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Review

Heterotrophic cultures of microalgae: Metabolism and potential products

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ABSTRACT

This review analyzes the current state of a specific niche of microalgae cultivation; heterotrophic growth in the dark supported by a carbon source replacing the traditional support of light energy. This unique ability of essentially photosynthetic microorganisms is shared by several species of microalgae. 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. Heterotrophic cultivation may allow large volume applications such as wastewater treatment combined, or separated, with production of biofuels. In this review, 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.

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1. Introduction

Large-scale microalgal production has been studied for decades (Becker, 1994; Lee, 2001), given the wide variety of practical and potential metabolic products, such as food supplements, lipids, enzymes, biomass, polymers, toxins, pigments, tertiary wastewater treatment, and "green energy" products that can be obtained. These products were achieved by cultivating the microalgae on diverse mineral media, organic substrates, and synthetic or real wastewaters (Pulz, 2001; de-Bashan et al., 2002, 2004; Pulz and Gross, 2004; Lebeau and Robert, 2006; Harun et al., 2010).

Today, the most common procedure for cultivation of microalgae is autotrophic growth. Because all microalgae are photosynthetic, and many microalgae are especially efficient solar energy convertors, microalgae are cultivated in illuminated environments naturally or artificially. Under autotrophic cultivation, the cells harvest light energy and use CO_2 as a carbon source. The introduction of sufficient natural or artificial light to allow massive growth and dense populations is the main objective and a limiting factor of the cultivation: the more light, up to a limit for the species, the better (Mandalam and Palsson, 1998; Yang et al., 2000; Suh and Lee, 2003). Therefore, as practiced with other microbial communities producing economic products, open ponds that mimic natural environments of microalgae are the most common option for mass cultivation (Oswald, 1992; Tredici, 2004).

Large open outdoor pond cultivation for mass algal production of single-cell protein, health food, and β-carotene (Borowitzka and Borowitzka, 1989; Wen and Chen, 2003; Carvalho et al., 2006; Chisti, 2007) is one of the oldest industrial systems since the 1950s (Oswald, 1992). Large open ponds can be built of glass, plastic, concrete, bricks, or compacted earth in a variety of shapes and sizes. The most common is the "raceway pond", an oval form resembling a car-racing circuit (Lee, 2001; Pulz, 2001; Chisti, 2007). These cultivation systems present relatively low construction and operating costs and the large ones can be constructed on degraded and nonagricultural lands that avoid use of high-value lands and crop producing areas (Chen, 1996; Tredici, 2004).

All these benefits notwithstanding, open ponds have several inherent disadvantages: (1) Poor light diffusion inside the pond, decreasing with depth. It is aggravated when cultivation is intensive and causes self-shading. Consequently, shallow depth is required for ponds and they have a low volume to area ratio; (2) Mono-cultivation of the desired microalgae is difficult to maintain for most microalgae species because of constant airborne contamination, except for extremophile species; (3) Environmental growth parameters of cultivation rely primarily on local weather conditions, which may not be controlled and make production seasonal; (4) Harvesting is laborious, costly, and sometimes limited by low cell densities; (5) Continuous and clean water is needed; and (6) Production of pharmaceutical or food ingredients is not feasible or is very limited (Chen, 1996; Tredici, 1999; Molina Grima et al., 1999, 2003; Lee, 2001; Pulz, 2001; Wen and Chen, 2003; Sansawa and Endo, 2004; Carvalho et al., 2006; Chen and Chen, 2006; Chisti, 2007; Patil et al., 2008).

To overcome inherent disadvantages of using open, less controlled environments, numerous closed photo-bioreactors (PBR) of various volumes and shapes have been designed (Molina Grima et al., 1999; Tredici, 1999, 2004; Tsygankov, 2000; Zhang et al., 2001; Barbosa, 2003; Suh and Lee, 2003; Zijffers et al., 2008). The principle final goal of any PBR is reduction in biomass production costs. This has been done by improving catalysts, shaping of the PBR, controlling environmental parameters during cultivation, aseptic designs, and operational approaches to overcome rate-limiting of growth, such as pH, temperature, and gas diffusion. Overcoming these limitations make monocultures and production of pharmaceutical and food goods possible (Cooney, 1983; Chen, 1996; Apt and Behrens, 1999; Pulz, 2001; Wen and Chen, 2003; Lebeau and Robert, 2006).

Similar to the open-pond concept, large-scale PBRs have three major disadvantages that make them uneconomical for low-cost end-products: At operational volumes of 50–100 L or higher, it is no longer possible to disperse light efficiently and evenly inside the PBR (Chen, 1996; Pulz, 2001); development of algal biofilm fouls PBR surfaces and thereby limiting light penetration into the culture. A high initial investment in infrastructure and continuous maintenance is required (Carvalho et al., 2006). Nonetheless, numerous applications of PBR for microalgae were proposed and were reviewed (Apt and Behrens, 1999; Lebeau and Robert, 2006; Muñoz and Guieysse,

2006; Moreno-Garrido, 2008; Brennan and Owende, 2010; Harun et al., 2010). Therefore, this approach will not be discussed in this essay.

A feasible alternative for phototrophic cultures in PBRs, but restricted to a few microalgal species, is the use of their heterotrophic growth capacity in the absence of light, replacing the fixation of atmospheric CO_2 of autotrophic cultures with organic carbon sources dissolved in the culture media. Heterotrophy is defined as the use of organic compounds for growth (Droop, 1974). Heterotrophs are organisms whose substrate and energy needs are derived from organic compounds synthesized by other organisms (Kaplan et al., 1986). The basic culture medium composition for heterotrophic cultures is similar to the autotrophic culture with the sole exception of adding an organic carbon (Tsavalos and Day, 1994). Mixotrophic growth regime is a variant of the heterotrophic growth regime, where CO_2 and organic carbon are simultaneously assimilated and both respiratory and photosynthetic metabolism operates concurrently (Kaplan et al., 1986; Lee, 2004). In some open-pond cultivations, organic carbon, such as acetate and glucose, are added continuously in small quantities. This is done to support higher microalgal biomass and simultaneously prevent excessive bacterial growth, which would be the outcome if the organic substrates were added in large quantity. Adding organic carbon substrate is usually done only during daytime hours, otherwise faster growing bacteria would outperform the microalgae under dark heterotrophic conditions. This fed-batch culture process is often limited to only one culture cycle to avoid bacterial contaminants from accumulating to unacceptable levels (Abeliovich and Weisman, 1978; Lee, 2001). Some microalgal species are not truly mixotrophs, but have the ability of switching between phototrophic and heterotrophic metabolisms, depending on environmental conditions (Kaplan et al., 1986).

The heterotrophic growth approach eliminates the two major deficiencies of illuminated autotrophic PBR: allowing the use of practically any fermentor as a bioreactor, such as those used for industrial production of medicines, beverages, food additives, and energy and yielding, as a major outcome, a significant reduction in costs for most processes (Gladue and Maxey, 1994; Lee, 1997). Cost effectiveness and relative simplicity of operations and daily maintenance are the main attractions of the heterotrophic growth approach. A side but significant benefit is that it is possible to obtain, heterotrophically, high densities of microalgae cells that provides an economically feasible method for large scale, mass production cultivation (Chen, 1996; Lee, 2004; Behrens, 2005). For example: under some heterotrophic cultures, the growth rate, the dry biomass, ATP generated by the supplied energy, and the effect on ATP yield (mg of biomass generated by each mg of consumed ATP), lipid content and N content are significantly higher than under autotrophic cultures and are mainly dependent on the species and strain used (Martínez and Orús, 1991; Chen and Johns, 1996a,b; Ogbonna et al., 2000; Shi et al., 2000; Yang et al., 2000, 2002; Behrens, 2005; Boyle and Morgan, 2009). Under some heterotrophic growth conditions, the microalgal biomass yields are consistent and reproducible, reaching cells densities of 50–100 g of dry biomass per liter (Gladue and Maxey, 1994; Radmer and Parker, 1994), much higher than the maximum 30 g l^{-1} of dry cell biomass in autotrophic cultures (Javanmardian and Palsson, 1991) and comparable to the 130 g l^{-1} of yeast dry biomass of commercial fermentors (Chen, 1996).

Heterotrophic cultures containing as large as 100,000 l can generate useful biomass reaching hundreds of kilograms. These large volumes and high productivity of cultures make the heterotrophic strategy far less expensive than the autotrophic approach (Radmer and Parker, 1994). For example, in Japan, biomass production of *Chlorella* spp. use heterotrophic cultures to generate ~500 ton of dry biomass, representing ~50% of total Japanese production of this algae (Lee, 1997). Mixotrophic cultivation was also shown to be a good strategy to obtain a large biomass and high growth rates (Ogawa and Aiba, 1981; Lee and Lee, 2002), with the additional benefit of producing photosynthetic metabolites (Chen, 1996).

Heterotrophic cultures have several major limitations: (1) There is a limited number of microalgal species that can grow heterotrophically; (2) Increasing energy expenses and costs by adding an organic substrate; (3) Contamination and competition with other microorganism; (4) Inhibition of growth by excess organic substrate; and (5) Inability to produce light-induced metabolites (Chen, 1996). Nonetheless, many recent studies show that heterotrophic cultures are gaining increasing interest for producing a wide variety of microalgal metabolites at all scales, from bench experiments to industrial scale (Apt and Behrens, 1999; Yang et al., 2000; Lee, 2001; Sansawa and Endo, 2004; Wen and Chen, 2001a, 2003; Li et al., 2007; Brennan and Owende, 2010).

This review critically 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 by-products that can be obtained from heterotrophic microalgal systems. Finally, we discuss some promising avenues of research.

2. Nutrient metabolism by microalgae in heterotrophic culture

2.1. *Key issues in heterotrophic growth of microalgae*

Heterotrophic cultivation is inappropriate for most microalgae and more species are obligate autotrophs than facultative heterotrophs (Lee, 2001; Behrens, 2005). Yet, some species are effectively grown in complete darkness and thus their cultures can be grown in conventional dark fermenters. Chen and Chen (2006) 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 (Droop, 1974). (c) Ability to adapt to fast

environmental changes, and (d) Capacity to resist hydromechanical stress inside the fermentors.

In a broad sense, all organisms, including microalgae, use the same metabolic pathways for respiration. As expected, the metabolism of microalgae generally resembles, with only minor differences, that of higher plants. However, it is impossible to precisely predict which specific substrates can be used or preferred by any given microalgae (Neilson and Lewin, 1974). During respiration, oxygen is consumed and CO_2 produced. The respiration rate of any organic substrate is intimately geared to growth and cell division. The rates of endogenous respiration and of O_2 uptake vary through the cell cycle (Lloyd, 1974). Dark respiration rates (mol O_2 mol carbon^{-1} d^{-1}) increase with growth rates. Under optimal conditions, respiration rates are about 20–30% of growth rates (Geider and Osborne, 1989). 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. Physiological regulation of respiration is assumed to be controlled by demand for the products of respiration metabolism, such as energy in the form of ATP and NADH and carbon skeletons provided by the organic substrate (Geider and Osborne, 1989). 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, since CO_2/C ratios for autotrophic growth are much lower than values for heterotrophic growth (Raven, 1976).

Independent of the supplied organic substrate or the microalgae species, growth rates are enhanced by higher levels of aeration (Griffiths et al., 1960). 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 (Wu and Shi, 2007). Species of the genera *Chlorella*, *Tetraselmis*, and *Nitzschia* grew at higher rates under heterotrophic compared to autotrophic systems (Endo et al., 1974; Day et al., 1991; Gladue and Maxey, 1994; Chen and Johns, 1995; Lee, 2001; Shi and Chen, 2002; Boyle and Morgan, 2009). 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 (Yang et al., 2000). 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 (Wilhelm et al., 2006).

