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Chapter

Isolation, Characterization, and Biotechnological Potential of Native Microalgae From the Peruvian Amazon

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Abstract

The objective of this chapter is to provide scientific information on basic aspects to be taken into account to achieve the successful isolation, biochemical, and molecular characterization and then to evaluate the biotechnological potential of native microalgae of the Peruvian Amazon. Recent investigations reported by our research team has demonstrated that the isolated native microalgae from the Peruvian Amazon have a great potential for the biotechnological production of biodiesel and nutraceuticals. This biotechnological potential was identified thanks to the application of various protocols that were standardized by the authors over the last 5 years. In conclusion, the native microalgae of the Peruvian Amazon have biotechnological potential and are therefore promising for the production of both biodiesel and nutraceuticals. Various species of microalgae were identified, isolated, cultured, and characterized using biochemical, nutraceutical, and molecular techniques, the isolation stage being the starting point to achieve various biotechnological applications. *Ankistrodesmus* sp. is one of the microalgae with potential for the production of biodiesel and microalgae such as *Haematococcus pluvialis*, *Scenedesmus* sp., and *Chlorella* sp., among others demonstrated a high potential for nutraceutical production. The stress conditions to which microalgae are subjected are being a determining factor for the production of biodiesel and nutraceuticals.

Keywords: biotechnological potential, biodiesel, nutraceuticals, Peruvian Amazon

1. Introduction

From the biotechnological point of view, the term microalgae refer to those microorganisms that contain chlorophyll-a and/or other similar pigments, which allow them to perform oxygenic photosynthesis. In this context, cyanobacteria or green-blue algae, prokaryotes, have traditionally been considered within the group of microalgae. According to this definition, photosynthetic bacteria are excluded,

since they do not contain chlorophyll-a and perform anoxygenic photosynthesis. Therefore, the term microalgae have no taxonomic meaning and within it, organisms with two different cell types are included: cyanobacteria that have prokaryotic cell structure and the remaining microalgae with eukaryotic cell structure [1]. Microalgae are characterized by accumulating triglycerides due to their photobiosynthetic capacity [2, 3], can sequester CO_2 from industrial sources [4] and demand less cropping area than traditional oleaginous plants [5]. In addition, microalgae can produce various substances of commercial interest, such as nutrients, food additives, drugs, and other substances [6, 7].

Due to this great biosynthetic diversity, isolates have been made and there are collections of microalgae in several institutions around the world [8]. An estimated 50,000 species have been identified and are kept in collections [9]. These only represent a small fraction of the enormous biodiversity of species that exist. Likewise, it is estimated that less than 10% have been evaluated for their biodiesel production capacity and only of some species their genomes have been sequenced [10, 11].

Therefore, in the Laboratory of Biotechnology and Bioenergetics of the Scientific University of Peru, efforts have been initiated to isolate and increase the diversity of the collections and be more likely to find ideal microalgae strains for the production of biodiesel, nutraceuticals, bioremediation, and other biotechnological applications. It should be noted that there are several methods of isolation, which depend on the dimensions of the microalgae, their mobility, and their morphology. The most commonly used methods are: (a) micropipette isolation, (b) on agar plates, and (c) serial dilutions. However, it is advisable to combine all these techniques to allow isolation and have unialgal cultures [12]. Finally, in this chapter, we will focus on the different isolation techniques, cultivation of freshwater microalgae, biochemical and molecular characterization to evaluate the biotechnological potential of native microalgae of the Peruvian Amazon based on our experience acquired over the years.

2. Isolation and culture techniques of native microalgae

2.1 Isolation techniques

The application of the different techniques of isolation in microalgae has as main objective to obtain a population of microalgae, starting from a single individual or clone (cells, filaments, colonies, and/or cysts) achieving unialgal cultures [3]. The use of the isolation technique depends on the dimensions of the microalgae, its mobility, and its morphology; however, according to our experience in this field, it is advisable to combine the different techniques [8]. The isolation techniques that the authors used are micropipette isolation and agar plate isolation, which are described below:

2.1.1 Isolation of microalgae with micropipette

This technique consists in isolating microalgae with the help of a Pasteur pipette with a reduced tip and/or with a capillary obtained by casting and then sterilized at 121°C for later use. Once the capillary is prepared and sterilized, a drop is removed from the natural collection and placed on a slide sheet, observed with the inverted light microscope or compound microscope to verify the presence of microalgae to be isolated. Under the microscope and with the help of the capillary, the desired microalgae are "trapped" and transferred to a slide sheet having a drop of sterile culture medium. This technique requires constant practice, since microalgal transfers must be

done quickly and carefully avoiding causing stress to the microalgae. The microalgae in isolation process can be continued transferring at least five times successively in drops of sterile culture medium, until a single type of microalgal cell is obtained. After obtaining the isolated microalgae, it is transferred to a test tube containing between 200 and 500 μL of sterile culture medium and grown for 7–10 days until microalgal growth is evidenced. Subsequently, the content is transferred to a test tube by adding fresh culture medium in order to continue with the culture. It is advisable to verify if the isolation was successful, that is to say that it is not contaminated with other species of microalgae, otherwise the isolation process must be repeated.

2.1.2 Isolation of microalgae in Petri dishes with agar

This technique is generally used when microalgae are 10 μm in diameter or less and consist of preparing Petri dishes with the appropriate culture medium to which 2% agar is added, and it is autoclaved at 120°C and 15 lb.ft.$^{-2}$ (1.1 kg.cm^{-2}) pressure for 15 min. Subsequently, it is left at room temperature and before it solidifies, they are emptied into the Petri dishes allowing their solidification. In Petri dishes with solid medium, 50–100 mL of the natural collection or microalgal suspension obtained using the capillary technique (to complement the isolation with capillaries) is added on the surface of the medium and with the help of the seeding handle or Drigalsky sterile handle homogenizes the suspension in the middle. It is immediately covered with the lid, sealed with parafilm, inverted, and cultivated for 5–10 days until observing the first colonies. The recommended culture conditions are photoperiod of 12 h light/12 h dark, light intensity of 100 μmol photons.m^2.s^{-1} and temperature of 25°C. Subsequently, observations are made on the inverted or compound microscope, and the microalgal colonies free of other microorganisms are selected with the help of the sowing handle and re-seeded in another Petri dish with culture medium. Repeating this procedure as many times as necessary to achieve a unialgal culture (**Figure 1**).

