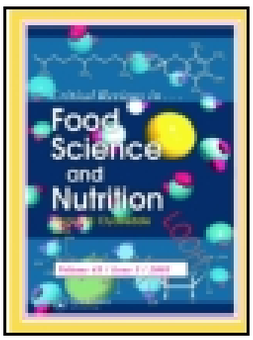

Critical Reviews in Food Science and Nutrition

ISSN: 1040-8398 (Print) 1549-7852 (Online) Journal homepage: https://www.tandfonline.com/loi/bfsn20

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To cite this article: Matheus Lopes Amorim, Jimmy Soares, Jane Sélia dos Reis Coimbra, Mauricio de Oliveira Leite, Luiz Fernando Teixeira Albino & Marcio Arêdes Martins (2020): Microalgae proteins: production, separation, isolation, quantification, and application in food and feed, Critical Reviews in Food Science and Nutrition, DOI: 10.1080/10408398.2020.1768046

To link to this article: https://doi.org/10.1080/10408398.2020.1768046

Published online: 28 May 2020.

CRITICAL REVIEWS IN FOOD SCIENCE AND NUTRITION
https://doi.org/10.1080/10408398.2020.1768046

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REVIEW

Microalgae proteins: production, separation, isolation, quantification, and application in food and feed

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ABSTRACT

Many countries have been experienced an increase in protein consumption due to the population growth and adoption of protein-rich dietaries. Unfortunately, conventional-based protein agroindustry is associated with environmental impacts that might aggravate as the humankind increase. Thus, it is important to screen for novel protein sources that are environmentally friendly. Microalgae farming is a promising alternative to couple the anthropic emissions with the production of food and feed. Some microalgae show protein contents two times higher than conventional protein sources. The use of whole microalgae biomass as a protein source in food and feed is simple and well-established. Conversely, the production of microalgae protein supplements and isolates requires the development of feasible and robust processes able to fractionate the microalgae biomass in different value-added products. Since most of the proteins are inside the microalgae cells, several techniques of disruption have been proposed to increase the efficiency to extract them. After the disruption of the microalgae cells, the proteins can be extracted, concentrated, isolated or purified allowing the development of different products. This critical review addresses the current state of the production of microalgae proteins for multifarious applications, and possibilities to concatenate the production of proteins and advanced biofuels.

KEYWORDS

Arthrospira; biorefinery; *Chlorella*; commercial production; downstream; microalgae farming

Introduction

Algae farming is a promising strategy that might attenuate the impacts of population growth and the increasing of anthropic emissions. Microalgae are a heterogenous group formed by photoautotroph microorganisms that can grow using anthropic emissions as sources of nutrients and convert small molecules, such as carbon dioxide or ammonium, in value-added macromolecules like proteins. Microalgae show high photosynthetic efficiency and growth rate that result in high productivities of carbohydrates, proteins or lipids (Abomohra et al. 2016; Chen et al. 2018; Su et al. 2017). Moreover, microalgae farming can be performed in lands with soil, water or climate that are not suitable for conventional crops. Therefore, microalgae do not compete directly with food crops, and may even help to treat water for agriculture, as well as assist in the distribution of income in arid regions (Badvipour, Eustance, and Sommerfeld 2016; Ncube, Ndlovu, and Tsegaye 2018; Trentacoste, Martinez, and Zenk 2015).

The first ventures of microalgae farming occurred in the second half of the 1900s in different countries for food production (Borowitzka 1999), and more recently, due to the dwindling of oil sources, a great interest arose to use microalgae as a feedstock for the production of advanced biofuels. It is expected that the intensification of the researches and advances in the microalgae-related technologies will allow the production of food, advanced biofuels and other biocompounds in biorefineries with low emission rates, or even zero-waste emissions in a more optimistic scenario (Mitra and Mishra 2019).

The development of economically feasible biorefineries is the keystone for the establishment of microalgae as a feedstock for multifarious applications. Microalgae farming is recent and still under development when compared to conventional agriculture. Moreover, microalgae production differs in many aspects from conventional crops; hence the transference of technology and equipment from the well-established agriculture processes for microalgae farms is drastically limited. Microalgae farming is costly because it is performed in specific and complex systems that keep the microalgal cells at high productivity rates of biomass and biocompounds. Those costs are also associated with the high energy consumption of some downstream processes like the biomass drying and lipid extraction (Dasan et al. 2019).

Despite the great potential presented by microalgae and a plethora of studies focused on the production of advanced biofuels, it still pivotal the reduction of costs of biomass production and processing. Indeed, microalgal advanced biofuels are not yet competitive when compared to other renewable sources used as raw material for the production of biofuels. Researches with microalgae are mainly focused

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on the production of biodiesel (Demirbas and Demirbas 2011; Kumar et al. 2016; Mata, Martins, and Caetano 2010), or pyrolysis of algal biomass to produce bio-oil (Biller et al. 2015; Demirbas 2011; Kumar et al. 2016). The underutilization of microalgae biomass and the low value-added of advanced biofuels are the main problem to produce microalgae solely for the production of advanced biofuels (Ursu et al. 2014; Wijffels, Barbosa, and Eppink 2010).

Microalgae are able to accumulate several biocompounds with potential for the production of food, green chemicals, advanced biofuels; such as carbohydrates, proteins, pigments, vitamins and antioxidants (Demirbas 2011). Thus, microalgae biorefining processes have been proposed as a very promising alternative to sequentially extract biocompounds and develop commercially feasible algae farms (Ansari et al. 2017; Vanthoor-Koopmans et al. 2013; Wijffels, Barbosa, and Eppink 2010). However, it is noteworthy that the commercial feasibility of some microalgae applications, like the production of advanced biofuels, may require the production of millions of tons a year, which may oversupply the market with other microalgal co-products which are not produced at large scale like the advanced biofuels (Benemann, Woertz, and Lundquist 2018). Therefore, a good alternative to improve the commercial attractiveness of algae farming is the development of biorefining methods that results in other biocompounds that fit in high-demand segments; such as proteins, that show a great diversity of applications in food and feed. Indeed, recent studies have been shown that the best sequence of biorefining microalgal biomass is certainly a primary extraction of proteins followed by the extraction of lipids and carbohydrates because this sequence diminishes the losses of proteins, and it may improve the recovery of solvents employed in the lipid extraction step (Amorim et al. 2020; Ansari et al. 2017).

Herein, it is first addressed the potential of microalgae for co-production of proteins and advanced biofuels (section "Microalgae as feedstock for co-production of proteins and advanced biofuels"). Then, the recovery of proteins from the microalgal biomass is extensively covered in order to highlight the possibilities to produce different proteinaceous products (section "Recovery of microalgae proteins"). Section "Microalgae proteins" covers the production of microalgal proteins, as well as their potential applications as food and feed. The perspectives about the expansion of the commercial production of microalgae are briefly addressed in section "Commercial production of microalgae", while different patent applications are presented in section "Patents". In conclusion, it is discussed about the challenges in production of microalgae proteins as food and feed (section "Current challenges in production of microalgae proteins as food and feed").

Microalgae as feedstock for co-production of proteins and advanced biofuels

Several microalgae strains show interesting phenotypic traits for advanced biofuels production what allured the attention of many researchers and companies during the 2000s. However, the establishment of a feasible supply chain and inclusion of biofuels in the energetic matrix of a country is a herculean task. For example, the production of bioethanol in the United States and Brazil began in the 1970s and still replacing just part of fossil fuels. Therefore, researches and perspectives related to the production of advanced biofuels using microalgae as raw material have been redirected for long-term results, and they are now funded mostly by governments and large energy companies with the goal of greenhouse gas reductions (Benemann, Woertz, and Lundquist 2018).

Unfortunately, the emission of greenhouses gases still increases as results of a global energy matrix mostly based on nonrenewable sources. The global energy matrix corresponded for more than 14 billion tons of oil equivalent in 2018, and it resulted in the emission of 32.9 billion tons of carbon dioxide (Enerdata 2019; REN21 2019). Many countries have adopted long-term plans to replace the nonrenewable energy sources by renewable ones to attenuate the negative effects of the emission of greenhouse gases. For instance, Denmark has been adopting policies to establish an energetic matrix based only on renewable energy sources by 2050 (REN21 2019). Due to the concern about the dependence of fossil fuels and their negative impact on the environment, the world experienced the largest investment in renewable energies during the 2010s, it was invested USD 289 billion in this segment in 2018 (REN21 2019). Moreover, efforts have been made, especially in the United States, to recognize microalgae as an agricultural crop that may benefit microalgae farmers in terms of legislation and access to finance as observed in the Agriculture Improvement Act of 2018.

However, microalgae hardly will be an exception among other biomasses that have been screened for the production of advanced biofuels, and more research is required for the development of feasible processes (Chen et al. 2018). A very promising alternative is the sequential fractionation of whole microalgae biomass in different classes of macromolecules using the biorefinery concept, which allows to expand and diversify the microalgae farms products (Ansari et al. 2017; Ursu et al. 2014; Wijffels, Barbosa, and Eppink 2010). Indeed, several microalgae strains have been proposed as robust platforms for the production of multifarious value-added products such as proteins, carbohydrates, antioxidants, essential fatty acids like polyunsaturated fatty acids (PUFAs), carotenoids (e.g., astaxanthin and β-carotene), chlorophylls, phycobiliproteins (e.g., phycocyanin and phycoerythrin), and polyhydroxybutyrate (Chew et al. 2017; García Prieto et al. 2017; Hu et al. 2018).

It is important to highlight that although several biocompounds can be extracted from microalgae biomass, the development of microalgae strains or biorefineries processes should consider the supply chain and demand dynamics for such biocompounds. A recent example occurred in the market of astaxanthin which is partially provisioned by the microalgae *Haematococcus*, and during the 2010s the supply of this carotenoid surpassed the demand and thus lowering its price (Benemann, Woertz, and Lundquist 2018). In this

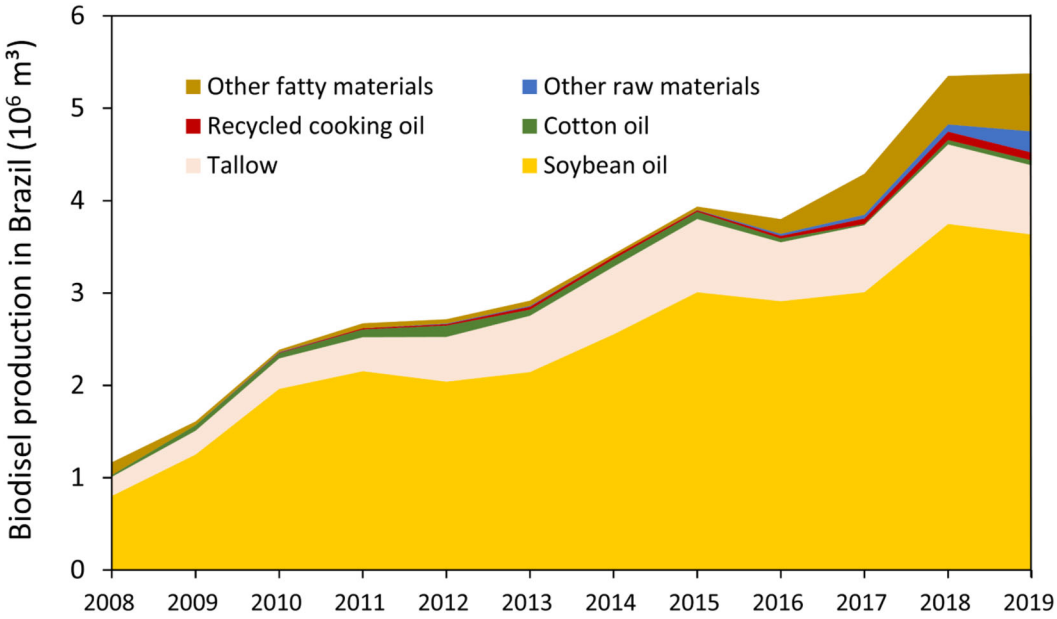

Figure 1. Biodiesel production in Brazil using different raw materials from 2008 to 2019. Adapted from ABIOVE (2020).

context, large-scale production of microalgae is essential to address the demand for advanced biofuels; and consequently, it is very important to identify potential markets for supplying microalgae by-products compatible with the global biofuels scale.

Thus, the production of proteins and advanced biofuels can be considered one of the most promising strategies of microalgae biorefineries. Microalgae like *Chlorella* (Illman, Scragg, and Shales 2000) and *Scenedesmus* (Amorim et al. 2020; Rocha et al. 2019; Soares et al. 2018) show a high content of proteins even when cultured for accumulation of C-rich biocompounds, and these compounds are also preferred for the production of advanced biofuels like bioethanol and biodiesel, resulting in residual biomass with a high content of proteins. Moreover, the production of protein for food and feed is several orders of magnitude bigger than the production of microalgae. The current protein demand for 7.3 billion inhabitants is estimated in approximately 202 million tons (Henchion et al. 2017), and approximately 800 million tons of cereals are used in animal feed (Makkar 2018). Thus, microalgae can be inserted in these markets as protein concentrates, feed formulations, or defatted microalgae (i.e., residual biomass obtained after the extraction of lipids).

