Heterotrophic Growth of Micro Algae

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Heterotrophic Growth of Micro Algae

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Abstract

Today cultured microalgae is used as producer of food ingredients such as omega-3 fatty acids or natural food colorants and dyes, food, fertilizer, bioplastics, chemical feedstock (raw material), pharmaceuticals, and algal fuel, and can also be used as a means of pollution control. Microalgae can grow 20 or 30 times faster than traditional food crops, and has no need to compete for arable land. Since micro algal production is central to so many commercial applications, there is a need for production techniques which increase productivity and are economically profitable. Where possible, heterotrophic growth overcomes major limitations of producing useful products from microalgae: dependency on light which significantly complicates the process, increase costs, and reduced production of potentially useful products. As a general role, and in most cases, heterotrophic cultivation is far cheaper, simpler to construct facilities, and easier than autotrophic cultivation to maintain on a large scale. This capacity allows expansion of useful applications from diverse species that is now very limited as a result of elevated costs of autotrophy; consequently, exploitation of microalgae is restricted to small volume of high-value products. In this literature, we present a general perspective of the field, describing the specific cellular metabolisms involved and the best-known examples from the literature and analyze the prospect of potential products from heterotrophic cultures.

Introduction

Recently, several photosynthetic microalgae have been identified as efficient biological systems for producing a wide variety of high-value chemicals and pharmaceuticals, such as phycobiliproteins, astaxanthin and polyunsaturated fatty acids (PUFAs) Consequently, several processes have been developed to obtain some of these compounds on a commercial scale; most of these developments are based on phototrophic growth using CO_2 as a carbon source [1,3]. Although most microalgae grow photoautotrophically, some are able to grow heterotrophically using organic substrates as sole carbon and energy sources The heterotrophic growth of microalga depends on the strain and culture conditions, and the consumption of the carbon source depends on the transport or diffusion of the carbon source across the membrane, and the enzymatic processes required for its incorporation into the central carbon metabolism. A heterotroph (heteros = “another”, “different” and trophe =

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"nutrition") is an organism that cannot fix carbon and uses organic carbon for growth [4]. Heterotrophs can be further divided based on how they obtain energy; if the heterotroph uses light for energy, then it is considered a photoheterotroph, while if the heterotroph uses chemical energy, it is considered a chemoheterotroph. In other word an organism that cannot synthesize its own food and is dependent on complex organic substances for nutrition.

Heterotrophic algae are algae that take up organic molecules as a primary source of nutrition. Compared to photoautotrophic growth, heterotrophic cultivation of microalgae eliminate light requirements, can significantly increase growth rates and cell mass, protein and lipid productivities [5–7]; bioreactor operation and maintenance is relatively simple and can be performed under strict axenic conditions; also cell masses obtained under heterotrophic conditions are higher because the energy density of the carbon source is higher in comparison with carbon dioxide [7] and cell densities can be increased using some culture strategies like fed-batch cultures, leading to a decrease in the costs of biomass harvesting [8,9]. One of the most notable advantages of the phototrophic cultivation is that under such condition microalgae fixes carbon dioxide and produces oxygen, contributing to the reduction of carbon emissions to the atmosphere [10]; while heterotrophic cultures use an organic carbon source, consumes oxygen and generates some CO_2 during this kind of cultivations. Furthermore, phototrophic cultures permit the use of non-potable water and not arable land and do not displace food crops cultures [10]. A microalga suitable for heterotrophic culture should have the following physiological abilities: divide and metabolize without light, grow on easily sterilized culture media, adapt rapidly to environmental changes and withstand the hydrodynamic stresses generated in stirred tank bioreactors and peripheral equipment [11, 12, 13].

A number of microalgae are capable of growing heterotrophically on organic substrates anf thus do not depend on sunlight for energy. Carbon in some form is necessary to provide the energy and carbon skeletons for cell growth. Heterotrophic algae derive energy from organic substrates [14]. Other carbon sources include carbohydrates such as fructose, sucrose, lactose and starch. C/N ration is an influencing factor with affects cellular lipid content as it controls the switch between lipid and protein syntheses. Nitrogen deficit (high C/N ratio) in the culture media triggers lipid accumulation [15]. Heterotrophic cultivation of some algae could result in higher biomass production and high lipid accumulation in cells. In microalgal culture, heterotrophic growth can be a cost effective alternative to photoautotrophic growth. This mode of culture eliminates the requirement for light and hence, offers the possibility of greatly increasing cell density and productivity. The heterotrophic growth processes can occur in open raceway ponds or closed bioreactor systems [20]. Different production schemes involving combinations of different growth regimes in various reactor configurations have been proposed in an effort to maximize biomass productivity. Although open raceway ponds require low energy inputs and lower capital costs, several issues such as contamination (i.e., by unwanted algal species as well as viral, bacterial and fungal pathogens) and low final biomass concentrations (often less than 1.0 g/L) increase production costs to levels that are still economically unviable [21]. Closed photobioreactors (CPBR) in different configurations have been proposed to address the contamination issues associated with open raceway systems. CPBRs also permit more stringent control of growth conditions and harvesting [22]. However, CPBRs often require extensive upfront capital investments compared to open raceway ponds and therefore face similar commercialization challenges. But in case of valuable product production it reduces the ultimate cost. Many photosynthetic microalgae have been reported to grow well in aerobic heterotrophic conditions like: *Chlorella protothecoides*, *C. vulgaris*, *C. sorokiniana*, *C. regularis*, and *C. pyrenoidosa*, *Scenedesmus sp*, *Haematococcus sp*, *Spirulina sp*, *Nitzchia laevis*, *Chlamidomonas reinhardtii*, *Scenedesmus obliquus*, *Synechocystis*, *Plectonema boryanum* and *Nostoc* etc. with the introduction of organic compounds like glucose, peptone and acetate [23]. However, the sugar based heterotrophic system frequently suffers from problems with contamination.