In addition to the initial parameters for heterotrophic cultivation listed earlier (Chen and Chen, 2006), the main practical key issues in large-scale heterotrophic cultures of microalgae are: (a) Good survival of the strain during cultivation, (b) Its robustness, (c) Overall low cultivation costs, reflected as the ability of the strain to efficiently use inexpensive, common carbon sources, tolerate environmental changes, and generate economical worth in the quantity of the metabolite(s), and (d) At the industrial level, the strains must also be easy to handle; its cell walls must withstand hydrodynamic and mechanical shear occurring in large bioreactors and it should produce high density biomass, all in minimally modified fermentors used for other microorganisms (Day et al., 1991; Gladue and Maxey, 1994; Chen and Chen, 2006). Consequently, these requirements reduce even further the microalgal strains that can be employed and use of available carbon sources. So far, the latter consists of glucose, glycerol, acetate, wastewater, and to a lesser extent, a few other organic carbon sources. Glucose is available to the great majority of heterotrophic algae and galactose and fructose are also somewhat used, but disaccharides are less generally available and, of the polyhydric alcohols, only glycerol is frequently used (Droop, 1974).

2.2. *Carbon metabolism*

2.2.1. *Assimilation of 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 (Griffiths et al., 1960). This may happen because glucose possesses more energy content per mol compared with other substrates. For example, glucose produces ~2.8 kJ/mol of energy compared to ~0.8 kJ/mol for acetate (Boyle and Morgan, 2009). 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 (Martinez et al., 1991) and protein, chlorophyll, RNA, and vitamin contents (Endo et al., 1974).

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 (Droop, 1974). Of the several pathways used by microorganisms for aerobic glycolysis (breakdown of glucose), apparently only two: the Embden–Meyerhof Pathway (EMP) and the Pentose Phosphate Pathway (PPP) have been shown in algae (Neilson and Lewin, 1974). Algae cannot metabolize glucose under anaerobic-dark conditions because insufficient energy is liberated during dissimilation of glucose and also retarded by low levels of the enzyme lactate dehydrogenase (EC 1.1.1.27), which is essential to complete the anaerobic fermentation process (Droop, 1974; Neilson and Lewin, 1974). Of 100% of glucose taken up by microalgae, about 1% remains as free glucose. More than 85% of the glucose is assimilated and converted to oligo-(mainly sucrose, ~50%) and polysaccharides (mainly starch, ~30%) (Tanner, 2000). Some microalgae species, such as *Prymnesium parvum* and *Dunaliella tertiolecta* are unable to assimilate glucose even though they possess the enzymes necessary for its metabolism (Neilson and Lewin, 1974).

Probably the most notable difference in glucose metabolism in heterotrophic growth of microalgae, in comparison with autotrophic glucose metabolism or other non-carbohydrate organic substrates, is that under darkness, glucose is mainly metabolized via the PPP pathway (Fig. 1, Table 1), while the EMP pathway is the main glycolytic process of cells in light conditions (Lloyd, 1974; Neilson and Lewin, 1974; Yang et al., 2000; Hong and Lee, 2007). Both pathways are carried out in the

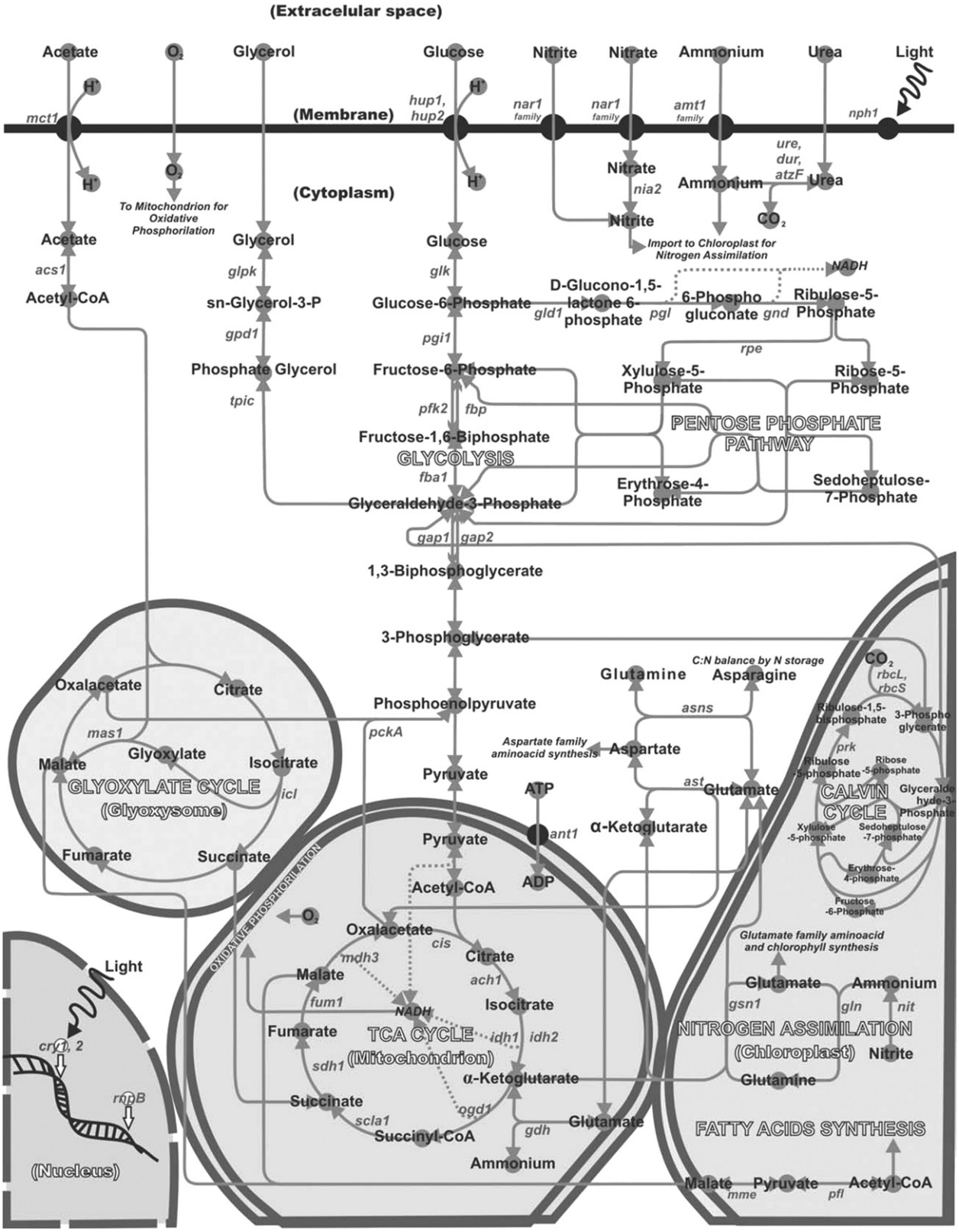

Fig. 1 – Heterotrophic metabolism in microalgae. The enzymes are indicated as "gene nomenclature" similar to Table 1. Only routes important for heterotrophic growth are shown.

Table 1 – Enzymes and proteins regulated in different heterotrophic regimens compared to autotrophic conditions.

Enzyme/Protein	E.C. #	Reaction/Function	Gene	Glucose assimilation	Acetate assimilation	Glycerol assimilation	Reference
EMP pathway (Glycolitic direction)							
Glucokinase	2.7.1.2	ATP + D-Glucose => ADP + D-Glucose 6-phosphate	*glk*	– »			Yang et al. (2000, 2002); Hong and Lee (2007)
Glucose-6-phosphate isomerase	5.3.1.9	D-Glucose 6-phosphate <=> D-Fructose 6-phosphate		« –			Yang et al. (2000, 2002)
6-Phosphofructokinase	2.7.1.11	ATP + D-Fructose 6-phosphate <=> ADP + D-Fructose 1,6-bisphosphate	*pfk2*	« –			Yang et al. (2000, 2002)
Fructose-bisphosphate aldolase	4.1.2.13	D-Fructose 1,6-bisphosphate <=> Glycerone phosphate + D-Glyceraldehyde-3-phosphate	*fba1*	« –			Yang et al. (2000, 2002)
Glyceraldehyde-3-phosphate dehydrogenase-NAD	1.2.1.12	D-Glyceraldehyde-3-phosphate + Orthophosphate + NAD+ => 1,3-Biphosphoglycerate + NADH + H+	*gap1*	–			Hilgarth et al. (1991); Yang et al. (2002)
6-phosphofructokinase	2.7.1.11	ATP + D-Fructose 6-phosphate <=> ADP + D-Fructose 1,6-bisphosphate	*pfk*	–			Yang et al. (2002)
EMP pathway (Gluconeogenesis direction)							
Glyceraldehyde-3-phosphate dehydrogenase-NADP dependent	1.2.1.59	1,3-Biphosphoglycerate + NADPH + H^+ => D-Glyceraldehyde-3-phosphate + Orthophosphate + $NADP^+$	*gap2*	↓		↓	Aubert et al. (1994); Yang et al. (2002)
Fructose-1,6-bisphosphatase	3.1.3.11	D-Fructose 1,6-bisphosphate + H_2O <=> D-Fructose 6-phosphate + Orthophosphate	*fbp*	–		↓	Aubert et al. (1994); Yang et al. (2002)
Phosphoenolpyruvate carboxykinase (ATP)	4.1.1.49	ATP + Oxaloacetate <=> ADP + Phosphoenolpyruvate + CO_2	*pckA*		»		Boyle and Morgan (2009)
Anaerobic fermentation							
D-lactate dehydrogenase (only present in *Prochlorococcus* spp.)	1.1.1.28	(D)-Lactate + NAD^+ <=> Pyruvate + NADH + H^+	*dlh*				Neilson and Lewin (1974); Garcia-Fernandez and Diez (2004).
Pentose phosphate pathway							
Glucose-6-phosphate dehydrogenase	1.1.1.49	D-Glucose 6-phosphate + $NADP^+$ <=> D-Glucono-1,5-lactone 6-phosphate + NADPH + H^+	*gld1* or *zwf*	↑»	»	↓	Aubert et al. (1994); Yang et al. (2000); Hong and Lee (2007); Boyle and Morgan (2009)
6-phosphogluconolactonase	3.1.1.3	D-Glucono-1,5-lactone 6-phosphate + H_2O <=> 6-Phospho-D-gluconate	*pgl*	»	»		Hong and Lee (2007); Boyle and Morgan (2009)
6-phosphogluconate dehydrogenase	1.1.1.44	6-Phospho-D-gluconate + $NADP^+$ <=> D-Ribulose 5-phosphate + CO_2 + NADPH + H^+	*gnd*	↑»	»	↓	Aubert et al. (1994); Yang et al. (2002); Hong and Lee (2007); Boyle and Morgan (2009)
Ribulose-phosphate 3-epimerase	5.1.3.1	D-Ribulose 5-phosphate <=> D-Xylulose 5-phosphate	*rpe* or *cfxE*	– »	»	↓	Aubert et al. (1994); Yang et al. (2002); Boyle and Morgan (2009)