A good example of successful co-production of biofuels and protein occurred in Brazil during the 2000s. Soybean oil and animal fats are currently co-products of the food and feed industry, and they corresponded in 2019 for more than 81% of feedstock used in Brazilian biodiesel production matrix that reached 5.3 million m^3 (ABIOVE 2020) (Figure 1). Indeed, the use of co-products was crucial for the establishment of feasible biodiesel industries in Brazil (Coelho and Goldemberg 2013; Stattman, Bindraban, and Hospes 2008).

Recovery of microalgae proteins

Recovery of crude protein from microalgae is frequently performed after the biomass production in open or closed cultivation systems. Recent attempts to develop microalgae strains able to secrete recombinant proteins in the culture medium have been showing promising results (Baier et al. 2018; Lauersen et al. 2015). However, microalgae strains developed for the secretion of specific recombinant proteins show a greater potential to produce biotechnological applications in specific niche markets, while crude proteins might be better produced using microalgae farming based on the biorefinery concept because requires robust processes with easy scalabilities aiming to attend the demand of the food and feed markets.

There are several routes for proteins and advanced biofuels production that can be adopted by microalgae farms (Figure 2). These routes allow the production of defatted high-protein meals, defatted low-protein meals, protein concentrates, protein isolates or purified proteins (Figure 2). However, the degree of complexity to recover proteins increases as the protein purity increases. Thus, the production of crude proteins or protein by-products requires a lower number of processing steps in comparison to the production of proteins with a higher degree of purity (Figure 2).

The downstream processing of microalgae begins with the biomass recovery from the liquid medium after the cultivation. The biomass recovery can be divided into the following stages: harvesting, thickening, dewatering and drying; and these stages allow the increasing of the total solids content by submitting the microalgal biomass to different processes of water removal (Borowitzka and Moheimani 2013). Thus, it is possible to extract proteins from the wet or dry biomass of microalgae which allows the use of different protein recovery strategies. The next step required for protein recovery is the disruption of the microalgae cells in order to release their intracellular components, like lipids and proteins, to the medium. The production of the defatted high-protein meal is simple and requires only that the roasting or drying of the defatted biomass in order to remove moisture and solvents used in the extraction of lipids (Figure 2).

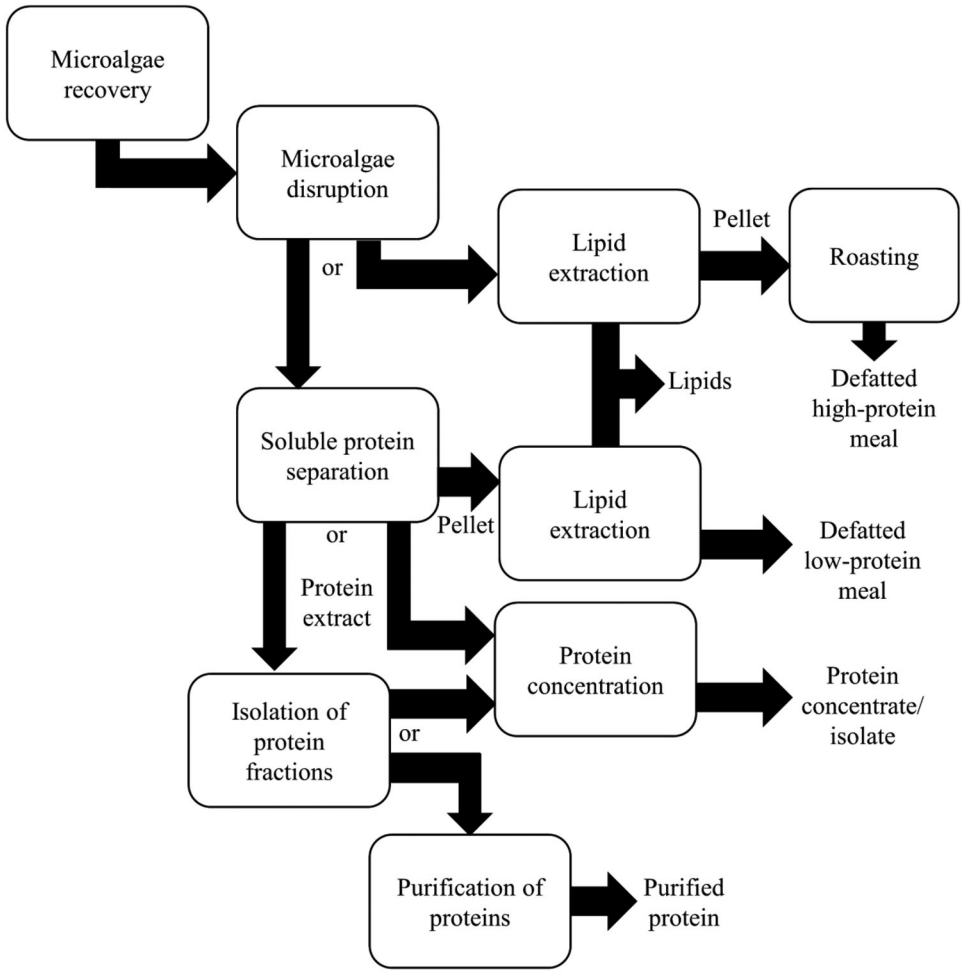

Figure 2. Basic scheme of routes proposed to obtain microalgae proteins.

Otherwise, the production of purified proteins is complex and requires four steps starting with the cell disruption, separation of soluble proteins, isolation of the protein fractions and purification of proteins as described as follows (Figure 2).

Biomass recovery

Microalgae cultures generally present biomass concentrations that range from 0.5 $g{\cdot}L^{-1}$ and 5 $g{\cdot}L^{-1}$ when they reach the stationary phase of growth in open tanks and photobioreactors, respectively (Vandamme, Foubert, and Muylaert 2013). The microalgal biomass should be preferably harvested in order to reduce storage and processing costs. The current harvesting strategies allow to concentrate the biomass up to 100 times, reaching concentrations close to 50 $g{\cdot}L^{-1}$ (Vandamme, Foubert, and Muylaert 2013).

Microalgae biomass are generally harvested by coagulation/flocculation methods. These methods allow microalgae to form large aggregates, thus increasing their size and facilitating the process of separating the cells by sedimentation (Sharma et al. 2013; Vandamme, Foubert, and Muylaert 2013). A microalgal cell normally shows negative surfaces that repel other cells, thus the microalgae remain in the water in the form of a colloidal suspension (Vandamme, Foubert, and Muylaert 2013). To circumvent this phenomenon, methods of chemical flocculation and autoflocculation are widely employed in microalgal harvesting (Sharma et al. 2013; Vandamme, Foubert, and Muylaert 2013).

Chemical flocculation is commonly performed with cationic polyelectrolytes, such as metallic salts and polyacrylamide based-polymers, that interact and neutralize the negative surface of microalgae cells (Pugazhendhi et al. 2019; Vandamme, Foubert, and Muylaert 2013). Despite the high efficiency of the cationic polyelectrolytes, these compounds are difficult to remove, and they contaminate the water and biomass. Thus, the use of cationic polyelectrolytes should be carefully evaluated in microalgae farms that produce biomass for food and feed (Vandamme, Foubert, and Muylaert 2013). There are safer alternatives, such as chitosan and cationic starch, that might be better alternatives to harvest microalgal biomass for food and feed (Vandamme, Foubert, and Muylaert 2013).

A similar issue is observed in microalgal harvesting methods based on autoflocculation. The autoflocculation of microalgae cells can be induced by the addition of calcium or magnesium under alkaline conditions (e.g., pH 9). Calcium and magnesium precipitate at high pH values, and these precipitates show a positive surface that may neutralize the electrical charge of the surface of microalgal cells (Spilling, Seppälä, and Tamminen 2011; Vandamme,

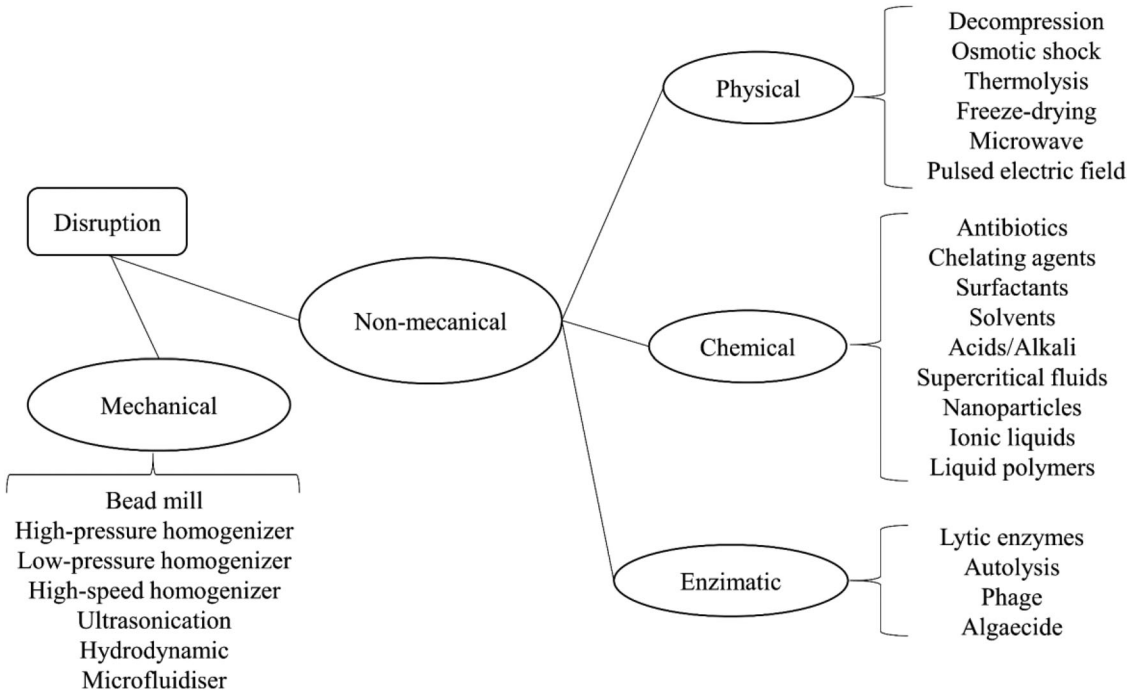

Figure 3. Microalgae disruption techniques. Adapted from Lee, Lewis, and Ashman (2012), Günerken et al. (2015), Lee et al. (2017), and Kumar et al. (2019).

Foubert, and Muylaert 2013). However, the addition of high doses of magnesium or calcium, as well as the pH adjustment to 9 or higher may result in microalgal biomass with a high mineral content requiring further treatment (Vandamme, Foubert, and Muylaert 2013).

In general, the other stages of biomass recovery (i.e., thickening, dewatering and drying) are less prone to change adequacy of the microalgal biomass as food and feed, because they are based on physical methods such as flotation, filtration, centrifugation and convective drying (Borowitzka and Moheimani 2013). It is possible to produce microalgae paste with a total solids content from 15% to 25% (wt/wt) applying dewatering methods. However, some dewatering methods are energetically costly, such as centrifugation, which increases the capital and operating costs (Sharma et al. 2013). The convective drying is used to produce the microalgae powder with a total solids content generally higher than 90% (wt/wt), and it is adequate to increase biomass stability by minimizing spoilage (Borowitzka and Moheimani 2013). Hence, the biomass recovery methods must be carefully chosen according to the application of the microalgal biomass and their associated costs, because the biomass recovery can represent up to 30% of the biomass production costs (Dasan et al. 2019; Gouveia et al. 2016; Ruiz et al. 2016).

Disruption of microalgae cells

Refineries processes are mostly based on the extraction or recovery of biomolecules present inside the microalgal cells. Thereby, it is necessary to lysate the cell and release the intracellular content to buffers or solvents in order to decrease the costs and improve the efficiency of the recovery and extractions processes (Becker 2007). In this sense, the main structure that can hinder the disruption efficiency is the cell wall of the microalgae (Field et al. 2017; Safi et al. 2013).

Microalgae show complex cell walls mainly composed of polysaccharides like cellulose, hemicellulose and β-glucan; furthermore, recalcitrant components like lignin and silica are also observed in red algae (Rhodophyta) and diatoms (Bacillariophyceae), respectively (Popper and Tuohy 2010). The different composition and structure of microalgae cell walls also reinforce the importance of screening for potential commercial strains that are easily disrupted by conventional methods. For instance, it was already shown that *T. suecica* is more susceptible to rupture than *Chlorella* sp. and *Nannochloropsis* sp. (Spiden et al. 2013).

