Green Energy and Technology

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Microalgae Cultivation Fundamentals

Yuan Kun Lee

Abstract Microalgal cultivation has attracted much attention in recent years, due to their applications in CO_2 sequestration, biofuels, food, feed and bio-molecules production. The general requirements for successful microalgal cultivation include light, carbon, macronutrients such as nitrogen, phosphorus, magnesium and silicates and several micronutrients. This chapter discusses the principles of microalgae cultivation with regards to essential requirements and growth kinetics.

Keywords Microalgae · Light · Nutrient supply · Culturing · Growth kinetics

1 Introduction

Microalgal cultivation has attracted much attention in recent years, due to their applications in CO_2 sequestration, biofuels, food, feed and bio-molecules production. Estimates of the number of algal range from 350,000 to 1,000,000 species, however only a limited number of approximately 30,000 have been studied and analysed (Richmond 2004). Microalgae are a diverse group of organisms that occur in various natural habitats. Many of the microalgae studied are photosynthetic, whilst only few of them are known to grow mixotrophically or heterotrophically (Lee 2004). The general requirements for successful microalgal cultivation include light (photosynthetic and mixotrophic), carbon, macronutrients such as nitrogen, phosphorus, magnesium and silicates and several micronutrients (species dependant) for their successful cultivation. This chapter will provide an overview of the fundamentals of microalgal cultivation.

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2 Illumination

Microalgal cultures receive light at their illuminated surface. The ratio between the illuminated surface area and volume of cultures (*s*/*v*) determine the light energy available to the cultures and the distribution of light to cells in the cultures. Generally, higher the *s*/*v* the higher the cell density and volumetric productivity could be achieved (Pirt et al. 1980). High cell density reduces the cost of harvesting, as well as cost of media. Thus high *s*/*v* photobioreactors (PBRs) are generally preferred. However it must be cautioned that high cell density may lead to shallow light-path. Thus turbulence in the systems must be sufficient to facilitate light supply to each of the cell in the culture system to sustain maximum photosynthetic activity and growth. This would lead to reduced volumetric productivity.

2.1 Light Absorption

The quantity of light energy absorbed by a photosynthetic culture is mostly determined by cell concentration and not the photon flux density. That is, most photons of low flux density could pass through a culture of low cell concentration, but all photons of high flux density could be captured by a culture of high cell concentration. Thus, cell concentration of a photosynthetic culture will continue to increase exponentially until all photosynthetically available radiance (PAR) impinging on the culture surface are absorbed. For example, a *Chlorella* culture with an optical absorption cross-section of 60 cm^2 mg^{-1} chlorophyll a, and chlorophyll a content of 30 mg Chl a/g-cell, will require 5.6 g-cell m^{-2} or 0.56 g-cells L^{-1} to absorb all available photons impinging on a culture of 1 m (wide) × 1 m (long) × 0.01 m (deep), irrespective of the photon flux density. Once this cell concentration is reached, biomass accumulates at a constant rate (linear growth phase, Pirt et al. 1980) until a substrate in the culture medium or inhibitors become the limiting factor.

2.2 Light Attenuation Through Mutual Shading

Once all the photons are absorbed by cells nearer to the illuminated surface, the cells located below this "photic zone" do not receive enough light energy for photosynthesis. This leads to the phenomenon of mutual shading. Thus, cell growth is limited to the photic zone.

Let us consider a monochromatic light impinges on a microalgal culture, where I_0 = incident photon flux density, I_t = transmitted photon flux density, a = absorbance coefficient or extinction coefficient of the culture at the wavelength,

L = light path, and x = cell density. The relationship of absorbance and cell density can be described by the Beer-Lambert Law:

$$\log(I_o/I_t) = axL \tag{1}$$

For a *Chlorella pyrenoidosa* culture with absorbance coefficient of 0.11 $m^2\ g^{-1}$ cell at the wavelength of 680 nm, 99 % of the light could only penetrate 3.6 cm into a culture of 0.5 g L^{-1}, and 1.2 mm into a culture of 15 g L^{-1} cell density. The final cell density in outdoor shallow algal pond cultures was about 0.5 g L^{-1} (Richmond 2004), whereas the highest cell density achievable in a simple batch culture in a narrow light path PBR could be >10 g L^{-1} (Cuaresma et al. 2009; Doucha and Livansky 1995; Pulz et al. 2013; Lee and Low 1991, 1992; Quinn et al. 2012; Ugwu et al. 2005). Hence in most algal cultures indoors and outdoors, a significant proportion of the algal cultures is kept in dark at any given time. As a consequence, cells circulating in the culture receive energy intermittently.

Turbulence facilitates cycling of cells between the photic and dark zones. It was indeed observed that the photosynthetic efficiency and biomass productivity of microalgal cultures (Hu and Richmond 1996; Vejrazka et al. 2012) of different cell densities were functions of the stirring speed or aeration rate. The mixing effect on the areal productivity of outdoor *Spirulina* cultures was also demonstrated (Richmond and Vonshak 1978). These studies suggest that long intermittent illumination (and/or dark phase) leads to lower photosynthetic efficiency and productivity. Improvement of both gas and nutrient mass transfer may also contribute to the enhanced biomass productivity.

3 Carbon Supply

In high *s*/*v* PBR where most of the algal cells receive sufficient light energy to sustain growth, CO_2 absorption, volumetric O_2 evolution, nutrient depletion and metabolite excretion proceed at high rates, which may determine the overall productivity of the culture (Pirt et al. 1980).

Inorganic carbon is usually supplied as CO_2 gas in a 1–5 % mixture with air. High CO_2 partial pressure is inhibitory to most algae (Lee and Tay 1991). Another mode of carbon supply is as bicarbonate. The balance between dissolved free carbon dioxide ($CO_{2(aq)}$), carbonic acid (H_2CO_3), bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) is pH and temperature dependent. Higher pH (alkaline) favours forward direction of the balance equation:

$$CO_{2(aq)} + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+ \leftrightarrow CO_3^{2-} + 2H^+ \tag{2}$$

The CO_2 absorption rate, i.e. the rate of CO_2 transfer from the gas to the liquid phase (R) is expected to accord with the mass transfer equation (Lee and Tay 1991),

$$R = K_L a(c_s - c) \tag{3}$$

where K_L is a constant, a = interfacial area, c_s = saturation concentration of CO_2, and c = concentration of CO_2 in bulk liquid. The term $K_L a$ is known as the "volumetric gas transfer constant". In solution, the CO_2 hydrates to some extent to form H_2CO_3. The equilibrium constant of the reaction $CO_2 + H_2O \leftrightarrow H_2CO_3$ is given as $K = [H_2CO_3]/[CO_2]$. The amount of carbonic acid or dissolved CO_2 present at equilibrium will be directly proportional to the partial pressure of CO_2. The equilibrium between the CO_2, bicarbonate and carbonate ions will depend on the pH according to the Henderson-Hasselbach equations,

$$\log[HCO_3^-]/[CO_2] = \text{pH} - pK_1 \tag{4}$$

and

$$\log\left[CO_3^{2-}\right]/[HCO_3^-] = \text{pH} - pK_2 \tag{5}$$

where pK_1 and pK_2 are the negative logarithms of the first and second dissociation constants of carbonic acid respectively and the square brackets denote concentrations. The values of pK_1 and pK_2 are respectively 6.35 and 10.3 at 25 °C, hence, at equilibrium when the pH = 6.35, $[HCO_3^-] = [CO_2]$, and when the pH = 10.3, $[CO_3^{2-}] = [HCO_3^-]$. Thus at the neutral pH of 7.5 at which the level of the $[CO_3^{2-}]$ can be discounted and only the $[HCO_3^-]$ and $[CO_2]$ need be considered.

It should be noted that only the free CO_2 concentration (c) enters into Eq. (2) and apparently the bicarbonate concentration should not affect the CO_2 absorption rate.

In a homogeneous culture which derives its CO_2 from the gas phase, at a constant pH the net rate of CO_2 accumulation is given by

$$dc/dt = K_L a(c_s - c) - \mu x/Y \tag{6}$$

where μ = specific growth rate, x = biomass concentration and Y = growth yield from CO_2. The interconversion rates of free CO_2 and bicarbonate ions are assumed to be equal because they are in equilibrium.

The maximum CO_2 absorption rate is given by

$$R_{max} = K_L a\, c_s = \mu x Y \tag{7}$$

And the volumetric CO_2 absorption coefficient is given by

$$K_L a = \mu x/c_s Y \tag{8}$$

The terms used in Eqs. (7) and (8) are similar to those used in Eqs. (3)–(6).

In a CO_2 limited culture with a P_{CO_2} of 0.05 atm (5 % CO_2 in air), it is assumed that Eq. (8) would apply since c_s would be about 10^{-3} M and generally for carbon substrate-limited growth c is of the order 10^{-5} M.

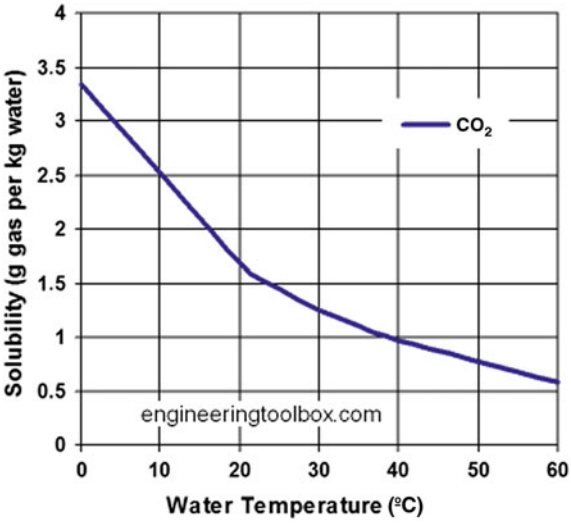

Fig. 1 The effect of water temperature on the solubility of carbon dioxide (www.engineeringtoolbox.com)

According to the theory outlined, although change in pH will affect the degree of conversion of CO_2 to HCO_3^-, this should have no effect on the CO_2 absorption rate. It is not to be excluded, however, that a pH change could affect the diffusivity of CO_2 and the reaction kinetics of CO_2 at the interface, both of which could affect K_L. Also the interfacial area (*a*) could be affected by the pH.

The effect of temperature on the overall solubility of CO_2 is depicted in Fig. 1: The higher the temperature the lower the CO_2 in the culture (www.engineeringtoolbox.com).

4 Oxygen Accumulation

Accumulation of photosynthetically generated O_2 is one of the main factors that limit the scale-up of enclosed PBR. Oxygen production is directly correlated with volumetric productivity, and dissolved O_2 concentrations equivalent to 4–5 times that of air-saturations are toxic to many algae (Richmond 1986). These dissolved O_2 concentrations could be easily reached in outdoor cultures, especially in tubes of small diameter (high *s*/*v*). At maximal rates of photosynthesis, a 1 cm diameter reactor accumulates about 8–10 mg O_2/L/min. In order to keep the O_2 level below the toxic concentration requires frequent degassing and thus one may have to resort to short loops or high flow rates (Pirt et al. 1983). Manifold systems and vertical reactors offer a significant advantage in this respect.

5 Nutrient Supply

Main considerations in developing nutrient recipes for algal cultivation and maintenance are as follows.

- Nitrogen source: Nitrate, ammonia and urea are widely used as the nitrogen source, depending on the ability of the alga to use the nitrogen substrate and culture pH. pH of a culture medium containing nitrate tends to increase due to the removal of proton (H^+), whereas medium containing ammonium tends to decrease due to the accumulation of H^+. The pH of a urea-containing culture maintains constant, in this case the algae must be able to produce urease to utilize urea. Algae contain 7–9 % nitrogen per dry weight. Thus to produce 1 g of cells in 1 L of culture, a minimum of 500–600 mg/L of KNO_3 is required.
- Minerals: These include potassium, magnesium, sodium, calcium, sulfate and phosphate. Sea water is often phosphate deficient.
- Trace elements: These include aluminum, boron, manganese, zinc, copper, iron, cobalt and molybdenum. For solubility of the mixture of trace elements, chelating agents such as citrate and EDTA are included.
- Vitamins: Some algae (e.g. *Euglena* and *Ochromonas*) require vitamins such as thiamin and cobalamin.
- Total salt concentration: Depending on the ecological origin of the alga, for example the green alga *Dunaliella* can only survive in a medium containing 0.5 M NaCl and the optimal salinity for growth is 2 M NaCl.
- pH: Most media are neutral or slightly acidic to prevent precipitation of calcium, magnesium and trace elements.

5.1 Common Culture Media

The following are common culture media used for the maintenance and cultivation of algae in laboratories (www.utex.org/prodmedia).

5.1.1 Bold Basal Medium

A medium commonly used for fresh water algae. It may be supplemented with soil extract for growing algae isolated from soil.

Base media	(i) $NaNO_3$	10 g/400 mL
	(ii) $MgSO_4 \cdot 7H_2O$	3 g/400 mL
	(iii) K_2HPO_4	3 g/400 mL
	(iv) KH_2PO_4	7 g/400 mL
	(v) $CaCl_2 \cdot 2H_2O$	1 g/400 mL
	(vi) NaCl	1 g/400 mL
Trace element solutions	(i) EDTA	50 g/L
	KOH	31 g/L
	(ii) $FeSO_4 \cdot 7H_2O$	4.98 g/L
	Water acidified with 1 mL H_2SO_4	
	(iii) H_3BO_3	11.42 g/L
	(iv) $ZnSO_4 \cdot 7H_2O$	8.82 g/L
	$MnCl_2 \cdot 4H_2O$	1.44 g/L
	MoO_3	0.71 g/L
	$CuSO_4 \cdot 5H_2O$	1.57 g/L
	$Co(NO_3)_2$	0.49 g/L

10 mL of each of the base medium (i)–(vi) and I mL of each of the trace element solution (i)–(iv) are made up to a final volume of 1 L. The medium may be solidified in 15 g/L agar.

5.1.2 N8 Medium for *Chlorella* and Other Green Algae

	mg/L
$Na_2HPO_4 \cdot 2H_2O$	260
KH_2PO_4	740
$CaCl_2$	10
Fe EDTA	10
$MgSO_4 \cdot 7H_2O$	50
Trace elements	1 mL
Trace element stock	
	g/L
$Al_2(SO_4)_3 \cdot 18H_2O$	3.58
$MnCl_2 \cdot 4H_2O$	12.98
$CuSO_4 \cdot 5H_2O$	1.83
$ZnSO_4 \cdot 7H2O$	3.2

5.1.3 Bristol Medium

For 1 L medium, to approximately 900 mL of dH_2O add each of the components in the order specified while stirring continuously. Bring total volume to 1 L with dH_2O.

	mL	Stock solution (mL)	Final concentration (mM)
$NaNO_3$	10	10 g/400 mL dH_2O	2.94
$CaCl_2 \cdot 2H_2O$	10	1 g/400 mL dH_2O	0.17
$MgSO_4 \cdot 7H_2O$	10	3 g/400 mL dH_2O	0.3
K_2HPO_4	10	3 g/400 mL dH_2O	0.43
KH_2PO_4	10	7 g/400 mL dH_2O	1.29
NaCl	10	1 g/400 mL dH_2O	0.43

5.2 Sterilization

Sterilization of culture media is mandatory, even if the algal cultures are not bacteria-free, to avoid additional contamination. All glassware, pipettes and media should be sterilized before use by autoclaving for 15 min at 15 pounds pressure. Seawater could not be autoclaved, for this would cause precipitation of salt from the medium, sterilization could be achieved through pasteurization and filtration sterilization (Little et al. 1987).

6 Culture Methods

6.1 Cultivation on Solid Media

Streaking method is commonly applied. This could be done on agar plate in petri dish (Fig. 2), or on agar slant in test tube (Fig. 3). The slant in test tube has a smaller surface area compared to the petri dish, thus allowing less growth. However it has the advantage of preventing drying thus the culture could be stored for relatively long term (6 months to a year).

To prepare an agar slant, the molten agar medium, after autoclaving, is introduced into a test tube. The test tube is tilted, the agar medium is allowed to cool and harden in a slant fashion.

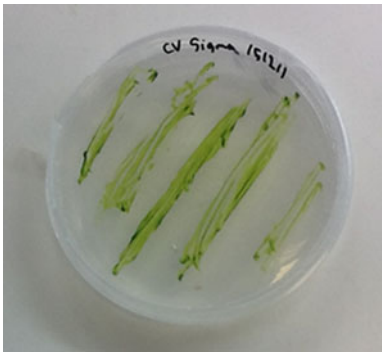

Fig. 2 Streak microalgal culture on agar plate

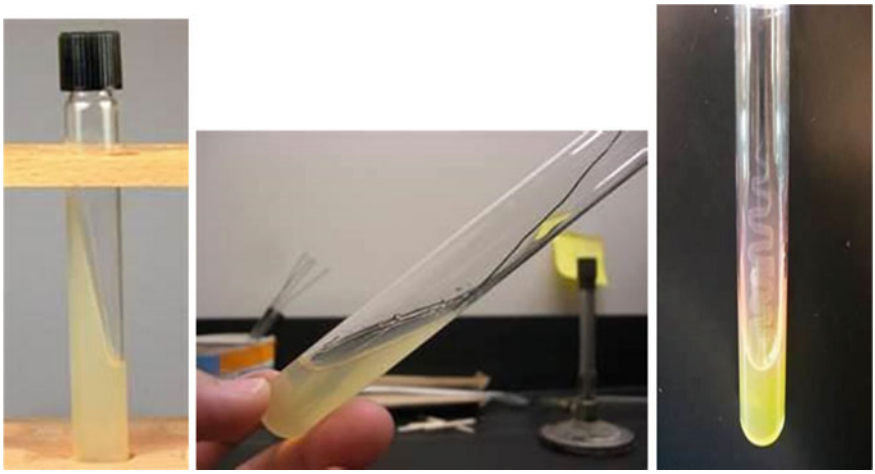

Fig. 3 Streak microalgal culture on agar slant

6.2 Cultivation in Liquid Culture Media

6.2.1 Batch Culture

This is the most common method for cultivation of microalgal cells. In a simple batch culture system, a limited amount of complete culture medium and algal inoculum are placed in a culture vessel and incubated in a favourable or otherwise defined environment for growth. Some form of agitation, such as shaking or impeller mixing, is necessary to ensure nutrient and gaseous exchange at the cell–water interface. The culture vessel can be a simple conical flask (Fig. 4) or an environment controlled fermentor.

Fig. 4 Batch cultivation of liquid microalgal culture in conical flask

In a photosynthetic or mixotrophic culture, CO_2 is supplied by either purging the conical flask with CO_2 enriched air (e.g. 5 % v/v CO_2 in air) and capped, or by gassing the culture continuously with CO_2 enriched air. The culture can be illuminated externally by either natural or artificial light sources, or through optical fiber, placed in the culture vessels.

Batch culture is widely used for commercial cultivation of algae for its ease of operation and simple culture system. Since the process is batch wise, there is low requirement for complete sterilization. When light is the rate-limiting energy source which impinges on the culture surface continuously, strictly speaking this is not a batch culture, but a constant volume fed batch system.

Mixing and Turbulence

One obvious reason for mixing is to prevent the microalgal cells from settling to the bottom of the culture system. Settling occurs when the flow is too slow and will be particularly severe in the culture region where turbulence is smallest (dead space). Clearly accumulation of cells at dead corner of the culture system will result in cell death and decomposition. This decreases the output, and adversely affects the quality of the product. In extreme cases, toxic materials might form with ramifications far greater than mere effect on decreased productivity.

Another reason to maintain high turbulence relates to the nutritional and gaseous gradients which are formed around the algal cells in the course of their metabolic activity. For example, active photosynthesis at midday creates extremely high concentration of dissolved O_2 which may reach over 400 % saturation, and is inhibitory to cell growth. Vigorous mixing decreases the O_2 tension in the culture (Richmond 1986).

A large raceway pond cannot be operated at a water level lower than 15 cm, otherwise a severe reduction of flow and turbulence would occur. The main objective for creating a turbulent flow in the culture relates to the phenomenon of

mutual shading (Cuaresma et al. 2009; Doucha and Livansky 1995; Pulz et al. 2013; Lee and Low 1991, 1992; Quinn et al. 2012; Ugwu et al. 2005).

A turbulent flow causes a continuous shift in the relative position of the cells with respect to the light zone. Thus, turbulence, in effect, causes the solar radiation impinging on the surface of the culture system to be distributed more evenly to all the algal cells. It is well documented that the photosynthetic system of the algal cells is saturated by high light within seconds, and the cells is able to use the light energy to perform photosynthesis in the dark for a brief period of time, such is the flashing light effect (Lee and Pirt 1981; Vejrazka et al. 2012).

The Paddlewheel has almost become the standard method of mixing in pond culture system, with one large paddlewheel per pond. It is a relatively expensive device, with a power demand of about 600 W for a pond of 100 m^2 (Richmond 2004).

Growth Phases

The different growth phases, which may occur in a batch culture, reflect changes in the biomass and in its culture environment.

Lag Phase

An initial lag phase where the specific growth rate is at the sub-maximum level may often be observed. The growth lag could be due to the presence of non-viable cells in the inoculum. The growth lag could also be the period of physiological adjustment due to changes in nutrient or culture conditions. For example, growth lag may be observed when shade-adapted cells are exposed to a higher irradiance. Lag phase may be prevented or reduced when cells at a late exponential growth phase cultured in a same culture medium and growth conditions are used as inoculum.

Exponential Phase

At the late lag phase, the cells have adjusted to the new environment and begin to grow and multiply (accelerating growth phase), and eventually enter the exponential (or logarithmic) growth phase. At the latter phase, cells grow and divide as an exponential function of time, as long as mineral substrates and light energy are saturated (Fig. 5), $IA > \mu_m X$. V/Y, where,

I Photon flux density in the photosynthetically available range (J m^{-2} h^{-1}),
A Illuminated surface area (m^2),
μ_m Maximum specific growth rate (h^{-1}),
X Biomass concentration (g m^{-3}),
V Culture volume (m^3),
Y Growth yield (g J^{-1})

Doubling Time, Specific Growth Rate and Output Rate

When the culture environment is favourable and all nutrients required for cell growth are present in a non-growth limiting quantity, i.e. at sufficiently high

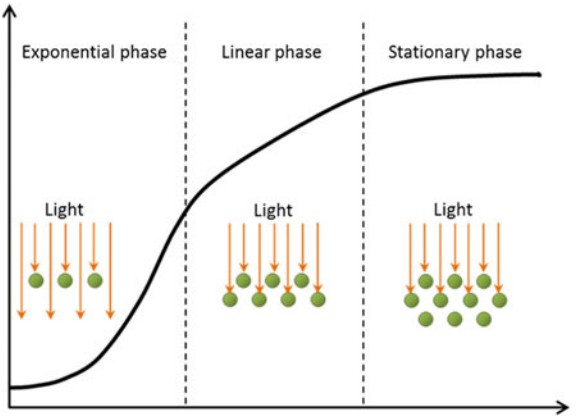

Fig. 5 The growth phases in a photosynthetic microalgal culture

concentrations so that minor changes do not significantly affect the reaction rate, most unicellular algae reproduce asexually. The size and biomass of individual cells increase with time, resulting in biomass growth. Eventually, the DNA content is doubled in quantity and cell division ensues upon complete division of the cell into two progenies of equal genome and of more or less identical size. Population number is thereby increased, and population growth is therefore referred to as increase in population of the number of cells in a culture.

The time required to achieve a doubling of the number of viable cells is termed doubling time (t_d). It is also termed generation time, as it is the time taken to grow and produce a generation of cells. The number of cells in an exponentially growing microbial culture could be mathematically described as follows:

$$2^0\mathrm{N}_0 \rightarrow 2^1\mathrm{N}_0 \rightarrow 2^2\mathrm{N}_0 \rightarrow 2^3\mathrm{N}_0 \rightarrow 2^\mathrm{n}\mathrm{N}_0 \tag{9}$$

N_0 Initial number of cells
N Number of doublings (generations)

Number of doublings (n) at a time interval t, is determined by the relation t/t_d. Thus, the number of cells (N_t) in an exponentially growing culture after being incubated for some time, t, can be estimated as follow:

$$\begin{aligned} N_t &= 2^{t/\mathrm{td}}\mathrm{N}_0 \\ N_t/\mathrm{N}_0 &= \ln 2 \cdot t/t_\mathrm{d} \end{aligned} \tag{10}$$

During the exponential growth phase, the growth rate of the cells is proportional to the biomass of cells. Since biomass generally can be measured more accurately than the number of cells, the basic microbial growth equations are often expressed in terms of mass. A biomass component such as protein may be used as an alternative to

direct weighing of biomass. Hence, Eq. (10) can be modified, by assuming the biomass concentration at time 0 (initial) and time t as X_0 and X_t, respectively:

$$\begin{aligned} &\ln(X_t - X_0)/t = \ln 2/t_{\mathrm{d}} \\ &\mathrm{d}(\ln X)/\mathrm{d}t = 0.693/t_{\mathrm{d}} \\ &\mathrm{d}(\ln X)/\mathrm{d}X \cdot \mathrm{d}X/\mathrm{d}t = 0.693/t_{\mathrm{d}} \\ &1/X \cdot \mathrm{d}X/\mathrm{dt} = 0.693/t_{\mathrm{d}} \\ &\mu = 0.693/t_{\mathrm{d}} \end{aligned} \tag{11}$$

where μ represents the specific growth rate (h^{-1}) of the culture. It defines the fraction of increase in biomass over a unit time, i.e. an increase of certain g-biomass from every g of existing biomass per hour. One should take note that the specific growth rate of a photosynthetic culture is the average growth rate of all cells present in a culture, but not necessarily the maximum specific growth rate of the individual cells, as each cell receives different photon flux density across the light gradient away from the illuminated surface (Pirt et al. 1980).

The expression of the rate of microalgal growth as a specific growth rate avoids the effect of cell concentration, i.e. the output rate of a culture at a concentration of 1 g/L is 1 g-biomass/L/h with a doubling time of 1 h, whereas the same culture with the same doubling time produces 10 g-biomass/L/h at a biomass concentration of 10 g/L.

Linear Growth Phase

In the light limited linear growth phase, the relationship between the biomass output rate and the light energy absorbed by the culture can be expressed as follows.

$$IA = \mu X \cdot V/Y \tag{12}$$

The above equation suggests that, if the value of growth yields (Y) for a particular microalga is a constant, the specific growth rate (μ) changes with changing cell concentration (X). The only specific growth rate that could be maintained at a constant value over a period of time is the maximum specific growth rate at light saturation. Thus, it is only meaningful to compare the biomass output rates (μX, g L^{-1} h^{-1}) of light-limited photosynthetic cultures.

At the cell level, the growth rate (μ) of a light-limited photosynthetic cell is determined by the photon flux density. The relationship between the photon flux density (I) and the specific growth rate has the form of the Monod relation.

$$\mu = \mu_{\mathrm{m}} I/(I + K_{\mathrm{I}}) \tag{13}$$

where,

μ_{m} Maximum specific growth rate,
K_{I} Light saturation constant, numerically equals the photon flux density required to achieve half of the maximum specific growth rate

In a study on the photosynthetic bacterium *Rhodopseudomonas capsulata*, the K_I for monochromatic light at 860 nm, in which photons are mainly absorbed by bacteriochlorophyll, was 25 μmol m^{-2} s^{-1} (Gobel 1978). The K_I value of carotenoid pigment (absorbing light at 522 nm) was 103 μmol m^{-2} s^{-1}. Apparently, bacteriochlorophyll has a higher affinity for light than carotenoids. The maximum specific growth rate of *Rhodopseudomonas* culture was, however, independent of the wavelength used. In addition, the light harvesting pigment content of the photosynthetic culture did not affect the affinity (K_I) or the uptake rate of light energy. The alteration in pigment content observed at different specific growth rates was interpreted as a physiological adaptation of the culture aimed at maximizing photon absorption.

Stationary Growth Phase

Eventually a soluble substrate in the culture medium is exhausted; the culture enters into stationary phase (Pirt et al. 1980). In this phase, photosynthesis is still being performed and storage carbon products, such as starch, neutral lipid, are accumulated.

6.2.2 Continuous Culture

In continuous flow cultures, fresh culture medium is supplied to the homogeneously mixed culture and culture is removed continuously or intermittently. The approach is based on the observations that substrates are depleted and products accumulate during growth, eventually, culture growth ceases due to depletion of the growth limiting substrate or accumulation of a growth-inhibiting product. To sustain cell growth, the growth-limiting substrate needs to be replenished and the growth inhibitory product needs to be removed or diluted by adding fresh culture medium (Pirt et al. 1980).

Principles of Continuous Flow Culture

For simplicity, let us assume that the medium feed rate and the rate of removal of culture (F) is the same, and the culture volume is a constant, V (Fig. 6). A peristaltic pump is most suitable for delivery of medium into the culture, for the mechanical parts are not in direct contact with the medium.

The culture could be removed by another peristaltic pump, or through an overflow located at the side of the culture vessel.

The increase in biomass in the culture can be expressed as follows:

$$\text{Net increase in biomass} = \text{Growth} - \text{Biomass removal}$$

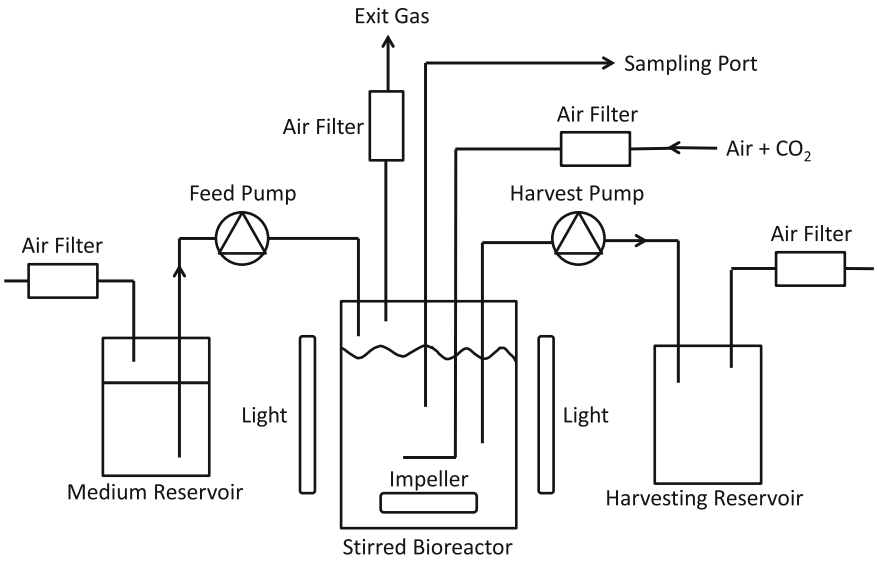

Fig. 6 Schematic illustration of a continuous flow culture setup

For an infinitely small time interval dt, this balance for the culture could be written as,

$$V\mathrm{d}x = V \cdot X\mathrm{d}t - FX\mathrm{d}t \tag{14}$$

where,

V Culture volume (m^3),
dx Increase in biomass concentration (g m^{-3}),
μ Specific Growth rate (h^{-1}),
X Biomass concentration (g m^{-3}),
dt Infinitely small time interval (h),
F Culture flow rate (m^3 h^{-1})

Thus,

$$\mathrm{d}x/\mathrm{d}t = (\mu - F/V) \cdot X \tag{15}$$

The term F/V represents the rate of dilution of the culture. For example, medium is added into and culture removed from a 5 L algal culture, at a flow rate of 10 L h^{-1}. The rate of dilution of the culture is 10/5 = 2 h^{-1}. That is, the culture is

diluted two times every hour. The F/V is termed dilution rate (D) with the unit of h^{-1}. Thus, the above equation could be written as,

$$dx/dt = (\mu - D)X \tag{16}$$

This equation suggests that at steady state, the specific growth rate equals the dilution rate ($\mu - D = 0$), $dx/dt = 0$. That is, no net increase in the biomass concentration takes place. This steady state condition is readily demonstrated experimentally.

The steady state is self-regulatory and history independent. That is, irrespective of the initial cell concentration and physiological state, the steady state is identical for a given set of conditions. In general, steady state of a chemostat (constant volume continuous flow culture) could be reached after four-volume changes of the culture, i.e. for a culture of 1 L volume; steady state could be reached after 4 L of fresh culture medium has been pumped through. Theory indicates that it is possible to fix the specific growth rate of an algal culture at any value from zero to the maximum, by adjusting the dilution rate of the culture.

In a light limited continuous flow culture, where all incident PAR is absorbed, the energy balance in the culture could be expressed as follows,

$$\begin{aligned}\text{Net increase in energy content} = {} & \text{Energy absorbed by biomass} \\ & - \text{Energy in outflow biomass}\end{aligned}$$

For an infinitely small time interval, dt,

$$V dE = IA dt - FX \cdot dt/Y$$

where, dE = Increase in energy content of the culture (J m^{-3})

$$dE/dt = IA/V - FX/YV$$

At steady state, $dE/dt = 0$ and $D = F/V$ hence,

$$DX = IA \cdot Y/V \tag{17}$$

This relationship implies that for any incident irradiance, the output rate of a light limited algal culture in steady state ($\mu X = DX$) should be a constant, if growth yield is a constant value (Fig. 7). It should be mentioned that at the extreme low dilution rates when the biomass concentration is high, the degree of cell shading of light could be significant, leading to increased maintenance energy in intermittent illumination (Lee and Pirt 1981) and lower biomass output rate as indicated in Fig. 7.

Any deviation from the constant value would suggest a change in the conversion efficiency of light energy to biomass. For example, in a light-limited photosynthetic culture where maintenance energy requirement (e.g. for motility, osmotic balance) is a significant fraction of the total energy uptake, the steady state biomass concentration and biomass output rate may dip towards the lower dilution rates (Fig. 8).

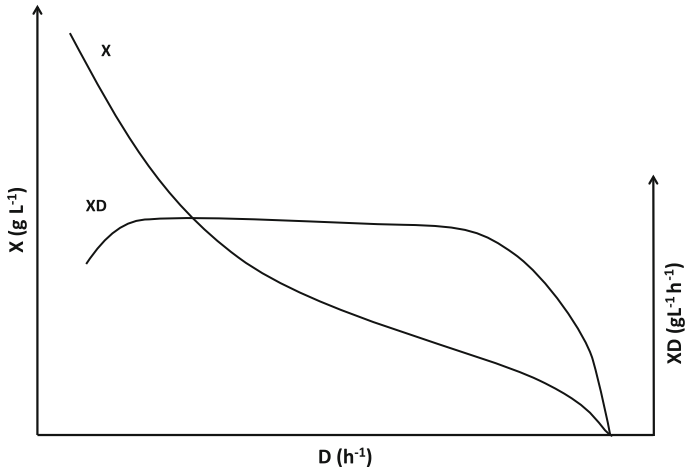

Fig. 7 Steady state biomass concentration (*X*) and biomass output rate (*XD*) as functions of dilution rate (*D*) in a continuous flow culture system at constant light intensity. The intercept on the *X*-axis denotes the Critical Dilution Rate (numerically equal the maximum specific growth rate)

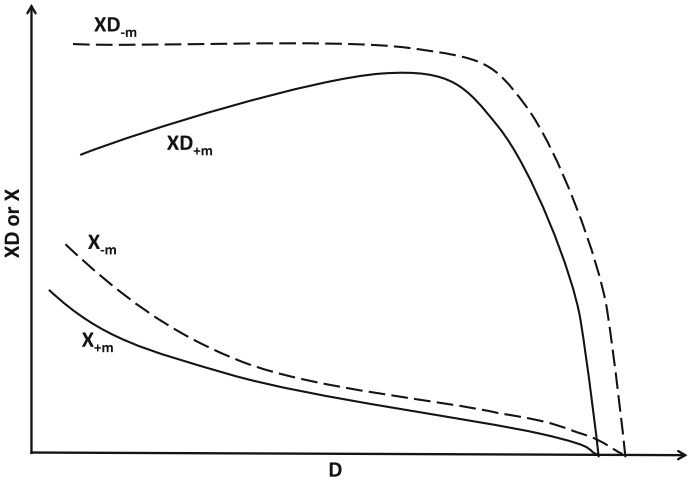

Fig. 8 Steady state biomass concentration (*X*) and biomass output rate (*XD*) as functions of dilution rate (*D*) in a continuous flow culture system at constant light intensity. $-m$ denotes negligible maintenance energy requirement and $+m$ denotes a significant maintenance energy requirement

6.3 Chemostat

The special type of continuous culture where the rate of addition of medium and the rate of removal of culture is the same, and culture volume is thus maintained at a constant level, is called chemostat (constant chemical environment). Chemostat is widely used in research, for it allows full adjustment of the cells' physiology to the prevailing culture conditions and maintaining the specific growth rates at pre-determined values. Culture parameters such as temperature, pH and substrate concentration could be readily adjusted and studied at fixed specific growth rates. In a simple batch culture, a change in a culture parameter leads inevitably to altered specific growth rate. Such a batch culture could not differentiate between the effects of culture parameters and the specific growth rate.

6.4 Fedbatch Culture

Unlike the conventional batch cultivation method where all the substrates are added at the beginning of the process, fed batch process is a batch culture with feeding of nutrients while the effluent is removed periodically. The volume of the culture could be variable, by feeding the complete medium (Variable Volume FBC) or constant (Constant Volume FBC), by feeding the growth-limiting substrate in the form of solid, concentrated solution, gas or light. In the case where at the end of a cultivation cycle, the culture is partially withdrawn and replaced with fresh complete medium, thereby initiating a new cycle. This is termed the cyclic FBC (Fig. 9).

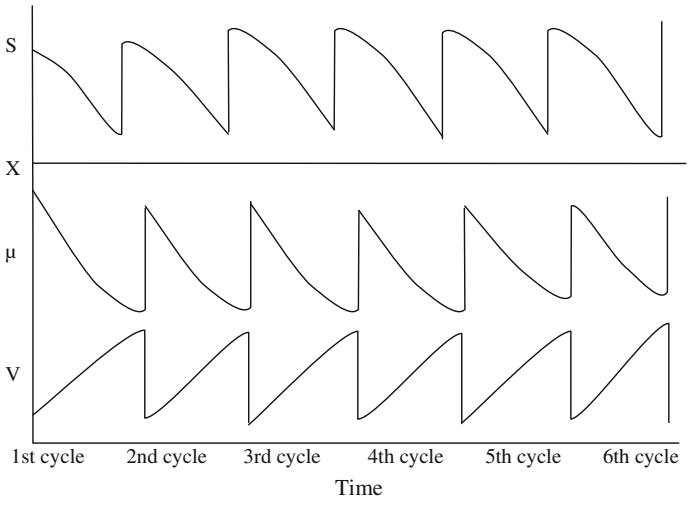

Fig. 9 Changes in the rate-limiting substrate concentration (S), biomass concentration (X), specific growth rate (μ) and culture volume (V) in a Cyclic Fed Batch Culture system

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Large-Scale Production of Algal Biomass: Raceway Ponds

Yusuf Chisti

Abstract Raceway ponds are widely used in commercial production of algal biomass. They are effective and inexpensive, but suffer from a relatively low productivity and vagaries of weather. This chapter discusses design and operation of raceways for large-scale production of algal biomass.

Keywords Microalgae · Raceway ponds · High-rate algal ponds · Biomass production

Nomenclature

A	Surface area of raceway (m^2)
C_x	Biomass concentration (kg m^{-3})
D	Dilution rate (h^{-1})
d_h	Hydraulic diameter of flow channel (m)
e	Efficiency of the motor, drive, and the paddlewheel
f_M	Manning channel roughness factor (s $m^{-1/3}$)
g	Gravitational acceleration (9.81 m s^{-2})
h	Culture depth in pond (m)
I_L	Local irradiance at depth L (μE m^{-2} s^{-1})
I_o	Incident irradiance on the surface of the pond (μE m^{-2} s^{-1})
K_a	Light absorption coefficient of the biomass (μE m^{-2} s^{-1})
K_i	Photoinhibition constant (μE m^{-2} s^{-1})
K_L	Light saturation constant (μE m^{-2} s^{-1})
L	Depth (m)
L_r	Total length of the flow loop (m)

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l_c Depth at which the local irradiance level is at the light compensation point (m)
P Power requirement for paddlewheel (W)
PAR Photosynthetically active radiation
P_a Areal productivity of biomass (kg m^{-2} d^{-1})
PVC Polyvinyl chloride
P_v Volumetric biomass productivity (kg m^{-3} d^{-1})
p Length as shown in Fig. 1 (length of pond) (m)
Q_f Feed flow rate (m^3 h^{-1})
q Length as shown in Fig. 1 (width of pond) (m)
Re Reynolds number defined by Eq. (3)
Δt Time interval (d)
u Flow velocity in channel (m s^{-1})
V_L Working volume of the raceway (m^3)
w Channel width (m)
X_f Peak concentration of biomass (kg m^{-3})
X_i Initial concentration of the biomass (kg m^{-3})
x_b Pseudo steady state biomass concentration in the pond (kg m^{-3})

Greek symbols

μ Viscosity of the algal broth (Pa s)
μ_{av} Depth-averaged specific growth rate in the illuminated volume (d^{-1})
μ_L Local specific growth rate at depth L (d^{-1})
μ_{max} Maximum specific growth rate (d^{-1})
ρ Density of algal broth (kg m^{-3})

1 Introduction

Raceway ponds, raceways, or "high-rate algal ponds", were first developed in the 1950s for treating wastewater. Since the 1960s, outdoor open raceways have been used in commercial production of microalgae and cyanobacteria (Terry and Raymond 1985; Oswald 1988; Borowitzka and Borowitzka 1989; Becker 1994; Lee 1997; Pulz 2001; Grima 1999; Borowitzka 2005; Spolaore et al. 2006; Chisti 2012). Such production does not use wastewater. This chapter discusses the biomass production in raceways as typically used in commercial processes and not in treating wastewater. The design and performance of raceways are discussed. The factors influencing the biomass productivity in raceways are analyzed. A raceway is an oblong and shallow recirculating pond with semicircular ends as shown in Fig. 1 (Chisti 2012). The flow and mixing are typically generated by a single slowly rotating paddlewheel (Chisti 2012).