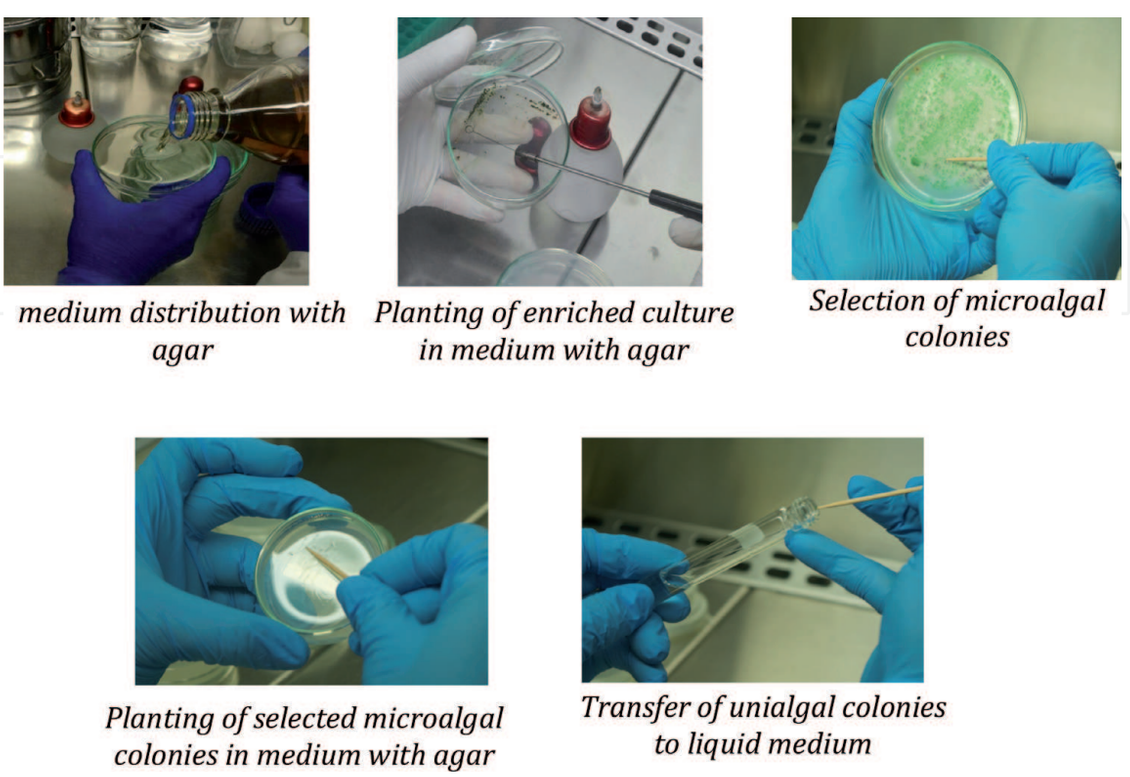


Figure 1.
Isolation of microalgae in Petri dishes with agar.

2.2 Microalgae cultivation techniques

Microalgae are characterized by the ability to synthesize various substances from water, CO_2, and minerals with the help of light energy (photosynthesis) [2]. In order to obtain these substances, culture conditions are required that guarantee a successful microalgal growth and its subsequent direct consumption as live food or nutritional supplements and indirectly for obtaining algal extracts (antibiotics, enzymes, essential fatty acids, among others) [13]. Currently, in the world, a large number of technologies and culture systems are used, especially those that are applied in laboratory conditions, which are addressed in detail in this chapter.

The culture conditions of the microalgae depend on the species to be cultivated and on the planned experimental tests. Various factors such as the composition of the culture medium, temperature, relative humidity, air flow, CO_2 concentration, lighting, among others influence the cultivation of microalgae. Initially, the microalgal suspensions are kept in test tubes of 10 or 15 mL at 25°C with a light intensity of 100 μmol photons.m^{-2}.s^{-1} with a photoperiod 12 h light/12 h dark. As the cell density of the cultures increases, they are transferred to 50, 100, and 250 mL flasks to volumes of 5 L or more (depending on the needs of microalgal biomass) for 4–8 weeks in an orbital shaker at 200 rpm or with constant aeration (**Figure 2**). If you do not have an agitator or air flow, it is recommended to shake manually 2 or 3 times a day. In addition, it is advisable to monitor the cultures daily by microscopic observations.

The amount of inoculum and cell density of the culture are important aspects in the cultivation of microalgae. For example, small inoculums and cultures with low cell densities may be lost due to photooxidation. Also, cultures with high cell densities are affected by the self-name effect. In addition, the inoculum must consist of cells of a single species and preferably in exponential growth phase [7, 13].

