Critical Reviews in Biotechnology

ISSN: (Print) (Online) Journal homepage: www.tandfonline.com/journals/ibty20

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To cite this article: Bermejo Elisabeth, Filali Rayen & Taidi Behnam (2021) Microalgae culture quality indicators: a review, Critical Reviews in Biotechnology, 41:4, 457-473, DOI: 10.1080/07388551.2020.1854672

To link to this article: https://doi.org/10.1080/07388551.2020.1854672

CRITICAL REVIEWS IN BIOTECHNOLOGY
2021, VOL. 41, NO. 4, 457–473
https://doi.org/10.1080/07388551.2020.1854672

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REVIEW ARTICLE

Microalgae culture quality indicators: a review

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ABSTRACT

Microalgae are photosynthetic microorganisms that have generated increasing interest in recent years due to their potential applications. Their biological capacity to grow faster than higher plants and their ability to convert solar energy into biomass and other bioactive molecules, has led to the development of various culture systems in order to produce different high-value products with commercial interest. The industrialization of the microalgae cultivation process requires the introduction of standardized quality parameters. In order to obtain bioactive compounds with high added value at a commercial level, it is necessary to sustainably produce biomass at a large scale. Such a process would imply specific stress conditions, such as variation in temperature, light or pH. These environmental conditions would make it more difficult to maintain the viability of the culture and protect the yield and condition of the target molecules. The physiological and biochemical impact of these stress factors on the microalgae biomass can be potentially measured by the presence and activity of various biochemical indicators called biomarkers. This review presents an overview of the main techniques that exist for assessing the "quality" of microalgae cultures through quantification of cell viability and vitality by monitoring specific markers indicative of the status of the culture.

ARTICLE HISTORY
Received 10 June 2020
Revised 17 September 2020
Accepted 26 October 2020

KEYWORDS
Microalgae; biomarkers; quality indicators; cell viability; analytic monitoring techniques

Introduction

Microalgae are a large and diverse group of unicellular eukaryotic microorganisms of different shapes and sizes (2–50 μm). The other principal group of photosynthetic microoganisms are cyanobacteria, which are prokaryotes. Photosynthetic microorganisms are found to be spread across many phyla and are able to grow in fresh, brackish or saltwater [1].

Microalgae play a key role in aquatic ecosystems as they are photosynthetic organisms responsible for 40% of global photosynthesis [2], producing almost half of the atmospheric oxygen on Earth at any one time. Their culture is simple and can be cost-effective as these organisms can grow photoautotrophically, using CO_2 and light as sources of carbon and energy respectively, and convert them into biomass and O_2.

The photosynthetic apparatus of microalgae is organized into special organelles called chloroplasts in the same way as it is in plants. They consist of a system of lipoproteic membranes (thylakoids), an aqueous phase or stroma, and different accessory light-gathering pigments.

The basic mechanisms of oxygenic photosynthesis are carried out by specific pigments, responsible for light capture, electron generation and adenosine triphosphate (ATP) generation. The electron is then transferred to the reaction center and, used to reduce the first acceptor of the electron transport chain during photosynthesis. Electron transport allows the final reduction of $NAD(P)^+$ from ferredoxin, giving rise to reducing power used for the assimilation of nutrients [3]. In photosynthesis, inorganic compounds containing C, N, P, and S (in the form of: CO_2, NO_3^-, PO_4^{3-}, and SO_4^-) are converted into organic matter through their assimilatory reduction, resulting in their incorporation into the carbon skeletons of metabolic intermediates and cell components. This process represents the main mechanism responsible for carbon fixation and oxygen release into the atmosphere [4]. Thus, all living organisms existing on Earth depend directly or indirectly on photosynthesis as an energy and carbon source for their metabolism and growth [5].

The biodiversity of microalgae is huge. It is estimated that there are more than fifty thousand different types of microalgal species; among them, only thirty

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thousand have been studied [6]. This diversity offers great potential that still needs to be exploited. The application of microalgae in the industry is increasing; microalgal biomass holds great promise as a source of alternative energy and as a rich source of different compounds of commercial interest [7].

Under stress and/or extreme conditions such as changes in temperature, high salinity or nutrients limitation, microalgae can produce biologically active and unique metabolites [8,9]. The production of bioactive compounds can be controlled through specific culture conditions [10–12].

Other advantages of microalgae cultivation are related to their ability to fully use solar energy and CO_2 to grow only photosynthetic biomass, resulting in higher growth rates through shorter growth cycles and the avoidance of root or stem formation as is the case in higher plants. They are responsible for reducing excess CO_2 in the atmosphere through photosynthetic bio-fixation [13,14], controlling the greenhouse effect and moderating global warming. Another advantage of microalgae cultivation is the potential use of areas where the climate is unsuitable for conventional farming; hence avoiding direct competition with traditional food production. Moreover, nitrogen and phosphorus can be removed from wastewater using microalgae in an efficient solar energy biomass conversion system [13].

Microalgae cultivation, as part of a biorefinery concept, is able to offer a broad range of different products with applications in food, pharmaceuticals, nutriceuticals, medicine and biofuels [12,15]. This approach can increase the economic sustainability of microalgal production [16].

Despite the centuries-old traditional uses of microalgae and cyanobacteria by some populations, large scale commercial production of microalgae only dates back to the 1950s [17,18]. The late start in the industrialization of microalgae is surprising when considering the aforementioned advantages (high surface productivity, use of non-productive land, reuse and recovery of waste nutrients, use of saline or brackish waters and reuse of CO_2). In recent years, extensive efforts have been initiated to achieve commercial-scale production [19]. The economic viability of the industry remains challenging due to the low biomass concentration that can be achieved in the microalgae cultures leading to a relatively costly harvesting and separation process for the microalgal biomass [20]. Microalgae are grown in a wide range of different culture systems, from open ponds to closed photobioreactors (PBRs). The main characteristics of open ponds, amongst which the most frequent type is the "raceway system," is that microalgal culture is directly exposed to the atmosphere. Raceway ponds are shallow ponds (between 10 and 50 cm deep) composed of two or more interconnected straight channels. They incorporate paddlewheels or pumps for moderate mixing and recirculation. The culture suspension is pushed or pumped around in a loop and it is directly illuminated by sunlight at its surface [21]. Nutrients are added to the water feed, which is used to replace the continuously harvested microalgae culture [22]. Open ponds are very economical to operate but despite their popularity for commercial microalgae cultivation, they present several drawbacks such as, low biomass productivity, poor species control and a high risk of contamination. In order to overcome the weaknesses of open raceway ponds, new closed systems, so-called photobioreactors, have been developed to support the large-scale cultivation of microalgae. In the case of pond systems, the sunlight-exposed area is equal to the ground area occupied by the pond. Photobioreactors are usually characterized by a larger light exposed surface than the ground area occupies. Therefore, improved use of light, both natural and artificial, is achieved by using photobioreactors, leading to an increase in the culture productivity [23,24].

