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Insights into upstream processing of microalgae: A review

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HIGHLIGHTS

- This review discusses the important steps of upstream processing in microalgae research.
- Critical aspects that influence microalgal cultivation and biomass production are discussed.
- Existing challenges and knowledge gaps are discussed with future recommendations.

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ABSTRACT

The aim of this review is to provide insights into the upstream processing of microalgae, and to highlight the advantages of each step. This review discusses the most important steps of the upstream processing in microalgae research such as cultivation modes, photobioreactors design, preparation of culture medium, control of environmental factors, supply of microalgae seeds and monitoring of microalgal growth. An extensive list of bioreactors and their working volumes used, elemental composition of some well-known formulated cultivation media, different types of wastewater used for microalgal cultivation and environmental variables studied in microalgae research has been compiled in this review from the vast literature. This review also highlights existing challenges and knowledge gaps in upstream processing of microalgae and future research needs are suggested.

1. Introduction

Microalgae are a diverse group of microorganisms that can be found in water, soil (Subashchandrabose et al., 2011), air (Sahu and Tangutur, 2015), trees bark microhabitats (Wicker and Bhatnagar, 2020), and in some cases, even on animals (Pauli et al., 2014). Microalgae are eukaryotic microorganisms containing chlorophyll *a* found as individual cells or small colonies (Phwan et al., 2018). As compared to other microorganisms and terrestrial plants, microalgae have several unique advantages. As primary producers and capable of performing photosynthesis, they absorb sunlight (photons) and assimilate carbon dioxide (CO_2) from the atmosphere for biomass production. On the other hand, the majority of bacteria and fungi without photosynthetic apparatus have to feed on organic matter. Unlike plants, the growth of microalgae

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is not limited to arable lands and fresh water (Sajjadi et al., 2018). Microalgae can be cultivated on unproductive lands, such as infertile, arid, semi-arid lands, and polluted soils that are not usable for conventional agriculture (Junying et al., 2013). Also, these microorganisms can grow in saline water and even in nutrient-enriched wastewater. Moreover, microalgae cultivation is not limited to seasons; it can be repeated year-around and can be harvested daily (Gouveia and Oliveira, 2009).

Because of the above-mentioned benefits and their numerous applications, microalgae have attracted the attention of researchers from various fields such as environmental sciences, biology, genetics, chemistry, chemical engineering, medicine, polymer science, agriculture, and aquaculture. Research on microalgae is diverse, from genomic investigation to wastewater treatment, from pharmaceutical extraction to bioenergy production, from CO_2 bio-mitigation to biofertilizer manufacturing, among others. Microalgae research mainly comprises of upstream, midstream, and downstream processes. Upstream processing focuses on microalgal cultivation and maximization of biomass production. Midstream processing aims to harvest microalgae from cultivation media, dry the collected biomass, and rupture the microalgal cell walls before the extraction process. Downstream processing targets the extraction and purification of the bioproduct(s) from microalgal biomass (Manirafasha et al., 2016).

Upstream processes are considered as the baseline in microalgae research. These processes are important, technically and economically, as they directly affect the quality and quantity of the produced microalgal biomass. Upstream processes have several main steps (e.g. bioreactor design, cultivation media preparation, CO_2 supplementation, and adjustment and control of environmental factors, etc.) that should be considered in microalgae research. Owing to the importance of determining steps in upstream process, they have been reviewed previously. For example, the design and operation of different types of photobioreactors for microalgal cultivation has been reviewed (Vo et al., 2019). Li et al. (2019) evaluated the application of industrial, agricultural, and municipal wastewaters for microalgal cultivation. In another study, influence of different factors such as light, nutrients, pH, and CO_2 on microalgal growth were investigated (Junying et al., 2013). In previously published articles, some important factors of upstream process along with midstream and downstream processes were discussed. For instance, cultivation (upstream) and harvesting (midstream) of microalgae for biodiesel and biofuel production (downstream) was reviewed by Yin et al. (2020).

Despite the availability of several review articles related to different aspects of microalgae cultivation, there is lack of a comprehensive review on the upstream processes in microalgae research. Previously published review papers do not address all steps of microalgal cultivation and usually, their content has focused on a specific research field such as wastewater treatment, biodiesel production, agriculture, biomedicine etc. Thus, the aim of this review is to provide insights into the upstream processing of microalgae and to highlight the advantages of each step. For this purpose, the main pillars of upstream process in microalgae research are discussed, including different cultivation modes, photobioreactor design, culture media preparation, microalgae supply, environmental factors, and microalgal growth monitoring.

2. Microalgae cultivation modes

Like all living cells, microalgae also need a source of energy and starting materials to maintain steady biosynthesis, growth, and cell division (Sun et al., 2018). Depending on the sources of carbon and energy used, microalgae are categorized into photoautotrophic, heterotrophic, mixotrophic, and photoheterotrophic (Fig. 1), as also reported elsewhere (Hu et al., 2018). It should be noted that heterotrophic and mixotrophic microalgae have the ability of photoautotrophic metabolism also. In fact, heterotrophic and mixotrophic as secondary metabolic pathways might be observed in some photoautotrophic microalgae species. Cultivation of microalgae occurs via four pathways namely

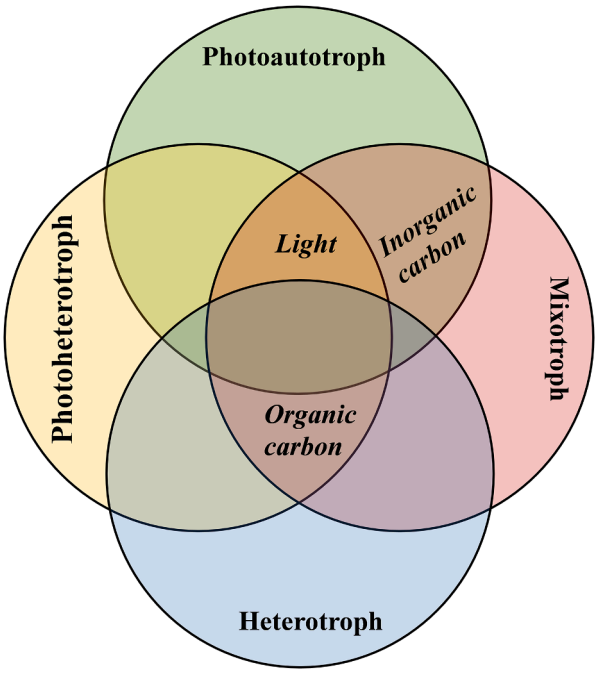

Fig. 1. Light, inorganic carbon, and organic carbon requirement for the photoautotrophic, heterotrophic, mixotrophic, and photoheterotrophic cultivation of microalgae.

photoautotrophic, heterotrophic, mixotrophic, and photoheterotrophic modes. Cultivation modes directly affect the requirements of the research and design, and the resulting growth of microalgae and biochemical composition of biomass. Therefore, it is one of the very first factors that needs to be determined in microalgae research.

2.1. Photoautotrophic cultivation of microalgae

The photoautotrophic process is the oldest and most common method of microalgae cultivation (Chew et al., 2018). Photoautotrophic microalgae biosynthesize organic matter by utilizing inorganic carbon as the source of carbon and light as a source of energy (Huang et al., 2010), forming chemical energy via photosynthesis. Eq. (1) shows biofixation of carbon and photosynthesis in organisms with chlorophyll *a*:

$$CO_2 + H_2O \rightarrow C_6H_{12}O_6 + O_2 \quad (1)$$

CO_2 and bicarbonate (HCO_3^-) are the foremost sources of carbon for cell growth of photoautotrophic microalgae (Kim et al., 2014). This implies that sequestration of CO_2 occurs via photoautotrophic cultivation mode. The ability of CO_2 biofixation by photoautotrophic algae has attracted the attention of researchers toward the development of carbon capture and utilization (CCU) strategies. CCU, as a distinguishing feature of microalgae, has two major benefits: it can assist in reducing greenhouse gas (GHG) emissions and, consequently, contribute towards mitigating climate change. In addition, the carbon captured by microalgae is fixed in their molecular structure, such as lipids, proteins, and carbohydrates, which can be utilized to produce many value-added biobased products (Subhash et al., 2017). Lower biological contamination risk is another advantage of the photoautotrophic cultivation mode. This is due to the absence of organic carbon in the photoautotrophic cultivation, which protects the medium against the heterotrophic bacteria. Hence, this cultivation mode is more appropriate for the outdoor cultivation of microalgae than other cultivation modes (Chew et al., 2018). The photoautotrophic mode is recommended for outdoor scale-up cultivation of microalgae, but its application is limited by light-dependency. Large-scale outdoor photoautotrophic cultivation with artificial light is expensive, therefore, finding a suitable location is

essential to optimize the process. Sunlight irradiation varies depending on the geographical region, season, and climatic condition. The lower biomass productivity of microalgae cultivated under the photoautotrophic mode as compared to the heterotrophic and mixotrophic cultivation modes is another drawback. Lower biomass productivity in the photoautotrophic cultivation mode is attributed to the self-shading effect on the microalgal vertical distribution that prevents light availability for denser cultivation (Nitsos et al., 2020). Hence, light as a single source of energy has a critical role in the successful implementation of photoautotrophic microalgae cultivation mode.