This article analyzes the processes and cases solely where heterotrophic cultivation of microalgae is possible to explore the potential and usefulness of this approach. It presents cases of autotrophic growth only for comparison or when similar mechanisms operate under autotrophic and heterotrophic conditions. It focuses on: (1) Basic metabolic processes of the microalgae; (2) Environmental parameters affecting growth and metabolism; (3) Kinetic parameters, such as specific growth rates and biomass production, and (4) Actual and potential end-products and byproducts that can be obtained from heterotrophic microalgal systems. Finally, we discuss some promising avenues of research.

Metabolisms of Heterotrophic Algae Culture

An interesting feature observed in heterotrophic cultures is that there is a decrease in the overall chlorophyll content of the algae in course of its growth. It has been reported that there is up to 94% chlorophyll loss under heterotrophic growth. This could be attributed to the fact that there is reduction in the chlorophyll synthesis as photosynthesis is inhibited and the carbon in its organic form is directly incorporated from the various sugars fed to the algae [24]. This causes the microalgal cells to adapt to this kind of carbon assimilation where the synthesis of the unutilized chlorophyll is down regulated to conserve energy. Hence the metabolic regulation starts the biodegradation of chlorophyll in these heterotrophic cultures [25,26]. Furthermore, as chlorophyll pose some interference during the trans-esterification process for biodiesel production, the reduction in chlorophyll content during heterotrophy only favors the biodiesel production from microalgal feedstock. Besides providing the advantage of eliminating the light requirements, heterotrophy also gives a cultivation process that is much easier to control. The addition of external organic carbon in required amounts also generates a CO_2 rich environment that promotes growth of algae [24]. Due to the high cell densities achieved at the end of the heterotrophic process, the biomass harvesting is also cost effective [27]. A close study needs to be done with heterotrophy to obtain an enhanced cultivation technique for microalgal growth.

Metabolism of Organic Carbon Source

Glucose

Glucose is the most commonly used carbon source for heterotrophic cultures of microalgae, as is the case for many other microbial species. Far higher rates of growth and respiration are obtained with glucose than with any other substrate, such as sugars, sugar alcohols, sugar phosphates, organic acids, and monohydric alcohols [28]. This may happen because glucose possesses more energy content per mol compared with other substrates. For example, glucose produces w2.8 kJ/mol of energy compared to w0.8 kJ/ mol for acetate [29]. Glucose promoted physiological changes in Chlorella vulgaris, which strongly affects the metabolic pathways of carbon assimilation, size of the cells, volume densities of storage materials, such as starch and lipids grains [30] and protein, chlorophyll, RNA, and vitamin contents [31]. Oxidative assimilation of glucose begins with a phosphorylation of hexose, yielding glucose-6-phosphate, which is readily available for storage, cell synthesis, and respiration. An equivalent of a single phosphate bond is required per mole of glucose assimilated into glucose-6-phosphate. In that process, an additional 30 equivalents of phosphate bonds are generated by aerobic oxidation of a mole of glucose [32]. Of the several pathways used by microorganisms for aerobic glycolysis (Break down of glucose), apparently only two: the Embdene Meyerh of Pathway (EMP) and the Pentose Phosphate Pathway (PP pathway) have been shown in algae [33]. Under complete darkness heterotrophic growth, glucose is mainly metabolized via PP pathway can be seen in Figure 1 [34].