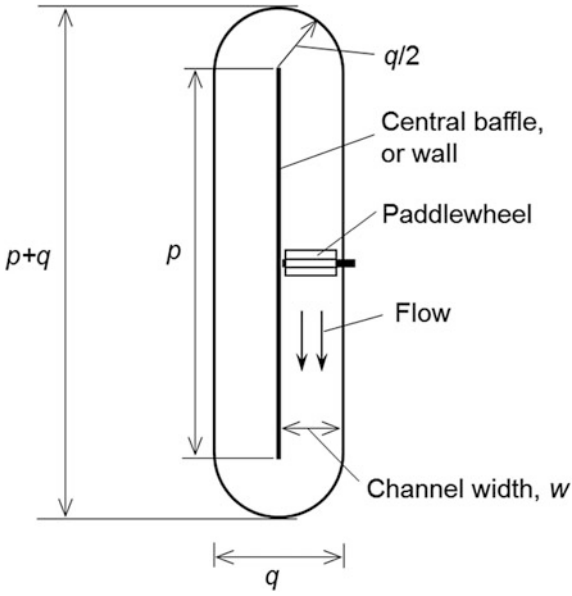

Fig. 1 A top view of a raceway pond as typically used for algal biomass production

2 Raceways

2.1 Typical Configuration

A raceway pond is a closed-loop flow channel with a typical culture depth of about 0.25–0.30 m (Fig. 1) (Becker 1994; Chisti 2007). A paddlewheel continuously mixes and circulates the algal broth in the channel (Fig. 1). An algal biomass production facility will typically have many ponds. The surface area of a single pond does not usually exceed 0.5 ha, but can be larger.

Raceways generally have a flat bottom and vertical walls. If the thickness of the central dividing wall (Fig. 1) is neglected, the surface area A of a raceway such as shown in Fig. 1, can be estimated using the following equation:

$$A = \frac{\pi q^2}{4} + pq \tag{1}$$

The p/q ratio can be 10 or larger (Chisti 2012). If this ratio is too small, the flow in the straight parts of the raceway channel begins to be affected by the disturbances caused by the bends at the ends of the channel. The working volume V_L is related to the surface area and the depth h of the culture broth, as follows:

$$V_L = Ah \tag{2}$$

The surface-to-volume ratio is always $1/h$. A lower depth increases the surface-to-volume ratio and this improves light penetration, but in a large pond the depth cannot be much less than 0.25 m for reasons discussed later in this chapter.

A compacted earth construction lined with a 1–2 mm thick plastic membrane may be used for the pond, but this relatively cheap setup is uncommon for biomass production. Ponds used to produce high-value biomass are often made of concrete block walls and dividers lined with a plastic membrane to prevent seepage. Membranes made of ultraviolet resistant polyvinyl chloride (PVC), polyethylene, and polypropylene are generally used and can last for up to 20 years (Chisti 2012). Depending on the end use of the biomass, special care may be required to use liners that do not leach contaminating and inhibitory chemicals into the algal broth (Borowitzka 2005). The pond design must consider the mixing needs; the feeding and harvesting of the algal culture; the carbon dioxide input; the drainage and overflow; and the cleaning aspects. The key aspects of design and operation are discussed here.

2.2 Culture Flow in the Raceway Conduit

The flow in a raceway conduit needs to be turbulent to keep the cells in suspension, enhance vertical mixing, prevent thermal stratification, and facilitate removal of the oxygen generated by photosynthesis. Whether the flow is turbulent depends on its Reynolds number, *Re*, defined as follows:

$$Re = \frac{\rho u d_{\mathrm{h}}}{\mu} \tag{3}$$

In Eq. (3), ρ is the density of the culture broth, u is its average flow velocity, d_h is the hydraulic diameter of the flow conduit, and μ is the viscosity of the algal broth. Typically, the density and viscosity of water at the operating temperature are taken to closely resemble the properties of a dilute algal broth (Chisti 2012). The hydraulic diameter d_h for use in Eq. (3) is defined as follows:

$$d_{\mathrm{h}} = \frac{4wh}{w + 2h} \tag{4}$$

In Eq. (4), w is the width of the channel (Fig. 1) and h is the average depth of the broth in it.

The flow in the channel is generally taken to be turbulent if the *Re* value exceeds 4000; however, the threshold of turbulence in channels is poorly defined and, therefore, a higher Reynolds number of about 8000 is used as safer criterion of turbulence (Chisti 2012). The flow in a rough channel becomes turbulent at a lower

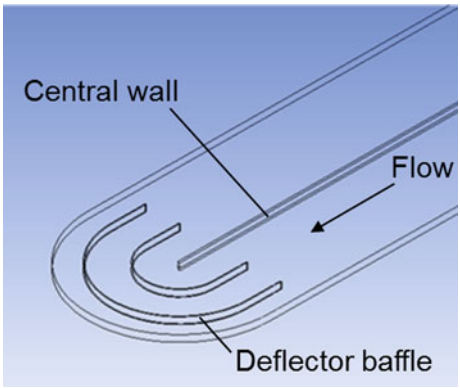

Fig. 2 A raceway pond with deflector baffles

value of Reynolds number compared to the case of a smooth channel. In practice, the average flow velocity in the channel is kept much higher than the minimum required to attain a Reynolds number value of 8000.

In ponds with semicircular ends (Fig. 1), curved baffles or flow deflectors are commonly installed at both ends (Fig. 2). The baffles ensure a uniformity of flow throughout the curved bend and minimizes the formation of dead zones (Chisti 2012). Dead zones adversely affect mixing, allow solids to settle, and cause unwanted energy losses (Chisti 2012). Other methods of preventing the development of the dead zones have been discussed in the literature (Chisti 2012; Sompech et al. 2012).

2.3 Power Consumption for Flow and Mixing

The power requirement P (W) for a paddlewheel to generate a flow of velocity u in the straight channel of a typical raceway is estimated using the following equation (Chisti 2012):

$$P = \frac{1.59A\rho g u^3 f_{\mathrm{M}}^2}{e d_{\mathrm{h}}^{0.33}} \tag{5}$$

where A (m^2) is the surface area of the pond, ρ (kg m^{-3}) is the density of the culture broth, g (9.81 m s^{-2}) is the gravitational acceleration, d_h is the hydraulic diameter of the flow channel, f_M is the Manning channel roughness factor, and e is the efficiency of the motor, drive and the paddlewheel. Typical values of f_M are 0.012 s $m^{-1/3}$ for compacted gravel lined with a polymer membrane and 0.015 s $m^{-1/3}$ for an unfinished concrete surface (Chisti 2012). The e value is about 0.17 (Borowitzka 2005) for a paddlewheel (Fig. 3) located in a channel with a flat bottom. The hydraulic diameter d_h is calculated using Eq. (4). Equation (5) does not account for

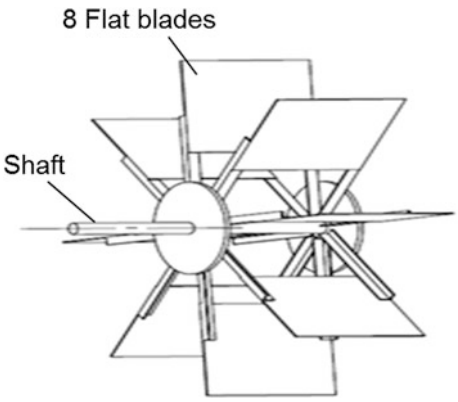

Fig. 3 A typical paddlewheel for mixing and recirculation of the broth in a raceway pond

head losses around bends, but can be used for estimating an approximate head loss in a raceway of the total channel loop length L_r (Chisti 2012).

As reflected in Eq. (5), the power required depends strongly on the flow velocity and, therefore, the flow velocity must remain at a low value that is consistent with a satisfactory operation (Chisti 2012). Although a flow velocity of 0.05 m s^{-1} is sufficient to prevent thermal stratification, a higher velocity of around 0.1 m s^{-1} is needed to prevent sedimentation of algal biomass (Becker 1994). In practice, a straight channel velocity of at least 0.2 m s^{-1} is required to ensure that the velocity everywhere in a raceway is above the necessary minimum value of 0.1 m s^{-1} (Becker 1994). Raceways for biomass production are frequently operated at a flow velocity of 0.3 m s^{-1} (Becker 1994). At this velocity, the Reynolds number in a 1.5 m wide channel with a broth depth of 0.3 m would be around 257,000.

The turnaround of the flow at the ends of the raceway contributes substantially to the total power consumption. Installation of suitably designed semicircular flow deflector baffles (Fig. 2) at the ends of the raceway is known to reduce the specific power consumption (Sompech et al. 2012; Liffman et al. 2013) relative to baffle-free operation, but contrary data have also been reported (Mendoza et al. 2013a). Design of raceway ends to minimize the power required for a given flow velocity is further discussed in the literature (Sompech et al. 2012; Liffman et al. 2013).

Under typically used conditions, the mixing of fluid between the surface and the deeper layers is poor (Chisti 2012; Mendoza et al. 2013b; Sutherland et al. 2014b; Prussi et al. 2014). This adversely affects productivity. In particular, poor mixing results in inadequate oxygen removal during period of rapid photosynthesis and an accumulation of dissolved oxygen to far above the air saturation concentration. Improving mixing substantially would require prohibitively high input of energy. Mixing requires energy and installation of devices to reduce the energy dissipation associated with the turnaround of flow at the ends of a raceway actually reduces mixing (Mendoza et al. 2013a; Prussi et al. 2014). Other measures have been suggested for improving the energy efficiency of raceway ponds (Chiaramonti et al. 2013).

Typically, the flow in raceways is characterized as being plug flow with little mixing occurring in the direction of flow. Most of the mixing occurs in the region of the paddlewheel and at the semicircular ends where the flow turns around. In a 20 m^3 raceway with a loop length of 100 m and total width of 2 m, operated at a water depth of 0.2 m, the power consumption of the paddlewheel ranged from 1.5 to 8.4 W m^{-3}, depending on the velocity of flow (Mendoza et al. 2013a). This was much greater than the range of 0.5–1.5 W m^{-3}, typical of larger commercial raceways. The 100 m raceway required between 15 and 20 flow circuits for 5 % deviation from the state of complete mixing in the configuration without the semicircular deflector baffles installed at the ends (Mendoza et al. 2013a). The mixing time ranged from 1.4 to 6 h (Mendoza et al. 2013a). With the deflector baffle installed, the mixing time was longer, with 30–40 flow circuits being required for 5 % deviation from complete mixing (Mendoza et al. 2013a). This was likely because the deflector baffles reduced the mixing potential of the semicircular ends. The specific power consumption increased if the depth of the fluid increased or decreased from 0.2 m (Mendoza et al. 2013a).

Computer simulations of pond fluid dynamics have resulted in design recommendations for minimizing energy consumption while achieving sufficient mixing to prevent sedimentation and dead zones (Sompech et al. 2012; Hadiyanto et al. 2013; Liffman et al. 2013; Prussi et al. 2014; Huang et al. 2015). The power consumption can be greatly reduced by lowering the channel flow velocity at night (Chisti 2012). The paddlewheel motor should always be sized for a flow velocity of at least 0.3 m s^{-1} and a further safety factor on power demand should be added (Chisti 2012). The motor and drive should allow a variable speed operation, unless the operational performance of a comparable raceway has been previously confirmed over an extended period (Chisti 2012). The drive mechanism should have a turndown ratio of at least 3:1 (Dodd 1986).

2.4 The Paddle Wheel

Paddlewheels (Dodd 1986) are generally believed to be the most effective and inexpensive means of producing flow in raceways. A raceway is typically mixed by a single paddlewheel to avoid interference between multiple paddlewheels (Dodd 1986). An eight-bladed paddlewheel (Fig. 3) with flat blades is generally used (Dodd 1986), but paddlewheels with curved blades are also in use. Other newer configurations of paddlewheels (Li et al. 2014) are being developed and may well be more efficient than the traditional paddlewheel of Fig. 3.

The raceway channel directly below the paddlewheel is generally flat, but a more efficient configuration with a curved pond bottom has been described (Dodd 1986; Borowitzka 2005; Chisti 2012). The load on the drive mechanism oscillates as the paddles of a conventional paddlewheel (Fig. 3) move in and out of the algal broth. The power demand and load oscillations may be reduced by displacing the paddles at mid channel by 22.5° (Dodd 1986). This also lowers the maintenance demands

(Dodd 1986). Paddlewheels are generally considered superior to pumps and propellers for driving the flow in a raceway pond.

2.5 Climatic and Topological Considerations

The geographic location (Dodd 1986; Oswald 1988) of a raceway-based production facility has the greatest impact on biomass productivity. The climatic conditions of the chosen location should be such that a consistently high biomass productivity is achieved throughout the year. The main factors influencing productivity are the average annual irradiance level and the prevailing temperature. Ideally, the temperature should be around 25 °C with a minimum of diurnal and seasonal variations (Chisti 2012). Other considerations are: the humidity and rainfall; the wind velocity; the possibility of storms and flood events; and the presence of dust and other pollutants in the atmosphere (Chisti 2012). Access to carbon dioxide and water of a suitable quality are important.

Freshwater is always needed to make up for evaporative loss and prevent an excessive rise in salinity (Chisti 2012). Evaporation rate depends on the local environment, especially on the level of irradiance, the wind velocity, the air temperature and the absolute humidity. An average freshwater evaporation rate of 10 L m^{-2} d^{-1} has been noted for some tropical regions (Becker 1994). This amounts to 0.01 m^3 m^{-2} d^{-1}, or 10 mm per day (Chisti 2012). The evaporation rate of seawater from a pond is generally a little less than the evaporation rate of freshwater under the same environmental conditions (Chisti 2012).

The price of land is a further factor to consider. Local topography and geology must be suitable for construction of raceway ponds (Dodd 1986).

2.6 Temperature and Productivity

The culture temperature strongly affects the algal biomass productivity and in some cases the biochemical composition of the biomass (Goldman and Carpenter 1974; Geider 1987; Raven and Geider 1988; James et al. 1989; Davison 1991). Furthermore, the daytime temperature history may affect the biomass loss by respiration during the subsequent night (Grobbelaar and Soeder 1985; Richmond 1990). Most algae grown in warm climates in raceways generally have an optimal growth temperature in the range of 24–40 °C (Chisti 2012). Optimal growth temperature typically spans several degrees, rather than being a sharply defined value.

The temperature in a raceway is governed by the sunlight regimen, evaporation, and the local air temperature. Temperature is typically not controlled, as doing so is impractical (Chisti 2012). Therefore, the temperature varies cyclically (Moheimani and Borowitzka 2007; Tran et al. 2014) with the day–night cycle and the amplitude of this cycle is affected by the season (Tran et al. 2014). In a tropical location with a

uniformly warm temperature during the year and a moderate diurnal variation, a high biomass productivity can be sustained year-round in a raceway without temperature control so long as the alga being grown has been adapted for the local conditions (Chisti 2012).

In temperate regions, the length of the growing season strongly influences the average annual algal productivity (Chisti 2012). In production of high-value products, implementing some level of temperature control may be feasible by recirculating the algal broth from the raceway through external heat exchangers (Chisti 2012), but this is rarely done.

Diurnal and seasonal variations in temperature in a raceway can be modeled reasonably well (James and Boriah 2010). In a tropical climate, because of evaporation and other heat losses, the diurnal variation in temperature is generally less than 10 °C (Chisti 2012). Growth may cease at the diurnal extremes of temperature, but algae generally survive short periods at up to 40 °C (Chisti 2012). Increasing temperature typically reduces the efficiency of photosynthesis as the rate of respiration increases faster with temperature compared to the rate of photosynthesis (Davison 1991; Pulz 2001).

2.7 The pH and Carbon Supply

Algal biomass typically contains 50 % carbon by weight. All carbon in photoautotrophically grown biomass comes from carbon dioxide or dissolved carbonate. Stoichiometrically, therefore, about 1.83 tons of carbon dioxide is needed to produce a ton of algal biomass (Chisti 2007). If carbon dioxide is consumed rapidly and not replenished, the pH becomes alkaline. A pH rise during periods of peak photosynthesis is commonly seen in raceways (Becker 1994; García et al. 2006; Moheimani and Borowitzka 2007; Craggs et al. 2014; Sutherland et al. 2014a) and is an evidence of carbon limitation. Carbon dioxide absorption from the atmosphere through the surface of a raceway is entirely insufficient to support photosynthesis during sunlight. This carbon deficit is accentuated during peak sunlight periods. A supply of carbon dioxide is necessary to avert carbon limitation and attain high biomass productivity. Carbon dioxide can be effectively supplied in response to a pH signal. The pH should be controlled well below eight by injecting carbon dioxide. An alkaline pH is not wanted as it results in generation of toxic ammonia from dissolved ammonium salts and this inhibits algal productivity. The generation of ammonia as a consequence of inadvertent rise in pH is best prevented by using nitrate as the source of nitrogen, although algae use ammonium more readily than nitrate. The carbon dioxide supply system should be designed to effectively control the pH during peak demand periods of high irradiance (Chisti 2012).

Microporous gas diffusers (Fig. 4) are used in raceways to provide carbon dioxide in the form of fine bubbles (Chisti 2012). Carbon dioxide diffusers are placed at intervals along the flow path at the bottom of the raceway channel (Chisti 2012). The diffusers should be easily removable from the gas distribution tubing for

Fig. 4 A microporous gas diffuser for dispersing carbon dioxide in a raceway culture. Courtesy of Mott Corporation, Farmington, CT, USA

cleaning and replacement (Chisti 2012). Between 35 and 70 % of the pure carbon dioxide sparged into a pond is lost to the atmosphere (Weissman et al. 1989). This translates to a significant monetary loss (Chisti 2012). For algae that grow at alkaline pH, inorganic carbon may be supplied as bicarbonate (Chi et al. 2011). Doing so may potentially reduce the cost of providing carbon (Chi et al. 2011). Growth under alkaline pH may not be possible for oceanic algae as marine salts precipitate at pH values of >8 (Chisti 2012). In most cases, the carbon dioxide supplied is actually taken up by the alga as bicarbonate.

Carbon dioxide requirements can be estimated from the expected biomass productivity of the raceway, accounting for the inevitable losses to the atmosphere as previously discussed (Chisti 2012). The demand for carbon dioxide varies with the rate of photosynthesis which is controlled by the irradiance. Therefore, the best strategy to ensure a sufficiency of carbon and minimize loss is to inject carbon dioxide in response to a signal from a pH controller (Chisti 2012). Periodic injection without automatic pH control may be feasible, depending on historical experience with a given alga and location (Becker 1994).

In principle, a suitably pretreated flue gas resulting from burning of fossil fuels can be used to provide relatively cheap carbon dioxide for growing microalgae, but most commercial algae production operations do not use it. Desulfurized flue gas from a coal fueled electric power plant typically contains 12–14 % carbon dioxide by volume, the rest being mostly water vapor and nitrogen (Chisti 2012). The flue gas must be free of heavy metals (Chisti 2012). Cooled desulfurized flue gas is a satisfactory source of inorganic carbon, but the flow rates required are substantially greater than if pure carbon dioxide is used (Chisti 2012). This is because absorption of carbon dioxide from flue gas into water is slower than absorption from pure carbon dioxide. Successful use of flue gas from diesel powered boilers has been reported for growing algae (de Godos et al. 2014; Tran et al. 2014). If carbon dioxide is fed in the form of flue gas, the loss to atmosphere is expected to be well above 80 % (Chisti 2012) although this may be reduced substantially by controlled feeding using a well-designed supply system (de Godos et al. 2014). Carbon dioxide absorption rate is pH dependent and is reduced at pH values less than 8. At

25 °C, the solubility of carbon dioxide in seawater is nearly half of its solubility in freshwater and this need to be considered in photoautotrophic production of marine algae (Chisti 2012).

2.8 Oxygen Inhibition of Production

Photosynthesis generates oxygen and is inhibited by an accumulation of dissolved oxygen in the culture broth (Shelp and Canvin 1980; Suzuki and Ikawa 1984; Molina et al. 2001). Other than agitation by the paddlewheel, no oxygen removal mechanism is used in a typical raceway. In some cases, the culture may be sparged with air to control buildup of oxygen. Despite a high surface area relative to the culture depth, the oxygen removal from raceway ponds is poor (Chisti 2012; Mendoza et al. 2013b) and the dissolved oxygen concentration increases dramatically during periods of peak photosynthesis. The paddlewheel assists with oxygen removal, but is mostly ineffective. As a result, the broth undergoes a diurnal change in concentration of dissolved oxygen (García et al. 2006; Moheimani and Borowitzka 2007). During peak sunlight, the level of dissolved oxygen may exceed 300 % of the level in air saturated water (Richmond 1990; Moheimani and Borowitzka 2007). Such high levels of dissolved oxygen can reduce the rate of photosynthesis (Becker 1994; Molina et al. 2001) and adversely affect the biomass productivity (Mendoza et al. 2013b). The composition of the algal biomass may also be affected by the concentration of dissolved oxygen (Richmond 1990).

Sparging of the pond with air may reduce the oxygen inhibition of photosynthesis, but requires energy. The energy associated with this sparging has been claimed to be compensated by improved biomass productivity made possible by a reduced inhibition by oxygen (Mendoza et al. 2013b). For a given fluid depth, a relatively small pond achieves better oxygen removal than a larger pond. This is because the proportion of the zone of good mixing and mass transfer in the vicinity of the paddlewheel is larger in a small pond compared to a larger one. This explains the sometimes reported better productivity of small ponds relative to the equally deep larger ponds placed under the same climatic conditions.

2.9 Culture Contamination

Open ponds are exposed to rain, dust, and other debris. Ponds may be placed within greenhouses, but this is not feasible for facilities occupying large areas. Other contamination issues include infestations of predators feeding on algae (Turner and Tester 1997; Richmond 1990); viral infections (Van Etten et al. 1991; Van Etten and Meints 1999; Wommack and Colwell 2000); and contamination by unwanted microalgae (Richmond 1990), fungi, and bacteria. The low peak alga concentration in a raceway accentuates the effects of predators and other unwanted

microorganisms (Chisti 2012). Filtration of water may help reduce the frequency of certain types of infestations, but filtration is expensive. The typically used microfiltration does not prevent contamination with viruses (Chisti 2012). Management practices can be used to reduce the frequency of culture contamination and failure (Chisti 2012). Predator control in raceways is potentially possible (Lass and Spaak 2003; Borowitzka 2005; Van Donk et al. 2011), but has not received much attention (Chisti 2012). Contamination with heterotrophic bacteria is inevitable (Erkelens et al. 2014) and not necessarily harmful, but may necessitate implementation of specific controls depending on the final application of the alga being grown.

2.10 Dependence of Photosynthesis on Culture Depth

Growth is driven by photosynthetically active radiation, or PAR, the component of the sunlight that is within the wavelength range of 400–750 nm. Although the peak sunlight level at solar noon at the surface of a raceway in a tropical location may be as high as 2000 μE m^{-2} s^{-1}, photosynthesis saturates at roughly 10–20 % of the peak PAR value. Therefore, the rate of photosynthesis does not increase beyond a PAR value of about 100–200 μE m^{-2} s^{-1} (Chisti 2012) and all the excess light is wasted. Nevertheless, an increasing incident irradiance level generally increases raceway productivity, as the local irradiance level in the broth declines rapidly with culture depth and a high surface irradiance generally means a larger illuminated culture volume.

Algal cultures become photoinhibited once the PAR value exceeds the saturation threshold. In a photoinhibited culture, the rate of photosynthesis actually decreases with a further increase in irradiance. During peak light, the culture near the surface of a pond is photoinhibited, but deeper layers of a dense culture are light limited. If the pond is sufficiently deep, or the culture sufficiently dense, the light will not penetrate the entire depth. In fact, most of the depth of a dense raceway culture is optically dark and contributes nothing to photosynthesis. Photosynthesis stops once the irradiance level declines to the light compensation point. The biomass at and below the light compensation point, consumes itself by respiration.

For a fixed incident light level I_o on the surface of the raceway, the irradiance declines rapidly with depth as the light is absorbed by the cells. The local irradiance I_L, at any depth L from the surface, is estimated by the following equation (Chisti 2012):

$$I_L = I_o e^{-K_a C_x L} \tag{6}$$

In the above equation, K_a is the alga-dependent light absorption coefficient of the biomass and C_x is the concentration of the biomass. The strong decline in local irradiance with depth is shown in Fig. 5 for various values of the biomass concentration in the culture.

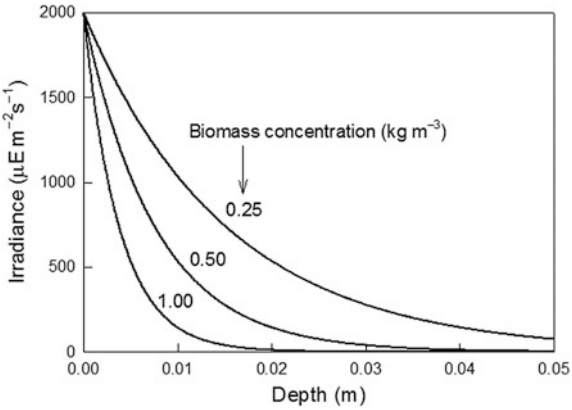

Fig. 5 Irradiance variation with depth in a 0.3 m deep raceway at various concentrations of the algal biomass in the broth. The local irradiance profiles were calculated for an alga with a K_a value of 2.632 μE m^{-2} s^{-1} and an incident irradiance of 2000 μE m^{-2} s^{-1} at the surface of the raceway

In a typical culture at a peak biomass concentration of about 0.5 kg m^{-3}, more than 80 % of the culture volume in a raceway is in the dark at solar noon. That is, the biomass in all this volume is actually consuming itself rather than photosynthesizing. In the same raceway, less than 4 % of the culture volume is photoinhibited; less than 3 % of the volume is light-saturated; and about 9 % of the culture volume is light limited (Chisti 2012).

2.11 Specific Growth Rate

The specific growth rate of a microalga in a pond varies with depth because the light intensity declines with depth (Fig. 5). If light is the only growth limiting factor, the specific growth rate μ_L at any depth L can be estimated from the local value of irradiance I_L (Eq. 6) at that depth (Chisti 2012). For example, the local growth rate may depend on local irradiance in accordance with the Haldane light-inhibited growth model, as follows:

$$\mu_{\mathrm{L}} = \frac{\mu_{\max} I_{\mathrm{L}}}{K_{\mathrm{L}} + I_{\mathrm{L}} + I_{\mathrm{L}}^2/K_{\mathrm{i}}} \tag{7}$$

where $\mu_{\max}$ is the maximum specific growth rate, K_L is the light saturation constant, and K_i is the photoinhibition constant. The values of the constants $\mu_{\max}$, K_L, and K_i depend on the alga and the culture temperature (Richmond 1990).

The depth-averaged specific growth rate μ_{av} in the illuminated volume of the pond may now be estimated (Chisti 2012), as follows:

$$\mu_{av} = \frac{1}{L}\int_0^L \mu_L \mathrm{d}L \tag{8}$$

or

$$\mu_{av} = \frac{1}{L}\int_0^L \left(\frac{\mu_{\max} I_L}{K_L + I_L + I_L^2/K_\mathrm{i}}\right)\mathrm{d}L \tag{9}$$

where L is the distance from the surface. Equation (8) is applicable so long as $L \leq l_c$ where l_c is the depth at which the local irradiance level is at the light compensation point (Chisti 2012). If the pond has a dark zone, the self-consumption of the biomass will occur in this zone and the actual average specific growth rate will be lower than the value calculated using Eq. (9).

So long as all the nutrients are provided in excess and the temperature and pH are satisfactory, the productivity of biomass depends only on the availability of sunlight. Light enters the pond only through its exposed surface. The light available per unit volume of culture declines if the depth of the culture is increased. Therefore, shallower ponds are more productive than deeper ponds so long as the growth is exclusively photoautotrophic.

Unfortunately, in a raceway spanning a large area, achieving a culture depth of less than about 0.25 m is impractical as a small tilt of the bottom relative to the horizontal causes a large difference in depth in different parts of a large pond. A perfectly flat bottom is difficult to construct. In addition, to drive the circulation, the paddlewheel must create a hydraulic pressure gradient, so that the depth of fluid in front of the paddlewheel is higher than the depth behind the paddlewheel (Chisti 2012). If the static depth of the culture is too small, the region behind the paddlewheel could become too shallow for stable recirculation to occur.

2.12 Cost of Construction and Operation

Plastic lined earthen raceways are apparently the least expensive to build. Unlined earthen ponds are used in wastewater treatment operations, but not generally considered satisfactory for producing algal biomass (Chisti 2012). For a 100 ha plastic lined pond of compacted earth, a construction cost of $69,500 per ha has been estimated (Benemann et al. 1987). This historical cost data corrected for inflation (Chisti 2012) provides a reasonable estimate of the current cost. A cost of $144,830 per ha was estimated for 2014. This estimate included the earth works, the plastic lining, the carbon dioxide supply tubing, inlets and outlets, the baffles, the paddlewheel and motor (Benemann et al. 1987). The cost would be higher if, for example, the ends of the raceway and the dividing baffle are designed to eliminate dead zones

(Chisti 2012; Sompech et al. 2012). Plastic lined concrete ponds are significantly more expensive than the plastic lined compacted earth ponds (Chisti 2012). For a 5 ha, 0.35 m deep, unlined pond of compacted earth, intended for use in wastewater treatment, an installed cost of NZ$89,600 (June, 2009) per ha has been reported (Craggs et al. 2012). In 2014, such a pond would cost US$74,260 per ha. This includes the earth works, the carbon dioxide distribution piping, the flow deflector end baffles, the pH controller and valves, the paddlewheel, and the motor.

Ponds enclosed in glass houses or plastic-covered greenhouses are relatively protected from contamination compared to open ponds and allow a better control of the growth environment (Chisti 2012). Such ponds may be suitable for high-value low-volume products such as nutraceuticals and have been commercially used (Becker 1994; Lee 1997).

The cost of producing dry *Dunaliella* biomass in outdoor commercial raceway ponds in Israel has been estimated to be about $18/kg (Ben-Amotz 2012). This included the cost of purchasing the carbon dioxide, recovering the algal biomass from the broth by continuous flow centrifugation in disc-stack centrifuges and subsequent drying of the biomass paste. Both drying and biomass recovery by centrifugation tend to be expensive (Chisti 2012). This notwithstanding, when applicable, the raceway-based production of biomass is generally claimed to be the least expensive production option.

3 Biomass Production in Raceways

A raceway may be operated as a batch culture, or a pseudo steady state continuous culture. In a batch process, the nutrient medium is placed in the raceway and inoculated with a culture of the chosen alga. The inoculum generally constitutes about 10 % of the operating volume of the raceway. The inoculum is generally grown (Fig. 6) in the same medium as used in the raceway and is in exponential

Fig. 6 An early monoseptic stage of production of an algal inoculum for a small raceway

phase of growth just prior to inoculation. The biomass concentration in the inoculum is generally at least 0.5 kg m^{-3} if the inoculum has been produced in a raceway. Often, the concentration is much higher if a photobioreactor is used to produce the inoculum. Multiple inoculum generation stages may be necessary for inoculating a large raceway. Other than carbon dioxide, air for possible oxygen removal, and the makeup water, nothing is added to the raceway during a batch operation. The biomass grows to peak concentration of about 0.5, or 1 kg m^{-3} in the best of circumstances (Borowitzka 2005). Productivity is enhanced by periodic dilution of the raceway culture to maintain the biomass concentration at less than the typical maximum value. Dilution improves light availability in the raceway.

In a sunny locale with a stable diurnal temperature of ~25 °C, an alga capable of rapid growth can attain an average annual dry biomass productivity of around 0.025 kg m^{-2} d^{-1} in a well-operated raceway (Chisti 2012; Mendoza et al. 2013a), but higher daily productivities have been recorded (Terry and Raymond 1985; Grobbelaar 2000; Moheimani and Borowitzka 2007) during suitable weather conditions. Biomass productivities in excess of 0.05 kg m^{-2} d^{-1} have been documented (Weissman et al. 1989). Of course, not all algae are equally productive (Chisti 2012). In mixotrophic growth as is commonly encountered in high-rate algal ponds treating wastewater (Craggs et al. 2012, 2014), dissolved organic compounds contribute to growth leading to a generally higher productivity than is possible with purely photoautotrophic growth. For example, in a 0.2 m raceway pond operated mixotrophically, Sing et al. (2014) observed a peak dry biomass productivity of 37.5 kg m^{-2} d^{-1} over an extended period.

A photoautotrophic culture requires supplementation with carbon dioxide to attain high biomass productivity. The carbon dioxide in the ambient atmosphere is insufficient to support productivities that are biologically feasible under otherwise nonlimiting conditions. In the ambient atmosphere without supplemental inorganic carbon, the productivity in a raceway may be <13 % of the productivity likely to be possible with an unlimited supply of inorganic carbon (Raes et al. 2014). The peak biomass productivity attainable in a typical non-limiting raceway is far lower than the limit imposed by the algal biology (Chisti 2012).

Although high biomass productivities are biologically attainable, vagaries of weather influence an exposed raceway so much so that the maximum annual average productivity may be reduced to only 0.01 kg m^{-2} d^{-1}, or less (Chisti 2012). As a result, a raceway can take 4–6 weeks from inoculation to attain the peak biomass concentration of around 0.5 kg m^{-3} (Pulz 2001). Once the peak biomass concentration is attained, all or most of the broth may be harvested in a batch process to recover the biomass (Chisti 2012). The residual broth may become the inoculum for the next batch, or an entirely fresh batch may be initiated after the raceway has been cleaned (Chisti 2012).

A continuous culture generally begins as a batch operation. Once the algal biomass has grown to a sufficient concentration, the operation is switched to a continuous flow mode. In continuous culture, the raceway is fed with the fresh medium at some specified flow rate. The feed point is typically located just forward of the paddlewheel. During feeding, the algal broth is withdrawn, or harvested,

from the raceway at a rate equal to the feed flow rate. Feeding and harvesting occur only during daylight and must stop at night, or the biomass may washout out of the raceway overnight. Prolonged continuous culture operation under stable weather and day–night cycle allows a pseudo steady state biomass concentration to be maintained in the raceway (Chisti 2012). The daytime feed flow rate Q_f must be such that the dilution rate D remains below the maximum specific growth rate (μ_{max}) of the alga under the specific conditions of operation of the raceway (Chisti 2012). The culture will washout if the dilution rate exceeds the maximum specific growth rate. The dilution rate D is calculated as follows:

$$D = \frac{Q_f}{V_L} \tag{10}$$

where V_L is the working volume of the raceway.

Irrespective of whether batch or continuous operation is used, up to 25 % of the biomass produced by the end of a daylight period may be consumed during the following night through respiration (Chisti 2012). The magnitude of this respiratory loss depends on the irradiance level during growth, the daytime temperature of growth, and the temperature during the night (Grobbelaar and Soeder 1985; Richmond 1990).

3.1 Biomass Productivity

The biomass productivity of a culture system is a measure of its ability to produce biomass. Productivity may be expressed either in volume terms, or in terms of the surface area of the culture pond. In a batch culture, the volumetric productivity (P_v, kg m^{-3} d^{-1}) of the biomass is determined as follows:

$$P_v = \frac{X_f - X_i}{\Delta t} \tag{11}$$

where X_i (kg m^{-3}) is the initial concentration of the biomass, X_f (kg m^{-3}) is the peak concentration of the biomass, and Δt (d) is the time interval between inoculation and the attainment of the peak biomass concentration (Chisti 2012).

In a continuous flow operation, the volumetric productivity of the biomass is calculated using the following equation (Chisti 2012):

$$P_v = \frac{Q_f x_b}{V_L} \tag{12}$$

where Q_f is the flow rate of the feed to the pond, x_b is the pseudo steady state biomass concentration in the broth leaving the raceway and V_L is the volume of the broth in the raceway.

The areal biomass productivity (P_a, kg m^{-2} d^{-1}) and the volumetric productivity (P_v, kg m^{-3} d^{-1}) of a raceway are related as follows (Chisti 2012):

$$P_{\mathrm{v}} = \frac{P_{\mathrm{a}}}{h} \tag{13}$$

where h is the depth in m. Productivity is high in a dilute culture, but declines rapidly as the biomass concentration increases. The maximum attainable biomass concentration in a raceway is of the order of 0.5–1.0 kg m^{-3}.

4 Concluding Remarks

Large-scale commercial production of algal biomass generally relies on open raceway ponds. This chapter outlined the raceway pond design, operation, and limitations. Raceway ponds require a relatively low investment in capital and, therefore, remain the production system of choice despite their low productivity.

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Large-Scale Production of Algal Biomass: Photobioreactors

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Abstract Photobioreactors have been used extensively for the cultivation of microalgae for a variety of applications from biofuels to high value products. The ability to cultivate monocultures of algae with high biomass yields and significantly smaller footprints has made photobioreactors a very attractive technology for specific applications. This chapter deals with photobioreactor design, application, efficiencies, and factors affecting their performance.

Keywords Microalgae · Photobioreactors · PBR productivity · Photoinhibition · Biomass production

Nomenclature

A	Local specific radiant energy absorbed (μmol s^{-1} kg^{-1})
a_{light}	Specific illuminated area for the photobioreactor (m^{-1})
C_X	Biomass concentration (kg m^{-3})
D	Dilution rate (h^{-1} or s^{-1})
E_a	Mass absorption coefficient (m^2 kg^{-1})
f_d	Design dark volume fraction of any photobioreactor (dimensionless)
G	Local irradiance (μmol s^{-1} m^{-2})
G_c	Compensation irradiance value (μmol s^{-1} m^{-2})
K	Half saturation constant for photosynthesis (μmol s^{-1} m^{-2})

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L Depth of the rectangular photobioreactor (m)
M_X C-molar mass for the biomass ($kg_X\ mol_X^{-1}$)
q Photon flux density on a given surface (PFD) ($\mu mol\ s^{-1}\ m^{-2}$)
Q Volume liquid flow rate ($m^3\ d^{-1}$)
r_X Biomass volumetric growth rate (productivity) ($kg\ m^{-3}\ s^{-1}$ or $kg\ m^{-3}\ h^{-1}$)
S_{light} Illuminated surface of the photobioreactor (m^2)
P_S Areal biomass productivity ($kg\ m^{-2}\ d^{-1}$)
P_V Volumetric biomass productivity ($kg\ m^{-3}\ d^{-1}$)
t Time (days or s)
V_r Photobioreactor volume (m^3)
x_d Diffuse fraction for incident PFD at any location (−)
z Depth of culture (m)

Greek Letters

α Linear scattering modulus (dimensionless)
β Inclination of the photobioreactor surface (rad)
γ Fraction for working illuminated volume in the photobioreactor (dimensionless)
δ Extinction coefficient for the two-flux method (m^{-1})
θ Incident angle (defined from the outward normal of the PBR) (rad)
λ Wavelength (m)
ρ_M Maximum energy yield for photon conversion (dimensionless)
τ_p Residence time (h)
τ_λ Absorption optical thickness, $\tau_\lambda = Ea_\lambda C_X / a_{light}$ (dimensionless)
φ'_X Biomass mole quantum yield for the Z-scheme of photosynthesis ($mol_X\ \mu mol_{hv}^{-1}$)

Subscripts

max Related to maximum available value
opt Related to the optimal value for residence time

Other

$$\langle X \rangle = \frac{1}{V} \iiint_V X \mathrm{d}V$$ Spatially-averaged property

$$\overline{X} = \frac{1}{\Delta t} \int_{\Delta t} X \mathrm{d}t$$ Time-averaged property

Abbreviations

PAR Photosynthetically active radiation
PBR Photobioreactor
PFD Photon flux density

1 Cultivation Systems

1.1 Requirements for Photosynthetic Growth and Possible Limitations

Photosynthetic growth in standard autotrophic conditions is based on the assimilation, under illumination, of inorganic carbon and mineral nutrients dissolved in the medium. Cultivation of photosynthetic microorganisms requires the following: (1) sunlight or an artificial light source, with an appropriate emission spectrum in the photosynthetically active radiation (PAR) region, ranging between 400 and 700 nm, (2) an inorganic carbon source, such as dissolved CO_2, (3) mineral nutrients including major nutrients such as N, S, P and micronutrients such as Mg, Ca, Mn, Cu, Fe, etc., and (4) a favorable set of culture conditions including pH, temperature, and dissolved oxygen.

Quantitatively, the main variables affecting photosynthetic growth and productivity of microalgae cultivation systems are (1) the incident light characterized by its photons flux density (PFD) with given angular distribution and spectrum, (2) the concentrations of various compounds in the liquid phase affecting growth such as dissolved inorganic carbon, dissolved oxygen, and growth mineral nutrients, and (3) culture conditions such as pH, temperature, and possible biological contamination. Designing and operating a microalgal cultivation system aims to optimize these conditions with the objective of maximizing growth. Control of these growth conditions is significantly more complex in industrial scale systems under outdoor conditions than in indoor benchtop systems. In fact, maintaining optimal growth conditions is very difficult to achieve for microalgae cultivation systems under highly variable outdoor conditions and scaling up the bioprocess features' major technical challenges discussed in this chapter.

1.2 Open Systems and Closed Photobioreactors

Open systems, such as natural ponds and raceways, are currently the most employed technology for outdoor solar cultivation. In fact, they have been used for several decades at industrial scale (Borowitzka 1999; Carvalho et al. 2006; Molina Grima et al. 1999; Morweiser et al. 2010; Pruvost 2011; Pulz 2001; Richmond 2004a; Ugwu et al. 2008). The main limitations of open systems are inherent to their operating principles. First, they are exposed to high risks of biological contamination by other microalgae species, bacteria, and/or predators owing to the direct contact of the culture with the atmosphere. Therefore, only resistant species can be cultivated for long periods of time. The large interface between the culture and the atmosphere also renders the control of culture conditions difficult. For example, it is difficult to maintain an optimal temperature although open systems are less subject to overheating than closed systems. In addition, the relatively low atmospheric CO_2 concentration generally results in small concentrations of dissolved carbon in the

culture medium, often insufficient to meet the needs of photosynthetic microorganisms in intensive biomass production. Thus, a carbon source can be added by injecting CO_2 gas (or chemical carbonate). However, a significant part of dissolved inorganic carbon is inevitably degassed into the atmosphere. In practice, this makes carbon limitation difficult to prevent entirely in open systems.

Closed systems, often called "photobioreactors" (PBRs), reduce risks of external contamination and provide better control of growth conditions. For example, CO_2 can be sparged into the PBRs. Then, the larger gas partial pressure in the bubbles and PBR headspace prevent carbon limitation. However, PBRs also suffer from several limitations inherent to their operating principles. First, culture confinement increases the risk of biofilm formation on the PBR walls. It leads to oxygen accumulation in the culture which can have possible toxic effects on photosynthetic growth. It may also cause overheating of the culture especially under solar radiation due to the large amount of infrared radiation absorbed by the culture medium (Borowitzka 1999; Carvalho et al. 2011; Grobbelaar 2008; Torzillo et al. 1996). Unlike challenges faced by open systems, those affecting closed PBRs can be overcome in part by appropriate engineering solutions such as optimizing mixing conditions to increase heat transfer and gas–liquid mass transfer or to prevent biofilm formation. Once all these challenges have been addressed, light remains the only limiting factor. In other words, the amount of light received and its use by the culture will determine the productivity of the system.