Glycerol metabolism							
Glycerol kinase	2.7.1.30	ATP + Glycerol <=> ADP + sn-Glycerol 3-phosphate	*glpk*	–		↑»	Neilson and Lewin (1974)
sn-glycerol-3-phosphate NAD^+ oxidoreductase	1.1.1.8	sn-Glycerol 3-phosphate + NAD^+ <=> Glycerone phosphate + NADH + H^+	*gpd1*	–		↑»	Neilson and Lewin (1974)
Triose-phosphate isomerase	5.3.1.1	Glycerone phosphate <=> D-Glyceraldehyde-3-phosphate	*tpic*	–		↑»	Neilson and Lewin (1974)
TCA cycle							
Citrate synthase	2.3.3.1	Acetyl-CoA + H_2O + Oxaloacetate <=> Citrate + CoA	*cis*		»	»	Neilson and Lewin (1974); Boyle and Morgan (2009)
Aconitate Hydratase	4.2.1.3	Citrate <=> Isocitrate	*ach1*		»		Boyle and Morgan (2009)
Isocitrate dehydrogenase-NADH dependent	1.1.1.41	Isocitrate + NAD^+ <=> α-Ketoglutarate + CO_2 + NADH + H^+	*idh1* or *icd*	– »			Yang et al. (2002).
Isocitrate dehydrogenase-NADPH dependent	1.1.1.42	Isocitrate + $NADP^+$ <=> α-Ketoglutarate + CO_2 + NADPH + H^+	*idh2*		»	»	Neilson and Lewin (1974); Boyle and Morgan (2009)
Ketoglutarate dehydrogenase	1.2.4.2	α-ketoglutarate + CoA + NAD^+ <=> Succinyl–CoA + CO_2 + NADH + H^+	*ogd1*		«		Neilson and Lewin (1974); Boyle and Morgan (2009)
Succinate–CoA Ligase (ADP forming)	6.2.1.5	ADP + Orthophosphate + Succinyl-CoA <=> ATP + Succinate + CoA	*scla1*		«		Neilson and Lewin (1974); Boyle and Morgan (2009)
Succinate dehydrogenase	1.3.5.1	Ubiquinone + Succinate <=> Ubiquinol + Fumarate	*sdh1*			»	Neilson and Lewin (1974)
Fumarate hydratase	4.2.1.2	Fumarate + H_2O <=> (S)-Malate	*fum1* or *citH*	– »	»	»	Neilson and Lewin (1974); Yang et al. (2002)
Malate Dehydrogenase-NADP dependent	1.1.1.37	(S)-Malate + $NADP^+$ <=> Oxaloacetate + NADPH + H^+	*mdh3*		»		Boyle and Morgan (2009)
Acetate assimilation and Glyoxylate cycle							
Acetyl-CoA synthetase	6.2.1.1	ATP + Acetate + CoA <=> AMP + Diphosphate + Acetyl-CoA	*acs1*		↑»		de Swaaf et al. (2003); Boyle and Morgan (2009)
Isocitrate lyase	4.1.3.1	Isocitrate <=> Succinate + Glyoxylate	*icl*		↑»		Neilson and Lewin (1974); Boyle and Morgan (2009)
Malate synthetase	2.3.3.9	Acetyl-CoA + H_2O + Glyoxylate <=> (S)-Malate + CoA	*mas1*		↑»		Neilson and Lewin (1974); Boyle and Morgan (2009)
Calvin–Benson Cycle (Carbon fixation)							
Ribulose bisophosphate carboxylase/oxygenase large subunit	4.1.1.39	D-Ribulose 1,5-bisphosphate + CO_2 + H_2O <=> (2) 3-phospho-D-glycerate	*rbcL*	↓			Yang et al. (2002)
Ribulose bisophosphate carboxylase/oxygenase small subunit	4.1.1.39	D-Ribulose 1,5-bisphosphate + CO_2 + H_2O <=> (2) 3-phospho-D-glycerate	*rbcS*	↓			Yang et al. (2002)
Phosphoribulokinase	2.7.1.19	ATP + D-Ribulose 5-phosphate <=> ADP + D-Ribulose 1,5-bisphosphate	*prk*	–			Yang et al. (2002)
Phosphoenolpyruvate carboxylase	4.1.1.31	H_2O + Phosphoenolpyruvate + CO_2 <=> Orthophosphate + Oxaloacetate	*ppc*	– »			Yang et al. (2002)

(continued on next page)

Table 1 (*continued*)

Enzyme/Protein	E.C. #	Reaction/Function	Gene	Glucose assimilation	Acetate assimilation	Glycerol assimilation	Reference
Fatty Acids Synthesis							
Malate dehydrogenase (oxalacetate decarboxylating)-NADP dependent	1.1.1.40	(S)-Malate + $NADP^+$ <=> Pyruvate + CO_2 + NADPH + H^+	*mme*		»		Boyle and Morgan (2009)
Pyruvate formate-lyase	2.3.1.54	CoA + Pyruvate <=> Acetyl-CoA + Formate	*pfl*		»		Boyle and Morgan (2009)
Transports							
Hexose/H^+ symport system 1		Transport hexoses and protons with a stoichiometry of 1:1 trough the cell membrane investing 1 ATP	*hup1*	↑			Komor and Tanner (1974); Sauer and Tanner (1989); Hilgarth et al. (1991)
Hexose/H^+ symport system 2		Transport sugars and protons with a stoichiometry of 1:1 trough the cell membrane investing 1 ATP providing higher spectrum of sugars specificity uptake	*hup2*	↑			Caspari et al. (1994)
Hexose/H^+ symport system 3		Transport sugars and protons with a stoichiometry of 1:1 trough the cell membrane investing 1 ATP providing higher spectrum of sugars specificity uptake	*hup3*	↑			Caspari et al. (1994)
Hexose transport system		Mitochondrial membrane hexose transport protein	*hxt1*	–			Merchant et al. (2007)
ATP/ADP mitochondrial translocator		Adenine nucleotide translocator; ATP/ADP translocase	*ant* or *aat*	–			Hilgarth et al. (1991)
Monocarboxylic/proton transporter		Protein that aids transport of monocarboxylic (such as acetate) molecules across the membrane	*mct1*		↑		Becker et al. (2005)
Ammonium transporter proteins (AMT1)		Proteins for ammonium transport across the cellular and chloroplastic membranes belonging to the ammonium transporter family 1(AMT1)	*amt1* (*a,b,c,d,e,f,g,h*)	–	–	–	Wilhelm et al. (2006); Fernandez and Galvan (2007)
Nitrate/nitrate transporter proteins (NAR1)		Proteins for high affinity nitrate/nitrite transport across the membranes belonging to the ammonium transporter family, also presente in chloroplast	*nar1*	↓	↓	↓	Kamiya (1995); Fernandez and Galvan (2007)
Nitrate/nitrate transporter proteins (NRT1,2)		Proteins for high affinity nitrate/ nitrite transport across the cellular membranes belonging to the transporter family	*nar1*, *nar2*	↓	↓	↓	Kamiya (1995); Fernandez and Galvan (2007)
Nitrogen assimilation							
Glutamine synthetase (GS)	6.3.1.2	ATP + L-Glutamate + NH_3 => ADP + Orthophosphate + L-Glutamine	*gln*	↑	–	–	Tischner (1984); Kaplan et al. (1986); Lu et al. (2005); Vanoni and Curti (2005)

Glutamate synthase–NADH deoendent (GOGAT)	1.4.1.14	L-Glutamine + α-Ketoglutarate + NADH + H^+ => (2) L-Glutamate + NAD^+	*gsn1*	–	–	–	Tischner (1984); Kaplan et al. (1986); Fernandez and Galvan (2007); Lu et al. (2005); Vanoni and Curti (2005)
Glutamate synthase–Ferredoxin dependent (GOGAT)	1.4.7.1	L-Glutamine + α-Ketoglutarate +(2) Reduced ferredoxin + (2) H+ => (2) L-Glutamate + (2) Oxidized ferredoxin	*gsf1*	–	–	–	Tischner (1984); Lu et al. (2005); Vanoni and Curti (2005); Fernandez and Galvan (2007)
Glutamate dehydrogenase-NADH dependent (GDH)	1.4.1.3	α-Ketoglutarate + NH_3 + NADH + H^+ <=> L-Glutamate + NAD^+ + H_2O	*gdh*	–	–	–	Lea and Miflin (2003); Lu et al. (2005)
Aspartate aminotransferase	2.6.1.1	Oxaloacetate + L-Glutamate <=> L-Aspartate + 2-Oxoglutarate	*ast*	–	–	–	Inokuchi et al. (2002); Coruzzi (2003)
Asparagine synthetase	6.3.5.4	ATP + L-Aspartate + L-Glutamine + H_2O <=> AMP + Diphosphate + L-Asparagine + L-Glutamate	*asns*	↓	↓	↓	Inokuchi et al. (2002); Coruzzi (2003)
Nitrate reductase–NADH dependent	1.7.1.1	Nitrate + NADH + H^+ => Nitrite + NAD^+ + H_2O	*nia2* or *nr*	↓	↓	↓	Kamiya (1995); Kamiya and Saitoh (2002)
Nitrite reductase–ferredoxin dependent	1.7.7.1	Nitrite + (6) Reduced ferredoxin + (6) H^+ => NH_3 + (2) H_2O + (6) Oxidized ferredoxin	*nit* or *nir*	«	«	«	Morris (1974); Kamiya (1995)
Urease	3.5.1.5	Urea + H_2O <=> CO_2 + (2) NH_3	*ure*	–	–	–	Kaplan et al. (1986); Oh-Hama and Miyachi (1992)
Urea amidolyase	6.3.4.6	ATP + Urea + HCO^{3-} <=> ADP + Orthophosphate + Urea-1-carboxylate	*dur*	–	–	–	Morris (1974); Kaplan et al. (1986); Oh-Hama and Miyachi (1992)
Allophanate hydrolase	3.5.1.54	Urea-1-carboxylate + H_2O <=> (2) CO_2 + (2) NH_3	*atzF*	–	–	–	Morris (1974)
Gene regulation							
Ribonuclease P	3.1.26.5	Endonucleolytic cleavage of RNA, removing 5′-extranucleotides from tRNA precursor	*rnpB*	–			Yang et al. (2002)
NPH1 Flavoprotein (Autophosphorylating serine–threonine protein kinase action)	2.7.11.1	Blue-light photoreceptors whit photoinhibitory action over *Hup1* gene. ATP + Protein <=> ADP + Phosphoprotein	*nph1*	– »			Kamiya and Kowallik (1987b); Christie et al. (1998); Kamiya and Saitoh (2002)
Cryptochromes 1 and 2 Flavoproteines (ATP binding/protein homodimerization/ protein kinase action)		Blue-light photoreceptors to photoinhibition of *Hup1* gene	*cry1, 2*	– »			Kamiya and Kowallik (1987b); Kamiya and Saitoh (2002)

↓ Reduction of enzyme concentration and/or gene expression level (mRNA concentration) compared to autotrophic cultures.
↑ Increase of enzyme concentration and/or gene expression level (mRNA concentration) compared to autotrophic cultures.
– Presence of enzyme or it's mRNA but no changes in their concentration level compared to autotrophic cultures.
« Reduction of metabolite flux rate of the reaction compared to autotrophic cultures.
» Increase of metabolite flux rate of the reaction compared to autotrophic cultures.