2.2. Heterotrophic cultivation of microalgae

In the heterotrophic cultivation mode, microalgae can grow in the absence of light. Heterotrophic microalgae species can provide the required carbon and energy for cellular metabolism through the consumption of organic carbon (Lam and Lee, 2012a). Heterotrophic microalgae have unique features. Biomass productivity in the heterotrophic cultivation mode is higher than that in the photoautotrophic cultivation mode. This is due to the light-independency of heterotrophic microalgae that facilitate high cell density without photoinhibition, a limiting factor in photoautotrophic mode (Chew et al., 2018). The feasibility of microalgae cultivation under dark conditions reduces the requirement of high surface to volume ratio, which eases the design of the heterotrophic microalgae bioreactor (Zhan et al., 2017). Overall, high biomass production and light-independency of the heterotrophic cultivation reduce production costs compared to the photoautotrophic cultivation. Nonetheless, the heterotrophic cultivation has several disadvantages that need to be considered. All microalgae can grow photoautotrophically, but few species can grow heterotrophically. Heterotrophic microalgae cannot consume CO_2, even though they generate CO_2 through the metabolism of organic carbon. Therefore, they are not useful in CO_2 mitigation research (Hu et al., 2018). The high risk of biological contamination with competing heterotrophic microorganisms, such as bacteria, yeast, and fungi, is another drawback of the heterotrophic cultivation mode, which could negatively affect biomass production and quality of the products of interest. Under heterotrophic conditions, other heterotrophic microorganisms compete with heterotrophic microalgae for the same sugar-based organic carbon substrates. Thus, the growth of microalgae is reduced in the presence of bacteria which have high growth rate and less doubling time (Hu et al., 2018). For the heterotrophic cultivation, everything in contact with microalgae such as reactor, supplied gases, and the medium, needs to be sterilized thoroughly to avoid culture contamination (Di Caprio et al., 2019). This problem is more significant for outdoor microalgae cultivation, including open ponds and raceways due to uncontrolled conditions (Bilad et al., 2014).

2.3. Mixotrophic cultivation of microalgae

Some microalgae species grow under mixotrophic conditions by using inorganic carbon and organic compounds simultaneously. Suitable microalgae for the mixotrophic cultivation have cellular apparatus for the photoautotrophic and heterotrophic metabolism, and based on the definition of mixotrophy, both inorganic and organic carbon are necessary for their growth. Although during mixotrophic cultivation, microalgae can grow photoautotrophically or heterotrophically, there is no apparent switch between heterotrophic and photoautotrophic metabolisms (Grobbelaar, 2013). Hence, switching between these two modes should not be confused with mixotrophy. The mixotrophic microalgae need illumination for biofixation of CO_2 through photosynthesis, and organic substrates for aerobic respiration while in total darkness, the metabolism turns to heterotrophy (Perez-Garcia and Bashan, 2015). Mixotrophic microalgae, possessing photoautotrophic and heterotrophic features, benefit from the advantages of photoauto- and heterotrophic modes. The combined use of CO_2, organic compounds and light is the distinguishing property of mixotrophic microalgae. This ability maximizes the usage of different resources to supply carbon and energy demands, and supports the requirements of both photoautotrophic and heterotrophic metabolisms. In the mixotrophic cultivation mode, the light requirement is lower than for photoautotrophic growth, which eliminates the associated light limitation. Some compounds, such as pigments, which are not produced in the heterotrophic cultivation mode due to the absence of light, are produced in mixotrophic cultivation (Lee, 2003). Like photoautotrophic microalgae, mixotrophic microalgae participate in CO_2 reduction via photosynthesis. The released CO_2 from respiration under heterotrophic metabolism is trapped and reused during photoautotrophic growth (Gaignard et al., 2019), which overall decreases CO_2 emissions compared to the heterotrophic cultivation mode. Ananthi et al. (2021) reported that biomass productivity in the mixotrophic cultivation mode is higher than in the photoautotrophic and heterotrophic cultivation modes. Li et al. (2014) found that the maximum dry weight of *Chlorella sorokiniana* cultivated mixotrophically was 2.4 and 5.2 times that of the same species cultivated heterotrophically and photoautotrophically, respectively. While the mixotrophic cultivation mode holds the advantages of both heterotrophic and photoautotrophic cultivation modes, microalgae cultivation via mixotrophic mode also encounters various disadvantages. Similar to the heterotrophic cultivation mode, the application of an organic substrate increases the cost of the mixotrophic cultivation. Consequently, the maintenance of axenic cultures in a pure state is difficult because of the sugar-based culture compounds, which increase the risk of contamination by unwanted heterotrophic microorganisms. Although mixotrophy reduces light-dependency, light is still a vital element for microalgae growth. Therefore, the mixotrophic cultivation requires organic substrates and sterilization (to prevent contamination) in addition to the presence of light that can increase the overall cost of the bioreactor design and operation. Moreover, only few microalgae species grow mixotrophically, which diminishes the research opportunity that can benefit from biodiversity. Details of several heterotrophic and mixotrophic microalgae species can be found in a review published by Hu et al. (2018).

2.4. Photoheterotrophic cultivation of microalgae

Photoautotrophy is also known as photo-organotrophy, photometabolism, or photo-assimilation (Chew et al., 2018). Photoheterotrophic microalgae like *Chlorella vulgaris* ESP-31 are a group of microalgae that require light as a source of energy, and organic carbon as a source of carbon (Yeh et al., 2012). Unlike photoautotrophs and mixotrophs, photoheterotrophs cannot metabolize CO_2. In contrast to heterotrophs, photoheterotrophs cannot grow on glucose without light. The photoheterotrophs use glucose as a building material, but not as the source of energy. In the light phase of photosynthesis, the energy of light is transformed into chemical energy of nicotinamide adenine dinucleotide phosphate (NADPH) and adenosine triphosphate (ATP). The synthesized ATP and NADPH are used in dark phase for the assimilation of glucose to biomass. In contrast to mixotrophs and heterotrophs, the generation of CO_2 is negligible in photoheterotrophs as the Calvin cycle is not active (Chojnacka and Marquez-Rocha, 2004). Photoheterotrophy is an expensive cultivation mode as microalgae need both organic carbon and illumination for growth and special design of photobioreactor is required for microalgal cultivation (Chew et al., 2018; Ananthi et al., 2021).

Considering the discussion presented above, it can be concluded that microalgae benefit from a worthwhile metabolic diversity. The diverse metabolic pathways enable microalgae to adapt to and use different sources of energy and carbon. Microalgal cultivation modes can be flexible depending on the availability of light, CO_2, and organic carbon (Fig. 1). Also, photoautotrophic, heterotrophic, mixotrophic, and photoheterotrophic metabolisms fit for different research goals and industrial applications of microalgae. Although photoautotrophy is the

dominant metabolic pathway, all other individual pathways have their own advantages which make them suitable for a variety of applications. Clear identification of metabolic pathways must be performed and required sources of energy and carbon should be provided for microalgae growth for achieving target outcomes.

3. Bioreactors for microalgal cultivation

In microalgae research, the selection of an appropriate vessel or container for microalgal cultivation is one of the important factors that need to be decided at an early stage. Typically, the term *bioreactor* is applied to the containers which support the growth of microalgae for biomass production and product formation. In this regard, photobioreactors refer to bioreactors which supply light for photoautotrophic microalgae that need light as a source of energy. Bioreactors with different sizes, shapes and materials are available for microalgal cultivation. The design can be as simple as handmade bioreactors to high-tech photobioreactors. Handmade bioreactors are mainly made of transparent glassware or polymeric materials such as polycarbonate, which can be assembled by researchers themselves or are manufactured and sold by local companies. For instance, Choi et al. (2019) designed a polymer film-based photobioreactor for microalgal cultivation. They developed a polypropylene-based bubble column photobioreactor (10 cm diameter and 120 cm height) for the cultivation of several microalgae species. Erlenmeyer flasks, bottles, or jars with different volumes have been widely used as bioreactors for microalgal cultivation. These are equipped with a tube for aeration and mixing of medium and covered by a cap, cotton stopper, aluminum foil, or parafilm to decrease evaporation and contamination (Aghaalipour et al., 2020; Supraja et al., 2020). Fig. 2(A) shows a schematic of a typical handmade bioreactor for microalgal cultivation designed by Daneshvar et al. (2019). The handmade bioreactor has one inlet for the injection of air to supply CO_2 and mixing power, one outlet for taking samples, and one small hole for gas venting (Fig. 2(A)). A syringe connected to the outlet tube facilitates the