During the last decade, many different types of photobioreactors have been developed (plate, tubular, panel, bubble column, etc.). However, mainly two types, tubular and panel bioreactors, are the most widely employed closed photobioreactors for algal production.

Nevertheless, despite all efforts to improve the culture of microalgae on a large scale, the economic sustainability of biomass and bioactive compound production remains limited. Process scale-up is considered as the main limitation for large scale production, which decreases the number of successful microalgae cultivation cases [25]. A necessity for process commercialization is the identification of suitable methods to monitor the quality of the microalgal cultures during and after production. These quality control methods must be fast, precise, cost-effective and easy to apply to minimize product and culture-viability losses.

This review article aims to highlight the value of the quality control of microalgae biomass for industrial applications. The most widely used methods to determine cell viability/death in microalgae cultures, as well as the main factors that cause cell damage, will be discussed. Finally, the importance of the detection of some bio-markers indicative of the status of the cultures will be taken into consideration.

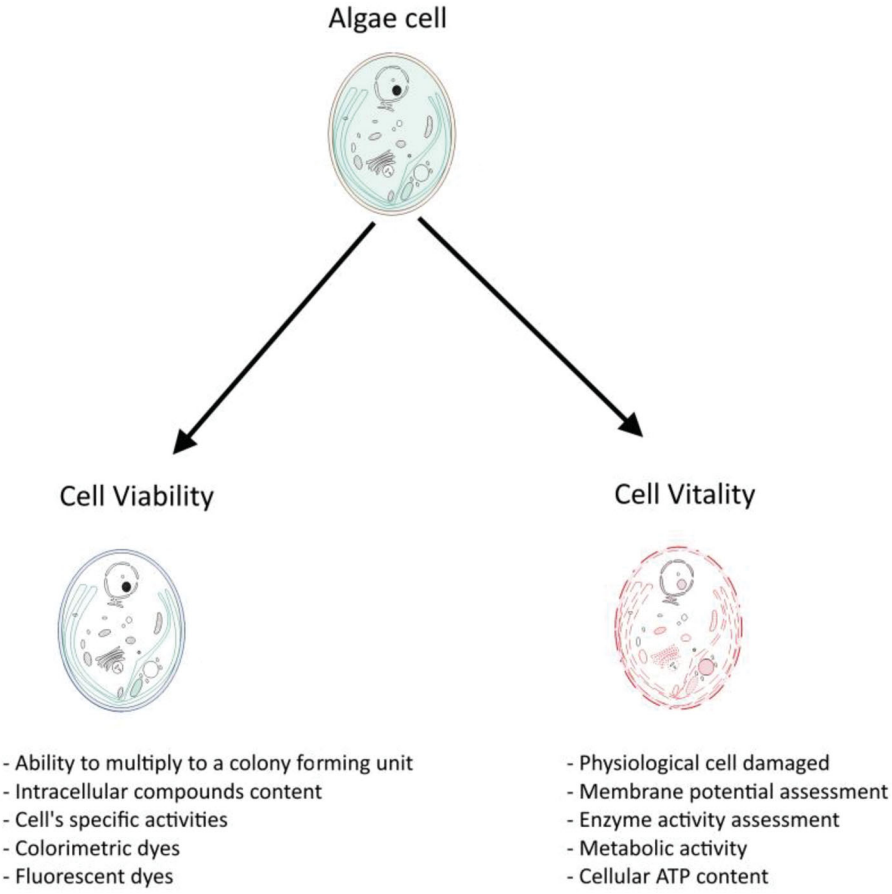

Figure 1. Comparison between cell viability and vitality.

Microbial culture-quality measurements

Cell viability measurements

The classic definition of viability in microorganisms is related to their ability to multiply through reproduction. As a definition, this seems to be clear, but it raises a number of questions: How long would we have to wait to declare an organism dead? To what growth conditions does the definition apply? Is an organism capable of metabolism, but deficient in the ability to replicate, considered as dead or alive?

A neighboring concept to viability is that of vitality where the key activity of the organism is considered (Figure 1). For instance, the baker's yeast's ability to produce CO_2 is often taken as a measure of vitality [26]. After all, the number of organisms capable of reproducing are not of strict interest to the baker but is the ability of a certain amount of culture to produce a defined volume of CO_2 in a predefined time.

When it comes to practical measurements of viability, this concept uncomfortably approaches the definition of vitality. This is because numerous viability measurement methods depend on the cell's specfic activities rather than its ability to replicate (Table 1).

The determination of the percentage of living, also named "viable," cells in the whole population of a culture is often necessary in microbiology in order to define the biological behavior of a population. The assessment of changes in cell viability has a great importance for the design and control of biotechnological processes including those involving microalge cultures. Microalgae cultures can be prone to "spontaneous crashes"; a phenomenon that is not compatible with industrialization. In large-scale systems, it is really difficult to have cultures with 100% viability due to factors such as, mechanical stress [27], UV-irradiation [28] or changes in temperature [29]. Thus, it is essential to be able to diagnose the health of the culture (cell viability) and to understand the phenomena (physical or biological) that affect it.

Cell viability determination is usually carried out by staining different components of the cells with specific dyes, indicating the percentage of inactive cells. For instance, to obtain the proportion of dead cells in a culture, dye exclusion methods are frequently used. These techniques are based on the assumption that living cells are impermeable to dyes whereas certain dyes, such us methylene blue, trypan blue, eosin, or nile blue,

Table 1. Different techniques used to evaluate the quality, in terms of cell viability and vitality, of microbial cultures.