Finally, solutions designed to overcome the different technical challenges previously mentioned result, most often, in increased cost and complexity. Current industrial scale biomass production is mainly performed in large open systems because they are easier to build and operate than PBRs. However, PBR technology offers higher potential for improvement in terms of productivity and efficiency. Great efforts are currently underway to develop new technologies devoted to industrial-scale production in PBRs.

1.3 Photobioreactors Principles

There exists a wide variety of PBRs technologies such as tubular, cylindrical, or flat panel systems, as illustrated in Fig. 1. This diversity of PBR designs results from various attempts to optimize light capture while satisfying other practical constraints related to (i) engineering design including system integration, scale of production, materials selection, and cost and to (ii) system operation concerned with CO_2 bubbling, oxygen removals, temperature and pH regulation, nutrient delivery, etc. Numerous reports and publications can be found in the literature on the various PBR technologies available. All of them have advantages and limitations in terms of control of culture conditions, culture confinement, hydrodynamics conditions, easiness to scale up, construction cost, biomass productivity, and energy efficiency (Borowitzka 1999; Carvalho et al. 2006; Grima et al. 1999; Morweiser et al. 2010; Pruvost 2011; Pulz 2001; Ugwu et al. 2008). Regardless of the PBR

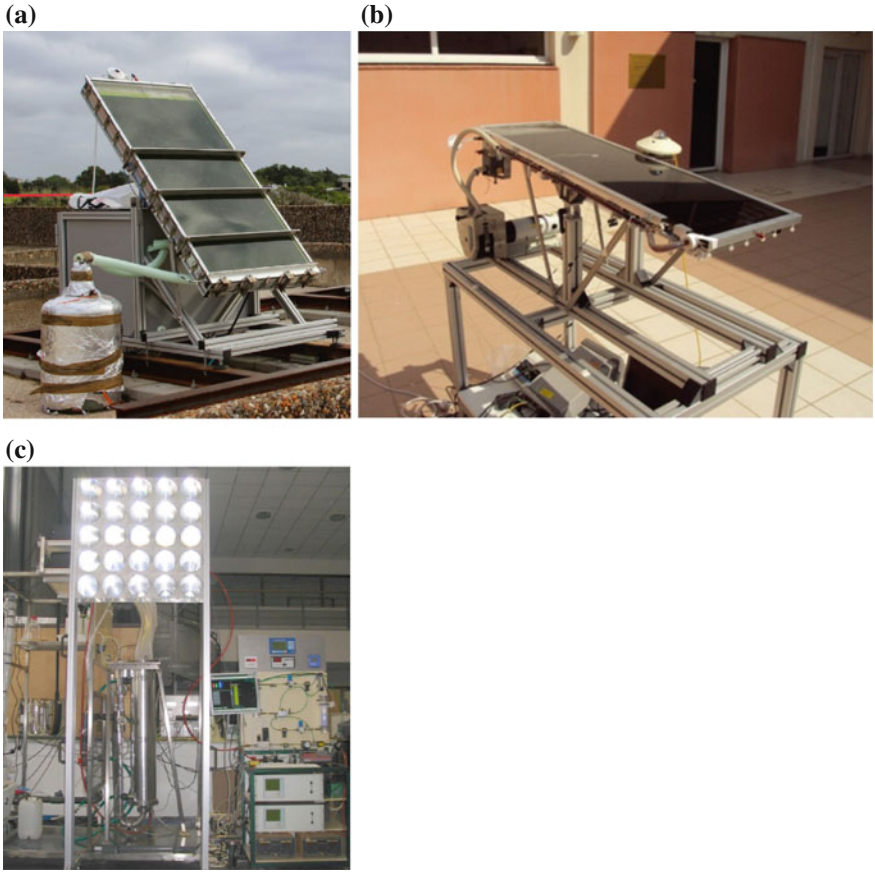

Fig. 1 Examples of solar photobioreactor technologies. **a** Flat panel solar PBR (GEPEA, University of Nantes, France). **b** AlgoFilm© solar PBR (ultrathin PBR) (GEPEA, University of Nantes, France). **c** DiCoFluV© solar photobioreactor with Fresnel lenses for sun capture and lateral diffusing optical fibers inside the reactor (Institut Pascal, Clermont-Fd, France)

concept, the goal is to provide sufficient control of the culture conditions to make the process only limited by the amount of light supplied and the photosynthetic process in the culture. The photon flux densities (PFDs) incident onto the PBR surface and locally available inside the culture are major parameters. Although maximizing light intercepted must be an obvious consideration of any microalgal cultivation system (as it is for any solar process), other constraints also have to be considered. For example, using the airlift method for mixing will preclude horizontal geometries. In addition, shading must be accounted for when arranging vertical or tilted systems on a given land area. Therefore, optimizing photobiological cultivation systems proves more complex than other traditional solar-driven processes, such as photovoltaic panels, where the amount of intercepted light is the only operating parameter of any given panel technology.

1.4 Surface and Volumetric Illuminations

Light can be supplied in two general ways, either by direct illumination of the cultivation system or by distributing light sources inside the culture volume. Then, one distinguishes between surface-illuminated and volumetrically illuminated systems, respectively. Most cultivation systems fall in the simpler surface-illuminated category (Carvalho et al. 2006; Morweiser et al. 2010; Richmond 2004a; Ugwu et al. 2008). As for any solar processes, various positioning options have been considered including systems positioned horizontally (Acién Fernández et al. 2001; Oswald 1988; Molina et al. 2001), vertically (Chini Zitelli et al. 2000, 2006; Pulz 2001), and in few cases, tilted (Doucha and Livansky 2006; Lee and Low 1991; Richmond and Cheng-Wu 2001). However, maximizing the incident solar radiation flux is not trivial. It obviously depends on the longitude and latitude of the system's location and on the day of the year. For example, horizontal systems are best suited for locations close to the Equator (latitude 0°). For higher latitudes, it is necessary to tilt the system exposed surface to maximize the amount of light collected. Roughly speaking, the optimum inclination angle with respect to Earth surface which maximizes light capture over the year on a fixed PBR corresponds to the latitude of the PBR location (Duffie and Beckman 2006; Hu et al. 1996; Pruvost et al. 2012; Richmond and Cheng-Wu 2001). Inclination angle can also be adjusted as a function of time to optimize light capture. For example, flat panel equipped with sun-tracking systems were tested by Hindersin et al. (2013). This method not only maximized light capture during the day but also prevented excessive incident irradiation on the systems around noon, by temporarily setting the illuminated surface of the PBR perpendicular to the sun collimated irradiation.

Volumetrically illuminated systems require more complex technologies than surface-illuminated systems. However, they enable the optimization of the light delivery and use in the culture. First, inserting light sources in the volume of the culture guarantees maximal use of the collected or emitted photons. Second, and more interestingly, internal lighting allows light to be "diluted." Increasing PFD leads to higher volumetric productivity but associated with a progressive decrease in the conversion yield, due to photosynthesis saturation. By diluting the light incident on the system's surface into the volume of the culture, a larger yield can be maintained. This is of particular interest in outdoor PBRs exposed to sunlight. In this case, solar radiation incident on a given surface is collected using, for example, a parabolic solar collector. It is then delivered to the culture in a controlled manner, using optical fibers (Cornet 2010; Csogör et al. 2001) or light guides (Pilon et al. 2011), for example. Because of the large PFD characteristic of solar conditions, an increase in surface productivity can be achieved. Note that the optical connection between the light collection device and the light delivery system needs to be carefully designed as it can be the source of major optical losses. Furthermore, light dilution can be combined with a solar tracking system, giving an additional possibility of optimization by maximizing light intercepted as the sun travels in the sky (Hindersin et al. 2013). A full description of such a principle has been described by

Cornet (2010) with a volumetrically lightened photobioreactor based on the "DiCoFluV" concept. Despite their promise, only a few examples of volumetrically illuminated PBRs can be found in the literature (Cornet 2010; Csogör et al. 2001; Hsieh and Wu 2009; Ogbonna et al. 1996; Zijffers et al. 2008). This is mainly explained by the increase in technological complexity and by the difficulty in scaling up PBR systems to large surface areas.

2 Photobioreactor Engineering and Scaling Rules

2.1 Maximizing Biomass Production

Most growth limitations previously mentioned can be avoided, or at least greatly reduced, thanks to proper engineering design of the cultivation system. This is especially true for nutrient and CO_2 limitations in closed PBRs. Note, however, that light limitation cannot be avoided due to the rapid light attenuation in the culture and to the large energy requirement of photosynthesis. This simple yet important observation is central to the optimization of microalgae cultivation systems. One major practical consequence involves the need to develop PBR with geometries able to optimize light supply to the culture. But, as detailed hereafter, working under light limitation will also facilitate the design and control of efficient processes. Light will be indeed the only parameter to control. This implies however that the effects of light on the process should be accurately taken into account.

2.2 Growth Limitations by Nutrient and Inorganic Carbon Sources

In order to prevent mineral limitation, the growth medium must contain all the necessary macro- and micronutrients in sufficient quantities based on the expected biomass concentration. Stoichiometric equations can be used for this purpose, or nutrient concentrations can be monitored and adjusted during cultivation. The interested reader is referred to studies in which this method has been applied to various species (Pruvost et al. 2009; Pruvost 2011).

Inorganic carbon source comes from CO_2 gas dissolved in the culture medium or from directly adding chemical carbonate compounds in the medium. In both cases, a minimum amount of total dissolved carbon (TDC) of about 5–10 mM is necessary to avoid carbon limitation on the microorganisms' growth. When using gaseous CO_2, the minimum TDC depends on the pH, the biological consumption rate, and the gas–liquid mass transfer rate. The latter is affected not only by the magnitude of the mass transfer coefficient $k_L a$ but also by the driving force of the carbon dissolution in water determined by thermodynamics equilibria of the chemical reactions involved.

TDC can be determined by performing a mass balance analysis on the cultivation process or by directly monitoring TDC using a total inorganic carbon measurement, for example (Degrenne et al. 2010). This is analogous to oxygen dissolution in aerobic cultivation of yeasts or bacteria. The main significant difference lies in the fact that CO_2 dissolution also affects the pH of the growth medium, i.e., excess supply of CO_2 leads to acidification of the medium. In turn, this influences the amount and type of dissolved carbon (CO_2, HCO_3^-, and CO_3^{2-}) in the culture. Overall, the carbon feeding strategy requires maintaining the pH optimal for growth while averting carbon limitation. This may not be trivial considering that mineral nutrient consumption during growth can also participate in the water chemistry of carbon. However, in most cases, simple CO_2 bubbling is usually sufficient for regulating both pH and TDC. This could be more difficult when ammonium is used as a nitrogen source since its consumption during photosynthetic growth also leads to acidification (Ifrim et al. 2014).

2.3 The Light-Limited Regime

The control of cultivation conditions such as pH and temperature can be challenging in practice, especially in outdoor conditions (Borowitzka 1999; Grobbelaar 2008; Richmond 2004a; Torzillo et al. 1996). These challenges, however, can be overcome with an adequate engineering and control of the cultivation system. If all cultivation conditions are kept at their optimal value, and nutrients are provided in adequate amounts, light-limited conditions should eventually occur. The light-limited regime has several major features. The first consequence is that, by definition, the culture is not subject to any other limitation. Thus, maximum biomass productivity can be achieved and is determined by the amount of light provided and its use by the culture (Takache et al. 2010; Pruvost 2011; Pruvost et al. 2011b, 2012; Pruvost and Cornet 2012). Any limitation other than light limitation would result in further decrease of biomass productivity while maximizing the PFD received onto the cultivation system increases the productivity. Note that this remains valid in the case of high PFD leading to photoinhibition of photosynthetic apparatus (PFD roughly superior to 400 $\mu mole_{hv}\ m^{-2}\ s^{-1}$). Special attention should be paid to light attenuation conditions to avoid or at least greatly reduce photoinhibition phenomena by operating the PBR to achieve complete light extinction in the culture, as described in detail in the next section. A second important consequence is that, in the light-limited regime, controlling the incident light and its effect on the process leads to the control of the entire cultivation system performance. This corresponds to the so-called «physical limitation» in chemical engineering, when the process is limited by one parameter whose control enables the control of the entire process. This last feature is essential to the efficient design and operation of photobiological cultivation systems.

2.4 Role of Light Attenuation Conditions and Absorption Rate

The light-limited regime is a necessary but not sufficient condition to obtain maximal biomass productivity in any given PBR. Appropriate light transfer (or light attenuation) conditions have to be established inside the culture volume (Cornet and Dussap 2009; Pruvost 2011; Takache et al. 2010). On the one hand, if the biomass concentration is too low, part of the incoming photons is not absorbed and instead is transmitted through the culture (Fig. 2b, case A). This results in a loss of biomass productivity. In addition, the light received per cell is large and may lead to further decrease in productivity due to the increased photosynthetic dissipation. It may also induce a decrease in algal pigment content and thus lead to further increase in light transmission. As a consequence, the system becomes highly unstable resulting usually in culture washout. Thus, such conditions should be avoided in microalgae cultivation, especially for large incident PFD, typically larger than 200 $\mu mole_{hv}\ m^{-2}\ s^{-1}$. On the other hand, if the biomass concentration is too large, a dark zone appears inside the culture (Fig. 2b, case B). This dark zone is the direct consequence of light extinction by cells suspension, whose effect can be positive in the case of high illumination conditions by reducing photoinhibition effect and then increasing process stability (Carvalho et al. 2011; Grima et al. 1999; Richmond 2004b). Note that for microorganisms with respiration activity under illumination such as eukaryotic microalgae, a dark zone in the culture volume promotes respiration resulting in a loss of biomass productivity. Therefore, achieving the maximum biomass productivity requires in this case the exact condition of complete absorption of the incident light (Takache et al. 2010), but without a dark zone in the culture volume, as illustrated in Fig. 2b, case C. This condition is often referred to as luminostat mode. Note that it should not be confused with turbidostat mode referring to a turbidity-based regulation of a continuous culture. This condition has also been introduced as the "$\gamma = 1$" condition where γ denotes the ratio of the volume of the PBR illuminated to the total volume of the culture (Cornet et al. 1994; Takache et al. 2010). For microorganisms with negligible respiration activity under illumination, such as prokaryotic cyanobacteria cells, fulfilling the condition of complete light absorption ($\gamma \leq 1$) will be sufficient to reach the maximum biomass productivity.

Another way to represent the strong correlation between light attenuation conditions and the associated biomass productivity is to calculate the rate of photons absorption per unit volume of culture denoted by $\langle A \rangle$. The latter can be obtained by considering the spectral specific absorption coefficient Ea_λ(in m^2/kg) of the cultivated species and the local spectral irradiance G_λ inside the culture of total volume V_R (Aiba 1982; Cassano et al. 1995; Kandilian et al. 2013):

$$\langle A \rangle = \frac{1}{V_R} \int_{\Delta\lambda} \iiint_{V_R} Ea_\lambda G_\lambda \mathrm{d}V \mathrm{d}\lambda \tag{1}$$

where $\Delta\lambda$ denotes the PAR range (400–700 nm).

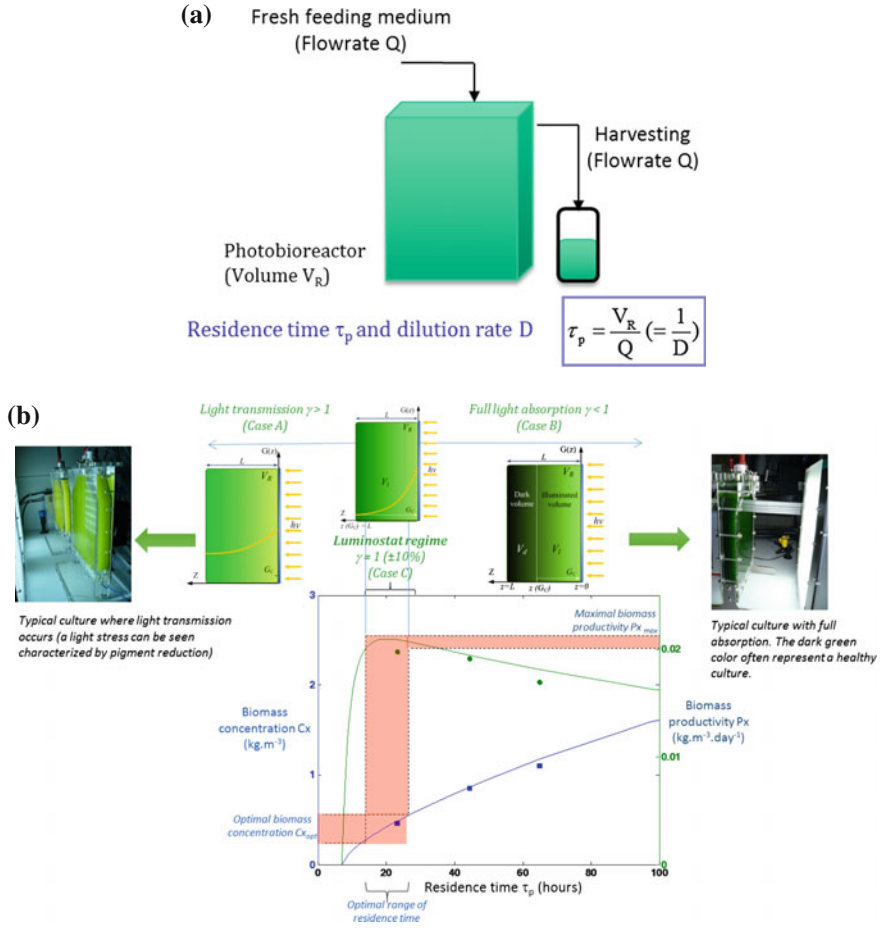

Fig. 2 Role of light attenuation conditions on biomass productivity in PBR. The illustration is here given for a PBR operated in continuous mode (**a**). The residence time (or dilution rate) applied allows controlling biomass concentration in the culture volume and then light absorption conditions, thus affecting the resulting biomass productivity. The relation with illuminated and dark zone repartition in the culture volume is given in (**b**), and the relation to the rate of photons absorption is given in (**c**). Both can be used independently to maximize biomass productivity of any cultivation system. Values are here given for Chlorella vulgaris (PFD = 200 μmole m^2 s, PBR depth = 0.04 m)

Increasing the biomass concentration in the cultivation system will result in a decrease of the rate of photons absorption by the cultivation system (Fig. 2c) due to stronger light attenuation and the resulting smaller irradiance G_λ. As a result, the maximum biomass productivity will be obtained for an optimal value of the rate of photons absorption, typically around 10–12 $\mu mole_{h\nu}\ g^{-2}\ s^{-1}$ (Fig. 2c). Note that this representation is consistent with the condition of luminostat regime ($\gamma = 1$), and

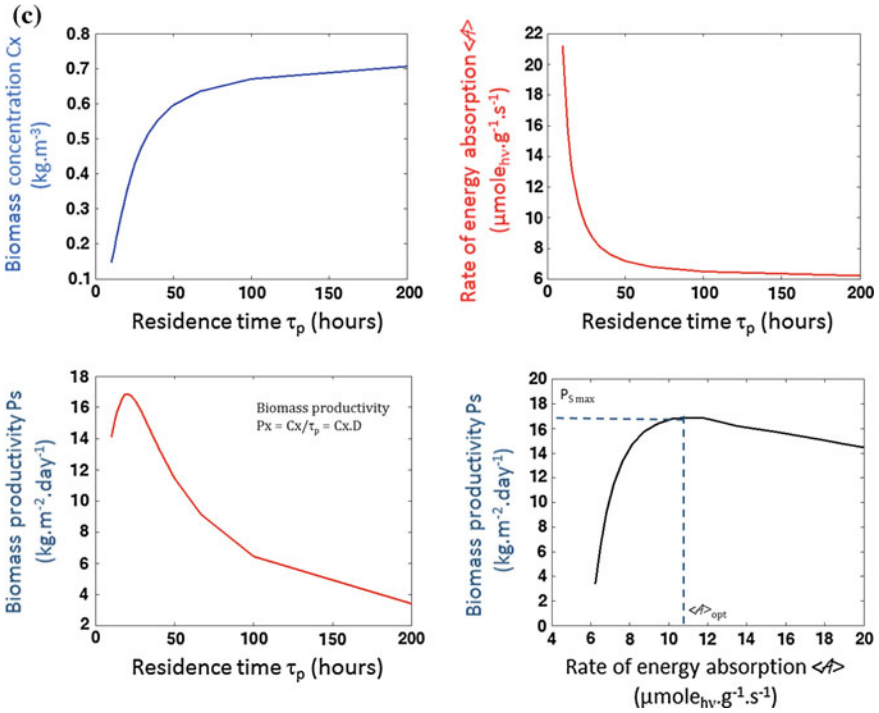

Fig. 2 (continued)

both approaches can be used to maximize the biomass productivity of any cultivation system.

One major consequence of the previous considerations is that, for any PBR and photosynthetic microorganism, light transfer from the collection site to the delivery inside the volume of the culture has to be taken into account. This analysis depends on the angular distribution of PFD, the PBR design, the biomass concentration, and the microorganisms' radiation characteristics. In practice, light attenuation conditions can be controlled by adjusting the biomass concentration. This can be achieved in continuous or semicontinuous cultivation mode, by modifying the residence time value τ_p of the microorganisms in the cultivation system represented by the dilution rate D such that $\tau_p = 1/D = V_r/Q$ where Q represents the medium flow rate through the PBR. The reader is referred to Takache et al. (2010) for a detailed example of such optimization in continuous mode. In batch mode, $\langle A \rangle$ depends on time due to the continuous increase in biomass concentration making difficult the control and optimization of light absorption. To that effect, a simple and robust control strategy of the incident light has been recently proposed and demonstrated with benchtop PBRs (Kandilian et al. 2014).

In the specific case of solar production, maintaining optimal light attenuation conditions, even in continuous or semicontinuous operations, is far from a trivial

task (Grognard et al. 2014; Hindersin et al. 2013; Pruvost et al. 2011a). The process is indeed strongly time dependent and driven by an uncontrolled and highly variable input, namely the solar incident flux. The growth kinetics of biomass is slow compared with the rapid variations in incident sunlight intensity and prevents the operator from establishing a luminostat regime. At best, a compromise needs to be found to determine the conditions approaching this ideal set point for most of the day. Whatever the case, light attenuation within the culture is not easy to determine. To do so, light transfer modeling is essential (Cornet et al. 1992a, b, 1995; Lee et al. 2014; Pilon et al. 2011; Pruvost and Cornet 2012; Pruvost et al. 2012). Furthermore, it can be associated with kinetics models of photosynthetic growth for a complete representation of the cultivation system characterized by its biomass concentration and biomass productivity. The reader is referred to the following studies for further information (Cornet 2010; Cornet and Dussap 2009; Lee et al. 2014; Takache et al. 2010).

2.5 PBR Efficiencies and Intensification Principles

Among the various criteria characterizing the performance of microalgal cultivation systems, biomass productivities per illuminated surface area and per unit volume of culture as well as light to biomass energy conversion efficiency are of primary relevance.

Surface biomass productivity P_s (in g m^{-2} d^{-1}) gives the area required to achieve a given amount of biomass produced per day. In light-limited regime, P_s depends only on the PFD received by the culture and on the photosynthetic conversion yield. Productivity is then a function of location, meteorological conditions, and of the system's ability to capture sunlight depending on its design, inclination, and orientation. A useful and simple engineering equation taking into account those parameters was proposed to estimate the maximum surface productivity $P_{s,max}$ of any PBR (ponds, flat plate, tubular, …) knowing only mean yearly solar information (Pruvost and Cornet 2012):

$$P_{s,max} = (1-f_d)\rho_M M_X \bar{\phi}'_X \frac{2\alpha}{1+\alpha}\left[\frac{\bar{x}_d K}{2}\ln\left[1+\frac{2\,\bar{q}}{K}\right] + (1-\bar{x}_d)\overline{\cos\theta}\,K\ln\left[1+\frac{\bar{q}}{K\overline{\cos\theta}}\right]\right] \quad (2)$$

where the parameters specific to a given microorganism species are (i) the linear scattering modulus α (default value 0.9), (ii) the molar mass M_X, typically around 0.024 kg_X/mol_X, (iii) the half saturation constant for photosynthesis K, usually about 100 μmol_{hv} m^{-2} s^{-1}, and (iv) ρ_M the maximum energetic yield for photon conversion ($\sim$0.8). The location, time of the year, and the ability of the cultivation system to collect light are accounted for by (a) $\bar{x}_d$ the fraction of diffuse radiation in

the total incident solar flux density (PAR), typically around 0.1–0.5 (b) the cosines of the incident angle θ onto PBR surface $\overline{\cos\theta}$ usually ranged between 0.4 and 0.7 and (c) the incident PFD $\bar{q}$ (all values here averaged on a given operating period). Here, f_d is the volume fraction of the PBR in the dark which cannot be lit by the incident or scattered PFD because of the system design, as for example when using a recirculating tank.

Equation (2) was originally developed and validated for cyanobacteria (Cornet 2010). It has also been validated for microalgae (Takache et al. 2010). It should be treated as a reliable and convenient tool for calculating the productivities of cultivation systems during design and operation of PBRs. Please note that the logarithmic relation in Eq. (2) accounts for the decrease in photosynthetic conversion efficiency with increasing PFD. Increasing PFD increases the surface productivity of the system but reduced its energy efficiency.

Volumetric biomass productivity P_v (i.e., g m^{-3} d^{-1}) gives the volume requested for a given production rate. Its maximum value $P_{V,max}$ can be estimated from Eq. (2) according to

$$P_{V,max} = \frac{P_{s,max} S_{light}}{V_R} = P_{s,max} a_{light} \tag{3}$$

This expression introduces an important parameter, $a_{light} = S_{light}/V_R$, corresponding to the specific illuminated surface to PBR volume ratio. In contrast to surface productivity which depends only on the ability of the system to collect light, volumetric productivity depends on the engineering design of the cultivation system represented by a_{light}. This parameter can cover two orders of magnitude in practice, ranging from 1 to 10 m^{-1} for systems presenting culture depths larger than 10 cm to values of 100 m^{-1} or larger for systems with thin culture less than 10 mm.

Equations (2) and (3) reveal that the volumetric productivity P_v increases with increasing a_{light} for constant surface productivity P_s which is independent of a_{light}. Increasing the incident PFD results in an increase in both surface and volumetric productivities providing that adequate light attenuation conditions prevail, as explained in Sect. 2.4. A typical example is given in Fig. 3 for realistic values of a_{light} corresponding to depths of culture commonly encountered in actual systems and in intensified technologies presenting ultrathin culture (see further description of AlgoFilm© technology).

Both surface and volumetric productivities represent the kinetics efficiencies of a cultivation system. Energy efficiency is also a relevant performance indicator for energy applications of PBRs. The rigorous thermodynamic efficiency (the exergetic yield of the PBR) is defined (Cornet et al. 1994) as the ratio of the volumetric chemical power produced over the volumetric light absorption rate $\langle A \rangle$ [Eq. (1)] and can be roughly assimilated to the photosynthetic conversion efficiency of the PBR (PCE). Whereas the kinetics performances increase with increasing PFD, the PCE decreases with PFD because photosynthesis rapidly saturates with light (Cornet 2010; Wilhelm and Selmar 2011). This consideration is the basis of the light dilution principle applied to the design of solar cultivation technologies aiming to

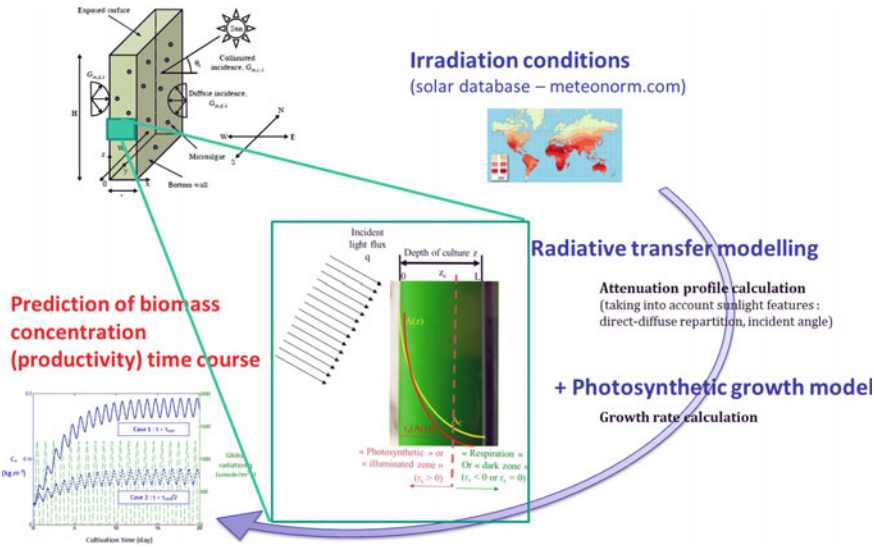

Fig. 3 Overview of the general modeling approach used to simulate solar PBR (for details, see Pruvost and Cornet 2012; Pruvost et al. 2011a, b, 2012)

optimize the rate of biomass produced per unit footprint of PBR. In this case, the surface area used for collecting the sunlight is smaller than the surface area illuminating volumetrically the culture inside the PBR. Their ratio is the so-called geometric dilution ratio which is less than unity. In other words, the PFD inside the culture is lower than the PFD of the captured sunlight. This concept leads then to larger PCE and larger biomass production rates per unit surface area used for collecting light.

3 The Specific Case of Solar Photobioreactor Engineering

3.1 The Use of Sunlight

Outdoor conditions and the use of sunlight as the primary energy source result in several challenges in the engineering design and control of outdoor cultivation systems. The amount of direct and diffuse solar incident irradiances as well as the strongly time-dependent incident PFD and the associated incident angle have been found to significantly affect the process efficiencies (Pruvost et al. 2011a, 2012). Consequently, although the luminostat regime is the ideal case leading to maximum biomass productivity, it cannot be maintained under solar conditions because of the rapid variations in light with time compared with the biomass concentration (Hindersin et al. 2013; Pruvost et al. 2011a, 2012). Thus, a compromise in design and operation has to be found. This can be achieved, in continuous PBRs, by

defining for example, a residence time that maximizes the yearly biomass productivity by controlling the temporal evolution of the biomass concentration and the light attenuation in the PBR. Moreover, oversaturating light can be received by cells, especially for high PFD typically larger than 400 μmole m^{-2} s^{-1}. Such PFD are commonly encountered in most Earth's location in the summer. It is known to impair culture health and possibly induce biological drift (i.e., photoinhibition) which can lead to process instability. Strong light attenuation in the PBR is known to have a positive effect as it decreases the amount of light energy received per cell along the depth of the PBR (Carvalho et al. 2011; Hindersin et al. 2013; Torzillo et al. 1996). Overall, these examples reveal that controlling the biomass concentration is a key aspect of optimizing the operation of solar PBR as it directly affects the PBR productivity and its stability.

3.2 Thermal Regulation Issues

Like in any biological process, temperature directly influences photosynthesis and microorganism growth. Particularly under solar illumination, closed PBRs tend to overheat and open systems may suffer from evaporation of water under strong incident irradiance. This can be attributed to culture confinement and to the strongly exoenergetic photosynthetic growth (Carvalho et al. 2011; Hindersin et al. 2013; Torzillo et al. 1996; Wilhelm and Selmar 2011). In fact, the thermodynamic efficiency over the PAR region of systems working with low light typical of artificial illumination (100–300 $\mu mole_{h\nu}$ m^{-2} s^{-1}) is in general below 5 % (Cornet 2010) and decreases to 2 % under large solar irradiance (>500 $\mu mole_{h\nu}$ g^{-2} s^{-1}). As a result, around 95 % of the captured light is converted into heat. In fact, under outdoor conditions, around 50 % of the energy in the solar radiation is contained in the near- and mid-infrared above 750 nm and directly participates in heating up the culture (Goetz et al. 2011; Hindersin 2013; Hindersin et al. 2013, 2014).

Thermal regulation of PBRs has been widely investigated as a major issue of solar microalgal cultivation (Borowitzka 1999; Grobbelaar 2008; Hindersin et al. 2013, 2014). Unfortunately, without proper thermoregulation, temperatures lethal to living microorganisms can easily be reached inside the PBR. For temperate climates during winter, excessively low temperatures can result in loss of biomass growth and productivity. Then, heating the culture can be beneficial (Hindersin 2013). The appropriate temperature window depends strongly on the species cultivated. However, it typically ranges between 10 and 30 °C.

Various solutions for heating or cooling PBRs are available, depending of the PBR technology, size, and location. Water cooling and/or heating by spraying on the PBR outside surfaces or by direct PBR immersion in a pool are often used (Borowitzka 1999). In temperate regions, cultivation systems can also be placed in greenhouses. Although efficient, those methods can increase the construction and operating costs and negatively impact the environmental footprint through excessive energy and water consumptions.

Although technical solutions currently exist, PBR temperature control remains a challenge under solar conditions, especially if cost-effective solutions with low energy consumption and year-round operation are sought. This can lead to the need for both cooling and heating. The engineering of the cultivation system is also relevant. For example, Goetz et al. (2011) experimentally and theoretically investigated the effect of various designs of a flat panel PBR. Depending on the configuration, the authors observed a decrease of up to one order of magnitude in the PBR energy consumption. IR filtering, for example, was found to be especially effective at reducing culture overheating. More recently, research efforts have investigated the integration of PBR technology into building façades. This integration offers various benefits regarding thermal management of both PBRs and buildings. Energy exchanges between the building and the PBRs can indeed be designed so as to cool or warm each one of the subsystems. For example, PBRs can filter sunlight in summer to reduce the thermal load on the building. In winter, excess thermal energy in the cultivation system can be used to warm the building. Finally, the added thermal mass of the building can be used to facilitate PBR thermal regulation regardless of the season.

Overall, thermal regulation of PBR depends on the location, the time of year, and on the strain cultivated. Cooling and/or heating requirements have to be estimated (usually in the range 50–200 W m^{-2}) and the associated thermal solutions should be defined and integrated at the early stage of the system's design. For climate with large variations in outdoor temperature and solar irradiation during the course of a year, it could be beneficial to cultivate different species with optimal growth at different temperatures (Hindersin 2013). This could lead to a significant decrease in energy needs.

3.3 Modeling for Solar PBR Optimization

Biophysical modeling of photobiological processes in solar PBRs aims to relate the various design and operating parameters to the productivity and efficiency of microalgal cultivation systems. Such modeling should account for the complex phenomena involved in the process and particularly the coupling between light transfer in the culture and photosynthetic growth. Several recent studies have modeled solar PBR operation with the aim to optimize productivities as a function of PBR design, location, and/or cultivated species (Pruvost et al. 2011a, 2012; Quinn et al. 2011; Slegers et al. 2011, 2013a, b).

Based on in-depth modeling efforts, engineering rules and formulae have been derived to design, optimize, and control PBRs in a predictive and rational way. Those tools are today available for both artificial light sources and sunlight and for systems based on either surface or volumetrically illuminated PBRs. The interested reader is referred to the manuscript by Pruvost et al. (2011a) for a complete description of solar PBR model and to those by Pruvost and coworkers (Pruvost et al. 2011b, 2012; Pruvost and Cornet 2012) for more detailed investigations.

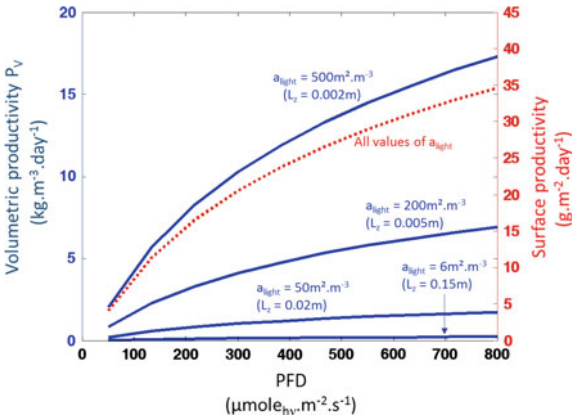

Fig. 4 Maximal volumetric and surface biomass productivities of the microalga *Chlamydomonas reinhardtii* as a function of incident PFD. Results are given for various typical values of illuminated surface-to-volume ratio (a_{light}). Corresponding depths of culture for flat panels are also given for information ($a_{light} = 1/L_z$)

In general, current models mainly aim at relating sunlight conditions obtained from meteorological database to growth kinetics so as to predict PBR performances, as illustrated in Fig. 4 (Pruvost et al. 2011a; Quinn et al. 2011; Slegers et al. 2011). These models can provide valuable predictions of productivity for PBR operated during an entire year. They can also assess the influence of various parameters such as PBR location, harvesting strategy, strains cultivated, and the effects of night and day cycles. However, they may be regarded as oversimplified considering the complexity and numerous parameters affecting PBR operation and productivity in outdoor conditions. There is clearly a need to pursue the efforts of developing a set of robust tools for solar cultivation optimization to achieve better accuracy and to extend their applicability to other challenges related to solar PBRs. For example, Slegers et al. (2013a) integrated, in its model of the process, a thermal model able to predict the temporal evolution of the culture temperature under solar conditions and assess its influence on growth. Temperature was found to strongly influence the growth rate. Simulations predicted the range of temperature for a given location as a function of culture volume and thermal inertia of the system. The effect of nonoptimal thermal regulation on the productivity was simulated.

3.3.1 Model-Based Design of Intensified PBR Technologies

Modeling can be used to simulate various configurations of PBR for the optimization of their design. This section presents two examples of solar technologies whose development was based on the general methodology described in the previous section. Those technologies, namely AlgoFilm© and DiCoFluV©, are also illustrated in Fig. 1.

First, the AlgoFilm© PBR is based on surface illumination principle. It aims to achieve a very high volumetric productivity and the maximum surface productivity achievable under direct illumination. According to Eq. (3) and Fig. 3, large volumetric productivity can be achieved if the system presents a very large illuminated surface to volume ratio a_{light}. Thus, AlgoFilm© PBR is based on a falling film concept with value of a_{light} of 470 m^2 m^{-3}, corresponding to 2.1 L per m^2 of illuminated surface area. To fulfill the light limitation condition and to guarantee maximum biomass productivities of the system, various optimization strategies have been explored including (i) PBR hydrodynamics, (ii) gas–liquid mass transfer optimization (CO_2 dissolution, oxygen removal), (iii) development of thermal regulation devices, and (iv) material selection to prevent biofilm formation. A volumetric productivity of 5.7 kg m^{-3} day^{-1} was experimentally achieved under solar illumination condition with a daily averaged PFD $\bar{q}$ = 270 $\mu mole_{h\nu}$ m^{-2} s^{-1} typical of the average irradiation conditions in France. Note that the maximum productivity predicted by the model was 5.5 kg m^{-3} day^{-1} (Le Borgne 2011). This confirmed the relevance of the modeling used during the design phase of the cultivation system.

Moreover, the DiCoFluV© concept (Cornet 2010) is based on volumetric illumination with optimized light dilution principle. First, the conception of the optimal layout for the optical fibers with lateral diffusion of light used inside the culture volume was obtained using the constructal approach (Bejan 2000; Bejan and Lorente 2012) and imposing a low PFD to achieve high thermodynamic efficiency (namely 15–18 % in the PAR). This required models of light transfer for simple one-dimensional (Cornet 2010) or complex three-dimensional PBR geometries (Dauchet et al. 2013; Lee et al. 2014). Second, the optimum solar capture area was determined using kinetic model coupling the local light absorption rate A with biomass growth rates based on yearly solar databases. The modeling effort led to a design with 25 Fresnel lenses for a total volume of 30 L (Fig. 1). The optimal light dilution factor of the incident PFD was found to be relatively constant for any location on earth. But clearly, this concept is more interesting for locations with strong direct illumination. Relatively large volumetric biomass productivities are possible because of the large illuminated surface a_{light} of roughly 350 m^2 m^{-3} compensating for the low incident diluted PFD. Nevertheless, this PBR is mainly conceived as an optimal surface biomass productivity concept capable of increasing the surface productivity by an order of magnitude (by unit footprint) in solar conditions compared to conventional direct illumination systems. This corresponds to the maximum thermodynamic efficiency of photosynthesis. Actual performance of the system depends on the optical efficiency of the capture/concentration/filtration/distribution of light inside the culture vessel. Nowadays, efficiencies between 5 and up to 30 % for the DiCoFluV© PBR have been reported, reducing the theoretical maximal surface productivities down to around 100 t_x ha^{-2} $year^{-1}$ at the Earth's equator (Cornet 2010).

3.3.2 Prediction of PBR Operating Conditions and Optimization of Biomass Productivity

Modeling can be used to simulate PBR operation under outdoor solar irradiation. Because of variations in the incident illumination, the PBR never reaches a steady state and biomass concentration continuously evolves with time. Figure 4 shows an overview of the general modeling approach used to simulate solar PBRs. By predicting the temporal evolution of the biomass concentration in response to irradiation conditions, modeling of solar PBR operation is especially useful as biomass concentration directly affects light attenuation conditions and the resulting biomass productivity (Hindersin 2013; Hindersin et al. 2014; Slegers et al. 2011; Pruvost et al. 2011a). The effects of the season and of day–night cycles on the process dynamics and biomass productivity can be accounted for. Various engineering parameters can also be easily simulated to identify their optimum values. This includes parameters related to PBR design such as PBR geometry, orientation and inclination, and operating parameters like the residence time applied onto the cultivation system for continuous or semicontinuous cultures (Grognard et al. 2014; Hindersin et al. 2014; Pruvost et al. 2011a, 2012).