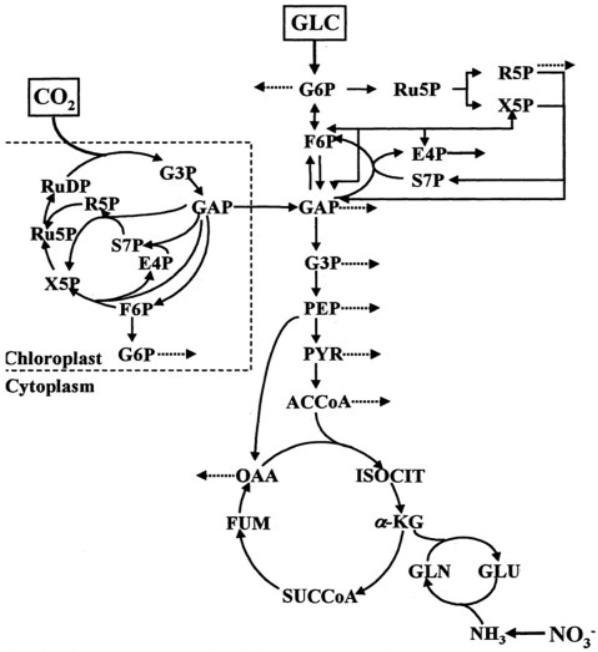

Figure 1: Glucose metabolism in heterotrophic microalgae. Compound abbreviations are following specified. GLC glucose, G6P glucose 6-phosphate, F6P fructose 6-phosphate, GAP 2 glyceraldehyde-3-phosphate, G3P glucose 3phosphate, PEP phosphoenolpyruvic acid, PYR pyruvate, ACCoA acetyl co-enzyme, ISOCIT isocitrate, α-KG alpha keto-glutamic acid, SUCCoA succinyl co-enzyme, FUM fumarate, OAA oxalo acetate, GLN glutamine, GLU glutamate, Ru5P ribose 5-phosphate, R5P ribulose 5-phosphate, X5P xylulose 5-phosphate, RuDP ribulose-1,5-diphosphate.

Carboxylic Acid (mainly acetate)

Uptake of dissolved carboxylic acids, such as acetic, citric, fumaric, glycolic, lactic, malic, pyruvic, and succinic under microalgal heterotrophic cultivation has been well known for decades [35]. Acetate (or acetic acid) is one of the most common carbon sources for many microbial species, including microalgae [32]. Once inside microalgal cells in the cytosol, the starting point for acetate assimilation is acetylation of coenzyme A by acetyl-CoA synthetase (EC 6.2.1.1) to form acetyl coenzyme A (acetyl-CoA) in a single-step catalyzed reaction using a single ATP molecule, as shown in Figure 1 [34]. Acetate (carried by coenzyme A) is generally oxidized metabolically through two pathways: (a) the glyoxylate cycle to form malate in glyoxysomes (specialized plastids in the glyoxylate cycle) and (b) through the Tricarboxylic Acid Cycle (TCA) to citrate in the mitochondria, which provides carbon skeletons, energy as ATP, and energy for reduction (NADH). However, acetate does not always promote growth. It could be toxic for many microorganisms at high concentrations, despite its common use for buffering high pH levels in bioreactors. Keeping the concentration of acetate at low levels is useful for the fed-batch configuration in cultures or pH-auxostat (pH is maintained as a constant).

Alcohols (mainly glycerol)

Heterotrophic growth using glycerol as a substrate has been demonstrated for several algae, despite the simplicity of glucose metabolism in microalgae. Most of these species occur naturally in habitats of somewhat elevated osmolarity, such as seawater and saline pounds. Glycerol as an osmoticum (a substance that has the capacity of raising the osmotic strength of the solution and consequently keeps the osmotic equilibrium in cells) is an economical carbon source for an energy supply and carbon requirements and is a very

compatible solute for enzymes and membranes, with almost no toxic effects even at high concentrations [36]. It is commonly used for long term preservation of microorganisms at low temperatures. Inside cells, glycerol is used as an osmo regulatory molecule. Glycerol is first phosphorylated using ATP and the glycerol phosphate is then oxidized to triose phosphate. Micro algae genomes contain genes encoding for glycerol kinase (EC 2.7.1.30), sn-glycerol-3-phosphate NAD oxidoreductase (EC 1.1.1.8) and and triose-phosphate (EC: 5.3.1.1) to convert glycerol into glyceraldehyde-3-phosphate and glycerate, which are intermediates in the EMP pathway of glycolysis to form pyruvate that enters the TCA cycle [33].

Metabolisms of Nitrogen Sources

After carbon, and apart from hydrogen and oxygen, nitrogen is quantitatively the most important element contributing to the dry matter of microalgal cells, accounting from 1 to 10% dry weight. Carbon and nitrogen metabolism are linked in microalgae because they share (a) carbon supplied directly from respiration of fixed CO2 (autotrophic growth) or assimilated organic carbon (heterotrophic growth) and (b) the energy generated in the TCA cycle and in the mitochondrial electron transport chain. The primary assimilation of inorganic nitrogen (ammonium) to form amino acids requires carbon skeletons in the form of keto-acids (2- oxaloglutarate and oxaloacetate) and energy in the form of ATP and NADPH to synthesize the amino acids glutamate, glutamine, and aspartate. In both autotrophic and heterotrophic growing cells, keto-acids, ATP, and NADPH are provided by the TCA cycle [37,38]. The metabolic pathways involved in nitrogen assimilation are depicted in Figure 2 [39].

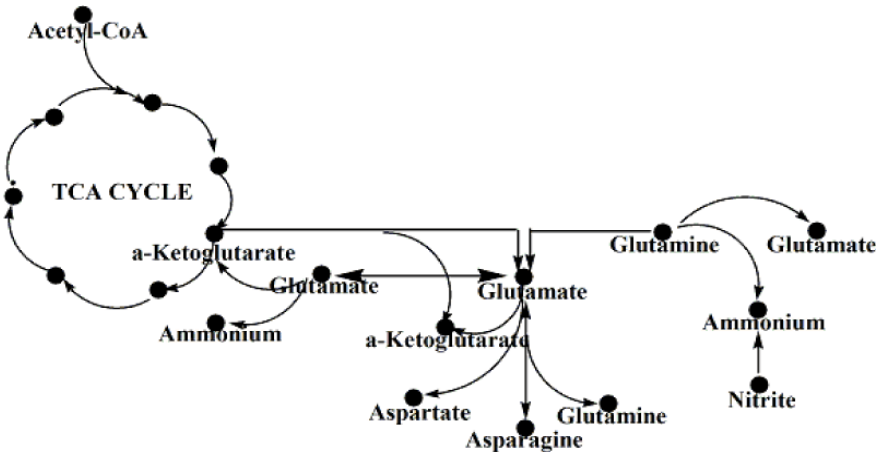

Figure 2: Typical nitrogen metabolic pathway in microalgae.