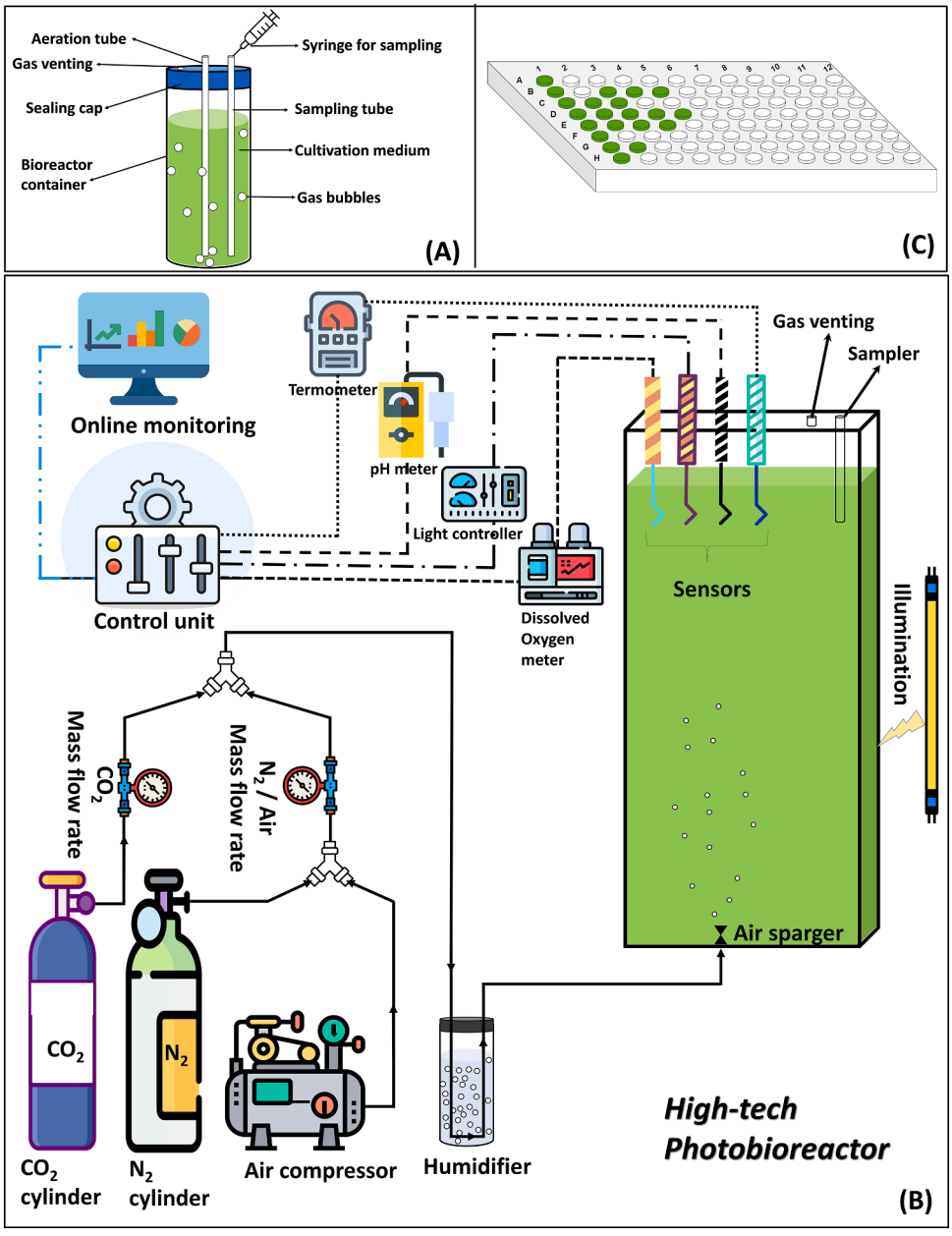

Fig. 2. Different types of bioreactors used for microalgae research: (A) Schematic overview of common handmade bioreactor, (B) High-tech photobioreactor with online monitoring system (Adapted from Naira et al., 2019), (C) Microplate for a small volume of microalgal cultivation.

collection of samples necessary for the evaluation of microalgal growth, biochemical analysis, and other specific measurements depending on the research objectives. In addition, the sealing cap reduces the evaporation rate and protects the culture from contamination better than other sealing options such as cotton, parafilm, and aluminum foils.

Although these simple bioreactors successfully assist in microalgal growth and biomass production and can meet the requirements of many research topics, they are not always appropriate. For example, in some experiments, pH level, CO_2 and O_2 concentrations, temperature etc. should remain constant. In this case, commercially available advanced bioreactors are required for controlling and monitoring sensitive parameters and to achieve optimal system performance during the experiments. These high-tech bioreactors can regulate parameters such as temperature, pH, O_2 and CO_2 pressure, mass and heat transfer, nutrient supply, hydraulic retention time, fluid velocity, shear stress, and cell growth (Mustafa et al., 2018). Depending on the model and application, advanced bioreactors can control several of the above-mentioned parameters. For instance, Li et al. (2003) used a stirred-tank fermenter model BiofloIII, New Brunswick Scientific, Edison, NJ, as a bioreactor for microalgal cultivation. They equipped the bioreactor with a pH sensor, pH meter, CO_2 mass flow controller, air mass flow controller, dissolved oxygen sensor, and oxygen meter. Typically, this kind of high-tech photobioreactor has a data acquisition board and supervisory computer for online monitoring of the parameters (Fig. 2(B)) (Naira et al., 2019). But handmade bioreactors can also be upgraded with individual sensors based on the requirements of the experiment. For example, Khichi et al. (2019) attached a pH and temperature probe, mass flow controller, and heating/cooling coil system to a photobioreactor to control the pH and temperature of the culture.

Microplates or multiwell plates have also been used for microalgal cultivation. Microplates can be considered as miniature bioreactor that are appropriate for the experiments with high numbers of treatments in tiny volume of microliters to milliliters. Fig. 2(C) shows a 96-well microplate containing 12 column and 8 rows. Dao et al. (2018) cultivated microalgae in a transparent 96-well microplate. Each well was filled with 100 μL of medium, and the microplate was sealed with a breathable sealing film. In another study, Kim et al. (2019) used a 96-well microplate with 200 μL working volume for microalgal cultivation. To isolate the experimental units, microplates were covered using a gas and light permeable membrane.

We would like to add to this discussion that the bioreactors which are used for microalgal cultivation are extremely diverse in shape, volume, and materials. Table 1 presents a list of bioreactors (and their working volumes) that have been used for the cultivation of different microalgae strains in synthetic media and wastewater. The information presented in Table 1 shows that the size of the bioreactors used in microalgae research varies from microliter (100 μL) to as large as thousands of liters (33,000 L). The volume of a research bioreactor does not limit the selection of microalgae species, cultivation modes, and cultivation media (Table 1). A microalgae research laboratory, that is called phycolab, may include different types of bioreactors for microalgal cultivation, depending on the requirements of the research. In microalgae research, usually, preliminary studies are performed in small bioreactors such as Erlenmeyer, bottles, or glass jars. As it has been illustrated in Fig. 3(A), these bioreactors can be arranged in a shelf, and illuminated by artificial lights. The optimized experimental conditions are used for scaling up the microalgal cultivation in larger bioreactors. Open ponds, raceways, tubular photobioreactors, and flat plate photobioreactors are used for large-scale microalgal cultivation. Open ponds and raceways are usually constructed using cement and polyvinyl chloride materials. These ponds are shallow (around 30 cm) such that more light can be absorbed maximizing the photosynthetic rate. A pedal is used to circulate and mix the culture medium in raceway ponds. Tubular photobioreactors are long tubes, made from glass or transparent materials. Usually, the diameter of the tubes is<10 cm for appropriate light penetration. Tubular photobioreactors might have horizontal or vertical forms. Horizontal tubes have panel-like system (tubes on the ground) and fence-like system (tubes parallel up together). Vertical tubes are divided into bubble and airlift columns. Flat plate photobioreactors with high surface area for light absorption are installed vertically or inclined toward light sources. Due to short light-path and efficient light penetration, rectangular photobioreactors are commonly used for microalgal cultivation. Similar to vertical photobioreactors, an air sparger connected to air pump is used to mix and circulate the medium in flat plate photobioreactors. Different types of photobioreactors and their properties have been discussed in detail by Chew et al. (2018). Fig. 3 shows four types of common bioreactors viz., bottle bioreactors (A), flat plate (B), helical (C), and airlift bioreactor (D) , which are used frequently for pilot-scale cultivation of microalgae. Appropriate aeration, illumination, medium circulation, and mass/heat transfer must be considered

Table 1
A list of bioreactors and their working volumes used in microalgae research.

Bioreactor	Working volume	Microalgae species	Cultivation medium	Reference
Microplates (96-well)	100 μL	*Scenedesmus* sp.	Modified BG11	(Dao et al., 2018)
Microplates (24-well)	150 μL	*Neochloris oleoabundans*	A seawater-type medium	(Santos et al., 2012)
Microplates (96-well)	200 μL	8 green microalgae	BG11 and f/2	(Kim et al., 2019)
Clear Multiwell Plate	4 mL	100 native microalgal strains	Sterile municipal wastewater or BBM	(Abdelaziz et al., 2014)
Erlenmeyer flask	80 mL	*Auxenochlorella protothecoides*	Tris-acetate-phosphate (TAP)	(Polat et al., 2020)
Erlenmeyer flask	100 mL	*Scenedesmus vacuolatus*	BG11	(Anand et al., 2019)
Transparent bottle	200 mL	*Chlorella vulgaris*	BBM medium	(Daneshvar et al., 2018b)
Flat-plate photobioreactors (transparent polymethyl methacrylate)	1.6 L	*Chlorella vulgaris* FACHB-31	BG11	(Chang et al., 2016)
Glass bottle photobioreactor	4 L	*Psammothidium* sp.	Allen Medium	(Aghaalipour et al., 2020)
Polymer film-based bubble column	5 L	Various microalgae species	TAP, BG11, f/2	(Choi et al., 2019)
Bubble-driven column photobioreactor	9.6 L	*Chlorella* sp. FC2 IITG	BG11	(Naira et al., 2019)
Column photo-bioreactors	30 L	*Nannochloropsis oculate*	Wright's cryptophyte	(Blockx et al., 2018)
Quartz columns photobioreactor	50 L	*Chlorella vulgaris*	Secondary effluents samples	(Almomani, 2020)
Flat-plate photobioreactor	550 L	*Scenedesmus* sp.	Nutrient-rich effluent from pretreated sewage	(Viruela et al., 2016)
Thin-film flat-plate photobioreactor (FPPBR)	13,000 L	*Chlorella* sp.	BG 11	(Yan et al., 2020)
Raceways	33,000 L	*Desmodesmus armatus*	–	(Corcoran et al., 2018)