Figure 5 shows typical model predictions of the daily surface biomass productivity P_S as a function of the residence time for a continuous flat panel PBR located in Nantes, France for two commonly used microorganisms, namely *Chlamydomonas reinhardtii* (microalgae, eukaryotic microorganism) and *Arthrospira platensis* (cyanobacteria, prokaryotic microorganism). Those simulations were conducted for

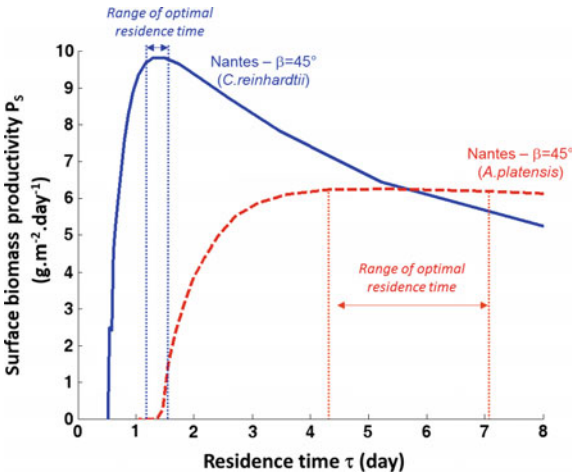

Fig. 5 Yearly average areal productivity of an inclined flat panel PBR (45°) as a function of the residence time applied on the cultivation system operated in continuous mode (Nantes locations, France). Values are given for the microalga *C. reinhardtii* and for the cyanobacteria *A. platensis,* illustrating the narrower range of residence time to maximize productivity for eukaryotic cells as explained by their sensitivity to dark volumes induced by too high values of residence time values

light-limited conditions with otherwise optimal growth conditions. An entire year of operation was simulated. Results for *C. reinhardtii* indicate that the maximum daily surface productivity could be achieved over a very narrow range of residence times. By contrast, the daily surface productivity for *A. platensis* reached a maximum for a large range of residence times. This was due to the fact that *A. platensis* is less sensitive to the presence of dark regions of the PBR which promote respiration activity (Gonzalez de la Vara and Gomez-Lojero 1986). On the other hand, dark volumes have a strong and negative influence on *C. reinhardtii* growth. Figure 5 also indicates that for either microorganisms, the productivity decreased sharply as the residence time decreased leading to culture washout.

Maximum biomass productivity can be easily achieved in continuous PBR exposed to artificial constant illumination by setting the biomass concentration corresponding to optimal light attenuation conditions (Takache et al. 2010). Under sunlight, the biomass growth rate is not sufficient to compensate for the rapid changes in sunlight intensity. Consequently, light attenuation conditions are never optimal. The optimal value of the residence time illustrated in Fig. 5 represents the best compromise to achieve maximum biomass productivity over the year of operation.

Because of variations in the incident irradiation, a wide range of light attenuation conditions can be encountered inside the culture volume during the course of a day. This can affect the process stability. For example, promoting small residence time to reduce the extent of dark zone favors low biomass concentration. This also reduces light attenuation and possibly impairs the process stability for periods where oversaturating light is encountered, such as at noon in the summer. A practical advice consists of promoting light attenuation by relying on large biomass concentration. For example, Hindersin et al. (2013) recommended a minimum value of biomass concentration for a given PFD incident on a solar PBR with sun-tracking capabilities, to maintain sufficient light attenuation. However, this approach results in a decrease in biomass productivity particularly for species with large respiration activity under illumination, as previously discussed. Again, a compromise has to be found, between the process productivity and its stability and robustness.

A first attempt to set rational strategies to achieve this compromise can be proposed. Light transmission through the PBR can be considered as an indicator of insufficient PFD attenuation, as light is not fully absorbed by the culture. Based on simulations of the process operation, the number of hours when light transmission through a continuous PBR occurs during a year of operation is calculated. Figure 6 shows the fraction of the time when light transmission is nonzero as a function of the residence time imposed to the cultivation system simulated in Fig. 5. It indicates that the number of hours when light transmission occurs is strongly influenced by the residence time because of its direct dependence on biomass concentration. For example, long residence time results in high biomass concentration and strong PFD attenuation. However, the evolutions of biomass productivity and light transmission with the residence time are different. For example, at optimal residence time for maximal biomass productivity of *C. reinhardtii*, the light transmission regime prevails 50 % of the time when the cultivation system is illuminated. This relatively

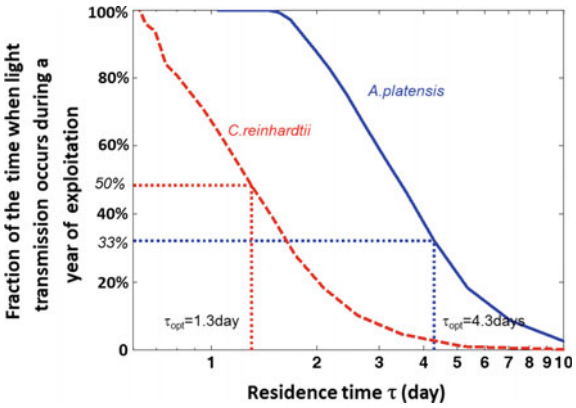

Fig. 6 Distribution of the number of hours per year when light transmission occurs, as a function of the residence time applied in the cultivation system (Nantes location, inclined PBR at 45°, *C. reinhardtii*). Values are normalized with respect to the total number of lightened hours in the year, i.e., 4355 h for Nantes location

large fraction would certainly lead to a significant risk of photoinhibition and culture drift, and potentially process instability and loss of efficiency. This risk can be reduced by increasing the residence time to obtain larger biomass concentration. For example, if the residence time is doubled, the PBR presents non-zero light transmission during only 15 % of illuminated hours, which can be considered acceptable as a first-order approximation. This corresponds to a limited loss of productivity of about 10–15 %. These results illustrate the need to find a compromise between maximizing biomass productivity and maintaining stable production conditions by setting the appropriate residence time and the associate biomass concentration in the cultivation system. Then, modeling seems especially useful since it can predict the evolution of the parameters defining the state of the culture so as to determine the optimum operating conditions leading to both a robust and efficient production.

3.3.3 Definition of Optimal Concentration to Maximize PBR Productivity

Biophysical models previously mentioned can be used to derive simple and practical analytical expressions and rules of thumb to determine the maximum surface and volumetric biomass productivities achievable by the PBR, thanks to Eqs. (2) and (3), respectively. Other models have also been developed to find the optimum PBR dimensions and/or microorganism concentration for a given species. The last two parameters have typically been treated and optimized separately (Pruvost et al. 2011a; Quinn et al. 2011; Slegers et al. 2011). However, based on the observation that PBR productivities were strongly influenced by light attenuation conditions,

Lee et al. (2014) used the concept of absorption optical thickness τ_λ defined as the product $\tau_\lambda = Ea_\lambda C_X / a_{light}$ (dimensionless). Here, Ea_λ is the specific absorption coefficient for a given species under the growth conditions considered, $C_X = C_X(t = 0)$ is the initial biomass concentration, and $a_{light} = S_{light}/V_R$ is the illuminated surface area per unit volume of PBR, also used in Eq. (3). Figure 7a shows the surface productivity P_s of horizontal PBR, growing *C. reinhardtii* and operated in batch mode during a summer day in Los Angeles, CA as a function of initial biomass concentration for different values of $a_{light} = 1/L$ where L is the depth

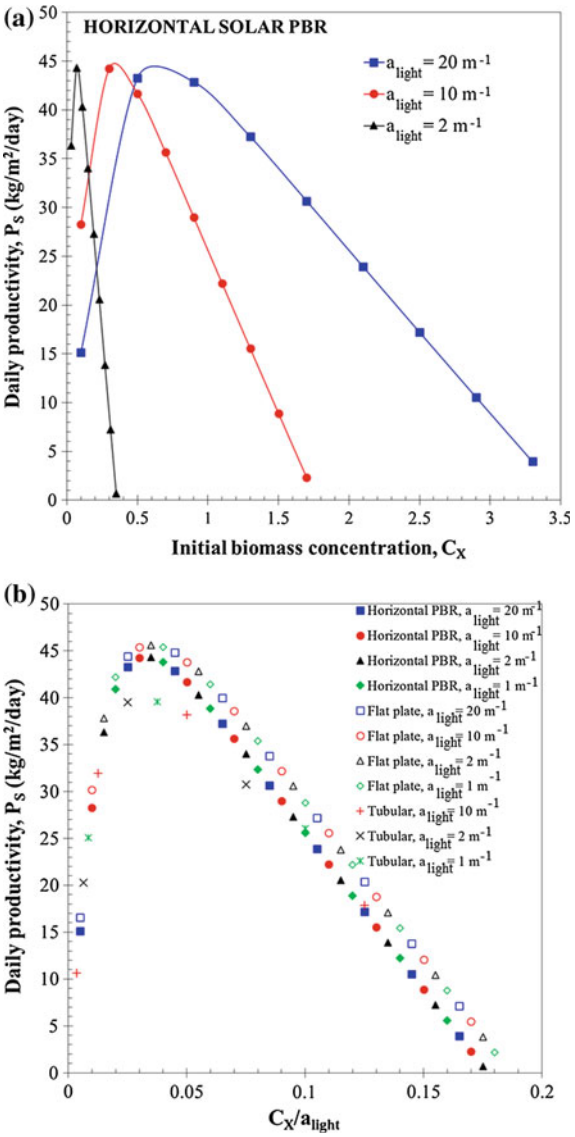

Fig. 7 Surface productivity for horizontal PBR presenting different values of depth L and of $a_{light} = 1/L$ as a function of (**a**) initial biomass concentration C_X and (**b**) for the ratio C_X/a_{light}

of the pond. It indicates that for a given depth, there exists an optimal initial concentration for which P_S is maximal. Figure 7b shows the same data but plotted as a function of C_X/a_{light} as well as those for flat plate and tubular PBRs. It is evident that the daily surface productivity P_s was a unique function of the ratio C_X/a_{light}. What is more, the function $P_s(C_X/a_{\text{light}})$ for *C. reinhardtii* was the same for outdoor horizontal PBR (open ponds), vertical flat plate, and tubular PBRs operated in batch mode. In addition, the validity of this approach was also established for experimental data (Pruvost et al. 2011b) and other simulation results based on different models (Pruvost et al. 2011a; Quinn et al. 2011; Slegers et al. 2011) for different microorganisms and PBRs operated in continuous mode. The PBR absorption optical thickness, represented by C_X/a_{light} for a given species, constitutes a convenient parameter for designing (via $a_{\text{light}} = S_{\text{light}}/V_R$) and operating (via C_X) these PBRs to achieve their maximum productivity $P_{s,\max}$ predicted by Eq. (2).

4 Conclusions

This chapter has reviewed the various parameters which one should consider in designing and operating large microorganism cultivation systems. Open systems constitute a simple and mature technology already deployed at industrial scale. By contrast, closed PBRs can be regarded as more complex systems, mainly due to the influence of light on the process. However, the main challenges have been identified and some robust engineering solutions have been recently proposed. Their use in the design and control of solar PBRs was illustrated in this chapter. Research efforts have to be pursued to develop solar PBR technologies in order to achieve their maximum theoretical performance. This is a prerequisite to compensate for the higher cost associated with the confinement of the culture to prevent contamination. These efforts should focus on the closely connected areas of biophysical modeling, engineering design, and operation and control.

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Commercial Production of Macroalgae

Delin Duan

Abstract Macroalgae, or seaweeds, are commercially important foods and sources of other products. The main seaweed producers are mostly in Asia, but significant production exists in central and south America. Biotechnology is expected to play an increasingly significant role in commercial production of macroalgae. This chapter provides an overview of biotechnology-based production of seaweeds.

Keywords Macroalgae · Seaweeds · Macroalgae biotechnology · Strain improvement

1 Introduction

Seaweed is favored seafood in some regions and is also used as feedstock for extracting fine chemicals. Among all the maricultured species, seaweeds now account for nearly 50 % of the total production (Table 1). The total global seaweed production continues to grow (Fig. 1). The main aquacultured seaweed genera are *Saccharina* (*Laminaria*), *Undaria*, *Pyropia* (*Porphyra*), *Eucheuma/Kappaphycus*, and *Gracilaria*, representing about 98 % of global seaweed production (Pereira and Yarish 2008). Commercial production of macroalgae is carried out mainly in Asia (Table 2) and Latin America (Table 3), where production is a relatively low-technology business with high labor demand. The main producing areas in Latin American are shown in Fig. 2. Attempts have been made to implement higher technology and less labor intensive production in tanks on land, but such attempts have had little commercial success.

Current seaweed production is valued up to US$12 billion, with food accounting for about 75 % of production, followed by hydrocolloids (alginate, agar, and carrageenan) with about 20 %. The remaining 5 % of production is used for various

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Table 1 World mariculture production of major species groups from 1996 to 2010 (from Chopin and Neish 2014)

Major mariculture species groups	Production (%)				
	1996	2000	2004	2008	2010
Molluscus	48.2	46.2	43.0	42.7	37.2
Seaweeds	44.0	44.0	45.9	46.2	50.9
Finfish	7.0	8.7	8.9	8.9	9.1
Crustaceans	1.0	1.0	1.8	1.8	1.8
Other aquatic animals (sea urchins, sea cucumbers)	–	0.1	0.4	0.4	1.0

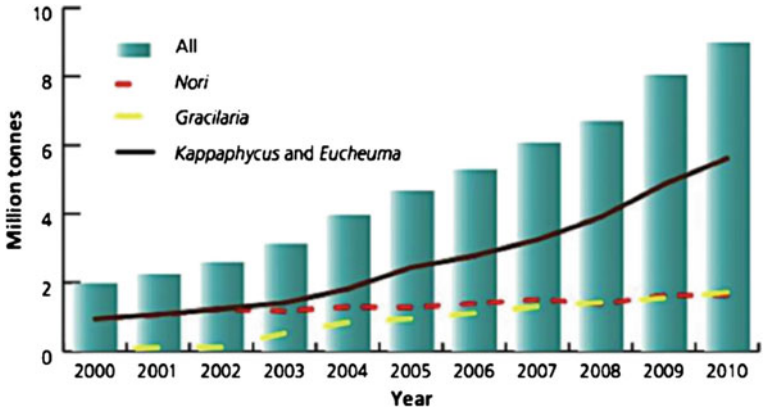

Fig. 1 Global production (fresh weight) of red seaweeds by genus. Reproduced from Hurtado et al. (2014)

Table 2 Aquaculture production of the five most important seaweed genera

Genus	Production (metric tons)	Value (10^3 US$)	Main producers
Saccharina	4,075,415	2,505,474.9	China (98.3 %), Japan (1.2 %), South Korea (0.5 %)
Undaria	2,519,905	1,015,040.5	China (87.1 %), South Korea (10.4 %), Japan (2.5 %)
Pyropia	1,397,660	1,338,994.7	China (58 %), Japan (25.6 %), South Korea (16.4 %)
Eucheuma/ *Kappaphycus*	1,309,344	133,325.2	Philippines (71.9 %), Indonesia (20.9 %), Tanzania (5.4 %)
Gracilaria	948,282	385,793.7	China (93.7 %), Vietnam (3.2 %), Chile (2.1 %)

Modified from Aquaculture Production 2004 (2006), FAO Yearbook, Fishery Statistics, vol. 98/2, Rome: FAO

Table 3 Harvested and cultivated seaweeds production (metric tons) in Latin America

Production method/country	2009	2010	2011
Seaweed harvested[a]			
Chile	456,184	380,742	417,965
Mexico	5814	6009	5721
Peru	3874	3836	5801
Seaweed cultivated[b]			
Brazil	520[c]	730[c]	730[c]
Chile	88,193	12,179	14,469

Reproduced from Rebours et al. (2014)
Sources IFOP Instituto de Fomento Pesquero (www.ifop.cl), SAGARPA Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pescay Alimentación
[a]Harvest data from Chile (IFOP), Mexico (SAGARPA), Peru (DIREPRO)
[b]Aquaculture data from FAO (18/12/2013)
[c]FAO estimates

products, such as fertilizers and animal feed additives. About 20 million tons of wet seaweed is used annually, mostly from cultivated plants but some from wild plants (FAO 2012).

In Southeast Asian countries with sub-tropical and tropical regions, the main seaweed production is carrageenophytes and agarophyte and much effort and cultivation refinement have occurred since 2000. *Kappaphycus-Eucheuma* has dominated the production (Fig. 3) of cultivated and harvested red seaweed biomass since 2004 (Hurtado et al. 2014). In 2010, according to the farming producers' shares, Indonesia was the top carrageenan seaweed produces in the region (60.5 %), followed by Philippines (31.9 %) and Malaysia (3.7 %) (FAO 2012). Latin American countries account for about 17 % of the global seaweed production, with Argentina, Brazil, Chile, Mexico, and Peru producing about 37 % of red seaweeds for phycocolloid extractions (Hayashi et al. 2014). In Latin America, Europe and Canada, seaweed industries rely on harvesting wild populations and production is limited by the ability of wild populations to recover.

Currently, the region with the largest production for seaweed is China (Tseng 2004), with total cultivated area of approximately 121,990 ha and annual cultivated output of over 1,856,800 tons. According to 2014 statistics, about 950,000 tons of the large brown magroalga kelp *Saccharian japonica* is produced annually, mainly in Shandong and Fujian provinces (Wu 1998) and there is significant production of *Sargassum fusiformis* (15,150 tons), *Pyropia* (*Porphyra*) (113,900 tons) and *Gracilaria (Gracilariopsis) lemaneiformis* (246,110 tons) (CFSY 2014). However, despite being the top seaweed producer, China still needs to import huge quantities from other countries to meet a huge and growing demand.

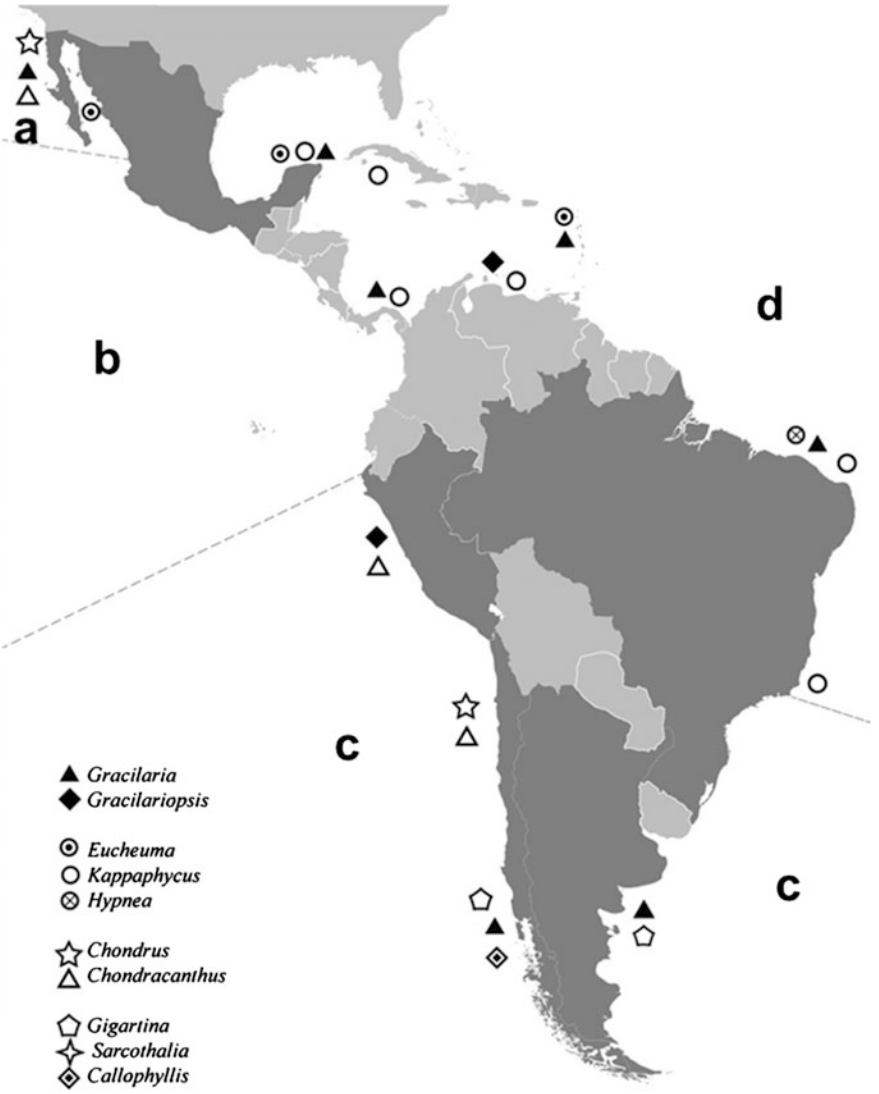

Fig. 2 Main economically important seaweeds cultured in Latin America in various biogeographical zones: **a** Temperate Northern Pacific; **b** Tropical Eastern Pacific; **c** Temperate South America; **d** Tropical Atlantic. Reproduced from Hayashi et al. (2014)

2 Advances in the Commercial Production of Macroalgae

Over the years, the use of products derived from seaweeds has increased and there have been major improvements in production, particularly in China. Such progress includes

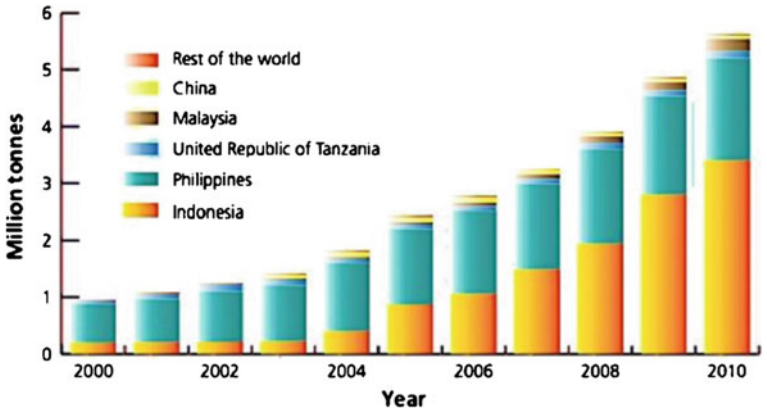

Fig. 3 Production of *Kappaphycus-Eucheuma* (fresh weight) by country. Reproduced from Hurtado et al. (2014)

2.1 *Enhanced Availability of Seaweed Seedstock and Improvement via Strain Selection and Hybridization*

A seaweed seedstock preservation system has been established to protect commercially valuable brown and red seaweeds cell lines such as *Saccharina* (*Laminaria*) (Fig. 4), *Undaria*, and *Pyropia* (*Porphyra*) (Fig. 5). This seedstock preservation system is undoubtedly important for seedling nurseries and seaweed reproduction (Tseng 1981). However, the application of good cultivars accounts for less than 5 % of commercial production, so there needs to be intensive work to develop commercial seaweed variety selection, hybridization and production. Successive selection and use of seaweed germplasm in production will cause regression and degradation of germplasm. Consequently, renewing and enlarging seedstocks will be necessary to refresh seaweed varieties and benefit seaweed production in terms of quantity and quality (Wu et al. 2004).

Fig. 4 Greenhouse nursery of *Saccharina* seedlings

Fig. 5 Propagation of *Pyropia* (*Porphyra*) cultivars

2.2 Increased Number of Commercially Cultivated Species

In view of the need for diversity, new types of seaweeds that have not yet been commercialized are being cultivated for trial and demonstration purposes, including *Sargassum thunbergii*, *S. naozhouense* and *S. fulvellum* (Hwang et al. 2006; Wang et al. 2006; Chu et al. 2011; Xie et al. 2013). Large-scale production of *Eucheuma/Kappaphycus* has begun relatively recently in Southeast Asia and there is low-level production of other species, including *Entermorpha*, *Monostroma*, *Ecolonia*, *Bangia* sp., *Costaria* (Fig. 6), *Caulerpa* and *Scytosiphon* (Ohno 1995; Kim et al. 1996; Choi et al. 2002; Fu et al. 2010; Guo et al. 2015; Zhuang et al. 2014).

Fig. 6 Gametophyte clones nursery in *Costaria* sporeling culture

2.3 Application of DNA-Marked Systems in Genetic Study and Application

As a prerequisite for using biotechnology in seaweed breeding and cultivation, effective molecular markers need to be developed. Detecting more genetic loci in a segregated population of seaweed should allow construction of a high density linkage map based on SSR (simple sequence repeats) and SNP (single nucleotide polymorphism) markers. Because of lower macroalgal evolutional position and marine environment influences, strain verification based on genotype is more convincing than the use of morphologic characteristics. However, despite difficulties, DNA markers can be effectively applied in seaweed germplasm identification, genetic variation and quantitative trait locus (QTL) analysis (Sun et al. 2006; Liu et al. 2010).

2.4 Improvements in Seedling Nursery Systems and Development of New Technologies for Intensive High-Efficiency Seedling Production

Until recently, nursery production of *Saccharina* (Fig. 4) and *Pyropia* seedlings has been time-consuming and laborious. Improvements in temperature and illumination control have shortened the duration of *Saccharina* nursery seedling production to 60 days. Similar integrated temperature-light control systems (Fig. 5) have allowed *Pyropia* (*Porphyra*) filaments to grow rapidly and consistently release a huge number of conchospores.

2.5 Expansion of Cultivation Areas from Shallow Seas to Deep Seas

The area devoted to seaweed aquaculture is continually expanding and commercial production is increasing steadily (FAO 2010, 2012). In China, production today is four times larger than ten years ago. Increased production is linked significantly to increased movement from the shallow sea to the deep sea areas (Fig. 7). New seaweed cultivation systems and shipping facilities have reduced the possibility of contaminating shallow seawater used for cultivation and have greatly improved seaweed food safety.

Fig. 7 Deep sea *Pyropia* cultivation in Jiangsu, China

2.6 *Polyculture of Commercial Seaweeds and Aquatic Animals*

Polyculture of species offers other exciting possibilities. Ploysculture of *Saccharina* with *Undaria* and aquatic animals such as scallop and abalones has been shown to be possible and can be advantageous. Alternate cultivation of *Saccharina* and *Gracilaria* on the same cultivation raft has been demonstrated and shown to enhance biomass production. Moreover, polyculture of aquatic animals and seaweed has the potential to alleviate eutrophication in shallow sea areas (Chirapart and Lewmanomont 2004; Zhang et al. 2005; Holdt and Edwards 2014).

2.7 *Improvements of Seaweeds Process Technologies and Development of Novel Foods and Drugs*

Substantially enhancing grower income and financial viability and innovative processing technologies increase the quality and diversity of products derived from various species, including seaweeds (Sasaki et al. 2004). The marine drugs, polysaccharide sulfate sodium (PSS), and fucose-containing polysaccharide sulfate (FPS or fucodian) are already made from the extract of *Saccharina japonica* and more drugs may be developed from other seaweed.

3 Concluding Remarks

Aquaculture of macroalgae has ecological benefits and may lower atmospheric carbon dioxide (Yue et al. 2014) in some situations. Future growth in production will occur through expansion of growth areas and through the use of cultivars enhanced via biotechnology. The diversity of the products derived from macroalgae will likely increase.

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Harvesting of Microalgal Biomass

Xianhai Zeng, Xiaoyi Guo, Gaomin Su, Michael K. Danquah, Xiao Dong Chen, Lu Lin and Yinghua Lu

Abstract Dewatering of microalgal broth is a key unit operation for producing algal fuels and other products. Dewatering is expensive and a factor hindering the commercialization of low-value algal products such as fuel oils. The extremely dilute nature of microalgae cultures coupled with the small size of the algal cells makes dewatering energy intensive. This chapter provides an overview of some of the key dewatering methods applicable to microalgal broths.

Keywords Microalgae · Dewatering economics · Dewatering technology · Drying · Flocculation · Flotation · Filtration

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1 Introduction

Microalgae are receiving increasing attention for various applications (Brennan and Owende 2010; Zeng et al. 2011). Harvesting, dewatering, or recovery of microalgal biomass from the culture broth, are essential steps in processes for producing products from microalgae. Dewatering can be energy intensive and typically requires removal of water so that a dilute suspension of algal cells with about 0.02–0.06 wt/vol.% biomass by dry weight is concentrated to 5–25 % suspended solids by weight (Uduman et al. 2010a). In some cases, multistep dewatering schemes are used. For example, biomass may be concentrated to 2–7 % suspended solids in an initial step followed by a second dewatering step which concentrates the slurry to 15–25 % solids by dry weight (Uduman et al. 2010a). Occasionally, a biomass drying step may be required subsequent to dewatering.

Many processes are available for dewatering a microalgae broth. These include centrifugation, flocculation, flotation, filtration, sedimentation, and electrophoretic separations. This chapter is focused on efficient and low-cost microalgae dewatering technologies with commercial prospects.

Dewatering is a major contributor to cost for producing algal biomass. This is because the initial broth is quite dilute, the size of microalgal cells is small, and the density of the cells is close to that of water, the suspending fluid (Danquah et al. 2009a, b; Uduman et al. 2010b; Zeng et al. 2012). Dewatering techniques such as centrifugation are highly effective and commercially used, but prohibitively expensive and energy intensive for use in production of low-value algal products. Efficacy of other methods such as flocculation and gravity sedimentation depends on the specifics of the algal species and the culture method used. Techno-economic analyses of dewatering methods indicate that no universal technology of choice exists for all applications. Issues such as energy consumption, capital cost, maintenance requirements, dewatering efficiency, the specifics of the microalga being dewatered and the end use of the biomass need to be considered in selection of appropriate dewatering method for a given scenario (de la Noue and de Pauw 1988; Uduman et al. 2010a; Zeng et al. 2011; Ahmad et al. 2011; Chen et al. 2011; Cerff et al. 2012; Lam and Lee 2012; Sharma et al. 2013).

2 Microalgae Dewatering Technologies

2.1 Flocculation

Flocculation is a process in which cells in suspension are made to agglomerate into larger particles (Veldkamp et al. 1997; Uduman et al. 2010a), or flocs that sediment easily compared to the microscopic cells. Flocculation is induced by adding a flocculating agent, or it may occur spontaneously as a consequence of a flocculating agent being produced by algal cells, or other contaminating microorganisms.

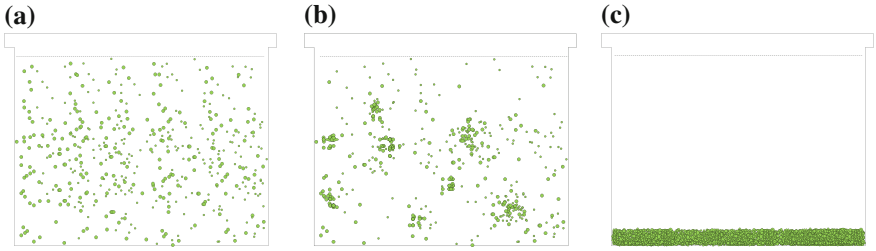

Fig. 1 Flocculation of microalgal broth: **a** dispersed and suspended cells; **b** agglomeration and floc growth; **c** sedimentation of flocs

Flocculants are generally added in the form of a solution. Flocculation is widely used specially in treatment of water and wastewater (Loh and Hubbard 2002).

Microalgae cells have a typical size range of 5–50 μm. Cells generally form a relatively stable suspension. Cells carry a net negative surface charge as a result of ionization of the functional groups on their cell wall (Tenney et al. 1969). Mutual repulsion between the negatively charged cells prevents them from agglomerating and settling out of suspension (Tenney et al. 1969; Uduman et al. 2010a). Addition of a flocculant to a suspension destabilizes it so that the particles agglomerate to generate flocs (Fig. 1). Some algal suspensions will autoflocculate at some stage of cultivation as a result of the flocculating activity of the metabolites being produced by the cells.

Flocculants are mainly of three types: inorganic flocculants, organic polymers or polyelectrolytes, and microbial flocculants (Uduman et al. 2010a). Salts of multivalent metals such as aluminum and iron are effective flocculants. Cationic polymers carrying multiple positive charges on the molecule are also quite effective in flocculating microalgae suspensions (Pushparaj et al. 1993). Microbial flocculants are produced by microorganisms. Microbial flocculants are mainly biopolymers.

Efficacy of flocculation of a microalgal broth depends on a number of factors including the type of flocculant, the concentration used, the morphology and surface charge properties of the algal cell, the pH of the broth and the ionic strength of the suspending medium (Oh et al. 2001). Compared to centrifugation and filtration, flocculation followed by gravity sedimentation is cheap, can treat large volumes and demands less energy (Pushparaj et al. 1993; Lee et al. 1998). Most algae can be harvested by flocculation. Although flocculation is effective, the biomass sludge recovered by sedimentation still contains a lot of water and further dewatering by other methods may have to be used. Some microalgal flocs can be difficult to sediment because their density is close to that of water and in sunlight the cells in the floc generate oxygen bubbles (Moraine et al. 1979), which tend to keep the flocs afloat.

2.2 Electrolysis

Electrolytic technologies are increasingly being used in microalgae harvesting (Mollah et al. 2004). Three common variants of electrolytic processes are

Table 1 Comparison of the three typical electrophoretic technologies

Dewatering process	Final solids content (% TSS)[a]	Energy usage (kWh/m^3)	Reliability	Limitations	References
Electrolytic coagulation	>95	0.8–1.5	Good	High energy cost; electrode depletion	Bektaş et al. (2004), Danquah et al. (2009a)
Electrolytic flotation	3–5	High	Poor	High energy cost; low efficiency	Rubio et al. (2002), Shelef et al. (1984)
Electrolytic flocculation	>90	~0.3	Very good	Cathode fouling	Danquah et al. (2009a), Edzwald (1995), Poelman et al. (1997)

[a]*TSS* Total suspended solids (wt/vol.%)

electrolytic coagulation, electrolytic flotation, and electrolytic flocculation (Mollah et al. 2004). The performance parameters of these processes are summarized in Table 1.

2.2.1 Electrolytic Coagulation

Electrolytic coagulation, or electrocoagulation, is an attractive alternative to traditional methods of flocculation. It has a relatively low energy demand and a high efficiency (Poelman et al. 1997; Alfafara et al. 2002; Gao et al. 2010a, b).

Electrocoagulation involves three stages: (1) generation of metal cationic coagulants (e.g., Al^{3+}) by dissolution of a reactive sacrificial anode; (2) destabilization of the cell suspension by the coagulant; and (3) aggregation of the destabilized particles to form flocs (Mollah et al. 2004; Bukhari 2008; Chen et al. 2011). Compared with conventional flocculation with inorganic salts, electrocoagulation has several advantages: (1) no anions such as sulfate and chloride are introduced in the system as would occur if an inorganic salt was used as flocculant; (2) the cationic coagulants produced by electrolytic oxidation of the anode are highly effective so that the dosage of the metal ion required may be less than if a salt was used; (3) a prior pH adjustment is not necessary and alkalinity is not consumed during the process as the OH^- ions are generated at the cathode; (4) the microbubbles of gases produced at the electrodes may function as flotation aids to enhance floc removal by flotation (Hosny 1996; Gao et al. 2010b).

2.2.2 Electrolytic Flotation

Electrolytic flotation is similar to electrocoagulation. However, in electrolytic flotation, the cathode is made of an electrochemically inert metal. As shown in

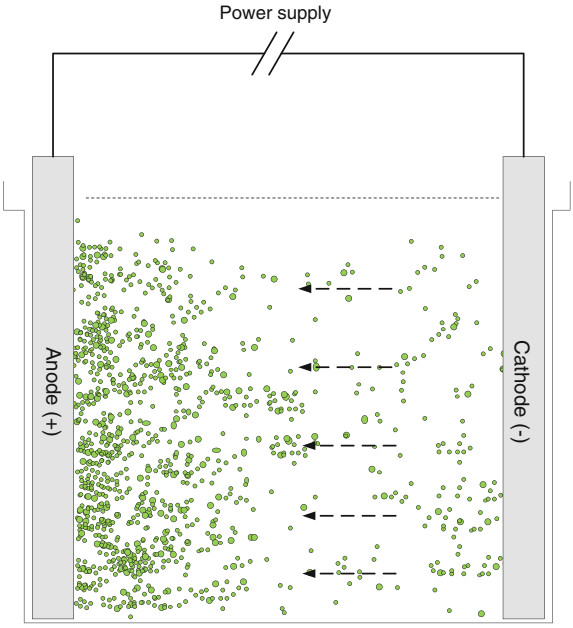

Fig. 2 Migration of microalgae cells in an electric field

Fig. 2, the negatively charged algal cells tend to migrate toward the anode in an electric field and aggregate (Aragón et al. 1992). The electrolysis of water generates microbubbles of gases at the electrodes. These bubbles entrapped in the microalgal floc carry them to the surface, where the biomass is skimmed off (Mollah et al. 2004).

The generation of bubbles at the electrodes drives flotation. As the rate of bubble generation is proportional to electric current, the power demand of an electroflotation process may be high. Electrolytic flotation might not be suitable in every application, but it is commonly used for dewatering broths of marine microalgae (Alfafara et al. 2002; Uduman et al. 2010a).

2.2.3 Electrolytic Flocculation

Electrolytic flocculation occurs without the addition of flocculants. The negatively charged algal cells migrate toward the inert anode. At the anode, the surface charge of the cells is neutralized to cause the cells to aggregate. Unlike in electrocoagulation, a sacrificial anode is not used. The gases produced at the electrodes through electrolysis of water facilitate the flotation of the flocs (Poelman et al. 1997). Biomass removal efficiency of 95 % or more may be achieved for diverse groups of microalgae (Poelman et al. 1997). The power consumption is of the order of 0.3 kWh/m^3. In view of high efficiency, low cost, and independence of sacrificial electrodes, electrolytic flocculation is commercially used especially if the biomass

is required to be free of added flocculants (de la Noue and de Pauw 1988). This may be the case if the biomass is for use as feed, food, or feedstock for extraction of products for food and pharmaceutical use. Unlike electrolytic flotation, electrolytic flocculation focuses on producing flocs and not on generating gases at the electrodes to cause flotation. Consequently, electrolytic flocculation requires much less energy than electrolytic flotation.

2.3 Gravity Sedimentation

Gravity sedimentation subsequent to flocculation is one of the cheapest techniques for solid–liquid separation and is widely used in water treatment processes. Sedimentation is generally not effective for microalgae unless the particle size has been increased by flocculation. A flocculated suspension may be readily concentrated by sedimentation in lamella separators or other sedimentation tanks. Lamella separators offer an increased settling area compared to conventional thickening tanks (Metting 1982; Uduman et al. 2010a). After sedimentation, the supernatant is decanted off to obtain a concentrated suspension of microalgal cells. Further dewatering may be required using methods such as centrifugation, filtration, or evaporation under sunlight. Gravity sedimentation can be slow, but it can be used with most algae after a flocculation treatment.

2.4 Magnetic Separation

Magnetic separation was originally developed for use in mining and metal processing industries. It is a rapid and simple method for efficiently removing magnetic or magnetized particles from a suspension by applying an external magnetic field (Li et al. 2009; Xu et al. 2011). Magnetic separation is potentially useful for recovering microorganisms from water so long as the cells can be made responsive to an external magnetic field (Chalmers et al. 1998; Lim et al. 2012). This may be possible by adding magnetic particles such as iron oxide to the cell slurry such that the cells adhere to the particles. Electromagnets may then be used to collect the particles with the adhering biomass (Fig. 3).

The adsorption of cells to magnetic particles is mainly a result of electrostatic attraction (Xu et al. 2011). In principle, magnetic separation can be low cost and highly efficient with a low energy demand. Magnetic separation for use with microalgae is being developed (Gao et al. 2009; Liu et al. 2009; Lim et al. 2012; Toh et al. 2012; Prochazkova et al. 2013). Impact of parameters such as the concentration of the magnetic particles and the broth pH, on the efficiency of magnetic separation has been discussed both for marine and freshwater microalgae (Cerff et al. 2012). Methods of separating the biomass from the magnetic particles need to be further developed. Under suitable conditions, a 5–10 min treatment resulted in

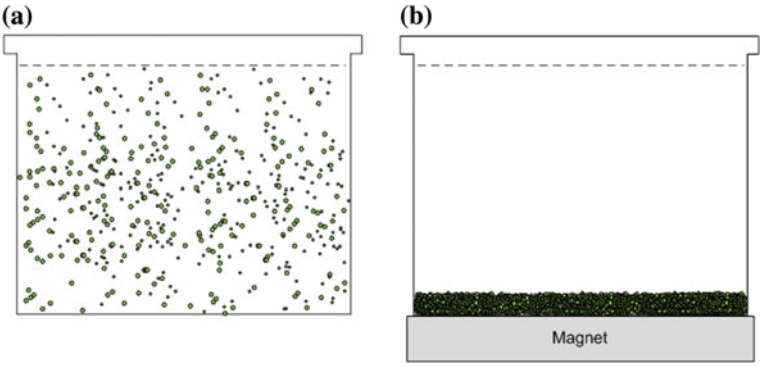

Fig. 3 Magnetic recovery of microalgae: **a** dispersed suspended cells; **b** sedimentation of cells by attraction to a magnet

biomass recovery efficiencies of >95 % (Xu et al. 2011; Prochazkova et al. 2013). After separation, the biomass could be recovered from the surface of the particles by treatment with sulfuric acid at 40 °C under the influence of ultrasound (Xu et al. 2011).

2.5 *Filtration*

In a filtration process, the solids are retained by a physical barrier that allows the suspending fluid to pass through (Uduman et al. 2010a). Various types of filtration processes can be used for dewatering a microalgal broth. These include magnetic filtration, vacuum filtration, pressure filtration, and cross-flow (or tangential flow) filtration (Show et al. 2013). At a fundamental level, all filtration processes remove solids by interception on a semipermeable barrier through which the fluid is driven by a pressure differential (Ríos et al. 2012). Biological solids such as cells often produce compressible cakes that offer increasing resistance to fluid flow as the differential pressure across the cake is increased. Also, suspensions of microorganisms have a tendency to foul the filtration medium and slow the filtration process (Ríos et al. 2012). Microalgae cause significant fouling of filtration membranes because of the extracellular polymers secreted by the cells. This also adversely affects the rate of filtration (Babel and Takizawa 2010).

2.6 *Evaporation and Drying*

Microalgal cells and many algal products are damaged by heat. Although heat sensitive materials are concentrated by evaporation of water in food and pharmaceutical

processing (Palen et al. 1994; Ribatski and Jacobi 2005b; Ribatski and Thome 2007), this technology is unproven for concentrating live cells. Furthermore, despite the improved energy efficiency of the modern evaporation technologies, they still require far more energy than many of the other dewatering processes.

Falling film evaporation is one technology that may be useful in some cases. Falling film evaporation is commercially well-established for concentrating milk, fruit juices, and other products (Ribatski and Jacobi 2005a; Quek 2011). Its use for concentrating microalgae broths has been discussed in the literature (Quek 2011; Zeng 2012; Zeng et al. 2014). In a falling film evaporator, the fluid being concentrated flows down the inner wall of a tall vertical tube as an annular film. The tube is surrounded by steam. Heat transfers from the steam condensing outside the tube through the wall to the algal broth. Water is removed from the broth by evaporation. Based on well-known energy balances, at least 1 kg of steam is required to evaporate a kg of water from the algal broth. In practice, because of heat losses, at least 1.1 kg of steam is needed to evaporate a kg of water. As a consequence, thin film evaporation is highly energy intensive and entirely unsuitable for dewatering algal biomass for production of fuels. In a fuel production process, the total energy recovered in the fuel must necessarily exceed the total fossil energy input to the process, or production will not be worthwhile.

