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Review

Growing phototrophic cells without light

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Abstract

Many phototrophic microorganisms contain large quantities of high-value products such as *n*-3 polyunsaturated fatty acids and carotenoids but phototrophic growth is often slow due to light limitation. Some phototrophic microorganisms can also grow on cheap organic substrate heterotrophically. Heterotrophic cultivation can be well controlled and provides the possibility to achieve fast growth and high yield of valuable products on a large scale. Several strategies have been investigated for cultivation of phototrophic microorganisms without light. These include trophic conversion of obligate photoautotrophic microorganisms by genetic engineering, development of efficient cultivation systems and optimization of culture conditions. This paper reviews recent advances in heterotrophic cultivation of phototrophic cells with an emphasis on microalgae.

Introduction

Photosynthesis is the process by which many types of bacteria, algae and vascular higher plants convert light energy into chemical energy of organic molecules (Lawlor 2001). In recent decades, some photosynthetic microorganisms, especially some prokaryotic and eukaryotic microalgae, have been identified as efficient biological systems for producing a great variety of high-value chemicals and pharmaceuticals such as phycobiliproteins, astaxanthin and polyunsaturated fatty acids (PUFAs) (Chen 1996). These findings lead to considerable interest in developing commercial production processes by microalgae. Most of the processes investigated to date, however, have been based on photoautotrophic growth (Olaizola 2003). Nevertheless, photoautotrophic growth is often limited by light deficiency due to mutual shading of cells. As a result, the productivity of a photoautotrophic system is low (Wen & Chen 2003). The requirements for more efficient, economical and controllable production systems make the development of heterotrophic growth process deeply desirable (Wen & Chen 2003). The aim of the present paper is to review the recent advances in heterotrophic cultivation of phototrophic cells for biomass and valuable products, in particular of microalgae.

Causes of obligate phototrophy

Photosynthesis is the process by which plants, cyanobacteria and some protistans use CO_2 and sunlight energy to produce organic materials, which is then converted by cellular respiration into ATP, the 'fuel' used by all living things. The first question in relation to heterotrophic cultivation of phototrophic microorganisms might be why many of these microorganisms could only grow photoautotrophically while some others could also grow well in the dark. A great deal of

evidence has showed that the 'dark metabolism' of photosynthetic plants and microalgae is essentially similar to that of non-photosynthetic organisms such as yeasts. Therefore, from a biochemical viewpoint, it would be expected that almost any substrate or intermediate in the major pathways of energy metabolism might substitute for the carbohydrate produced by photosynthesis (Lewin 1962). However, such an expectation is fulfilled only partially, and to varying degrees, in various species of microalgae. Obligate photoautotrophy might be one of the most puzzling characteristics found among microalgae and appears to be widespread among the diatoms and dinoflagellates (Lewin 1962).

Although no satisfactory explanation for obligate photoautotrophy among microalgae has been provided since its discovery (Benedict 1978, Wood *et al.* 2004), two interesting principal hypotheses are proposed based on studies on different phototrophic microorganisms. In some microalgae, a lack of efficient uptake of essential substrates, especially sugars, into the cells might be the major reason for their obligate phototrophy (Lewin 1962, Zhang *et al.* 1998). A deficiency of glucose-concentrating capacity was demonstrated in *Nostoc* sp. PCC7118, making the organism only able to grow photoautotrophically (Beauclerk & Smith 1978). In contrast, the presence of an active glucose uptake system was described for *Nostoc* MAC (PCC 8009) and *Plectonema boryanum*, both of them capable of phototrophic and heterotrophic growth (Beauclerk & Smith 1978, Raboy & Padan 1978). More convincing evidence was reported that an obligate photoautotrophic microalga *Phaeodactylum tricornutum* could grow heterotrophically when a single gene (*Glut1*) that encoded the glucose transporter protein (Glut1) was introduced into this alga (Zaslavskaia *et al.* 2001).