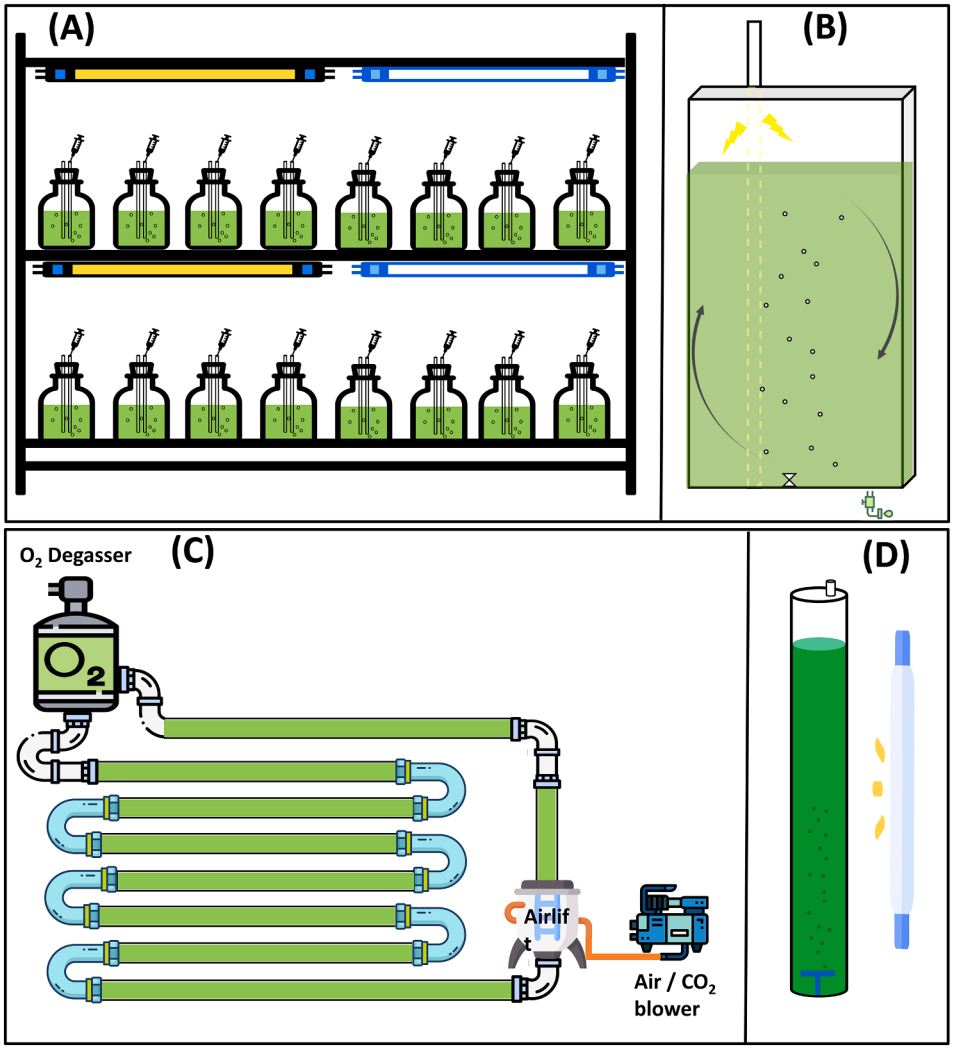

Fig. 3. Common lab-scale bioreactors for microalgae cultivation: (A) bottle bioreactors, (B) flat plate bioreactor, (C) helical bioreactor, and (D) airlift bioreactor.

carefully in designing of larger bioreactors.

4. Culture media and nutrients supplementation

Culture media are solutions containing essential nutrients that are needed by microalgae to maintain a steady state, good health, and growth (Procházková et al., 2014; Grobbelaar, 2013). Nutrients are categorized into macronutrients, micronutrients, and trace elements depending on their required amount for optimal growth. The first group includes elements, such as carbon (C), hydrogen (H), oxygen (O), nitrogen (N), and phosphorous (P), that microalgae need in higher amount (g/L) in the cultivation media. Lower concentrations (mg/L or less) of micronutrients such as cobalt (Co), zinc (Zn), manganese (Mn), and barium (Ba) in cultivation media are sufficient for microalgal growth and biomass production (Grobbelaar, 2013).

Formulated media and different wastewaters (usually enriched in nitrogen and phosphorus compounds) are frequently used as culture media to supply nutrients for microalgal growth (Table 1). Formulated media are synthetic broth with recommended concentrations of micro- and macro-nutrients. These media have been extensively tested for the cultivation of different freshwater and marine microalgal species (e.g. f/2 medium developed by Guillard (1975)), institutes (Culture collection of Algae at The University of Texas, UTEX), or commercial groups (The Culture Collection of Algae and Protozoa, CCAP). As stated in Table 2, formulated media are popular by their abbreviates or commercial names such as BBM (Bold's Basal Medium), f/2 (Guillard), and BG11 (Blue-Green), in microalgae research community. Each synthetic medium comes with specific instructions including the names of components (macro- and micronutrients) and their concentrations (mass concentration or molarity), which describes the stepwise solution preparation (Polat et al., 2020).

Formulated media could be applicable as non-specific media, therefore, being useful for the cultivation of many microalgal species or they might be designed for a specific group of microalgae. For example, Bold's Basal Medium (BBM) and Guillard (f/2) are two commonly used formulated media for the cultivation of a diverse group of freshwater and marine microalgae, respectively. Blue-Green (BG11) is an appropriate medium for the cultivation of cyanobacteria, but it is also extensively used for the cultivation of microalgae (Enamala et al., 2018). On the other hand, Zarrouk and f/2 + Silicon (Si) (Guillard's medium for diatoms) are specialized media for the cultivation of *Spirulina* sp. (cyanobacteria) and diatoms, respectively (Araújo and Souza-Santos, 2013; Costa et al., 2018). A list of commonly used formulated media for the cultivation of cyanobacteria, freshwater and marine microalgae, and their properties have been reported by Geada et al. (2017). Procházková et al. (2014) introduced 30 elements as sources of macro- and micronutrients for the photoautotrophic cultivation of microalgae. Chemical compounds containing these elements and their concentrations in a few popular media are presented in Table 2. It should be noted that supplying the essential elements is not limited to the compounds presented

Table 2
Elemental composition of four well-known formulated cultivation media of microalgae (Adapted from Grobbelaar, 2013 and Procházková et al. (2014)).

Element	Compounds*	BBM	f/2	BG11	Zarrouk
C	CO_2, HCO_3^-, CO_3^{2-}	Aeration	Aeration	Aeration	Aeration
O	O_2, H_2O	H_2O	H_2O	H_2O	H_2O
H	H_2O	H_2O	H_2O	H_2O	H_2O
N	$NaNO_3$	0.25 g/L	0.075 g/L	1.5 g/L	2.5 g/L
Na	NaCl	0.025 g/L	–	–	1 g/L
	Na_2CO_3	–	–	0.02 g/L	–
	$NaHCO_3$	–	–	–	16.8 g/L
K	KOH	0.031 g/L	–	–	–
	K_2SO_4	–	–	–	1 g/L
Ca	$CaCl_2{\cdot}2H_2O$	0.084 g/L	–	0.036 g/L	0.08 g/L
P	K_2HPO_4	0.075 g/L	–	0.04 g/L	0.5 g/L
	KH_2PO_4	0.175 g/L	5.65 mg/L	–	–
S, Mg	$MgSO_4{\cdot}7H_2O$	0.075 g/L	–	0.075 g/L	0.2 g/L
Cl	as Na^+, K^+, Ca^{2+} or NH_4^+ salts	–	–	–	–
Fe	Fe-ammonium citrate	–	–	0.006 g/L	–
	$FeSO_4{\cdot}7H_2O$	4.98 mg/L	–	–	0.01 g/L
	$FeCl_3{\cdot}6H_2O$	–	3.15 mg/L	–	–
Zn	$ZnSO_4{\cdot}7H_2O$	8.82 mg/L	0.022 mg/L	0.222 mg/L	0.222 mg/L
Mn	$MnCl_2{\cdot}4H_2O$	1.44 mg/L	0.18mg/L	1.81 mg/L	1.81 mg/L
B	H_3BO_3	11.42 mg/L	–	2.86 mg/L	2.86 mg/L
Mo	$Na_2MoO_4{\cdot}2H_2O$	–	0.006 mg/L	0.391 mg/L	–
	MoO_3	0.71 mg/L	–	–	0.01 mg/L
Cu	$CuSO_4{\cdot}5H_2O$	1.57 mg/L	0.01 mg/L	0.079 mg/L	0.08 mg/L
Co	$Co(NO_3)_2{\cdot}6H_2O$	0.49 mg/L	–	0.0494 mg/L	–
	$CoCl_2{\cdot}6H_2O$	–	0.01 mg/L	–	–
Br		as Na^+, K^+, Ca^{2+} or NH_4^+ salts		Not applied in these media	
Si		$Na_3SiO_3{\cdot}9H_2O$		Not applied in these media	
V		$Na_3VO_4{\cdot}16H_2O$		Not applied in these media	
Sr		as sulfates or chlorides		Not applied in these media	
Al		as sulfates or chlorides		Not applied in these media	
Rb		as sulfates or chlorides		Not applied in these media	
Li		as sulfates or chlorides		Not applied in these media	
I		as Na^+, K^+, Ca^{2+} or NH_4^+ salts		Not applied in these media	
Se		SeO_3^{2-}, SeO_4^{2-}		Not applied in these media	
	Citric acid	–	–	0.006 g/L	–
	Vitamin B12	–	0.0005 mg/L	–	–
	Vitamin B1	–	0.1 mg/L	–	–
	Biotin	–	0.0005 mg/L	–	–
	EDTA	0.05 g/L	4.16 mg/L	0.001 g/L	0.01 g/L
	pH	–	–	7.4	7.5

in this table, and it depends on the recipe of the media. Based on the information provided in Table 2, we can summarize that the concentrations of some compounds such as vitamins are needed at low concentrations (mg/L) in culture media. Usually in microalgae laboratories, the culture media are prepared by diluting highly concentrated solutions of individual compounds.

'Modified media' is another term that is commonly used when composition of medium is changed slightly by increasing or decreasing the original concentrations of compound(s) in culture media. Modified media are used to enhance microalgal biomass production, to stimulate the production of special metabolites, or to study the effects of nutrient deficiency or deprivation based on the requirement of experimental design. For example, Anand et al. (2019) used modified BG-11 media at various concentrations (10 – 100 mM) of NaCl, $MgCl_2{\cdot}6H_2O$, and $CaCl_2{\cdot}2H_2O$ for cultivation of *Scenedesmus vacuolatus*. They tested salinity-driven stress as a biodiesel trigger to enhance lipid production in microalgae. The same mixture of nutrients that has been suggested for the photoautotrophic cultivation of microalgae (Table 2) can be used for the heterotrophic, mixotrophic, and photoheterotrophic cultivation modes after the addition of organic carbon sources such as glucose, acetate, or glycerol (Perez-Garcia and Bashan, 2015). Smith et al. (2020) used original f/2 and modified f/2 (f/2 medium containing Si) for the photoautotrophic cultivation of green microalgae and diatoms (marine microalgae that need silicon to grow), respectively. Glucose, glycerol, and acetate as organic carbon sources were added to f/2 medium for the heterotrophic cultivation of the same microalgae.