When denaturation is not a concern, the microalgal biomass may be dewatered by thermal drying. For this, the dilute slurry is first concentrated by other means such as centrifugation and microfiltration and subsequently dried by thermal methods. Thermal drying methods include tray drying, drum drying, and spray drying. Drying typically reduces the moisture content of the biomass to 12–15 % by dry weight (Shelef et al. 1984).

In some applications, the preconcentrated or thickened biomass is freeze-dried. Freeze-drying does not denature the product, but is extremely expensive (Molina Grima et al. 2003) and therefore suitable only for really high-value products. Spray drying, freeze-drying, and drum drying of microalgae produce satisfactory results in terms of the uniformity of the biomass powder and the storage stability of the dried product (Ben-Amotz and Avron 1987).

3 Comparison of Dewatering Techniques

The performance of some of the algal dewatering methods is summarized in Table 2. Centrifugation is a generally robust technology that is commercially used, but is suitable only for high-value applications as it requires a substantial capital investment and the operating cost is high. Energy consumption of centrifugation is high.

Filtration is another efficient dewatering method that is commercially used in some algal processes, although it may not be universally applicable. Energy requirement of filtration are associated with the need to generate flow and pressure drop across the filter medium. The filter medium needs to be replaced periodically. Filters such as belt filters can be operated continuously.

Table 2 Performances of some dewatering technologies

Dewatering process	Suitable initial cell concentration (wt/vol.%)	Final solids contents (% TSS)[a]	Energy usage (kWh/m^3)	Reliability	Advantages	Limitations
Flocculation (Bilanovic et al. 1988; Danquah et al. 2009a)	≤10	20–30	Very low	Excellent	Low cost, low energy usage and high yield	Slow process resulting in low productivity; the flocculants may contaminate product
Centrifugation (Danquah et al. 2009a; Heasman et al. 2000)	≤30	10–22	8	Excellent	Fast, efficient	High energy usage; high capital costs
Filtration (Bernhardt and Clasen 1994; Danquah et al. 2009a)	≤10	1–40	0.4–2.16	Good/Excellent	Fast, efficient	Filters need to be replaced periodically
Gravity sedimentation (Uduman et al. 2010a)	≤10	0.5–1.5	0.1	Poor	Low cost, low initial capital investment	Slow; poor reliability
Flotation (Uduman et al. 2010a)	1–10	1–6	≥10	Good	Efficient	High energy usage; high loss of biomass
Electrophoresis (Bektaş et al. 2004; Uduman et al. 2010a)	0.5–10	1–5	Very high	Excellent	High selectivity	High maintenance cost; high energy usage
Falling film evaporation	5–20	10–25	~24	Good	Attains high solids concentration; suitable for high-value, heat-stable products	High energy usage; high capital investment; not applicable to heat sensitive products

[a]*TSS* Total suspended solids (wt/vol.%)

Flocculation and its variants (e.g., bioflocculation, electrofloccualtion) are generally well established. Conventional flocculation methods are efficient, have a low energy demand (Uduman et al. 2010a; Wijffels and Barbosa 2010) and require a low initial capital investment. Flocculation is commonly used as a pre-concentration step, especially when the residual flocculant in the biomass is acceptable. Flocculated biomass is often recovered as thickened slurry by gravity sedimentation in various kinds of sedimentation tanks. Gravity sedimentation is cheap. The initial investment in lamella separators and sedimentation tanks is small. The maintenance requirements are low. Gravity sedimentation of algae without the aid of flocculants is not generally feasible (Edzwald 1993).

Flotation is a potentially useful biomass recovery method, but appears to be less used in commercial processes. Flotation has a high energy demand as it requires creation of fine and stable gas bubbles. The energy and maintenance requirements of this system can be high. Electrophoretic dewatering methods are relatively new, but are gaining interest.

Methods such as falling film evaporation are energy intensive and not suitable for highly thermally labile products. Falling film evaporation is not commercially used in microalgae processing, but is commonly used to concentrate food products such as milk and fruit juice.

4 Conclusion

Dewatering is essential for producing all kinds of algal products. Many of the effective dewatering methods are well-established, but newer methods such as magnetic-based separation are emerging. Genetic engineering of algae can potentially provide highly specific and inexpensive methods of dewatering, but not much appears to have been done in this area.

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Extraction and Conversion of Microalgal Lipids

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Abstract Efficient downstream processing is crucial for successful microalgal biodiesel production. Extraction of lipids and conversion of lipids are the main downstream steps in microalgal biodiesel production process. This chapter provides the overview of the conventional as well as novel extraction and conversion technologies for microalgal lipids. The extraction and conversion technologies have to be environmentally friendly and energy efficient for sustainable and economically viable microalgal biodiesel production.

Keywords Microalgae · Lipid · Biodiesel · Extraction · Transesterification

1 Introduction

Microalgal lipids can be converted to a renewable alternative biofuel, i.e., biodiesel. Fast growth rates, substantial lipid accumulation, suitable lipid profiles, minimal arable land requirement, utilization of wastewater as growth medium, CO_2 sequestration, and use of residual biomass make microalgae an excellent biodiesel feedstock. Various strategies can be applied such as nutrient stress and alteration of cultivation conditions to enhance the lipid accumulation capability of microalgae

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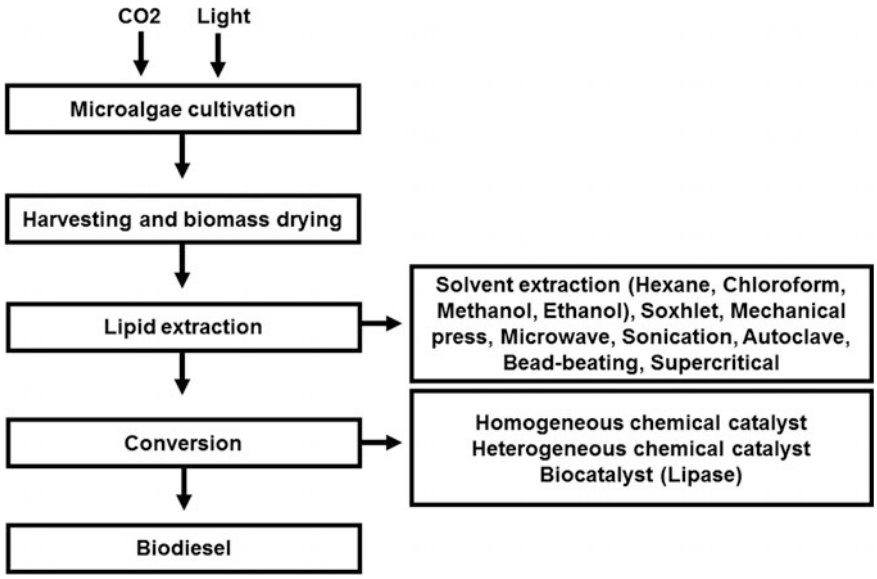

Fig. 1 Microalgal biodiesel synthesis process flow: different extraction and conversion techniques

(Rawat et al. 2013; Singh et al. 2015). Biodiesel synthesis from microalgae is a multistep process. The process involves cultivation of microalgae for biomass generation, harvesting of biomass, extraction of lipids, and conversion of lipids to biodiesel as major steps (Fig. 1). Much focus has been given to the upstream development such as cultivation of microalgae and enhancing the lipid yields. Downstream processes such as harvesting, extraction, and conversion are still, however, considered major bottlenecks for commercial-scale biodiesel production process.

Extraction of microalgal lipids and its conversion to biodiesel have proven to be challenging and are associated with environmental and economic concerns. There are no available technologies which are mature enough to be applied at commercial scale. Extraction of cellular lipids from microalgae faces challenges of cell wall disruption, high cost, and environmental concerns due to use of toxic solvents amongst others. Solvent extraction is widely used for lipid recovery from microalgae (Mubarak et al. 2015). Cell disruption techniques such as microwave, sonication, bead beating, autoclaving, etc. are generally coupled with solvent extraction to promote the process efficiency. Conversion of microalgal lipids to biodiesel can be achieved by transesterification. In transesterification, lipids are converted to fatty acid alkyl esters in the presence of a suitable acyl acceptor and catalyst. Glycerol is formed as a byproduct in this process. Transesterification can be accomplished using various homogeneous or heterogeneous chemical catalysts or lipase as a biocatalyst (Sharma et al. 2011). Efficient, environmentally friendly,

and economical lipid extraction and conversion techniques are the foremost requisites for the sustainable biodiesel production from the microalgae.

2 Microalgal Lipids for Biodiesel Production

A steady supply of lipid feedstock is pertinent for the commercial-scale biodiesel production. The quantity and quality of the lipid feedstock is of vital importance for the ease of production and final quality of biodiesel. Microalgal lipids have shown both the substantial quantitative yields as well as suitable composition, to make it an excellent feedstock for biodiesel production. Various microalgal strains have been studied to assess their potential as a biodiesel feedstock and have yielded varying lipid content and profiles. The reported lipid contents of microalgae vary greatly and are strongly dependent on the strain and cultivation conditions. Widely studied microalgal genera such as *Chlorella, Dunaliella, Nannochloropsis, Scenedesmus, Neochloris, Nitzschia, Porphyridium, Phaeodactylum,* and *Isochrysis* yield lipid content in the range of 20–50 % (Amaro et al. 2011). High lipid productivity is important to ensure the economic viability of microalgal biodiesel production. Various stress conditions like nitrogen limitation, light, CO_2, etc. are known to enhance the lipid accumulation in microalgae, and however may negatively affect biomass production and thus lipid productivity (Singh et al. 2015).

The major constituents of microalgal oils are neutral lipids, polar lipids, and some amount of hydrocarbons, sterols, waxes, and pigments (Sharma et al. 2012). The neutral lipids are the best suited for the biodiesel synthesis due to their ease of conversion to fatty acid alkyl esters (FAAE). Neutral lipids function as the energy storage components of microalgal cells and composed mainly of triglycerides (TAG) and some amount of free fatty acids (FFA). The polar lipids are responsible for structural functions (phospholipids in cell membrane) and physiological roles such as cell signaling (sphingolipids) (Sharma et al. 2012). Triglycerides are the suitable for conversion to biodiesel via base catalyzed transesterification, whilst free fatty acids can be converted to biodiesel via esterification prior to transesterification or acid/lipase catalysis. Microalgal lipid accumulation can be enhanced by altering the nutrient composition in the media or cultivation conditions.

Fatty acid composition of lipids is an important criterion for selection of suitable microalgal strains for biodiesel synthesis. Fuel properties of the biodiesel are primarily influenced by the carbon chain length, degree of unsaturation, and percentage composition of saturated and unsaturated fatty acid in microalgal lipids. Microalgal lipids are composed of saturated, monounsaturated, and polyunsaturated fatty acids. Many of the lipid-accumulating microalgae have been shown to comprise C14:0, C16:0, C18:1, C18:2, and C18:3 as major contributing fatty acids of their lipids, which are considered to be suitable for good quality biodiesel (Song et al. 2013). Table 1 shows fatty acid profile and lipid content of various microalgal strains studied for biodiesel production.

Table 1 Fatty acid profile and lipid content of different microalgae

Microalgal strain	Lipid content (%)	Fatty acid composition (%)									Reference
		C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	SFA	PUFA	
Scenedesmus sp.	16	–	15.62	4.06	2.97	15.23	7.00	22.99	18.59	56.86	Talebi et al. (2013)
Chlorella vulgaris	17.3	–	14.55	1.183	10.51	23.62	13.80	32.10	25.06	70.7	Talebi et al. (2013)
Chlorella vulgaris	20.82	0.78	36.97	5.10	0.5	4.96	4.38	8.42	–	–	Song et al. (2013)
Staurastrum sp.	10	4.97	40	16.50	–	–	–	–	–	–	Song et al. (2013)
Chlorella sp.	30	0.30	0.41	2.41	25.94	3.82	0.47	0.99	–	–	Praveenkumar et al. (2012)
Monoraphidium sp. KMN5	20.84	–	41.03	–	17.67	10.16	3.03	1.53	58.7	16.26	Tale et al. (2014)
Scenedesmus sp. KMN4	28.63	–	41.27	–	20.53	9.21	1.91	0.57	61.82	12.77	Tale et al. (2014)
Nannochloropsis gaditana.	–	3	16	17.1	–	3.9	8	9	19.4	68.3	Hita Peña et al. (2015)
Scenedesmus sp. ISTGA1	20	–	14.6	12.1	21.2	24.9	8.8	4.2	35.8	54.3	Tripathi et al. (2015)
Ankistrodesmus falcatus KJ671624	23.3	–	–	21.15	–	28.18	19.25	14.33	–	–	Singh et al. (2015)
Neochloris oleoabundans	29	0.43	19.35	1.85	0.98	20.29	12.99	17.43	20.76	64.6	Gouveia et al. (2009)

3 Lipid Extraction Techniques

Lipid extraction from microalgae is a crucial step in biodiesel synthesis. The techniques of lipid extraction from microalgae studied by researchers include various physical and chemical methods. Solvent extraction is a most widely used method and can be coupled with cell disruption techniques to intensify the process for improved yields. Various cell disruption methods investigated include autoclaving, osmotic shock, microwave-assisted extraction, and sonication-assisted extraction (Halim et al. 2012; Mubarak et al. 2015). Novel methods of lipid extraction such as supercritical fluid (SCF) extraction, electrochemical extraction, high pressure extraction, etc. are more recent developments. Ideally, lipid extraction should be high yielding, energy efficient, time saving, and environmentally friendly with usage of nontoxic chemicals and easy handling. The other important aspect of the extraction technique is that it should not have deteriorating effects on the quality of lipids. The efficiency of lipid extraction techniques depends on the number of factors such as microalgal strain, cell wall structure, their lipid content, quality of lipids, etc. Table 2 depicts the various techniques and solvents used for extraction of lipids from the microalgae.

3.1 Conventional Lipid Extraction Techniques

Conventional lipid extraction methods such as solvent extraction, soxhlet, and mechanical press extraction are in practice, since the biodiesel started gaining the interest of researchers and industries. These methods are widely used at industrial scale for the extraction of oil from edible and nonedible plant seeds. Due to the smaller cell size and cell wall composition of microalgae, physical methods have been found to be inefficient. The conventional methods are low yielding and time consuming. These drawbacks negatively affect the economics of microalgal biodiesel.

3.1.1 Soxhlet Extraction

The soxhlet technique is the most widely employed organic solvent extraction strategy for the extraction of oil from different plants and microalgal strains. In the soxhlet technique, oil and fat from the biomass matrix are concentrated by repeated washings with an organic solvent, typically *n*-hexane or petroleum ether, under reflux in a specialized apparatus called soxhlet extractor. This method is used extensively by oil industry (Kirrolia et al. 2013). This method is temperature dependent as variations in extraction temperature affect the lipid yield. Temperatures ranging from 30 to 60 °C enhance the yield of lipids (Fajardo et al. 2007). However, temperatures above 70 °C decrease lipid yield due to loss of thermolabile ingredients by oxidative degradation. Soxhlet extraction is simple and nonlabor-intensive process which is economical and scalable. Soxhlet extraction is,

Table 2 Extraction of lipids from microalgae using different cell disruption techniques and solvents

Microalgae	Cell disruption	Solvents (ratio)	Lipid yields (%)	Reference
Scenedesmus sp.	Microwave	Chloroform: ethanol (1:1)	29.65	Guldhe et al. (2014)
Botryococcus sp.	Microwave	Chloroform: methanol (1:1)	28.6	Lee et al. (2010)
Chlorella sp.	Microwave	Chloroform: methanol (2:1)	38	Prabakaran and Ravindran (2011)
Nannochloropsis	Microwave	Chloroform: methanol (2:1)	32.8	Koberg et al. (2011)
Chlorella sp.	Sonication	Chloroform: methanol (2:1)	40	Prabakaran and Ravindran (2011)
Scenedesmus sp.	Sonication	Chloroform: ethanol (1:1)	19.85	Guldhe et al. (2014)
Botryococcus sp.	Sonication	Chloroform: methanol (1:1)	8.8	Lee et al. (2010)
Chlorella sp.	Autoclave	Chloroform: methanol (2:1)	24	Prabakaran and Ravindran (2011)
C. vulgaris	Autoclave	Chloroform: methanol (1:1)	10	Lee et al. (2010)
Botryococcus sp.	Bead beating	Chloroform: methanol (1:1)	28.1	Lee et al. (2010)
Scenedesmus sp.	Osmotic shock	Chloroform: methanol (1:1)	7.9	Lee et al. (2010)
Schizochytrium limacinum	Soxhlet	*n*-Hexane	45	Tang et al. (2011)
Scenedesmus caucus	–	*n*-Hexane	4	Shin et al. (2014)
Nannochloropsis oculata	–	Dichloromethane	9	Liau et al. (2010)
Nannochloropsis oculata	–	*n*-Hexane	5.79	Liau et al. (2010)
Nannochloropsis oculata	–	Petroleum ether	8.2	Converti et al. (2009)

however, a time-consuming method and generally needs large amounts of solvents. The lipid yields from soxhlet extraction are low, which could be because of poor extraction of polar lipids.

3.1.2 Mechanical Press

Mechanical press is a physical extraction technique which utilizes mechanical pressure to break and compress out oil from the dry biomass. This method is

common for extraction of oils from the plant seeds. For its efficient performance this technique needs large amount of biomass (Harun et al. 2010). The small cell size of some algae renders the process ineffective for microalgal lipid extraction. This technique is slow and tedious compared to other methods of oil extraction and does not recovery the total lipid content as in organic solvent extraction. The benefit of mechanical pressing is that the technique maintains the chemical composition of lipids as no chemicals and/or extra heat is applied.

3.1.3 Solvents for Lipid Extraction

Use of the organic solvents to extract lipids from microalgae is the most employed technique. Lipids are soluble in organic solvents, and thus they are employed for lipid extraction process. Organic solvents such as *n*-hexane, methanol, ethanol, and mixed polar/nonpolar chemical solvents (e.g., methanol/chloroform and hexane/isopropanol) are effective for extraction of the microalgal lipids, but yield factor depends on microalgal strain and its lipid content (Halim et al. 2012). Microalgal lipids are of two types viz., neutral lipids where the carboxylic group end of the fatty acid molecule bonded to an uncharged head group (e.g., Glycerol) and polar lipids where fatty acid carboxylic group head is attached to a charged head group (e.g., Phosphate complex). Polar lipids are divided into two parts that are phospholipids and glycolipids. Neutral lipids (storage lipids) take the function of energy storage, while the polar lipids (membrane lipids) are involved in structural components of cell such as cell membrane. The basic concept of lipid extraction by solvent is like dissolving like. The solvents used for lipid extraction are polar and nonpolar or mixture of both in particular ratios. The polar solvents extract the polar lipids, while the nonpolar solvents extract the neutral lipids. The mixture of polar and nonpolar solvents can extract both neutral lipids and polar lipids (Halim et al. 2012).

Nonpolar organic solvents (viz., hexane, chloroform) penetrate the cell membrane and interact with neutral lipid present in the cytoplasm (Halim et al. 2012). The solvent–lipid interaction is reported to be due to the van der Waal's forces that lead to the formation of organic solvent and neutral lipid complex. The concentration gradient drives this complex to diffuse across the cell membrane. The neutral lipids get extracted from the cell to dissolve in the organic solvent that is of nonpolar nature. However, some amounts of neutral lipids found in cytoplasm are present as complex with polar lipids (i.e., phospholipids, glycolipids, sterols, carotenoids). The complex is said to be strongly linked by hydrogen bonds with proteins in the cell membrane which can be broken using polar organic solvents (e.g., methanol, ethanol). Hence, to break the membrane-based lipid–protein association, it is necessary to use both the nonpolar and polar organic solvents. Thus, the use of mixture of polar and nonpolar solvents like chloroform and methanol is advocated by many researchers for efficient lipid extraction (Guldhe et al. 2014; Lee et al. 2010). When *n*-hexane was used by Keris-Sen et al. (2014) for

lipid extraction from a mixed culture (i.e., *Scenedesmus* sp., *Chlorococcum* sp.) the yield was 24 %. While Guldhe et al. (2014) applied mixture of chloroform and ethanol (1:1 v/v) for lipid extraction from *Scenedesmus obliquus* and Lee et al. (2010) applied mixture of chloroform and methanol (1:1 v/v) for lipid extraction from *Botryococcus* sp., the yields were 29.65 and 28.1 %, respectively.

The major drawback of using solvents is their hazardous nature and adverse effects on the environment. Some of the organic solvents like chloroform and methanol are highly toxic in nature, and could pose a serious threat while handling. Researchers are focusing on the use of greener solvents and minimizing the amount of solvents needed for extraction. The solvent extraction method can be linked with efficient cell disruption technique to improve the lipid extraction yields.

3.2 Process Intensification by Cell Disruption

To alleviate the drawbacks of conventional lipid extraction techniques, various cell disruption methods are coupled with solvent extraction to intensify the extraction process. These techniques facilitate the easy contact of the solvents and lipids in the microalgal cells. These techniques are associated with the advantages of high lipid yields, minimum solvent requirement, and reduced time of extraction. Microwave, sonication, autoclaving, bead beating, etc. are the cell disruption techniques commonly used for microalgal lipid extraction.

3.2.1 Microwave

Microwave is highly efficient cell disruption technique with reported high lipid yields from the microalgae (Koberg et al. 2011). Microwave energy generated in this technique causes rotation of the molecular dipole and results in disrupting the weak hydrogen bonds. This phenomenon increases the movement of dissolved ions that facilitate the diffusion of solvent resulting in effective lipid extraction. In the microwave technique the heating is efficient as the electromagnetic waves interact with cell matrix at the molecular level. Also in this technique both the mass and heat transfer take place from inside the cell toward the outside solvent. These reasons make the microwave-assisted lipid extraction from microalgae a highly efficient process. Prabakaran and Ravindran (2011) used microwave technique for extraction of lipids from *Chlorella* sp. with a lipid yield of 38 %. Koberg et al. (2011) obtained 32.8 % lipid yield when microwave technique is applied for extraction of *Nannochloropsis* sp. The advantages of microwave-assisted cell disruption techniques are rapid, minimal solvent consumption, high lipid yield, and easily scalable. However, this technique is energy intensive as the electromagnetic waves are generated for cell disruption.

3.2.2 Sonication

In sonication, the cavitation impact generated due to sound waves causes the cell disruption of microalgae. At the point when ultrasound is transmitted to the media cavitation bubbles are formed. These microbubbles moving in the media collapse with each other and implode generating chemical and mechanical energies in the form of heat, free radicals, stun waves, etc. This energy generated from cavitation bubbles disrupts the cell envelope, facilitating the solvent and cellular lipid interaction and easy mass transfer (Guldhe et al. 2014). Sonication technique provides the high lipid yield and greater penetration of solvents into the microalgal cells. When lipids from *Chlorella* sp. were extracted using sonication-assisted solvent extraction the lipid yield obtained was approximately 40 % (Prabakaran and Ravindran 2011). This technique does not involve the input of external heating source and thus operates at moderate temperatures. Generating the ultrasound waves is energy intensive and this technique requires sophisticated instrumentation which could be cost intensive.

3.2.3 Autoclaving

Autoclaving has also been investigated for the extraction of lipids from microalgae (Mendes-Pinto et al. 2001). The lipid yields from extraction by autoclave technique are lower than the microwave or sonication techniques. In microwave technique the cell disruption is caused by the heating at molecular level inside the cell, while in autoclaving the heat is diffused from the surroundings into the cell, which makes the cell disruption inefficient. Lee et al. (2010) in their comparative study of different cell disruption techniques found 10 % lipid yield while using autoclave as a cell disruption technique for extraction from *Chlorella vulgaris*. Autoclaving is also an energy-intensive process.

3.2.4 Bead Beating

Bead beating or milling causes cell disruption by the grinding of beads against the microalgal cells inside the vessel. The bead mills are of two types, viz., shaking vessels and agitating beads. In the first type cell disruption is caused by shaking the whole vessel filled with microalgal cells and beads. In the second type the vessel is fixed but provided with the rotary agitator, which facilitates the grinding by beads. The efficiency of bead beating depends upon number of factors such as size and structure of the beads, the velocity and configuration of the agitator, and time (Halim et al. 2012). The bead mills can be designed in horizontal or vertical orientation and can be easily scaled up from laboratory scale to industrial scale. The only constraint with this method is that it is economically viable with the high concentrations of microalgal biomass (100–200 g l^{-1}) (Show et al. 2014).

Table 3 Extraction of lipids from microalgae by supercritical fluid extraction method

Microalgae	Solvent	Conditions (Temp, pressure, time)	Lipid yields (%)	Reference
Schizochytrium limacinum	CO_2 and ethanol	40 °C, 35 MPa, 30 min	33.9	Tang et al. (2011)
Pavlova sp.	CO_2	60 °C, 306 bar, 6 h	34	Cheng et al. (2011)
Nannochloropsis sp.	SC–CO_2	55 °C, 70 MPa, 6 h	25	Andrich et al. (2005)
Chlorococcum sp.	SC–CO_2	60 and 80 °C, 30 MPa, 80 min	81.7	Halim et al. (2011)

3.3 Supercritical Lipid Extraction

Supercritical liquid extraction is the recent technology that can possibly replace the conventional organic solvent extraction. In this technique, specific pressure and temperature are maintained above the critical temperature and pressure of the liquid to attain the supercritical solvent properties. The microalgal biomass is exposed to SCF under specific temperature and pressure conditions, which causes dissolution of lipids from sample into the SCF. SCFs can be tuned by adjusting the temperature and pressure conditions, to preferentially extract the neutral lipids from microalgal biomass (Halim et al. 2012). Supercritical properties of liquid facilitate its rapid penetration in the cell matrix and efficient extraction of lipids. Thus, this lipid extraction technique is rapid and highly efficient. Supercritical properties of solvent are the function of density, which can also be adjusted by controlling the temperature and pressure. The lipid obtained from the supercritical extraction does not need to undergo solvent recovery step as they are free from solvents. Supercritical CO_2 ($SCCO_2$) is the commonly used solvent for extraction of microalgal lipids. $SCCO_2$ has critical pressure (72.9 atm) and moderate temperature (31.1 °C), which makes it a suitable solvent for lipid extraction without thermally degrading the lipid components (Mubarak et al. 2015). Advanced instrumentation is required for controlling the temperature and pressure at critical levels which could be energy and cost intensive. Table 3 depicts the extraction of lipids from microalgae using SCF.

3.4 Effect of Preceding Processing Steps on the Lipid Extraction

The steps in the biodiesel production process preceding lipid extraction such as harvesting also have an impact on the lipid extraction of microalgae. Some of the recent approaches to the harvesting of microalgae have been shown the influence on

the lipid yields during the extraction. Harvesting processes like ozoflotation, electrolyte-assisted electrochemical harvesting, etc. aid in weakening of the cell wall, and thus facilitate the easy cell disruption and higher lipid yields. Komolafe et al. (2014) have reported that the treatment of microalgae, *Desmodesmus* sp. with ozone during harvesting (ozone flotation), led to the formation of higher content of saturated fatty acids in the oil. The ozoflotation of microalgae has been reported to aid in disruption of the microalgal cells and improves the lipid extraction process. Misra et al. (2014) investigated the electrochemical harvesting process for the *Chlorella sorokiniana*. In their study they found that when electrolyte (NaCl) is applied to intensify the electrochemical harvesting process, the lipid yields were also increased. The electrolyte aided in the weakening of the cell wall by osmotic shock and thus resulted in the higher lipid recovery yields. The right combination of preceding step with efficient extraction technique or merging this technique could make the biodiesel production process sustainable and economical.

4 Novel Lipid Extraction Techniques and Recent Advances

The quest for efficient, scalable, energy, and cost-effective lipid extraction technology is still ongoing. Researchers are investigating several novel aspects of production such as novel extraction techniques, cell disruption methods, wet biomass extraction, and use of green solvents. Steriti et al. (2014) applied hydrogen peroxide (H_2O_2) and ferrous sulfate ($FeSO_4$) to disrupt the cell wall of *Chlorella vulgaris* and found increased lipid yield of 17.4 % compared to 6.9 % without any cell disruption. In the electrochemical lipid extraction process, the electrical field develops a potential difference across the membrane. This potential difference causes the breakdown of membrane and makes the cell wall permeable for lipid extraction (Daghrir et al. 2014). Pulse electric field is an attractive lipid extraction technique where microalgal cell disruption is caused by the electroporation method. The main advantage of this process is that it can be applied directly to the microalgal culture, and as a result the harvesting and drying of biomass can be skipped (Flisar et al. 2014). Taher et al. (2014) investigated the extraction of lipids from wet biomass of microalgae *Scenedesmus* sp. using enzymes lysozyme and cellulose. Ionic liquids are considered as the green solvents because of their non-volatile characteristic, synthetic flexibility, and thermal stability. Kim et al. (2012) extract lipid from *Chlorella vulgaris* using mixture of ionic liquids and methanol, which enhance the overall yield of lipid from 11.1 to 19 % compared to original lipid extraction techniques of Bligh and Dyer method. These novel techniques have shown promising potential in the quest for sustainable, economical, and efficient lipid extraction method.

5 Microalgal Lipids Conversion to Biodiesel

Conversion of microalgal lipids to biodiesel is commonly accomplished by transesterification or alcoholysis of triglycerides with acyl acceptor employing a suitable catalyst that yields fatty acid alkyl esters (FAAE) and glycerol. Commonly employed acyl acceptors are short-chain alcohols like methanol and ethanol, while the catalysts used are either homogeneous and heterogeneous chemical catalysts or enzyme biocatalysts (lipase) (Table 4). This three-step process first converts triglycerides to diglycerides, then diglycerides to monoglycerides, and finally monoglycerides to glycerol with yield of a monoalkyl ester of fatty acid in each of the three steps. Stoichiometrically, three moles of alcohol are required for converting one mole of triglyceride into biodiesel. Normally, more than three moles of alcohol are added to shift the equilibrium reaction in the forward direction.

5.1 Chemical Catalysis

Catalysts play an important role in the synthesis of biodiesel. For the completion of the transesterification in acceptable timeframes, either homogeneous or heterogeneous catalyst is employed. The types of catalyst used are acidic, alkaline, or enzyme. Commonly employed acid homogeneous catalysts include sulfuric acid. The commonly employed alkaline homogeneous catalysts are potassium hydroxide, sodium hydroxide, and sodium methoxide. The heterogeneous catalyst includes a wide range of compounds. These include Brønsted or Lewis catalysts. The catalyst used in transesterification more often depends on the free fatty acid (FFA) content of the feedstock. For alkaline transesterification, the oil must contain low amounts of FFA. It is reported that the FFA should be lower than 4 mg $KOHg^{-1}$ for efficient alkaline transesterification (Sharma et al. 2008). Microalgal lipids have been found to generally contain higher amounts of free fatty acids. Thus, either acid catalyst or two-step method of acid esterification followed by transesterification by alkaline catalyst are the suitable strategies for microalgal lipid conversion (Singh et al. 2014). Heterogeneous catalysts have the advantage of fast reaction rates and reuse over the homogeneous catalysts. Miao and Wu (2006) studied conversion of *Chlorella protothecoides* lipids using sulfuric acid catalyst and the biodiesel yield obtained was 60 %. When heterogeneous Al_2O_3-supported CaO catalyst was employed for the conversion of lipids extracted from *Nannochloropsis oculata* biodiesel, yield of 97.5 % was observed (Umdu et al. 2009).

5.2 Biocatalysis

Biocatalytic conversion is greener approach as it is associated with the advantages of less wastewater generation and low energy input. Lipases are the enzymes which

Table 4 Conversion of microalgal lipids to biodiesel by different catalytic methods

Microalgae	Catalyst	Catalyst loading (% wt/oil wt)	Molar ratio Alcohol: oil	Reaction conditions [temperature (°C), time (h), stirring (RPM)]	Biodiesel yield (Y)/conversion (C) %	Reference
Chlorella protothecoides	Sulfuric acid	100	56:1	30, 4, 160	Y ≈ 60	Miao and Wu (2006)
Oedogonium sp.	Sodium hydroxide	–	–	–, 3, 300	Y > 90	Hossain et al. (2008)
Spirogyra sp.	Sodium hydroxide	–	–	–, 3, 300	Y > 90	Hossain et al. (2008)
Nannochloropsis oculata	Al_2O_3 supported CaO	2	30:1	50, 4, 1100	Y = 97.5	Umdu et al. (2009)
Chlorella protothecoides	*Candida* sp. 99–125 sp. lipase	30	3:1	38, 12, 180	C = 98.15	Xiong et al. (2008)
Chlorella pyrenoidosa	*Penicillium expansum* lipase (PEL)	20	3:1	50, 48	Y = 90.7	Lai et al. (2012)

can be employed as the catalyst for transesterification of microalgal lipids. The enzyme-catalyzed reactions are less energy intensive than the chemical-catalyzed reactions as they can be carried out at moderate temperatures. The lipase-catalyzed conversion yields high-quality products, i.e., biodiesel and glycerol. Lipases are capable of catalyzing both the transesterification and esterification reactions which make them advantageous over most of the chemical catalysts as they can be used for feedstocks with high free fatty acid content (Guldhe et al. 2015a). Lipases can be used in two ways, i.e., extracellular and intracellular (whole cell catalyst) (Guldhe et al. 2015b). The main drawback associated with the enzyme catalysts is their high cost. However, immobilization of enzyme makes its reuse possible and thus improves the overall economics. Separation and purification of product is easier in the enzyme catalysis compared to chemical catalysis. The short-chain alcohols used in reaction and glycerol produced have a negative impact on the activity of lipase used for transesterification. Greener enzyme catalysis and sustainable microalgal feedstocks hold promising potential for environmentally friendly biodiesel production. This area is scarcely studied and needs further investigation to alleviate the constraints of lipase-catalyzed biodiesel conversion. Xiong et al. (2008) applied the *Candida* sp. 99–125 sp. lipase for the conversion of lipids extracted from *Chlorella protothecoides*, and observed 98.15 % biodiesel conversion.

5.3 Solvents Used in Conversion

The widely used acyl acceptors in transesterification reactions are short-chain alcohols like methanol and ethanol. The alcohol and oil in the reaction mixture form a two-phase system. Low dissolution rate of oil in the alcohol causes slower reaction rates. To increase the reaction rate solvents are added to the transesterification reaction mixture. A solvent in the reaction mixture increases the solubility of the reactants and also aids in the proper mass transfer and thus increases the reaction rate. Tetrahydrofuran (THF), diethyl ether, hexane, and tert-butanol are the solvents commonly employed in the transesterification reaction (Lam et al. 2010). The ideal solvent should be inert and nontoxic, and should be easily recovered after the completion of reaction. For high quality of the biodiesel, the solvent should be completely removed from it. The solvents can be used for transesterification catalyzed by either chemical catalyst or biocatalyst.

SCFs can also be applied as a solvent for transesterification reaction. SCFs form a single-phase system which drives the reaction at faster rates. With supercritical methanol a noncatalytic method of biodiesel production can be developed. In this method supercritical methanol and oil form a single phase and reaction is completed in short duration of time. In other mode SCFs can be used as solvents for transesterification reaction in the presence of catalyst. Taher et al. (2011) have advocated the enzymatic production of biodiesel from microalgal in supercritical CO_2. The supercritical condition leads to faster completion of reaction. Another advantage

with this method is the ease in separation of products. However, the high temperature and pressure requirements to maintain the supercritical conditions make this process an energy-intensive route. The supercritical method also leads to simultaneous extraction of lipid from algal biomass and transesterification of the lipids to biodiesel. The optimum yield of FAAE was reported to have obtained at 265 °C, and at pressure of ca. 80 bar using microwave reactor. Supercritical method has been reported to be suitable for noncatalytic transesterification of wet microalgae by Kim et al. (2013). At a high temperature when supercritical methanol is used, the water–methanol mixture in wet microalgae exhibits both hydrophobic and hydrophilic characteristics which will reduce the reaction time as well as product separation. It has, however, been reported that in situ transesterification will not be feasible when the water content is in excess of 31.7 % (Kim et al. 2013). The reason attributed for this is hydrolysis during transesterification.

6 Influence of Microalgal Lipids on Biodiesel Properties

The lipid profile has an important role to play in the characteristics of biodiesel. Fatty acid composition of lipids in microalgae has an influence over fuel properties such oxidative stability, cetane number, viscosity, iodine value, cold flow properties, etc. Long chain length and low degree of unsaturation are considered good for oxidative stability and high cetane number (Shekh et al. 2013). The feedstock which is rich in monounsaturated fatty acids is considered suitable for synthesis of biodiesel as it improves the cold flow property of the fuel. A substantial amount of saturated fatty acid content in the feedstock is equally important to impart oxidation stability in the fuel. As there is an inverse relationship between the cold flow property and the oxidation stability of the fuel, it has been reported that a high content of oleic acid (a monounsaturated fatty acid) could lead to strike a balance between these two properties (Tale et al. 2014). Tale et al. (2014) reported a high content of oleic acid for the five microalgae studied. The unsaturated fatty acid (comprising monounsaturated and polyunsaturated fatty acid) content in three strains of *Chlorella* species (isolate: KMN 1, KMN 2, KMN 3) ranged from 31.62, 31.26, and 35.01 %, respectively. The saturated fatty acids in the species were 40, 43.92, and 47.48 %, respectively. The species *Scenedesmus* sp. and *Monoraphidium* sp. possessed comparatively lesser content of unsaturated fatty acids of 12.77 and 16.26 %, respectively, and a much higher content of saturated fatty acids (61.82 and 58.7 %, respectively). It has been reported that the appropriate balance of UFA and SFA will be suitable for the production of biodiesel.

Cheng et al. (2014) reported that polyunsaturated fatty acids in microalgal lipids are prone to degradation and autoxidation when subjected to high temperature during either extraction of lipid or transesterification. This could further lead to loss of FAAE yield. Cheng et al. (2014) have thus suggested comparatively lower temperature (at 90 °C) of transesterification of lipids in wet microalgae by microwave instead of high temperature operation (at 250 °C) using supercritical alcohol.

Table 5 Fuel properties of biodiesel produced from different microalgae

Biodiesel characteristics	Units	ASTM 6751	EN 14214	*Chlorella protothecoides*	*Scenedesmus* sp.	*Nannochloropsis* sp.	*Dinoflagellate*
Cetane number	–	Min 47	Min 51	–	–	–	–
Calorific value	MJ kg^{-1}	–	–	41	–		–
Density	kg m^{-3}	860–900	860–900	0.864	0.852	0.854	0.878
Methyl ester content	%	–	Min 96.5	–	91.0	92.2	96.6
Linolenic acid methyl ester content	%	–	Max 12	–	8.26	–	–
Acid value	mgKOH g^{-1}	Max 0.8	Max 0.5	0.374	0.52	0.46	0.44
Iodine number	g100 g^{-1}	–	Max 120	–	–	–	–
Cold filter plugging point (CFPP)	°C	–	–	–11	–	–	–
Oxidative stability	h	Min 3	Min 6	–	5.42	1.93	1.02
Sulfur	wt%	Max 0.05	–	–	0.02	0.06	0.04
Reference	–	–	–	Miao and Wu (2006)	Chen et al. (2012)	Chen et al. (2012)	Chen et al. (2012)

Cheng et al. (2014) have also suggested that a high concentration of sulfuric acid as catalyst could promote polymerization of double bonds in the unsaturated fatty acids. Patil et al. (2013) have reported microwave-assisted transesterification of dried biomass of microalgal using supercritical ethanol. Table 5 depicts the fuel properties of biodiesel produced from different microalgal strains.

7 Challenges in Lipid Extraction and Conversion

The major challenge for lipid extraction and biodiesel conversion techniques is their economical viability. Biodiesel is a bulk commodity which is considered as a low-cost product compared to pigments and other therapeutic proteins from the microalgae. Thus for its successful industrialization, the crucial steps like extraction of lipids and conversion to microalgal biodiesel have to be economical. The other bottlenecks are the environmental implications and the scalability of the techniques. The commonly used solvents applied for the lipid extraction and conversion process are toxic and volatile in nature, which could be hazardous as well as threat to the environment. The wastewater generated during the conversion process contains acidic or basic catalyst, solvent, glycerol, etc. The techniques like microwave and sonication for extraction have shown excellent results in terms of yields. But yet these techniques have not been investigated at the industrial-scale microalgal biodiesel production plant. Applications of the heterogeneous chemical and biocatalysis are considered as the environmental friendly and efficient conversion methods. The fuel properties of microalgal biodiesel are still a concern. These fuel properties have to comply with the specification set by the international standards. However, the recent investigations and technical advances in the microalgal lipid extraction and biodiesel conversion processes have shown potential to alleviate these challenges toward the sustainable microalgal biodiesel production.

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Techno-economics of Algal Biodiesel

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Abstract Algal biodiesel production presents a possible carbon-neutral source of transportation fuel. Whilst algal biodiesel circumvents some of the issues arising from the use of crop- and waste–biomass-based fuels, the lack of commercial success raises questions regarding the feasibility of the process. Numerous economic and environmental impact assessments have produced highly variable results, predicting costs from as little as 0.42–72 USD L^{-1}. A meta-analysis of these assessments reveals that areal productivity and provision of nutrients, as well as energy and water usage, are the key challenges to algal biodiesel production. A consideration of maximum achievable photosynthetic activity indicates that some scope exists for increasing areal productivity; hence, the factors influencing productivity are discussed in detail. Carbon dioxide supply may represent the single most important challenge to algal biodiesel, while recycling of other nutrients (specifically nitrogen and phosphate) is essential. Finally, a careful balance must be struck between energy and water consumption; this balance is primarily influenced by bioreactor design. It is unlikely that algal biodiesel will supply a substantial portion of the world's transportation energy demand, but it may fill niche markets such as aviation fuel. Process economics are enhanced by integrating biodiesel production into a biorefinery, producing a suite of products.

Keywords Microalgae · Net energy ratio · Techno-economics · Feasibility

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1 Introduction

The need for renewable energy has prompted the investigation of biologically derived renewable fuels. First-generation biofuels rely on food crops and are in direct competition with food production for arable land. The use of corn ethanol has already impacted food prices (Zilberman et al. 2012) and the first-generation biofuels pose a serious threat to food security (Escobar et al. 2009). Second-generation biofuels avoid these issues by utilizing nonedible (primarily lignocellulosic) biomass as feedstocks. Second-generation biofuels are limited by unreliable biomass supply rates and low conversion efficiency (Sims et al. 2010). Algal-based "third generation" biofuels seem to overcome these challenges and potentially fulfill all sustainability requirements (Brownbridge et al. 2014). Algae are extremely versatile, with the ability to adapt to a range of environments and to produce different forms of biofuels, including bioethanol, biohydrogen, and biodiesel (Jones and Mayfield 2012). Rapid growth rates and high lipid yields make algal biomass ideally suited to biodiesel production (Chisti 2007).