However, impermeability of essential nutrients should not be the unitary factor in obligate photoautotrophy of all organisms, because some phototrophs do incorporate organic compounds into cell carbon (Zhang *et al.* 1998). It was hypothesized that a biosynthetic or degradative lesion in central metabolism would seem probable being a primary cause of obligate photoautotrophy (Wood *et al.* 2004). For example, an incomplete tricarboxylic acid (TCA) cycle might be a vital reason in some phototrophic microorganisms. The TCA cycle not only provides ATP as energy but also generates precursors such as α-ketoglutaric acid for biosynthesis. Some obligate photoautotrophic microalgae were shown to possess an incomplete TCA cycle due to the absence of α-ketoglutarate dehydrogenase (Benedict 1978). The unavailability of this key enzyme was also observed in various thiobacilli and some methanotrophs. In such a case, the TCA cycle is used mainly to channel carbon into central biosynthetic pathways for biosynthetic purposes (Figure 1) but cannot provide ATP (Wood *et al.* 2004).

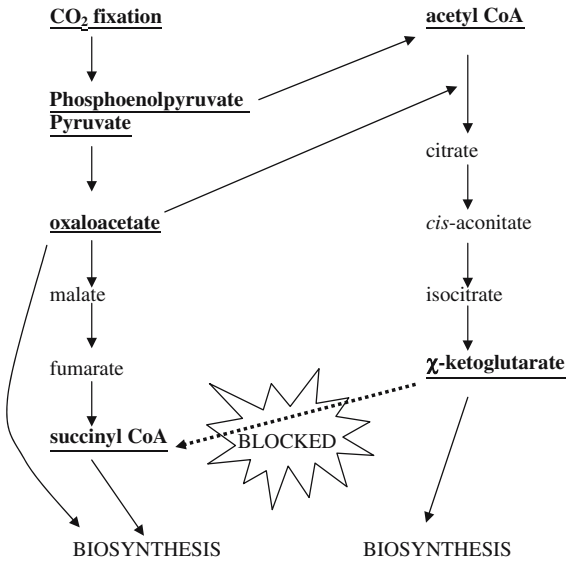

Fig. 1. Simplified schematic to illustrate the incomplete TCA cycle in obligate photoautotrophs (Wood *et al.* 2004).

Obligate phototrophy possibly has different or multiple reasons in different organisms. This topic requires further research by combining knowledge and techniques of biochemistry, molecular biology and bioinformatics (Wood *et al.* 2004).

Trophic conversion of obligate photoautotrophic microalgae

Although the universal reason for obligate phototrophy of microalgae is still unknown, an attempt to convert photoautotrophic microalgae into facultative heterotrophs still continues due to rapidly developing metabolic engineering techniques, the