Interestingly, the average energy for disruption of an individual cell of *T. suecica* was determined as 17.4 pJ, which is equivalent to a specific disruption energy of 673 $J \cdot kg^{-1}$ of dry microalgal biomass (Lee, Lewis, and Ashman 2013). That study showed the importance of the development of efficient technologies for the disruption of microalgae cells because the energy required is much lower than the energy employed in a disruption process. An efficient mechanical cell disruption technique like the hydrodynamic cavitation has a specific energy requirement of approximately 5 orders of magnitude higher (i.e., 33 $MJ \cdot kg^{-1}$) than the average energy required for disruption of individual *T. suecica* cells (Lee, Lewis, and Ashman 2012).

A great number of techniques have been evaluated for the efficient disruption of microalgal cells, and they are divided into mechanical and nonmechanical methods. The different mechanical and nonmechanical methods can be applied individually or in combinations (Figure 3) (Günerken et al. 2015; Lee, Lewis, and Ashman 2012; Lee et al. 2017; Middelberg 1995). However, it is unlikely that a specific disruption technique fits most of the microalgae

species and commercial applications since each technique shows advantages and disadvantages (Table 1). A very important parameter for the adoption of a disruption technique in a downstream process of large microalgae farms resides in its scalability. Thus, mechanical techniques such as high and low-pressure homogenizers, and bead mills show great potential when compared to other disruption techniques (Table 1).

Mechanical disruption of microalgal cells

In general, mechanical techniques are often employed in the disruption of microalgal cells because their effectiveness is less affected by the different species of microalgae (Lee, Lewis, and Ashman 2012). Mechanical disruption of microalgal cells is frequently performed in high-pressure homogenizers or bead mills (Table 1). However, the mechanical disruption techniques are generally inefficient in terms of energy consumption, and the control of the temperature might be required in some cases to avoid the degradation of thermo-unstable compounds or releasing of lytic biocompounds like proteases (Dong et al. 2016; Günerken et al. 2015).

Disruption of the microalgal cells using high-pressure homogenizers consists of pumping the harvested biomass (slurry) through a valve that collides with an impact ring. The cells are disrupted due to the high pressure of the fluid that is accelerated on the stationary surface of the valve (shear forces), and by the occurrence of cavitation resulting from the pressure drop induced by the shear stress (Günerken et al. 2015; Halim et al. 2012; Lee et al. 2017). However, the pressure applied by these homogenizers is highly dependent on the microalgae species, as well as the biomass concentration and the growth conditions employed in the cultivation of the strain (Günerken et al. 2015). The main disadvantages of high-pressure homogenizers are the control of the temperature, production of particulates, and release of different biocompounds which leads to increased costs for the isolation of products of interest (Günerken et al. 2015).

Bead milling consists of the transference of kinetic energy to beads made of steel, glass or ceramic. The beads are placed in a chamber containing the microalgae biomass, and the disruption of cells occurs by compaction and shear forces resultant from multiple collisions of the beads (Chisti and Moo-Young 1986). Bead milling shows greater efficiency and energy consumption when compared to the high-pressure homogenizers, ultrasound, and chemical hydrolysis techniques (Doucha and Lívanský 2008; Günerken et al. 2015; Lee et al. 2017; Postma et al. 2015; Safi et al. 2015). The high energy consumption observed in bead milling often requires the cooling of the equipment during its operation because of the shear stress produced (Doucha and Lívanský 2008).

Nonmechanical disruption of microalgal cells

Nonmechanical disruption techniques consist of the release of compounds from biomass using chemical agents, enzymes or physicochemical methods (Gerken, Donohoe, and Knoshaug 2013; Lai et al. 2014; Lai et al. 2016). In general, nonmechanical disruption techniques are less aggressive than mechanical techniques, so they are employed in recovery or extraction of biocompounds which might degrade under the harsh conditions of the mechanical disruption techniques (Günerken et al. 2015). Nonmechanical disruption techniques are used to perforate or increase the permeability of the cell wall (Vogels and Kula 1992). However, certain chemical agents employed in the nonmechanical disruption might contaminate or degrade target compounds, such as the use of detergents and EDTA that affect the lipid extraction yield from microalgae (Lee, Lewis, and Ashman 2012).

Microalgae disruption using enzymes require low energy and mild operational conditions, but the high costs of enzymes might limit the adoption of this technique in some commercial segments of microalgae products since cocktails of enzymes might be required for efficient hydrolysis (Gerken, Donohoe, and Knoshaug 2013; You et al. 2011). On the other hand, the use of enzymes might simplify further downstream processes like protein isolation. Enzymes show great specificity for cellular components which results in a selective disruption of cells and a well-controlled release of undesired biocompounds as well (Demuez et al. 2015; Günerken et al. 2015). In general, enzymes require longer residence times and well-controlled parameters for high conversion rates when compared to mechanical methods.

The disruption of microalgae cells using chemicals can be performed using a plethora of chemicals such as antibiotics, chelating agents, chaotropic agents, detergents, solvents, hypochlorites, acids and alkalis. However, the selectivity and efficiency of such chemicals vary according to the composition of the cell wall (Keris-Sen and Gurol 2017; Lai et al. 2016; Safi et al. 2014; Safi et al. 2015; Scholz et al. 2014). For example, the presence of algaenan (sporopollenin) in the cell wall of some microalgae species hinder the dispersion of proteins, since this polymer is highly inert to chemical agents (Gerken, Donohoe, and Knoshaug 2013; Safi et al. 2014).

Sodium hydroxide is frequently employed as a nonmechanical and low-cost disruption technique of microalgae cells due to its large production and distribution; furthermore, sodium hydroxide penetrates the crystalline structure of the cellulose and disperses the proteins of the cell wall (Safi et al. 2014; Safi et al. 2015). Protein recovery using sodium hydroxide can be improved using mild temperatures, like 60 °C, and high alkaline conditions as pH above 11 (Amorim et al. 2020; Gerde et al. 2013). However, the degradation of proteins and reduction of the nutritional value of the microalgae by-products can occur under such conditions as a result of Maillard reactions, protein denaturation, cross-linking, and hydrolysis, as well as amino acid degradation (Becker 2013; Gerde et al. 2013).

Separation of microalgal proteins

Depending of disruption technique applied to the microalgae cells, the medium can present proteins, fine particles, intact cells, disrupted or damaged cells, and other undesired compounds and by-products such as nucleic acids and ribosomes (Law et al. 2018; McMillan et al. 2013; Safi et al.

Table 1. Main techniques used for disruption of microalgae cells.

Group	Technique	Disruption mechanism	Factors influencing disruption efficiency	Advantages	Disadvantages	References
Mechanical	Bead mill	• Compaction • Shear stress	• Size and material of beads • Milling speed • Temperature, concentration and viscosity of the microalgae culture	• Efficient disruption • Easy scale-up • High productivity	• Moderate energy consumption • Low selectivity • Severe conditions • Temperature increase • Formation of particulates	Lee, Lewis, and Ashman 2012; Günerken et al. 2015; Doucha and Lívanský 2008; Lee et al. 2017
	High-pressure homogenizer	• Shear stress • Cavitation	• Applied pressure • Pressure drops after valve • Medium temperature • Number of passes • Flow rate • Valve design	• Efficient disruption • Easy scale-up	• Moderate energy consumption • Low selectivity • Severe conditions • Temperature increase • Formation of particulates	Günerken et al. 2015; Lee, Lewis, and Ashman 2012; Safi et al. 2014; Lee et al. 2017
	High-speed homogenizer	• Shear stress • Cavitation	• Design and size of rotor/stator • Medium viscosity • Flow rate • Cell concentration	• Simple • Efficient disruption • Accepts high solid-loading	• High energy consumption • Low selectivity • Severe conditions • Difficult scale-up	Günerken et al. 2015; Lee, Lewis, and Ashman 2012
	Ultrasonication	• Cavitation • Shockwave propagation • Formation of free radicals	• Medium temperature • Medium viscosity • Application time	• Simple • Scaling-up possible	• Low disruption efficiency • Low selectivity • Severe conditions • Temperature increase	Günerken et al. 2015; Zheng et al. 2011; Halim et al. 2012; Lee et al. 2017
	Low-pressure homogenizer	• Shear stress	• Number of passes • Cell concentration • Applied pressure • Valve design	• Efficient disruption • Low energy • Easy scale up	• Low selectivity • Temperature increase	Martins, Vieira, et al. 2019
Nonmechanical	Microwave	• Wall disruption due to vaporization of water inside the cells	• Water activity in cells • Cell concentration • Application time • Frequency applied	• Short processing time • Accepts higher cell concentration • Easy scale-up • Lower power consumption • Efficient disruption	• Moderate energy consumption • Low selectivity • Protein denaturation	Günerken et al. 2015; Lee, Lewis, and Ashman 2012; Zheng et al. 2011; Lee et al. 2017
	Pulsed electric field	• Membrane electroporation	• Electric field strength • Electric field pulses • Cell concentration • Size of cells • Application time	• Proteins with greater stability • Easy scale up • Soft conditions • Greater selectivity • No particulate formation	• Moderate energy consumption • Medium should be free of ions • Medium cannot be conductor • Not able to recover chloroplast proteins	Grimi et al. 2014; Coustets et al. 2015; Günerken et al. 2015; Lee et al. 2017; Carullo et al. 2018
	Enzymatic	• Interaction between enzyme and cell wall substrate	• Type of enzyme • Enzyme concentration • Cell concentration • Temperature • Pressure • Time • Buffer type and concentration	• Greater selectivity • Soft conditions • Low energy consumption • No particulate formation	• Slow process • Costly • Product inhibition • Difficult scale-up	Günerken et al. 2015; Zheng et al. 2011; Lee et al. 2017
	Ionic liquid	• Solubilization of lignocellulosic materials in cell wall	• Ionic liquid class • Reaction time • Reaction temperature	• Negligible vapor pressure • Thermal and chemical stability • Recovery and reuse • Greater selectivity • Low energy consumption	• High cost	Jeevan Kumar et al. 2017; Zhu et al. 2019; Liu et al. 2019
	Chemicals	• Interaction between reagent and cell wall substrate	• Shaking • Type of reagent • Cell wall composition • Reagent concentration • Cell concentration • Temperature • Time	• Greater selectivity • Moderate conditions • Low energy consumption	• Slow process • Contamination by reagent • Product degradation • Limited protein recovery	Chisti and Moo-Young 1986; Günerken et al. 2015; Safi et al. 2015

(*continued*)

Table 1. Continued.

Group	Technique	Disruption mechanism	Factors influencing disruption efficiency	Advantages	Disadvantages	References
	Osmotic shock	• Elevation of osmotic pressure	• Type of salt • Salt concentration • Presence and composition of the cell wall • Type of microalgae	• Simple • Low energy consumption • Soft conditions • Easy scale-up	• Low disruption efficiency • High cost for salt recovery • Protein precipitation	Lee et al. 2010; Yoo et al. 2012; Lee et al. 2017

2015; Scopes 1994). The separation of microalgae proteins from cellular debris is mainly based on their dispersibility in water and the separation of the protein-rich aqueous phase from the solid phase (Carullo et al. 2018; Safi et al. 2014; Safi et al. 2015; Unterlander, Champagne, and Plaxton 2017; Ursu et al. 2014). Centrifugation is largely employed in the separation of the protein-rich aqueous phase from the solid phase (Coustets et al. 2015; Gerde et al. 2012; Gerde et al. 2013; Safi et al. 2014; Safi et al. 2015; Sattayasai 2012). However, the parameters settled for separation of the microalgal proteins by centrifugation, like high acceleration (e.g., 10,000 g) and low temperatures, might limit the scaling of this technique to microalgae farms (Coustets et al. 2015; Gerde et al. 2012; Gerde et al. 2013; Safi et al. 2014; Safi et al. 2015; Sattayasai 2012; Unterlander, Champagne, and Plaxton 2017; Ursu et al. 2014). Centrifugation also results in protein-rich supernatant with a high content of chlorophyll because this nonpolar pigment remains adhered to fine particles that remain in the aqueous phase, probably in the form of colloidal dispersion (Gerde et al. 2012; Safi et al. 2014). Alternatively, the separation of the protein-rich liquid phase can be achieved by filtration and ultrafiltration (Sattayasai 2012; Ursu et al. 2014).

Thus, it is important to improve the dispersion of proteins in the aqueous phase in order to achieve an efficient separation. The dispersibility of proteins in water depends, basically, on the relationship between protein-protein and protein-water interactions, and the factors that increase protein-water interactions or decrease protein-protein interactions induce the protein dispersion (Damodaran, Parkin, and Fennema 2007). The most important parameters for efficient dispersion of proteins are the ionic strength and pH (Arakawa and Timasheff 1985; Jubeau et al. 2013; Stack et al. 2018). At low ionic strengths, charged ions reduce the dielectric constant of water and reduce the salt bridges interactions between proteins, increasing the interactions water-protein and the protein dispersibility, a phenomenon called salting in. However, in systems with high concentrations of salts, the proteins compete with the salts for water molecules reducing protein-water interactions and protein dispersibility, this is called salting out (Damodaran, Parkin, and Fennema 2007; Pylaeva, Brehm, and Sebastiani 2018).