Metabolisms of Ammonium

Ammonium is the most preferred nitrogen source for algae. It is also the most energetically efficient source, since less energy is required for its uptake. Under autotrophic and heterotrophic conditions, ammonium is transported across the membranes by a group of proteins belonging to the Ammonium Transporter Family (AMT), a group of evolutionarily related proteins commonly found in bacteria, yeast, algae, and higher plants [40]. Several ammonium transporters, all belonging to the AMT family, have been identified in diatoms [41]. Ammonium is present in all compartments of the cell. Its concentration varies, depending on several factors including the concentration of ammonium in the neighboring compartment(s), the diferences in pH, and electrical potential between compartments. In compartments where ammonium is not metabolized, such as the vacuole, the concentration of ammonium may approach its equilibrium value. In compartments in which ammonium is metabolized, such as the cytosol and plastids, the steady-state concentration of ammonium may be much lower than the predicted equilibrium [42].

Assimilation metabolism of ammonium under either authotrophic or heterotrophic conditions is catalyzed by glutamine synthetase (GS; EC 6.3.1.2), which produces glutamine, and glutamate synthase (GOGAT; EC 1.4.1.14), which produces two molecules of glutamate from glutamine plus one molecule of a-ketoglutarate [43-46]. Alternatively, ammonium is incorporated into glutamate by the reversible reductive amination of a-ketoglutarate, which is catalyzed by glutamate dehydrogenase (GDH, EC 1.4.1.2) [47]. Heterotrophic growth conditions do not affect uptake rates of ammonium and the expression of nitrogen assimilation enzymes but mixotrophic regimen does. For example, adding acetate to autotrophic *Scenedesmus obliquus* affects its rates of ammonium uptake. In autotrophy, uptake is 17.8 mmol $cell^{-1}$ min^{-1} and is similar to that in heterotrophy (17.4 mmol $cell^{-1}$ min^{-1}), but this is ~4 times lower than occurring under mixotrophy (65.9 mmol $cell^{-1}$ min^{-1}) [48]. However, when the pH of the culture and other growth conditions are controlled, ammonium is a reliable nitrogen source [49]. For example, *P. tricornutum* grew well after adjusting the initial pH to 8 and a fed-batch configuration was used [50]. Another option to control pH and use ammonium as a nitrogen sources is to add a buffer.

Factor Related In Heterotrophic Growth of Microalgae

Heterotrophic cultivation is inappropriate for most microalgae and more species are obligate autotrophs than facultative heterotrophs [51,52]. Yet, some species are effectively grown in complete darkness and thus their cultures can be grown in conventional dark fermenters. Chen and Chen (2006) [53] listed the required initial characteristics that a microalgae species must have to be useful for heterotrophic cultivation: (a) Faculty of cell division and active metabolisms in absence of light. (b) Ability to grow in culture media with easy-to-sterile organic substrates where energy required for heterotrophic growth must be supplied by oxidation of part of the organic substrate [32]. (c) Ability to adapt to fast environmental changes, and (d) Capacity to resist hydro-mechanical stress inside the fermentors. However, it is impossible to precisely predict which specific substrates can be used or preferred by any given microalgae [33]. During respiration, oxygen is consumed and CO_2 produced. The respiration rate of any organic substrate is intimately geared to growth and cell division. Under optimal conditions, respiration rates are about 20%-30% of growth rates [54]. In microalgae, dark respiration of an organic substrate assimilated from the medium has rates varying from 0.01 to 0.6 d_1. This dark respiration plays two major roles in microalgae: (a) It serves as the exclusive source of energy for maintenance and biosynthesis under dark environment and (b) It provides essential carbon skeletons for biosynthesis under any growth condition. Under heterotrophic growth conditions, respiration rates equal or exceed the theoretical minimum cost of biomass synthesis. Values for CO_2 evolved per carbon (C) incorporated into new biomass (CO_2/C) equaled 0.4-1.4 for several *Chlorella* species and diatoms. This indicates that biomass synthesis during heterotrophic growth conditions can proceed at nearly the maximal theoretical efficiency.

Oxygen supply is a key factor in heterotrophic cultivation of microalgae. For example, the limitation of oxygen in a culture may reduce the specific growth rate of *Chlorella spp.* and thus lower the productivity of biomass when cell density is high [55]. Additionally, under cyclic cultures of autotrophic/ heterotrophic conditions, cell production of biomass of *Chlorella* is about 5.5 times higher than under autotrophic cycles alone, where cells were producing 16 times more ATP under heterotrophic culture [56]. In diatoms, heterotrophic growth is linked to their ability to maintain photosynthesis under dark environments using chloro-respiration to protect cells from photo damage after light returns; heterotrophic growth in this case is aided by high lipid accumulation, a product of reduced carbon in the absence of light [40].