cytosol and are functional in microalgae cells. However, the PPP pathway might have a higher flux rate than the other, depending on light and the presence of glucose. Some examples illustrate the process. In complete darkness and using glucose as sole carbon source, the PPP pathway in *Chlorella pyrenoidosa* (renamed *Chlorella sorokiniana*) accounts for 90% of glucose metabolic flux distribution via glucose-6-phosphate dehydrogenase (EC: 1.1.1.49) and the reaction catalyzed by glucose-6-phosphate isomerase (EC: 5.3.1.9) of the EMP pathway is totally "shifted down" (Yang et al., 2000). In heterotrophic culture of the cyanobacteria *Synechocystis* spp., the PPP was the major pathway of glucose catabolism via glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (EC: 1.1.1.44) (Yang et al., 2002; Hong and Lee, 2007). However, the EMP glycolytic pathway is not completely shifted down, only the key reactions catalyzed by glucose-6-phosphate isomerase (EC: 5.3.1.9), 6-phosphofructokinase (EC: 2.7.1.11), and fructose-bisphosphate aldolase (EC: 4.1.2.13) are affected in glucose assimilation, the other reactions of this pathway remain active such as in autotrophic growth (Yang et al., 2000, 2002; Hong and Lee, 2007). The Tricarboxylic Acid Cycle (TCA) and mitochondrial oxidative phosphorylation maintain high activities in cultures of *C. pyrenoidosa*, such as in illuminated environments, which indicate a minor effect of light on these pathways in this microalga species (Yang et al., 2000; Hong and Lee, 2007). At the same time, the flux through the pentose phosphate pathway during illumination was very small, resulting from light-mediated regulation. Heterotrophic culture of *C. pyrenoidosa* generated more ATP from the energy supplied as glucose than the autotrophic and mixotrophic cultures with energy supplied as light (Yang et al., 2000). In *Synechocystis* spp., a multi-level regulatory mechanism of the enzymes required for glucose metabolism depends on the energy source available to the cells and this depends on environmental conditions, transcription rates, metabolites availability, and flux requirements. In heterotrophic cultures, the expression of the genes *rbcL* (codes for ribulose bisophosphate carboxylase/oxygenase large subunit, EC: 4.1.1.39) and *gap2* (glyceraldehyde-3-phosphate dehydrogenase-NADP; EC: 1.2.1.59), were down-regulated about two-fold by a light-regulated transcription mechanism, while the gene *gnd* (6-phosphogluconate dehydrogenase, EC: 1.1.1.44) was up-regulated about 60% in response to an apparent flux of substrate product of that enzyme because the system requires more of the product of that enzyme. In contrast, the expression of the genes *prk*, *fbp*, *rnpB*, *glk*, *gap1*, *ppc*, *pfk*A, *icd*, *fum1* (that codes respectively for phosphoribulokinase – EC: 2.7.1.19, fructose-1,6-bisphosphatase – EC: 3.1.3.11, ribonuclease P – EC: 3.1.26.5, glucokinase – EC: 2.7.1.2, glyceraldehyde-3-phosphate dehydrogenase – EC: 1.2.1.12, phosphoenolpyruvate carboxylase – EC: 4.1.1.31, 6-phosphofructokinase – EC: 2.7.1.11, isocitrate dehydrogenase – EC: 1.1.1.41, and fumarate hydratase – EC: 4.2.1.2) are not affected by the presence or absence of light or glucose, proving that also in cyanobacteria the TCA and many reactions of the EMP are actively independent of the energy and carbon sources for the culture (Yang et al., 2002). Compared to the mixotrophic condition, the mRNA levels of all the genes were not up or down-regulated significantly during autotrophic growth. The protein expression pattern under the autotrophic condition was very similar to that in the mixotrophic condition; this means that the presence of glucose under illuminated conditions did not significantly alter the protein expression profiles (Yang et al., 2002). Compared to *Synechocystis* spp., other marine cyanobacteria, such as *Prochlorococcus* spp. have an incomplete TCA cycle metabolism lacking key enzyme genes such as those encoding for malate dehydrogenase (EC: 1.1.1.37) and succinyl coA–ligase (EC: 6.2.1.5). However some strains of this genus possess alternatively the enzyme D-lactate dehydrogenase (EC: 1.1.1.28) that allows recovery of NAD^+ produced by glycolysis, while transforming pyruvate to lactate under anoxic environments (Garcia-Fernandez and Diez, 2004).

Chlorella cells possess an inducible active hexose/H^+ symport system responsible for uptake of glucose from the medium (Tanner, 1969; Komor, 1973; Komor and Tanner, 1974, 1976). This mechanism transports sugars and protons with a stoichiometry of 1:1 (Komor et al., 1973) and the cell invests one molecule of ATP per molecule of sugar transported (Tanner, 2000). The transporter is completely inactive for all fluxes: influx, efflux, and exchange flux, between environment and cytosol when the intracellular pH is below 6.0 and is optimally active at an extracellular pH of 6.0 (Komor et al., 1979). In *C. vulgaris* growing with glucose as the inducer, the minimum time necessary to induce synthesis of the hexose/H^+ symport system proteins is 15–18 min (Haass and Tanner, 1974; Komor and Tanner, 1974). Induction of the transporter is achieved by D-glucose, D-fructose, and D-galactose, but not by pentoses, sucrose, D-manose, disaccharides, or sugar alcohols (Komor et al., 1985).

The gene corresponding to the hexose/H^+ symport system protein is the Hexose Uptake Protein Gene (*hup1*) (Sauer and Tanner, 1989). The HUP1 protein is a member of the Major Facilitator Superfamily (MFS) of transporter proteins. The structure of the hexose/H^+ symport system was reviewed in detail (Caspari et al., 1994; Tanner, 2000). The mRNA of the *hup1* gene, absent in photosynthetically-grown cells, appears within 5 min after sugar is added (Hilgarth et al., 1991). The *hup1* gene, the ATP/ADP translocator mitochondrial gene (*aat*), and the glyceraldehyde-3-phosphate dehydrogenase gene (*gap1*) are activated when autotrophically grown *Chlorella kessleri* cells switch to heterotrophic growth in the presence of D-glucose. Uptake mutants ($HUP1^-$) do not respond to application of sugars in this way (Hilgarth et al., 1991). *Chlorella* cells in general possess two more hexose transporter genes (*hup2* and *hup3*), co-induced by D-glucose. The other sugars provide a higher spectrum of sugars specificity uptake (Caspari et al., 1994). In *Chlamydomonas reinhardtii*, the *hxt1* gene is codified for the hexose transporter protein through which glucose is transported through chloroplastic membranes (Merchant et al., 2007).

Light plays a major role in glucose uptake. In *C. vulgaris* cells, light inhibits expression of the hexose/H^+ symport system. The blue end of the visible spectrum is very effective at inhibiting the uptake of hexoses where the red end is only slightly effective (Kamiya and Kowallik, 1987a). Because a similar photo-inhibiting effect occurred in a non-photosynthesizing mutant of *C. vulgaris*, this suggests that photosensitivity is independent of photosynthesis and is performed by the blue-light photoreceptors flavoproteins NPH1 and cryptochromes 1 and 2 (Kamiya and Kowallik, 1987b; Kamiya

and Saitoh, 2002). For *Chlorella* cells growing with glucose, the presence of blue light controls numerous metabolic reactions, such as inhibiting uptake of glycine, proline, and arginine, and ammonia, but enhancing uptake of oxygen and nitrate through activation of nitrate reductase (EC: 1.7.1.1) (Kamiya, 1995; Kamiya and Saitoh, 2002). Under complete darkness, glucose in the medium induces expression of two transmembrane amino acid transport systems. One is for transport of neutral amino acids, such as alanine, proline, serine, and glycine and the other is for transport of the basic amino acids, arginine and lysine. This induction enhances uptake of these amino acids about 5–10 times faster than the rates of uptake reported for any other plant cell in higher plants or algae (Cho et al., 1981). These enhancing effects were not observed under red or far-red illumination (Kamiya and Saitoh, 2002). When microalgae species and strains are able to grow under mixotrophic regimes, specific growth rates of the mixotrophic cultures is approximately the sum of the growth rates of cells under phototrophic and heterotrophic conditions. Consequently, only algal strains that are not sensitive to photoinhibition are suitable for mixotrophic cultivation (Lee, 2004).

Although it is generally agreed that glucose can serve as a common carbon source, the precise effects of glucose on metabolism of microalgae varies greatly and these contradictions may lead to the conclusion that glucose uptake depends mainly on the quantity of light and the species of microalgae that is used. Several examples on the effect of glucose on oxygen, pH, and substrate concentration can illustrate this point.

Depletion of glucose and fructose in cultures of *Galdieria sulphuraria* was accompanied by a rapid increase in concentration of dissolved oxygen in the culture resulting from diminished respiration rate caused by complete depletion of the original carbon source. An intermittent feeding method for the microalga was proposed. After the dissolved oxygen tension increased to >10%, a new batch of substrate was added. The concentration of sugar was kept sufficiently low to serve as the growth-limiting factor, although the total amount of glucose that was added was large (Schmidt et al., 2005). For *Schizochytrium limacinum*, the concentration of oxygen had no effect on growth. Differences in sugar consumption and dissolved oxygen in the medium could be attributed to the pH of the culture and to the strains that were used (Chi et al., 2007). For *C. vulgaris* growing on sufficient glucose, the hexose/H^+ symport system is induced to promote the alkalinization of the culture media by a net movement of protons accompanied glucose uptake. Since other sugars can be used, the velocity of the increase in pH depends on the concentration and type of sugar used (Komor and Tanner, 1974). Commonly, under low hexose concentrations, a decrease in pH and sugar consumption occurs. Sugar consumption is apparently linked to a net movement of protons in sugar translocation through the membrane, yielding a pH-shift (Komor and Tanner, 1974, 1976; Komor et al., 1985). Consequently, high concentrations of glucose and glycerol have been shown to inhibit microalgal growth, at least for a considerable period of time. This is the underlying reason of the adoption of the feed-batch configuration of bioreactor operation. This proposed configuration can maintain a constant low substrate concentration and avoid, in practical and easy ways, adverse effects on growth and end-product yields (Tan and Johns, 1991; Cerón García et al., 2000; Wen and Chen, 2000; Schmidt et al., 2005; Xiong et al., 2008).

This proposal creates a dilemma: How low is low? It is likely that this depends on the microalgal species and specific growth conditions. For example, to promote cellular growth of *C. vulgaris* and *Scenedesmus acutus*, the initial concentration of glucose should be limited to 10 g l^{-1} and 1 g l^{-1}, respectively (Ogawa and Aiba, 1981). For optimal growth of *Chlorella saccharophila*, a concentration of glucose of 2.5 g l^{-1} is required and inhibition occurred at concentrations >25 g l^{-1}; inhibition of *C. sorokiniana* occurs at concentrations >5 g l^{-1} (Tan and Johns, 1991). *Chlorella protothecoides* has been cultivated at concentrations as high as 85 g l^{-1} to obtain an optimal yield of biomass (Shi et al., 1999). In trials with *Nitzschia laevis*, yields decreased as concentration increased from 1 to 40 g l^{-1} (Wen and Chen, 2000). *G. sulphuraria* grown with high concentrations of glucose or fructose up to 166 g l^{-1} (0.9 M) continued to thrive, but higher concentrations inhibited growth (Schmidt et al., 2005).

In summary, information on the concentration of glucose required for optimal metabolic growth is too scattered to reach a definite conclusion. The answer may be related to specific combinations of factors, with the microalgal species as the main factor and cultivation and environmental conditions as secondary factor. Consequently, each combination of factors may lead to different consumption levels. From the data published, it appears that glucose might be considered a "preferred substrate" for heterotrophic cultivation of microalgae. Microalgal cells grown on other substrates require a lag period (an acclimation period) to develop the specific transport systems necessary for uptake of other substrates. Consumption of "less preferred" substrates is aborted because the enzymes that catalyze uptake of an alternate substrate cannot be synthesized in the presence of the "preferred" substrate (Lewin and Hellebust, 1978; Ratledge et al., 2001; Narang and Pilyugin, 2005). This lag phenomenon may not always occur because other factors, such as the strain used, bioreactor configuration, and environmental conditions will have a profound impact on uptake of alternative substrates.