The apparent promise of algal biodiesel is in stark contrast to the lack of commercial success. To date, no facility is producing biodiesel derived from algae on an industrial scale. The scientific community is divided as to whether any further resources should be spent on the concept (see, for instance, the articles "The nonsense…" and "The rationality…" [of biofuels] (Michel 2012; Horta Nogueira et al. 2013). Large-scale algal biofuel production faces a number of technical and economic challenges (Brownbridge et al. 2014), including high energy demands, operating expenses, and capital investment (Richardson et al. 2012; Rawat et al. 2013).

Economic assessments of algal biodiesel vary across a large range of estimates, hindering commercial interest (Liu et al. 2012). Projected costs for algal biodiesel range from 0.42 (Nagarajan et al. 2013) to 72 USD L^{-1} (Harun et al. 2011). Costs could be decreased by producing biodiesel as one of the commodities in a suite of different bioproducts in a biorefinery approach (Wijffels and Barbosa 2010), including (i) high-value coproducts such as pigments (Ferreira et al. 2013); (ii) bioremediated wastewater (Park et al. 2011; Pittman et al. 2011); (iii) ethanol or methane produced by the fermentation or digestion of spent biomass, respectively (Jones and Mayfield 2012); and (iv) carbon credits. Algal biodiesel has the potential to be a carbon-neutral fuel, but current analyses indicate greenhouse gas (GHG) emissions comparable to crop-based biofuels (Liu et al. 2012). A recent innovative study speculated on the future role of algal biodiesel with respect to carbon policies (Takeshita 2011). Strict regulations would decrease the use of fossil fuels and, subsequently, decrease the availability of anthropogenic carbon dioxide point sources for enhanced algal cultivation, potentially rendering algal biodiesel unfeasible. On the other hand, if carbon restrictions are held at moderate levels, algal biodiesel is predicted to be essential to the future energy mix.

One of the major technical arguments against algal biodiesel is the low efficiency of photosynthesis. The maximum theoretical efficiency for the photosynthetic conversion of water and carbon dioxide to glucose and oxygen, using the sun's light spectrum, is approximately 11 % (Walker 2009). The Shockley–Queisser limit for single-junction photovoltaic (PV) cells on the other hand is close to 30 % (Shockley and Queisser 1961); this limit can be raised using multi-junction cells. The key difference between PV cells (and other major sources of renewable energy such as wind- and hydropower) and microalgae is energy storage: whereas PV cells generate electricity that must be connected to a grid or secondary storage device, photoautotrophs store energy in chemical bonds. Energy storage is especially important when considering transportation energy, which currently accounts for approximately 20 % of global energy consumption (U.S. Energy Information Administration 2014). The gravimetric energy density of liquid fuels ($\sim$46 MJ kg^{-1} for biodiesel) is an order of magnitude greater than hydrogen ($\sim$6 MJ kg^{-1} at 70 MPa, including the required container) and more than a hundred times greater than lithium ion batteries ($\sim$0.6 MJ kg^{-1}) (Zhang 2011). Current electrical vehicles would need a 670 L battery to have a range similar to that of a diesel car with a 46 L tank (Eberle and von Helmolt 2010). Liquid biofuels are essential to applications which require high energy densities; aviation fuels in particular present a potential niche market for algal biofuels (Chisti 2013a).

Hydrogen represents the current energy storage alternative. A recent study compared the annual production of hydrogen by the electrolysis of water, driven by PV cells, to the annual biomass production by photosynthesis. It was found that the PV-electrolysis system had an energy efficiency of 11 %, whereas the annual biomass energy efficiency was 3 % (excluding downstream processing) (Blankenship et al. 2011). Current PV systems exceed the theoretical upper limit of algal biodiesel production efficiency and the PV-photosynthesis efficiency gap is set to grow in the future. While many see this as convincing evidence against the feasibility of algal biofuels, others argue for the use of genetic modifications, synthetic biology, and cell-free technologies (Blankenship et al. 2011; Zhang 2011). These approaches require significant further research.

In spite of the apparent inefficiencies, biofuels have an important role in the future energy economy, in part due to their immediate application in internal combustion engines. Unlike hydrogen, the global biodiesel market and infrastructure is well established (Sims et al. 2010). The International Energy Agency Technology Roadmap (International Energy Agency 2011) predicts that biofuels will constitute approximately 27 % of transportation fuels by 2050 and specifically emphasizes the need for the commercial deployment of advanced biofuel production facilities, including algal biodiesel. The success of algal biofuel depends on a holistic methodology, illustrated in Fig. 1. Biodiesel is a single product in an integrated biorefinery, and it will fill a niche, medium-term role in the future energy mix. This chapter investigates the major challenges and opportunities in the techno-economics of algal biodiesel.

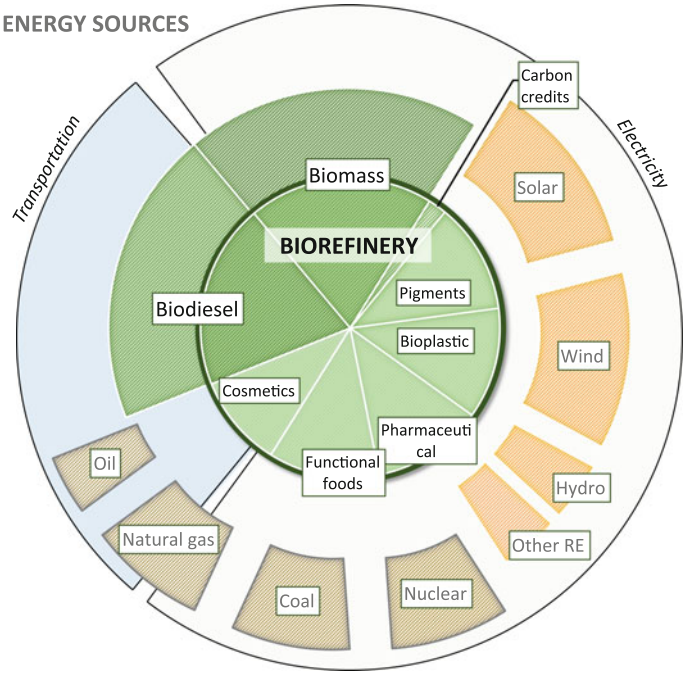

Fig. 1 Qualitative pie chart illustrating the integrated approach to biodiesel. Algal biodiesel is a single product from an integrated biorefinery. Both biodiesel and spent biomass represent future renewable energy sources, but biodiesel is one of the only future energy sources appropriate for transportation

2 Economic Assessment

Numerous studies have attempted to develop an economic model for algal biofuel production [recent reviews include Brownbridge et al. (2014), Nagarajan et al. (2013) and Williams and Laurens (2010)]. A list of recent economic analyses, with key assumptions, is presented in Table 1. Estimated costs of algal biodiesel production range from 0.42 to 7.50 USD L^{-1} in open ponds and 1.25 to 72 USD L^{-1} in closed PBRs. Low-cost estimates follow from optimistic assumptions, e.g., Nagarajan et al. (2013) estimated a final cost of algal biodiesel in the range of 0.42–0.97 USD L^{-1}, assuming growth rates between 30 and 60 g m^{-2} day^{-1} and a lipid content of 50 %, neither of which have been achieved independently or in combination in long-term outdoor cultivation. The wide range of cost estimates is due to the limited availability of industrial information regarding this immature technology (Brownbridge et al. 2014), and presents a major challenge to economic analyses.

Brownbridge et al. (2014) conducted an in-depth sensitivity analysis and found that the majority of the process cost is in the production of algal biomass, harvesting, and the price of inputs (e.g., fertilizer), while the total cost was most

Table 1 Recent (post-2010) cost estimates of algal biodiesel production along with key assumptions

Reference	Scale	Cultivation method	Biomass productivity	Lipid content	USD per kg algal biomass	USD per L algal lipid or biodiesel
Williams and Laurens (2010)			103 t ha^{-1} $year^{-1}$	35 %		1.52[b]
Amer et al. (2011)	500 ha	PBR	40–90 g m^{-2} day^{-1}	40 %		25–33
Amer et al. (2011)	500 ha	Ponds	24 g m^{-2} day^{-1}	40 %		4–7.50
Davis et al. (2011)	10 MG lipid $year^{-1}$ (1950.58 ha)	Ponds	25 g m^{-2} day^{-1}	25 %		2.25
Davis et al. (2011)	10 MG lipid $year^{-1}$ (1950.58 ha)	PBR	1.25 kg m^{-3} day^{-1}	25 %		4.78
Harun et al. (2011)		PBR				47–72
Norsker et al. (2011)	2100 t $year^{-1}$ (100 ha)	Ponds	21 t ha^{-1} $year^{-1}$		6.57	
Norsker et al. (2011)	4100 t $year^{-1}$ (100 Ha)	Tubular PBR	41 t ha^{-1} $year^{-1}$		5.51[a]	
Norsker et al. (2011)	6400 t $year^{-1}$ (100 Ha)	Flat-panel PBR	64 t ha^{-1} $year^{-1}$		7.91[a]	
Acién et al. (2012)	30 m^3	PBR	90 t ha^{-1} $year^{-1}$		89[a]	
Acién et al. (2012)	200 t $year^{-1}$	PBR	90 t ha^{-1} $year^{-1}$		16.2[a]	
Delrue et al. (2012)	333.3 ha	Ponds	20–30 g m^{-2} day^{-1}	20–50 %		1.3–2.53[a]
Delrue et al. (2012)		PBR	0.65–1.95 kg m^{-2} day^{-1}	20–50 %		2.29–4.34[a]
Richardson et al. (2012)	10 MG lipid $year^{-1}$	Ponds	Based on Davis et al. (2011)	Based on Davis et al. (2011)		3.54

(continued)

Table 1 (continued)

Reference	Scale	Cultivation method	Biomass productivity	Lipid content	USD per kg algal biomass	USD per L algal lipid or biodiesel
Richardson et al. (2012)	10 MG lipid $year^{-1}$	PBR	Based on Davis et al. (2011)	Based on Davis et al. (2011)		8.95
Nagarajan et al. (2013)	400 ha	Ponds	30 g m^{-2} day^{-1} or 60 g m^{-2} day^{-1}	50 %		0.42–0.97
Brownbridge et al. (2014)	100,000 t $year^{-1}$	PBR	80–120 t ha^{-1} $year^{-1}$	20–40 %		1.25–2.5[a]

[a]Converted from reported currency into USD using the average exchange rate in the year of publication acceptance
[b]Converted from USD/kg using a density for biodiesel of 0.9 kg L^{-1}

sensitive to variations in algal lipid content, annual areal productivity, plant production capacity, and the price of carbon. Davis et al. (2012) attempted to combine data from the resource assessment, techno-economic, and life-cycle analysis models of major US national laboratories into a harmonized baseline model representing near-term production of algal biodiesel. They found that models are most sensitive to estimates of algal productivity, lipid content and composition, and downstream processing.

The cost of CO_2 supply is a major challenge to economic feasibility (Williams and Laurens 2010). It is often assumed that the algal plant will be situated close to a point source of waste CO_2 and therefore carbon supply will be free. In reality, there are significant costs associated with transport and distribution of gas, technical problems related to the effect of flue gas contaminants on algal productivity, and sustainability concerns regarding the availability of CO_2 point sources in the future (Chisti 2013a).

Water, energy, and nutrient usage and recycle affect cost and environmental impact. Brownbridge et al. (2014) based their economic analysis on the assumption that the oil-extracted algal biomass would be gasified to produce further crude oil and electricity, while many others have included anaerobic digestion (AD) as a method to recycle nutrients (Harun et al. 2011; Sun et al. 2011; Davis et al. 2012; Nagarajan et al. 2013), and some consider both options (Delrue et al. 2012). Although there is extensive published knowledge and experience on the application of AD to human and animal waste, there is relatively little information on its application to algal biomass (Sialve et al. 2009; Ward et al. 2014).

Until recently, reports consistently pointed to harvesting as a major area of uncertainty that has large economic implications (Williams and Laurens 2010), but recent studies indicate progress in this area. Davis et al. (2012) assumed a 95 % harvesting, 85 % extraction, and 78–85 % conversion efficiencies, resulting in an overall efficiency of $\sim$65 % conversion of algal lipids to fuel. There is potential for substantial improvements in economics if the conversion efficiency can be improved. Harvesting methods such as flocculation followed by centrifugation (Nagarajan et al. 2013), settling followed by dissolved air flotation and centrifugation (Davis et al. 2012), belt filter press (Delrue et al. 2012), and OriginOil's 'Electro Water Separation' technology (Brownbridge et al. 2014) have been proposed as viable options.

New technologies with the potential to revolutionize biomass production have been explored, but are yet to be demonstrated at scale. For example, Norsker et al. (2011) estimate the production cost to be between 5.5 and 7.9 USD kg^{-1} biomass dry weight, but with improvements in solar radiation through site selection, a reduction in the flow velocity of mixing, free sources of nutrients, and CO_2, and an increase in photosynthetic efficiency of 60 %, the price of biomass production could be reduced to around 1.7 USD kg^{-1} dry weight for open ponds and 0.9 USD kg^{-1} dry weight in closed reactors—a price at which production of bulk commodity products (e.g., chemicals, fuels, materials, feed) becomes feasible. The likelihood of these improvements all occurring on a sufficiently large scale is uncertain.

Brownbridge et al. (2014) summarized the overall sentiment of the literature with the conclusion that "with current technologies, prices, and forecasts it is unlikely that a plant that has algal biodiesel as its primary product can be commercially feasible." Accurately assessing the economic feasibility of algal biodiesel in a biorefinery setting requires a fundamental understanding of the limiting factors, which literature indicates to be areal productivity, carbon dioxide and nutrient supplies, and energy requirements in cultivation as well as downstream processing.

3 Environmental Impact Assessment

One of the main driving forces behind research and development of algal biodiesel is its potential as an environmentally sustainable transport fuel (Slade and Bauen 2013). Rigorous analysis of the production process is required to determine the environmental impacts of algal biodiesel before it can be deemed sustainable. A popular method for assessing the environmental impact is life-cycle analysis (LCA). Many LCAs have been performed for algal biodiesel production under a variety of conditions and assumptions. These studies show that global warming potential, carbon footprint, greenhouse gas emissions, nutrient use, water consumption, and fossil energy requirements are the primary environmental concerns. Table 2 summarizes LCA results from a selection of reports on algal biodiesel.

Algae capture CO_2 during photosynthesis, which is released back into the atmosphere on combustion of algal biodiesel. Thus, the production of algal biodiesel can result in a carbon-neutral CO_2 cycling system at best, having no net effect on atmospheric CO_2. Biomass cultivation (mixing, pumping, and aeration), harvesting, and drying constitute the major fossil energy requirements for algal biodiesel production (Slade and Bauen 2013) and contribute to GHG emissions. Energy requirements may be offset by anaerobic digestion and combustion of spent algal biomass after lipid extraction. If fossil fuels are utilized in the production of algal biodiesel, more CO_2 will be emitted than consumed during algal cultivation. Similarly, if CO_2 from industrial flue gas is fed to algal cultures, this CO_2 will be released on combustion of the biodiesel and so will not result in a net reduction of fossil carbon and GHG emissions (Stephenson et al. 2010; Chisti 2013a; Azadi et al. 2014), although the associated carbon cycling may facilitate enhanced energy provision per unit CO_2 emissions formed. Understanding the relationship between CO_2 generation, supply, and consumption in the algal biofuel process is critical.

In addition to methane from anaerobic digestion, other coproducts include syngas, ethanol, glycerol, and nutrient-rich feed. The reduction in GHG emissions and other environmental burdens associated with producing these coproducts from algae as opposed to fossil sources has a significantly positive outcome on the overall LCA (Batan and Quinn 2010; Sander and Murthy 2010; Stephenson et al. 2010). AD, with associated recycle of nitrogen and phosphorus in the digestate, could aid in the reduction of eutrophication which occurs when nitrogen or phosphate is released into the environment, changing the natural concentration of

Table 2 Summary of several life-cycle analyses of algal biodiesel production by transesterification with a focus on carbon footprint/greenhouse gas emissions

Reference	Process description	LCA detail	Greenhouse gas emissions	Other impacts
Azadi et al. (2014)	Raceway, paddlewheel speed reduced at night Clarification and centrifugation Gas-fired- or solar and gas-fired-drying Wet or dry extraction Spent biomass used for biogas/syngas	GHG emissions (CO_2) from energy, fertilizer, construction, chemicals Sensitivity analyses for: – oil extraction – bio-energy from spent biomass	Total (wet extraction + anaerobic digestion): 85 g MJ^{-1} (CO_2) Total(wet extraction + hydrothermal gasification): 40 g MJ^{-1} (CO_2)	
Batan and Quinn (2010)	Sparged polyethylene PBR bags CO_2 from flue gas, mixed with air at 2 % Centrifugation and solvent extraction Transport	"Strain-to-pump" GHG emissions (CO_2, CH_4, N_2O) Biomass and glycerin coproducts	Total: −59.49 g MJ^{-1} (CO_2) Negative CO_2 output due to algal CO_2 capture, displacement of petroleum, and displacement of coproducts	
Clarens and Resurreccion (2010)	Raceway Additional CO_2 sparged in Wastewater effluent comparison Flocculation and centrifugation	Cradle-to-gate for dry biomass (not biodiesel) Functional unit: 317 GJ biomass energy	Total: 0.567 g MJ^{-1} (CO_2)	Water use: 0.004 m^3 MJ^{-1} Eutrophication potential: 1.04 g MJ^{-1} (PO_4) Land use: 1.3 ha MJ^{-1}

(continued)

Table 2 (continued)

Reference	Process description	LCA detail	Greenhouse gas emissions	Other impacts
Sander and Murthy (2010)	Raceway (inoculated from PBR) CO_2 from flue gas Wastewater for nutrients Chamber filter press and centrifugation Natural gas-fired dryer Solvent extraction (hexane) Transport	"Strain-to-pump" Coproduct allocation: – spent biomass to ethanol – distillers dry grain (DDGS)	Growth: 0.00 g MJ^{-1} (CO_2) Harvest (filter press): 241.81 g MJ^{-1} (CO_2) Separation: 6.33 g MJ^{-1} (CO_2) Biodiesel conversion: 3.18 g MJ^{-1} (CO_2) Coproduct allocation: −273.60 g MJ^{-1} (CO_2) Total (filter press): −20.9 g MJ^{-1} (CO_2) Total (centrifuge): 135.71 g MJ^{-1} (CO_2)	
Stephenson et al. (2010)	Raceway or tubular PBR Flue gas (12.5 % CO_2) Two stage nitrogen replete/deficient strategy Flocculation Homogenization and solvent extraction Anaerobic digestion	Cradle-to-gate Coproduct allocation: – Methane – Glycerol	Total (tubular PBR): 315 g MJ^{-1} (CO_2) Total (raceway): 18.86 g MJ^{-1} (CO_2) Cultivation has highest CO_2 emissions.	
Campbell et al. (2011)	Raceway CO_2 from flue gas (15 %) Flocculation, floatation, and centrifugation Anaerobic digestion	System boundaries exclude construction of production facilities	Total (30 g m^2/d): −25.86 g MJ^{-1} (CO_2) Total (15 g m^{-2} day^{-1}): −11.82 g MJ^{-1} (CO_2)	

nutrients, and particularly dissolved oxygen, in surrounding rivers and lakes and threatening ecosystems. Fresh water is a very scarce commodity, and overuse has serious environmental and social sustainability issues.

4 Key Challenges to Feasibility of Algal Biodiesel

The economic and environmental impact of algal biodiesel is influenced by the use of technology, biology, and process design to address a set of key technical challenges. These challenges include (i) areal productivity, limited by light utilization; (ii) nutrient supply, including carbon, nitrogen, and phosphate; and (iii) water and energy consumption.

4.1 Areal Productivity

Algal productivity is influenced by the characteristics of the strain as well as environmental parameters such as nutrient availability, temperature, pH, and salinity, but is fundamentally limited by light availability. Available sunlight provides the ultimate constraint to biofuel production and, as such, arguments against the feasibility of biofuels usually refer to "maximum theoretical productivity" based on light utilization. This maximum productivity is dependent on the insolation, photosynthetic efficiency, and bioreactor design; an understanding of these factors will illuminate the discussion.

4.1.1 Insolation

The amount of solar energy (SSE) reaching the earth's surface is the total insolation (measured in W m^{-2}). SSE is abundant (120×10^3 TW) but diffuse, with annual average intensities of 200–280 W m^{-2} in regions of interest. Insolation is significantly less than the extraterrestrial irradiance due to atmospheric light scattering and absorption. Insolation data are used to evaluate the potential productivity of a site. Satellite-derived data for insolation calculations are freely available from the NASA Langley Research Center Atmospheric Science Data Center Surface Meteorological and SSE web portal supported by the NASA LaRC POWER Project (https://eosweb.larc.nasa.gov/sse/) (NASA 2013). Once a site has been selected, the only method of enhancing solar insolation is by adjusting the tilt of the insolated plane. This is impossible for open raceway ponds, but is an important consideration for closed (specifically flat panel) PBRs. See RETScreen International (2005) and Duffie and Beckman (2013) for an introduction to interpreting and applying insolation data in the assessment of SSE sources.

4.1.2 Photosynthesis

Only a portion of the solar irradiance spectrum is utilized during photosynthesis. A large amount of energy is absorbed by atmospheric gases, most importantly, oxygen and ozone, resulting in distinct absorption bands in the terrestrial insolation spectrum. Insolation spectrum data are freely available from ASTM (2003).

Green algae host two separate photoreaction centers activated by 680 and 700 nm wavelength photons, respectively (Zhu et al. 2008). Light-harvesting complexes allow photosystems to utilize energy from photons with shorter wavelengths by resonance energy transfer (RET), but photons with longer wavelengths have insufficient energy to cause excitation in the photoreaction center. Due to this limitation, photosynthetically active radiation (PAR) is most often defined as the insolation in the 400–700 nm bandwidth, with some sources adjusting the limits to 380–740 nm. The energy contained in the 400–700 nm range is approximately 42 % of the total insolation energy (using data provided by ASTM (2003)). Bacteria often contain photosystems with the ability to absorb near-infrared light with wavelengths up to 1000 nm, thereby increasing the useable light spectrum (Blankenship et al. 2011).

Light-harvesting complexes can absorb photons with shorter wavelengths and higher energy content than the 680/700 nm photons required by the reaction centers, but the excess energy is dissipated as heat during RET. This process represents a significant energy loss: given the terrestrial insolation spectrum (ASTM 2003), RET amounts to a decrease in energy of approximately 20 %.

The photosynthetic machinery of green algae and higher plants requires at least eight photons to fix a single carbon dioxide molecule (Zhu et al. 2008), or 48 photons to form glucose (that is, 24 at 680 nm and 24 at 700 nm), in a process commonly known as the Z-scheme. The 48 mole of photons contain 8323.40 kJ of energy, while the heat of combustion of glucose is 2805 kJ $mole^{-1}$, resulting in a 66 % energy reduction when operating in optimal light utilization conditions. This energy reduction is intrinsic to the photosynthesis Z-scheme.

4.1.3 Absorption and Photoinhibition

Not all of the incident lights over a given area of algal culture reach the photosynthetic machinery of an algal cell. A proportion is lost due to light scattering and reflection on the reactor surface, as well as absorption by non-photosynthetic material. The absorption coefficient is dependent on the wavelength (Hoepffner and Sathyendranath 1993). The total PAR is often weighted by $a(\lambda)$ to yield the photosynthetically useable radiation (PUR) (Morel 1978) or the yield photon flux (YPF) (Barnes et al. 1993), which provides a more realistic approximation to the photosynthetic action spectrum. The relationship between PUR and PAR is dependent on many factors including the water column depth, biomass concentration, and the state of photoacclimation. Spectrally resolved models have been developed to account for these effects (Behrenfeld and Falkowski 1997;

Kyewalyanga et al. 1997). All these aspects can (and should) be controlled by PBR design, but it is impossible to assign a fixed value to the ratio PUR:PAR. Zhu et al. (2008) used a value (without explanation) of 10 % for "inefficient absorption," the same value is adopted here, but significant variation is likely.

It is well known that the rate of photosynthesis is linearly proportional to the absorbed photon flux in the light-limited regime, but a maximum fixation rate is achieved once the irradiation crosses a certain threshold (light saturated regime). A further increase in light intensity can cause photoinhibition. The relationship between photon flux density and photosynthetic rate is commonly referred to as the photosynthesis-irradiance (PE) response curve. The PE curves have been studied intensively by MacIntyre and Kana (2002).

Algae can modify their PE characteristics to avoid photoinhibition in a process known as photoacclimation, primarily by modifying pigment content. Photosynthesis utilizes light most efficiently in the light-limited regime, and thus it is this region that is of interest to PBR design. In the light-limited region, the photosynthetic rate is approximately linearly proportional to the photon flux, with proportionality constant $\alpha = a\phi_m$, where a is the specific absorption coefficient and ϕ_m is the quantum yield (MacIntyre and Kana 2002). Both the quantum yield and the absorption coefficient are nearly constant in the light-limited region, although the quantum yield can decrease at higher levels of photon flux due to non-photosynthetic absorption. The quantum yield is limited to a maximum of 0.125 mol of oxygen produced per mole of photons absorbed due to the photosynthesis Z-scheme, as discussed above.

4.1.4 Photorespiration and Respiration

Algae consume energy and produce carbon dioxide by photorespiration as well as mitochondrial respiration. Photorespiration occurs when oxygenation instead of carboxylation is catalyzed by the Rubisco enzyme. At atmospheric conditions (380 ppm CO_2 and 21 % O_2), photorespiration can result in an approximately 49 % decrease in energy efficiency (Zhu et al. 2008). However, Rubisco is highly selective toward carbon dioxide and photorespiration can effectively be eliminated by maintaining a high CO_2 concentration around the enzyme (Sousa 2013).

Respiration is necessary to perform a host of biological activities. Some 10 % of the oxygen generated by photosynthesis is used in respiration; higher reported values can often be attributed to the presence of respiring bacteria (Talling 1957; Li et al. 2003). As with photorespiration, respiration can be minimized by decreasing oxygen concentration.

4.1.5 Theoretical Areal Productivity

The primary factors influencing the efficiency of light utilization are illustrated in Fig. 2. Approximately, 89 % of the energy lost can be attributed to the inherent

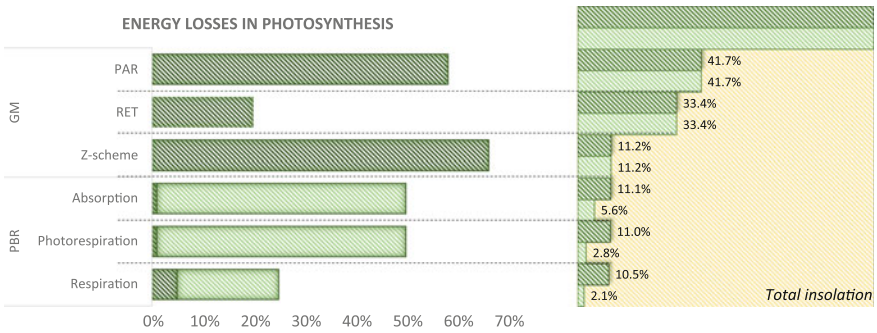

Fig. 2 Factors influencing energy loss in photosynthesis. *Left* The total percentage of energy lost due to individual processes. Energy losses due to the photosynthetic machinery are fixed and can only be addressed through substantial genetic modification (GM). The energy losses due to photorespiration, respiration, and inefficient/non-photosynthetic absorption are dependent on environmental factors; estimates of minimum and maximum losses are shown. These effects can be addressed through PBR design. *Right* Cumulative effect of energy losses on total energy efficiency, showing an estimated final efficiency between 3 and 11 %

photosynthetic machinery. Genetic modification is required to address these issues, and a number of suggestions are made in the literature. Notably, Chen and Blankenship (2011) suggest expanding the PAR range through the incorporation of red-shifted chlorophylls, which will result in the capture of a major portion of the solar irradiance spectrum. However, absorbed photons with wavelengths longer than 700 nm cannot be utilized by algal reaction centers. To overcome this problem, some authors suggest coculture with bacterial species able to utilize photons with wavelengths up to 900 nm (Pilon et al. 2011). Blankenship et al. (2011) suggested a complete re-engineering of photosystem I to absorb photons close to 1100 nm.

Other factors can be minimized through PBR design. Eliminating photorespiration could double photosynthetic efficiency: PBR oxygen concentrations typically exceed saturation levels (Molina et al. 2001), and strategies for degassing have been explored (Sousa 2013). Respiration is an essential cellular process and cannot be removed completely, but ratios of respiration to photosynthesis of as low as 5 % have been observed in nature (Talling 1957). Similarly, inefficient- and non-photosynthetic absorption could potentially be minimized if PBRs can be designed to operate purely in the light-limited regime. Proper PBR design can increase the efficiency of light utilization from 3 to 11 %, but this is still significantly lower than current PV cell efficiencies.

4.1.6 Biomass and Lipid Productivity

The photosynthetic efficiencies discussed above pertain to the conversion of SSE into chemical energy contained in glucose, which does not translate directly into areal biomass productivity. The biomass productivity (g m^{-2} day^{-1}) can be

calculated through an elemental carbon balance: Carbonaceous Biomass Accumulation = Carbon Fixation − Respiration = 0.4 × Net Glucose Production (the mass fraction of carbon in glucose is 0.4). The net glucose production is determined using the photosynthetic efficiencies above, and the areal biomass productivity is calculated using the following equation:

$$\frac{dX'}{dt} = \left(\frac{0.4}{f_C}\right) \times \left(\frac{\eta_P I}{\Delta H_c}\right)$$

where dX'/dt is the rate of biomass accumulation per unit area (g m^{-2} s^{-1}), $f_C \approx 0.5$ is the mass fraction of carbon in the biomass (Mirón et al. 2003), ΔH_c is the heat of combustion of glucose (15.57 kJ g^{-1}), η_P is the photosynthetic efficiency, and I is the insolation (W m^{-2}). The $0.4/f_C$ term varies between 65 and 90 %, depending on the biomass composition. An annual average insolation of 250 W m^{-2} and photosynthetic efficiency of 3 or 11 % will result in a biomass productivity of 16 or 61 g m^{-2} day^{-1}, respectively.

Comparison in Table 1 shows that estimates of actual productivities are in general much lower than the theoretical limit. This indicates that there is substantial room for improvement in PBR design to minimize the productivity gap. The decreased productivity can be attributed partly to seasonal variation. Algal productivities were estimated to range from 6.2 (winter) to 16.5 g m^{-2} day^{-1} (spring) in the region of the US most suitable for algal production (Davis et al. 2012). Seasonal variations are often neglected in economic models, but affect process cost greatly. Facilities large enough to accommodate peak season productivities are oversized for the rest of the year. It is essential that data on algal productivities in large-scale outdoor facilities over seasonal variations be generated, both from experimental observations and using the techniques described above, and made available to allow R&D to consider seasonal costs, energy consumption, and productivities rather than annual averaged values (Davis et al. 2012). Site and strain selection is critical in minimizing seasonal fluctuations.

The feasibility of algal biodiesel is heavily dependent on biomass productivity as well as lipid content and composition. In the Davis et al. (2012) economic model, an estimated algal productivity of 25 g m^{-2} day^{-1} and a lipid content of 25 % was used. However, these assumptions resulted in higher estimates of lipid productivity than many developers felt justified at the time. Reducing productivity to a more realistically achievable season-dependent annual average of 13.2 g m^{-2} day^{-1} resulted in a selling price of 19.6 USD gal^{-1} (5.2 USD L^{-1}). Williams and Laurens (2010) suggest that 35 % lipid appears to be a maximum realistic target. They also point out that an increase in lipid content results in a decreased fraction of other cell components (e.g., protein, nucleic acid, and carbohydrate), which in turn leads to reduced growth rates. Correlating growth and lipid content data from a variety of studies (e.g., see the analysis of Griffiths and Harrison 2009), it was estimated that an increase in lipid content from 15 to 30 % would lead to a reduction in growth rate of 50 % or more. In addition, non-triacylglycerides unsuited to biodiesel production can account for a sizeable portion of the lipid fraction (Davis et al. 2012).

These factors are often unaccounted for in models that predict a concurrently high lipid content and growth rate. Genetic modification of strains to improve productivity, particularly with concomitant high triacylglycerol content, and aid recovery of lipids, could have major impacts on algal biodiesel feasibility (Chisti 2013a). Robustness of such modifications, particularly for systems not operated as monoseptic, also needs consideration.

Seasonal variations and lipid conversion rates notwithstanding the theoretical limit to productivity are nearly 200 % greater than that which is currently achieved. Insolation and the fundamental limits to photosynthetic efficiency do not render algal biodiesel infeasible, as is commonly believed, and there is great scope for improvement in areal productivity.

4.2 Nutrient Supply: Carbon, Nitrogen, and Phosphate

Generating algal biomass requires, at minimum, the stoichiometric equivalent of its average elemental composition: $CH_{1.7}O_{0.4}N_{0.15}P_{0.0094}$ (Oswald 1988). CO_2 is often the major or only source of carbon in algal cultivation (Chisti 2007). Algae utilize CO_2 from the air, but supplementary CO_2 in cultivation systems is essential for commercially viable growth and lipid productivities (Brennan and Owende 2010; Pate et al. 2011). Purchasing supplementary CO_2 can increase operating costs by 3–10 % (Takeshita 2011; Chisti 2013a; Nagarajan et al. 2013; Richardson et al. 2014). The cost and availability of CO_2 is affected by policies aiming to stabilize atmospheric CO_2 and the environmental impacts of CO_2 release. Sequestration of captured CO_2 will be an essential means of meeting climate stabilization constraints in the next 50–100 years. Production of biodiesel, which is eventually be combusted, at best results in carbon-neutral cycling.

Generating 1 t of algal biomass requires approximately 1830 kg of CO_2, 50–80 kg of nitrogen, and 5 kg of phosphate (Chisti 2007; Pate et al. 2011; Borowitzka and Moheimani 2013). Therefore, nitrogen and phosphate are two additional nutrients of particular concern due to the volumes required, cost, and long-term sustainability (Chisti 2013a). Species selection can affect nutrient requirements (Harrison et al. 2013). Selection of algal species with a low nitrogen content, or species that can maintain biomass productivity with an enhanced lipid content under nitrogen limiting conditions, could assist in minimizing the nitrogen input required. The application of nitrogen limiting conditions, known to enhance lipid content of many algal species (Griffiths and Harrison 2009; Griffiths et al. 2012), can reduce the nitrogen use of the overall process. Lardon et al. (2009) estimated that the energy required for provision of fertilizer can be reduced, under nitrogen-limited conditions, to 6–9 % of total process energy (compared to 15–25 % under nitrogen sufficient conditions). Despite potential reductions in nutrient requirements, feed and fertilizer requirements are central to assessing algal biodiesel feasibility.

4.2.1 Availability

Atmospheric CO_2 exists in abundance. Algae are more efficient at fixing CO_2 than terrestrial plants, which results in higher lipid yields (Chisti2013a; Borowitzka and Moheimani 2013). However, atmospheric CO_2 levels remain insufficient for growing large concentrations of algae. Genetic engineering could be used to improve algal carbon concentrating mechanisms (Savile and Lalonde 2011), but gas–liquid CO_2 mass transfer remains limiting. Reports indicate that heterotrophic cultivation can result in very high biomass and lipid production (Rosenberg et al. 2008; Chisti 2013a); however, the organic carbon is ultimately derived from plants and so reintroduces many of the limitations to terrestrial plant-derived biodiesel (including land use, water, and productivity), unless it can be sourced from waste resources.

Industrial waste, such as flue gas, represents an inexpensive source of concentrated CO_2. Flue gas contains up to 20 % CO_2 from conventional sources, far exceeding atmospheric concentrations of 0.036 % (Brennan and Owende 2010). This decreases cost but restricts the location of the algae facility to the vicinity of CO_2 point sources. Reports indicate that the amount of CO_2 required to produce meaningful amounts of algal biodiesel far outweighs the CO_2 availability at point sources (Pate et al. 2011; Chisti 2013a). According to an analysis by Pate et al. (2011), 140 million tons of CO_2 are required to produce 40 billion L year^{-1} of algal biodiesel (only 2–3 % of liquid fuel consumption in the USA), but only 193 million tons of CO_2 emissions are available at stationary sources in the region best suited to algal cultivation in the USA. As production of energy from renewable sources is set to increase, CO_2 point sources such as coal burning power stations will decrease, further limiting CO_2 availability (Chisti 2013a). On the other hand, CO_2 will become available from new sources such as anaerobic digesters and bioethanol production plants (Takeshita 2011). These may also represent cleaner and more concentrated CO_2 sources.

Atmospheric nitrogen is almost unlimited, but current production methods of fixed nitrogen are energy intensive (Chisti 2013a). The provision of nutrients, particularly nitrogen, to bioprocesses not only represents a major cost of the process, but also greatly increases the carbon footprint and fossil fuel requirement due to the manufacture of ammonia, urea, or nitrate fertilizers (Harding 2009). The provision of fertilizer has been estimated to account for 15–25 % of the total energy requirement per unit algal biodiesel (Lardon et al. 2009). Clarens and Resurreccion (2010) attribute up to 50 % of the energy requirement for biomass production in open ponds to nutrient provision. In addition, at current production levels, any significant use of fertilizer for production of fuel would reduce the availability of fertilizer for food agriculture.

In contrast to CO_2 and nitrogen, the global supply of phosphate is scarce and finite. Due to its nongaseous environmental cycle, there is no means of production other than mining of phosphate rock, a nonrenewable resource. It is estimated that terrestrial global reserves may be depleted in 50–100 years (Cordell et al. 2009).

Due to its finite supply, there is an absolute necessity to reclaim phosphate from waste sources and recycle phosphate within all agricultural processes.

4.2.2 Carbon Dioxide Mass Transfer

In addition to the limitations of CO_2 supply to the cultivation plant, another key consideration is the rate of CO_2 transfer from the gas phase into the liquid culture. In raceway ponds, a paddlewheel creates turbulence that enhances CO_2 absorption from the surrounding air. However, the mass transfer in raceways is low in comparison to other PBRs, and is one of the limiting factors of these systems (Mata et al. 2010; Brennan and Owende 2010; Christenson and Sims 2011). Bubbling CO_2 into the ponds increases mass transfer and improves productivity (Brennan and Owende 2010), but much of the CO_2 is lost to the atmosphere due to short bubble residence times (Mata et al. 2010; Christenson and Sims 2011). Christenson and Sims (2011) suggest using rotating gas–liquid contactors to increase mass transfer.

Closed PBRs commonly use gas sparging to provide CO_2. CO_2 losses from aeration are lower in closed PBRs compared to open systems, and mixing and mass transfer tend to be superior (Mata et al. 2010; Singh et al. 2011). In this analysis, the combined consideration of gas–liquid mass transfer and CO_2 uptake rates by the algae is essential to maximize the efficiency of CO_2 utilization (Langley et al. 2012). The compression of gas for sparging is energy intensive and thus limits the feasibility for large-scale algal biodiesel production. Carvalho et al. (2006) report the use of an open gas exchange unit at the bottom of a flat-panel reactor for gas–liquid mass transfer. However, this system is open to contamination, which is usually one of the advantages of closed PBRs. Carvalho et al. (2006) describe membrane aeration in which CO_2 diffuses through a silicone or hollow-fiber membrane tubing. This prevents CO_2 losses that occur with bubbling, and also allows for accurate control of transfer rates and the use of pure CO_2. However, large membrane surface areas are required which contributes to the cost of cultivation. High salt media, as used with marine algae, limits membrane diffusion, and bacterial cells can cause fouling (Carvalho et al. 2006). Further development of low energy PBRs with high mass transfer will greatly enhance the feasibility of algal biodiesel.

4.2.3 Alternative Fertilizer Sources

There are three approaches to reducing the need for fertilizers in algal biodiesel production: (1) using a source of waste nutrients, e.g., domestic wastewater, (2) using nitrogen-fixing algae or bacteria, and (3) recycling the nutrients in the non-lipid portion of the biomass. Wastewater sources are commonly suggested as a means of supplying cheap nutrients to algal culture, but wastewater streams vary in composition over time, making reliable production challenging, and the nutrient

concentrations are often far below that required for optimal algal growth (Peccia et al. 2013). Wastewaters frequently contain toxic levels of ammonia (Borowitzka and Moheimani 2013) and pose a high risk of contamination with unwanted algae and other eukaryotes, bacteria, and viruses that may compete with or reduce the productivity of oleaginous algae (Peccia et al. 2013). Most problematically, the volumes of waste nutrient sources available globally will constrain the amount of biofuel produced (Chisti 2013a; Borowitzka and Moheimani 2013). It is likely that waste sources of nutrients (including sewage, urine, manure, waste biomass, ash, and bone meal) will be in high demand for other bioprocesses, as well as conventional agriculture, in the future.

Eukaryotic microalgae cannot utilize N_2 directly (Peccia et al. 2013). The ability to fix nitrogen could potentially be engineered into a lipid producing algae, or a naturally nitrogen-fixing strain of cyanobacteria could be used as the fuel producer. Alternatively, a coculture or series of cultures could be developed utilizing cyanobacteria (e.g., *Anabaena* or *Synechococcus*) or other nitrogen-fixing bacteria (e.g., *Rhizobium*) (Dawson and Hilton 2011; Borowitzka and Moheimani 2013). This kind of artificial symbiosis between oleaginous strains of microalgae and ammonium-producing bacteria has been demonstrated (Lipman and Peakle 1925; Ortiz-Marquez et al. 2012), and genetic modification to enhance nitrogen fixation has been successful in *Anabaena* (Chaurasia and Apte 2011). Nitrogen fixation as well as O_2 removal is energetically costly operation for the organism, indicating that an engineered strain would require an increased photosynthetic capacity in order to maintain both nitrogen fixation and lipid production (Peccia et al. 2013; Chisti 2013a).

4.3 Nutrient Recycling

Efficient recycling of CO_2, nitrogen, and phosphates within the process is likely to be an absolute requirement for sustainable production of algal biofuels (Chisti 2013a). This precludes the production of coproducts such as food, feed, or fertilizer that would result in net export of large amounts of nutrients from the process, unless the income or reduction in alternative production of those products offsets the energy and fertilizer use in the algal process.