Growth Characteristics of Heterotrophic Microalgae

The ability of a number of microalgal species to grow with organic carbon substrate has been demonstrated Previously [32,57]. However, the number of current commercially

important microalgae that are capable of growth on organic carbon substrates in the dark, and where experience of fed batch cultivation has been gained, is very limited. Both the (growth) kinetic and stoichiometric characteristics of these microalgae, along with values for other microalgal species obtained from batch cultures, are summarized in Table 1 [58]. This table encompasses fast-growing species with a specific growth rate higher than 0.09 h−1 (e.g. *Chlorella*, *Crypthecodinium*, *Nitzia*, *Prototheca spp.*) and species that grow at about half the rate, but where a lot of cultivation experience is available (e.g. *Galdieria*, *Haematococcus*, *Nannochloropsis* or *Schizochytrium spp.*). These specific growth rates correspond to doubling times of between 7 and 15 h. Interestingly, the *Chlorella* genus exhibits a wide range of growth rates with glucose, which vary with species and growth conditions, such as temperature, pH or dissolved oxygen concentrations [59]. Furthermore, heterotrophic growth of *Dunaliella sp.* and *Nannochloropsis sp.* is possible but is not practicable due to its very slow growth [57].

Species		μ_{max} (h^{-1})	$Y_{x/s}$ ($gCDWg^{-1}$)	T (°C)	pH	Carbon sources	s_{inhib} (gl^{-1})	Products
Chlorella vulgaris	P/H	0.180	0.55–0.69	36	6.0 – 7.5	Glucose (acetate, glutamate, lactate)	n.a.	Biomass
Crypthecodinium cohnii	H	0.089	0.56	25	7.2	Glucose (acetate)	>20	DHA
Dunaliella sp.	P/H	< 0.010	n.a.	26	7.5–8.3	Acetate, lactate, glucose, glutamate, glycerol	n.a.	Biomass beta-carotene
Euglena gracilis	P/H	0.045	0.43	25	2.8–3.5	Glucose, (acetate, alanine, aspartate, asparagine, ethanol, glutamate)	n.a.	Alpha-tocopherol
Galdieria sulphuraria	P/H	0.045–0.048	0.48–0.50	42	2	Glucose	>200	Phycocyanin
						Sugar beet molasses (fructose, sucrose)	>350	
Nannochloropsis oculata	P/H	< 0.007	n. a.	26	7.5–8.3	Glucose (ethanol)	n.a.	Biomass, EPA
Nitzschia alba	H	0.106	n. a.	30	n. a.	Lactate, succinate	n.a.	Biomass, EPA
						Glucose, glutamate		
Nitzschia laevis	H	0.017	0.44	20	8.2	Acetate, glucose	n.a.	EPA
Prototheca zopfii	H	0.330	0.81	21	7.2	Glucose (acetate)	n.a.	L-Ascorbic acid
Scenedesmus actus	P/H	0.040	n.a.	30	6	Glucose	>1	Biomass
Schizochytrium sp.	H	0.071	0.42	27	7	Glucose	>200	PUFA, DHA, GLA
Tetraselmis sueica	P/H	0.028	0.41	25	7.5	Acetate (glucose, glutamine, lactate)	n.a.	Lipids, PUFA n-3 HUFA

Table 1: Growth characteristics of microalgae in heterotrophic batch cultures.

μ_{max} maximum specific growth rate, $Y_{x/s}$ biomass yield determined in batch culture at given temperature (T), s_{inhib} substrate concentration resulting in a decrease of the specific growth rate and/or biomass yield with the particular substrate, *H* heterotroph, *P* (facultative) photoautotroph, *n.a.* not available, *DHA* docosahexaenoic acid, *EPA* eicosapentaenoic acid, *GLA* gamma-linolenic acid, *HUFA* highly unsaturated fatty acids, *PUFA* polyunsaturated fatty acids.

Cultivation of Microalgae in Stirred Bioreactors

The microalgal species which are currently attracting commercial interest grow under heterotrophic conditions and perform efficiently in conventional bioreactors in a similar manner to bacteria or yeast [60]. Such sophisticated, safe and controllable bioreactor systems are used to produce novel high-value compounds with microalgae. In contrast, established microalgal products are mostly manufactured by traditional outdoor photoautotrophic technologies [61]. If a product is unique or is not obtainable in the desired quality or quantity

by other means (such as extraction from animal or plant material or chemical synthesis), the superior heterotrophic growth characteristics become less critical. Performance losses may also be acceptable in cases where patent infringements need to be prevented. In order to be optimally suited for cultivation in stainless steel stirred bioreactors, a particular microalgal species should meet a number of desirable criteria showed in Table 2 [58]. The primary prerequisite is the ability to grow heterotrophically in an inexpensive, well-defined mineral medium with a high degree of resistance to mechanical and chemical stress.