Nutrient-enriched wastewaters are another low-cost and freely available medium that can provide required macro- and micronutrients in addition to water for microalgal growth. Contrary to formulated media, concentrations of nutrients in wastewater are unknown. When evaluating the suitability of wastewater as microalgal cultivation media, the concentrations of nutrients, specifically nitrogen (N), phosphorus (P), and carbon (C) must be quantified. The threshold concentrations of these nutrients in wastewater should be considered to avoid microalgal growth limitation. The elemental ratio of C:N:P in microalgal biomass are approximately 106:16:1, which is known as the Redfield ratio (Grobbelaar, 2013). However, the Redfield ratio is used to estimate the limitations of essential nutrients in microalgal cultivation medium, but it cannot be generalized as an optimum value for all microalgal species. Depending on the species, the ratio of N:P in wastewater can vary from 8 to 45 (Salama et al., 2017). In this regard, various types of domestic, agricultural, and industrial wastewaters have been tested for the cultivation of numerous freshwater and marine microalgal species (Table 3). The characteristics of the wastewater used for microalgal cultivation are usually provided in research articles. The main characteristics include physical and chemical properties (e.g., pH, total suspended solid, color, and electrical conductivity) and the concentrations of nutrients (e.g., NH_4^+, NO_2^-, NO_3^-, and PO_4^{3-}). For example, Ansari et al. (2019) measured several characteristics of municipal wastewater including pH, color, odor, temperature, electrical conductivity, total dissolved solids, salinity, dissolved oxygen, chemical oxygen demand, biochemical oxygen demand, alkalinity, NH_4^+, NO_2^-, NO_3^-, PO_4^{3-}, Fe, Zn, Na, and Mg. The utilization of wastewater as a medium for microalgal cultivation has several advantages including i) low-cost production of microalgal biomass; ii) recovery of nutrients from wastewater; and iii) treatment of wastewater for safe discharge (Salama et al., 2017). Wastewater as cultivation medium has been utilized specifically for wastewater treatment (Li et al., 2019), bioenergy production (Ananthi et al., 2021), and CO_2 sequestration (Razzak et al., 2017). It should be noted that microalgal biomass produced in wastewater cannot be used in food, cosmetics, medicine production etc. for human consumption due to the risk of contamination of biomass produced in wastewater with organic and inorganic pollutants and microbes, which could be detrimental to human health. Although the biomass produced in formulated media can be used for different applications and human consumption, mass production in synthetic media is expensive compared to wastewater.

Table 3
Different types of domestic, agricultural, and industrial wastewaters used for microalgae cultivation (Units of N, P, and C compounds: mg/L).

Wastewater	N	P	C	Microalgae	Biomass production	References
Molasses wastewater	32.50 TN	2.42 TP	3770 COD	*Monoraphidium* sp.	1.21 g/L	(Dong et al., 2019)
Petrochemical wastewater	31.27 TN	1.95 TP	671.30 COD	*Tribonema* sp.	4.4 g/L	(Huo et al., 2019)
Swine wastewater	510 TN	76.10 TP	5200 COD	*Chlorella sorokiniana* AK-1	8.08 g/L	(Chen et al., 2020)
Domestic Wastewater	52 – 93 TN	13.40 – 28.50 TP	140 – 210 COD	*Chlorella variabilis*	0.99 g/L	(Tran et al., 2020)
Raw biogas slurry	271.45 TN	51.92 TP	997.23 DIC	*Chlorella* sp.	0.53 g/L	(Yan et al., 2016)
Municipal wastewater	52.20 NH_4^+	8.47 PO_4^{3-}	400 COD	*Scenedesmusobliquus*	0.88 g/L	(Ansari et al., 2019)
Mixture of black water and gray water	95 TN	12 TP	700 COD	*Spirulina platensis*	0.81 g/L	(Zhou et al., 2017)
Centrate wastewater	64–289 TN	68–142 TP	1014–4611 COD	*Chlorella vulgaris*	2.2 g/L	(Ren et al., 2017)
Hydrocarbon wastewater	63.50 TN	17 TP	285 COD	*Spongiochloris* sp	8.51 g/L	(Abid et al., 2017)
Secondarily treated urban wastewater	20.09 TN	1.55 TP	70 COD	*Scenedesmus obliquus*	1.4 g/L	(Álvarez-Díaz et al., 2017)
Seafood wastewater	243.9 NH_4^+	69.80 PO_4^{3-}	610 HCO_3^-	*Chlorella vulgaris*	0.49 g/L	(Nguyen et al., 2019)
Tannery wastewater	103.80 TN	1.83 PO_4-P	814 COD	*Tetraselmis* sp. consortium	1.40 g/L	(Pena et al., 2020)
Dairy wastewater	86.0 TN	8.75 PO_4^{3-}	170.11 TOC	*Tetraselmis suecica*	0.58 g/L	(Daneshvar et al., 2019)
Textile wastewater	373.6 NO_3^-	78.70 PO_4^{3-}	42.44	*Micractinium* sp.	1.35 g/L	(Oyebamiji et al., 2019)
Industrial wastewater	153.1 NH_4-N	11 PO_4-P	72 TOC	*Chlorella vulgaris*	1.52 g/L	(Yadav et al., 2019)

Therefore, formulated medium and wastewater as cultivation media have their own advantages and disadvantages in microalgae research.

5. Adjustment of environmental factors governing microalgal growth

Several environmental factors, such as pH, temperature, irradiation, and aeration need to be adjusted before cultivation of microalgae (Table 4). These parameters not only affect the growth of microalgae, but also influence the biochemical composition of microalgal biomass. The individual and combined effects of these factors on microalgal growth have been researched and reviewed extensively. After determining the cultivation mode, bioreactor selection/design, and preparation of cultivation media, environmental factors must be adjusted, before inoculation of microalgae. Below, we review and summarize the most important environmental factors which affect microalgal growth and biochemical composition.

5.1. Light

Microalgae have different types of pigments, such as chlorophyll *a* (all microalgae), carotenoids, phycoerythrin (red microalgae), and

Table 4
A list of environmental variables as reported in different studies in microalgae research.

Microalgae	Light intensity μmol m^{-2} s^{-1}	Photoperiod (L:D)	Light wavelength	Light source	pH	CO_2 %	Temp. °C	Salinity	Aeration L min^{-1}	Maximum algal yield g L^{-1}	Reference
Chlorella sorokiniana	1000–3000	–	–	Natural irradiance	5.0 and 6.5	–	23.1–30.8	–	5	0.7	(Liu et al., 2020)
Eustigmatos vischeri	300	24:0	–	–	–	1	25	–	–	8.08	(Xu et al., 2020)
Scenedesmus obliquus	100	12:12	–	–	7.2	–	25	–	–	0.897	(Qu et al., 2020)
Chlorella sp. *and Nannochloris oculata*	100	–	400–750 440–500 500–550	LED	–	1	25	–	–	–	(Yuan et al., 2020)
Tisochrysis lutea	60	24:0	627	LED	8.7	0.6	25	40–50	1	1.5	(Fret et al., 2020)
Nannochloropsis oceanica	200 to 636	24:0	450–620	LED	7.8		15–30		1	–	(Sá et al., 2020)
Botryococcus braunii	133 to 348	-	–	LED	8	5	27	–	–	2.52	(Khichi et al., 2019)
Scenedesmus obliquus	280	24:0	–	LED	6.8	0.04–34	27	–	0.1	>2	(Molitor et al., 2019)
Chlorella sorokiniana	175	14:10	–	–	7	3	25	–	0.5	11.5	(Vasconcelos Fernandes et al., 2015)
Chlamydomonas reinhardtii	110.3	24:0	–	Cool-white fluorescent	7.2	0.04	25	–	–	0.19	(Patel et al., 2015)
Nannochloropsis salina	90–120	16:8	–	–	7.5		23	20	–	–	(Fagerstone et al., 2011)
Nannochloropsis oculata	–	–	–	–	8.5	–	–	30	5	0.25	(Blockx et al., 2018)
Pavlova lutheri	90–130	24:0 12:12	–	White fluorescent	5–10	–	28	15–40	–	–	(Shah et al., 2014)
Botryococcus braunii	0–2000	14:10	–	Cool-white fluorescent	–	0.04–50	5–45	0.4–18.1	–	3.3	(Yoshimura et al., 2013)
Chlorella sp.	300–900	12:12 14:10 16:8	460,660	LED	6.42	–	25	–	–	0.532	(Yan et al., 2016)
Phaeodactylum tricornutum	500	24:0	–	–	8	5	20	–	–	~ 0.75	(Choi et al., 2019)

phycocyanin (cyanobacteria) (Detweiler et al., 2015). Photosynthetic microalgae can convert the energy of light to chemical energy using these pigments. Therefore, except for the heterotrophic cultivation, all other modes (photoautotrophic, mixotrophic, and photoheterotrophic cultivation modes) must have a light supply as the primary energy source for microalgal growth. Natural sunlight, and fluorescent and LED lights have been utilized as lighting system for microalgae cultures (Table 4). Ordinary fluorescent lights, which are conventionally used in microalgae research, irradiate indivisible growth-efficient and growth-inefficient wavelengths (Ra et al., 2016). LEDs produce a narrower spectral range, which are more compatible with the absorption bands of microalgae pigments (Hsieh-Lo et al., 2019). Despite stimulating microalgae cultures with fluorescent lights or LEDs, the optimization of the lighting conditions is one of the key factors for achieving the highest growth rate of microalgae. Light intensity, wavelength, and photoperiod (lightning time) are three important characteristics of light that can significantly affect the growth of microalgae in photosynthetic cultures. Light intensity is the amount of light received on the surface per second (μmol m^{-2} s^{-1}). Table 4 presents a wide range of light intensities (from < 100 μmol m^{-2} s^{-1} to > 1000 μmol m^{-2} s^{-1}) that have been tested for microalgal growth.