Culture-quality measurement techniques	Cell viability	Cell vitality	Reference
Dye exclusion methods	Dead cells are stained as certain dyes penetrate the damaged cytoplasmic membranes	–	[30,31]
Fluorescent dyes			
Esterase enzymes activity (FDA, Calcein-AM, CMFDA, H_2DCFDA)	Living cells can be detected by fluorescence or absorbance as their esterase activity convert FDA into fluorescein	Cellular vitality (fluorescence intensity) is related to the esterase activity of the cells	[32–35,78–80]
PI	Damaged cells nucleic acids are stained	–	[37–43]
SYTOX Green	Damaged cells nucleic acids are stained without overlapping chlorophyll autofluorescence	Cells vitality (fluorescence intensity) is related to their membrane potential and integrity	[44,67,81,82]
DiBAC4(3)	–		
Serial Dilution Culture Most Probable Number	Viable cells are evaluated through the presence or absence of growth in serial dilutions of a sample	–	[49–55,60–62]
LDH enzyme determination	Dead cells are related to the concentration of lactate dehydrogenase in the culture broth	–	[71]

selectively stain dead cells as they penetrate the damaged cytoplasmic membranes [30,31].

Fluorescent dyes are also used to measure the viability of cultures, fluorescein diacetate (FDA) being the most common fluorescence-based technique to assess microalgal viability [32–35]. Only living cells are able to convert FDA into fluorescein through their esterase activity, rendering it detectable by fluorescence or absorbance measurement. By counting the stained cells by microscopy, the viability can be estimated. Fluorescent based methods have high sensitivities and can be automated. However, FDA rapidly leaks from cells making the method variable. To determine whether inhibition of FDA fluorescence was due to reduced retention inside the microalgae cell or to inhibition of intracellular esterases, Franklin et al. used a nucleotide-binding stain–propidium iodide (PI)–, to assess membrane integrity [36]. PI enters cells with damaged membranes staining nucleic acids. Thus, it is able to discriminate between live, viable (non fluorescent) cells, and nonviable (fluorescent) cells. Due to this feature, PI has been widely used to indicate dead cells [37–43]. However, this dye has maximum fluorescence intensity over 600 nm that overlaps with autofluorescence of microalgae pigments, which makes the results not totally accurate unless compensation is used (e.g. an extra dye). Sato et at. demonstrated the suitability of a dual-fluorescence viability assay using SYTOX Green [44]. This dye offers the advantage that it becomes fluorescent once in contact with DNA without overlaping chlorophyll autofluorescence. Thus, SYTOX Green fluorescence and autofluorescence can be used simultaneously as markers for dead and live cells. Despite the fact that staining techniques allow simple and direct measurements, they can involve microscopic enumeration, which is time-consuming and prone to operator error. Cells stained with fluorescent dyes can also be counted by flow cytometry. This technique is particularly useful since it can differentiate between those particles that auto-fluoresce, such as photosynthetically active microalgae containing healthy chlorophyll, and those that do not fluoresce, such as dead cells containing degraded chlorophyll. Despite that, microbiology laboratories are not commonly equipped with flow cytometers due to the high cost of this type of equipment. Another drawback is related to the fact that it requires sophisticated data analysis and is restricted to liquid samples. In order to avoid using expensive equipment, Capasso et al. developed a new viability evaluation technique based on spectrophotometric measurements [45]. It consisted of the enzyme mediated reduction of tetrazolium salt (MTS) by actively growing cells to a colored product (formazan) [46]. The authors demonstrated that the number of viable cells of two different organisms (*Dunaliella* and *Spirulina*) was directly related to formazan-related color development. Nevertheless, as the activity of the cells may also vary with the physiological state of the viable microalgae, this technique makes it difficult to accurately estimate the fraction of viable cells in a culture (e.g. if stressed viable cells produce less formazan than unstressed viable cells, then the test could underestimate true viability).

A benchmark test in microbiology for the quantitative estimation of cell viability is the Serial Dilution Culture Most Probable Number (SDC-MPN) technique. This method, developed for interpreting the results of clinical analyses of bacteria [47], is based on diluting

the culture and was used with phytoplankton for more than a century [48]. This assay is based on assessing the presence of viable cells in the serial dilution of a sample through the presence or absence of growth. Therefore, as the dilution increases, the probability of a single viable cell remaining in the culture decreases and, thus, the probability of detecting growth in the sample. The determination of the MPN of cells in the undiluted sample can be calculated statistically from the number of samples in which there is evidence of growth in a set of replicate dilutions [49].

Several studies of microalgae and cyanobacteria, in which the SDC-MPN method was used in different contexts, have been reported. For instance, this technique has been used to identify cyanobacteria, diatom, raphidophyte, and dinoflagellate propagules from soils and sediments [49–54] and to select subpopulations of phytoplankton difficult to grow [49,55–60]. It has also been used more recently to assess the viability of cells after an ultraviolet-C (UVC) treatment [61,62].

According to the study carried out by Cullen and MacIntyre, one critical point to take into account is to evaluate if no-growth is due to a real absence of viable cells, or to an error caused because the experiment was not conducted for long enough [60]. This potential error of the SDC-MPN method can be avoided under laboratory culture conditions by including a positive control incubated under conditions that are known to lead to active growth.

When wishing to determine cell death, it is essential to define fast and appropriate methodologies to diagnose the process of cell lysis. Factors such as the presence of pathogens (e.g. viral infection), physiological stress, auto-mortality or programmed cell death (PCD), can lead to cell lysis [63–66]. Environmental stress conditions are known to produce reactive oxygen species in cellular compartments, resulting in oxidative damage and apoptosis. It has been reported that the process of cell lysis due to environmental stress factors takes place in three steps: (1) the cell-membranes integrity is compromised, (2) photosynthetic pigments are degraded, and (3) genomic DNA is fragmented [67]. Another mechanism for cell death is that of PCD and it is independent of environmental factors. PCD is defined as the mechanism through which an independent cell reacts to external or internal signals, firstly genetically, and then biochemically carrying out its own destruction. A PCD type known as apoptosis is characterized by particular requirements, from a morphological and a biochemical point of view, such as condensation and margination of chromatin, DNA cleavage while the cytoplasm and organelles remain unaltered, and the contribution of a group of proteases, known as caspases, as main regulators [68]. In contrast to the apoptosis process, necrosis is characterized by a loss of membrane integrity, cell swelling, and lyses (Figure 2). The existence of PCD mechanisms in unicellular organisms is still controversial and unclear because unlike multicellular organisms, it results in complete loss of the organism [70]. Thus, it is difficult to confirm the process of cell death.

