 cells (*143*).

Other Interesting Species. Another wide group of microalgae have already been screened for interesting functional compounds. Among them, PUFAs are probably the most important group. The production of PUFA-enriched organisms can be of great interest considering that some of them, for instance, α-linolenic acid, EPA, docosapentaenoic acid (DPA), or docosahexaenoic acid (DHA), have demonstrated positive effects on the reduction of coronary diseases (*144*). Besides, it has also been demonstrated that some fatty acids from algae can have certain antiviral activities (*145*). In this respect, *Phaeodactylum tricornutum* can have around 30−45% of PUFAs, among which EPA can account for up to 20−40% of the total fatty acids in this microalga (*33*). Besides, other minor PUFAs are present, such as palmitoleic, 9,12-hexadecadienoic, and 6,9,12-hexadecatrienoic acids (*146*). On the other hand, the yellow-green microalga *Monodus subterraneus* is one of the most promising microalgal sources of EPA. Depending on the growing conditions, the proportion of EPA in this species ranges from 20 to 37% of total fatty acids (*34*). Thus, these two species could be important natural sources for bioactives such as EPA and other PUFAs that could be potentially used as functional ingredients. *Schizochytrium* is a microalgae rich in DHA (*147*−*149*). According to Nakahara et al. (*150*), after culture optimization conditions, the DHA content of the lipid was 34% of total fatty acids. Interestingly, the use of microalgae as a source for functional ingredients has been demonstrated by Valencia et al. (*151*) who obtained dry fermented sausages enriched in DHA. This was attained by incorporating *Schizochytrium* sp. oil to the formulation of the original product. The result of this study confirmed that algae oil could

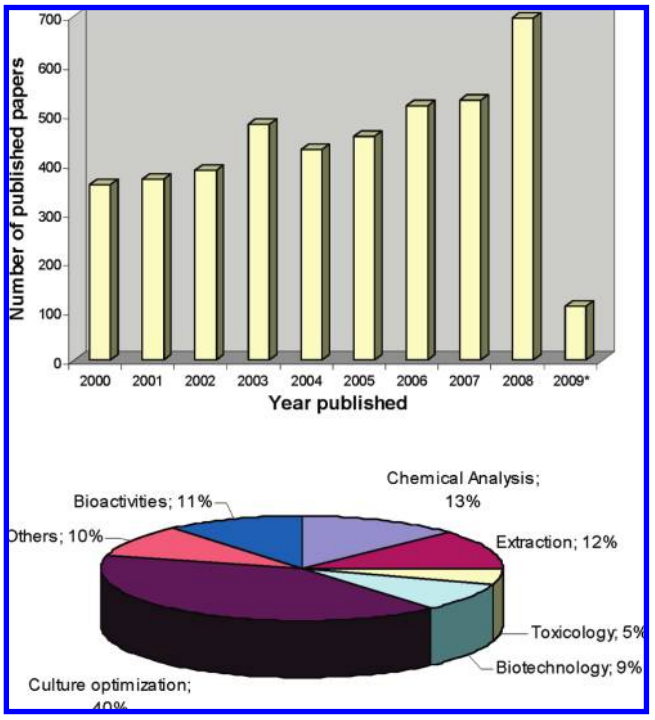

Figure 1. (**Top**) Number of published works per year in the range 2000−2009. A bibliographic search was carried out in the www.isiknowledge.com database using the general term "microalga*" (data up to March 2009). (**Bottom**) Papers published for each marked category during the period 2000−2009 according to the bibliographic search performed in the FSTA database using the term "microalga*".

be used as a functional ingredient in sausages. The resulting products showed a better n6/n3 ratio than traditional sausages.

Another microalga that has received some attention is *Botryococccus braunii*. Tonegawa et al. (*152*) investigated the pigment composition of this green microalga concluding that the intracellular carotenoids were composed of neoxanthin, loroxanthin, violaxanthin, lutein, α-carotene, and β-carotene while the main component of the extracellular carotenoids was a ketocarotenoid, echinenone.

The PLE extraction of *Phormidium* was evaluated to obtain interesting bioactives (*51*). Those extracts showed both antioxidant and antimicrobial activities, attributed to the carotenoids present and to different terpenes and fatty acids, respectively, which were identified using different chromatographic methods (*51*). Another less studied microalga (*Coelastrella striolata* var. multistriata) was proposed as a source of functional compounds that could be employed in several applications, such as feed supplements, sources of antioxidants, or as a dye (*153*). It was demonstrated that under particular growing conditions, this microalga was able to accumulate ca. 5.5% of secondary carotenoids per g of biomass, including canthaxanthin, astaxanthin, and β-carotene. Other carotenoids have also been isolated and purified by HSCCC from *Chlorococcum* (astaxanthin) (*154*) and *Microcystis aeruginosa* (zeaxanthin) (*155*).