The relationship between light intensity and photosynthetic rate is shown by photosynthetic light-response curve. This curve has three phases, namely light-limitation, light-saturation, and photoinhibition phase. Light limitation and photoinhibition are the two phases, capable of decreasing or even terminating the microalgal growth. Light limitation occurs in cultures with insufficient light intensity or high cell density. This could arise due to the self-shading effect in reactors with a high biomass concentration that reduces the amount of light, penetrating through the bioreactor, and negatively affects photon absorption and photosynthetic efficiency (Holdmann et al., 2019). In the light-limitation phase, increase in the light intensity enhances microalgal growth up to the area of light saturation. Further increase in light intensity in this phase does not affect microalgal photosynthesis, while photoinhibition occurs at light intensities higher than saturation point. Intensive irradiation at photoinhibition phase damages photosystem II and decreases microalgal growth significantly, or collapses the culture (Hsieh-Lo et al., 2019). The approximate light intensities for light-limitation, light-saturation, and photoinhibition phases are up to 300 μmol m^{-2} s^{-1}, 300–1600 μmol m^{-2} s^{-1}, and > 1600 μmol m^{-2} s^{-1}, respectively (Straka and Rittmann, 2018).

The light spectrum of solar radiation consists of diverse wavelengths of energy, most of which cannot be utilized by microalgae. Microalgae can use visible wavelengths from 400 to 700 nm through photosynthesis. This narrow spectrum is called photosynthetically active radiation (PAR) range (Vadiveloo et al., 2015) and includes 400–500 nm and 600–700 nm wavelengths (blue and red, respectively) which is the appropriate range for optimal microalgal photosynthesis, and 500–600 nm and 700–800 nm (green-yellow and far-red, respectively) are the transmitted or reflected wavelengths (Ramanna et al., 2018). Each pigment has major absorption bands that can absorb specific wavelengths of PAR. For example, the major absorption bands of chlorophyll *a*, chlorophyll *b*, and carotenoids are 450–475 nm (blue of blue-green), 630–675 nm (red), and 500–600 nm, respectively (Teo et al., 2014). Zhao et al. (2013) reported the highest dry weight of *Chlorella* sp. as 412.93, 470.74, 518.43, and 560.79 mg/L under red light irradiation with intensities of 800, 1200, 1600, and 2000 μmol m^{-2} s^{-1}, respectively. Fozer et al. (2019) found a higher photosynthetic efficiency under mixed color irradiation than under monochromatic irradiation. They reported the highest biomass productivity of 60.4, 50.0, 41.2, 40.3, 33.4, 31.7, and 29.86 mg/L/d under purple (626 nm, 470 nm), blue-green (525 nm, 470 nm), yellow (626 nm, 525 nm), white (626 nm, 525 nm, 470 nm), blue (470 nm), red (525 nm), and green (626 nm) illumination, respectively.

In case of outdoor cultivation, microalgae receive a light based on natural day-night rhythm. Under controlled laboratory conditions, the duration of lighting or photoperiod can vary from 0 (the heterotrophic cultivation mode) to 24 h. Microalgal growth has been evaluated under different photoperiod cycles (e.g., 12:12, 14:10, 16:8, and 24:0h light: dark) (Table 4). For example, the highest cell density of the microalga *Nannochloropsis* sp. (3.0×10^7 cell/mL) was observed in a 24:0 h photoperiod (Wahidin et al., 2013). Cell density decreased from 2.1×10^7 to 1.3×10^7 cell/mL when the photoperiod decreased from 18:06 to 12:12 h. In another study, the specific growth rates of *Chlorella vulgaris* were found to be 1.20, 1.8, and 1.7 /d at photoperiods of 24:00, 16:08, and 12:12 h (light:dark), respectively (Atta et al., 2013). It should be noted that 24 h lighting is not necessary for the continuous growth of all microalgae species, and the optimum photoperiod depends on the light intensity and the microalgal strains (Lam and Lee, 2012b).

5.2. The pH of cultivation medium

The pH of cultivation medium is another factor that significantly influences microalgal metabolism and growth. The pH value controls the acid-base balance in the cultivation medium, and affects the solubility and availability of different forms of inorganic carbon (CO_2, bicarbonate, and carbonate) and nutrients (phosphates and ammonium/ammonia) as well as their liquid–gas transfer phenomena (Rossi et al., 2020). For example, a high pH (>9.75) is favorable for ammonia volatilization in which ammonium (NH_4^+) is converted to ammonia (NH_3) gas (Lu et al., 2019). In addition, changing the pH of the medium affects the physiology and morphology of microalgae by activating the permeability of the membrane cell for certain ions, and consequently affecting microalgal growth and biochemical composition (Liang et al., 2011). Moreover, the pH of the cultivation medium is considered as a tool for controlling biological contamination in wastewater. This is because a cultivation medium with pH higher than 9 inhibits the growth of indigenous bacteria present in wastewater (Lu et al., 2019). Also, during cultivation of microalgae, e.g., the green alga, *Haematococcus pluvialis*, an acidic pH of 4 is recommended to avoid lethal fungal contamination of the culture (Hwang et al., 2019).

Based on microalgal growth performance, the pH of cultivation medium can be classified as fatal, tolerable, or optimal. Extremely low (acidic) and high (basic) pH values are fatal for most microalgae species. For example, Sakarika and Kornaros (2016) studied the growth of *C. vulgaris* at pH values in the range of 3–11. Lysis of microalgal cells was observed at highly acidic (e.g., 3 and 4) and basic (e.g., 11) pH after two days of cultivation. Microalgae were reported to grow in the pH range from 5 to 8, while based on growth parameters, the optimum pH was found to be between 7.5 and 8. In another study, Bartley et al. (2014) investigated the effects of pH, in the range from 5 to 10, on the growth and lipid accumulation of microalgae, *Nannochloropsis salina*. Highest growth rates of 95.6×10^6 and 92.8×10^6 cells/mL were reported at optimum pH of 8 and 9, respectively. Therefore, most microalgal species grow well in cultivation media with pH ranging between 7 and 9 (Aishvarya et al., 2015), but such narrow range of pH cannot be applied for cultivation of all microalgae species under controlled conditions. The optimum, acceptable, and lethal ranges of pH depend on microalgal species and cultivation conditions. It has been observed that some microalgal species can tolerate extraordinary acidic or basic pH. For instance, *Dunaliella salina* grows well in a pH close to 11.5, while the optimal pH of *Dunaliella acidophila* is between 0.0 and 3.0 (Sakarika and Kornaros, 2016). Hence, the optimum pH range for cultivation of the same commercial and well-known microalgal species can thus be found from previous studies. For newly isolated microalgae strains which have less available information, the cultivation conditions need to be evaluated and optimized under controlled laboratory conditions.

5.3. CO_2 supplementation

Approximately 50% of the dry weight of microalgal biomass is composed of carbon, which is mainly derived from CO_2 (Bilad et al.,

2014). Cultivation media with low concentrations of CO_2 negatively affects the synthesis of vital enzymes involved in carbon metabolism, such as ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) and carbonic anhydrase reactions (Chang et al., 2016). A carbon-limited environment also restricts the synthesis of microalgal pigments. In a study conducted by Miller and Holt (1977), the color of *Synechococcus lividus,* cultivated under CO_2 deprivation, changed to yellow after 96 h due to the loss of pigments. The cells rapidly produced chlorophyll *a* and C-phycocyanin after injecting CO_2 into the culture. Therefore, it is necessary to supply CO_2 for the healthy growth of microalgae and to maximize biomass yield.

Atmospheric air with an approximate CO_2 concentration of 0.04% is frequently used for microalgal cultivation. Although aeration of the culture with ambient air can provide the required CO_2 for the growth of most microalgal species, some strains grow better at higher CO_2 concentration. In this regard, higher concentrations of CO_2 can be provided by mixing CO_2 gas with atmospheric air. Zheng et al. (2012) applied a mixture of compressed air and different concentrations of CO_2 for the cultivation of *C. vulgaris*. Maximum biomass concentrations of 2.71, 3.32, 3.76, 2.59, and 0.65 g/L with 0.03 (ambient air), 1, 5, 10, and 15% of CO_2, respectively were reported. However, microalgal growth was inhibited after 10 days in the medium with 0.03% CO_2 as compared to the medium with 5% CO_2, which could be attributed to insufficient carbon. Higher concentrations of CO_2 (above the optimum range) can also be harmful to microalgae. Inhibition of growth of *Chlorella* sp. at 10 and 15% CO_2 concentrations was observed by Chiu et al. (2008). This is due to the decrease in pH in the cultivation medium with a high concentration of CO_2, which can negatively affect the activities of key photosynthetic enzymes such as ribulose 1,5-bisphosphate carboxylase-oxygenase (Zheng et al., 2012). Pure CO_2 has also been used as a carbon source for the cultivation of microalgae (Wang et al., 2019). Pure CO_2 can be supplied commercially via high-pressure cylinders. The flow rate of CO_2 can be adjusted using a flow meter, and the pressure can be monitored online in case of high-tech bioreactors.

Flue gas is another source of CO_2 that can provide the required carbon for microalgal growth. Flue gas containing 6%−15% CO_2 can also be used as a low-cost source of carbon for microalgal cultivation (Abd Rahaman et al., 2011). Biomass productivity of *Scenedesmus* sp. cultivated in media fed with ambient air containing 10% CO_2 and flue gas containing 5.5% CO_2 was found to be 217.50 and 203 mg/L/d, respectively (Yoo et al., 2010). In another study (Yadav et al., 2019), the highest biomass productivity of *Chlorella* sp. and *Chlorococcum* sp. in cultivation media aerated with flue gas (containing 5% CO_2) was found to be 208.93 and 105.42 mg/L/d, respectively, which were significantly higher than the ones obtained from cultivation media aerated with ambient air (114.79 and 60.45 mg/L/d). Integration of microalgal biomass production using flue gas not only provides CO_2 for microalgal growth, but also contributes towards controlling CO_2 emissions and mitigating climate change. Flue gas has high temperature that needs to be reduced before injecting to the microalgal cultivation media. When replacing flue gas with atmospheric air and pure CO_2, the effects of toxic gases and substances, such as CO, NO_x, SO_x, C_xH_y, heavy metals, and particulate matter should be considered (Van Den Hende et al., 2012).