Methods that allow the quantification of dead and/or lysed cells must also be considered as a tool to be used in microalgae bioprocess development and research to determine the state of the culture. The most commonly used methods for assessing cell death (dye exclusion methods) are unable to detect cell lysis. In this connection, Gallardo-Rodríguez et al. proposed a cost-effective, rapid and sensitive method for assessing cell death in microalgae cultures through indirect determination of the lactate dehydrogenase (LDH) enzyme released into the culture after the loss of the cell membrane integrity [71]. They based their method on the assumption that when the cell membrane integrity is compromised, intracellular materials may be released into the medium. Absorption fluorimetry and subsequent quantification of the concentration of these compounds in the culture broth could indicate the magnitude of the cell lysis.

The aforementioned techniques to determine cell viability or death are highly dependent of the conditions. All methods have their own weaknesses and their aplication depends on the type of microorganism, the availability of equipment, and the accuracy required from the measurement. For industrial application a method has to be inexpensive to apply, reproducible and rapid. When considering these constrains, spectorphotometry and flurometry may be suitable techniques.

Cell vitality measurements

As previously mentioned, in microbiology vitality measurements and viability determinations are not so distinct from each other (Table 1).

The previously mentioned methods for cell viability evaluation in microalgae cultures (Section ***Cell viability measurements***) only estimate either the living or the dead proportion of cells in a population. Apart from these, another category of cells can be present; cell damage is due to the toxic environmental factors, that are not toxic enough to kill the organism. These factors can produce alterations at morphological, intracellular or metabolic levels that disable cell division but do not completely disactivate the cells metabolically. Due to a natural and

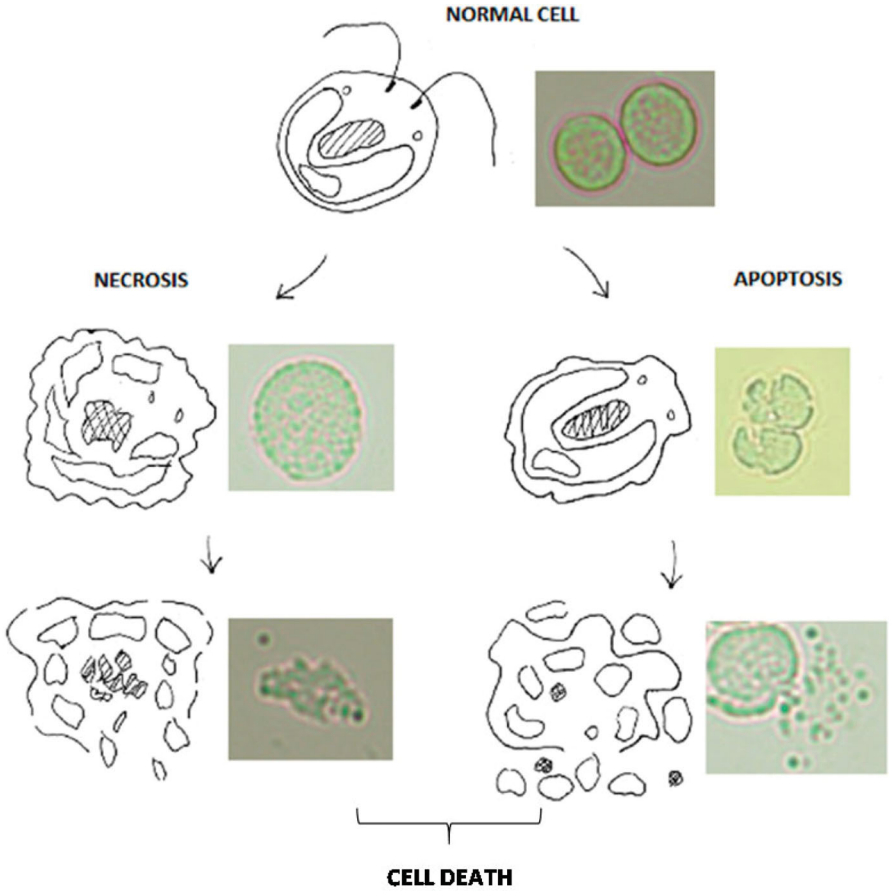

Figure 2. Schema of necrosis and apoptosis phenomenon's, of *Chlamydomonas reinhardtii* cells, induced by UV radiation. Adapted from Sydney et al. [69].

unavoidable cell death rate, a vital but non-viable culture is undesired for industrial production. Such cultures may start well but would eventully become totally inactive due to the accumulation of dead cells.

A second place were the issue of vitality becomes key is during storage; after biomass production it is not desirable that the target molecules should be degraded or lost. Here the cells must remain vital but their ability to reproduce (viability) is not necessarily required. Any optimized biotechnological production process must also take into account optimization of the post-production conditions, including storage and processing. A good example of these types of processes can be encountered in the dairy industry.

Methods used to assess cell vitality focus on different aspects of the physiological state of the cells and they can be divided into three groups: (1) membrane potential assessment [72,73]; (2) cellular ATP content determination [74]; and (3) enzyme activity assessment. Such enzymes include: esterases [75], oxidoreductases [46], or other different redox enzymes [76].

Culture viability and vitality can be both measuerd using certain fluorescent probes, often used in combination with epifluorescence microscopy. These have been described for microalgae vitality measurements [35,77]. The most commonly used fluorescent probes for this type of microorganisms is the FDA probe, based on esterase activity. As previously mentioned in Section *Cell viability measurements*, the fluorescein may rapidly leak out of the cells and can lead to a visible loss of vitality. Other esterase probes have been studied such as: Calcein-AM, used to assess the vitality of virus-infected cells of the phytoplankton species *Phaeocystis pouchetii* and *Micromonas pusilla* [78] and CMFDA, are used for monitoring phytoplankton cells in growth experiments [79]. A green fluorescent and charged Calcein, which is membrane-impermeable, is produced when the acetoxymethyl (AM) ester of Calcein-AM is hydrolyzed. In the case of CMFDA, its chloromethyl group undergoes a covalent reaction with thiols such as glutathione inside the cell, becoming fluorescent green when the acetates are cleaved by the intracellular esterases. H_2DCFDA is another membrane-permeable esterase probe that produces a green fluorescent color in the cells when esterases remove the acetate groups. The oxidation reaction takes place by

reactive oxygen species (ROS) [80]. This dye can be equally used to assess oxidative stress.