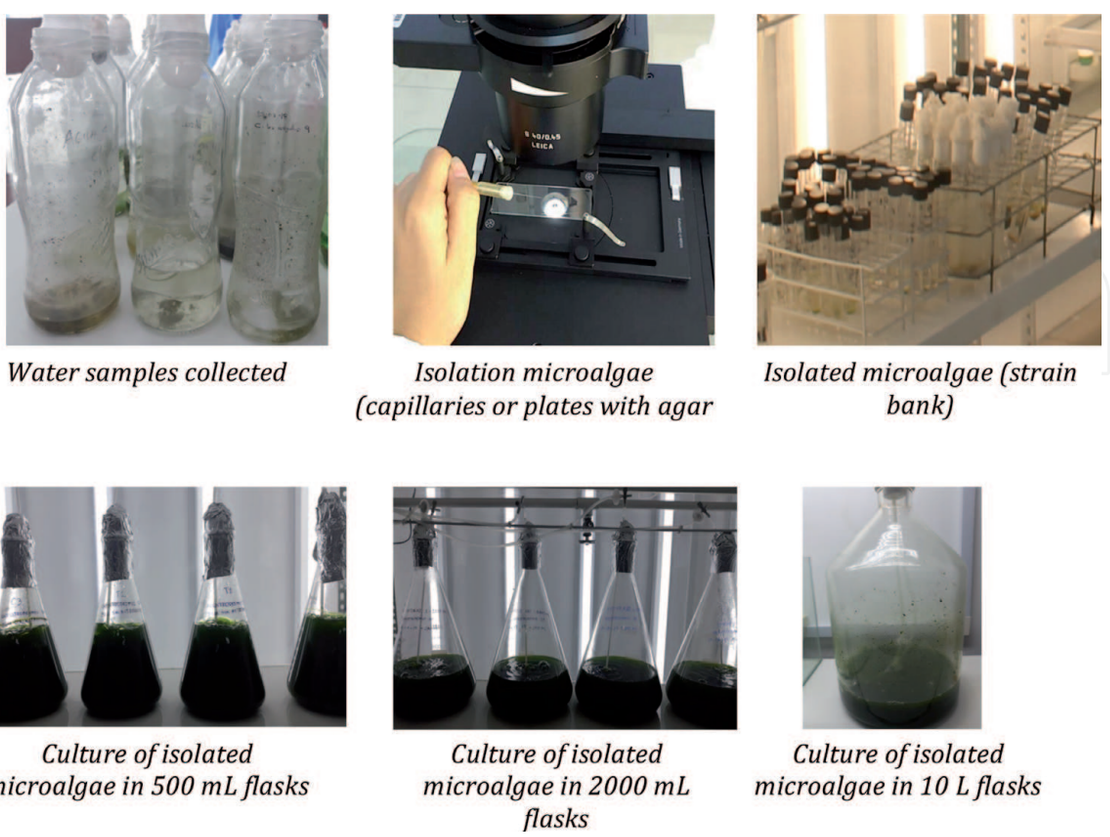


Figure 2.
Microalgae culture process under laboratory conditions.

2.2.1 Culture media used

The culture of microalgae under laboratory conditions requires culture medium, this being an aqueous solution that transports the nutrients necessary for its growth, such as water, light, CO_2, and mineral salts; among which are mainly some source of nitrogen and a source of phosphorus. The requirements of certain minerals vary widely between species and type of study. However, the supply of culture medium and nutrient concentrations must be directly related to the production of biomass so that it is necessary to periodically add enough to avoid the decrease in the productivity of the biomass or even some dysfunction of the culture due to photoinhibition.

The use of the culture medium depends on the type of microalgae to be cultivated, since there are different compositions and even with some modifications with which excellent results were achieved in the growth of these microorganisms. However, it is important to take into account certain considerations when preparing the culture medium, such as pH of the medium, hardness and salinity of the water, validity of the reagents, etc. The culture media that the authors generally used were Chu-10, BG-11, Beijerinck, and Bold Basal (see composition in **Table 1**).

For example, to prepare the Chu-10 medium, stock solutions are required, as detailed in **Table 2**.

Each of the stock solutions are added in a ratio of 1:10 v/v. Likewise, before weighing the reagents, it is advisable to perform simple calculations that guarantee the good preparation of the culture medium. For example, to prepare a stock solution of 100 mL of calcium chloride ($CaCl_2.2H_2O$), 3.67 g of this salt is required and flush up to 100 mL with distilled water.

2.2.2 Microalgal growth evaluation

It is recommended that microalgal growth records be interdiary. Commonly, the 0.1 mm deep Neubauer counting chamber is used to count microalgae from 2 to 30 mm and cultures of densities between 5×10^4 and 5×10^7 cells/mL. For this type of record, a sample of the culture (~100 mL) is taken, 50 mL of lugol is added and gently homogenized. About 50 mL of the mixture is placed in the Neubauer counting chamber that has the coverslip, allowed to stand for 5–10 min for the sample to stabilize. The cells are then counted using a microscope using a manual cell counter (**Figure 3**). It is advisable to double count in each of the fields (upper and lower chamber) for each of the samples. Once the average number of microalgal cells is obtained, the calculation is carried out with the following equation (Eq. 01):

$$N\ (\text{cells/mL}) = Pnc \times Fd/6 \times 10^6 \quad (1)$$

where N = number of cells/mL, Pnc = average number of cells obtained from the four fields of the Neubauer counting chamber, Fd = dilution factor (250,000 for small microalgae such as *Chlorella* sp. and *Euglena* sp. 10,000 for larger microalgae such as *Scenedesmus* sp., and *Ankistrodesmus* sp.).

A second method to evaluate the microalgal growth used in our laboratory is using the Nanodrop 2000 C UV/visible spectrophotometer. This equipment can measure accurately and reproducibly up to 2 mL of concentrated cultures. The system retains the sample between two optical fibers thanks to the surface tension [14]. The procedure is simple and consists of the following steps: (1) in the software that controls the spectrophotometer, the option to read cells at 680 nm (wavelength absorbed by chlorophylls a and b) is selected, (2) the baseline reading is performed (bleaching) by placing 2 mL of the culture medium in the sensor, and (3) the same volume of the microalgal culture is read.

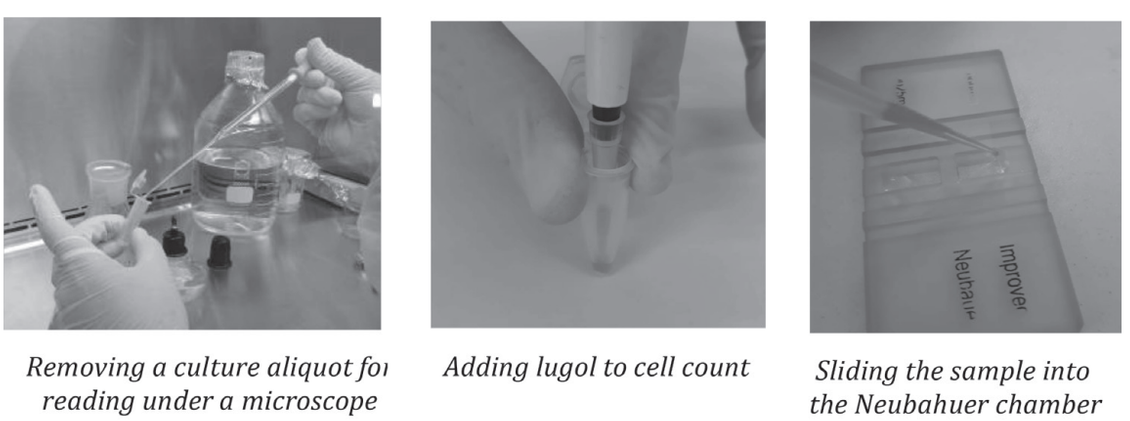
Chemical components	CHU-10	BG-11	Beijerinck	Bold basal
$NaHCO_2$	12.6 g	—	—	—
$NaNO_3$	85 g	1.5 g	—	1.5 g
KH_2PO_4	—	—	—	1.05 mg
K_2HPO_4	8.7 g	40 mg	1.18 g	0.45 mg
$MgSO_4.7H_2O$	36.9 g	75 mg	20 mg	0.45 mg
$CaCl_2.2H_2O$	36.7 g	36 mg	10 mg	1.2 g
NO_3NH_4	—	—	150 mg	—
PO_4H_2K	—	—	907 mg	—
NaCl	—	—	—	0.15 mg
$NaCO_3$	—	20 mg	—	—
$Na_2SiO_3.9H_2O$	28.4 g	—	—	—
HCl (1 mol/L)	0.05 mL	—	—	—
NaEDTA	50 mg	1.04 g	5 mg	50 mg
KOH	—	—	—	31 mg
Citric acid	—	6 mg	—	—
Ferric ammonium citrate	3.35 g	6 mg	—	—
pH	7.5	7.4	6.8	6.6
Total volume	1 L	1 L	1 L	1 L
H_2BO_3	618 mg	2.86 g	1 mg	11.42 mg
$MnCL_2.4H_2O$	12.6 mg	1.81 g	0.15 mg	1.44 mg
$ZnSO_4.7H_2O$	44 mg	0.22 g	2.2 mg	8.8 mg
$NaMoO_4.2H_2O$	12.6 mg	0.39 g	—	—
$CuMoO_4.2H_2O$	19.6 mg	79 mg	0.15 mg	1.57 mg
$Co(NO_3)_2.6H_2O$	—	49.4 mg	—	0.49 mg
H_2SO_4	—	—	—	1 mg
$FeSO_4.7H_2O$	—	—	—	4.98 mg
MoO_3	—	—	—	0.71 mg
$Mo_7O_{24}(NH_4)_6.4H_2O$	—	—	0.10 mg	—
$CoCl_2$	20 mg	—	—	—
Distilled water	1.0 L	1.0 L	1.0 L	1.0 L