2.2.2. *Assimilation of glycerol*

Heterotrophic growth using glycerol as a substrate has been demonstrated for several algae, despite the simplicity of glucose metabolism in microalgae (Table 1). Most of these species occur naturally in habitats of somewhat elevated osmolarity, such as seawater and saline pounds (Neilson and Lewin, 1974). 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 (Richmond, 1986). It is commonly used for long-term preservation of microorganisms at low temperatures.

Microalgae can produce glycerol as part of the glycerolipid metabolism because it is a product of hydrolysis of many lipids that are glyceryl esters of fatty acids (León and Galván, 1999). A few species can assimilate glycerol from the medium, where it increased their growth rate and induced specific biochemical and structural changes in their photosynthetic system, such as reduction of cell phycoerythrin content, degree of tylakoid

packing, number of tylakoids per cell, and the size of Photo System II particles (Lewitus et al., 1991).

When plant cells are growing on a glycerol substrate, it enters the cell by simple diffusion (Neilson and Lewin, 1974). Inside cells, glycerol is used as an osmoregulatory molecule. Glycerol is first phosphorylated using ATP and the glycerophosphate is then oxidized to triose phosphate. Plant cells possess 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 (Neilson and Lewin, 1974). Glyceraldehyde-3-phosphate may also be formed by reduction of 3-phosphoglycerate, a key intermediate of the Calvin–Benson cycle of photosynthesis. It has been demonstrated that sn-glycerol 3-phosphate inhibits the reversible glycolytic pathway, as expected in the gluconeogenesis pathway. Aubert et al. (1994) suggest that the pentose phosphate pathway is also inhibited when glycerol is the unique carbon source (Fig. 1). Glycerol can be photometabolized (photoheterotrophy) by some algae species, such as *Agmenellum quadruplicatum*, *Goniotrichium elegans*, *Navicula pelliculosa*, *Nostoc* sp. These species can only assimilate glycerol as a carbon source, in the presence of light and without an external supply of CO_2 (Ingram et al., 1973; Kaplan et al., 1986).

Glycerol and light were used as substrates for cultivation of mixotrophic microalgae, yielding significant positive results. For example, in a culture media supplemented with 0.1 M glycerol and 165 μmol photons m^{-2} s^{-1}, *Phaeodactylum tricornutum* increased its growth 74% more compared to autotrophic culture. However, a pronounced lag phase occurred, as explained above for growth on substrates other than glucose (Cerón García et al., 2000). *Nannochloropsis* sp., *Rhodomonas reticulate*, and *Cyclotella cryptica* seem to prefer glycerol over glucose or acetate by using mixotrophic metabolism and positively responding to environmental changes, such as when a nitrate is added to the medium (Wood et al., 1999).

When *C. vulgaris* was immobilized in alginate with *Azospirillum brasilense* (a microalgae growth-promoting bacteria; MGPB) and grown autotrophically on synthetic wastewater growth medium (SWGM), *A. brasilense* mitigated environmental stress for the microalgae (de-Bashan et al., 2002). In another study, major cell growth occurred at pH 8 for *A. brasilense* immobilized with *Chlorella*, compared to *Chlorella* grown alone under autotrophic conditions (de-Bashan et al., 2005). In similar experiments using joint immobilization carried out under heterotrophic conditions, an eight-fold increase in the growth of *C. vulgaris* in SWGM containing 0.17 M glycerol after culturing for 24 h, compared to cultures with *C. vulgaris* immobilized alone under the same conditions, where there was no growth. Similar growth was obtained at pH 8 compared to pH 6 and 7 (Escalante F.M.E., unpublished data). This suggests that *A. brasilense* plays a major role in changing the metabolic behavior of *Chlorella* under autothrophic or heterotrophic conditions.

In conclusion, although glycerol can serve as a substrate for heterotrophic growth, knowledge of metabolism under heterotrophic conditions is limited. With a potential for biodiesel production from microalgae (discussed later) where glycerol is a major by-product and a substrate of the process, this niche probably will be revived.

2.2.3. Assimilation of 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 (Bollman and Robinson, 1977). Acetate (or acetic acid) is one of the most common carbon sources for many microbial species, including microalgae (Droop, 1974). Under dark, aerobic conditions, eukaryotic cells uptake acetate using the monocarboxylic/proton transporter protein that aids transport of monocarboxylic molecules across the membrane. This protein is a member of the Major Facilitator Superfamily mentioned earlier (Becker et al., 2005). 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 Fig. 1 (Droop, 1974; de Swaaf et al., 2003; Boyle and Morgan, 2009).

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) (Table 1) 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). Many of the intermediates of both cycles are the same metabolites (Neilson and Lewin, 1974; Ahmad and Hellebust, 1990; Boyle and Morgan, 2009). In general, microalgae that grow by assimilating acetate must possess a glyoxylate cycle pathway to efficiently incorporate acetyl groups of acetyl-CoA to carbon skeletons. The operation of the glyoxylate cycle requires synthesis of isocitrate lyase (EC 4.1.3.1) and malate synthetase (EC 2.3.3.9). Both enzymes are induced when cells are transferred to media containing acetate (Neilson and Lewin, 1974; Boyle and Morgan, 2009). In *C. vulgaris*, isocitrate lyase, the key enzyme of the glyoxylate cycle, is synthesized constitutively, but the glyoxylate cycle is functional only during growth on acetate (Harrop and Kornberg, 1966). In *Scenedesmus obliquus*, activity of isocitrate lyase showed nearly a four-fold increase in activity after 24 h in the dark in the presence of acetate. Under heterotrophic conditions, isocitrate lyase activity increased as a function of increasing acetate concentration (Combres et al., 1994). In *Chlorella* spp. growing on acetate in the dark, the glyoxylate cycle enzymes are induced but not the TCA cycle enzymes; the latter pathway remains active, but its activity is not enhanced. Light and glucose suppress the formation of isocitrate lyase (Goulding and Merrett, 1967). In assimilation by the glyoxylate cycle, 4 mol of acetate are required per mole of glucose-6-phosphate that is synthesized, one of the four being consumed in the process (Droop, 1974).

In *C. reinhardtii* growing on acetate, the oxidative pentose phosphate pathway is also active, providing reducing power as NADPH for cytosol (Boyle and Morgan, 2009). Additionally, mitochondrial and chloroplastic electron transport chains are active in these cells and have a close interaction through the glycolytic pathway (Rebeille and Gans, 1988).

When sodium or potassium salt of acetate is used as a substrate, the pH rises. This happens because the remaining Na^+ or K^+ couples with hydroxyl ions (OH^-) or other

anions to form alkalis. This phenomenon also occurs if reactors are pH-neutralized with alkali. Since metallic hydroxides are stronger than organic acids, the media must be neutralized or at least brought to a non-inhibitory pH level by adding an acid, acetic acid for instance, into the cycle (Ratledge et al., 2001). 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, with the exception of *Chlamydomonas mundana*, which grew rapidly using acetate (Maciasr and Eppley, 1963; Wood et al., 1999). 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). In this way, as long as acetate is consumed, more acetic acid is added to the reactor, which avoids the rise in pH at the time that more acetate is added (Wood et al., 1999; Zhang et al., 1999; de Swaaf et al., 2003; Sijtsma et al., 2005). Although acetate fermentation in pH-auxostats is linked to succinic acid production that also inhibits growth, addition of propionate was suggested to provide oxaloacetate to the cells and improve cellular growth (Fig. 1). No explanation was provided on the mechanism by which propionate would alleviate inhibition of acetate in microalgae growing in this substrate. However, since propionate is a precursor of oxaloacetate, a possible explanation might be found in work with the bacterium *Pseudomonas citronellolis*. One of the requirements for efficient oxidation of the carbon source through the TCA cycle is a proper balance between oxaloacetate and acetyl-CoA. It is likely that elevated concentrations of acetyl-CoA in cells cultured with acetate would inhibit oxaloacetate decarboxylase. Hence, to maintain the supply of oxaloacetate, it is necessary to metabolize the acetate via the TCA cycle. Addition of propionate to the culture medium would lead to oxaloacetate production by alternative pathways (O'Brien and Taylor, 1977). For further details on acetate metabolism, see Sijtsma et al. (2005).

Several examples on growing microalgae on acetate are known. *Euglena gracilis* strain L incorporates acetate efficiently under light but not in the dark (Cook, 1967). On the other hand, *E. gracilis* var. *bacillaris* incorporates acetate in the dark when its concentration is below 5 g l^{-1}. This strain was able to use a variety of substrates in heterotrophic cultures, such as acetate, sucrose, ethanol, amino acids, butyric acid, among a few other organic substrates (Cook, 1968). *Crypthecodinium cohnii* is able to grow in heterotrophic cultures with acetate concentrations as high as 1 g l^{-1}. No higher concentrations were tested (Vazhappilly and Chen, 1998). Another study with this strain achieved good growth when cultured in a pH-controlled, pH-auxostat with 8 g l^{-1} of sodium acetate (Ratledge et al., 2001). In a recent study (Perez-Garcia et al., in press), when 10 g l^{-1} sodium acetate was added to municipal wastewater with *C. vulgaris* for tertiary treatment, significant heterotrophic growth occurred; however, this did not happen when calcium acetate was added.

It seems that as long as the level of acetate is low and remains low, several microalgae can use it as its sole carbon source. This is specifically important because acetate is a readily available and inexpensive substrate derived from many industrial applications and its use does not impose severe restrictions on culturing microalgae.

2.2.4. *Wastewater and other carbon sources for heterotrophic growth of microalgae*

One commonly proposed application of autotrophically grown microalgae is wastewater treatment (de la Noüe and Proulx, 1988; de la Noüe et al., 1992; Oswald, 1992; Cañizares et al., 1994; Gonzalez et al., 1997; Lee and Lee, 2001; de-Bashan et al., 2002, 2004; Hernandez et al., 2006). The major advantages of these treatments are that additional pollution is not generated when the biomass is harvested and efficient recycling of nutrients is possible (de la Noüe et al., 1992). To date, this has hardly been tested under heterotrophic conditions (de-Bashan and Bashan, 2010). Nonetheless, *Chlorella* spp. and strains of *Scenedesmus* were isolated from wastewaters kept in the dark and in oxidation ponds (Abeliovich and Weisman, 1978; Lalucat et al., 1984; Post et al., 1994). *C. pyrenoidosa* growing in sterilized sewage were able to use some of the organic matter, as indicated by a decrease in soluble BOD and dissolved volatile solids in cultures of short retention times. Use of organic compounds was influenced by the supply of CO_2 to the culture; decrease in the organic matter per unit of cell weight produced was greater when the supply of CO_2 was low (Pipes and Gotaas, 1960).