Considering another mode of action of vitality probes, the anionic oxonol DiBAC4(3) is among the methods used to assess cell vitality, this time, based on membrane potential and integrity. This probe enters the membrane once its depolarization has taken place, increasing the intensity of the fluorescence. Another membrane integrity probe is the aforementioned SYTOX Green (Section *Cell viability measurements*). This vitality probe has been tested in several microalgae [67,81,82]. Further, optical measurements may include cell auto-fluorescence, the intensity of vital stains, fluorescent products of viability stains, and forward and side light scatter [83].

Flow cytometry has also been used in combination with fluorescent probes [49,78,84]. The advantage of this technique is that it is capable of measuring multiple optical parameters simultaneously on a single-cell basis.

Many methods exist to measure viability and vitality and their suitability depends on the exact aplication. For industrial applications, viability is more commonly used than vitality to assess the impact that some factors might cause in the microbial community of a culture.

The importance of the quality control for industrial production of microagal biomass

Industrial processes for microalgal biomass production are highly dependent on the appropriate choice of the species for the desired application. The selection of the algal species, with specific biochemical properties, imposes certain considerations with respect to intrinsic environmental characteristics (the sensitivity and robustness of these microorganisms is strongly dependent on the environmental conditions) but also on the adequate production system. The application field restricts and directs the choice of organisms and technologies applied in each stage of the industrial process (Figure 3). The factors to consider are algal strains and their biochemical composition, the cost of land, availability of energy, type of water and nutrients, as well as seasonal environmental condition (in the case of outdoor culture) and the type of valuable target molecules [85]. As previously mentioned, microalgae produce a large range of intracellular molecules of interest through photosynthesis. The ability to accumulate these internal molecules differs in various algal species and is highly dependent on the growth condition. The process has to be tuned with the adapted algal strain most relevant to the desired valuable end-product [86]. The biochemical content of microalgae varies according to operational parameters such as the type of culture system as well as its growth medium composition in terms of mineral content [85]. Biomass quality control is a key issue for any biorefinery concept. Indeed, some specific actions need to be set up in order to guarantee the quality and certification of the biomass produced. Monitoring chemical, physical, biochemical and microbiological aspects are important troughout the culture process, especially when producing food-grade microalgae biomass [87]. These analyses ensure adequate quality management and biochemical composition uniformity of the end-product. They can be performed in the on-site laboratory and need to be validated by occasional analysis from independent certified laboratories [88]. The integration of the quality management approach (involving amongst others, hazard analyses and critical control points -HACCP- practices) is crucial in industrial processes in order to ensure the regulatory standards of the nutritional aspects of the biomass produced. Besides the importance of the biomass quality in terms of physiological integrity and biological properties, the microalgae biomass is subject to contamination risks such as heavy metals (from the materials used during the construction of the culture systems and the processing equipment), sodium content (due to the raised concern about foods containing high concentrations of calcium and sodium), bacterial contaminats, infesters (viruses), predators (protozoa), competitors (other algae) and potentially alien algal toxins (phytotoxins, hepatotoxin -microcystin- and neurotoxin -anatoxin-a- production) [88]. Biomass production under sterile or axcenic conditions is of great importance to control the microbial load in an industrial operation. For this, an appropriate and well-controlled production and biomass processing environmental must be in place [89]. The quality criteria of microalgal products are usually defined by the end cutomers and consumers [90]. Quality control through process assurance can be enhanced through two important parameters: the choice/design of the culture system and the control of environmental conditions. This, in turn, has a high impact on the physiological integrity of the cells (mortality, cell damage, etc.) and of their biochemical metabolism (bioaccumulation of intracellular molecules). One of the classic indicators of low biomass quality is a high level of chlorophyll degradation leading to the formation of phaeophytins, chlorophyllides and pheaophorbides [90]. Consequently, a measure of chlorophyll degradation could be used as an indicator of cellular health and damage in photosynthetic organisms.