Table 1.
Composition of microalgae culture media.

However, the spectrophotometer absorbance readings are not sufficient, it is necessary to determine their correlation with the number of microalgal cells per milliliter of culture. This is done through a standard curve of absorbances versus cell count for each species under study. To prepare the standard curve, 100 mL of the microalgal culture (in the logarithmic growth phase) is transferred to a microtube, and serial dilutions are made (1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128) with the culture medium. The absorbance at 680 nm in the spectrophotometer is measured from each dilution, and the cell number was determined using a Neubauer counting chamber. The absorbance values and the corresponding microalgae cell number data/mL, are entered in two columns in a Microsoft Excel® spreadsheet. Select both columns, insert the scatter plot, add

Stocks	Components	g/100 mL de H_2O
1	$CaCl_2.2H_2O$	3.67 g
2	$MgSO_4.7H_2O$	3.69 g
3	$NaHCO_3$	1.26 g
4	K_2HPO_4	0.87
5	$NaNO_3$	8.50 g
6	$Na_2SiO_3.9H_2O$	2.84 g
7	Ferric citrate solution	3.35 g/1000 mg water
8	Micronutrient solution	—
	NaEDTA	50.0 mg
	H_2BO_3	618.0 mg
	$CuSO_4.5H_2O$	19.6 mg
	$ZnSO_4.7H_2O$	44.0 mg
	$CaCl_2.6H_2O$	20.0 mg
	$MnCL_2.4H_2O$	12.6 mg
	$NaMoO_4.2H_2O$	12.6 mg

Table 2.
Composition of stock solutions of the Chu-10 medium.



Removing a culture aliquot for reading under a microscope

Adding lugol to cell count

Sliding the sample into the Neubahuer chamber

Microalgal growth lectur using a manual contometer

Microalgae observed during cell count by microscopy

Figure 3.
Microalgal growth evaluation process.

the trend line. In the trend line options select "Lineal", activate "present equation in the graph", and "present R^2 value in the graph". This will give us an equation of a straight line and the corresponding R^2 value (should be > 0.98). For example, for a strain of *Chlorella* sp. from our isolated microalgae culture collection, it has been determined that the R2 value was 0.99 and the following equations were obtained (Eq. 02 and 03):

$$A = (3 \times 10^9)\ (n) - 0.0025 \quad (2)$$

$$n = A + 0.0025/(3 \times 10^9) \quad (3)$$

where A is the absorbance at 680 nm and n is the microalgae cell number/mL.

Finally, it is important to consider some aspects that could contribute to the cultivation of microalgae.

- It is important to keep the crops in proper condition because they will be useful for future research. Likewise, it is advisable to use plastic tips with a filter to avoid contamination of a microalgal culture with other strains or species of microalgae.
- The maintenance of the strains can be done in liquid or solid medium.
- Periodically (15–20 days), culture medium (liquid medium) should be added under sterile conditions.
- The plates or flasks must be labeled with the codes and/or names of the microalgal strains, date of inoculation among other relevant data.

3. Techniques for biochemical and molecular characterization

3.1 Techniques for biochemical characterization

The biochemical characterization of the microalgae developed in our laboratory is based on the following analyzes:

3.1.1 Total lipid extraction

Total lipid extraction according to Yu et al. [15]. It consists of transferring the dry biomass to mortars for crushing with 8 mL of a mixture of chloroform:methanol (2:1). The extract obtained is transferred to 2 mL microtubes, and 100 μL of 0.9% NaCl is added for every 1000 μL of extract. The solution is homogenized in vortex for 30 sec and centrifuged at 23,000 × *g* at 4°C for 5 min. The chloroform phase is filtered with 0.45 μm syringe filters and transferred to beakers of known weight. Cellular debris and other components are retained in the intermediate phase (aqueous and chloroform phases) and are treated several times with the lipid extraction solution (chloroform:methanol) after homogenization in the vortex and centrifugation. All extracts with organic solvents are filtered and transferred to the same beaker. The organic solvents are evaporated from the beaker in a hotplate at 50°C for 4 h. Then the lipid components retained in the beaker are dried at 50°C for 4 h. Finally, the beaker is tempered to 25°C, and its weight is determined. The amount of total lipids obtained is determined by weight difference of the beaker with and without the lipids. With the following equation:

$$\text{Total lipid content (\%)} = (P_L/P_M) \times 100 \quad (4)$$

where P_L is the dry weight of total lipids and P_M is the dry weight of microalgae.

3.1.2 Qualitative determination of total lipids

The intracellular triglycerides of the microalgae are detected by the fluorescence they emit when interacting with Nile Red [16]. For this, the cells are stained with 2 mg/mL of Nile Red (dissolved in acetone) for 15 min and photographed using a trinocular microscope of Carl Zeiss-AxioLab.A1 epifluorescence and a real-time AxioCamERc 5 s digital camera. The images are obtained with a magnification of 1000× with visible light and epifluorescence (excitation: 510–560, emission: 590) [17].