	Prerequisites/benefits	Constraints
Bioreactor cultivation	Performance independent of climate Reduced downstream costs Enhanced productivity and/or titer Control of substrate concentrations Scalable process strategies Use of multi-purpose bioreactors Low land requirement Indoor and cGMP operation	High oxygen demand Sophisticated substrate feed control Rheological limitations (at high viscosity) Critical/toxic levels of metabolites High costs for (new) equipment
Culture media	Energy of light not required Defined (mineral) and inexpensive Easy to sterilize Non-corrosive (low salinity, acidity) Contamination protection (due to high salinity, extreme pH levels, high temperature > 40 °C)	Enhanced risk of contamination (organic carbon substrate, temperature, pH) Corrosion (high salinity, critical pH) Expensive ingredients (vitamins, amino acids) Non-defined composition (e.g. yeast extract)
Species	Available as axenic culture Reasonable specific growth rate Mechanical resistance Temperature achievable with conventional cooling (25–40 °C) Robust and resistant (to long periods of refrigeration, freezing, repeated cultivation, sudden condition changes)	Surface adhesion Aggregate formation Secretion of viscous metabolites Osmotic stress (at substrate over-dosing) Intracellular product harvest (hampered by rigid cell walls)

Table 2: Benefits and constraints of heterotrophic cultivation in stirred bioreactors.

Axenic Culture

An additional crucial prerequisite is the requirement for a monoculture in a long-term bioreactor operation. To date, this is still hampered by the dearth of axenic (pure) cultures of species isolated from the environment. In heterotrophic cultures, the advantage of preventing the growth of contaminants through selective photoautotrophic conditions is not a possibility. Thus, any (minor) contamination introduced with the inoculum could easily outgrow the desired microalgal species.

Composition of Culture Media and Micro algal Biomass

Culture Media

For natural phytoplankton (representing a heterogeneous consortium of microalgae), the proportions of the elements are typically derived from the 'Redfield ratio' dating back to 1934, suggesting a molar ratio of $C_{106}N_{16}P_1$ as described in Falkowski (2000) [62]. This has recently been further extended to include other important elements [63, 64]. For heterotrophic cultures of *Chlorella vulgaris*, a molar stoichiometry of $C_{3.96}H_{7.9}O_{1.875}N_{0.685}P_{0.0539}K_{0.036}Mg_{0.012}$ was determined [65], and this has been reflected in optimized media compositions for biomass production in high-cell-density fed batch processes. All of the major molecules in microalgae (i.e. proteins, carbohydrates, and lipids) contain carbon as the principal element, with oxygen, hydrogen and nitrogen at lower, or even zero, concentrations. Typically, in media for heterotrophic cultures that support optimal growth, all of the constituents are supplied in stoichiometric excess to the organic carbon source. Applying stoichiometric principles to an established medium for photoautotrophic cultures of *Chlorella* spp. [66], the medium was shown to be deficient in iron, magnesium, sulphur

and nitrogen. When optimised, a fivefold increase in biomass concentration was achieved [67]. For most of the microalgal species capable of heterotrophic growth, glucose or acetate is an adequate source of energy and carbon. In particular, glucose is used for the production of high-value compounds where the processes need to be reproducible for prospective regulatory approval for pharmaceutical manufacture. Nitrate, ammonia and/or urea are the preferred nitrogen sources at a bioreactor scale [68]. Tryptone, glycine and yeast extract have also been evaluated for their potential to enhance growth or product formation [69]. Moreover, growth data suggest that nitrogen source preference might vary between the species [69, 70]. Yeast extract, a complex component with a high carbon content, is not defined at the single-element level but is frequently used as a source of nitrogen, amino acids, vitamins and trace elements [71].

Biomass Composition

The growth regime together with the microalgae strain and supplied organic substrate greatly influences the molecular composition of microalgae biomass. The composition of biomass is usually reported as the percentage of biomass per dry weight of protein, lipids or carbohydrates or any other specific molecule. Table 3 [72] presents percentage rate of dry weight.

Compound (%)	Photo-autotrophic	Heterotrophic	Mixotrophic
Protein	29-60	10-40	32-40
Carbohydrates	10-20	15-45	10-30
Lipids (Total)	3-28	25-60	11-58
Ash	6.36±0.05	5.93±0.04	7.0±0.02
Other (nucleic acid and pigments)	10.42±0.65	11.20±0.61	9.14±0.78

Table 3: Biomass composition range obtained in photo-autotrophic, heterotrophic and mixotrophic cultivation of microalgae.

Advantage and Limitation of Heterotrophic Cultivation of Microalgae

Advantage

The advantages of heterotrophic cultivation of microalgae, in comparison with photo-autotrophic cultivation are the following:

(a). Higher growth rate and biomass density (also called biomass productivity)
(b). Higher lipid content per dry weight of cells (lipid productivity)
(c). Higher biomass productivity in per area of culture
(d). Cheaper and simple bioreactor design
(e). Easer scaling up process
(f). The possibility to manipulate biomass composition by changing the culture medium's organic substrate that stimulate specific metabolic and biosynthetic pathways and
(g). Potential to remove organic carbon and several types of nitrogen and phosphorus compounds from waste water

Limitation

Heterotrophic cultures have several major limitations:

(a) There is a limited number of microalgal species that can grow heterotrophically

(b) Increasing energy expenses and costs by adding an organic substrate

(c) Contamination and competition with other microorganism

(d) Inhibition of growth by excess organic substrate and

(5) Inability to produce light-induced metabolites.