After extraction of lipids (largely carbon and hydrogen), the residual nutrient-rich biomass can be fed to an AD, which produces methane for the generation of heat and power, as well as CO_2 and a liquid effluent rich in ammonia and phosphates (Sialve et al. 2009; Cai et al. 2013; Sheets et al. 2014; Ward et al. 2014). The liquid AD effluent currently appears to be the most feasible option for nutrient recycling (Chisti 2013a). The CO_2 produced by the AD and methane combustion could be fed to the algal culture, effectively recycling the majority of carbon not exported in the lipid fraction (Fig. 3).

Thermo-catalytic conversions (e.g., hydrothermal liquefaction or gasification) are alternatives to AD in recycling nutrients, but these thermal technologies are

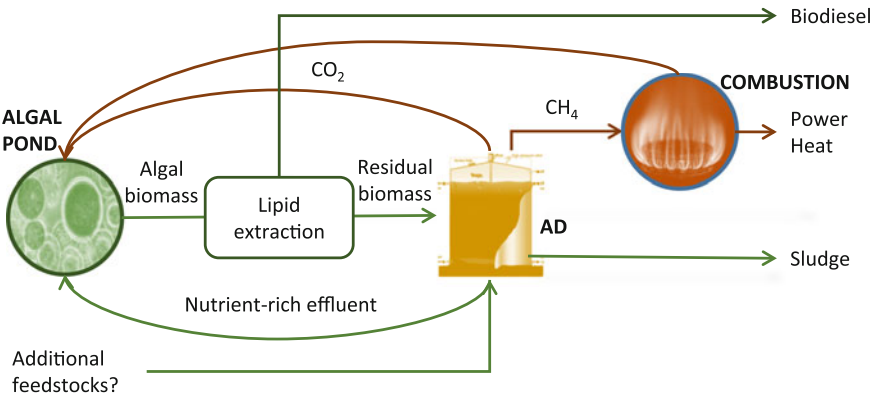

Fig. 3 Integration of anaerobic digestion with algal biodiesel production, indicating potential recycle of nutrients and CO_2

more often performed on whole biomass, rather than biomass residue from lipid extraction, because the benefits lie in reducing the capital and life-cycle costs associated with lipid extraction and conversion (Peccia et al. 2013). Hydrothermal liquefaction has been shown to convert 80 % of the energy in algal biomass to biocrude and generate a waste stream containing the majority of nitrogen and phosphate that has potential to be recycled (Valdez et al. 2012).

Nutrient recycling is critical to successful algal biodiesel production. However, product export and system inefficiencies will require constant input of carbon, nitrogen, and phosphate. CO_2 supplies are the limiting factor, particularly at the scales required to ensure algal biodiesel contributes to the future energy mix.

4.4 Water and Energy Consumption

The production of algal biofuels, whether in closed reactors or open ponds, requires large amounts of water and energy. Water is used in algal cultivation for replacing evaporative and process losses, with smaller amounts used in biodiesel production (Harto et al. 2010). At scales large enough to make a significant contribution to the future world energy mix, the supply of freshwater quickly becomes limiting, particularly in warm areas with high insolation (Borowitzka and Moheimani 2013). It is very unlikely that freshwater species will be used to produce algal biofuels in significant quantities, unless grown in wastewater, where production will be limited by wastewater availability. Even if marine algal species are used, the freshwater required to replace that lost to evaporation and therefore to prevent an increase in the salinity of the medium will become a limiting factor (Chisti 2013a).

The energy used to cultivate algae and produce biodiesel must be significantly lower than the energy that can be harnessed from the resulting biodiesel,

i.e., a favorable net energy recovery. If fossil fuel energy is used in the process, the environmental impacts of this must be weighed against the benefits of the resulting algal biodiesel. According to a review of studies on algal biodiesel sustainability, major contributing factors to energy intensity include PBR design, nutrient source, dewatering and biomass drying, and lipid extraction (Lam and Lee 2012; Harrison et al. 2013).

4.4.1 Algal Cultivation

In regions of high insolation, evaporation rates are generally greater than 1.5 m $year^{-1}$ or approximately 4 mm day^{-1} (Borowitzka and Moheimani 2013). It will be advantageous to minimize evaporative water loss by careful selection of location. The major factors determining evaporation rate are solar irradiance, temperature, wind speed, and humidity (Chisti 2013b). Temperature and irradiance are likely to be chosen for optimal algal productivity rather than minimizing water loss (higher algal productivity per unit volume also assists in decreasing the water requirement per unit product); the key criteria for site selection in terms of evaporation are therefore low wind speeds and high humidity. Evaporation could potentially be controlled or contained to an extent using closed reactors or covered ponds, with a concomitant increase in capital costs. There is also a difficult balance between minimizing evaporation and maximizing mass transfer of CO_2 into and O_2 out of the culture medium.

It is likely that replacement of evaporative water will need to be with saline water, leading to a gradual increase in the salinity of the culture medium. Algal cells will incur a metabolic cost at high salt concentrations due to energy required to export excess salt or produce compounds that maintain the osmotic balance (Chisti 2013a). If the increase in salinity affects microalgal performance, a portion of the pond water must either be purged regularly, or the entire pond contents replaced, leading to loss of nutrients and increased costs. Some success has been achieved in finding algal species that are tolerant of a wide range of salinities so that productivity can remain high while the pond salinity increases (Sing et al. 2014).

Currently, algal cultivation requires the majority of share of the energy input in biodiesel production (Stephenson et al. 2010; Harun et al. 2010; Slade and Bauen 2013). Several studies indicate that raceway ponds have substantially lower energy requirements than tubular PBRs, the latter frequently demonstrating unfavorable net energy ratios (NER) (more energy being used to produce the algae than can be harvested from the biodiesel) (Stephenson et al. 2010; Lam and Lee 2012; Rawat et al. 2013; Slade and Bauen 2013). Jones and Harrison (2014) discuss key factors affecting NER in vertical tubular reactors which may allow improved NER with improved reactor design and operation. Specifically, the NER is sensitive to the CO_2 partial pressure and can be lowered substantially by sparging with enriched CO_2. Power requirements for circulating the algal culture (paddle wheels, pumps, aeration) usually account for the majority of the energy demand. Stephenson et al. (2010)

suggest alternative PBR designs, such as flat panels or oscillating reactors to improve the NER of algal biodiesel production from closed systems. However, as long as extensive sparging is required for mixing and/or gas transfer, energy requirements will remain a critical consideration. Jorquera et al. (2010) demonstrated a much-improved NER for a flat-panel reactor in comparison to a tubular PBR, due to the substantially lower power requirements for pumping air (2500 W m^{-3} for tubular PBR; and 53 W m^{-3} for flat-panel PBR).

4.4.2 Downstream Processing

Downstream processing usually makes a smaller contribution to the NER of algal biodiesel production in comparison to cultivation, with the exception of drying (Harun et al. 2010; Slade and Bauen 2013). Centrifugation and filtration are energy-intensive harvesting methods (Lam and Lee 2012), but gravity sedimentation, flocculation, and flotation have minor energy requirements, usually attributed to pumping (Sander and Murthy 2010; Stephenson et al. 2010; Brennan and Owende 2010; Campbell et al. 2011; Rawat et al. 2013). Lam and Lee (2012) report energy consumption for bioflocculation to be 9.8 kJ kg^{-1} biodiesel.

A major cause of water loss is associated with harvesting. Minimizing the loss of water in the harvested algal biomass is limited by the economic and energetic feasibility of achieving efficient solid–liquid separation of small algal cells from the medium. Based on an algae plant producing 100,000 bbl of lipids $year^{-1}$, Borowitzka and Moheimani (2013) calculated evaporative water loss to be between 490 and 1307 ML and harvest loss between 13,415 and 35,772 ML. In contrast, Harto et al. (2010) calculate the evaporative water loss (average 165 L L^{-1} fuel) to be much greater than the process water consumption (average 50 L L^{-1} fuel) in open ponds.

Water inhibits lipid extraction and transesterification (Griffiths et al. 2010), thus necessitating drying. Many energy analyses assume the use of solar drying, and so do not discuss the large energy demand associated with other drying methods. Solar drying is only a realistic option under ideal conditions in sunny climates (Brennan and Owende 2010; Lam and Lee 2012), and prohibits water recycling. Sander and Murthy (2010) report in their case study that drying in a natural gas-fired dryer accounted for 69 % of the total energy consumption in the biodiesel production process. Using biodiesel conversion methods that are not inhibited by wet biomass is important for improving sustainability in terms of both water and energy usage (Azadi et al. 2014). Alternative methods of lipid extraction and biodiesel production that require only partial or no drying, such as supercritical fluid extraction, in situ transesterification, and hydrothermal liquefaction (Lam and Lee 2012), are especially attractive.

Lipid extraction accounts for 5–10 % of the energy requirement for biodiesel production (Sander and Murthy 2010; Stephenson et al. 2010). Solvent extraction is

the most commonly used method, but requires dry biomass (Lam and Lee 2012). Supercritical fluid extraction can be achieved using wet biomass, and thus eliminates the large energy burden associated with drying. This method requires heat and compression energy (Rawat et al. 2013). A commonly used fluid is supercritical CO_2 due to its low critical temperature. However, the energy requirement for capturing CO_2 from the atmosphere and recompressing it after each extraction needs to be assessed (Lam and Lee 2012).

Transesterification of the algal oil into biodiesel accounts for a small portion of energy requirements (0.036 J J^{-1} fuel) (Lardon et al. 2009; Sander and Murthy 2010). Acid–base and heterogeneously catalyzed transesterification are the conventional methods, but in situ transesterification is an emerging technology that has several advantages. In situ transesterification allows extraction and conversion to occur in one step in which biomass is used directly for conversion to diesel, removing the energy demands associated with lipid extraction as well as biomass drying (Griffiths et al. 2010; Lam and Lee 2012; Rawat et al. 2013). This may impact the application of the residual biomass.

Water may be recycled after the biomass has been harvested and processed, but recycling introduces additional challenges. Any soluble organic waste products, pond debris, or additives (e.g., chemical flocculants used in harvesting) will accumulate, potentially leading to reduced productivity. Contaminants such as competitors, predators, and pathogens (e.g., viruses) will be recycled if not removed. This may call for additional treatment or sterilization of the recycled medium, which inevitably increases energy requirements and cost (Williams and Laurens 2010).

The total water footprint of algal biodiesel has been estimated by Harto et al. (2010) to be between 216 and 656 L L^{-1} fuel in open ponds, based on conditions in the southwest US, with reduction to between 30 and 63 L L^{-1} fuel in closed systems. Yang et al. (2011) calculated a water requirement of 591 L L^{-1} fuel, assuming that all the water from harvesting was recycled. This is lower than estimates for ethanol from sugar beet or sugar cane (1388 and 2516 L L^{-1}, respectively, Gerbens-Leenes et al. 2009) but higher than that for cellulosic ethanol (between 356 and 423 L L^{-1}, Harto et al. 2010) and significantly higher than that for fossil fuels (2–6 L L^{-1}, Harto et al. 2010). Improvements in algal productivity per unit volume or an increase in cell density would reduce the water footprint per liter of fuel (Yang et al. 2011); however, algal cell density and culture surface area to volume ratio are constrained by the necessity to provide adequate sunlight to all cells.

Many studies have assessed the NER of algal production processes. The results are best illustrated in Fig. 4, which shows a NER ranging between 0.12 and 5.92. As discussed above, the choice of bioreactor (open ponds, flat or tubular PBRs) seems to be the most important factor influencing the NER. Water and energy requirements clearly illustrate the difficulty in choosing between open ponds and closed PBRs. Energy gains from the use of open ponds must be carefully balanced against water and CO_2 losses, as well as decreased productivity.

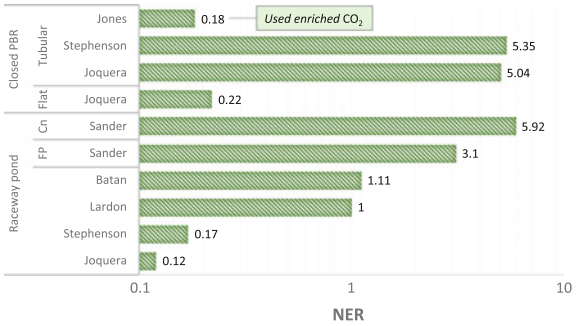

Fig. 4 Comparison of net energy ratios (NER; energy input into process: energy recovered from biodiesel produced) from a selection of life-cycle analyses for algal biodiesel production. *RP* raceway pond; *PBR* Photobioreactor; *Flat* flat panel. The *graph* shows NER below 1 (positive energy balance) for most biodiesel processes that use raceway ponds; the harvesting method (*FP* filter press; *Cn* centrifugation) plays a substantial role in final energy balance; and flat-panel reactors have potential for low NER

5 Conclusions

The composition of 1 L of algal biodiesel in terms of nutrients, solar and fossil energy, and water, as described in the previous sections, is illustrated in Fig. 5. A clear trade-off exists between open and closed systems in terms of NER and

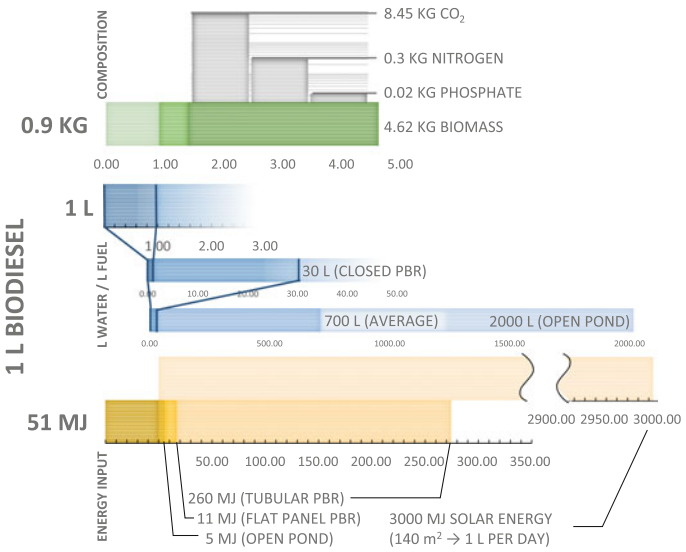

Fig. 5 Biofuel composition inputs, assuming 3 % photosynthetic efficiency, 0.5 g carbon per g biomass, 30 % lipid content, 65 % lipid to fuel conversion, 250 W m^{-2} insolation. Fossil energy inputs based on NER as discussed above, water usage as discussed above

water usage, although flat panel closed PBRs show great potential. The required SSE input is extremely large, with nearly 140 m^2 required to produce 1 L biodiesel day^{-1}, given an average daily insolation of 250 W m^{-2}. Approximately, 8.45 kg of CO_2 is required to produce 1 L of biodiesel; considering that the US requires approximately 2 trillion L of transportation fuel per year, this number quickly becomes staggering.

If algal biodiesel were to fill a niche in the liquid fuels market, such as aviation fuel, these limitations do not eliminate its potential as a feasible renewable liquid fuel source (Lundquist et al. 2010; Norsker et al. 2011; Chisti 2013a). Algal biodiesel is superior to first-generation biofuels for aviation fuel due to the high energy density and low freezing point (Brennan and Owende 2010). The 2008 demand for aviation fuel was 89 billion L $year^{-1}$. Using conservative estimates of CO_2 availability at point sources from Pate et al. (2011), 40 billion L of biodiesel can be produced from algae, supplying 45 % of aviation fuel demand. The production would require 1.5 Mha of land and, if open raceway ponds are used, close to 80,000 ML of water day^{-1}.

Aside from limitations of scale, the immediate determinant of the economic feasibility is a direct comparison between the cost of algal biodiesel and the price of competing fuels. The price of crude oil has varied between 70 USD bbl^{-1} (0.44 USD L^{-1}) and 114 USD bbl^{-1} (0.71 USD L^{-1}) over 2014, while the retail price of fossil-derived diesel in the US has averaged 1.02 USD L^{-1} over 2014 (Ameritrade et al. 2014; InfoMine 2014). Comparing predicted costs (Table 1) in light of the limitations discussed (max 30 % lipid content, <60 g m^{-2} day^{-1} biomass), and removing outliers, it seems feasible that biodiesel can be produced at between 2 and 3 USD L^{-1}.

Although the cost of producing biofuels may currently be higher than the cost of petroleum-based fuel, the cost of fossil fuels is artificially low as the environmental and social costs of burning fossil fuels are unaccounted for, particularly the impact of rising CO_2 levels in the atmosphere. The price of fossil fuels must include the cost of carbon mitigation; this will contribute substantially toward the competitiveness of sustainable liquid fuels. As liquid biofuels seem to be an essential part of the future energy mix, at least in the short term, the key question becomes twofold: how does the cost of producing algal biodiesel compared to the cost of producing liquid fuels from other sources? which feedstock(s) are most sustainable and cost effective for the production of biofuels? Although the cost of production of algal biodiesel may be higher, and the production process less well developed than that of terrestrial crop plants, crop plants suffer from issues regarding food security and sustainability. Major advantages of algal biodiesel include potentially higher productivity per unit area, production of only oil-containing biomass (i.e., no roots, leaves, and stems when only seeds are required), minimal loss of fertilizer due to runoff, potential to enhance carbon mass transfer through dissolution in liquid medium, and reduced competition with food crops for arable land, although competition at scale for fresh water and nutrients remains.

The economic and energetic feasibility of algal biodiesel will be greatly improved by applying the biorefinery concept in terms of utilizing all component

parts of the biomass generated (Wijffels et al. 2010; Williams and Laurens 2010; Harrison et al. 2013). Potential coproducts along with algal oil for biodiesel production include proteins for animal feed, carbohydrates for bioethanol, and high-value pigments. Coproducts resulting in a net export of nutrients, e.g., food, feed, or fertilizer, will only be feasible if it offsets the use of alternative sources, e.g., traditional feed or fertilizer, sufficiently to account for the inputs required to the algal process.

A great deal of uncertainty remains regarding the techno-economic feasibility of algal biodiesel. There are several key factors, highlighted by a review of the literature, to bear in mind when developing processes and models for algal biodiesel production. Progress in downstream processing has the potential to reduce energy consumption, but the algal cultivation NER remains a critical factor, especially in closed systems, and particularly in terms of the energy required to mix and sparge reactors. Complete drying of algal biomass is unlikely to be financially or energetically viable, and therefore conversion to biodiesel and extraction or production of any coproducts must be done using wet biomass. It is unlikely that it will be feasible to use freshwater for algal cultivation or replacing evaporative and process losses, and therefore there should be a focus on halotolerant production strains and minimizing water loss. Recycling of nitrogen and phosphate within the process will be critical and requires development and demonstration of cost- and energy-efficient mechanisms to achieve this. Additionally, coproducts leading to net export of nutrients from the process need to be carefully evaluated in terms of feasibility. Finally, major challenges appear to be in securing sufficient, inexpensive CO_2 supply at geographically suitable locations, balancing energy and water usage, and decreasing the gap between current and theoretical photosynthetic efficiencies.

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Fuel Alcohols from Microalgae

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Abstract Biosolvents such as acetone, butanol and ethanol are attractive biofuels which can reduce our dependence on fossil energy. Butanol is of particular interest as it can directly replace gasoline and be distributed using the current fuel infrastructure. Fuel alcohols are produced by fermentation of sugars obtained from starch. Starch and sugar feedstock for producing alcohols are currently obtained from crop plants. Microalgae are primitive plants that can accumulate large quantities of starch under suitable conditions. Use of algae as a source of starch for producing fuels overcomes many of the limitations associated with the conventional sources of starch. This chapter is focused on production of fuel alcohols from microalgae via the starch route.

Keywords Microalgae · Acetone · Fuel alcohols · Bioethanol · Biobutanol

1 Introduction

Biofuels are currently sourced from crops such as corn, soybean, and sugarcane. These crop-derived fuels have been linked to deforestation, water shortages and resource competition with food supply (Gouveia and Oliveira 2009; Costa and De Morais 2011). Microalgae offer an alternative to biofuels derived from higher plants. Under suitable conditions, microalgae can be grown rapidly without requiring arable land and potable water (Li et al. 2008; Gouveia and Oliveira 2009; Singh et al. 2011; Nguyen 2012). Suitably grown microalgae biomass can contain 50–60 % carbohydrates by dry weight. This can be converted to sugars and

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fermented by a diverse range of microorganisms to biofuels. Biofuels obtained from microalgae have the potential to stimulate domestic energy economy and reduce the need for non-renewable fossil-based fuels (Ellis et al. 2012). This chapter is an overview of the current production strategies for biofuels from microalgae. Implications and future applications of algal fuels are discussed.

The demand for clean, renewable, and sustainable biofuels continues to increase as petroleum prices remain volatile and global greenhouse gas (GHG) emissions continue to increase (Greenwell et al. 2009). Annual GHG emissions grew by 80 % between 1970 and 2004 (Greenwell et al. 2009) in pace with a rising worldwide demand for fossil energy (Singh and Gu 2010). The US currently consumes approximately 20 % of the crude oil used globally (EIA 2011b). Of this, 72 % is used for transportation purposes (EIA 2011a). Crude oil reserves are being depleted at a rate of approximately 85–90 million barrels of oil per day. With the worldwide proven oil reserves estimated at 1.3 trillion barrels, crude oil will likely be depleted within the next 50 years (Abdullah et al. 2007; EIA 2011b). The current heavy dependence on petroleum-based fuels is not sustainable due to diminishing crude oil reserves, fluctuating fuel costs and the adverse environmental impact of fossil fuel use (Chisti 2007; Pienkos and Darzins 2009; Demirbas and Fatih Demirbas 2011). Alternative fuels that can be produced renewably and sustainably are needed in preparation for a future without the fossil fuels.

During 1978–1996, the US Department of Energy (DOE) supported the Aquatic Species Program to specifically research the use of microalgae as a source of oil for making biodiesel. The high cost of biodiesel production from algal biomass and DOE budgetary constraints resulted in termination of this program (Sheehan et al. 1998). Recent increases in the price of crude oil, environmental concerns, and the need to reduce the US's dependence on imported oil have revived interest in microalgae as a source of renewable liquid fuels and other bioproducts (DOE 2010). Interest increased further after the enactment of the Energy Independence and Security Act (EISA) which mandated production of 36 billion gallons of renewable fuels in the US by the year 2022 (DOE 2010).

The algae industry is currently estimated to be worth $1.4 billion (http://www.nationalalgaeassociation.com/). Microalgae are viewed as an important feedstock for future production of renewable fuels. Algae capture sunlight and store it as chemical energy in the form of starch, oil and other chemicals (Li et al. 2008; Demirbas 2010; Ellis et al. 2012). Microalgae play a vital role in recycling carbon in the biosphere by converting carbon dioxide into organic compounds through photosynthesis (Christenson and Sims 2012) while producing oxygen.

Microalgae have potential advantages over other energy crops for production of renewable fuels: (1) microalgae have higher growth rates than terrestrial crops; (2) microalgae can be grown on non-arable or marginal lands with various qualities of water; (3) microalgae require little maintenance; (4) depending on growth conditions, microalgae generate high concentrations of energy rich compounds such as starch and lipids; and (5) microalgae do not require diverting food resources to energy production (Chisti 2007; Amin 2009; Gouveia and Oliveira 2009; Azócar et al. 2010; DOE 2010; Huang et al. 2010; Mata et al. 2010; Wijffels et al. 2010). In

addition, displacement of fossil fuels with microalgae derived fuels may contribute to reduced emissions of carbon dioxide (Singh et al. 2011).

In addition to fuels, algal biomass can be a source of other high-value byproducts and animal feed. Such byproducts can contribute to global food/feed supply and positively impact the economics of algal biomass production (Olivares and Sayre 2012). Furthermore, algae can be used to capture and recycle nutrients contained in municipal and farm wastewaters to produce value-added outcomes (Olivares and Sayre 2012).

2 Utility of Microalgae

Microalgae have been estimated to produce 30–100 times more energy per hectare compared to terrestrial crops (Greenwell et al. 2009; Demirbas 2010). Other attractive features of microalgae were mentioned earlier in this chapter.

Microalgae can be grown in such a way that the biomass produced is rich in carbohydrates. Carbohydrates can be hydrolyzed to their monomeric sugars for use in fermentations to produce fuel alcohols. Pentose and hexose sugars of microalgal carbohydrates (Table 1) can be consumed by other microorganisms to produce fuels via the pentose phosphate pathway and the Embden-Meyerhof-Parnas pathway, respectively (White 2006; Ezeji et al. 2007). In many cases, starch is the main carbohydrate in microalgae (Table 2) (Siaut et al. 2011; John et al. 2011). Once the intracellular starch is recovered from the cell, it can be readily broken down by amylase enzymes to glucose. Depending on the culture conditions, the carbohydrate content of the biomass may exceed 60 % of dry weight. The stored starch content in the biomass can be enhanced by controlling the supply of nitrogen and iron during cultivation (Liu et al. 2008; Dragone et al. 2011; Suali and Sarbatly 2012), for example. Other factors may also influence the starch content.

2.1 Extraction of Microalgal Carbohydrates

Starch and other carbohydrates stored within the algal cells and are not readily accessible to hydrolytic enzymes. Usually, the intracellular carbohydrates must first

Table 1 Sugar release from microalgae after acid hydrolysis

Sugar	Dry weight (DW) %
Total measured sugar	17.94
Glucose	11.49 ± 0.10
Xylose	0.79 ± 0.05
Galactose	4.42 ± 0.08
Mannose	1.24 ± 0.11

Modified from Laurens et al. (2012)

Table 2 Starch content of various microalgae species

Algal strain	Starch (% of dry weight)	Reference
Anabaena cylindrical	30.0	Zhu (2014)
Aphanizomenon flosaquae	23.0	Zhu (2014)
Chlamydomonas reinhardtii	53.0	Kim et al. (2006)
Chlorella pyrenoidosa	26.0	Zhu (2014)
Chlorella sp. TISTR 8262	21.5	Rodjaroen et al. (2007)
Chlorella sp. TISTR 8485	27.0	Rodjaroen et al. (2007)
Chlorella sp. TISTR 8593	22.0	Rodjaroen et al. (2007)
Chlorella vulgaris IAM C-534	37.0	Hirano et al. (1997)
Chlorella zofingiensis	28.0	Zhu (2014)
Chlorococcum sp. TISTR 8583	26.0	Rodjaroen et al. (2007)
Chlorococcum sp. TISTR 8973	16.8	Rodjaroen et al. (2007)
Euglena gracilis	18.0	Zhu (2014)
Nostoc maculiforme TISTR 8406	30.1	Rodjaroen et al. (2007)
Nostoc muscorum TISTR 8871	33.5	Rodjaroen et al. (2007)
Nostoc paludosum TISTR 8978	32.1	Rodjaroen et al. (2007)
Nostoc piscinale TISTR 8874	17.4	Rodjaroen et al. (2007)
Nostoc sp. TISTR 8872	30.7	Rodjaroen et al. (2007)
Nostoc sp. TISTR 8873	32.9	Rodjaroen et al. (2007)
Oscillatoria jasorvensis TISTR 8980	9.7	Rodjaroen et al. (2007)
Oscillatoria obscura TISTR 8245	12.6	Rodjaroen et al. (2007)
Oscillatoria okeni TISTR 8549	8.1	Rodjaroen et al. (2007)
Oedigonium sp.	33.6 (total sugar)	Hossain et al. (2008)
Oscillatoria sp. TISTR 8869	19.3	Rodjaroen et al. (2007)
Phormidium angustissimum	28.5	Rodjaroen et al. (2007)
Porphyridium cruentum	57.0	Zhu (2014)
Scenedesmus acuminatus TISTR 8457	7.3	Rodjaroen et al. (2007)
Scenedesmus acutiformis TISTR 8495	16.4	Rodjaroen et al. (2007)
Scenedesmus acutus TISTR 8447	18.6	Rodjaroen et al. (2007)
Scenedesmus arcuatus TISTR 8587	12.9	Rodjaroen et al. (2007)
Scenedesmus armatus TISTR 8591	15.4	Rodjaroen et al. (2007)
Scenedesmus dimorphus	52.0	Zhu (2014)
Scenedesmus obliquus TISTR 8522	23.74	Rodjaroen et al. (2007)
Scenedesmus obliquus TISTR 8546	23.4	Rodjaroen et al. (2007)
Scenedesmus sp. TISTR 8579	20.4	Rodjaroen et al. (2007)
Scenedesmus sp. TISTR 8982	13.3	Rodjaroen et al. (2007)
Scenedesmus, Chlorella, Ankistrodesmus, Micromonas, and *Chlamydomonas*	35.0 (total sugar)	Ellis et al. (2012)
Spirogyra sp.	43.3 (total sugar)	Hossain et al. (2008)
Spirulina fusiformis	37.3	Rafiqul et al. (2003)
Spirulina platensis	14	Zhu (2014)
Synechococcus sp.	15	Zhu (2014)

Modified from John et al. (2011)

be extracted from the cells by mechanical, chemical, or enzymatic pretreatment (John et al. 2011; Ellis et al. 2012; Potts et al. 2012). Once the starch is released and hydrolyzed, fermentable sugars are produced (John et al. 2011). Release and hydrolysis may be combined in a single treatment step. Some microorganisms possess the necessary enzymes to both hydrolyze, or saccharify, the released starch to sugars and ferment these to alcohols and other solvents.

3 Alcoholic Fermentation

Fermentation is an anaerobic microbial process that converts sugars to alcohols, or other products such as acetone. Diverse microorganisms including bacteria (Ellis et al. 2012), yeasts, and filamentous fungi can ferment sugars (Harun et al. 2010; Harun and Danquah 2011; John et al. 2011; Zhu 2014). The reaction scheme for ethanol production from glucose is as follows (White 2006):

$$\underbrace{C_6H_{12}O_6}_{\text{Glucose}} + 2ADP + 2P_i \rightarrow 2\underbrace{C_2H_6O}_{\text{Ethanol}} + 2CO_2 + 2ATP$$

where ADP is adenosine diphosphate, P_i is phosphate and ATP is adenosine triphosphate. Based on the theoretical yield, 1 kg of glucose can provide 0.51 kg of ethanol and 0.49 kg of CO_2 (Zhu 2014).

A variety of sugars can be fermented depending on the microorganism used. Fermenting microbes include *Saccharomyces cerevisiae*, *Zymomonas mobilis*, various *Clostrdium* spp. and various *Thermoanaerobium* species (Kim and Gadd 2008). Depending on the microorganism, a fermentation may proceed via a branched pathway that produces multiple products such as acetate, lactate, and hydrogen (White 2006; Kim and Gadd 2008). Alternatively, a fermentation may proceed via a linear pathway as in *Saccharomyces* and *Zymomonas*, to ultimately lead to ethanol, carbon dioxide and water (Kim and Gadd 2008).

3.1 Microalgae to Ethanol

Of the various potential routes for producing fuel alcohols from microalgae, production of bioethanol from starch appears to be of greatest interest simply because ethanol production from glucose via yeast fermentation is well understood (Harun et al. 2010; Harun and Danquah 2011). Bioethanol derived from corn starch and sugarcane molasses is a well-established biofuel as it mixes well with gasoline and requires no engine modification for use. Ethanol has an energy content of 26.4 MJ/kg and an octane rating of 129 (Amin 2009). Compared with this, gasoline has an energy content of about 44.4 MJ/kg and a typical octane rating of 91,

although this varies. Ethanol is typically blended with gasoline at a level of 10 %. This E-10 blend, or gasohol, has an energy content of about 42 MJ/kg and an octane rating around 93–94 (Amin 2009).

Many microalgae are capable of producing ethanol from intracellular stored starch under anaerobic conditions in the dark (Hirano et al. 1997). Hirano et al. (1997) discussed ethanol production from 250 strains of microalgae, with *Chlamydomonas reinhardtii* converting starch to ethanol with a yield of 0.3–0.4 g ethanol/g starch. This approaches the theoretical yield of ethanol on starch of 0.56 g/g (Hirano et al. 1997). Ueno et al. (1998) reported on ethanol production under dark anaerobic conditions in the marine alga *Chlorococcum littorale*. About 27 % of the starch was consumed within 24 h, generating 450 μmol ethanol/g alga by dry weight at 30 °C. Acetate, hydrogen, and carbon dioxide were produced in addition to ethanol (Ueno et al. 1998).

Estimates suggest that 5000–15,000 gallons of ethanol per acre can be produced annually from microalgae feedstocks. This productivity is orders of magnitude greater than for the other common feedstocks. For example, the annual ethanol yield (gallons/acre) from other feedstocks is: 430 for corn, 277 for wheat, 714 for sugar beet, 354 for cassava and 1150 for switch grass (Nguyen 2012).

3.2 Microalgae to Acetone, Butanol, and Ethanol

Acetone-butanol-ethanol (ABE) fermentation is carried out by anaerobic *Clostridia* such as *C. beijerinckii*, *C. acetobutylicum*, *C. saccharoperbutylacetonicum*, and *C. saccharobutylicum* (Lee et al. 2008). The product contains acetone, butanol and ethanol in the mass ratio of 3:6:1 (Potts et al. 2012). The ABE fermentation was first used during World War I to produce acetone for manufacturing explosives. The history of this process has been reviewed in the literature (Jones and Woods 1986; Lee et al. 2008; Ni and Sun 2009). The need for renewable fuels and solvents has reawakened interest the ABE fermentation.

Biobutanol is an attractive liquid transportation fuel that can directly replace gasoline. Unlike ethanol, butanol is readily separated from the fermentation broth by distillation. The energy density of butanol is 29.2 MJ/L, or comparable to gasoline (32.3 MJ/L), but greater than that of anhydrous ethanol (19.6 MJ/L) (Lee et al. 2008; Steen et al. 2008; Wu et al. 2008; Green 2011). Butanol is said to be a superior fuel alternative to ethanol as ethanol absorbs water and cannot be used in the existing fuel infrastructure due to its corrosive nature (Steen et al. 2008). Ethanol has a lower energy density relative to butanol and is more expensive to recover than butanol, from the fermentation broth by distillation.

Acetone is an industrial solvent and it is used make various other chemicals (Jones and Woods 1986; Wu et al. 2008). Acetone has an energy density of 23.2 MJ/L, making it more energy dense than ethanol but less so than butanol and gasoline (Wu et al. 2008). Acetone can be used as a fuel additive (Wu et al. 2008).

ABE fermenting microorganisms initially ferment sugars to produce acids such as acetic acid and butyric acid. The pentose and hexose sugars are metabolized via the pentose phosphate pathway and the Embden-Meyerhof-Parnas pathway, respectively (White 2006; Ezeji et al. 2007; Tashiro and Sonomoto 2010). Solvents are produced once the concentration of the undissociated acids reaches a critical point of 57–60 mmol/L (Maddox et al. 2000; Tashiro and Sonomoto 2010; Potts et al. 2012). Concentration of the undissociated butyric acid is the primary trigger for solvent formation. A butyric acid concentration of 6 mM is said to be the minimal concentration needed to trigger this metabolic switch (Hüsemann and Papoutsakis 1988). Solvent production, or solventogenesis, continues until inhibitory concentrations of acids or ABE accumulate in the medium during a batch fermentation. This inhibitory effect can be minimized by removing the ABE as it is formed during a fermentation. This can be done for example by using pervaporation or gas stripping of ABE (Liu et al. 2005; Ellis 2013).

ABE can be produced from a variety of feedstocks. These feedstocks include microalgae biomass (Ellis et al. 2012) and biomass of macroalgae, or seaweeds (Potts et al. 2012). Among other feedstocks, ABE production has been demonstrated from cellulose, glycerol, glucose, sucrose, lactose, xylose, xylan, starch (Andrade and Vasconcelos 2003; Tashiro and Sonomoto 2010), hardwood, domestic organic waste, agricultural waste, corn fiber, whey, and sago starch (Tashiro and Sonomoto 2010). *Clostridia* are able to produce a variety of carbohydrate hydrolyzing enzymes including α-amylase, α-glucosidase, β-amylase, β-glucosidase, glucoamylase, pullulanase, and amylopullulanase. This hydrolytic capability facilitates the degradation of complex polymers to their respective monomers, which are then transported into the cell and metabolized. The ability to hydrolyze and metabolize a variety of substrates makes ABE fermenting clostridia attractive for biofuel production from microalgae (Qureshi et al. 2006; Ezeji et al. 2007; Ellis 2013).

The fermentation of carbohydrates contained in algal biomass to prospective fuels such as acetone, butanol, and ethanol has been achieved using saccharolytic *Clostridium* spp. (Efremenko et al. 2012; Ellis et al. 2012; Potts et al. 2012). Using cyanobacterial biomass pretreated with dilute acid and heat, Efremenko et al. (2012) demonstrated the production of butanol and ethanol. The final concentrations were 0.43 g/L for butanol and 0.29 g/L for ethanol. The fermenting microorganisms was *C. acetobutylicum* (Efremenko et al. 2012). Using *Clostridium saccharoperbutylacetonicum*, the acid pretreated biomass could provide a final total ABE concentration of 2.74 g/L (Ellis et al. 2012). The total ABE concentration could be raised to 7.27 g/L by supplementing the biomass hydrolysate medium with 1 % glucose. Supplementation of the medium with hemicellulases and cellulases increased the final total ABE concentration to 9.74 g/L (Ellis et al. 2012). With enzyme supplementation, the total ABE yield was 0.311 g/g biomass substrate and the volumetric productivity was 0.102 g/L h (Ellis et al. 2012). Without pretreatment and any supplementation, the cyanobacterial biomass afforded at total final ABE concentration of 0.73 g/L (Ellis et al. 2012; Ellis 2013).

4 Other Applications/Products of Microalgae

Commercial biofuels are not currently made from microalgae, as production is expensvie. Nevertheless, microalgae are used to commercially produce many relatively high value products. Carotenoids such as β-carotene and astaxanthin are produced using microalgae and so are certain long-chain polyunsaturated fatty acids for use as human nutritional supplements (Borowitzka 1995, 2013). Numerous other bioactives have the potential to be produced using microalgae (Borowitzka 2013). Microalgae are used in treatment of wastewater, especially for absorbing nitrates and phosphates from water. In view of their great biodiversity and increasing prospects of being genetically engineered, microalgae have the potential to provide other novel products (Pulz and Gross 2004).

Future biorefineries may be developed around microalgal biomass (Ellis et al. 2012; Anthony et al. 2013) to provide a great diversity of products (Fig. 1). Microalgae have the potential to provide biohydrogen, biosyngas, and oil to replace fossil fuel (Demirbas 2010).

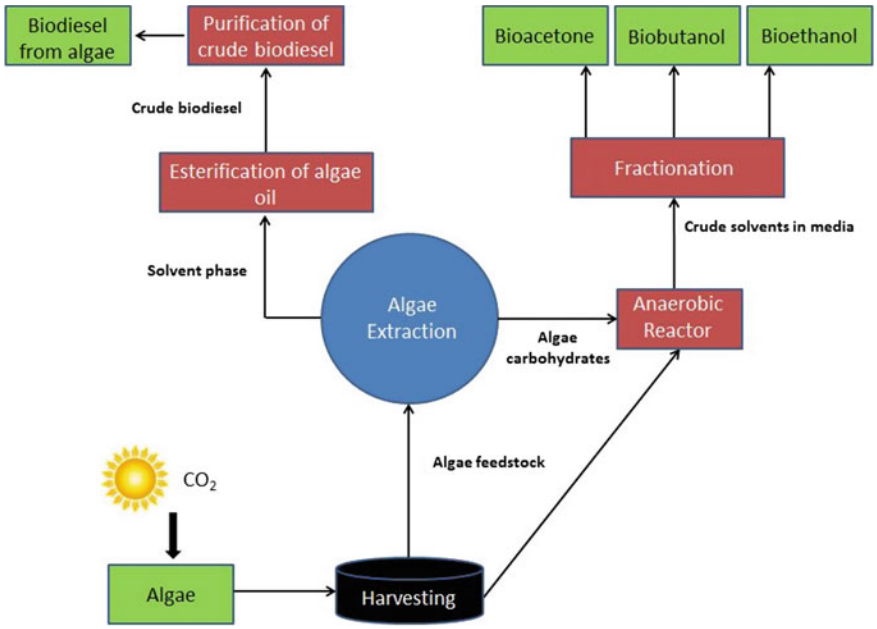

Fig. 1 A microalgae-based biorefinery concept for producing multiple products from a single feedstock. Modified from Anthony et al. (2013)

5 Concluding Remarks

Microalgae are potentially a good source of renewable carbohydrates that can form the basis of an algal fuel alcohol industry. The technology for converting starch and other carbohydrates to fermentable sugars and subsequent fermentation to ethanol and butanol, is largely established. If algal biomass with a high starch content can be generated inexpensively and sustainably, and the accessibility of the starch to hydrolytic enzymes is improved, algal fuel alcohols have a real potential. Compared to higher plants, algae offer many advantages as feedstocks for producing renewable fuels.

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Microalgae for Aviation Fuels

Dato' Paduka Syed Isa Syed Alwi

Abstract Aviation industry consumes over 5 million barrels of oil per day. The adverse effects of refining and burning oils can be potentially curbed by replacing it with renewable biofuels from microalgae. Microalgae are primitive plants with the ability to convert carbon dioxide, water, and sunlight to oils that can be made into aviation fuels.

Keywords Microalgae · Aviation fuels · Bioremediation · Jet fuels

1 History of the Aviation Industry

Although the first powered flight by Wright brothers occurred in 1903, the commercial airline industry based on fixed wing aircraft started nearly a decade later, around 1916. Improved aircraft and management practices have resulted in tremendous growth in air traffic (Ferreira 2001). The early aircraft had internal combustion piston engines similar to those used in automobiles today. The need for increased power led to the development of specialized engines. In the 1940s, the turbine engine was introduced and kerosene was the fuel in the first turbine engines. Later, kerosene was replaced by more specialized jet fuels (Hemighaus et al. 2006). Aviation industry is now an essential element of modern society. There is no alternative to aviation for rapid transportation of passengers and cargo on a global scale.

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2 Flying in the Twenty-First Century

Today the global aviation has established transport networks carrying about 2.3 billion passengers yearly (ICAO 2010) and is essential to modern quality of life and economic development. Aviation has seen the fastest growth rates of all the various transport modes. According to International Civil Aviation Organization, passenger air traffic is expected to keep increasing at a rate of 4.7–5.2 % annually over the coming 10–15 years (ICAO 2010). The global economic impact of aviation is estimated at US$3560 billion, or 7.5 % of the world's gross domestic product. Aviation supports nearly 32 million jobs globally. According to the Intergovernmental Panel on Climate Change (IPCC), air transport's contribution to climate change amounts to 2 % of the man-made carbon dioxide emissions and this may rise to 3 % by 2050 (ATAG 2009).