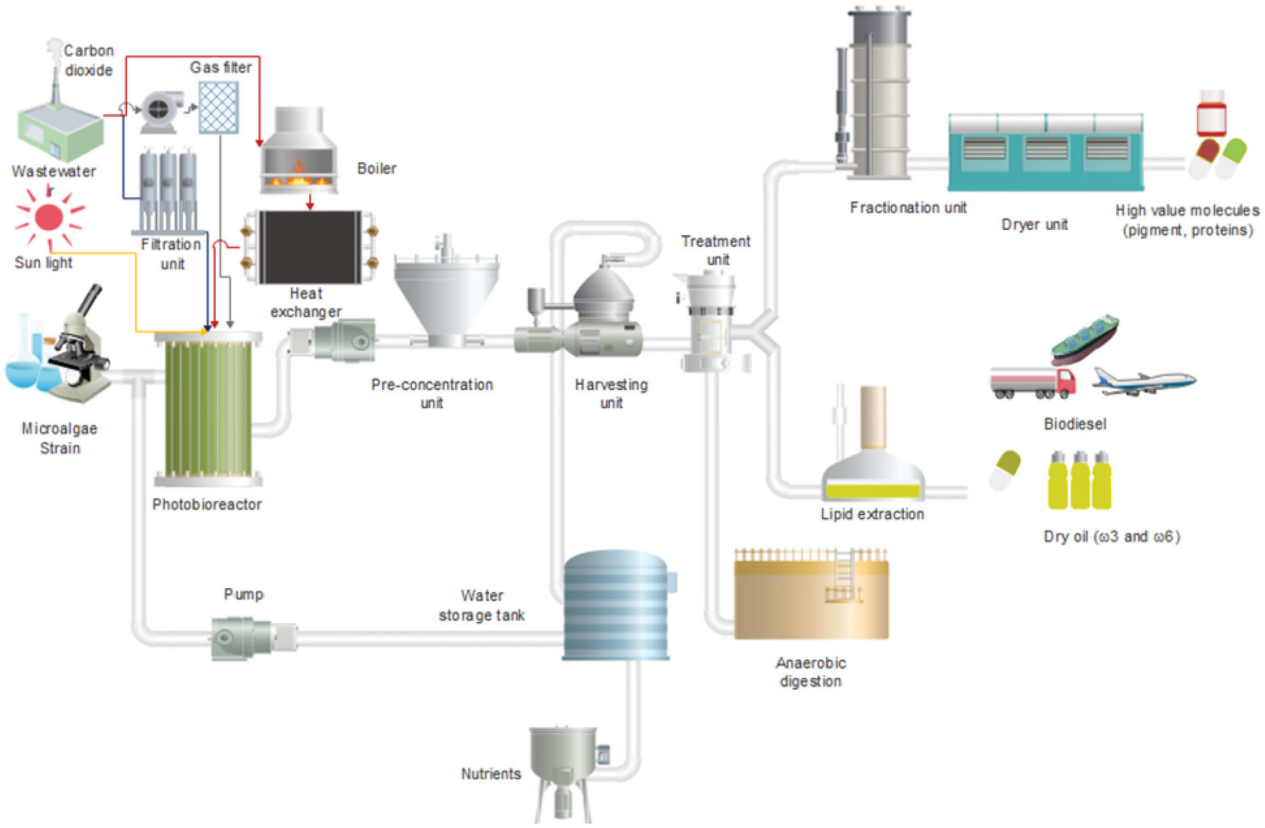

Figure 3. General microalgae production process flow.

Overall, it can be concluded that the regular control of microalgae culture quality is an indispensable condition for the success of any industrial application. The main parameters that might cause cell damage in a microalga are described below. These could constitute key parameters that could be monitored for quality reasons.

Principal factors of microalgae damage

Under abiotic stress, microalgae undergo significant metabolic and physiological changes in an attempt to survive (Table 2). Microalgae exposed to factors such as nutrient starvation, temperature variation, or ultraviolet radiation, increase their intracellular content of lipids, carbohydrates, carotenoids, and antioxidant enzymes. If the stress is too intense and/or persistent, the microalgal growth ceases and leads to apoptosis [103–105].

Temperature

Temperature is one of the most important factors that influences the production of biomass, as well as internal compounds, by its influence on the enzymatic reactions [106]. The optimum growth temperature for mesophilic microalgae is between 20 °C and 25 °C, although this range can vary depending on the growth medium composition and the species being employed. Temperatures below 16 °C can retard/reduce the growth rate of microalgae, while temperatures in excess of 35 °C are lethal for many algal species [99].

The microalgae growth temperature and their lipid profile correlate; for instance, the polar lipid content increases when the temperature decreases. Whereas, a temperature increase can lead to a higher accumulation of non-polar lipids (TAGs) [91]. Increasing the production of unsaturated fatty acids such as PUFAs, which maintains membrane fluidity at low temperatures, is a mechanism for the algae to adapt to such temperatures. Temperature is also known to influence the carotenoid content. An example is the effect of temperature on β-carotene accumulation in *Dunaliella salina* [107].

Light intensity and oxygen accumulation

Light is another major factor that influences the biochemical composition of the biomass due to its effect on photosynthesis [108]. Remarkable changes in the biomass and biomolecules profile of microalgae have been reported when algae cells are stressed with various light intensities and spectra. A reduction in the total lipids content of the microalga *Pavlova lutheri* due to an increase of light intensity has been reported [109]. A more intense light overcomes the need for a

Table 2. Main stress factors in microalgae cultures and changes observed in their biochemical composition.

Stress factor	Biochemical changes observed	Reference
Temperature		
below 25°C	Increase in PUFAs content (EPA and DHA).	[91,108]
	Low chlorophyll and high β-carotene accumulation in *D. salina*.	
above 25°C	Increase in TAGs content.	
High light intensity	Increase in TAGs content.	[92–94]
	Decrease in PUFAs content.	[95]
	Induction of the xanthophylls cycle in *C. reinhardtii*	[96]
	High levels of β-carotene accumulation in *D. salina*.	[107]
Oxygen accumulation	Decrease in the chlorophyll content.	[97]
pH		
optimum for biomass productivity	Maximum lutein content	[116]
alkaline	Decrease in membrane lipid content and accumulation of TAGs.	[98]
acid	High content of PUFAs and EPA.	[99]
Nutrients starvation (N, P)	Reduction in photosynthetic pigments and proteins	[117]
	Accumulation of lipids or carbohydrates.	[100–102]

high chloroplastidial activity. Consequently, a decrease in the content of such lipids is observed under intense light as these are major components of chloroplasts. An increase of neutral lipid content (TAGs) with a simultaneous decrease of polar lipids (PUFAs) has been observed in many species such as: *Chlorella sp., Monoraphidium sp.*, *Scenedesmus obliquus*, and *Nannochloropsis gaditana* under high light intensity [92–94]. The explanation may be related to the fact that the ROS, formed under higher light intensities, modifies the biochemical composition of chloroplasts to such unfavorable conditions through decreasing PUFAs synthesis. The photosynthetic potential of algae decreases as the light intensity increases (Figure 4) and this could indicate that fewer thylakoid membranes are required [110].

Another microalgal response due to light stress, is the induction of carotenoid synthesis in order to protect the cells from photodamage. Depending on the light source, light intensity or wavelength, and the microalgal strain, the mechanism of induction may follow different metabolic pathways. For example, the sudden exposure of *Chlamydomonas reinhardtii* to high light intensity led to the induction of the xanthophylls cycle [96].

When oxygen accumulates during photosynthesis, as it happens in closed photobioreactors, photoinhibition and photorespiration processes can occur. These processess both lead to a decrease in the photosynthetic efficiency yield, and the generation of ROS that can damage cellular components [97].

pH

pH is a particularly relevant growth indicator in the absence of strong buffering in microalgal cultures. Effective pH control in microalgae cultures, often by CO_2 injection, is an efficient way to produce algal biomass. Such a method effectivily maintains the optimal carbon species distribution in growth media [111,112]. Consequently, as the pH increases, carbonate concentration increases and bicarbonate and molecular CO_2 decrease, and vice-versa. However, at neutral pH values, any CO_2 consumed by photosynthesis can be replenished from a large pool of bicarbonate, since this is the predominant carbon source at neutral pH.

Some microalgae have adapted to low CO_2 s through the development of CO_2-concentration mechanisms (CCM) [113,114] which enables them to acquire and concentrate inorganic carbon from the environment, leading to efficient carbon dioxide utilization during photosynthesis [115].