3.1.3 Protein and carbohydrate

Protein determination is performed according to Lowry [18], and for carbohydrate evaluation, the Dreywood method [19] prior acid hydrolysis with 2 N HCI.

3.1.4 Humidity and ashes

The moisture content of the sample is determined weighing 0.1 g of the microalgae and dried in a vacuum oven at a temperature of 105°C for 16 h and at a pressure less than 0.1 bar, the result is expressed as a percentage.

The most common method to determine ashes is mucin calcination at temperatures between 500 and 600°C. Water and volatile substances are evaporated, while organic substances are incinerated in the presence of oxygen from the air to produce CO_2 and nitrogen oxide [20]. Most minerals are converted to oxides, sulfate, phosphate, chloride, and silicate.

$$\text{Ash } \% = [(P1 - P2) \times 100]/(P - P2) \quad (5)$$

where P is the weight in grams of the capsule plus that of the sample, P1 is the weight in grams of the capsule plus ashes, and P2 is the weight in grams of the empty capsule.

In general, microalgae have variations in the content of their biochemical parameters. For example, *Chlorella lewinii* showed a higher protein content (31.2%), *Ankistrodesmus* sp., a higher total lipid content (39.5%), and *Acutodesmus obliquus* a higher percentage of carbohydrates (49.6%) compared to other freshwater microalgae [21]. These parameters vary even more in conditions of physiological stress to which microalgae are subjected, as evidenced in the species of microalgae that accumulated a greater amount of total lipids (mg/g dry biomass) when grown in media without nitrogen; *Ankistrodesmus nannoselene* (316 mg/g dry biomass), *Ankistrodesmus* sp. (263.6 mg/g dry biomass), and *Scenedesmus* sp. (243.3 mg/g dry biomass), with respect to *Scenedesmus quadricauda* and *Chlorella* sp. which showed lower lipid content. Likewise, *Ankistrodesmus* sp., *A. nannoselene*, and *Scenedesmus* sp. showed statistically significant differences in total lipid content when grown in media with and without nitrogen, while in *S. quadricauda* and *Chlorella* sp., no significant differences were observed. However, the ash and moisture content remain very low [17]. Therefore, it is a fact that microalgae increase their lipid content when subjected to stress conditions in particular under nutrient restrictions [22].

These results suggest that some microalgae species have the ability to modify lipid metabolism in response to changes in environmental conditions, such as mentions Thompson [23] and Guschina and Harwood [24], producing large quantities of microalgal biomass but with relatively low lipid contents [25]. In essence, the production of biomass and microalgal triglycerides compete for photosynthetic assimilation, often requiring reprogramming of physiological pathways to stimulate lipid biosynthesis, which allows microalgae to withstand adverse conditions [26].

3.2 Techniques for molecular characterization

3.2.1 DNA and RNA extraction and purification

The hereditary basis of all living organisms is its genomic DNA, which contains the encoded information that is transmitted from generation to generation [27]. The first step of molecular biology studies and DNA recombination techniques begins with the extraction and purification of nucleic acids (DNA and RNA). The objective of all extraction methods is to obtain purified nucleic acids sufficient for downstream applications. Quantity and quality of extracted nucleic acids are especially important as these factors generally determine whether subsequent molecular techniques are successful. Inadequate methods, therefore, can compromise subsequent procedures for which much labor, time, and money are invested.

The specific procedure of nucleic acid extraction depends largely on the type of sample to be processed but generally consists of three steps: disintegration of cells or tissues (cell lysis), inactivation of intracellular nucleases, and separation of nucleic acids from other cellular components. RNA extraction is not always a simple process; however, since it is less stable than DNA, and the presence of pollutants such as RNAase, proteins, polysaccharides, and genomic DNA can complicate procedures [28]. Additionally, it has been reported that the presence of these contaminants may interfere with the amplification of nucleic acids [29]. Countless protocols now exist for obtaining nucleic acids that range from inexpensive home-brew protocols to commercial kits to complete automation. Each laboratory has generally optimized a few commonly used techniques, and their use is dictated on the time and money available for each research project.

In this chapter, we focus on the extraction and purification of DNA and RNA in freshwater microalgae, which is based on our experiences acquired over the better part of the last decade. Microalgae have attracted world-wide interest in the field of biotechnology due to their current and potential products of commercial interest such as biofuel, nutrients, food additives, and drugs [30].

3.2.1.1 Materials

3.2.1.1.1 Biological material

Approximately, 100–500 mg of microalgal biomass is necessary to achieve the best results. Given the amount of biomass, a large and active microalgae culture is required. Harvest must be carried out at 4°C and stored immediately at −80°C. In the case of RNA extraction, it is advisable to utilize liquid nitrogen to avoid RNA degradation.

3.2.1.1.2 Material and equipment of laboratory

For the extraction and purification of DNA, verify that materials and equipment are available (**Table 3**).

3.2.1.2 DNA extraction and purification

Extraction consists in the isolation of the total dissolved genomic DNA. The first step is the rupture of the plasma membrane in the case of animal cells, and the cell wall in the case of plant cells, managing to release the DNA, and the second is its precipitation. After these extraction steps are finished, agarose gel electrophoresis allows to visualize the genomic DNA bands and quantify approximately the size of the DNA obtained, by direct comparison with a marker whose

Materials	Equipment
Micropipettes of variable volume: 0.5–10, 10–100 and 100–1000 μL Eppendorf of 0.2, 1.5, and 2.0 mL Mortar and pestle Parafilm Magnetic stirrer Wash bottle or Pizetas Plastic tips with and without filter of 1–10, 20–200, 100–1000 μL Graduated Test Tubes of 25, 50, 100, 500, and 1000 mL Beakers of 50, 250, 500, and 1000 mL Flasks of 50, 250, and 500 mL	Autoclave (Yamato SM 510) Stove (Ecocell 111) Water bath (Labnet) Centrifuge (Hettich) Microcentrifuge (Spectrafuge Labnet) Water distiller (Barnstead Fistreem III Glass Still) Analytical balance (Sartorius) Gel Imaging System (BiodocAnalyze Biometra) Power source and horizontal electrophoresis system Spectrophotometer UV/Vis nanodrop 2000c pH-metro (Thermo Scientific) Water purifier (EASY pure RoDi Ultrapure) Vórtex-T Genie 2 (Scientific Industries) Dry block heater (Labnet).