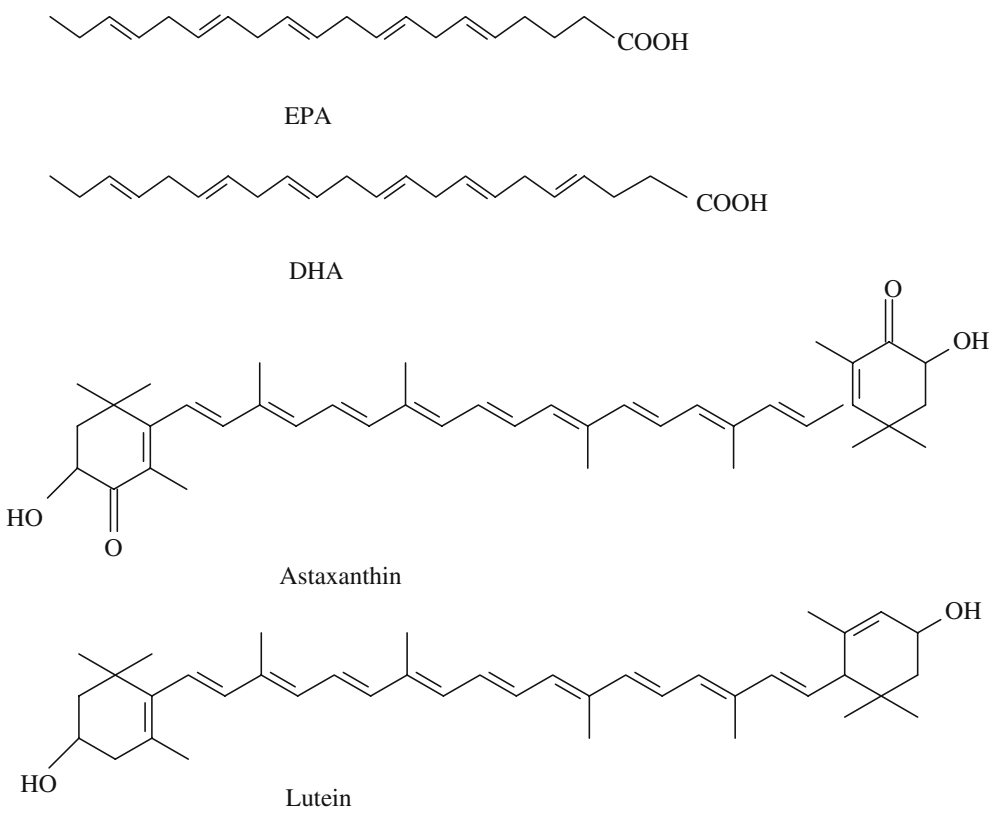

Fig. 2. Structures of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), astaxanthin and lutein.

potential application of microalgal biomass and their valuable products, the numerous disadvantages of photoautotrophic culture and the high efficiency and low cost of heterotrophic growth of microalgae in conventional microbial fermentors (Hallmann & Sumper 1996, Apt & Behrens 1999, Borowitzka 1999, Fischer *et al*. 1999). Zaslavskaia *et al*. (2001) reported the trophic conversion of an obligate photoautotrophic diatom *Phaeodactylum tricornutum* into a facultative heterotroph by genetic engineering. In the study, the coding region of a single gene (*Glut1*) that encodes the glucose transporter protein (Glut1) from human erythrocytes was inserted into the *P. tricornutum* transformation vector pPha-T1. The plasmid was then introduced into the alga and the Glut1 protein was expressed successfully in the transformants. The subcellular location of the Glut1 protein in transformant was identified via the GFP-tagging technique (Zaslavskaia *et al*. 2001). The heterologous protein was correctly targeted and inserted into diatom membranes, the exclusive location for the functionality of the glucose transporter. As a result, the engineered microalga exhibited a high rate of glucose uptake and was able to grow at normal rates and to higher cell densities in the dark than those obtained by mere photoautotrophic growth (Zaslavskaia *et al*. 2001). This was an important step toward establishing genetic engineered diatoms as useful organisms for industrial fermentation technology. The problem of light deficiency existing in large scale cultivation of obligate photoautotrophic organisms might be solved by genetic engineering, which in the future will allow their full potential for biotechnological applications to be harnessed (Apt & Behrens 1999).

Heterotrophic microalgae and their high-value products

Although most microalgae grow photoautotrophically, some of them are able to grow heterotrophically

using organic substrates as sole carbon and energy sources (Chen 1996, Lee 2001). Heterotrophic culture has several advantages including elimination of light limitation, high degree of process control, and low costs for harvesting biomass because of higher cell densities achieved (Barclay *et al*. 1994). A microalga suitable for heterotrophic culture should have four essential characteristics:

- the ability to divide and metabolize without light;
- the ability to grow on inexpensive and easily sterilized media;
- the ability to adapt rapidly to the new environment and
- the ability to withstand hydrodynamic stresses in fermentors and peripheral equipment (Chen 1996, Wen & Chen 2003).