Growth characteristics and removal of nutrients from synthetic wastewater with high acetate and propionate concentrations were investigated under heterotrophic and mixotrophic conditions for *Rhodobacter sphaeroides*, *C. sorokiniana*, and *Spirulina platensis*. Heterotrophic cultures of *R. sphaeroides* and *C. sorokiniana* produced the best results under dark conditions but *S. platensis* required light. Neither growth nor removal of nutrients by the cells were affected in synthetic wastewater with as high as 10 000 μg l^{-1} acetate, 1000 μg l^{-1} propionate, 700 μg l^{-1} nitrate and 100 μg l^{-1} phosphate (Ogbonna et al., 2000). Recently, Perez-Garcia et al. (in press) found that adding several carbon sources to municipal wastewater that normally do not support microalgal growth allowed heterotrophic growth of *C. vulgaris*. Growth effects, in declining order, was Na-acetate, D-glucose, D-fructose = fulvic acid, Na-citrate = lactic acid = acetic acid, malic acid, and L-arabinose.

Other carbon sources such as sucrose, lactate, lactose, and ethanol have been tested under heterotrophic microalgae cultures with negative results in growth and metabolite production (Theriault, 1965; Lewin and Hellebust, 1978; Ogbonna et al., 1998; Schmidt et al., 2005; Wang and Peng, 2008). It appears that microalgae do not have invertase to assimilate sucrose. Komor et al. (1985) report that disaccharides connected to carbon 1 (sucrose) or carbon 4 (maltose) are not transported; consequently, sucrose uptake by *C. pyrenoidosa* (Theriault, 1965) and *Chlorella zofingiensis* (Wang and Peng, 2008) is poor. Schmidt et al. (2005) show that *G. sulphuraria* had significant growth on a sucrose substrate, but only at pH 2. It is likely that sucrose was hydrolyzed into glucose and fructose, which are readily assimilated by microalgae.

While several carbon sources were proposed for heterotrophic growth of microalgae, practical evaluation of the carbon sources show that only a few substrates are supported by solid evidence. Those include glucose, glycerol, and acetate in wastewater. None of the other carbon sources tested supported sufficient growth. At this juncture, there are no other candidates for additional studies of growth. What is still pending is information about industrial wastes such as

molasses, vinegar, pharmaceutical by-products, and paper mill effluents. They contain the assimilated low molecular substrates that microalgae can use and can be mixed with water to create a substrate for microalgae. If feasible, these mixtures could be designated "prepared wastewater substrate".

2.3. Metabolism 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. This excludes diatoms, where silicon plays the more important role instead of nitrogen (for review, Martin-Jézéquel et al., 2000) and nitrogen-deficient microalgae that accumulate oils or polysaccharides (Kaplan et al., 1986). Carbon and nitrogen metabolism are linked in microalgae because they share (a) carbon supplied directly from respiration of fixed CO_2 (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 (Huppe and Turpin, 1994; Inokuchi et al., 2002; Lea and Miflin, 2003; Fernandez and Galvan, 2007). Very small quantities of keto-acids were found in *Chlorella* spp. when grown autothrophically, but the levels were much higher under heterotrophic conditions and nitrogen starvation (Millbank, 1957). Respiration rates appear to be limited indirectly by the supply of inorganic nitrogen through the demand of carbon skeletons. This happens following conditions in which intracellular carbohydrate energy reserves can accumulate, such as under limited nitrogen when carbon is not a limiting factor (Geider and Osborne, 1989).

In general, nitrogen has a marked positive effect on growth and a negative effect on lipid accumulation. Microalgae are able to assimilate a variety of nitrogen sources, mainly ammonia (NH_4^+), nitrate (NO_3^-), and urea, as well as yeast extract, peptone, amino acids, and purines (Oh-Hama and Miyachi, 1992; Armbrust et al., 2004; Chen and Chen, 2006; Wilhelm et al., 2006; Ganuza et al., 2008). The metabolic pathways involved in nitrogen assimilation are depicted in Fig. 1.

2.3.1. Assimilation 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 (Syreth and Morris, 1963; Goldman, 1976; Kaplan et al., 1986; Shi et al., 2000; Grobbelaar, 2004; Wilhelm et al., 2006).

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 (Wilhelm et al., 2006). Several ammonium transporters, all belonging to the AMT family, have been identified in diatoms (Allen et al., 2005). An ample array of transporters for inorganic nitrogen compounds have been identified in *Chlamydomonas* sp., 8 putative ammonium transporters and 13 putative nitrate/nitrite transporters (Fernandez and Galvan, 2007). Ammonium transporters can be divided into two distinct systems: a high affinity system regulated by the nitrogen status of cells and a low-affinity system that exhibits a linear increase in activity in response to increases in ammonium concentration (Howitt and Udvardi, 2000). There are exceptions. Ammonium transporters in *Cylindrotheca fusiformis* and *P. tricornutum* are not only up-regulated by nitrogen limitation, but are also expressed at a higher level when grown on nitrate, compared to ammonium (Hildebrand, 2005).

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 (Howitt and Udvardi, 2000). Dark respiration of nitrogen-starved microalgae cells is correlated with inorganic nitrogen assimilation. Ammonium-enhanced respiration continued until either ammonia concentration in the suspending medium dropped to an undetectable concentration or intracellular carbohydrate energy reserves were almost completely exhausted. Addition of glucose will allow ammonium assimilation to continue, as well as amino acid and protein synthesis (Geider and Osborne, 1989).

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 α-ketoglutarate (Tischner, 1984; Kaplan et al., 1986; Lu et al., 2005; Vanoni and Curti, 2005) (Fig. 1). Alternatively, ammonium is incorporated into glutamate by the reversible reductive amination of α-ketoglutarate, which is catalyzed by glutamate dehydrogenase (GDH, EC 1.4.1.2) (Inokuchi et al., 2002). The GS/GOGAT pathway is regarded as the primary pathway for ammonium assimilation, while the GDH pathway plays an insignificant part in the formation of glutamate. However, the evidence suggests an important role for GDH as a catabolic shunt to ensure that nitrogen metabolism does not affect mitochondrial function and to enable synthesis of nitrogen-rich transport compounds during nitrogen remobilization (Lea and Miflin, 2003). Additionally, GDH is believed to be active under conditions of stress (Lu et al., 2005). Glutamine synthetase, known for its high affinity for ammonia and its ability to incorporate ammonia efficiently into amino acids (Miflin and Habash, 2002) is an important enzyme in any photosynthetic organism, even under heterotrophic metabolism. This enzyme plays a dual role by providing glutamine for biosynthesis and by assimilating ammonia (Rahman et al., 1997; de-Bashan et al., 2008). Following incorporation of ammonium into glutamate through either the GS/GOGAT cycle or GDH, nitrogen is

distributed to the other amino acids, much of it through transamination with oxaloacetate by aspartate aminotransferase (EC: 2.6.1.1) to yield aspartate. Through an ATP-dependent reaction catalyzed by asparagine synthetase (EC: 6.3.5.4), an amino group from glutamine is transferred to a molecule of aspartate to generate a molecule of glutamate and asparagine. Glutamine, glutamate, aspartate, and asparagine provide the building blocks for the synthesis of organic nitrogen compounds, such as amino acids, nucleotides, chlorophylls, polyamines, and alkaloids (Inokuchi et al., 2002; Coruzzi, 2003).

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 μmol cell^{-1} min^{-1} and is similar to that in heterotrophy (17.4 μmol cell^{-1} min^{-1}), but this is ~4 times lower than occurring under mixotrophy (65.9 μmol cell^{-1} min^{-1}) (Combres et al., 1994). *R. sphaeroides* and *C. sorokiniana* showed acceptable growth in darkness in synthetic wastewater supplemented with 10 g l^{-1} acetate and containing 400 mg l^{-1} of ammonia, while *S. platensis* was completely inhibited under these conditions (Ogbonna et al., 2000). The nutritional status of the cells affects ammonium uptake. Nitrogen-limited *C. sorokiniana*, without organic carbon in the medium, exhibited respiratory oxygen consumption (70%) and photosynthetic oxygen evolution (17%), of cells with sufficient nitrogen. Cells with sufficient nitrogen absorbed NH_4^+ in light at a linear rate, but absorption was totally inhibited by darkness. In contrast, cells with limited nitrogen absorbed NH_4^+ at almost similar rates in light and darkness (Di Martino Rigano et al., 1998). *C. kessleri* successfully removes high concentrations of ammonium or nitrate from synthetic wastewater that is supplemented with glucose (Lee and Lee, 2002).

A preference for ammonium has clearly been demonstrated for *Chlorella* spp. and *Dunalliela* spp., which can use a large variety of organic and inorganic nitrogen compounds, mainly ammonium and nitrate salts, and sometimes urea (Morris, 1974; Kaplan et al., 1986). For example, *C. sorokiniana* has as much as seven ammonium-inducible chloroplastic GDH isozymes composed of varying ratios of α- and β-subunits (Miller et al., 1998), indicating a wide spectrum of adaptation to different environmental conditions. When ammonium and nitrate are supplied together, *Chlorella* spp. preferentially uses ammonium first, which is incorporated into the organic compounds produced by the microalgae. *C. protothecoides*, *N. laevis*, and *P. tricornutum* exhibit a preference for nitrate or urea over ammonium. This happens when the pH is lowered by consumption of ammonium that induced severe reduction of growth and biomass yields when pH was not controlled (Yongmanitchai and Ward, 1991; Shi et al., 2000; Wen and Chen, 2001a,b; Lee and Lee, 2002).

However, when the pH of the culture and other growth conditions are controlled, ammonium is a reliable nitrogen source (de-Bashan et al., 2005). For example, *P. tricornutum* grew well after adjusting the initial pH to 8 and a fed-batch configuration was used (Cerón García et al., 2000). Another option to control pH and use ammonium as a nitrogen sources is to add a buffer. Using the same species, adding Tris buffer to the culture medium avoided a severe drop in pH; yet, some inhibition of growth occurred even when the buffer alleviated the side effects of ammonium consumption or pH drop (Yongmanitchai and Ward, 1991). However, the acidophilic microalga *G. sulphuraria* could be efficiently cultured with ammonium at the expected lower pH because of its natural capacity to grow under these conditions (Schmidt et al., 2005). A practical approach to solve the pH problem of avoiding the adverse effects of ammonium consumption of microalgal cultures is the use of a pH-auxostat feed-batch system (Ganuza et al., 2008).

In conclusion, regardless of the negative effects on microalgal growth in ammonium-supplemented media, it is still the preferred nitrogen source if the environmental parameters for proper development of the culture are controlled.

2.3.2. *Assimilation of nitrate and nitrite*

Nitrate is a major source of nitrogen with a strong impact on metabolism and growth of plants in general. To assimilate nitrate, plant cells transport it across the membrane and then reduce it to ammonia, in the process, consuming large amounts of energy, carbon, and protons (Crawford et al., 2000; Fig. 1). Contrary to the drop in pH observed with ammonium, nitrate consumption causes an increase in pH.

Studies of higher plants and microalgae suggest that only two enzymes, nitrate reductase (NR; EC 1.6.6.1-3) and nitrite reductase (NiR; EC 1.7.7.1), work sequentially to catalyze nitrate to ammonium (Kaplan et al., 1986; Fernandez and Galvan, 2007). Assimilatory NR catalyzes the reduction of nitrate to nitrite, using reduced pyridine nucleotides as physiological electron donors (Gewitz et al., 1981; Nakamura and Ikawa, 1993). NiR catalyzes the resulting nitrite; reduction from nitrite to ammonium uses ferredoxin as the electron donor in a reaction that involves the transfer of six electrons (Lopez-Ruiz et al., 1991; Fig. 1). NiR is a chloroplastic enzyme, while NR is located specifically in the cytoplasm and in pyrenoids of green algae (Fernandez and Galvan, 2007; Inokuchi et al., 2002).