The pH adjustment is a simple and low-cost process with great scalability that improves the dispersion of proteins. The degree of dispersibility of proteins decreases as the pH is near their isoelectric points which results in greater interactions between these proteins. The opposite phenomenon is observed for pH values that are too different from the isoelectric points of the proteins which results in electrostatic repulsions increasing their dispersibility (Damodaran, Parkin, and Fennema 2007; Safi et al. 2015; Ursu et al. 2014). Most of the proteins of commercial microalgae show a specific range of isoelectric point which is between 5 and 7 in *H. pluvialis* (Ba et al. 2016), and 4 to 5.5 (major group) and 6 to 8 (minor group) in *C. vulgaris* (Ursu et al. 2014); while the isoelectric point of *A. platensis* is lower and ranged from 2.8 to 3.5 (Chronakis et al. 2000). Thus, it is possible to disperse most of the microalgae proteins by the increasing of the pH, and higher protein recovery yields can be achieved combining the pH adjustment with the mechanical techniques of cell disruption (Ursu et al. 2014) and temperature (Amorim et al. 2020; Gerde et al. 2013). However, the adoption of harsh conditions in protein extraction should be carefully evaluated. It was already shown that protein extraction of *C. vulgaris* at high pressure assisted by alkaline pH affected the protein folding leading to protein aggregation and loss of emulsifying properties (Ursu et al. 2014).

The three-phase partitioning system is a recent technique that has been proposed for separation of microalgae proteins. The three-phase partitioning fractionates the microalgae components in nonpolar (upper phase) and polar phases (lower phase), while proteins were kept in the middle phase through a combination of ammonium sulfate for protein precipitation, and *t*-butanol to increase the protein buoyancy. The use of optimized conditions of three-phase partitioning resulted in a protein extract of *Chlorella pyrenoidosa* with a concentration of 78 % (wt/wt) (Waghmare et al. 2016). However, the high concentration of ammonium sulfate (20–50%, wt/wt) and solvents were required to achieve the high protein yields reported in that study. The reduction of chemical inputs is essential for the adoption of the three-phase partitioning technique in the commercial production of microalgae proteins.

Concentration of microalgal proteins

The concentration of microalgal proteins by dewatering might be required for the development of different commercial applications such as protein concentrates and isolates. There are several methods of protein concentration, and an adequate method should be chosen according to the protein characteristics (Scopes 1994). The precipitation of proteins is often performed using trichloroacetic acid or ammonium sulfate, but these chemicals remain in the protein concentrate and their removal might be required for some applications, as in food and feed (Gerde et al. 2013; Pohanish 2002; Sato, Gonmori, and Yoshioka 1999; Scopes 1994).

The ultrafiltration is a technique without salt or acid addition which is adequate for protein concentration. This

technique is based on the use of semipermeable membranes in which small molecules (e.g., water and salts) can flow through, but larger molecules such as proteins are retained by the membranes (Cheryan 1998; Scopes 1994). Another chemical-free technique largely employed in protein concentration is the freeze-drying that consists on remove water, at low temperature and pressure, by sublimation (Gerde et al. 2013; Field et al. 2017; Matejtschuk 2007; Schwenzfeier, Wierenga, and Gruppen 2011; Ursu et al. 2014). Freeze-drying increases the protein stability by reducing the residual water content, which allows the storage of the freeze-dried proteins at room temperature with minimal losses in the protein activity (Kerr 2007; Matejtschuk 2007). However, the salt content of the microalgae protein extract should be evaluated for the adoption of the freeze-drying technique. Freeze-drying increases the concentration of salts of the protein extract after the water removal, which may change the ionic strength and pH resulting in denaturation of proteins (Matejtschuk 2007).

Spray-drying is a very promising technique for the production of microalgal proteins for feeding. In this technique, the microalgal protein extract is sprayed and contacted with hot air (60–250 °C) rapidly which evaporates water, producing dried protein-rich particles that are then separated from the hot air stream (Anandharamakrishnan, Rielly, and Stapley 2007; Mujumdar 2014). Due to the fast contact with hot air, the spray-drying technique allows the drying of heat-sensitive products, and it is widely used for the production of whey proteins, vitamins, enzymes, instant coffee, teas and soups (Anandharamakrishnan, Rielly, and Stapley 2007; Mota et al. 2018; Mujumdar 2014).

Isolation of microalgal protein fractions

Isolation of protein fractions is technically feasible, but its complexity entails higher costs. Therefore, it is not commonly found microalgae protein isolates in the market, and certainly, the isolation of protein fractions is economically unattractive for feed segment. However, the use of microalgae protein fractions in nutritional supplementation might be promising and might allow the supply of high-value-added products.

Protein isolation is based on the separation of proteins in extracts that share similar physical characteristics, such as dispersibility, density or size (Sattayasai 2012; Scopes 1994; Walker 2010). This step is common before obtaining purified proteins because this procedure restricts the number of proteins present in the medium, which facilitates the purification process (Sattayasai 2012; Scopes 1994).

Most methods used to isolate and fractionate proteins are based on changes in dispersibility. As stated earlier, the factors that most affect protein dispersibility are ionic strength and pH. These factors are widely used for the separation of proteins that have similar dispersibility in a specific pH and ionic strength (Damodaran, Parkin, and Fennema 2007; Sattayasai 2012; Waghmare et al. 2016; Walker 2010). In addition, the fractionation and isolation of proteins can be performed based on their density using the gradient centrifugation technique. In this technique, proteins are dispersed in a liquid medium with a density gradient and centrifuged in sequence, the proteins with similar densities can be recovered according to the density gradient of the liquid (Hinton and Dobrota 1978; Ohlendieck 2010; Sattayasai 2012). The gradient density can be obtained using sucrose and Ficoll® (a highly branched neutral polymer) (Ohlendieck 2010; Sattayasai 2012). Another physical property of proteins that allow their isolation is the size using the techniques of ultrafiltration and ultrafiltration-centrifugation wherein the protein concentrate is submitted to a pressure or centrifugal field, respectively, and only water and smaller molecules can flow through the membrane pores, thus proteins can be separated as a function of the pore size of a membrane (Cheryan 1998; Sattayasai 2012; Scopes 1994).

Purification of microalgal proteins

It is unlikely that the purification of microalgal proteins, as well as the isolation of microalgal protein fractions, will be inserted in large microalgae biorefineries as the main process due to their complexity and high costs because high-purity proteins are not required for the production of regular food and feed. Indeed, purification of microalgae proteins might fit for small niche markets like specific branches of the health market. As such, the purification of microalgal proteins for obtaining active peptides that may have health benefits like the angiotensin-I converting enzyme inhibitory peptides from *Nannochloropsis oculata* and *S. obliquus* that show antihypertensive activity (Montone et al. 2018; Samarakoon et al. 2013).

Single proteins with high purity from 90% to 98% can be produced using the current purification processes (Walker 2010). High-purity proteins are frequently used in scientific research for the determination of their amino acid profile, three-dimensional structures and activities (mainly antigens); and these processes of protein purification are very important for therapeutic applications in order to satisfy safety criteria (Durbin and Feher 1996; Walker 2010).

The main technique employed in the purification of proteins is chromatography (Sattayasai 2012; Walker 2010). Chromatographic methods applied to protein purification use molecular exclusion, ion-exchange, affinity and hydrophobic interactions (Sattayasai 2012; Scopes 1994; Walker 2010). The molecular exclusion chromatography allows the separation of proteins according to their size and shape using silica, dextran, agarose, polyacrylamide and polyvinyl chloride as stationary phases (Agyei, Potumarthi, and Danquah 2013; Sattayasai 2012; Wilson 2010). Ion-exchange chromatography allows the separation and purification of proteins based on the magnitude of their net electric charge (Agyei, Potumarthi, and Danquah 2013; Sattayasai 2012; Wilson 2010). The electric charge of the ion exchangers should be made according to the pH that the protein is more stable and soluble. The stationary phases commonly used in ion-exchange chromatography are polystyrene,

cellulose and agarose with the sulfonic and quaternary ammonium ionic groups (Wilson 2010).

The affinity chromatography is an alternative to separate complex mixtures and produce proteins with a high degree of purity conserving its structure and functions (Agyei, Potumarthi, and Danquah 2013; Sattayasai 2012; Wilson 2010). The principle of this technique is simple and it is based on the interaction of the protein with a binder of the stationary phase. The protein is retained at the stationary phase matrix, while other compounds are carried out with the eluent, and the proteins are released from the column using an eluent that is able to carry the purified proteins (Sattayasai 2012; Walker 2010; Wilson 2010). However, the affinity chromatography requires specific stationary phases which increases the operational costs.

Finally, in chromatography by hydrophobic interactions the separation of proteins occurs by hydrophobicity derived from hydrophobic amino acids on the surface of each type of protein (Agyei, Potumarthi, and Danquah 2013; Sattayasai 2012; Walker 2010; Wilson 2010). However, the majority of hydrophobic groups of proteins are within their structures, which may decrease the efficiency of the purification process (Wilson 2010). To circumvent this phenomenon, salts can be added to the medium in order to expose the hydrophobic groups of the proteins to the matrix of the stationary phase. Therefore, the purification of proteins based on chromatographic methods is a costly and complex process that requires careful development of a specific method of purification according to the characteristics of the target protein and protein extract or isolate.

Protein quantification in microalgae farms

Routine analysis for quality control of protein in microalgae farms is essential for monitoring of the biomass production and subsequent downstream processes. Protein quantification can be performed either by evaluating the enzyme activity or estimation of the crude protein content (Scopes 1994). A reliable and accurate method for protein quantification is based on the hydrolysis of proteins in amino acids and their quantification is performed using chromatographic methods; however, this method is costly and time-consuming (Walker 2010). For routine analysis, a detailed analysis of proteins at the amino acid level is certainly superfluous, and other methods that fit better in microalgae farms should be adopted such as total nitrogen (Kjeldahl), ultraviolet absorbance, bicinchoninic acid, biuret, Bradford, and Lowry assays (Georgiou et al. 2008; Mota et al. 2018; Sattayasai 2012; Scopes 1994; Walker 2010).

Methods commonly employed in the quantification of proteins in microalgae are the total nitrogen, Lowry and Bradford assays (Barbarino and Lourenço 2005; Mota et al. 2018). The total nitrogen method is widely used for the determination of protein content in foods (FAO 2002), and a conversion factor of nitrogen to protein, which varies from 5.3 to 6.38, is used depending on the type of food (AOAC 2016; FAO 2002). For foods in which this factor is unknown, the average conversion factor of 6.25 if often adopted by considering 16% of nitrogen in proteins and the content of nonproteins nitrogen is not significant (FAO 2002; Lourenço et al. 2004). However, plants and microalgae present a large amount of nonprotein nitrogen compounds depending on the species, variety or strain, stage of growth and cultivation or production conditions (Lourenço et al. 2004; Mota et al. 2018). Thus, the nitrogen-protein conversion factor should be calculated for microalgae strains produced under specific conditions using amino acid digestion and chromatography prior to the adoption of the total nitrogen method in routine analyses (Lourenço et al. 2004; Mota et al. 2018; Safi et al. 2013).

The Bradford method is based on the absorbance of the binding of Coomassie brilliant blue to proteins, specifically in arginine residues, and to a lesser extent, in histidine, lysine, tyrosine, tryptophan and phenylalanine residues. Thus, the Bradford method is strongly influenced by the amino acids in a protein (Barbarino and Lourenço 2005). In addition, many compounds interfere in the Bradford method such as surfactants (sodium dodecyl sulfate and Triton X-100®), commercial detergents, alkaline extraction buffers like sodium hydroxide and tris(hydroxymethyl)aminomethane (Lucarini and Kilikian 1999). Absorbances of protein samples reacted with the Bradford reagent are compared to an analytical curve prepared with known concentrations of a specific protein, generally bovine serum albumin, for estimation of the total or crude protein content. The Bradford method is adequate for proteins with a molecular mass higher than 3 to 5 kDA, not being adequate for quantitation of small peptides and amino acids (Lucarini and Kilikian 1999).

Lowry method consists of a copper-catalyzed reaction of the Folin-Ciocalteau reagent and proteins, and the absorbance is compared with an analytical curve of known protein concentrations (Barbarino and Lourenço 2005). The Lowry method reacts with peptide bonds but is also very sensitive to amino acid residues tyrosine and tryptophan, and to a lesser extent, cysteine and histidine (Lucarini and Kilikian 1999). Thus, this interaction with peptide bonds during the Lowry method results in a reaction that is less influenced by the amino acid residues in comparison to the Bradford method. In addition, the Lowry method might show interference by amino acid derivatives, ammonium sulfate, amino acids, buffers, surfactants, chelating agents, lipids, nucleic acids, and salts (such as phosphates, potassium and magnesium) (Lucarini and Kilikian 1999).