Metabolic Products and Processes Using Heterotrophic Culture of Microalgae

The main driving force to grow microalgae commercially is harvesting metabolic products, feed for marine and terrestrial organisms, food supplements for humans, or to use the microalgae for environmental processes, such as wastewater treatment, fertilization of soils, biofuels, and phytoremediation of toxic wastes. The main attractiveness of heterotrophic cultivation is that it is potentially substantially cheaper.

Lipids

Several species of microalgae can be induced to overproduce specific fatty acids through relative simple manipulations of the physical and chemical properties of their culture medium. Accumulation of lipids in the microalgae cells, as well as for other oleaginous microorganisms (high oil producers), depends on diverse factors. These include growth temperature, pH, nutritional imbalances of carbon, nitrogen, phosphorous, and silicate, the growth regime (autotrophic, mixotrophic, or heterotrophic), the age of the culture,andthe specificmicroalgal strain [73, 16, 74]. For example, the lipid content in heterotrophically grown cells of *C. protothecoides* is as high as 55%, a quantity that is up to four times greater that autotrophically grown cultures under otherwise similar conditions [75]. In heterotrophic cultures, accumulation could be attributed to consumption of sugars at a rate higher than the rate of cell generation, which would promote conversion of excess sugar into lipids [76-78]. This process is often accomplished in two steps: exponential cell division leading to decreased growth from limits of nutrients, thereby leading to accumulation of lipids [79]. It might not be only related to higher lipid-synthesizing enzymes under nitrogen starvation, but to the cessation of other enzymes associated with cell growth and proliferation and operation of enzymes specifically related to accumulation of lipids [77, 80]. Another proposed mechanism for accumulating lipids under heterotrophic conditions used *E. gracilis* as a model. Under nitrogen starvation accumulation of lipids is attributed to mobilization of lipids from chloroplast membranes as chloroplastic nitrogen is relocated by 1, 5-biphosphate carboxylase/oxygensae (E.C. 4.1.1.39, Rubisco) [81]. After nitrogen starvation, microalgae, such as *C. pyrenoidosa*, *C. sorokiniana*, *Nitzschia alba*, *Skeletonema costatum*, *C. conhii*, accumulate large amounts of lipids, and diatoms respond to depleted silicates by accumulating lipids [40].

Polyunsaturated Fatty Acids

Long-chain polyunsaturated fatty acids (eicosapentaenoic acid, EPA, u-3, C20:5 and docosahexaenoic acid, DHA, u-3, C22:6) are two important fatty acids in early and old age metabolism in humans. They have been used in prevention and treatment of human diseases such as heart and inflammatory diseases and as nutritional supplements in humans and marine organisms in aquaculture. The nitrogen source affects production of EPA by the diatom *N. laevis* in heterotrophic cultures where nitrate and urea are preferred N sources for cell growth and EPA content. Tryptone and yeast extract were found to enhance EPA production [83]. Temperature also influences the fatty acids profile. When temperature is below the optimal growth temperature for the microalgae, more unsaturated fatty acids are metabolized, and the reverse effect occurs at higher temperatures. Reducing temperature by 10-15°C leads to a decrease in membrane fluidness. To compensate for decreasing fluidness, over-expression of the genes for desaturases (acyl-CoA desaturases, acyl-ACP desaturases, and acyl-lipid desaturases) promote desaturation of the membrane lipids. Use of acetic acid as a carbon source for heterotrophic production of DHA in batch-fed cultures of high cell density of *C. cohnii* resulted in much higher lipid and DHA contents than in cultivation with glucose [83, 78].

Biodiesel

Biofuels from microalgae is an attractive option for microalgae biotechnology. Compared to all other applications, it is one of the most attractive, given the high prices of crude oil.

Biodiesel is a suitable substitute for petroleum-based diesel fuel because of its multiple advantages for machines and the environment. heterotrophic microalgae cultivation represents a good source of LCFA [84]; so far, it is a less popular avenue for biodiesel production from microalgae. *C. protothecoides* is a suitable microalga for biodiesel production, heterotrophically using organic carbon sources. This species was able to produce quantities of lipids reaching ~50% of its dry weight. Enzymatic transesterification (converting lipids to biodiesel) was catalyzed by lipase, and the conversion rate reached close to 100% in several trials. One of the potential carbon sources for producing biodiesel heterotrophically is glycerol. Currently, glycerol is an inexpensive and abundant carbon generated as a by-product of biodiesel fuel production. Development of processes to convert this crude glycerol into higher-value products is needed. Given the highly reduced nature of carbon atoms in glycerol, fuel and reduced chemicals can be generated at higher yields than those obtained from common sugars, such as glucose [85, 86]. For example: *Schizochytrium limacinum* produced palmitic acid (16:0) as w45e60% of their dry weight when supplied with glucose, fructose, or glycerol [87, 88], which could potentially be used for biodiesel production.

Pigments

Naturally, all pigments are produced under autotrophic growth conditions, but surprisingly some are produced, and in large quantities, under heterotrophic dark conditions.