Table 3.
Material and equipment of laboratory for the extraction and purification of DNA.

band size is previously known, and the amount based on the intensity of the band in the gel. Visualization is possible thanks to the use of fluorescence emission markers under UV light. There are various ways to extract DNA. Therefore, depending on the nature of the species, the most suitable total DNA isolation protocol is selected.

3.2.1.2.1 Reagents and solutions

For the visualization of DNA, verify that the following are available (**Table 4**).

3.2.1.2.2 Methodology

Approximately 100 mL of liquid biomass from culture harvested by centrifugation at 1900 × *g* for 10 min was used. Genomic DNA was extracted using a modified version of the CTAB method as described by Doyle and Doyle [31]. Briefly, microalgae cells were completely ground by hand using a mortar and pestle containing 50 mg of sterilized sand and 3 mL of extraction buffer (300 mM Tris-HCl pH 8.0, 50 mM EDTA, 2 M NaCl, 2% cetyltrimethylammonium bromide, 3% polyvinylpyrrolidone (MW

Reagents and solutions
Sterilized sand. Extraction buffer: Tris-HCl 300 mM, pH 8.0, ethylenediamine tetraacetic acid (EDTA) 50 mM, NaCl 2 M, cetyltrimethylammonium bromide to 2, 3% polyvinylpyrrolidone (MW 40,000), and 2% de β-mercaptoethanol. Phenol/chloroform/isoamyl alcohol (25:24:1, v/v). Isopropanol 70% alcohol RNase treated sterilized water Absolute ethanol TE buffer: Tris-HCl 10 mM, pH 8.0, EDTA 1 mM Agarose gel Ethidium bromide

Table 4.
Reagents and solutions for the extraction and purification of DNA.

40,000) and 2% β-mercaptoethanol). Homogenized cells were incubated at 70°C for 30 min with gentle inversion every 2 min. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, v/v) was then added followed by centrifugation at 20,000 × *g*. The aqueous supernatant was transferred to a new microtube, and an equal volume of chloroform/isoamyl alcohol (24:1, v/v) was added followed by centrifugation at 20,000 × *g*. The aqueous supernatant was transferred to a new microtube and mixed with 0.1 volumes of 3 M sodium acetate (pH 5.2), and an equal volume of chilled isopropanol was added to precipitate the DNA that was pelleted by centrifugation at 15,000 × *g*. The DNA pellet was washed with 70% alcohol, air dried, and then dissolved in 100 μL of sterilized water treated with RNase A at 40°C for 30 min and then extracted with chloroform/isoamyl alcohol and DNA precipitation with absolute ethanol. Finally, the air-dried DNA pellet was dissolved in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and stored at −20°C. Once the purified microalgal DNA has been obtained, the following quality control methods are recommended.

Electrophoretic analysis allows nucleic acid molecules to be separated by size that is dependent on the density of the matrix used (19–21). High molecular weight DNA should produce a single, bright band with little to no smearing. For microalgae, samples were resolved by standard gel electrophoresis using 1.2% agarose gels [32] stained with ethidium bromide and visualized under ultraviolet light.

Spectrophotometric analysis allows the determination of the concentration and purity of extracted DNA [33]. It is important to identify low quality or contaminated samples early in the process as common contaminates such as proteins, phenols, polyphenols, and carbohydrates, can negatively affect downstream procedures. Absorbance measurements are commonly conducted at 230, 260, and 280 nm, because carbohydrates and polyphenols absorb at 230 nm, nucleic acids at 260 nm, and proteins at 280 nm. To verify the quality and quantity of genomic DNA were evaluated by standard OD measurement [32] using a Nanodrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

3.2.2 RNA extraction and purification

To perform studies at the molecular level, fast, simple, economical, reproducible, and high-performance protocols must be available for the extraction and purification of high-quality total RNA [34]. To cover these gaps in this chapter of the book, a standardized protocol is described to purify the total RNA of Amazonian oilseed microalgae, considering that the purity and integrity of the total RNA is essential to study the genetic expression at the level of individual genes or at the transcriptomic level.

3.2.2.1 Reagents and solutions

For the extraction and purification of RNA, verify that the following materials and equipment are available (**Table 5**).

3.2.2.2 Methodology

The most important process is to break the cell wall of the microalgae and for this you need to freeze the cells with liquid nitrogen and crush it with mortar and pestle. If liquid nitrogen is not available, crushing can be done with sterile sand, which is the case in our experience. Subsequently, the integrity of RNA must be ensured, by removing the other cellular components present in the cell, with the help of extraction buffers. As to our experience with the standardization of the RNA extraction protocol, the procedure was as follows.

Reagents and solutions
Hexadecyltrimethylammonium bromide (CTAB)
Tris base
Ethylenediaminetetraacetic acid (EDTA)
Sodium chloride (NaCl)
Polyvinylpyrrolidone (PVP)
Sodium dodecyl sulfate (SDS)
Sarkosyl, β-mercaptoetanol
Activated carbon
Absolute ethanol
Ethanol 70%
Lithium chloride (LiCl)
Sodium hydroxide (NaOH)
Hydrochloric acid (HCl)
Diethyl pyrocarbonate (DEPC)
Proteinase K
Chloroform
Isoamyl alcohol
Ethidium bromide
Boric acid
Formamide
Agarose
Bromophenol blue
H_2O ultrapure
Hydrogen peroxide
Sodium hypochlorite

Table 5.
Reagents and solutions for the extraction and purification of RNA.