For quick routine analysis, Bradford and Lowry-based methods have advantages because they are faster and cheaper. However, due to the variety of interfering compounds, the results obtained by these methods may not be accurate. Thus, in an established microalga farm whose production process is standardized, it is recommended for reliable results to use the total nitrogen method in which the conversion factor is determined in advance.

Microalgae proteins

Proteins production in microalgae

Microalgae proteins are present in different parts of the cell like cytoplasm, organelles, plastids, cell wall, and nucleus

(Safi et al. 2015). However, microalgae show a great variation of the number of potential protein-coding genes in their genomes. For example, the cyanobacterium *Arthrospira platensis* and the green microalgae *Haematococcus pluvialis* show, respectively, 6630 and 18,545 protein-coding genes, while *Chlorella* strains show 9349 to 10,240 of these genes (Fujisawa et al. 2010; Luo et al. 2019; Wu et al. 2019). Interestingly, the number of protein-coding genes is not directly correlated to the protein content. For example, microalgae with less protein-coding genes like *Arthrospira* (El-Kassas, Heneash, and Hussein 2015) and *Chlorella* (Liu and Hu 2013), can show high contents of total proteins (e.g., 60–70% on a dry weight basis) in comparison to *Haematococcus pluvialis* that show a protein content of 26% on a dry weight basis (Ba et al. 2016).

An important protein in photosynthetically organisms is the ribulose bisphosphate carboxylase-oxygen (Rubisco). Rubisco is the main enzyme involved in the fixation of inorganic carbon during photosynthesis, and it is found in a protein complex denominated pyrenoid in the microalgae chloroplasts (Kuchitsu, Tsuzuki, and Miyachi 1988). Moreover, Rubisco has been proposed as a potential source of protein for nutrition and a functional ingredient (Di Stefano et al. 2018). Even though microalgae express Rubisco, it was only recently reported that several microalgae did not show a high content of this enzyme (Losh, Young, and Morel 2013). Thus, wild microalgae strains might be not a potential bioresource to produce Rubisco as a protein source. On the other hand, an example of microalgae that accumulates a high content of a single protein is *A. platensis* that show a high content of the protein C-phycocyanin; this phycobiliprotein plays an important role in light-harvesting and shows applications in food, medicine and biotechnology (Eriksen 2008).

Protein productivity in microalgae can be improved by adopting specific cultivation strategies (Rocha et al. 2019; Zeng et al. 2012), and several parameters regulate the protein production and they should be adjusted for each microalgae strain. However, microalgae show a high content of proteins when cultured under nonstress conditions, while different conditions like halostress (Rocha et al. 2019) and nutrient starvation (Fazeli Danesh et al. 2017) induce the accumulation of carbon-rich molecules like lipids and carbohydrates. The most important parameter for high-protein production in microalgae farms is nitrogen (Perez-Garcia et al. 2011). For example, green algae show a nitrogen requirement that corresponds for 5–10% of their biomass, hence the culture media developed for microalgae are formulated with nitrogen between 5 and 50 $mmol \cdot L^{-1}$ to avoid starvation of this macronutrient (Becker 1994).

The nitrogen source is also important for microalgae farms based on the production of proteins. High consumption of nitrogen is expected in these segment of microalgae farms, thus the use of commercial-grade and low-cost nitrogen sources like agricultural fertilizers (Soares et al. 2018) is crucial for feasible microalgae farms. Moreover, some nitrogen sources like ammonium might be toxic (Soares et al. 2018), and the use of urea (Batista et al. 2019) might be preferred for the nutrition of some microalgae strains.

Other important parameters to increase the protein productivity in microalgae are the light quality and intensity, nutrients, temperature, halostress, carbon supply and source, and climate condition (Chronakis and Madsen 2011; Kose and Oncel 2015; Lupatini et al. 2017; Wan et al. 2011). Nutrients, including CO_2, should be adjusted according to the nutritional requirements of the microalgae strain, usually, 1.8 kg CO_2 per kg of biomass (Chisti 2007). The optimum temperature for microalgae cultivation varies according to the species, but usually is between 20 °C and 25 °C (Ras, Steyer, and Bernard 2013; Thompson, Guo, and Harrison 1992). In such a way, some regions might require the use of robust strains that are able to show high productivity of proteins during the winter. Indeed, microalgae strains are already been screened for the production of advanced biofuels using crop rotation and season biomass production strategies (Dahlin et al. 2018).

Interestingly, microalgae can be cultivated in water sources which are not adequate for conventional agriculture, including saline and brackish waters (Rocha et al. 2019). Although cultivation in wastewater is a feature of microalgae farms when associated with halostress, the production of microalgae proteins decreases because salts trigger the lipid accumulation (Rai, Gautom, and Sharma 2015; Rocha et al. 2019). Therefore, the use of saline and brackish waters might be limited for some microalgae strains that show a high protein production in such water sources, like the marine microalgae *Tetraselmis suecica* (Fabregas et al. 1984).

Cultivation systems and strategies are also important to produce biomasses with a high content of proteins and the establishment of feasible microalgae farms. For example, cultivations of *Scenedesmus* using semi-continuous strategies resulted in biomasses with twice the protein content when compared to cultivations performed in a single batch (Rocha et al. 2019). In terms of cultivation, the open systems are largely adopted for commercial applications such as circular and raceway ponds, because they are cheaper and more scalable than closed cultivation systems (Liu and Hu 2013). Microalgae farms based on closed cultivation systems, like tubular photobioreactors, can be a better alternative when it is necessary to avoid contamination of the biomass or achieve very high biomass productivities (Ugwu, Aoyagi, and Uchiyama 2008).

Microalgae proteins for nutrition

Several microalgae species show very interesting nutritional compositions when compared with soybean, corn and wheat (Table 2). These grains are essential for the nutrition of the human population and livestock. The microalgae *Arthorspira maxima*, *Synechococcus* sp., *Chlorella vulgaris*, *Dunaliella salina*, *H. pluvialis*, and *Scenedesmus obliquus* show a very high content of proteins and similar energetic values in comparison to soybean, corn and wheat (Table 2) (Lum, Kim, and Lei 2013; Rocha et al. 2019). Microalgae proteins are pointed as a potential source for nutrition and they are similar to

Table 2. Nutritional composition of grains, microalgae and cyanobacteria (% in dry mass).

Source	Protein (%)	Carbohydrate (%)	Lipid (%)	Energetic value* ($MJ \cdot kg^{-1}$ dried)
Soybean	37	30	20	21.6
Corn	10	85	4	18.5
Wheat	14	84	2	18.5
A. maxima	60–71	13–16	6–7	20.4
C. vulgaris	51–58	12–17	14–22	22.3
D. salina	26–29	16	18–25	16.8
H. pluvialis	48	27	15	21.7
S. obliquus	50–56	10–17	12–14	19.8
Spirogyra sp.	6–20	33–64	11–21	17.6
Synechococcus sp.	73	15	11	24.0

*Gross value estimated as described by Lee and Putnam (1973): 23.43 kJ per gram of protein, 17.15 kJ per gram of carbohydrates and 38.91 kJ per gram of lipid.
Adapted from Lum, Kim, and Lei (2013) and Becker (2013).

Table 3. Amino acid profile of different microalgae proteins and proteins from different sources used in food, in addition to the standard recommended by FAO ($g \cdot 100\,g^{-1}$ of protein).

Source	Ile*	Leu*	Val*	Lys*	Phe* + Tyr	Met* + Cys	Trp*	Thr*	Ala	Arg	Asp	Glu	Gly	His*	Pro	Ser
FAO/WHO	4	7	5	5.5	6	3.5	1	—	—	—	—	—	—	—	—	—
Egg	6.6	8.8	7.2	5.3	10	5.5	1.7	5	—	6.2	11	12.6	4.2	2.4	4.2	6.9
Soybean	5.3	7.7	5.3	6.4	8.7	3.2	1.4	4	5	7.4	1.3	19	4.5	2.6	5.3	5.8
Corn	3.4	11.2	4.9	3.2	8.9	3.3	4.4	3.7	7.2	5.1	6.8	17.6	4.1	—	8.5	4.6
C. vulgaris	3.8	8.8	5.5	8.4	8.4	3.6	2.1	4.8	7.9	6.4	9	11.6	5.8	2	4.8	4.1
D. bardawil	4.2	11	5.8	7	9.5	3.5	0.7	5.4	7.3	7.3	10.4	12.7	5.5	1.8	3.3	4.6
S. obliquus	3.6	7.3	6	5.6	8	2.1	0.3	5.1	9	7.1	8.4	10.7	7.1	2.1	3.9	3.8
A. maxima	6	8	6.5	4.6	8.8	1.8	1.4	4.6	6.8	6.5	8.6	12.6	4.8	1.8	3.9	4.2
A. platensis	6.7	9.8	7.1	4.8	10.6	3.4	0.3	6.2	9.5	7.3	11.8	10.3	5.7	2.2	4.2	5.1
Aphanizomenon sp.	2.9	5.2	3.2	3.5	2.5	0.9	0.7	3.3	4.7	3.8	4.7	7.8	2.9	0.9	2.9	2.9

Ile, isoleucine; Leu, leucine; Val, valine; Lis, lysine; Phe, phenylalanine; Tyr, tyrosine; Met, methionine; Cys, cysteine; Trp, tryptophan; Thr, threonine; Ala, alanine; Arg, arginine; Asp, aspartic acid; Glu, glutamic acid; Gly, glycine; His, histidine; Pro, proline; Ser, serine.
* Amino acids not produced by the human being (essential amino acids), being necessary to acquire them through the feeding.
Adapted from Becker (2007) and Keeney (1970).

proteins obtained from vascular plants (Becker 2013; Chew et al. 2017). However, microalgae species produce different protein pools and have intrinsic characteristics that may facilitate or hinder access to these proteins during feeding. Thus, the use of microalgae proteins for food and feed requires further detailed evaluation other than productivity surveys.

Quality of microalgae protein for nutrition

An important parameter for the evaluation of protein quality is the determination of the amino acid profile. Special interest is given to the essential amino acids because they are not produced by the human body, hence they are obtained by feeding (Damodaran, Parkin, and Fennema 2007). The essential amino acids are isoleucine, leucine, valine, lysine, phenylalanine, tyrosine, methionine, cysteine, tryptophan, threonine and histidine. The Food and Agriculture Organization of the United Nations (FAO/WHO) proposed a reference standard with the recommended content of the essential amino acids in a protein or a mixture of proteins (Table 3). The amino acid profiles of egg and soybean showed adequate contents for all essential amino acids, while the cyanobacterium *Aphanizomenon* sp. showed low contents of these amino acids (Table 3). The amino acid profiles of *C. vulgaris* and *Dunaliella bardawil* were slightly deficient for isoleucine and tryptophan, respectively; while *S. obliquus*, *A. maxima*, and *A. platensis* were deficient for two or three essential amino acids (Table 3). Microalgae show different amino acid profiles when cultured under different conditions (James, Al-Hinty, and Salman 1989), thus the optimization of microalgae cultivation conditions might allow the production of proteins with the adequate amino acid profile.

Even though the essential amino acid profile is a key factor for the evaluation of protein quality. Other parameters like protein bioavailability and digestibility are also important, and several factors might influence the digestibility of a protein, such as conformation, antinutritional factors and the downstream process used for its extraction (Damodaran, Parkin, and Fennema 2007). Thus, different indices have been proposed to facilitate the evaluation of protein quality and they are based on the content of essential amino acids and their bioavailability (Damodaran, Parkin, and Fennema 2007).

The Protein Digestibility Corrected Amino Acid Score (PDCAAS) was adopted by FAO/WHO in 1991 (FAO 2011a; Schaafsma 2000). PDCAAS consists of the ratio of the limiting essential amino acid (i.e., the amino acid with the largest difference in comparison to the reference) of the protein and the amount of the amino acid in the FAO/WHO reference. This ratio is multiplied by the digestibility of the protein (Equations 1 and 2) (Damodaran, Parkin, and Fennema 2007; FAO 2007; Schaafsma 2000).

$$\text{PDCAAS}\ (\%) = \frac{\text{mg of limiting amino acid in 1 g of test protein}}{\text{mg of same amino acid in 1 g of reference protein}} \times \text{TD} \quad (1)$$

$$TD = \frac{I \times (F_N - F_{EN})}{I} \times 100 \quad (2)$$

where TD is the fecal true digestibility, I is the total nitrogen intake, F_N and F_{EN} are the total fecal nitrogen and endogenous fecal nitrogen, respectively.