Some efforts have been made to culture microalgae without light in order to produce biomass, which is used as nutritional supplements or as aquacultural feed additives (Borowitzka 1997, Harel *et al*. 2002). In Japan, 50% of *Chlorella* biomass produced commercially is now grown heterotrophically in fermentors (Lee 1997). Moreover, some attempts have also been made to culture microalgae heterotrophically for nutritional and pharmaceutical products such as PUFAs and carotenoids (Ip & Chen 2005a, Wen & Chen 2003). Production of these two high-value bioactive compounds in heterotrophic culture on a large scale is very promising (Ip & Chen 2005a, Wen & Chen 2003). In addition, heterotrophic high cell density fed-batch culture of the red alga *Galdieria sulphuraria* was recently investigated for phycocyanin production (Schmidt *et al*. 2005).

Polyunsaturated fatty acids

In recent years, the therapeutic significance of *n*-3 PUFAs has been clearly indicated by clinical and epidemiological studies (Kris-Etherton *et al*. 2004, Ruxton *et al*. 2005). The most important compounds of *n*−3 PUFAs are eicosapentaenoic acid (EPA; 20:5 *n*−3) and docosahexaenoic acid (DHA; 22:6 *n*−3). Their chemical structures are shown in Figure 2. Health benefits of PUFAs have been observed in several areas including prevention of cardiovascular and inflammatory diseases, function in brain development and application in keeping mental health (Ruxton *et al*. 2004).

Several heterotrophic microalgae for EPA and DHA production are listed in Table 1. Wen & Chen (2002a) reported a cell density of 40 g l^{-1} and a high EPA yield of 1112 mg l^{-1} with *Nitzschia laevis* grown in a perfusion process. Barclay *et al*. (1994) reported a cell density of 30 g l^{-1} in stirred tank fermentors with another diatom, *Nitzschia alba*. The productivity and yield of EPA were 250 mg l^{-1} d^{-1} and 675 mg l^{-1}, respectively. *Schizochytrium* sp., *Ulkneia* sp. and *Crypthecodinium cohnii* are currently used commercially for the heterotrophic production of DHA, though details of the culture processes have not been published (Barclay *et al*. 1994, Jiang *et al*. 2004, Ratledge 2004, Sijtsma & de

Table 1. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) productivities of heterotrophic microorganisms.

	Strain	Device	Culture mode	Cell concentration (g l^{-1})	EPA/DHA productivity (mg l^{-1} d^{-1})	EPA/DHA yield (mg l^{-1})	Reference
EPA	*Ulkneia* sp.	Flask	Batch	4.7	6.5	14	Fan *et al*. (2001)
	Nitzschia alba	Fermentor	Fed-batch	30	250.0	675	Barclay *et al*. (1994)
	Nitzschia laevis	Flask	Batch	9	28.0	280	Wen & Chen (2001b)
	Nitzschia laevis	Fermentor	Fed-batch	22.1	49.7	695	Wen *et al*. (2002)
	Nitzschia laevis	Fermentor	Continuous		73.0		Wen (2001)
	Nitzschia laevis	Fermentor	Perfusion	40	74.1	1112	Wen & Chen (2002a)
DHA	*Crypthecodinium cohnii*	Fermentor	Batch	27.7	456.0	1400	de Swaaf *et al*. (1999)
	Crypthecodinium cohnii	Fermentor	Fed-batch	83	1276.4	11 700	de Swaaf *et al*. (2003a)
	Schizochytrium mangrovei	Flask	Batch	13.3	1282.6	2779	Fan *et al*. (2001)
	Schizochytrium sp. SR21	Fermentor	Batch	59.2	2976	15 500	Yaguchi *et al*. (1997)

Swaaf 2004, Cohen & Ratledge 2005). Fed-batch cultivation of *C. cohnii* on ethanol for DHA production was recently reported, and the dry biomass and DHA yield were 83 and 11.7 g l^{-1}, respectively (de Swaaf *et al.* 2003a).