In a mortar previously cooled to −20°C for 30 min, 0.25 g of microalgal biomass is added, add 2.5 mL of the extraction buffer [Tris-HCl 300 mM pH 8,0, EDTA 100 mM, NaCl 2 M, CTAB 2.25%, SDS 0.75%, Sarkosyl 0.13% and PVP 3%, water treated with DEPC (up to ~ 90% of the total volume), measure and adjust the pH (NaOH or HCl) and autoclave] and 100 μL of 2-mercaptoethanol, 20 μL of proteinase K (10 mg/mL), 50 μL of activated carbon (10 mg/mL), and 100 mg of sterile sand. Thoroughly crush the samples for ~8 min and transfer it to a 2 mL microtube, add chloroform:isoamyl alcohol (24:1) in a ratio of 1:1 v/v, homogenize in the vortex and centrifuge at 21,000 × *g* per 10 min at 4°C. Put the supernatant (700 μL) in a microtube, add 0.3 volumes of absolute ethanol, gently homogenize by inversion 8–10 times. Then, add equal volume of chloroform:isoamyl alcohol (24:1), homogenize in the vortex, and centrifuge at 21,000 × *g* for 10 min at 4°C. Transfer the supernatant to a microtube and repeat the previous step. To precipitate the RNA, transfer the supernatant to a microtube, add lithium chloride (LiCl) until a final concentration of 2.5 M is achieved, incubate at −20°C for 2 h or −80°C for 1 h, and centrifuge at 23,000 × *g* for 30 min at 4°C. Discard the supernatant, resuspend the RNA precipitate with 500 μL of 2 M LiCl, incubate at −20°C for 10 min, and centrifuge at 23,000 × *g* for 10 min at 4°C, repeat this step once more. Then wash the precipitated RNA by resuspending it with 500 μL of 70% ethanol and then with 500 μL of absolute ethanol, interspersing with centrifugation steps at 21,000 × *g* for 8 min at 4°C. Finally, discard the absolute ethanol, dry the precipitated RNA in a Thermo block at 45°C for 5 min, resuspend with 30–60 μL of TE buffer (10 mM Tris pH 8.0, 1 mM EDTA), and to stock at −80°C. Once the purified microalgal RNA is obtained, it is recommended to perform complementary analyzes:

- Electrophoretic analysis: it is a method that allows to determine the integrity of the purified RNA, which consisted in putting in a 0.2 mL microtube 5 μL

of the total purified RNA and 5 μL of the seeding buffer (contains 6% SDS, 0.5% bromophenol blue, 0.5% ethidium bromide, 10 μM EDTA, and 95% formamide), homogenize them by pipetting 3–5 times and then incubate in a water bath at 70°C for 10 min and cool on ice for 5 min, centrifuge for 5 s at room temperature, sow the treated sample on the agarose gel and perform the electrophoretic run for 30–60 min at 100 volts, and finally, observe the RNA bands in the photo documentation system.

- Spectrophotometric analysis: this analysis allows us to determine the concentration and purity of the purified total RNA [33]. The Nanodrop 2000c was used to perform the spectrophotometric analysis, the first step is to select the nucleic acid reading (RNA) option, subsequently, the blank reading (bleaching) is carried out by placing 2 μL of the TE buffer on the sensor and the same volume of the purified RNA is read, subsequently, the absorbance results are recorded at 260, 280, the quality ratios (A_{260}/A_{230} and A_{260}/A_{280}) and the RNA concentration in ng/μL.

3.2.3 De novo assembly and annotation of the microalgae transcriptome

Transcriptomics studies the level of expression of all transcribed genes (mRNAs, non-coding RNAs, small RNAs, etc.) in a cell or tissue. Transcriptomic analysis is often focused on the study of mRNA populations, which are molecules with encoded information for the synthesis of all proteins. Therefore, with this type of analysis, you can reconstruct the multiple metabolic pathways and physiological processes that are active, because these proteins are those that participate in fundamental processes as diverse and complex as transport, intracellular signaling, defense, enzymatic catalysis, among others.

There is a great variety of techniques used in transcriptomics, which allow quantifying millions of RNA molecules at the same time; this thanks to the recent development of massive sequencing technologies and the implementation of different bioinformatics tools, and it is now possible to analyze the transcriptome of any organism, even species that do not have sequenced genomes. Massive sequencing technologies differ in their details, but typically consist of three similar stages. These stages are: (1) template preparation, (2) clone amplification, and (3) cyclic rounds of parallel and massive sequencing.

However, Illumina sequencers are the ones that generate the most data at low costs, so these machines currently dominate the market [35]. The sequencing process with these platforms consists in the clonal amplification of DNA fragments linked to adapters on the surface of a glass sheet. In all Illumina models, the overall error rate is less than 1% and the most common type of error is substitution [36, 37]. Once the data are obtained, which are millions of short sequences (100–200 bp), they must be pre-processed, assembled, and finally their functional annotation.

Preprocessing or "cleaning" consists in eliminating erroneous sequences, low quality sequences, and technical sequences (adapters, primers, etc.). This is a process where bioinformatics programs are used, for example, the frequently used bioinformatics program for this cleaning process is the Trimmomatic and CutAdapt [38, 39] are two commonly used programs.

Subsequently, the sequences are assembled, that is to say, correctly join the short DNA fragments (100–200 bp) to assemble the thousands of transcripts that commonly have sizes from 1000 to 3000 bp. To achieve this purpose, there are different bioinformatics programs such as ABySS, ALLPATHS, SHORTY, Velvet, Oases, SOAPdenovo, Trinity, CAP3, among others [40, 41].

Finally, after assembling the transcripts, they proceed to their functional annotation, that is, based on their homology with sequences, previously reported and stored in the biological databases, the molecular and cellular function of each of the transcripts is deduced. For this bioinformatic analysis, the scientific community frequently uses the Blast2GO program [42]. This program also allows us to rebuild all metabolic pathways.

4. Biotechnological potential for biofuel and nutraceutical production

Climate change, the growing global energy demand, the increase in the cost and depletion of fossil fuels, high rates of child malnutrition, among others are problems that require medium- and long-term solutions. To mitigate these problems, in the different countries, several legal devices are available with the purpose of promoting and diversifying the energy matrix based on renewable sources such as biofuels and are considered alternatives to venturing into the production of functional (nutraceutical) foods, as it happens in our country. However, many times due to low domestic production volumes (approx. 20% of demand), these measures are forcing the importation of both biofuels and various food products from other countries. In the case of biofuels in our country, its production depends mainly on crops of large areas of oil plants such as oil palm, white pine nut, and other species, thus contributing to a loss of biodiversity and a decrease in ecosystem services [43]. In the case of functional or nutraceutical foods, it is common to find them under different commercial presentations (syrups, capsules, powders, etc.) imported from abroad that do not allow increasing the supply in each country. Therefore, to minimize these negative impacts, it is urgently necessary to have other alternative sources of biofuel and nutraceutical production.

An excellent alternative is offered by microalgae, which are organisms that play a key role in aquatic ecosystems because of their photosynthetic capacity, and that in aerobic conditions can fix carbon dioxide just like plants [44]. Among the compounds of most interest obtained from microalgae, carotenoids, biodiesel, phycobiliproteins, lipids, polysaccharides, and compounds with biological activity stand out. Currently, researchers from around the world have demonstrated the potential of several microalgae species (*Chlorella minutissima*, *Thalassiosira fluviatilis*, and *T. pseudonana*) [45]. Our team has carried out studies with *Ankistrodesmus* sp., using different culture media, registering a higher total lipid production of 263.6 mg/g dry biomass with 68.56 ± 2.35 palmitic acid under physiological stress conditions [46], as raw material to produce biodiesel.