The Digestible Indispensable Amino Acid Score (DIAAS) was proposed as a more precise protein quality index than the PDCAAS. The DIAAS considers the digestibility of each essential amino acid separately (Equation 3), while PDCAAS considers the digestibility of the protein in the form of nitrogen. Therefore, the DIAAS provides a better representation of the digestibility of each amino acid during digestion (FAO 2011a).

$$\text{DIAAS}\ (\%) = \frac{\text{mg of digestible dietary amino acid in 1 g of the dietary protein}}{\text{mg of the same dietary amino acid in 1 g of the reference protein}} \times 100 \quad (3)$$

In addition to the true digestibility, another index that accurately evaluates protein bioavailability is the Biological Value (BV), which in addition to fecal nitrogen also accounts for the losses of nitrogen in the urine (Equation 4) (Damodaran, Parkin, and Fennema 2007).

$$\text{BV}\ (\%) = \frac{I - (F_N - F_{EN}) - (U_N - U_{EN})}{I - (F_N - F_{EN})} \times 100 \quad (4)$$

where F_N is the total fecal nitrogen, F_{EN} is the endogenous fecal nitrogen, U_N is the total nitrogen lost in urine, and U_{EN} is the endogenous nitrogen lost in urine.

Evaluation of the response of ingested protein on the gain of animal weight is easily obtained using the Protein Efficiency Ratio (PER) (Becker 2013; Damodaran, Parkin, and Fennema 2007). PER consists of a relationship between the weight gain and the amount of protein ingested (Equation 5) (Becker 2013; Damodaran, Parkin, and Fennema 2007).

$$\text{PER} = \frac{\text{weight gain (g)}}{\text{protein intake (g)}} \quad (5)$$

Finally, a common index also used to evaluate the protein quality is the Essential Amino Acid Index (EAAI), which correlates the content of each essential amino acid of a protein and the content of the same amino acid in the egg protein (Equation 6) (Tabarsa et al. 2012). The closer the protein grade is to 100, the higher is its quality (Kent et al. 2015). However, the EAAI does not allow infer about the variability that can occur between amino acids or protein digestibility.

$$\text{EAAI}\ (\%) = \frac{\text{mg of essential amino acid in 1 g of sample protein}}{\text{mg of same amino acid in 1 g of egg protein}} \times 100 \quad (6)$$

Table 4 presents the protein indexes EAAI, PER, BV, and PDCAAS of the microalgae *Scenedesmus* sp., *Dunaliella* sp., *Arthrospira* sp., *Chlorella* sp. in comparison to the following conventional protein sources used as food and feed: egg, soybean, wheat, bovine milk and meat. *Arthrospira* sp. showed low EAAI and PDCAAS which were similar to wheat. These low scores are probably because the EAAI and PDCAAS consider only the limiting essential amino acids, and microalgae proteins generally show a low content of sulfur-containing amino acids (Table 3). In general, the whole microalgae biomasses, but not the protein extracts or isolates, are used to evaluate the BV and PER. The main problem related to the use of the whole microalgae biomass as feed is because several animals do not digest the recalcitrant cell wall of some microalgae (Becker 2007); hence the use of protein extracts and isolates might improve the protein quality indexes, as well as the screening of strains that are easily digested by animals.

Table 4. Scores of some protein indices.

Source	PDCAAS (%)	BV (%)	PER	EAAI (%)
Egg	118	94	3.9	100
Milk (bovine)	121	90	3.1	92
Meat (bovine)	92	74	3.0	86
Soybean	91	74	2.3	85
Wheat	42	65	1.5	63
Scenedesmus sp.	—	60–81	1.1–2.1	71
Dunaliella sp.	—	—	0.77	98
Arthrospira sp.	48	51–82	1.8–2.2	64
Chlorella sp.	—	53–80	0.8–2.2	85

Adapted from Becker (2013), Clément, Giddey, and Menzi (1967), Damodaran, Parkin, and Fennema (2007), Fabregas and Herrero (1985), Hoffman and Falvo (2004), Kent et al. (2015), Lubitz (1963); Oser (1959), Schaafsma (2000), and Waghmare et al. (2016).

Microalgae toxicity

The great diversity of microalgae species and the screening of potential strains for commercial applications require close attention to potential risks in their consumption as food and feed. *Arthrospira*, *Chlorella*, *Dunaliella*, *Haematococcus*, and *Schizochytrium* are "Generally Recognized as Safe" by the United States Food and Drug Administration (Chacón-Lee and González-Mariño 2010). However, a recent study reported that 4 of 18 dietary supplements containing *Arthrospira* and *Aphanizomenon flos-aquae* showed microcystins at levels that exceed the tolerable daily intake value of 0.04 μg·kg bodyweight^{-1} established by the World Health Organization (Roy-Lachapelle et al. 2017). Toxins in algae dietary supplements occur for three main reasons (Roy-Lachapelle et al. 2017): (1) cultivation of toxin-producing microalga like the strain *A. flos-aquae* DC-1 (Zhang et al. 2016); (2) contamination of the microalga culture by a toxin-producing microalga; (3) use of water sources that contain toxins. Thus, it is essential the adoption of strict control of quality, constant monitoring of the algae-based supplements, and establishment of the maximum intake of the different microalgae toxins to ensure the safety of these products (Roy-Lachapelle et al. 2017).

In general, toxin-producing microalgae are cyanobacteria or dinoflagellates (Camacho et al. 2007; Carmichael and Boyer 2016; Qian, Kang, and Ryu 2015). The fast growth of toxin-producing cyanobacteria and dinoflagellates in seas and rivers cause the death of marine animals, and it can lead to the intoxication and death of birds and humans (Richmond and Hu 2013). This phenomenon is known as

Table 5. Toxin-producing algae and the effect of their toxins on health.

Health effects	Microorganisms	Group	Toxin	Type	References
Permanent short memory loss Brain damage Lethargy Partial hoe Death	*Pseudo-nitzschia* spp. *Halamphora coffeaeformis* *Nitzschia navis-varingica*	Diatom	Domoic acid	Neurotoxin	Ajani et al. 2016; Tenorio et al. 2016; Grattan, Holobaugh, and Morris 2016
Vomit Diarrhea Tumorigenesis	*Prorocentrum lima* *Dinophysis acuta* *Protoceratium reticulatum*	Dinoflagellate	Okadaic acid Dinofisistoxins	Genotoxin Cytotoxin	Luo et al. 2017; Nielsen et al. 2016; Qian, Kang, and Ryu 2015
Nausea Diarrhea Sensory disorders Allodynia in cold Low mortality	*Gambierdiscus spp.*	Dinoflagellate	Ciguatoxins Gambierol	Neurotoxin	Roué et al. 2016; Qian, Kang, and Ryu 2015; Grattan, Holobaugh, and Morris 2016
Itching Cellular rupture Fish mortality	*Karlodinium veneficum* *Amphidinium sp.*	Dinoflagellate	Karlotoxins	Cytotoxin	Cai et al. 2016; Qian, Kang, and Ryu 2015
Breathing problems Eye irritation Nausea Diarrhea Loss of motor coordination	*Karenia brevis*	Dinoflagellate	Brevetoxins	Neurotoxin	Watkins et al. 2008; Gold et al. 2013; Grattan, Holobaugh, and Morris 2016
Nausea Diarrhea Headaches Cognitive dysfunction Death	*Microcystis aeruginosa* *Microcystis viridis* *Microcystis botrys* *Microcystis novacekii*	Cyanobacteria	Microcystins Nodularins	Cytotoxin Neurotoxin Hepatotoxin	Bulc Rozman, Jurič, and Šuput 2017; Carmichael and Boyer 2016
Muscle spasms Difficulty breathing Convulsions Death	*Dolichospermum flos-aquae* *Oscillatoria spp.* *Planktothrix spp.*	Cyanobacteria	Anatoxin-a Homoanatoxin-a	Neurotoxin	Carmichael and Boyer 2016
Mouth and lips paresthesia Tongue immobilization Asthenia Cognitive dysfunction Difficulty breathing Nausea Death	*Aphanizomenon flos-aqua*	Cyanobacteria	Aphantoxins	Neurotoxin	Zhang et al. 2013

the harmful algal bloom. Toxin-producing algae and the effect of their toxins on health are shown in Table 5.

Notwithstanding that most of the edible microalgae do not produce toxins, commercial microalgae can be a potential source of purines found in nucleic acids (Becker 2013; Henderson and Paterson 2014; Moudříková et al. 2017) and heavy metals (Sandau, Sandau, and Pulz 1996). The excessive intake of purines by humans and other monogastric animals (e.g., birds) can lead to high concentrations of uric acid in plasma and excreta, and inflammatory diseases such as gout and kidney problems (Choi, Liu, and Curhan 2005). Microalgae can be a potential source of heavy metal because some species, like *C. vulgaris* and *A. platensis*, show a great capacity to remove and accumulate heavy metals from the environment (Sandau, Sandau, and Pulz 1996).

Concerning the quality of algae products for food and feed, it is essential the development of process and quality control practices. Thus, the quality of the water source used in the cultivation of the microalgae should present low content of heavy metals, toxins, predators, and other microorganisms (Becker 2013; Chu and Phang 2019). Another possibility to improve the use of microalgae as food and feed is the biorefining of the biomass to produce high-quality protein extracts and isolates (Becker 2013).

Microalgae applications in food

Microalgae have been used as food in the form of powders, tablets, capsules, or liquids (Brennan and Owende 2010; Spolaore et al. 2006; Wells et al. 2017). The production of microalgal food is oriented to health benefits associated with the consumption of these microorganisms, such as anti-inflammatory, brain development, hypocholesterolemia, antioxidant, blood cell formation and pro-vitamin A (de Jesus Raposo, de Morais, and de Morais 2013; Wells et al. 2017). Moreover, microalgae farming can be performed in lands with soil, water or climate that are not suitable for conventional crops which may allow the production of food and energy in regions wherein people are experiencing hunger (FAO 2009; FAO 2011b; Stamer 2015).

The inclusion of microalgae biomass in the formulation of foods is also a promising strategy to innovate and improve the nutritional values of several products. The use of microalgae in the bakery segment has been showing promising results. For instance, *C. vulgaris* and *A. platensis* were used to increase the iron and selenium content of breadsticks, as well as improve their stabilities in terms of color and texture (Uribe-Wandurraga et al. 2019). The use of *Isochrysis galbana*, *T. suecica*, *Scenedesmus almeriensis*,

and *Nannochloropsis gaditana* as new functional ingredients in the formulation of wheat bread did not change the textural parameters of bread such as hardness, chewiness, and resilience (García-Segovia et al. 2017). The astaxanthin-rich biomass of *H. pluvialis* was also used as a functional ingredient in the formulation of wholemeal flour cookies improving their glucose release and antioxidant properties (Hossain et al. 2017).

The capacity of microalgae to accumulate large amounts of carotenoids and vitamins, like *Haematococcus* and *Arthrospira*, make them very promising to aggregate value in conventional food products. Carotenoids are natural pigments with antioxidant activity and they are associated with the prevention of degenerative diseases and certain types of cancer (Stahl and Sies 2005). It has been shown that *A. platensis* is able to produce and accumulate large amounts of vitamins A, B1, B2, B12, and E (Becker 2013). However, some studies have reported that the predominant form of vitamin B12 produced by *Arthrospira* is not well absorbed by humans and thus it is not a good source of this vitamin (Watanabe 2007).

A recent study showed that protein-rich extracts of *Chlorella protothecoides* have promising sensory properties which are the prerequisite for their incorporation in foods (Grossmann et al. 2020). Many alternative proteins such as pea, potato or rice proteins show low dispersibility of proteins and adverse taste profile at low pH hindering their use in the formulation of beverages (Grossmann et al. 2020). Other foods wherein microalgae were successfully used improving the nutritional or sensorial properties are the formulation of yogurt with *A. plantensis* (Barkallah et al. 2017), and pasta with *C. vulgaris* and *A. maxima* (Fradique et al. 2010). Moreover, microalgae like the *Schizochytrium limancinum* can be fed to dairy cows to increase the content of long-chain n-3 fatty acids and aggregate further human health benefits to dairy products like milk and cheese (Till et al. 2019).

Microalgae proteins also show great potential for techno-functional applications. Some studies have observed that microalgae proteins exhibit high foaming, emulsifying and stabilizing capacities (Law et al. 2018; Nirmala, Prakash, and Venkataraman 1992; Schwenzfeier et al. 2013; Ursu et al. 2014), that confer certain advantages in comparison to plant proteins that are generally poor in techno-functional properties (McCarthy, O'Callaghan, and O'Brien 2013).

Microalgae applications in feed

Feeding is the main operational cost in the farming of cows and calves, milk and hogs, and corresponded to 68%, 75%, 52% of the total costs of production in the United States in 2018, respectively (ERS - USDA 2018). The costs of feeds are highly impacted by nitrogen sources like proteins. Nitrogen-based nutrition is important to upregulate physiological and biochemical processes that convert feed into protein, and it is very important in the production of animals for slaughter, and milk in dairy cows (Gilbert 2004; Hof, Tamminga, and Lenaers 1994; Rezaei et al. 2013). An important parameter for the adoption of novel protein sources in feed formulations is the amino acid profile. Microalgae, like *C. vulgaris* and *A. platensis*, show amino acid profiles that show similarities to soybean which is currently the main source of protein used in feed (Table 3).