Environmental variables affect nitrate assimilation. Darkness may have a negative effect on assimilating nitrates. Most algae assimilate nitrate more rapidly in the light than in the dark. A direct photochemical reduction of nitrate and nitrite has been observed in chloroplasts. Light reduces cofactors such as flavoproteins, ferredoxins, and pyridine nucleotides, which then become used as electron donors for nitrate and nitrite reduction (Morris, 1974). Heavy metals affect nitrate assimilation in *C. reinhardtii*. Consumption of nitrate was not inhibited by metal concentrations below 100 μM. However, concentrations exceeding 150 μM of Cd^{2+}, Cu^{2+}, or Zn^{2+} induced inhibition of 75%, whereas Fe^{2+} or Co^{2+} did not significantly affect uptake of nitrate. Among the enzymes of nitrogen assimilatory pathways, exposure of cells for two days to 100 μM Cd^{2+} did not affect ferredoxin-nitrite reductase (EC 1.7.7.1), ferredoxin-glutamate synthase (EC 1.4.7.1), or NADH-glutamate synthase (EC 1.4.1.14) activities, but inhibition of glutamine synthetase activity (EC 6.3.1.2) of 45% occurred (Devriese et al., 2001).

In most microalgae, nitrate reductase is fully expressed in cells growing where the sole nitrogen source is nitrate and it is repressed in cells growing in media containing excess ammonium or a mixture of ammonium and nitrate (Gewitz

et al., 1981; Di Martino Rigano et al., 1982; Sherman and Funkhouser, 1989; Cannons and Pendleton, 1994). This further explains the preference of microalgae species like *Chlorella* for ammonium and supports the theory that environmental factors must be controlled for proper use of ammonium by microalgae, as explained above.

2.3.3. *Assimilation of urea and organic nitrogen*

Consumption of organic nitrogen by microalgae occurs under autotrophic and heterotrophic conditions. All of the organic nitrogen substrates capable of supporting growth under light conditions are also able to do so in the dark. Growth yields with organic nitrogen compounds were generally comparable to those obtained with nitrate or ammonia, although the growth rates varied greatly, depending on the organic nitrogen source, the carbon source, and the strain (Neilson and Larsson, 1980). Growth under heterotrophic conditions with glucose and acetate has been conducted in three microalgae. *Selenastrum capricornutum* was grown on glucose and urea, glycine, alanine, arginine, asparagines, and glutamine as the organic nitrogen substrate. *Chlorella* sp. used urea, glycine, glutamate, glutamine, asparagine, ornithine, arginine, and putrescine. *E. gracilis* grew on acetate, used glycine, alanine, and glutamine. Urea and glutamine are the most widespread organic nitrogen sources that support growth in algae (Morris, 1974; Neilson and Larsson, 1980).

Some *Chlorella* spp. can also use urea as the sole source of nitrogen. It is usually hydrolyzed into ammonia and bicarbonate before its nitrogen is incorporated into cells. In microalgae, two enzymes can metabolize urea, urease (EC: 3.5.1.5) and urea amidolyase (also called urea carboxylase, UALse, EC: 6.3.4.6), but most *Chlorella* spp. apparently lack urease (Kaplan et al., 1986; Oh-Hama and Miyachi, 1992) and metabolize urea by UALse.

The catabolic pathway by UALse is followed by allophanate lyase (EC: 3.5.1.54) that catalyzes hydrolysis of allophanate, resulting in hydrolysis of urea to ammonia and bicarbonate (Morris, 1974). Regardless of the activity, using urea is far less important in the growth cycle of *C. vulgaris* than ammonium and nitrate.

From the data we have so far on nitrogen metabolism under heterotrophic conditions, it is clear that the order of using a nitrogen source by most microalgal species is, in declining order: ammonium > nitrate > nitrite > urea, where special care with the concentration of ammonium is a major consideration.

3. 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. Many initiatives have been investigated to produce future products from microalgae, mostly at an experimental stage (Table 2). Ecological uses of microalgae are not discussed in this review.

3.1. *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. By manipulating fatty acid content, microalgae represent a significant source of unusual and valuable lipids and fatty acids for numerous industrial applications (Behrens and Kyle, 1996). 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, and the specific microalgal strain (Ratledge and Wynn, 2002; Wen and Chen, 2003; Chisti, 2007). 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 (Xu et al., 2006).

In general, accumulation of lipids in yeast and filamentous fungi is associated with exhausting a key nutrient for the microorganisms, usually nitrogen. After the nutrient becomes limited or exhausted, carbon uptake continues and is accumulated as lipids. It might be the same for microalgae (Zhekisheva et al., 2002; Merzlyak et al., 2007).

Several proposals to explain the mechanism of accumulating lipids were suggested. In the marine *Crypthecondium conhii* and freshwater *C. sorokiniana* accumulation of lipids may not be dependent on nitrogen exhaustion but on an excess of carbon in the culture media. Hence, in autotrophic or 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 (Chen and Johns, 1991; Ratledge and Wynn, 2002; de Swaaf et al., 2003). 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 (Leman, 1997). 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 (Ratledge and Wynn, 2002; Ganuza et al., 2008).

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) (Garcia-Ferris et al., 1996). This proposal is supported by the fact that development of chloroplasts is dependent on nitrogen. Chloroplast breakdown for the internal supply of nitrogen for the cell under nutrient reduction under dark conditions leads to cell survival and growth in the face of prolonged nutrient shortage if an external carbon source is not supplied.

But limited nitrogen is not always linked to lipid accumulation. Under nitrogen starvation, the diatoms *Achnanthes brevipes* and *Tetraselmis* spp. accumulate carbohydrates (Guerrini et al., 2000; Gladue and Maxey, 1994). This mechanism supports protein synthesis until the nitrogen supply in the medium is restored (Guerrini et al., 2000; Granum et al., 2002;

Table 2 – Potential metabolic products obtainable by heterotrophic cultivation of microalgae.

Product	Microalgae species	Significant technical details	Representative references sample
Lipids in general	*Chlorella vulgaris*, *C. saccharophila*, *C. protothecoides*, *C. sorokiniana*, *C. pyrenoidosa*, *Crypthecodinium cohnii*, *Cylindrotheca fusiformis*, *Euglena gracilis*, *Navicula incerta*, *Nitzschia alba*, *N. laevis*, *Schizochytrium* sp., *Skeletonema costatum*, *Tetraselmis suecica*	Up to 4 times higher quantity than under autotrophy; Accumulation probably by similar mechanisms as in autotrophy; Associated with exhausting of a key nutrient for the microalgae, usually nitrogen or silicate (in diatoms); Sugars play a determinant role on the type of lipids accumulated into the cells	Day et al. (1991); Chen and Johns (1991); Tan and Johns (1991, 1996); Gladue and Maxey (1994); Garcia-Ferris et al. (1996); Jiang et al. (1999); Wen and Chen (2000); Ratledge and Wynn (2002); de Swaaf et al. (2003); Wilhelm et al. (2006); Xu et al. (2006); Ganuza et al. (2008)
Polyunsaturated fatty acids	*Crypthecodinium cohnii*, *Nitzschia laevis*, *N. alba*, *Pavlova lutheri*, *Schizochytrium limacinum*, *Tetraselmis suecica*	Production high in diatoms; Production control by lowering temperature	Day et al. (1991); Gladue and Maxey (1994); Tatsuzawa and Takizawa (1995); Wen and Chen (2000, 2001a,b); Zhu et al. (2007)
Biodiesel	*Chlorella protothecoides*	Very limiting published data; Comparable to oil-based diesel;	Wen and Chen (2003); Xu et al. (2006); Chisti (2007, 2008); Xiong et al. (2008)
Pigments – phycocianin	*Galdieria sulphuraria*, *Spirulina platensis*	Auxiliary pigment to chlorophyll, improve the use of light energy. Can be produced in carbon-limited but nitrogen-sufficient heterotrophic cultures	Schmidt et al. (2005); Sloth et al. (2006)
Carotenoids – Xanthophylls	*Chlorella pyrenoidosa*, *Chlorella protothecoides*, *Chlorella zofingiensis*, *Haematococcus pluvialis*, *Dunaliella* sp	Pigments that protect chlorophyll against photo damage. Lutein can be produced heterotrophically, with glucose as a C source, and urea as N source. Astaxanthin heterotrophic production is associated to nitrogen starvation at very high C/N ratios	Theriault (1965); Tripathi et al. (1999); Ip and Chen (2005a,b); Wang and Peng (2008); Shi et al. (1997, 1999, 2000)

Wilhelm et al., 2006). Accumulation of lipids in diatoms is related to depletion of silicates because of their dependence on silica for growth (Roessler, 1988; Wen and Chen, 2000, 2003; Wilhelm et al., 2006).

In any of the above cases, the energy storage molecules, lipids, or carbohydrates, are accumulated. 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. In general, this behavior is most probably a survival response until restoration of less nutritionally stressing conditions. As an example in open environments, accumulation of lipids is favored when light is the source of energy. It has been demonstrated that storage products are depleted for energy supply according to their energy content, from lipids to carbohydrates to proteins (Wilhelm et al., 2006). In conclusion, despite the different mechanisms proposed for energy storage compounds, depletion of nitrogen or silicate favors lipid accumulation. Thus, the C:(N or Si) ratio becomes a determining factor in accumulation of lipids and lipid profiles.

After nitrogen exhaustion, the remaining sugars play a determining role on the type of lipids accumulated into the cells. Saturation of the fatty acids is directly dependent on the amount of excess sugar and on the autotrophic or heterotrophic conditions (Tan and Johns, 1991; Wood et al., 1999; Wen and Chen, 2000). As the concentration of sugar increases, the fatty acid becomes more saturated (Wood et al., 1999). For example, *C. saccharophila*, *C. vulgaris*, *N. laevis*, *Cylindrotheca fusiformis*, *Navicula incerta*, and *Tetraselmis suecica* accumulate more lipids under heterotrophic than under autotrophic conditions, mainly in the form of triglycerides that provide more energy from oxidation than polyunsaturated fatty acids and therefore, provide superior energy storage (Day et al., 1991; Tan and Johns, 1991, 1996; Gladue and Maxey, 1994). Conversely, autotrophic cultures form more highly unsaturated fatty acids (polar lipids) (Tan and Johns, 1991, 1996; Wen and Chen, 2000) (Fig. 1). In cultures of *N. laevis*, a cause for variations in accumulating lipids is the source of nitrogen, with ammonia slightly favoring saturated and monounsaturated fatty acids (C14:0, C16:0, C16:1) and nitrate and urea promoting polyunsaturated fatty acids (C20:4 and C20:5) (Wen and Chen, 2001b). Despite varied lipid profiles of specific strains, microalgae mainly accumulate the following fatty acids: (C): 14:0, 14:1, 16:0, 16:1, 18:0, 18:1, 18:2, 18:3, 20:4, 20:5, 22:5, 22:6 (Vazhappilly and Chen, 1998; de-Bashan et al., 2002); therefore, the microalgae are industrially important for medicines and nutritional supplements for humans and animals (polyunsaturated fatty acids), pigments, and lately, biofuels, mainly biodiesel (Wen and Chen, 2003; Kulkarni and Dalai, 2006; Chisti, 2007; Del Campo et al., 2007).

3.2. *Polyunsaturated fatty acids*

Long-chain polyunsaturated fatty acids (eicosapentaenoic acid, EPA, ω-3, C20:5 and docosahexaenoic acid, DHA, ω-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. Because the common source for EPA and DHA, fish oil, fails to meet the increasing demand for purified EPA and DHA, alternative sources such as microalgae that contain large quantities of high-quality EPA and DHA are considered a potential source of these economically-important fatty acids, especially under heterotrophic conditions that reduced the costs of production (Barclay et al., 1994; Vazhappilly and Chen, 1998; Apt and Behrens, 1999; Wen and Chen, 2003; Sijtsma and de Swaaf, 2004; Sijtsma et al., 2005; Chi et al., 2007).