Carotenoids

Carotenoids are a class of natural fat-soluble pigments found principally in plants, algae and photosynthetic bacteria, where they play critical roles in the photosynthetic process. Carotenoids also exist in some non-photosynthetic microorganisms, where they carry out a protective function against damage by light and oxygen. Although animals appear to be incapable of synthesizing carotenoids, many animals can incorporate carotenoids from their diets (Britton 1995).

Carotenoids may serve several important functions. The most widely studied and well-understood nutritional role of carotenoids is their provitamin A activity. Carotenoids also play an important potential role in human health by acting as biological antioxidants to protect cells from the damaging effects of reactive oxygen species. Other health benefits of carotenoids that might be related to their antioxidative potential include enhancement of immune system function, protection from sunburn, and inhibition of the development of certain cancers (Nishino *et al.* 2002, Sies & Stahl 2004, Stahl & Sies 2005). In addition, some carotenoids are widely used for the pigmentation of animal tissues and products, as well as for the coloration of foods, drugs and cosmetics (Guerin *et al.* 2003, Johnson 2004).

In recent years, research on the production of carotenoids from heterotrophic microalgae has mainly focused on the two highly valuable carotenoids: astaxanthin and lutein. Chemical structures of astaxanthin and lutein are shown in Figure 2, and the heterotrophic microalgae being studied for carotenoid production are shown in Table 2. With glucose as carbon source and peroxynitrite as biosynthesis inducer, 12.6 mg astaxanthin l^{-1} was produced in a heterotrophic culture of *Chlorella zofingiensis* (Ip & Chen 2005b). Shi *et al.* (2002) reported a high lutein yield of 225.3 mg l^{-1} with *Chlorella protothecoides* in heterotrophic fed-batch culture (Table 2).

Modes of heterotrophic culture of microalgae

Batch culture

Batch culture is a common technique to grow cells, especially at laboratory scale. Due to its simplicity and low cost, batch culture is commonly use in preliminary laboratory studies to investigate the effects of medium components and environmental factors on biomass and product yields (Yokochi *et al.* 1998, de Swaaf *et al.* 1999). Wen & Chen (2001b) reported optimization of EPA production by *N. laevis* in heterotrophic batch culture with a response surface methodology. After concentrations of NaCl, $CaCl_2$ and pH and temperature were identified as critical factors by Plackett-Burman design,

Table 2. Astaxanthin and lutein productivities of heterotrophic marine microorganisms.

	Strain	Device	Culture mode	Cell concentration (g l^{-1})	Astaxanthin/ lutein productivity (mg l^{-1} d^{-1})	Astaxanthin/ lutein yield (mg l^{-1})	Reference
Astaxanthin	*Chlorella zofingiensis*	Flask	Batch	10.2	0.7	10.3	Ip & Chen (2005a)
	Chlorella zofingiensis	Flask	Batch	7.5	1.3	11.8	Ip & Chen (2005c)
	Chlorella zofingiensis	Flask	Batch	7.3	1.4	12.6	Ip & Chen (2005b)
	Haematococcus pluvialis	Flask	Batch		0.8	9.0	Kobayashi *et al.* (1997)
Lutein	*Chlorella protothecoides*	Fermentor	Batch	19.6	11.3	83.8	Shi *et al.* (2000)
	Chlorella protothecoides	Fermentor	Fed-batch	46.9	22.7	225.3	Shi *et al.* (2002)

these variables were further optimized using a central composite design. EPA yield of 280 mg l^{-1} was obtained in shake-flask culture, which was 1.6-fold higher than that prior to optimization (Wen & Chen 2001a,b).