Table 6. Minerals present in soy, corn and some microalgae ($mg \cdot kg^{-1}$ dry mass).

Source	Ca	Mg	P	K	Cu	Fe	Mn	Se	Zn
Soybean	1730	—	5664	15896	10.8	85.3	23.8	0.5	29.2
Corn	193	880	2800	3700	1.6	—	3.7	0.001	14.2
C. vulgaris	3600	1100	2800	4000	21.9	198	34.6	0.6	25.4
A. platensis	7220	670	—	8920	69.6	1116	54.5	0.124	240
M. reisseri	3000	900	3100	5100	14.5	256.8	32	0.5	11.5
N. bacillaris	1600	800	2800	4400	22	800.4	17.4	*	10.9
Tetracystis sp.	2000	500	2700	4000	12.8	883.6	19.4	0.6	11

*Value below limit of detection

Adapted from Bøhn et al. (2014), Jiao et al. (2012), Tibbetts et al. (2015), Campanella Crescentini, and Avino (1999), and Vyn and Tollenaar (1998).

In addition, the microalgal biomass is a potential source of minerals (Table 6). *C. vulgaris*, *A. platensis*, *Micractinium reisseri*, *Nannochloris bacillaris*, and *Tetracystis* sp. show very high contents of iron in contrast to soybean (Table 6). Iron plays an important role in respiration, oxygen transport, acid-base balance, and energy metabolism in animals (Tibbetts et al. 2015). Microalgae also show adequate ratios of calcium and phosphorus from 0.6 to 1.3 which is more balanced than soybean and corn, which show calcium and phosphorus ratios of 0.3 and 0.1, respectively (Table 6). The imbalance of the calcium and phosphorus ratio in animal feeding leads to reduced growth, poor feed conversion, anorexia, and skeletal malformations (Tibbetts et al. 2015).

Microalgae biomass have been successfully employed in feed formulations for different animals like cattle, fish, goat, lamb, poultry, pigs and rabbits (Figure 4 and Table 7). In general, microalgae are added to feed formulations at concentrations up to 15%, and the use of higher concentrations of microalgae biomass results in less feed intake by some animals due to the lower palatability of the feed (Austic et al. 2013; Furbeyre et al. 2017; Ginzberg et al. 2000; Norambuena et al. 2015; Peiretti and Meineri 2008; Venkataraman, Somasekaran, and Becker 1994). Feed formulations are generally made with *A. platensis* and *C. vulgaris* (Table 7). In a general way, the use of microalgae in feed formulations improves the weight gain, milk production, and increase the accumulation of PUFAs in tissues, egg and milk (Figure 4 and Table 7).

For example, the daily inclusion of 5–10 g of *C. vulgaris* in the diet of goats (ruminant animals) increased the intake of feed, and improved the digestibility, rumen fermentation and milk yield (Kholif et al. 2017). Feed formulation with *C. vulgaris* also improved the fatty acid profile of the goats increasing the fatty acids associated with health benefits like unsaturated fatty acids and conjugated linoleic acid, while a decrease in the saturated fatty acids was observed (Kholif et al. 2017).

The total substitution of soybean meal by microalgae biomass was already evaluated in the feeding of dairy cows in order to reduce the dependence of the European Union for protein feed imports (Lamminen et al. 2019). The dairy cows were fed with silage and *C. vulgaris*, *A. platensis* or a

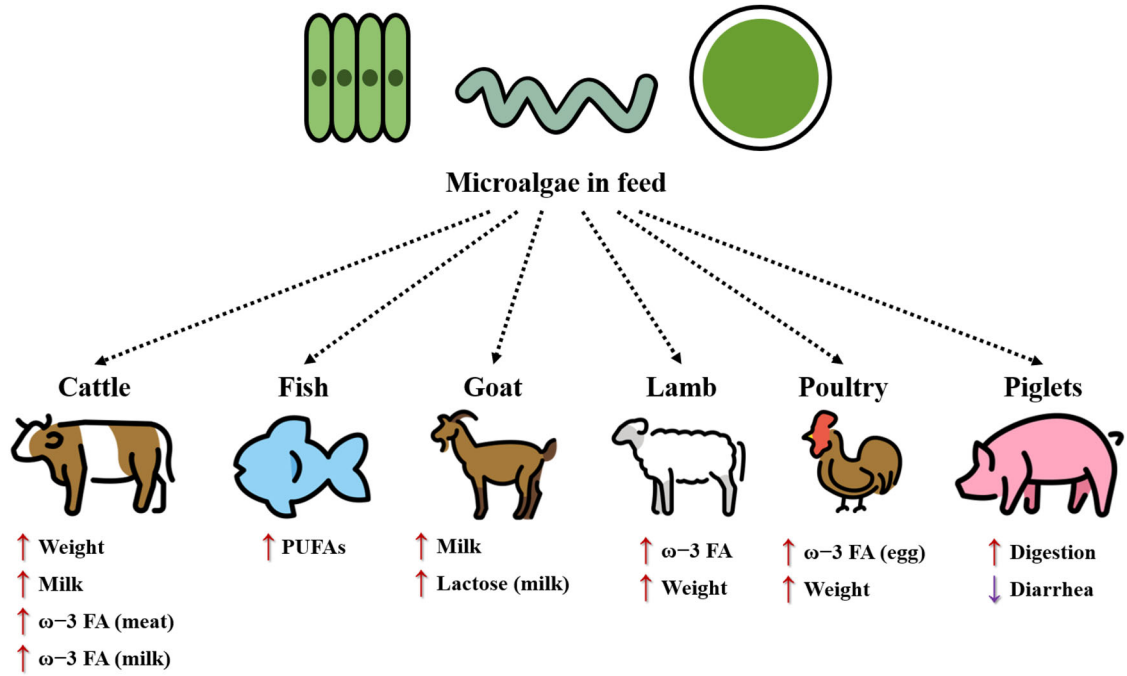

Figure 4. Microalgae used in the formulation of feed and their effect on the nutrition of different animals. Image sources: Sofie Ascherl and Selina Bauder used under license CC BY-SA 4.0.

mixture (1:1) of *C. vulgaris* and *N. gaditana*, and production of milk with a higher content of unsaturated fatty acids was observed in comparison to the fed containing soybean meal. The mixture of *C. vulgaris* and *N. gaditana* resulted in a higher relation of Ω-3 to Ω-6 fatty acids when compared to diets with *A. platensis*, *C. vulgaris* and soybean meal. However, it was observed that the cows preferred the silage, reducing the consumption of microalgae in comparison to the control group fed with soybean meal. This was probably due to the low palatability of microalgae in the concentrate. Thus, it is necessary to screen and develop strains with improved palatability or adding additives that may increase their palatability to improve the consumption of microalgae in feed formulation (Robertson, Gordon, and Pérez-Barbería 2006).

In fact, the whole microalgae biomass can be used in the formulation of feed, especially *A. platensis* that shows a very high content of protein (EL-Sabagh et al. 2014; Furbeyre et al. 2017; Lamminen et al. 2019; Venkataraman, Somasekaran, and Becker 1994). However, microalgae can be processed using the biorefinery concept aiming for better use of the biomass according to their biochemical composition (Ursu et al. 2014). For instance, microalgae have been proposed as a source of lipids for advanced biofuels production, but lipids respond to 20–50% of the biomass (Hu et al. 2008). Thus, the defatted biomass of microalgae obtained after the lipid extraction that contains proteins has been proposed as a source of proteins for animal feed (Austic et al. 2013; Ekmay et al. 2014).

The defatted biomass of *Desmodesmus* sp. and *Staurosira* sp. were used in feed formulations for weanling pigs and broiler chickens (Austic et al. 2013; Ekmay et al. 2014) (Table 7). The feeding of broiler chickens with a formulation containing defatted biomass of *Staurosira* sp. with a crude protein content of 19% resulted in reduced growth and loss of productivity due to the deficiency of amino acids and low level of crude protein in comparison to soybean meal, hence further supplementation with amino acids was required (Austic et al. 2013). Conversely, the use of defatted biomass of *Desmodesmus* sp. with a crude protein content of 31% in feed for weanling pigs and broiler chickens resulted in similar or higher growth performance in comparison to the control feed lacking the defatted biomass of this microalga (Ekmay et al. 2014). These previous studies clearly show the potential of the defatted biomass as a protein source for feed production.

Commercial production of microalgae

The food market unveiled the commercial potential of microalgae and it still driving their production in different countries. Microalgae production is constantly increasing at impressive rates over the last decades (Figure 5). Noteworthy, between 1999 and 2004 the market experienced a 7-fold expansion that resulted in a production of 7000 tons of microalgae biomass per year (Brennan and Owende 2010; Enzing et al. 2014; Fernández, Sevilla, and Grima 2013). Recent estimates show that yearly production of microalgae ranged between 19,000 and 20,000 tons between 2016 and 2018 (Benemann, Woertz, and Lundquist 2018; Transparency Market Research 2016). A fast-paced growth is projected for 2024 when it is expected production of 27,500 tons per year with a market value of USD 1.1 billion (Transparency Market Research 2016).

Even though 50,000 species of oxygenic photosynthesizers microorganisms are conveniently grouped as microalgae, only a few members have been cultivated at the commercial level (Figure 6). The cyanobacterium *Arthrospira*, and the

Table 7. Microalgae used in the formulation of feed and their effect on the nutrition of different animals.

Animal	Microalgae tested	Observation	References
Calf	*A. platensis*	Added to milk: 2, 6, and 25 g·day^{-1} 6 g·day^{-1} showed the highest weight gain 25 g·day^{-1} showed the lowest conversion efficiency	Heidarpour and Eghbalsaied 2011
Cattle	*S. limacinum*	Added to feed: 15 g·kg^{-1} and 30 g·kg^{-1} of cattle mass Animal meat presented higher Ω-3 fatty acid content Light seaweed flavor the greater the presence of Ω-3 fatty acid in muscle Remaining sensory characteristics poorly affected	Rodriguez-Herrera et al. 2018
Dairy cow	*A. platensis*	Added to the feed: 200 g·day^{-1} Tested animals presented higher milk production Animals tested showed superior lactose content	Kulpys et al. 2009
Dairy cow	*A. platensis; C. vulgaris; Microalgae mixture* (*C. vulgaris* and *N. gaditana*)	Substitution of soybean in concentrate feed No difference in dry mass intake but higher intake of silage in microalgae diets Higher fat content in milk of cows fed with microalgae Higher relation of Ω-3 to Ω-6 fatty acids, and higher production of eicosapentaenoic acid in milk of cows fed with *C. vulgaris* + *N. gaditana*	Lamminen et al. 2019
Fish	*Entomoneis* spp.	Added to feed: 2.5% and 5% There was no growth difference between the fish tested and the control Fishes tested showed higher concentration of PUFAs	Norambuena et al. 2015
Goat	*C. vulgaris*	Added to feed: 5 and 10 g·day^{-1} Higher feed intake in animals with microalgae in the diet Increase in milk production Increase in total solids content and lactose in milk	Kholif et al. 2017
Lamb	*Schizochytrium* sp. (DHAGold®)	Added to feed: 2% Higher concentration of PUFAs, especially Ω-3 fatty acids No nutritional difference found in muscles	Díaz et al. 2017
Lamb	*A. platensis*	Added to feed: 0.1 g·kg^{-1} of the animal Higher feed intake and higher body mass gain Higher cholesterol and glucose levels	EL-Sabagh et al. 2014
Laying hens	*Porphyridium* sp.	Added to feed: 5% and 10% Higher content of PUFAs in eggs of animals tested with 10% Eggs showed darker yellow coloration	Ginzberg et al. 2000
Piglet	*Desmodesmus* sp. (defatted biomass)	Added to feed: 10% No differences to control	Ekmay et al. 2014
Piglet	*A. platensis; C. vulgaris*	Added 1% replacing the antibiotic colistin after weaning Diarrhea incidence reduced in dietary with *C. vulgaris* Greater villous height Greater digestibility in tract	Furbeyre et al. 2017
Poultry	*A. platensis*	Added to the feed: 14% and 17% Greater weight gain in animals fed with 17% Meat with color more intense	Venkataraman, Somasekaran, and Becker 1994
Poultry	*Staurosira* sp. (defatted biomass)	Added to feed: 7.5% and 10% Feed formulation containing 10% *Staurosira* sp. was not well accepted Feed formulation containing 7.5% *Staurosira* sp. requires amino acid supplementation	Austic et al. 2013
Poultry	*Desmodesmus* sp. (defatted biomass)	Added to feed: 15% Greater growth in the animals	Ekmay et al. 2014
Rabbit	*A. platensis*	Substitution to the feed: 5%, 10%, and 15% Substitution did not differ from the control for final weight and weight gain Substitution with 10% led to higher feed consumption	Peiretti and Meineri 2008