Due to its low solubility in water, CO_2 easily escapes from the cultivation media through aeration. Replacement of CO_2 with other solid or liquid carbon sources has been extensively investigated for the cultivation of microalgae. Besides CO_2, bicarbonate-based compounds with high water solubility (9.21% (w/w) at room temperature) are considered as the main forms of inorganic carbon for microalgal cultivation (Kim et al., 2019). Kim et al. (2019) showed that sodium bicarbonate ($NaHCO_3$) salt, extracted from flue gas by electrochemical CO_2 mineralization, could support the growth of different species of freshwater, marine microalgae, and cyanobacteria.

Based on the above discussion, it can be concluded that ambient air, enriched ambient air with pure CO_2, pure CO_2, flue gas, and mineralized CO_2 compounds are the main sources of inorganic carbon that can be utilized for microalgal cultivation. Aeration of microalgal culture using ambient air is done more often because it is inexpensive, easily accessible, and available. However, low concentration of CO_2 of atmospheric air and gas escaping from the culture (due to low solubility) might limit the growth of microalgae. The advantages of other carbon sources with higher concentrations of CO_2 and solubility in water can enhance microalgal growth. It is worth noting that apart from the carbon sources (air, pure CO_2, flue gas, or bicarbonate compounds), the pH of the cultivation medium strongly influences the abundance of carbon species. CO_2, bicarbonate, and carbonate are the dominant species at pH $<$ 6, 7–10, and pH $>$ 10, respectively (Pedersen et al., 2013). Therefore, in addition to the selection of an appropriate source of carbon, the adjustment of pH is also necessary to maximize the growth of microalgae.

5.4. Aeration

Aeration of the culture is another important factor that affects the growth of microalgae and biomass yield. Usually, cultivation medium is aerated using an air compressor (Guo et al., 2015), air pump (Supraja et al., 2020) or by agitation or shaking of the medium (Nedbal et al., 2020). In cases of air injection, units such as *L*/min and *vvm* are used to denote the magnitude of the aeration. In *vvm*, the first *v* represents the volume of air (L), the second *v* represents the volume of medium (L), and *m* is minute (min). Different aeration rates from 0.1 to 10 L/min have been tested on microalgae cultures (Table 4), however, optimum rates depend on microalgae strains and the volume and shape of bioreactors (Barbosa et al., 2003). Features such as weight, size, density of microalgae cells, and tolerance to shear stress influence the optimum mixing and aeration rate. An aeration rate higher than the optimum value could damage the microalgae cells due to shear force effects, increasing evaporation and the operation costs (Guo et al., 2015). Han et al. (2015) investigated the effect of different aeration rates (0.067–0.333 vvm) on microalgal growth. A maximum dry weight of microalgae (1.24 g/L) was reported at 0.2 vvm aeration. The lowest and highest aeration rates were found to negatively affect the growth of microalgae due to insufficient mixing and cell damage, respectively.

As discussed in Section 5.3, aeration assists in proper supply of CO_2 for microalgal growth. In addition to carrying CO_2 to the cultivation medium, aeration provides mixing power and forms a turbulent flow in the culture and closed photobioreactor (Zhao et al., 2011). The created turbulent flow and bubbles enhance mass transfer between the gas (CO_2) and liquid (culture medium) phases, which improves the diffusion of CO_2 for photosynthesis (Zhao et al., 2011). Appropriate mixing culture by optimized aeration also distributes microalgae cells throughout the bioreactor homogeneously improving lighting conditions by exposing cells from dark zones to illustrated zones (Zhao et al., 2011). Moreover, proper mixing of the cultivation medium prevents nutrients, light, and temperature gradients as well as microalgal sedimentation in the culture broth (Guo et al., 2015). Therefore, aeration has a critical role in microalgal growth, and it needs to be optimized in microalgal cultivation at different scales.

5.5. Temperature

Temperature is also a critical factor for microalgal cultivation. Temperature directly affects the metabolism, nutrient uptake, CO_2 biofixation, photosynthesis, and growth rate (Subhash et al., 2014). In addition to growth, temperature also influences the physiology and biochemical composition of microalgae including the quality and quantity of microalgal lipids (Teng et al., 2020). Gonçalves et al. (2019) investigated the effect of temperature (between 20 and 36 °C) on the biochemical composition of *Pseudoneochloris marina* (a green microalgae). Temperature was found to significantly affect the amount of carbohydrates and saturated fatty acids of microalgal biomass. Therefore, it is necessary to optimize the temperature of cultivation medium

for optimum growth of microalgae.

The adaptation and response of microalgae to different temperatures are closely related to the origin of the microalgal species (Chokshi et al., 2015). Some species can tolerate extremely low and high temperatures. For example, *Chlamydomonas nivalis*, known as snow algae, has been isolated from the low-temperature environments of Antarctica (Fujii et al., 2010). Other microalgae and cyanobacteria such as *Cyanidium caldariu*, *Synechococcus elongatus*, and *Chlorella* sp. have shown maximum tolerance at 60 °C, 60 °C, and 45 °C, respectively (Kumar et al., 2011). *Cyanidiium caldarim*, *Galdieria partita*, and *Cyanidioschyzon melorae* were able to grow at 50 °C (Kurano et al., 1995). It should be noticed that most of the microalgal species cannot tolerate extremely low or high temperatures. Cultivation of most commercial and isolated microalgal species has been performed at temperatures between 20 and 28 °C (Table 4). Although microalgal strains might grow in a wide range of temperature conditions (Chokshi et al., 2015), the maximum growth rate of each microalgae species is obtained at the optimum temperature. Higher and lower temperatures than the optimum can negatively affect the growth of microalgae and biomass production. It is also important to know that microalgae tolerate lower temperatures better than higher temperatures. Microalgae can sustain a decreased growth up to 15 °C below the optimum temperature, however, only a few degrees higher than the optimum temperature can lead to microalgal cell death (Enamala et al., 2018).

The optimum range of temperatures for cultivation of microalgae have been reported as 18 to 30 °C (Vuppaladadiyam et al., 2018), 15 to 26 °C (Hosseini et al., 2018), and 20 to 30 °C (Enamala et al., 2018). Different optimum temperatures have been reported for the cultivation of microalgae under different cultivation conditions such as indoor/outdoor cultivation, open/closed systems, daytime, and light intensity. According to the information, presented in Table 4, it can be pointed out that cultivation of most microalgae species has been successfully performed at 25 °C. This implies that many microalgal species can grow well at room temperature. However, temperature of culture needs to be adjusted and controlled in case of species which are sensitive to temperature.

6. Microalgae supply

Providing microalgae seeds is the next step after the preparation of the bioreactor, media, and adjustment of environmental factors in the microalgae cultivation process. Microalgae seeds can be obtained from culture collections, or they can be isolated from natural water bodies and wastewater drainages. Culture collections are resource centers which store living microorganisms and their biological materials, such as cells. These centers, administered by the government, universities, or companies, handle, preserve, and provide microalgae to academics, and private and public industries to support their research and commercial activities (de Oliveira Lourenço, 2020; DUYGU et al., 2017). Pure cultures of phytoplankton, zooplankton, bacteria, fungi, and yeasts are available as axenic cultures for microalgae research (Table 5). These collections can supply a starter culture of different species of microalgae, cyanobacteria, and diatoms either in liquid medium or on an agar slope. Algal resource centers not only provide pure cultures as reference strains for research, but also conserve microalgal species (de Oliveira Lourenço, 2020). Microalgae culture collections can also provide useful information about the isolator, origin of isolation, appropriate cultivation media, and optimum culture conditions that can facilitate the microalgae cultivation (Schulze et al., 2019).

Microalgae can also be isolated from different environments, such as freshwater (lakes and rivers), brackish and marine waters (seas and oceans), soil, and wastewater drainages (Table 5). Research and industrial applications of indigenous microalgae are highly recommended due to the tolerance and compatibility of the latter with local geographical, climatic, and ecological conditions (Duong et al., 2012). These species can grow under harsh conditions, including hypersalinity, low or high temperatures, pH, and nutrient deficiency. For example, de Morais and Costa (2007) isolated microalgae from ponds or lakes around coal or oil-fired thermoelectric power plants. The combustion gases-adapted microalgal species were found to grow efficiently under specific conditions prevalent in those areas.

In nature, microalgal cells are found together with other microorganisms or microalgal strains. Several techniques including single-cell isolation, serial dilutions, medium enrichment, micromanipulation, atomized cell spray, and fluorescence activated cell sorting using flow cytometry have been introduced for the isolation of microalgae (Ghosh et al., 2016; Pereira et al., 2011). Plating (streak, spread, and pour plate) is a common method for the isolation of single colonies of microalgae from collected samples. Serial dilution is another simple isolation technique that decreases the concentrations of unwanted microorganisms (e.g., fungi and bacteria) and magnifies axenic cultures in higher dilution tubes (Barten et al., 2020). It should be noted that pure cultures cannot be isolated by applying a single isolation method, however, a combination of isolation techniques could be more successful in

Table 5
A list of supplied microalgae and their suppliers for lab-scale research.