Fed-batch culture

A fed-batch culture is essentially a batch culture supplied with nutrient medium continuously or intermittently during cultivation. This culture system for heterotrophic microalgae possesses several advantages including high cell density and minimization of substrate inhibition (Chen 1996). Wen & Chen (2002a,b) employed fed-batch culture to grow *N. laevis* heterotrophically for EPA production. Mixture of glucose, nitrate, tryptone and yeast extract at an optimal ratio of 32:1:2.58:1.29 (w/w) was supplemented continuously to the fermentor. The final cell concentration and EPA yield obtained were 22.1 g l^{-1} and 695 mg l^{-1}, respectively, both of which were much higher than those achieved in batch cultures (Wen *et al.* 2002). Fed-batch system was also successfully employed for heterotrophic cultivation of other microalgae such as *C. protothecoides* (for lutein production), *C. cohnii* (for DHA production) and *G. sulphuraria* (for phycocyanin production) (de Swaaf *et al.* 2003a,b, Schmidt *et al.* 2005, Shi *et al.* 2002). In the fed-batch culture of *C. cohnii*, a high DHA yield of 11.7 g l^{-1} was achieved with ethanol as carbon source (de Swaaf *et al.* 2003a).

Continuous culture

Continuous culture is basically a method of prolonging the growth phase of a microorganism in batch culture, which involves feeding with fresh nutrients and at the same time removing spent medium plus cells from the system. Growth and environmental factors are kept constant. It is a good system to grow heterotrophic microorganisms at high cell densities and to study the growth and physiological behavior of microorganisms. A continuous process was developed for EPA production by *N. laevis* in the dark, and the highest EPA productivity of 73 mg l^{-1} d^{-1} was obtained at a feed glucose concentration of 20 g l^{-1} and a dilution rate of 0.5 d^{-1} (Wen & Chen 2002b). Continuous culture was also successfully employed for growing a genetically important green microalga, *Chlamydomonas reinhardtii*, using acetate as the sole carbon and energy source. Because acetate was an inhibitor to the growth of the alga at high concentrations, the highest cell concentration achieved was only 1.5 g l^{-1} at a feed acetate concentration of 3.4 g l^{-1} (Chen & Johns 1996).

Perfusion culture

Perfusion culture, denoted as continuous culture with cell-recycle, is rarely reported for heterotrophic microalgal cultivation (Wen & Chen 2003). In contrast to continuous culture where steady state is maintained by constant dilution of the culture, the cells are physically retained in the vessel in perfusion culture. Cell concentration may increase constantly until it achieves a high cell density, or toxic metabolites are accumulated to a certain concentration in the culture. This culture technique has proved to be an efficient way to enhance cell density and yield of valuable products. Perfusion culture was also successfully employed for the heterotrophic cultivation of *N. laevis* for EPA production. As the result, a high cell dry weight concentration of 40 g l^{-1} and a high EPA yield of 1112 mg l^{-1} were achieved (Wen & Chen 2002a).

Factors influencing heterotrophic cultivation of microalgae

In heterotrophic culture of microalgae, both cell growth and biosynthesis of products are significantly influenced by medium nutrients and environmental factors. The major medium nutrients include carbon source, nitrogen source and phosphorus. The major environmental factors include cultural temperature, salinity (especially for marine microalgae), pH, and dissolved oxygen level. Detailed investigation is needed to establish the most suitable medium and environmental conditions for specific species.

Medium nutrients

Carbon sources are the most important element for heterotrophic culture of microalgae because they serve as both energy and carbon skeletons for