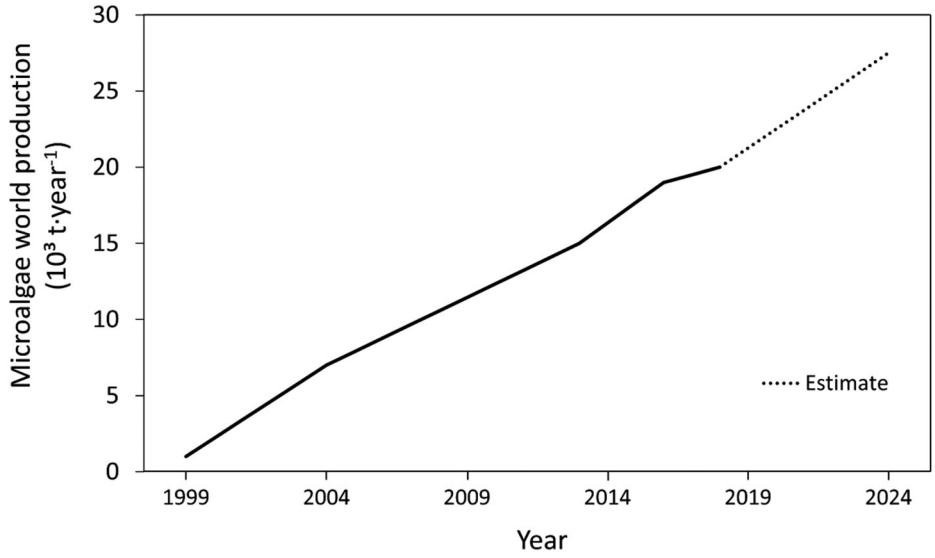

Figure 5. Microalgae world production growth. Adapted from Brennan and Owende (2010), Benemann (2013), Enzing et al. (2014), Transparency Market Research (2016), and Benemann, Woertz, and Lundquist (2018).

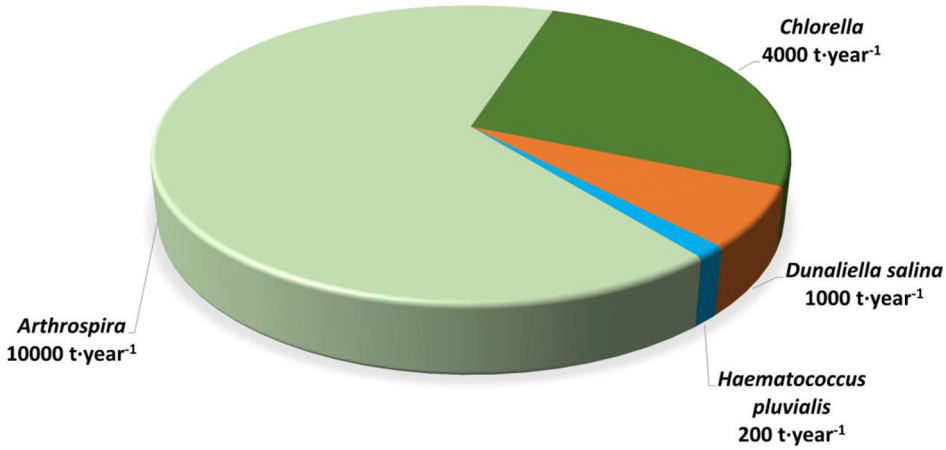

Figure 6. Main microalgae produced in the world in 2013. Adapted from Benemann (2013).

green microalgae *Chlorella* and *Dunaliella* are commercially produced as a functional food, while high-value-added foods like astaxanthin and *β*-carotene are produced from the green microalgae *Haematococcus* and *Dunaliella*, respectively (Brennan and Owende 2010; Yan et al. 2016). Many other microalgae genus have been screened and improved for large scale production like *Tetraselmis* (Benemann, Woertz, and Lundquist 2018), *Euglena* (Schwartzbach and Shigeoka 2017), *Nannochloropsis* (Ma et al. 2016), and *Scenedesmus* (Rocha et al. 2017; Silva et al. 2020).

Patents

Microalgae show great potential for commercial production and as a result, patents have been granted by national and regional offices to different countries, at least 5750 patents are related to processes and strategies for microalgae. Patent applications already cover many potentials uses of microalgae proteins such as food, nutraceuticals, feed, recombinant proteins, and protein recovering processes (Table 8).

For instance, the patent numbers US8552160B2 and US8741629B2 claimed methods based on a biorefinery concept of selective extraction and fractionation of microalgae proteins using single or multistep processes. These methods allow efficient separation of microalgae proteins for the production of feed and resulted in a lipid-rich deproteinized biomass that can be used in biofuel production. The patent BR1020180049739 describes a method for microalgae disruption without excessive fragmentation of cells, aiming the extraction and concentration proteins of free of cell debris. This patent claimed a device for disruption of microalgae cells at low-pressure homogenization and generates enough shear stress to disrupt microalgae cells at working pressures below 200 bars. The reduction of energy consumption also can be achieved by the development of more efficient and cleaner cultivation systems. The patent BR1020180118463A2 claimed an automated mixing system for open tanks based on solar energy as the only source of energy required for its operation.

The patent WO2011057406A1 proposed a method to produce protein concentrates and isolates using phytases and

Table 8. Patents application with potential use in the food and feed market.

Patent number	Status	Claim	References
US8552160B2	Granted	Methods for extraction and fractionation of proteins from microalgae biomass	Kale 2013
US8741629B2	Granted	Method for extraction of protein globulins from an intact microalgae biomass	Kale 2014
EP1433463B1	Granted	Use of microalgae proteins, or their derivatives, in cosmetics for the retention of moisture in the skin and hair	Hagino and Saito 2010
WO2017137668A1	Pending	Method for protein enrichment of a microalga, *Chlorella protothecoides*, cultivated under heterotrophic conditions	Le Ruyet et al. 2017
WO2013086302A1	Pending	Methods for extraction of peptides and amino acids from algae using water at subcritical temperature	Kumar and Hatcher 2013
WO2014027871A1	Pending	Methods of biomass disruption by applying rotating induced magnetic field, and isolation of bioproducts accumulated in the algal cells	Bendikiene, Romaskevicius, and Kiriliauskaite 2014
EP3024923B1	Granted	Method for maintaining the quality and stability of protein-rich microalgae biomass (>50% protein) over time by post-harvest fermentation	Druon, Patinier, and Toursel 2014
WO2015006541A2	Pending	Composition of animal feed with grains, a source of nonalgal proteins, microalgae, protease and oil	Lei 2015
WO2015171950A1	Pending	Method for providing animal feed composed by 65% of defatted microalgae	Piechocki et al. 2015
WO2016009146A1	Pending	Method for preparing a protein isolate from microalgae (*Chlorella*) through triple-phase separation followed by membrane separation or protein precipitation	Patinier 2016
WO2011057406A1	Pending	Method for production of protein concentrates and isolates from microalgae through the removal of fiber using low g-force centrifugation	Qingnong 2011
EP2658959A1	Granted	Method for growing, harvesting, disruption and isolation of biocompounds of interest in microalgae, such as proteins, lipids, PUFAs, vitamins, pigments and minerals	Milos et al. 2013
US4915961A	Granted	Food with possible health benefits containing *Dunaliella* powdered microalgae packaged with plastic wrap	Tanaka 1990
BR1020180049739	Pending	Method and device for microalgae disruption using low pressure homogenizer technique	Martins, Vieira, et al. 2019
BR1020180118463A2	Pending	Automated method and device for agitation of microalgae culture medium in an open tank using solar energy as energy source	Martins, Mendes, et al. 2019

cellulases followed by centrifuging at low g forces to separate the solid fiber from protein slurry. The protein slurry is filtered for the production of protein concentrate. On the other hand, the protein isolate is obtained from the permeate in the filtration step, then the system is subjected to pH adjustment to collect precipitated proteins. Patent WO2016009146A1 claimed a method for production of protein isolates of *Chlorella*. The method of the patent WO2016009146A1 consists in the mechanical disruption of *Chlorella* cells using a ball mill, that resulted in a stable emulsion. Then, the emulsion was destabilized by enzymatic digestion followed by the addition of ethanol that resulted in a tertiary system wherein the upper phase was rich in lipids, the middle phase contained soluble compounds, and the lower was composed by the cellular debris. The protein isolates were obtained from the middle phase by filtration (microfiltration and ultrafiltration) or precipitation (pH adjustment and centrifugation), followed by pasteurization and spray dring.

Current challenges in production of microalgae proteins as food and feed

Several microalgae strains show promising traits for the development of microalgae farms and biorefineries. Moreover, investments have been made with a focus on the long-term potential of microalgae as a novel crop in modern agriculture. The recognition of microalgae as an agricultural crop also may benefit microalgae farmers in terms of legislation and access to finance. Yet, most of the studies with microalgae have been conducted at a laboratory scale, and breakthrough discoveries are required to boost the production of microalgae at a commercial scale. A current challenge in the commercial use of microalgae is the reduction of the high costs of production and extraction of biocompounds when compared to other biomasses sources that have been rationally produced, domesticated and improved by humankind since the Neolithic era (Chung et al. 2017). A reduction of at least one order of magnitude is necessary to improve the commercial competitiveness of microalgae as raw material for the production of food, feed and biofuels (Benemann 2013).

Microalgae production costs are mainly associated with the cultivation (upstream processes), and harvest and processing of biomass (downstream processes). One of the main upstream costs that need to be reduced is that of nutrients for the crop, reaching values as EUR 79 per kilo of dry mass (Gouveia et al. 2016). Studies for the reuse of the culture medium have already been carried out, as well as the use of wastewater. However, the use of wastewaters may lead to contamination of microalga products limits their use in the food segment (Gouveia et al. 2016). Another important point related to the upstream costs is the farming system, photobioreactors allow better control of growth parameters but present high operating costs (Zittelli et al. 2013). Thus, it is necessary to improve cultivation systems of open tanks and raceways, and develop monitoring systems that allow

adequate control of large production units. The automation of the microalgal cultivation systems certainly will increase biomass productivity and reduce cultivation costs.

Microalgae recovery represents one of the main costs of the downstream processing, which can represent up to 30% of the total cost of microalgae production (Dasan et al. 2019; Gouveia et al. 2016; Ruiz et al. 2016). Among the various methods used to concentrate microalgae during harvesting, the flocculation-based methods present lower costs (Gerchman et al. 2017; Ríos et al. 2013). Inorganic salt flocculants like $Al_2(SO_4)_3$, $Fe_2(SO_4)_3$, and $FeCl_3$ are widely used in water treatment, but they are less efficient than polymeric flocculants (e.g., polyacrylamides, modified starches and chitosan) to flocculate microalgae cells (Gerde et al. 2014). Despite polyacrylamides show high recovery efficiency in flocculation of microalgae cells, low production costs and low toxicity in humans and animals, the polyacrylamides have low biodegradability. Moreover, it is important to check the presence of residual acrylamide monomers in the composition of polyacrylamides, because acrylamide monomers are potent neurotoxins (King and Noss 1989; Pugazhendhi et al. 2019). Thus, the use of modified low-cost starches seems promising because they show high efficiency, high biodegradability and low toxicity (Gerde et al. 2014; Pugazhendhi et al. 2019). Another challenge in downstream processing of microalgae is the development of low-cost and efficient methods for releasing of biocompounds because the current methods are based on mechanical disruption and present high energy consumption and low selectivity, what could increase the costs to recover, concentrate and purify specific biocompounds (Günerken et al. 2015).

Surely, the commercial production of microalgae requires the development of robust processes using the biorefining concept to aggregate value to the main biocompounds present in the microalgae biomass. It was already shown that the successful inclusion of biofuels in the energetic matrix of a country requires the commercial production of other high-valued added by-products such as proteins. Thus, the use of microalgae by-products as an input for the production of food and feed is the key to improve the viability of microalgae farms. Animals such as calves, cattle, dairy cows, fishes, goats, lambs, laying hens and poultry showed a good response to feeding with microalgae, either with higher productivity or higher nutritional quality in the final product. However, some studies have shown that the use of microalgae biomass or defatted microalgae biomass in animal feed requires further improvements, especially in order to improve their palatability. Thus, it is important that further studies be carried to evaluate the performance of the use of microalgae proteins in feed, and also to improve the palatability of the feed to reduce possible rejection by animals.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by Petróleo Brasileiro S/A (Petrobras) under grant 4600545175; Brazilian National Council for Scientific and Technological Development (CNPq) under grants 307147/2015-0 and 155994/2018-2; Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) under grant APQ-01357-14; Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brazil (CAPES) under grant 001.

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