Microalgae	Origin	Supplier	Country	Reference
Scenedesmus quadricauda and *a Tetraselmis suecica*	Culture Collection of Algae and Protozoa (CCAP)	Culture collection	Scotland	(Daneshvar et al., 2019)
Chlorella vulgaris FACHB-31	Institute of Hydrobiology, Chinese Academy of Sciences	Culture collection	China	(Chang et al., 2016)
Chlorella minutissima and *Synechococcus subsalsus*	Oceanographic Institute of the University of São Paulo (USP)	Culture collection	Brazil	(Costa et al., 2018)
Chlorella vulgaris, *Scenedesmus obliquus*, *Psammothidium* sp., and *Monoraphidium contortum*	The microalgae stock cultures of the Biology Department of Gazi University	Culture collection	Turkey	(Aghaalipour et al., 2020)
Chlorella sorokiniana SAG 211–8 k	SAG Culture Collection of Algae	Culture collection	Germany	(Holdmann et al., 2019)
Nannochloropsis salina (1776)	Provasoli-Guillard National Center for Culture of Marine Phytoplankton (NCMA)	Culture collection	United States	(Fagerstone et al., 2011)
Chlorella vulgaris	UTEX Culture Collection of algae, University of Texas at Austin	Culture collection	United States	(Almomani, 2020)
Haematococcus pluvialis	National Institute for Environmental Studies	Culture collection	Japan	(Hwang et al., 2019)
Acutodesmus dimorphus	Industrial effluents	Isolation	India	(Chokshi et al., 2015)
Eustigmatos vischeri JHsu-01	Subtropical lake	Isolation	China	(Xu et al., 2020)
100 native microalgal strains	Freshwater lakes and rivers	Isolation	Canada	(Abdelaziz et al., 2014)
Chlorella sp. FC2 IITG	Local freshwater pond	Isolation	India	(Naira et al., 2019)
Scenedesmus sp.	Secondary settler of the Carraixet wastewater treatment plant	Isolation	Spain	(Viruela et al., 2016)
Scenedesmus obliquus	Open pond at wastewater treatment plant	Isolation	South Africa	(Ansari et al., 2019)

isolating pure microalgae. For example, the capillary method, micromanipulation, and UV radiation might be needed after serial dilution to obtain axenic cultures (Ghosh et al., 2016; Pereira et al., 2011; Andersen, 2005).

The isolated microalgae are identified using molecular and morphological techniques for taxonomic classification and named using the binomial nomenclature system. Several identification methods, as simple as optical microscopy, and high-tech methods such as scanning electron microscopy (SEM), matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS), reverse dot blot hybridization (RDBH), high-performance liquid chromatography (HPLC), nuclear magnetic resonance spectroscopy (NMR), mass spectrometry, infrared spectroscopy, and X-rays have been used to identify microalgae (Ghosh et al., 2016). Among other techniques, 16 s or 18 s RNA sequence analysis is one of the most reliable for the identification of newly isolated species/strains. The ribosomal genes are the most conserved region of DNA in all cells and microalgal species, and are valuable tools for determining the phylogeny of the species (Ghosh et al., 2016; Maid and Zetsche, 1991). Culture collections provide certified microalgal species with accurate and safe information for users. Once microalgal species are isolated, identified and maintained in culture collection, these are available for immediate use. Therefore, culture collections provide faster and easier access to microalgae.

For successful microalgal application, selection of suitable microalgal strain for a specific cultivation purpose (e.g., reduction of CO_2; and acquisition of microalgal biomass as a feedstock for biofuels, materials, food, and feed) is another important consideration in the upstream processes. A specific microalgal species might be more appropriate for a specific research because of its ability or feature related to high growth rate, unique metabolite production, robustness, genetic manipulation, or composition of biomass. For instance, oleaginous microalgae such as *Nannochloropsis* sp. are more suitable for lipid extraction and biodiesel production (Liu et al., 2017). *Haematococcus* sp. is well-known as a natural source of astaxanthin. Spi*rulina* sp. has been widely investigated for its use as a food ingredient because of its high protein content. Likewise, diatoms (*Thalassiosira pseudonana*), source of natural mesoporous silica, have shown ability as drug delivery tools in biomedicine research (Delalat et al., 2015). Microalgae species such as *Tetraselmis suecica* and *Isochrysis galbana* are widely used as biofeed in aquaculture research (Fitzer et al., 2019). Therefore, a careful literature review can significantly help in the selection of more suitable microalgae species while addressing specific research problems.

7. Microalgal growth monitoring

Evaluating microalgal growth can be considered the last step of upstream processes in microalgae research. Analysis of microalgal growth can be performed at certain time intervals or during the end of the experimental period. Evaluating microalgal growth is important at least from two perspectives: (1) a direct index that monitors the performance of cultivation systems toward selection of an appropriate microalgal species, cultivation media, and optimized cultivation conditions; and (2) the amount of the produced microalgal biomass, which is critical for the implementation of consequent mid- and downstream processes in microalgae research. Measurement of microalgal dry mass, counting the number of microalgal cells, and the value of optical density are frequently used for calculating microalgal growth (Moheimani et al., 2013). Microalgal dry mass as a direct tool is the most accurate method for measuring microalgal growth. In this method, the solid phase (microalgal biomass) is separated from the liquid phase (cultivation media), and the weight of biomass is measured after drying. Centrifugation and filtration are frequently used for the separation of microalgal biomass from a certain volume of culture. The speed and frequency of rotation (revolutions per minute, rpm), and the mesh size are important factors that affect the efficiency of cell separation and filtration, respectively. Centrifugation speed of 3000–8000 rpm, and a revolution time of 5–8 min has been proposed for the centrifugation of microalgal biomass (Liao et al., 2014; Daneshvar et al., 2018a). Pre-dried and pre-weighed membrane filters (0.45 μm) or filter paper are used for the separation of microalgal cells (Li et al., 2003). Subsequently, the collected biomass is dried in an oven or freeze-dried until the microalgae weight becomes constant. Oven-drying is conducted in a temperature range of 60 °C to 110 °C for 2 to 24 h (Tang et al., 2012; Santana et al., 2017).

Counting the number of microalgal cells using a hemocytometer chamber is another commonly used method for measuring microalgal growth. Hemocytometer counting chambers are microscope-slide-sized base plates that were originally designed to count the blood cells. Although there are various brands of hemocytometer chambers (i.e., Thoma, Bürker, Bürker-Türk, and Fuchs-Rosenthal) with some differences in their design, the principle of counting cells is the same. Usually, the counting chamber has a grid of specified dimensions (1 mm × 1 mm squares). Each square is divided into smaller squares (0.05 mm × 0.05 mm). The big (1 mm^2) and small (0.0025 mm^2) squares have the same depth of 0.1 mm, therefore, the volume of each square can be calculated from its fixed dimension and depth. To count the cells, a small drop of the solution containing microalgae is seeped into the chamber underneath the coverslip, allowing the cell suspension to be drawn out by capillary attraction. The microalgal cells can be counted with a light optical microscope at 10x to 40x magnification. Finally, the concentration of cells can be presented as the number of cells per unit volume of culture (μL or mL) (Moheimani et al., 2013). It should be noted that it is not necessary to count all the 1 mm^2 cells for a statistically significant count. Depending on the concentration of microalgal cells in the sample, a subsample of type 2 (0.2 mm × 0.2 mm) or type 3 (0.05 mm × 0.05 mm) can be selected to calculate the concentration of cells.

Sometimes due to low volume of culture, high number of samples, or time limitation, determination of dry biomass or counting the number of microalgal cells are not appropriate methods for measuring microalgal growth. Measuring optical density (OD) is an alternative indirect measurement when the volume of culture is low (e.g., μL to mL) for evaluating microalgal growth (Santos et al., 2012). Absorbance of light by the microalgae suspension can be related directly to dry mass or cell numbers using a suitable standard curve with predetermined values at various wavelengths (650–750 nm) using spectrophotometer (Wang et al., 2020; Wu et al., 2012; Almomani, 2020; Hosoglu et al., 2020; Fagerstone et al., 2011). Microalgae OD in a small volume (μL) of culture, such as 96-well plates, can be performed using a microplate reader (Abdelaziz et al., 2014). It is recommended to dilute highly dense microalgae suspensions (wavelength > 1.00 nm) to avoid light absorption errors (Daneshvar et al., 2018b).

From the above discussion, it can be concluded that the direct measurement of dry weight and cell numbers are accurate and reliable to evaluate the growth of microalgae. However, these methods might not be applicable in low volume of microplates or whenever the concentration of cells is very low in culture. In such cases, microalgal growth can be indirectly calculated by the measurement of optical density. However, measurement of optical density is less accurate than the measurement of dry weight and cell numbers of microalgae.

8. Research needs and future directions

In recent years, the potential of microalgae has been increasingly exploited in numerous research fields including environmental science, biology, genetics, chemistry, chemical engineering, medicine, polymer science, agriculture, and aquaculture for diverse purposes. Increased interests in microalgae market opportunities have led to fast-evolving scientific research in the microalgae domain. Therefore, familiarization with the upstream processes is necessary. In this regard, all activities related to microalgal cultivation and biomass production should be considered as a part of upstream processing. This review paper discusses the main factors in microalgal cultivation including the cultivation

modes, bioreactors design, preparation of culture media, effect of environmental factors, supply of microalgae seeds, and monitoring of microalgal growth. The detailed information provided in this review, related to each of the above-mentioned determining factors can be beneficial in exploring upstream processing in microalgae research. There are many research avenues that can be explored further, for example, very little information is currently available on photo-heterotrophic cultivation of microalgae. A future research direction could be the identification of specific requirements of photo-heterotrophic cultivation mode. Research on developing appropriate bioreactors for photoautotrophic, heterotrophic, mixotrophic, and photoheterotrophic cultivation modes is necessary to facilitate cultivation of microalgae with different metabolic pathways. Developing new culture media, suitable for little-known microalgal species, can enhance the research opportunities. Additional investigations to solve illumination problem are required as it is one of the main obstacles in microalgae research on pilot-scale especially, in countries with less sunlight (e.g. Nordic countries). The main elements of upstream processes, as discussed in this review, should be expanded by coherent studies to establish an organized protocol for microalgal cultivation. Considering the high potential for microalgae research and its industrial applications, training in microalgal cultivation should be promoted by relevant authorized research institutes. For instance, researchers and students could be educated in the field of microalgal biotechnology / biorefineries through workshops or academic courses at universities and research institutes.

9. Conclusions

Careful assessment of underlying steps and aspects is critical to leverage the full potential of microalgae during upstream processing. In this review, efforts have been made to highlight the importance of vital steps of the upstream processing which are essential to make the process more efficient. Selection of suitable strain of microalgae for specific purpose, cultivation media, designing of bioreactors, environmental factors are some of the critical aspects that influence the microalgal biomass production, and these have been thoroughly discussed in this review. Additionally, this review has holistically addressed the technological challenges that can influence the performance of upstream processing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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