Regarding the effect of pH on the biochemical composition of microalgae, Fernández-Sevilla et al. have reported that in general, the maximum lutein productivity is achieved at the optimum pH for biomass productivity [116]. In the case of lipid composition, pH variations in the culture medium can alter the content of total fatty acids, as it was observed in *Pinguiococcus pyrenoidosus*, with the highest content of PUFAs and EPA at a pH value of 6 [99]. Another example is *Chlorella sp.*, in which a decrease in the membrane lipid content with simultaneous accumulation of TAGs was directly related to alkaline pH stress [98].

Nutrients starvation

Nutrient starvation (especially nitrogen and/or phosphorus) negatively affects the viability of microalgae cultures. The absence of nitrogen or phosphorus is known to cause a reduction in the cellular concentration of photosynthetic pigments and cellular proteins, thus making photosynthetic fixation of CO_2 difficult [117]. Under such conditions, the carbon flux fixed from photosynthesis is mainly used for the synthesis of carbohydrates and lipids, rather than proteins synthesis and cellular proliferation [104]. This explains why the accumulation of lipids and carbohydrates is one of the main responses of numerous microalgae incubated under nitrogen- or phosphorus-limiting conditions

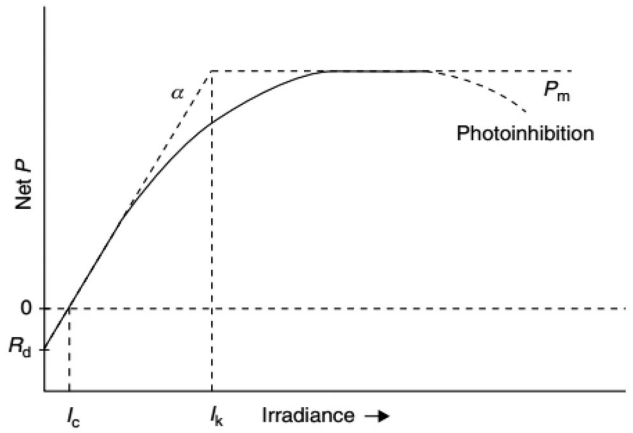

Figure 4. Photosynthesis vs. Irradiance (P-I) curve. R_d: respiration rate; I_c: compensation irradiance; I_k: saturation irradiance; I_{sat}: light saturation point; P_m: light-saturated rate [97].

[100–102]. Magnesium, which has a central position in the chlorophyll molecule, is also an important nutrient for the growth and the photosynthetic activity of microalgae [118].

The physiological effects of the main process parameters on microalgae growth and which targets molecule production, have been examined. Certain biomakers could provide a rapid method for detecting changes in culture physiology. These could be used for process control in an industrial setting.

Biomarkers: indicators of microalgal culture status

Biomarkers are defined as quantitative indicators of changes in a biological system that can be due to exposure to different environmental factors. The potential of some of these biomarkers as tools for process control are discussed below.

The cell membrane is the physical, chemical and biological interface with the outside environment. Thus, its integrity can be considered as an indicator of the culture status since cells with damaged membranes are, or will soon be, dead [119]. As discussed in Section ***Microbial culture-quality measurements***, cell membrane integrity can be measured in many methods by using selective stains in conjunction with microscopy. However, these techniques have the limitation of a potential overestimation of the number of viable cells because lethal stress may not lead to immediate cell membrane disintegration.

Living-cells convert nutrients and molecules into energy-rich compounds that can be used to ensure essential cellular activities such as reproduction, signaling, transport and motility.

In addition to being used as an indirect way to assess cellular vitality, the production of cell metabolism by-products can be considered to be biomarkers (Table 3). Their measurement can also provide relevant information on the deviation from optimal conditions [120].

ATP is a key high-energy molecule in the cell and should not be found in free form in the outside environment. The presence of ATP can be related to the number of living cells [121,122]. In a study carried out by Torres et al., they reported that in a culture of *Phaeodactylum tricornutum*, the cellular ATP content decreased as cadmium concentration increased, varying from 34.86 nmol ATP in health cultures to 18.58 nmol ATP in stressed cultures [123]. The method that has been used in industry for the detection of micoorganisms requires the addition of an ATP-releasing reagent to lyse the cells and release ATP which, in the presence of luciferase, reacts with the substrate D-luciferin to produce light. Thus, the intensity of light can be measured in relative light units (RLU) and can indicate the number of microorganisms present [124]. In addition, the presence of free ATP in the culture medium could be an indicator of cell lysis, and poor cell viability.

Conducting ATP assays, as an approach to measuring cell viability, has the disadvantage that the amount of these molecules can be overestimated, since exogenous ATP, already present in the medium, is also measured. Some commercial kits, such as CheckLite (Kikkoman, Japan), can avoid this limitation by enzymatically removing exogenous ATP.

Chlorophyll is another molecule that can be used to evaluate the status of a microalgae culture. This pigment, which is an effective indicator of the physiological state of the photosynthetic apparatus, can be conveniently deactivated and degraded by changes in temperature and pH since it is very sensitive to these factors [125,126]. A recent study, carried out by Takahashi, assessed a new method to quantify a *Chlorella* population and its status based on chlorophyll integrity, using an image-based cell counter with a fluorescent filter for chlorophyll detection [127]. The author observed that cells treated with high temperature signigicantly altered their fluorescence from high to low intensity, concluding that this method is able to evaluate the status of the culture. The production of fluorescence by chlorophyll is one of the forms of dissipation of excess light energy by the chloroplast, this being a useful tool for evaluating the photochemical potential of photosystem II (PSII), and also for monitoring potential cellular stress. From a quantitative point of view, chlorophyll fluorescence can be measured as an indirect indicator of cell viability using the pulse amplitude modulation (PAM) technique. PAM

Table 3. Biomarkers indicative of the status of microalgae cultures and main measurement methods.