Nostoc spp. is a microalga that has been reported to show antitumor activity (*156*). This effect may be due to polysaccharides. Kanekiyo et al. (*157*) isolated and characterized a novel antiviral polysaccharide, nostoflan, from *Nostoc flegelliforme*. Nostoflan exhibited a potent antiherpes simplex virus type a (HSV-1) activity. Besides, this polysaccharide showed potent antiviral activities against HSV-2, human cytomegalovirus, and influenza A virus, but no activity against adenovirus and coxsakie virus was observed.

THE POTENTIAL OF SOME MICROALGAE AS NATURAL SOURCES OF FUNCTIONAL INGREDIENTS

The research work described above has to be considered as an example, not pretending to be exhaustive since the research conducted in the past few years related with microalgae is really huge, including biotechnology, processing, extraction of bioactives, chemical composition, toxicity, etc. In **Figure 1**, the trend of published papers on this topic is shown. The top figure shows the total number of published works dealing with microalgae, whereas the bottom figure shows the particular contribution of each particular field in the food-related investigations concerning microalgae. What seems clear in light of the data presented is that the interest is enormous and growing. There might be a number of reasons for that: First of all, the number of available species is huge, and the chance of finding some new interesting species is enormous (*158*). Also, microalgae can be grown under certain controlled conditions that can help, not only to produce but also to accumulate, some strong biologically active compounds in large amounts, behaving like authentic natural reactors at a large scale. Examples can be found in *D. salina*, which is regularly used at an industrial scale and cultivated under particular conditions to maximize the β-carotene production. This characteristic, common to every microalga, is one of the most interesting when proposing their use as a source of functional ingredients for the food industry.

Thus, the knowledge of the chemical composition of the different microalgae species is mandatory as a first step (considering an screening methodology) since it will help to target the valuable compounds, antioxidants, suphated polysaccharides, PUFAs, etc., in the studied microalga. As a second step, the growing conditions could be optimized to maximize the production of the compound of interest. Moreover, there are some parameters that can be optimized that effectively control the growing conditions: salinity, radiation (luminosity), and nutrient availability. Because algae must adapt rapidly to the new environmental conditions to survive, they produce a great variety of secondary (biologically active) metabolites, with structures that cannot be found in other organisms.

The next step, once the biomass in the target compound (or compounds) is enriched, is to optimize the conditions to extract the valuable components with high yield and activities. Therefore, it is necessary to know not only the selectivity of the process but also the impact of such processes in the global definition of a sustainable process; aspects related to the extract such as yield, quality, and bioactivity should be considered but also other factors such as sustainability, environmental pollution, residues, cost effectiveness, etc. should also contribute to the final selection of the most appropriate extraction process. In this review, green processes have been considered based on the use of sub- and supercritical fluids like CO_2, ethanol, water, and combinations. The development of such processes is a bet that is becoming everyday more and more urgent in our society.

Another important aspect related to microalgae research for valuable compounds is to asses the toxicity of substances or compounds that may accumulate and that could affect the safe use of microalgae and their resultant extracts in products for human and animal use. Some microalgae are very well-known by producing some toxic compounds, some of them actually very dangerous (*159*, *160*). However, most of the valuable species do not produce such toxic compounds and, in fact, have been used as foods for different cultures and in the food industry.

As mentioned at the beginning of this discussion, the number of papers related to the search of bioactives from microalgae is increasing (see **Figure 1**), demonstrating the interest in the field. However, more multidisciplinary research is needed considering, in an integrated way, all of the above-mentioned aspects: chemical composition, biotechnology, extraction, bioactivity, and toxicity. However, also, to effectively transfer the research into a practical field in which the bioactives can be really used as food ingredients in the functional food market, the real functional activity of the extracts/compounds should be determined. Later on, the in vivo effects that could be provided by these compounds should be tested and proved. Finally, to be employed as ingredients in food, different studies should be carried out with the purpose of testing if their activity is maintained after the manufacturing and cooking processes. This kind of procedure is probably more developed in the plant field (*17*). However, similar developments could be performed with microalgae-derived compounds/products in the near future (*151*).

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