cell growth. Glucose and acetate are two major carbon sources for microalgae growing in the dark (Ratledge *et al*. 2001, Wen & Chen 2000). Glucose was employed as single carbon source in the heterotrophic culture of *C. zofingiensis* (Ip & Chen 2005a), *Haematococcus pluvialis* (Kobayashi *et al*. 1997) and *C. protothecoides* (Shi *et al*. 1999). Both Ratledge *et al*. (2001) and de Swaaf *et al*. (2003b) reported the utilization of acetic acid as carbon source in the production of DHA with *C. cohnii* in fed-batch culture. When the feeding rate was automatically controlled via the culture pH, a feed consisting of acetic acid (50% w/w) resulted in a higher overall volumetric productivity of DHA than a feed consisting of 50% (w/v) glucose. The DHA productivity was increased to 1152 mg l^{-1} d^{-1} when a feed consisting of pure acetic acid was employed (de Swaaf *et al*. 2003b). The superiority of acetic acid to glucose for DHA production in this alga may be explained by the metabolism of acetyl-CoA, which is the basic building block of fatty acid synthesis. The conversion of glucose into acetyl-CoA needs several steps while acetate may be directly activated to acetyl-CoA by a one-step action catalyzed by acetyl-CoA synthetase in *C. cohnii* (de Swaaf *et al*. 2003b). Moreover, in this culture, the pH was maintained at a constant value by addition of acetic acid, which also provided an additional carbon source in a controlled manner. This strategy might efficiently avoid the accumulation of excessive acetic acid in medium, and thus could prevent the inhibition effects of acetate at high concentrations (Ratledge *et al*. 2001). Heterotrophic microalgae were also able to utilize other carbon sources such as ethanol, glycerol and fructose, depending on the microalgal species used (Yokochi *et al*. 1998, de Swaaf *et al*. 2003a).

Besides carbon, nitrogen is an essential macronutrient for algal growth. There are two kinds of nitrogen sources: simple nitrogen sources including nitrate and urea and complex sources including yeast extract, tryptone and corn steep liquor. Complex nitrogen source might be superior to simple nitrogen source in heterotrophic culture of microalgae, since they also provide amino acids, vitamins and growth factors (Vogel & Todaro 1997). In the culture of *C. protothecoides* for lutein production, the effects of nitrate, ammonium and urea were compared. The highest yield of both biomass and lutein were achieved when urea was used as nitrogen source (Shi *et al*. 2000). A combination of 0.62 g nitrate l^{-1}, 1.6 g tryptone l^{-1} and 0.8 g yeast l^{-1} extract was identified as the most suitable nitrogen source for *N. laevis* cell growth and EPA production (Wen & Chen 2001a).

Initial carbon to nitrogen (C/N) ratio in the medium also has significant impact on microalgae. Biosynthesis of lipids, fatty acids and carotenoids were affected by C/N ratio (Choi *et al*. 2002, Tripathi *et al*. 2002, Wen & Chen 2003). When *C. zofingiensis* was cultured in darkness, a high C/N ratio of 180 in the medium could enhance the biosynthesis of secondary carotenoids including astaxanthin (Ip & Chen 2005a).

Phosphorus is another important nutrient for algal growth. In the cultivation of microalgae, the phosphorus source is mainly supplied in the form of orthophosphate (PO_4^{3-}). Phosphate is responsible for the energy transfer of the cells and the formation of cell membranes and nucleic acids. Moreover, phosphate also plays significant roles in the syntheses of valuable products such as astaxanthin and PUFAs. A recent study indicated that high biomass (3.5 g l^{-1}) and astaxanthin (15 mg g^{-1}) were achieved when *H. pluvialis* was grown in the PO_4^{3-}-deficient medium (Brinda *et al*. 2004). In another alga, *Chlorococcum* sp., cell growth was saturated at a relatively high PO_4^{3-} level while the secondary carotenoids were preferably accumulated at a relatively low PO_4^{3-} level (Liu & Lee 2000). The effects of phosphate on PUFA biosynthesis differed in different species. For example, EPA yield of *P. tricornutum* was enhanced at a high phosphate concentration but in the culture of *Pythium irregulare*, a high initial phosphate concentration resulted in a low EPA yield (Stinson *et al*. 1991, Yongmanitchai & Ward 1991).

In addition to carbon, nitrogen and phosphorous, the requirement of special nutrients for specific species in heterotrophic cultivation should be noted. For example, silicon is necessary for diatoms to form their frustules. Lipid contents of several marine diatoms, however, increased with a reduced silicate supply. Wen & Chen (2000) reported an increase of EPA content of diatom *N. laevis* under heterotrophic culture when silicate became a limiting factor. Another example is that iron plays an important role in the formation of pigments such as carotenoids in algal cells.