Biomarkers	Indicative of	Measurement methods	Reference
ATP	The presence of free ATP in the culture medium could be an indicator of cell lysis	Bioluminescence assay	[121]
Chlorophyll	The physiological state of the photosynthetic apparatus. Indirect indicator of cell viability	Cell counter with chlorophyll detector PAM	[127,128]
Lipids	The appearance of free fatty acids or malondialdehyde as products of lipids degradation is indicative of potential oxidative damage to cell membrane and eventually cell death	TLC/BF_3 method Thiobarbituric acid reactive substances (TBARS) method	[135–138]
DNA	DNA presence in the culture medium and DNA degradation into oligonucleosomal fragments are indicative of cell lysis	Competitive PCR DNA laddering / comet assay	[139,141–143]
Caspase-like activity	Increasing the caspase-like activity is indicative of cell death	Caspase colorimetric activity assay kits Aspartate containing caspase substrates method	[145–147]
RNA	A decrease in the RNA content or the presence of RNA degradation products in the culture medium are indicative of cell death	RNA gel blot and dot blot analysis	[150]

fluorometers can determine the maximum photosynthetic efficiency or the máximum quantum yield (F_v/F_m) of microalgae cultures resulting in values between 0 and 1, which correspond to 0–100% viability. The maximum values of the F_v/F_m ratio usually range between 0.7 and 0.8 in health, nonstressed microalgae [128].

Cellular lipid composition can vary according to the microalgae growth stage and the cell cycle. he most common measurements for lipids detection and quantification are those based on dyes such as, Nile Red and BODIPY 505/515 [129,130]. These two dyes are used to assess especially the neutral lipid content of microalgae. In addition, Nile Red exhibits high affinity, specificity and sensitivity to the degree of hydrophobicity of lipids, which results in a shift of the emission spectrum from red to yellow in the presence of polar and non-polar lipids, enabling a distinction between them [131–133]. Changes in environmental conditions (e.g. temperature, light, and pH) and nutrient availability affects the concentration of the internal compounds. When a culture is subjected to stress, changes in lipid metabolism is a key adaptation mechanism to protect the cell [134]. Nervetheless, under persistent unfavorable conditions, oxidative stress can lead to lipid peroxidation, eventually resulting in membrane disintegration and cell death. For instance, the appearance of free fatty acids (FFAs), as products of lipids degradation, could be a consequence of deficient storage conditions and/or long storage periods, triggered by the undesired activity of lipases. Several methods have been used to measure FFAs [135,136]. Malondialdehyde (MDA) is a product of lipid peroxidation which could be also used to evaluate the potential oxidative damage to cell membrane [137,138].

DNA presence in the growth medium is another potential indicator of cell lysis in microorganisms [139]. Apart from a simple presence or absence of DNA in the supernatant, DNA degradation into oligonucleosomal fragments is a hallmark of cell lysis. This phenomenon has been demonstraed for animal apoptotic cell death and may also occur during plant necrosis. Unfortunately, this event does not always occur in unicellular microalgae. In *Micrasterias denticulata*, DNA degradation was not found to be related to cell death [140]. In contrast, DNA analysis in some studies carried out with microalgae such as *Chlamydomonas reinhardtii, Dunaliella tertiolecta* and *Chlorella saccharophila* demonstrated the dependency of DNA fragmentation on cell death [141–143].

A biochemical change that has been characterized in higher plants and animals as indicators of cell mortality is the activation of caspase proteases, which are considered essential enzymes in the apoptosis process. Chlorophytes and phytoplankton possess homologous caspases with functional differences, called metacaspases and caspase-like enzymes [144]. In this sense, some studies carried out with microalgae such as, *Micrasterias denticulate*, *Dunaliella tertiolecta*, *Dunaliella viridis*, and *Chlorella saccharophila* demonstrated that cell death was related to an increase in caspase-like activity [140,145–147].

It has been widely reported that RNA is degraded rapidly during the cell autolysis of yeasts [148,149]. Besides, a study carried out by Vayda and Yuan with the antarctic red algae *Plocamium cartilagineum* showed that the amount of intracellular RNA decreased dramatically with lower growth temperatures [150]. The authors related this to heat stress, a decrease in the amount of RNA, and the presence of RNA degradation products (ribonucleotides, ribonucleosides, and bases) in microalgae culture supernatants, might be used as a biomarker to determine the status of the biomass.

The assessment of biomarker responses throughout the culture process provides early warning signals that predicts when a microorganism will not be able to withstand a certain stress, since changes in such responses often precede changes in the cellular content of a culture. In this respect, some studies have demonstrated that the responses of different biomarkers to a particular stress in a microalga culture were detected prior to changes in the cell density [151–153]. Bearing this in mind, measures can be taken in advance such as either harvesting the culture, or ceasing the stress in order to continue with the growth and obtain a higher amount of biomass. In addition to improving cell density, it affords considerable time and money savings!

Conclusions

Microalgae culture remains a biotechnological process of high potential, the application of which is still retarded by a number of technical challenges. One of the lacking technologies for the industrialisation of microalgae products is a suitable quality control tool. Such a tool has to be rapid, portable and easy to be applied by laymen. The quality of microalgae biomass is a crucial aspect for large-scale industrial production, particularly in sectors involving biological and bioactive end-products. Algal biomass quality control approaches increase the stability and efficiency of the production process in terms of biomass target molecule productivities. Monitoring the quality of a culture allows a retroactive approach to predict and potentially react against specific environmental conditions or the presence of contamination. Microalgae cultures with 100% viability are unlikely to exist due to the effect of different stress factors during growth. Indeed, the production of several target molecules involves stressing the algal population. Hence, for the success of any microalgae culture at the large-scale, it is essential to have techniques which allow the assessment of its quality, in a fast, precise, and cost-effective manner. Several methodologies have been described for the evaluation of the culture status by measuring cell viability and vitality.

Preservation of membrane integrity is an accepted criterion for cell viability. Measurable changes in membrane permeability includes dye exclusion methods and lactate dehydrogenase release. SDC-MPN is a reference method for determining culture viability but offers limited potential for monitoring the growth of different microalgae cultures industrially; this method is too slow to give results!

The most promising assays are those based on the measurement of biomarker activities that are associated with culture status. Several biomolecules present in microalgae, namely chlorophyll, ATP, lipid oxidation products, DNA, RNA, and caspase-like proteases, can be used as indicators of culture condition. In this context, methods based on measurements of these compounds, must be considered as potential tools of great importance for determining the quality of microalgae cultures.

For any quality control system, what is key is to have a marker that correlates well with the quality parameter of interest and to be able to monitor markers over time in order to ensure product reproducibility.

Acknowledgments

This work has been supported financially by the European Union under the Interreg NWE-programme (IDEA Project: www.nweurope.eu; contract number NWE 669).

Disclosure statement

No potential conflict of interest was reported by the author(s).

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