Carotenoids

Carotenoids from microalgae have been used for commercial purposes. Carotenoids are lipid-soluble pigments composed of isoprene units that are widely distributed in various classes of microalgae. Carotenoids are divided into two groups: those containing only hydrocarbons (not oxygenated) and xanthophylls that contain oxygen molecules [89]. Among the xanthophylls (zeaxanthin, canthaxantin, and lutein), lutein is considered the principal useful pigment of the group. It has high nutritional value and low toxicity and is used as a pigment for animal tissues (chicken skin and egg yolks coloring), food, cosmetics, and pharmaceutical products, such as an effective agent for prevention and treatment of a variety of degenerative diseases [90, 91]. Lutein is an intracellular product of *Chlorella*. This genus is used for production of lutein. Photoautotrophic systems produce low biomass; hence, heterotrophic cultivation represents an alternative. Increasing glucose concentration increases lutein production, but urea is currently the optimal source of nitrogen [90, 92-94]. Astaxanthin production by microalgae increases under stress conditions and is present in the esterified form and stored in lipid bodies outside the chloroplast, which enables green algae to accumulate a considerable amount [95]. *Haematococcus pluvialis* is the main producer of astaxanthin under autotrophic conditions but *C. zofingiensis* is superior in yield when heterotrophically cultivated with glucose [96]. The red microalgae *G. sulphuraria* can produce phycocyanin in carbon-limited but nitrogen-sufficient heterotrophic cultures; the content increases in the stationary phase. Although production of phycocyanin in this microalga is lower than in *S. platensis*, its ability to grow heterotrophically makes it a potential supplier of this pigment [97]. Another study found that this microalga produced more phycocyanin in heterotrophic batch-fed cultures of *G. sulphuraria* than is commonly attained in outdoor, sunlight-dependent cultures of *S. platensis* [98]

Waste Water Treatment

As mentioned earlier, tertiary wastewater treatment by microalgae is an old idea that so far has very limited application. This is directly related to the costs involved in treating very large volumes of wastewater in a timely manner under autotrophic conditions [99]. Heterotrophic wastewater treatment is a novel idea that so far has been studied at the laboratory scale. The most efficient carbon source for using *C. vulgaris* to treat wastewater heterotrophically was calcium acetate (Perez-Garcia et al., in press). Subjecting the

autotrophic, immobilized microalgae-bacteria system for wastewater treatment [100-102] to heterotrophic conditions revealed even higher potential of the system to eliminate nutrients [103]. The new data cannot provide a solid prediction about the practical potential of this approach.

Genetic and Metabolic Engineering for Improving Existing Heterotrophic Microalgae

Genetic and metabolic engineering can be used to expand heterotrophic capabilities of strains to growth on a specific organic substrate or developing a metabolic pathway for a novel product synthesis by inserting the genes for these pathways. There is an increasing interest in use transgenic microalgae as green cell factories capable of producing biofuels and valuable proteins and carbohydrates [72]. Now-a-days few microalgae, including the green algae *Chlamydomonas reinhardii*, *Nannochloropsis gaditana*, and *Osterococcus tauri* or the diatoms *Phaeodactylum triconutum* are currently established as platforms for genetic and metabolic engineering [72]. Successful metabolic engineering of strains involves the sequencing of a wild type strain, a systematic description of the organism genotype and phenotype using "omics" techniqes, then the application of molecular and genetic techniques for metabolic engineering. Metabolic engineering of a specific strain has one of the following goals: a. expand metabolic capabilities by adding new genes to an organisms, b. add genes encoding for transporters, c. increase formation of a pre arabinose, citrate, fructose, malate, lactic acid, lactose, peptone, urea, fulvic acids, ethanol, methanol, and sucrose have been tested for heterotrophic cultivation of microalgae. Chlorella vulgaris culture on the above mentioned carbon sources reached significantly lower biomass concentration in comparison to culture on acetate or glucose [72].

Concluding and Future Prospects

Cultivation of microalgae that are primarily photosynthetic under heterotrophic dark conditions for production of economically useful metabolites or technological processes is a tempting option, given significant reductions in complexity of cultivation and costs. Because heterotrophic growth consumes simple, cheap, and available carbon sources (glucose, acetate, glycerol) that are commonly used by fermentation industries for other aims, it is predicted that adoption of this approach is an easy, uncomplicated task. Fortunately, some of the most common and best-studied microalgae, such as Chlorella, are also heterotrophs. This information can jump start research in heterotrophy, which is probably quite prevalent among microalgae [104,105]. Furthermore, with current developments in genomics, bioinformatics analyses, and genetic and metabolic engineering, new approaches in microalgae biotechnology, including heterotrophy, have emerged [106,107]. As a result of genetic engineering, some obligate photoautotrophs were transformed to heterotrophy through the introduction of sugar transporters. *Volvox carteri* was one of the first green algae to be transformed with the hexose/Hþ symporter gene derived from *Chlorella sp*. [108]. These examples of a simple genetic transformation of single gene of a sugar transporter in the outer membrane of microalgae show the feasibility to convert microalgae from a photoautotrophic into a heterotrophic organism when sugar is present in the absence of light. Such genetic engineering is probably acceptable by society because microalgae cultivation can be independently managed without risk of environmental contamination.

Heterotrophic cultivation of microalgae is a niche of microalgae research field. Yet, the potential of expansion because of the advantages it offers are limitless. Only time will prove if this strategic approach will catch up with the industry.

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