Addition of 0.2 mM Fe^{2+} to the culture medium containing 0.1 mM H_2O_2 resulted in a 9% increase in the content of astaxanthin in *Chlorococcum* sp. as compared with the control culture supplemented with 0.1 mM H_2O_2 alone (Ma & Chen 2001).

Environmental factors

Temperature is a very important environmental factor affecting algal growth and the formation of temperature-dependent components such as lipids, fatty acids and secondary carotenoids. Jiang & Chen (2000a) reported a lower specific cell growth rate and higher DHA content at 15 °C than at 30 °C when *C. cohnii* was cultured heterotrophically. Low temperature also led to high PUFA content in *N. laevis* (for EPA production) and another *C. cohnii* species (de Swaaf *et al.* 1999, Wen & Chen 2001b). The relatively high PUFA content at low temperature might be explained by the fact that the algae need to produce more PUFAs to maintain proper cell membrane fluidity. Another reason might be that low temperature led to high level of intracellular molecular oxygen and hence improved the activities of the desaturases and elongases involved in the biosynthesis of PUFAs (Jiang & Chen 2000a). However, it should be noticed that the effects of temperature on cell growth and PUFA production may not be always the same as mentioned above (Wen & Chen 2003). Therefore they should be studied carefully for individual microalgal species.

Medium salinity (in terms of NaCl) may also influence the microalgal physiological properties. The requirement of salinity varies from species to species. In the heterotrophic culture of the marine diatom *N. laevis*, 8 g NaCl l^{-1} was suitable for cell growth and 14 g NaCl l^{-1} was the best for EPA production (Wen & Chen 2001b). The effects of salinity on cell growth and DHA content of three *C. cohnii* strains were investigated and the results showed that *C. cohnii* ATCC 30556 and ATCC 50051 had both the highest cell dry weight and the highest DHA yield at 9 g NaCl l^{-1} while *C. cohnii* RJH had both the highest specific growth rate and DHA content at 5 g NaCl l^{-1} (Jiang & Chen 1999).

Just as salinity, pH and dissolved O_2 are also important factors affecting the heterotrophic cultivation of microalgae. Jiang & Chen (2000b) investigated the effect of pH on cell growth and DHA production in *C. cohnii* ATCC 30556, and found that the suitable initial medium pH value was 7.2, where the highest specific growth rate, highest cell dry weight concentration, highest degree of fatty acid unsaturation and highest DHA proportion were obtained. In the heterotrophic culture of *Chlorella sorokiniana*, high aeration obviously enhanced cell growth, fatty acid yield and the synthesis of unsaturated dienoic and trienoic fatty acids, but decreased cell lipid content (Chen & Johns 1991). In the cultivation of *C. cohnii* in flask, cell growth was improved significantly by high agitation speed (de Swaaf *et al.* 1999).

Conclusions and prospects

The advantages of heterotrophic culture have led to the attempts to commercially cultivate phototrophic cells in the dark. There might be different and/or multiple reasons for obligate photoautotrophy in different organisms. Further investigation needs to be done to reveal the mechanism of obligate photoautotrophy, which will be valuable for further development of heterotrophic cultivation of phototrophic organisms. The successful trophic conversion of *P. tricornutum*, an obligate photoautotrophic microalga, through metabolic engineering marks an important step toward establishing microalgae as important organisms in the fermentation industry, though the real industrial application has not been demonstrated. The application of genetic engineering approach may be an efficient way to enable phototrophic cells to grow without light. From an application point of view, further research should also focus on screening phototrophic microorganisms for strains which have high contents of valuable products and can grow well in the dark. In addition, an in-depth investigation of the cultural strategies is necessary in order to develop a successful heterotrophic high-cell density culture of phototrophic microorganisms.

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