Under autotrophic conditions long-chain fatty acids are assembled from a successive coupling of carbon–carbon bounds from acetate and malonyl-ACP (acyl-carrier protein), beginning with acetyl-CoA as the initial substrate and ending with acyl-ACP. Acetyl-CoA is produced from pyruvate generated during glycolysis or from free acetate taken into plastids, probably activated by acetyl-CoA synthetase in the stroma. For a typical C18 fatty acid, 16 molecules of NAD(P)H are required. In the dark, the pentose phosphate pathway is the producer of the reduced NADPH (Somerville et al., 2000).

As mentioned earlier, under heterotrophic conditions, the production of saturated fatty acids is favored, while highly polyunsaturated fatty acids (C16:3 and C18:3) content are mainly produced under autotrophic conditions. However, the production of polyunsaturated fatty acids, EPA and DHA, is higher in dark cultures of the diatoms *Tetraselmis* spp., *N. laevis*, and *N. alba* (Day et al., 1991; Gladue and Maxey, 1994; Wen and Chen, 2000, 2003; Chen et al., 2007). It was further shown that microalgae-based heterotrophic production systems can exhibit ω-3 fatty acid productivities that are two to three orders of magnitude greater than those of outdoor autotrophic pond systems. Additionally, long-chain ω-3 fatty acid productivities reported for the microalgae fermentation systems are one to two orders of magnitude greater than productivities reported for fungal or bacterial systems (Barclay et al., 1994).

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 (Wen and Chen, 2001a). Temperature also influences the fatty acids profile (Fig. 1). 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. However, no change in total fatty acid production occurs (Tatsuzawa and Takizawa, 1995; Quoc and Dubacq, 1997; Sakamoto and Bryant, 1997; Zhu et al., 2007). It was suggested that the lipid 1-oleoyl-2-palmitoylmonogalactosyl-sn-glycerol is involved in the regulation of membrane fluidity during temperature acclimation of the cyanobacteria *Anabaena variabilis*. This compound increases with increasing temperature and decreases with declining temperature (Sato and Murata, 1986). Another plausible explanation for instaurations is the positive effect of low temperature on increasing the molecular oxygen level in cells and promoting the activity of desaturases and elongases for fatty acids biosynthesis (Richmond, 1986; Wen and Chen, 2003; Chen and Chen, 2006).

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 (Jiang and Chen, 2000; de Swaaf et al., 2003). This difference may be related to the biochemistry and subcellular location of acetyl-CoA metabolism. It is likely that, similar to yeasts, the mitochondrial pyruvate–dehydrogenase complex is the main source of acetyl-CoA during growth with glucose. The fatty acid synthetase complex of *C. cohnii* was shown to be cytosolic (Sonnenborn and Kunau, 1982), which suggests that, similar to yeasts (Ratledge and Evans, 1989), lipid synthesis in this microalga occurs in the cytosol. This implies that, during growth with glucose, export of acetyl-CoA from the mitochondrial matrix to the cytosol is required to make it available for lipid synthesis. In contrast, acetate can be directly activated to acetyl-CoA by the action of acetyl-CoA synthetase (de Swaaf et al., 2003).

In summary, studies show that heterotrophic production of EPA and DHA is feasible at larger production scales than in autotrophic regimes, but this can be accomplished with a few microalgae.

3.3. 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. Currently, biodiesel production depends mainly on vegetable oils, such as canola, soybean, sunflower, and palm containing long-chain fatty acids (LCFA) and to a small extent on animal fat and oil recycling. This implies seasonal availability and large expansion of farmland at the expense of food crops. This is a major limitation and sufficient reason to search for other sources of LCFA. The current type of production of biodiesel is not sustainable because of the inherent conflict with food supply and threat to food security.

Biodiesel from microalgae is an attractive, feasible alternative mainly because some microalgae species can significantly increase production of lipids and cultivation of microalgae is now possible through cheaper heterotrophic cultivation. Strains can be genetically engineered to produce the desired fatty acids without negative effects on the environments. Microalgae, potentially in the longer term, offer the greatest opportunities compared to oilseed crops. Productivity of many microalgae exceeds the best producing oil crops where oil content of many microalgae strains under heterotrophic conditions is usually 80% of its dry weight and, their production and processing into biofuels, is economically effective, uses currently available technology, and is environmentally sustainable because their production is not seasonal and the product can be harvested daily. Current mass production of microalgae requires significantly less land area than crop-based biofuels and releases fewer pollutants to the environment. Because biofuels from microalgae was recently reviewed so extensively from every angle (Chisti, 2007, 2008; Li et al., 2008a,b; Sharif Hossain et al., 2008; Song et al., 2008; Khan et al., 2009; Huang et al., 2010; Mata et al., 2010; Sivakumar et al., 2010), this review will briefly present only a few examples, mostly from China with heterotrophic cultivation. While heterotrophic microalgae cultivation represents a good source of LCFA (Wen and Chen, 2003); 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. The biodiesel was comparable to oil-based diesel and complies with the US Standard for Biodiesel (Li et al., 2007; Miao and Wu, 2006; Xu et al., 2006; Xiong et al., 2008). Using *C. protothecoides* biodiesel was produced from hydrolysate of the Jerusalem artichoke tuber under heterotrophic conditions, with significant cost reduction. Accumulated lipid *in vivo*, with lipid content as high as 44% of dry mass was obtained and converted to biodiesel. Unsaturated fatty acid methyl esters constituted >82% of the total biodiesel content, of which the chief components were cetane acid methyl, linoleic acid methyl, and oleic acid methyl esters (Cheng et al., 2009).

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 (Yazdani and Gonzalez, 2007; Murarka et al., 2008). For example: *Schizochytrium limacinum* produced palmitic acid (16:0) as ~45–60% of their dry weight when supplied with glucose, fructose, or glycerol (Yokochi et al., 1998; Chi et al., 2007), which could potentially be used for biodiesel production.

In summary, production of biodiesel by heterotrophic microalgae is a very new field of research, with little solid information available, apart from commercial promises, to indicate the true commercial potential of this source. Considering metabolism in microalgae, cheap carbon sources yielding promising amounts of long-chain fatty acids make this an attractive venue for future research.

3.4. Pigments

In addition to the main photosynthesis pigment chlorophyll, microalgae contain auxiliary photosynthetic pigments to improve use of light energy (phycobiliproteins) and protection against solar radiation (carotenoids) (Cohen, 1986; Pulz and Gross, 2004; Del Campo et al., 2007). Naturally, all pigments are produced under autotrophic growth conditions, but surprisingly some are produced, and in large quantities, under heterotrophic dark conditions.

3.4.1. 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 (Cohen, 1986). The green algae class *Chlorophyceae*, contain α and β-carotenes and the xanthophylls: lutein, zeaxanthin, violaxanthin, and neoxanthin (Theriault, 1965; Cohen, 1986). For example, autographically grown *Dunaliella* sp. is the richest source of lutein. It contains up to 14% dry weight and is used as a food supplement. Natural colorants have become increasingly important because of regulations limiting synthetic additives; with microalgae as a source, carotenoids are one of the major fields of exploitation of microalgal biotechnology (Theriault, 1965; Cohen, 1986; Pulz and Gross, 2004; Borowitzka, 2005; Lebeau and Robert, 2006; Del Campo et al., 2007).

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 (Shi et al., 1997; Pulz and Gross, 2004). Lutein is an intracellular product of *Chlorella*. This genus is used for production of lutein, mainly *C. protothecoides* and, to a lesser extent, *C. pyrenoidosa* and *C. vulgaris*. 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 (Theriault, 1965; Shi et al., 1997, 1999, 2000).

Astaxanthin is a red ketocarotenoid colorant used in the cosmetic, therapeutic, and food industries. In aquaculture, it has been used to increase growth and survival of aquatic animals and as a colorant of tissues (farmed salmon, shrimp, lobster, trout, and fish eggs) to provide a pinkish-red color to the tissue that is appealing to consumers. Its strong antioxidant character makes it a nutraceutical product (food or nutritional supplement that may improve health) and may prevent some cancers (Hagen et al., 2001; Ma and Chen, 2001; Pulz and Gross, 2004; Del Rio et al., 2005; Ip and Chen, 2005a; Wang and Peng, 2008). 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 (Wang and Peng, 2008). *Haematococcus pluvialis* is the main producer of astaxanthin under autotrophic conditions but *C. zofingiensis* is superior in yield when heterotrophically cultivated with glucose (Ip and Chen, 2005a). Biosynthesis of astaxanthin in *C. zofingiensis* starts in the early exponential phase and is a growth-associated metabolite (product that is produced only during active growth); therefore, it depends on assimilation of the carbon source. Its production was associated, similar to lipid accumulation, to nitrogen starvation at very high C/N ratios (Ip and Chen, 2005a; Wang and Peng, 2008) and mainly to the presence of oxidative stress that is essential for promoting the formation of several secondary carotenoids, including astaxanthin. Such oxidative treatments employ the hydroxyl radical (OH supplied by H_2O_2) (Ip and Chen, 2005b). Addition of reactive nitrogen species, such as peroxynitrite and nitryl chloride induced similar effects (Ip and Chen, 2005c).

The blue photosynthetic pigment, phycocyanin, is found in a few cyanobacteria and microalgae; its main source is the cyanobacterium *S. platensis*. It is used as a fluorescent marker in diagnostic histochemistry and as dye in food and cosmetics. 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 (Sloth et al., 2006). 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* (Schmidt et al., 2005). If the heterotrophic process is scaled up, the reduction of cost using *G. sulphuraria* would be significant.

In summary, although pigments are traditionally thought to be the outcome of metabolisms associated with exposure to light, the capacity of some microalgae to produce some of them in the dark under specific growth conditions opens a line of research that is barely explored.

3.5. *Wastewater 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 (de-Bashan and Bashan, 2010). 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 (de-Bashan et al., 2002, 2004; Hernandez et al., 2006) to heterotrophic conditions revealed even higher potential of the system to eliminate nutrients (Perez-Garcia et al., 2010). The new data cannot provide a solid prediction about the practical potential of this approach.

4. Concluding remarks 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 (Tuchman, 1996; Hellebust and Lewin, 1977). Furthermore, with current developments in genomics, bioinformatics analyses, and genetic and metabolic engineering, new approaches in microalgae biotechnology, including heterotrophy, have emerged (Hong and Lee, 2007; Boyle and Morgan, 2009).

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/H+ symporter gene derived from *Chlorella* sp. (Hallmann and Sumper, 1996). Similar trophic conversions have also been carried out in *C. reinhardtii* (Doebbe et al., 2007) and the diatom *P. tricornutum* (Zaslavskaia et al., 2001). 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; thus, these mutants can be employed in metabolic production of products, such as hydrogen by *C. reinhardtii* (Doebbe et al., 2007).

Adoption of heterotrophy for large-scale industrial processes, such as wastewater treatment and biofuels production, is somewhat more problematic and lies in the more distant future. Because microalgae cultivation alone cannot sustain biofuel production with the current cultivation technologies, perhaps combining two processes, wastewater treatment followed by biofuel production from the residual mass will yield a product that would make development of these technologies economically acceptable.

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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Appendix. Supplementary material

Supplementary data related to this article can be found online at doi:10.1016/j.watres.2010.08.037.

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