Likewise, it is demonstrated that the determination of the fatty acid profile is important to assess the potential for the quality and production of biodiesel in microalgae, since the quality of biodiesel depends on the type of fatty acid present in each microalgal cell. As also, it is necessary to know the number of cetones, oxidative stability and could-flow, which depend on the length of the hydrocarbon chains of saturated and unsaturated fatty acids (UFA) [47]. Studies carried under conditions of nitrogen limitation in the culture, the microalgae showed variation in the composition of saturated fatty acids (SFA) with monounsaturated fatty acids (MUFA). And that 20–30% are SFA and 69–80% are UFA, the range of SFA/UFA being in the microalgae evaluated between 0.25 and 0.45 [17]. Also, it was reported that palmitic (C16:0), oleic (C18:1n-9), linoleic (C18:2n-6), and γ-linolenic acids (C18:3n-3) were the most abundant fatty acids and showed greater variations within and between the species studied [46].

Regarding the nutraceutical potential of microalgae, it can be mentioned that in the early 50s, humans began using microalgae in their diets, mainly as dietary

supplements (protein and vitamin, in the form of powder, capsules, pills or tablets). These are usually incorporated into foods such as pasta, cookies, bread, candies, yogurts, soft drinks, among others. It is currently estimated that approximately 30% of the microalgae produced in the world is used in human nutrition due to its high protein content [48]. Therefore, *Arthrospira*-based nutraceutical compounds with anti-cancer protection properties are being commercialized due to their high content of β-carotene [12], the prevention of malnutrition in vitamin A and blood sugar levels, the stimulation of certain prostaglandins, prevention of degenerative diseases, and accelerated wound healing [48].

Recent research has shown that human consumption of microalgae is limited to few species due to strict control over food safety, commercial factors, market demand, and specific preparation for consumption, since the most prominent genera are *Chlorella*, *Arthrospira,* and *Dunaliella*, which are marketed as a food supplement [49, 50]. Studies on nutraceutical substances have been carried out almost exclusively in algae and microalgae, identifying more than 600 naturally occurring carotenoids in plants, animals, and fungi, of which 400 have been isolated and characterized [51], but of these, only a small number are commercially used among them B-carotene and astaxanthin, being only two species of marine microalgae recognized commercial sources of carotenoids: *Dunaliella salina* and *Haematococcus pluvialis* [52]. Recently, our team has registered that *Spirulina maxima* presented 269.54 ± 0.021 μg/g dry mass of β-carotene and *Scenedesmus* sp. presented 15.29 ± 0.01 μg/g dry mass.

In addition, microalgae have the ability to increase the nutritional content of traditional foods and even positively affect both human health and animal health. This is due to its original chemical composition, since the high protein content in some species is one of the main reasons to consider them as a source of unconventional protein. In turn, the amino acid profile of almost all microalgae is more favorable than compared to conventional sources. The carbohydrates in the microalgae can be found in the form of starch, glucose, sugars or other polysaccharides. Its digestibility is high, so there is no limitation of use in food preparations for both animals and humans [53, 54].

Many microalgal species are marketed for their medicinal value, as *Chlorella* protects against kidney failure and promotes the growth of *Lactobacillus*. In addition, it favors the decrease in blood cholesterol concentration [49, 55], increases the level of antioxidants in the body [56], and stimulates collagen synthesis, supporting tissue regeneration and wrinkle reduction [50]. *Dunaliella* is marketed for its high content of β-carotene [49, 55]. *Haematococcus pluvialis* is the only microalgae that have been commercially exploited for the production of astaxanthin [57, 58]. Today, microalgae are used to feed livestock, and human consumption is increasing, particularly in food supplements [59]. Algae dietary supplements can be particularly useful for supporting some diets.

Therefore, the microalgae native to the Peruvian Amazon have been characterized in order to determine their application and use from a biotechnological point of view, due to the abundant source of antioxidants that microalgae possess, which makes them candidates for use in biological processes (aging), as well as in the degenerative changes of different organs [60]. In addition, the beneficial effects of nutraceuticals have been attributed to polyphenols, polyunsaturated fatty acids, terpenes, chlorophyll, and accessory pigments of the photosynthetic apparatus of microalgae and are therefore considered excellent sources of proteins, small peptides, and amino acids that contribute to providing high amount of phosphorus to fight hypertension and lower cholesterol levels, help in the formation and regeneration of blood cells together with iron and stimulate the release of substances that control satiety.

In that sense, nutraceuticals currently play an important role in the daily life of a large number of people, being more prevalent in those with chronic diseases and high overall mortality. The various reasons for promoting the consumption of these products vary according to age, sex, nationality, customs, and existence of comorbidities [61]. Due to these interesting qualities, in recent years, research has been carried out with various species of algae for various biotechnological applications. As a result, today the biotechnology of microalgae has gained relevance due to the wide range of applications derived from its use, from biomass production for food, its use in aquaculture, as biofertilizer, to obtaining products of therapeutic or industrial value. Therefore, the success of microalgal biotechnology lies in choosing the correct species with relevant properties that, under specific culture conditions, produce the compounds of interest [48]. The applications range from the production of simple biomass for animal feed or for the production of valuable products for ecological applications. Due to the enormous biodiversity of microalgae and recent developments, this group of organisms represents one of the most promising sources for new products and applications. With the investigation of sophisticated crops, strain search and microalgal biotechnology, the demands of the food and pharmaceutical industries can be met [53, 54].

5. Conclusions

The native microalgae of the Peruvian Amazon have biotechnological potential and are therefore promising for the production of both biodiesel and nutraceuticals. Various species of microalgae were identified, isolated, cultured, and characterized using biochemical, nutraceutical, and molecular techniques, the isolation stage being the starting point to achieve various biotechnological applications. *Ankistrodesmus* sp. is one of the microalgae with potential for the production of biodiesel and microalgae such as *Haematococcus pluvialis*, *Scenedesmus* sp., and *Chlorella* sp., among others demonstrated a high potential for nutraceutical production. The stress conditions to which microalgae are subjected are being a determining factor for the production of biodiesel and nutraceuticals.

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Conflict of interest

The authors declare no conflict of interest.

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