3 Fuels That Mostly Fly the Sky

Aviation is powered by petroleum fuels. Liquid hydrocarbons offer the best combination of energy content, availability, price, ease of handling, and distribution. Aviation can be divided into two categories, commercial and military aviation (Penner et al. 2000). The different types of commercial and military jet fuels in use are shown in Table 1. All jet fuels (mostly grades of kerosene) are derived from petroleum. Kerosene is obtained by catalytic refining of petroleum. The commercial aviation fuels Jet A-1 and Jet A are kerosene-type fuels. Jet B is a blend of gasoline

Table 1 Types of commercial and military jet fuels (Elmoraghy et al. 2012)

Civil/commercial jet fuels
Jet A—A kerosene-type fuel with a flash point of above 38 °C (100 °F) and a maximum freezing point of −40 °C. Available mostly in the United States
Jet A-1—A kerosene grade suitable for most turbine engine aircraft. A flash point of above 38 °C (100 °F) and a maximum freezing point of −47 °C. A net heat of combustion of at least 42.8 MJ/kg. Standard specification jet fuel used worldwide
Jet B—A distillate spanning the naphtha and kerosene fractions of petroleum and having a superior cold weather performance. A freezing point maximum of −50 °C. A net heat of combustion of at least 42.8 MJ/kg. Has a higher flammability than Jet A and Jet A-1. Significant demand only in very cold climates
Military jet fuels
JP-4—The military equivalent of Jet B with added corrosion inhibitors and anti-icing compounds
JP-5—High flash point kerosene
JP-8—The military equivalent of Jet A-1 with added corrosion inhibitors and anti-icing chemicals

and kerosene, but is rarely used in view of its lower flash point. JP-5 and JP-8 are chemically enhanced fuels with antioxidants, dispersants, and/or corrosion inhibitors to meet the requirements of specific applications.

The kerosene-type fuel Jet A is used mostly in the United States while the rest of the world uses the Jet A-1, also a kerosene-type fuel. The choice of Jet A in the USA is driven by price and availability. Jet A-1 has a lower maximum freezing point of −47 °C compared to Jet A (−40 °C). The lower freezing point makes Jet A-1 more suitable for long international flights over polar routes and during winter, but Jet A-1 is more expensive than Jet A. The Jet B is used in some parts of Canada and Alaska because it is particularly suited to cold climates.

4 Search for Alternative Jet Fuels

The aviation industry is undergoing rapid growth in air travel demands. The industry consumes over 5 million barrels of oil per day (OECD 2012). This is creating challenges to security of fuel supplies and minimizing environmental impact.

Jet fuel is the biggest cost to the aviation industry. Jet fuel accounts for 10 % of the global transport fuel use and contributes 2 % of the world's carbon dioxide emissions. The carbon dioxide emissions from jet fuel use exceed 707 million tones. In 2010, the airlines spent US$140 billion on fuel, or more than 30 % of their operating costs (ATAG 2011). Expenditure on fuel is forecast to increase to (IATA 2010). In the USA, the jet fuel consumption rose more than 2.8-fold from 1984 to 2010. The United States is the largest single market for jet fuel and consumes about 37 % of the worldwide totally (Hemighaus et al. 2006).

In view of the high cost of jet fuel, its environmental impact and possible decline in future stocks, the aviation industry has been supporting the development of alternative jet fuels (Free Enterprise 2012). The Commercial Aviation Alternative Fuels Initiative (CAAFI) has brought together many interested parties including the Aerospace Industries Association and the Airports Council International-North America (ACI-NA), and the Federal Aviation Administration (FAA).

Any alternative fuel must meet certain essential criteria. Most importantly, it must be a "drop-in" fuel that can be blended with existing fuels or replaces them directly without requiring any major change to aircraft design and performance and the present fuel distribution infrastructure. An alternative fuel must be capable of being produced in the desired quantity relatively inexpensively and with a lower life-cycle impact compared to the existing fuels.

Many alternative fuel options have been suggested, including synthetic fuels (e.g., synthetic paraffinic kerosene), biofuels (bioethanol, biobutanol, biodiesel, biokerosene, biojet fuel), and cryogenic fuels (liquid hydrogen and liquid methane). Fuels such as the cryogenic fuels may have a potential in the longer term, but will require changes to aircraft design and development of suitable new technologies (Daggett et al. 2008). On the other hand, some of the biofuels may provide a near

term drop-in solution. Production of synthetic replacements is associated with increased emissions of carbon dioxide compared to the existing fuels (Sohi 2010).

5 Jet Fuel from Bio-based Sources

Production of jet fuel from various biological resources has received much attention. Potentially, suitable biofuel blends may be made sustainably from vegetable oils, animal fats, sugars, and waste biomass. Numerous tests have been conducted with biojet fuel blends where the biofuel component was sourced from oils of jatropha, camelina, and waste cooking oil. Such biojet fuels are associated with reduced emissions compared to the standard jet fuels and they are potentially drop-in replacements for conventional fuel. They are wholly compatible with the existing engines and fuel distribution systems and meet the same performance criteria as the traditional fuels. In addition, because these fuels can be domestically produced they have the potential to improve the security of fuel supply.

Biodiesel is viewed as a possible alternative to conventional petroleum-derived diesel for automobiles and is being considered as an aviation fuel as well. Bioethanol blended with gasoline and biodiesel blended with petroleum diesel are other fuel options that have been tested. Production of bioethanol depends solely on the ability to produce sufficient corn and sugarcane. Ethanol contains about 35 % oxygen by weight (Hemighaus et al. 2006) and therefore has a lower energy content per unit mass than the conventional jet fuel. Furthermore, ethanol is significantly more volatile than jet fuel and boils at a specific temperature (78 °C) (Hemighaus et al. 2006) whereas jet fuel boils over a broad range of approximately 150–300 °C. Ethanol has a significantly higher heat of vaporization than hydrocarbons of jet fuel due to its intermolecular hydrogen bonding. These properties impact its atomization and vaporization behavior in the combustor.

Soybean oil is the major source of biodiesel in the US, although other vegetable oils are also used. Rapeseed oil is commonly used in Europe to make biodiesel. In Asia, palm oil and coconut oil are mainly used (Hemighaus et al. 2006). A number of processes have been developed to convert vegetable oils into jet fuel. The best-developed technology for this is "hydroprocessing." This process adds hydrogen to the parent molecule while removing oxygen from it and breaks the larger hydrocarbon molecules into smaller molecules characteristic of jet fuels. According to Honeywell (UOP 2014), variants of this hydroprocessing technology can convert nonedible natural oils and waste oils to jet fuels meeting all the critical specifications. The resulting fuel is claimed to have higher energy density than conventional jet fuel allowing aircraft to fly farther on a given amount than if the same quantity of jet fuel was used.

Such renewable aviation fuels are molecularly identical to conventional jet fuel, but are not made from petroleum. Some of them have the potential to reduce carbon dioxide emissions by up to 80 % compared to conventional fuels, based on a total life-cycle assessment.

Extensive research on using oils derived from microalgae as a source of biodiesel as well as other fuels (gasoline, jet fuel) is underway. Biodiesel has a slightly lower energy content per unit mass than conventional jet fuel. Biodiesel is biodegradable, has good lubricity properties and is essentially free of sulfur and aromatics. If algae-based biodiesel can be produced in sufficient quantity, sufficiently cheaply and demonstrably sustainably, then it may displace jet fuel.

6 Microalgae as Alternatives to Jet Fuel

Microalgae are miniature primitive plants which are capable of producing lipids and hydrocarbons rapidly via photosynthesis (Sivakumar et al. 2012). This has generated a lot of interest in microalgae as a source of biodiesel and other liquid fuels. Converting algal oils to jet fuels has been shown to be possible. Production of algal fuels may reduce carbon dioxide emissions relative to production of energetically equivalent quantity of petroleum fuel. Many other advantages have been claimed for algal fuels (Aresta et al. 2005; Rakopoulos et al. 2006; Demirbas 2007). Although production has been shown to be possible, issues of economics, sustainability, environmental impact, and scalability of production continue to dog algal biofuels.

Algal oil can be converted to jet fuel with the correct physical characteristics by controlling and modifying processes such as hydrotreatment and refining. The stumbling block is the ability to produce algal oil cheaply and in large quantity.

Making a biofuel competitive with fossil fuels remains a challenge (Schneider 2006). Often, the biofuels tend to be more expensive than an energetically equivalent amount of a petroleum-derived fuel. A sustainable supply of huge quantities of the biomass feedstock needed to produce the fuel is difficult to achieve without impacting the supply of crops for food and animal feed. Algae may have advantages relative to higher plants as a biomass feedstock for biofuels, but their production remains expensive.

Numerous research laboratories and companies such as Sapphire Energy, Heliae, Phycal, Cellana, Solazyme, Algaetech International, and General Atomics, are attempting to produce renewable aviation fuels, or biojet fuels, from algae. Production has been shown to be technically possible and test aircraft have flown on such fuel. Nevertheless, commercialization remains well into the future.

7 Bioremediation and Wastewater to Biojet Fuel by Algaetech International, Malaysia

A large quantity of municipal and industrial wastewater is generated particularly in large cities and must be treated prior to reuse, or discharge into the environment. At the same time, the demand for freshwater free of pollutants is increasing. These

factors combined are placing increasing emphasis on reuse and recycling of wastewater after suitable treatment.

Much of the municipal wastewater is amenable to biological treatment, or bioremediation (Barrington et al. 2009), that removes pollutants or converts them into harmless products. Microorganisms are already widely used in biotreatment of wastewater. Microalgae have the potential to further improve some of the existing treatment processes. Specifically, microalgae can absorb nitrate and phosphate pollutants from wastewater. Algae are also quite effective in removing heavy metals from water by adsorption to biomass (Megharag et al. 2003) and provide affordable treatment options.

Algae for production of biofuels and other products can potentially be grown using the nutrients in the wastewater. Dissolved organic carbon, phosphates, and nitrates provide nutrients for algal growth. Depending on the growth scenario, some or all of the carbon required to make the algal biomass may be derived from the carbon dioxide absorbed from the atmosphere, or a supplemental supply of carbon dioxide. Removing the algal biomass from the water often leaves it sufficiently clean for discharge to the environment. Any heavy metals initially present in the wastewater are removed with the biomass, but the biomass sludge may be toxic if it contains a large quantity of certain heavy metals.

Fuels derived from microalgae are potentially carbon neutral and low in sulfur. Algae grown using wastewater on nonarable land avoid competition with agriculture. Depending on conditions, some algae grow sufficiently rapidly to double their biomass in under 24 h (Chisti 2007). In addition, algae are capable of producing energy-rich oils similar to the conventional vegetable oils (Rodolfi et al. 2009).

Algaetech International Sdn Bhd (AISB), Malaysia, is attempting to become the leading algae company in South Asia using innovative algae production technologies. AISB is developing "waste-to-fuel" algal technologies in collaboration with the European Aeronautic Defence and Space Company (EADS), France, and Aerospace Malaysia Innovation Center (AMIC), Malaysia. AISB is using algae for bioremediation of wastewater to use the biomass to produce algal lipids for making jet fuels. Microalgae are being assessed for growth in different wastewaters and environments, to establish feasibility of such processes. The physiochemical and biological properties of various municipal and agricultural processing wastewaters from Malaysia are being assessed in combination with suitable algae. Locally acclimatized strains of the microalgae *Chlorella* sp. (COO2), *Scenedesmus* sp. (DOO1) and *Selenastrum* sp. (TOO1) are being evaluated. In small-scale (15 L) operations, these algae were shown to produce oils in multiple cycles of growth.

8 Global Biofuel Mandatory Requirement

An aviation fuel must meet the mandated specifications. There are three major specifications for commercial jet fuels, The UK MOD (Ministry of Defence) Defense Standard 91-91 Jet A-1 is used in most of the world for Jet A-1 fuel.

The ASTM D1655 Jet A is used for Jet A fuel in the US. GOST 10227 TS-1 fuel specification is used in Russia and other countries in the Commonwealth of Independent States (Hemighaus et al. 2006). Other countries often have their own specifications, but these generally align with one of the other major specifications. The specifications of ASTM and MOD are the result of a cooperative process involving aircraft engine and airframe manufacturers, additives suppliers, national aviation regulatory agencies, and fuel suppliers. Eventually, the engine and airframe manufacturers specify the fuel properties required for safe and reliable operation of their equipment. These requirements are embodied in a fuel specification that is part of an aircraft's type certificate issued by the national aviation regulatory authorities.

Jet fuels with a high aromatics content do not burn as cleanly as fuels with a lower aromatics content. Therefore, the fuel specifications include a maximum acceptable aromatics concentration. Boiling range is another of the many specifications that are required to be met (Hemighaus et al. 2006). Biojet fuels require extensive testing to assure that they meet all the relevant mandated standards. Meeting the relevant standards has been shown to be possible.

9 How Close Are We to Sustainable Biojet Fuels?

There is a clear need for alternative jet fuels. Such fuels must meet many demanding criteria: a low-cost high-volume production capacity that does not compete for water, land, and fertilizers for food production; a lower environmental impact than that of existing fuels; a manageable ecological impact of production; and a positive socioeconomic impact of production on local stakeholders. No single alternative fuel appears to meet all these criteria and therefore the challenge remains.

Many countries, the supranational bodies such as the European Commission, the airlines, and the aircraft producers appear to be committed to sustainably produced alternative aviation fuels (OECD 2012). The challenges are daunting and no clear alternative fuel appears to be on the horizon in the near term.

According to the International Energy Agency (IEA), an all-time global peak was reached in conventional production of petroleum at around 70 million barrels per day in 2006 (OECD 2012). Further increases in petroleum demand may be met until 2035 through exploitation of unconventional resources such as oil sands and deep oil (OECD 2012). At some point in the future, the aviation industry will be facing a major energy crisis unless renewable fuels are developed and brought on stream. Many predictions have been made about the future contribution of biojet fuels, but nothing is certain. For example, according to Air Transport Action Group (ATAG 2009), the use of biojet fuels, including the algae-derived fuels, is expected to increase from less than 1 % in 2012 to 50 % of the aviation fuel use in the year 2040.

10 Concluding Remarks

Production of jet fuels from various bio-based sources has received considerable attention. Among the various sources, oils derived from microalgae are considered as having important advantages for making jet fuels compared to oils obtained from crop plants. Some microalgae may contain as much as 60 % oil by dry weight in the biomass. Potentially, algae can provide as much as 140,000 L of oil per hectare annually under optimum conditions. In theory, oils derived from microalgae have the potential to replace much more petroleum fuel than oils from crop plants ever could. Oil yields from crops are much lower compared to the yields from microalgae. Furthermore, oil production from microalgae can make use of wastewater and its nutrients, without encroaching on arable land required for production of food and feeds. Microalgal oil can be converted to jet fuel using various chemical technologies. In view of the potential of algal oils, Algaetech International SDN BHD, Malaysia, is one of the several companies developing technologies for commercializing jet fuels derived from microalgae.

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Biohydrogen from Microalgae

Alexandra Dubini and David Gonzalez-Ballester

Abstract This chapter provides an overview of the current state of knowledge of the mechanisms involved in biohydrogen production from microalgae. The known limitations linked to photohydrogen productivity are addressed. Particular attention is given to physiological and molecular strategies to sustain and improve hydrogen production. The impact of different nutrient stresses and the effect of carbon supply on hydrogen production are discussed. The genetic and metabolic engineering approaches for increasing hydrogen production are outlined.

Keywords Microalgae · Photohydrogen productivity · Chlamydomonas · Biophotolysis

1 Introduction

Hydrogen gas (H_2) is a promising option for satisfying the world's energy requirements and replacing the nonrenewable and carbon-based fuels. H_2 is a clean fuel that generates only water vapor on combustion. H_2 has the highest energy content (122 kJ/g) on a per unit weight basis compared to the other fuels (Das and Veziroglu 2008). However, H_2 is difficult to store and distribute as a fuel and its production is expensive. Currently, nearly all H_2 used commercially is derived from fossil fuels through steam methane reforming (SMR) technologies (Lam and Lee 2011). SMR processes are energy intensive and depend on the nonrenewable carbon-based fuels.

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Biological production of H_2 has attracted much attention as a potential large-scale source of a sustainable supply of energy. Some bacteria, cyanobacteria, and microalgae are able to produce H_2 from water and organic substrates using solar energy. Some microorganisms can produce H_2 via photosynthesis and/or fermentation; although fermentation provides better rates of H_2 production it requires the use of organic wastes. Photobiological production of H_2 without the involvement of organic feedstocks may ultimately hold more promise as it is not restricted by a supply of organic substrates that may have other competing uses. Although H_2 yields from biological processes have been improved through physiological manipulation and metabolic engineering, these processes remain financially nonviable for commercial use. This is partly due to the low yield of the process, a consequence of the strong inhibitory effects of oxygen (O_2) on the production of H_2.

This chapter discusses the different production pathways used by green algae to produce H_2. The factors limiting the development of a sustainable production system and the strategies used in improving the H_2 production rates are discussed. The focus is mainly on H_2 production in *Chlamydomonas reinhardtii* (Chlamydomonas throughout). The genome of this alga has been sequenced and a substantial amount of information is available on its genetics and metabolism (Merchant et al. 2007; Harris et al. 2008). Multiple "omics" studies have been published about Chlamydomonas (Miura et al. 2004; Bolling and Fiehn 2005; Mus et al. 2007; Harris et al. 2008; May et al. 2008; Matthew et al. 2009; Chen et al. 2010; Doebbe et al. 2010; Gonzalez-Ballester et al. 2010; Terashima et al. 2010; Castruita et al. 2011). All this has established Chlamydomonas as a model organism for study of H_2 production. This alga is able to grow heterotrophically using acetate as the sole carbon source and also possess a versatile fermentative metabolism (Mus et al. 2007; Dubini et al. 2009; Catalanotti et al. 2012; Magneschi et al. 2012). Consequently, Chlamydomonas may be used to produce H_2 under both autotrophic and heterotrophic conditions.

2 Hydrogen Production Pathways in Chlamydomonas

Chlamydomonas can produce H_2 via three metabolic pathways (Fig. 1) that are activated under different conditions although they all require anaerobiosis to release H_2. Two of these pathways are light dependent, while the third involves fermentation (Florin et al. 2001; Ghirardi et al. 2009a). The two hydrogenases (H_2 ases, Fig. 1) in the chloroplast of Chlamydomonas can evolve H_2 under anoxia through the above-mentioned three pathways via a common branch point of ferredoxin (FDX), the sole natural electron (e^-) donor to the hydrogenases (Roessler and Lien 1984a; Meuser et al. 2012; Winkler et al. 2010; Peden et al. 2013).

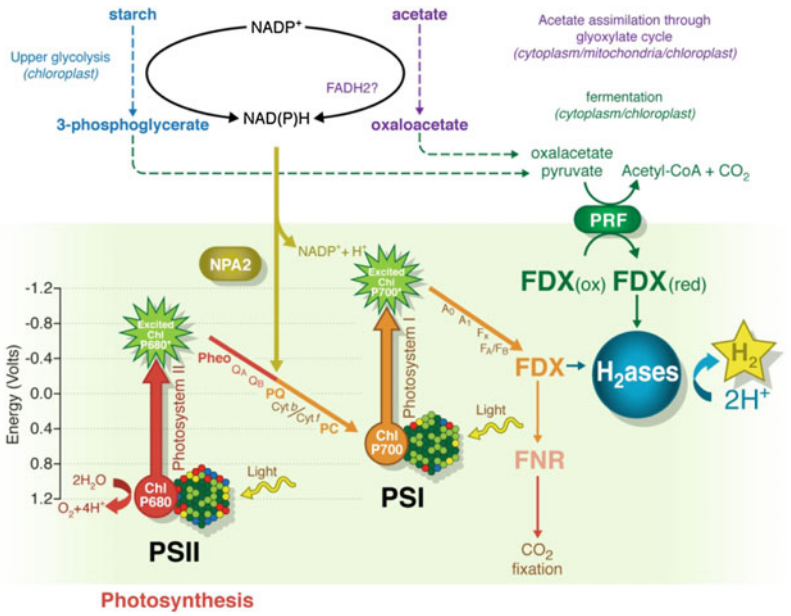

Fig. 1 Biohydrogen production pathways in Chlamydomonas. The photosynthetic chain is represented by the so-called Z-scheme. Three H_2 production pathways exist in the cell. The two photoproduction pathways use low-potential electrons generated from either (1) water oxidation by photosystem II (PSII) activity, or (2) by the non-photochemical reduction of plastoquinone by NDA2. Both the PSII-dependent (*red*) and the PSII-independent (*yellow*) pathways require reduction of the plastoquinone pool and PSI activity (*orange*). Electrons excited to higher energy (lower potential) by the PSI are able to reduce FDX, the physiological electron donor to hydrogenase. In the PSII-independent pathway, the NADH formed by the catabolism of organic substrates (starch degradation in blue; acetate assimilation in purple) can be used for reduction of the plastoquinone pool. In addition, dark fermentation of pyruvate and oxaloacetate catalyzed by PFR (*green*) is used to reduce FDX and mediates the observed production of H_2. Adapted from the Chlamydomonas Sourcebook (Harris et al. 2008) (Color figure online)

2.1 Photobiological Hydrogen Production Pathways

2.1.1 Direct Biophotolysis (PSII-Dependent Pathway)

The direct biophotolysis pathway is also known as the PSII-dependent pathway. In this, H_2 photoproduction is achieved via linear electron transfer (LET) through the photosynthetic apparatus involving both photosystems (PS), PSI, and PSII. This pathway converts the light energy into chemical energy to split water and release O_2 following the *Z*-scheme (Fig. 1). This process takes place in the thylakoid membranes and is initiated by light absorption by PSII. Once PSII is excited, electrons are transferred through the photosynthetic chain, sequentially reducing the plastoquinone (PQ) pool, the cytochrome *b6f* (cyt*b6f*), the plastocyanin (PC), and the

light-oxidized PSI. Light absorption by PSI generates excited electrons at P700, which are subsequently captured by FDX. Electrons are finally donated to the ferredoxin NADPH oxidoreductase (FNR) to produce NADPH for CO_2 fixation under the normal photosynthetic conditions. However, under anaerobic conditions and in the absence of CO_2, the FDX can direct the electrons to the hydrogenases and cause a release of H_2 (Roessler and Lien 1984a; Happe and Naber 1993; Winkler et al. 2010; Peden et al. 2013). This reaction is coupled to water splitting, a process that involves electron transfer steps and uses four photons to generate one molecule of O_2, four protons (H^+), and four electrons from two molecules of water:

$$2\,H_2O + 4\ \text{photons} = 4\,H^+ + 4\,e^- + O_2 \quad (1)$$

Two of the protons recombine with two electrons to produce H_2, as follows:

$$2\,H^+ + 2\,e^- + 2\ \text{photons} = H_2 \quad (2)$$

The overall reaction, the sum of Eqs. (1) and (2), is the following:

$$2\,H_2O + 8\ \text{photons} = O_2 + 2H_2 \quad (3)$$

Eight photons are used because PSI and PSII are involved. Two H_2 molecules are produced.

The herbicide DCMU inhibits PSII and is commonly used to estimate the contribution of the PSII-dependent pathway to the total production of H_2. The direct biophotolysis pathway contributes up to 90 % of the total electron flux used in photoproduction of algal H_2, but this contribution depends on the specific strain being used, the culturing and test conditions, and the protocol used in inducing H_2 production (Laurinavichene et al. 2004). As this pathway requires LET, it is modulated by the factors affecting photosynthesis, as discussed in Sect. 4. A major drawback of this pathway is the incompatibility of simultaneous production of H_2 and O_2, as algal hydrogenases are strongly inhibited by O_2.

2.1.2 Indirect Hydrogen Production (PSII-Independent Pathway)

Indirect H_2 production refers to the PSII-independent pathway that uses electrons from NAD(P)H that are transferred directly to the PQ pool, bypassing PSII (Mus et al. 2005; Baltz et al. 2014). This process depends on the non-photochemical reduction of the PQ pool, mediated by NDA2, NADH dehydrogenase type II in Chlamydomonas (Jans et al. 2008; Desplats et al. 2009; Mignolet et al. 2012; Baltz et al. 2014). Subsequently, the electrons are transported through the photosynthetic chain, reducing PC and the light-oxidized PSI. As described for the PSII-dependent pathway, the light absorption and charge separation at PSI generate additional electrons that reduce FDX. FDX can then donate electrons to the hydrogenases.

The most common source of PQ reductants is the glycolytic degradation of glucose (or starch) (Fig. 1, yellow pathway). This indirect pathway allows for an efficient separation of the production of O_2 and H_2 as it does not involve PSII activity. The maximal rates of H_2 production obtained through the PSII-independent pathway are around 10 times lower than the rates obtained via the PSII-dependent pathway (Cournac et al. 2002; Antal et al. 2009; Chochois et al. 2009). Several metabolic factors are believed to limit the PSII-independent pathway, including the rate of hydrolysis of starch; the rate of subsequent reduction of the PQ pool; and the molecular processes competing with hydrogenases for electrons (e.g., PTOX, Plastid Terminal Oxidase activity and CEF, Cyclic Electron Flux) (Cournac et al. 2002).

2.2 Fermentation-Dependent Pathway

The third H_2 production pathway is linked to fermentation and is activated under dark anoxia. In the dark, fermentation results in the degradation of starch reserves (Gfeller and Gibbs 1984). In this case, the electrons needed by the hydrogenases are derived from the decarboxylation of pyruvate to acetyl-CoA mediated by the pyruvate ferredoxin oxidoreductase (PFR). This results in the reduction of FDX (Fig. 1) (Noth et al. 2013; van Lis et al. 2013). Formate, acetate, and ethanol are produced as major fermentation products, and H_2 and CO_2 are emitted as minor products (Gfeller and Gibbs 1984). Chlamydomonas is a rare example of a eukaryote that has homologs of all the four predominant enzymes used in the fermentative metabolism of pyruvate. These include the pyruvate formate lyase (PFL), PFR, lactate dehydrogenase (LD), and pyruvate decarboxylase (PDC) (Atteia et al. 2006; Mus et al. 2007).

The Chlamydomonas PFL and PFR enzymes likely act in concert to balance the conversion of pyruvate into acetyl-CoA with the production of formate and CO_2, respectively. In addition to pyruvate, oxaloacetate may be used as a substrate by PFR, thus contributing reductant for the production of H_2 (Noth et al. 2013). As pyruvate and oxaloacetate are also the end products of lipid and amino acid catabolism, the PFR-dependent oxaloacetate degradation could in principle support H_2 production (Noth et al. 2013). H_2 production under dark anaerobiosis, although very low compared to the other production processes, is a part of the cell acclimation process to enable it to survive and balance its redox status under prolonged anaerobiosis.

3 Physiological Strategies to Sustain Hydrogen Production

The strategies used to obtain and sustain algal H_2 production share some common features. Establishment of anaerobic/hypoxic condition in the cultures is a prerequisite to produce H_2. Induction of anaerobiosis in photosynthetic microorganisms is

not a trivial task, especially under light which results in photosynthetic production of O_2. Typically, to obtain H_2 production the level of the photosynthetic activity must be reduced and/or the rate of mitochondrial respiration must be increased. When mitochondrial respiration activity exceeds photosynthetic activity (and more specifically the PSII activity), the O_2 levels in a culture drop to values compatible with the expression of the hydrogenase gene and the hydrogenase enzyme activity. Although hypoxia/anaerobiosis is the first requirement to produce H_2 in algae, it is not sufficient. Cell metabolism, especially chloroplast metabolism, needs to be such that electrons and protons accumulate in the stroma, as these are the substrates for the hydrogenase. Normally, the electrons and protons are provided either directly by the photosynthetic activity or indirectly by reductants such as NAD(P)H and flavin adenine dinucleotide ($FADH_2$). These reductants are less in demand by the cell metabolism under the conditions of H_2 production and accumulate in the chloroplast. The catalytic activity of certain enzymatic reactions (e.g., PFR) can also provide electrons to the hydrogenases.

To sustain H_2 production, the above-mentioned conditions (i.e., a low O_2 concentration and accumulation of electrons or reducing equivalents) need to be maintained for prolonged periods of time without affecting the cell viability. The most common physiological strategies used for producing H_2 in green algae are summarized in Sects. 3.1 and 3.2.

3.1 Nutrient Stress (S, N, P)

In general, an insufficiency of a macronutrient (e.g., S, N, P), or nutrient stress, results in cessation of cell growth as essential molecules cannot be synthesized. Rates of carbon fixation and anabolic metabolism decline and, as a result, there is a decreased demand for NAD(P)H. This results in its accumulation in the chloroplast and prevents the reoxidation of the photosynthetic electron chain. Therefore, more light-derived electrons potentially become available for hydrogenases. A short-term, nutrient stress may also cause an increase in the synthesis of certain metabolites, especially starch and lipids, which are later mobilized as sources of energy and reduced equivalents during prolonged stress. During the mobilization phase of internal metabolites (e.g., starch, lipids, and proteins), the chloroplasts are supplied with an extra pool of reductants and this mobilization phase often matches the H_2 production phase.

In the chloroplast, an excess of NADPH can be dissipated by the activity of NDA2 contributing to an over-reduction of the photosynthetic chain and facilitating PSII-independent H_2 production (Jans et al. 2008; Desplats et al. 2009). Furthermore, during most nutrient stresses, the algal photosynthetic machinery undergoes rearrangement and activates the so-called non-photochemical-quenching (NPQ) mechanisms to cope with the excess light that the cells can no longer efficiently use. Among other consequences, the PSII activity declines and so does the photoproduction of O_2. The reduced O_2 levels and the over-reduction of the

photosynthetic electron chain, can favor hydrogenase activity. Although some of the responses to a macronutrient stress are general, stress due to each specific macronutrient has its metabolic particularities, or specific responses, that make them more or less suitable for H_2 production.

3.1.1 Sulfur Deprivation

Sulfur (S) deficiency is probably the most commonly used strategy for sustaining H_2 production. In 2000, Melis et al. (2000) showed that Chlamydomonas was capable of producing H_2 in media devoid of S. S deficiency has a broad impact on cell metabolism (Bolling and Fiehn 2005; Timmins et al. 2009; Chen et al. 2010; Gonzalez-Ballester et al. 2010; Toepel et al. 2013) although one factor is considered crucial to elicit H_2 production: a partial inactivation of PSII and O_2 evolution. Also, an initial phase of starch accumulation is thought to be important for enhancing H_2 production by S deprivation. Later on, this starch will be degraded and will provide the substrates for respiration and contribute additional reductants for the PSII-independent pathway.

The rate of photosynthetic O_2 evolution in Chlamydomonas declines by 75 % after 1 day of S starvation in acetate-containing media and is further reduced after 4 days (Wykoff et al. 1998). Electron flow is inhibited at the PSII level and PSII centers undergo a rapid degradation (Melis et al. 2000; Zhang et al. 2002), but the PSI activity is essentially unchanged in S-starved cells. The photosynthetic machinery then transition to state 2 in which CEF is induced and this further reduces PSII activity and O_2 production (Wykoff et al. 1998; Fianzzi et al. 2002). During this process, the mitochondrial activity is not severely affected and this favors a rapid depletion of O_2 from a sealed culture if acetate is present in the medium (Melis et al. 2000). It generally takes between 1 and 3 days for a light-saturated S-depleted culture to become anaerobic in the presence of acetate (Melis et al. 2000; Fouchard et al. 2005).

Chlamydomonas cultures subjected to S deprivation undergo several physiological stages. First, about 30–40 % of PSII is irreversibly inactivated shortly after the initiation of the S deprivation. This favors a net consumption of O_2 in the culture if acetate is present (Wykoff et al. 1998; Antal et al. 2003). Also, in aerobic Chlamydomonas cultures an initial increase in the cellular starch content occurs after 24 h of S deprivation. Acetate in the culture medium can facilitate this process (Melis et al. 2000). Once the O_2 is completely consumed, there is rapid and almost complete inactivation of the remaining active PSII centers (Antal et al. 2003). This inactivation is reversible and the PSII centers can undergo gradual reactivation as soon as H_2 production begins. Likely, this is because the hydrogenase activity leads to reoxidation of the photosynthetic electron transport chain and causes a partial reactivation of PSII activity. Although, there is a mutual feedback between PSII reactivation by H_2 production and the hydrogenase activity itself, since the O_2 produced by PSII activity inhibits hydrogenase activity. Thus, about 20 % of the PSII centers remain active during the anaerobiosis phase (Antal et al. 2011;

Volgusheva et al. 2013). Once anaerobic conditions are established, the acetate uptake ceases and starch reserves are consumed by respiration and fermentation (Melis et al. 2000).

The herbicide DCMU significantly reduces H_2 photoproduction in S-deprived cells (Ghirardi et al. 2000; Antal et al. 2003, 2009), suggesting that the predominant H_2 production pathway during S deprivation relies on the low residual activity of PSII. About 70 % of the H_2 production is estimated to be PSII-dependent. The remaining H_2 production (about 30 %) may be via the PSII-independent pathway. Evidence for PSII-independent H_2 production during S deprivation was provided by Mignolet et al. (2012). They reported that an *NDA2* mutation resulted in a 30 % loss in H_2 production. Also, an increased rate of electron flow from stromal reductants such as NAD(P)H, to the PQ pool, has been directly observed (Antal et al. 2006). The degradation of the previously accumulated starch has been proposed as the main source of ATP and NAD(P)H during the anaerobic phase of S deprivation. Moreover, the existence of fermentative metabolism in illuminated S-deprived cultures is revealed by the accumulation of succinate, formate, and ethanol. This also suggests the possible existence of fermentation-derived H_2 production coupled to the oxidation of pyruvate to acetyl-CoA (Kosourov et al. 2003; Timmins et al. 2009).

3.1.2 Nitrogen Deficiency

Nitrogen (N) deficiency is also able to elicit H_2 photoproduction although this phenomenon has been much less studied than H_2 production under S deprivation (Aparicio et al. 1985; Philipps et al. 2012). Similar to S deficiency, the N deficiency results in a decline in the photosynthetic activity and an accumulation of starch and lipids in the presence of acetate (Martin and Goodenough 1975; Peltier and Schmidt 1991; Work et al. 2010). However, N deprivation induces a much slower decline in PSII activity and this results in a more prolonged aerobic phase and a delayed H_2 production relative to the case for S deficiency. The H_2 production rates are also lower relative to those obtained under S deficiency, but the reasons for this are not clearly understood (Philipps et al. 2012).

Notably, the herbicide DCMU does not significantly influence H_2 yields in N-depleted cultures. This suggests a minor contribution of the PSII-dependent pathway to H_2 production during N deprivation, unlike the case for S-depleted cultures (Philipps et al. 2012). Transition to state 2 also occurs in N-starved cells and, therefore, a significant contribution of PSII-independent pathway to H_2 production is expected. Interestingly, N-depleted cells can accumulate starch and lipids more efficiently than the S-depleted cells, although the starch reserves are not mobilized during H_2 production, therefore, starch degradation does not limit H_2 production during N starvation (Philipps et al. 2012). Under these conditions, protein degradation has been suggested as the major source of reductive equivalents (Aparicio et al. 1985). H_2 production in N-depleted cells occurs also during the dark, contrary to the case for the S-depleted cells. In the dark, the N-depleted cells

produce similar levels of ethanol as the S-depleted cells, but less of formate is produced. This suggests a higher activity of the PFR enzyme and, consequently, a higher level of fermentative H_2 production in N-depleted cells.

3.1.3 Phosphorous Deficiency

Only one report of the effect of phosphorous (P) deprivation on H_2 production appears to exist (Batyrova et al. 2012). As do S and N deficiencies, a P deficiency causes a decline in PSII activity and a switch to state 2, resulting in the establishment of culture anaerobiosis in the presence of acetate (Wykoff et al. 1998). However, the inactivation of PSII centers does not occur as rapidly as under S deficiency and the production of O_2 declines more slowly relative to the case for S-starved cells. Chlamydomonas cells are capable of storing P in the form of polyphosphate (Siderius et al. 1996; Komine et al. 2000) and this may explain the slow inactivation of PSII during P deprivation: the cells can use the stored P reserves for days before experiencing any real P deficiency. This is not so in the cases of S and N deficiencies. The possible effects of stored P have been circumvented by suspending very low concentrations of cells in P-deficient media (Batyrova et al. 2012).

As in S-deprived cultures, the acclimation of algal cells to P-deprived conditions is accompanied by an accumulation of starch during the aerobic phase and its degradation during the H_2 production phase. However, there is no evidence that reductants derived from starch provide electrons to the hydrogenases via the PSII-independent pathway. The relative contributions of PSII-dependent and PSII-independent pathways to H_2 production in P-depleted Chlamydomonas cultures are not known.

3.1.4 Differences in Hydrogen Production with Different Nutritional Stresses

Understanding the precise mechanisms that influence H_2 production under different nutrient stresses can help in understanding this process. Although algal cultures depleted of S, N, and P display similar physiological responses (e.g., PSII inactivation, switch to state 2, starch accumulation, O_2 depletion), the physiology of H_2 production under the different stresses is different. For example, PSII inactivation and O_2 depletion seem to occur much faster in S-stressed cultures compared to cultures depleted in P and N. The slow response to P depletion is partly attributed to the internal reserves of P that delay the onset of a severe P stress. The slow response to N depletion is not easily understood as N is quantitatively more abundant in a cell than in S and N depletion results in a rapid loss of culture viability than S depletion (Cakmak et al. 2012). In theory, therefore, a rapid inhibition of PSII is expected in N-stressed cultures compared to the S-stressed cultures, but this is contrary to empirical observations. A possible explanation for this anomaly is that

under N deficiency, unlike with S deficiency, the mitochondrial phosphorylation is also severely compromised and this would slow down the O_2 consumption.

The S-depleted cultures produce H_2 mainly through the PSII-dependent pathway because of the residual-PSII activity present under anaerobiosis. This suggests that the S-depleted cells can reach a steady state with a low LET activity that can last for several days. On the other hand, N-depleted cultures produce H_2 mainly through the PSII-independent pathway, indicating that PSII activity and LET must be severely compromised during the anaerobic phase. Although a deficiency of all three nutrients elicits starch accumulation, mobilization of starch does not occur under N deficiency, unlike in the cases of S and P deficiencies. Hence starch accumulation does not contribute to, or enhance, H_2 production in N-depleted cells. In N-depleted cells, the degradation of other cellular components such as proteins may be important for providing the chloroplast with reduced equivalents. In the case of S-depleted cultures and possibly the P-depleted cultures, starch accumulation is important, but not essential, for enhancing the photoproduction of H_2.

In terms of photoproduction of H_2, the physiological state reached during S deficiency is more interesting than the one obtained under N deficiency. This is because S-depleted cells do not depend on mobilization of internal metabolites to produce H_2 as much as N-depleted cells do and this is reflected in a more sustained H_2 production by the S-depleted cells. Little is known about the light-dependent pathways governing P-stressed production of H_2.

Specific nutrient stress responses may be of interest for understanding the physiological differences related to H_2 production. For example, some S deprivation regulatory mutants show a light-dependent bleaching phenotype and death during S deprivation (Davies et al. 2006; Gonzalez-Ballester et al. 2008). This has been linked to a failure of the mutants to downregulate photosynthetic electron flow out of PSII (Wykoff et al. 1998) and indicates that specific photoadaptive responses are critical for the survival of S-deficient cells. In the absence of S, the rapid degradation of PSII (Melis et al. 2000; Zhang et al. 2002) has been traditionally linked to the inability to repair the photodamage to the D1 proteins due to the downregulation of the de novo protein synthesis. However, this nonspecific phenomenon should be even more prominent in the case of N depletion because protein turnover is further compromised in such cells than in the S-depleted cells. As explained by Malnoe et al. (2014), the D1 degradation during S deprivation is a fine-tuned process, controlled by the FstH protease, and it occurs also in the dark, contrary to the D1 degradation observed under P deficiency, or under photoinhibitory conditions. This suggests that D1 degradation in S-depleted cells is not just a consequence of functional damage but an S-specific acclimation response. It would be of interest to verify if the activity of the FstH protease is somehow under the control of the S deprivation regulatory elements and whether H_2 production is influenced by S depletion in a FstH-deficient mutant.

The NPQ mechanisms occurring during stress conditions are critical for downregulation of the photosynthetic activity and prevention of photodamage. The latter has been shown to impact H_2 production (Nguyen et al. 2011). Few photosynthesis-related transcripts are known to increase their abundance during

S deprivation (Gonzalez-Ballester et al. 2010). Among them is the *LHCBM9* transcript. This transcript is barely detectable in Chlamydomonas cells grown in a nutrient-replete medium, but increase by more than 1000-fold during S deprivation and is the second most abundant mRNA in S-depleted cells. This increase is specific to S deprivation and has not been seen in cases of N/P deprivation or in S deficiency regulatory mutants (Gonzalez-Ballester et al. 2010). It has been shown that mutants impaired in *LHCBM9* expression undergo a more pronounced oxidative damage due to singlet O_2 and a diminished capacity to photoproduce H_2 during S deprivation (Grewe et al. 2014).

Finally, CEF and other electron dissipating mechanisms can compete with hydrogenases for the electrons present in the over-reduced photosynthetic electron chain (Kruse et al. 2005; Antal et al. 2009; Tolleter et al. 2011). A comparative analysis of the activities that compete with hydrogenases for the electrons (e.g., NPQ mechanisms, plastid terminal oxidase (PTOX) activity, and CEF) under given conditions may help in explaining the peculiarities of the physiological responses to the different nutritional stresses.

3.2 Supply of Reduced Carbon Source

Photoproduction of H_2 in some algae adapted to light anaerobiosis has long been known to be enhanced by the presence of acetate in the media (Jones and Myers 1963; Healey 1970; Klein and Betz 1978; Bamberger et al. 1982; Gibbs et al. 1986). These studies established that under light, algae can consume all of the available acetate and subsequently produce H_2 and CO_2. In the dark however, acetate has no effect on H_2 production and acetate consumption is not detected. Notwithstanding these observations, little information exists on the effect of acetate on the production of H_2 in nutrient-replete media (Wang et al. 2011). The effect of acetate has been mostly indirectly studied in S-depleted media. A majority of the studies performed with S-depleted Chlamydomonas cells were in the presence of acetate. Some reports exist on H_2 production by S-deprived Chlamydomonas cells under autotrophic conditions (Fouchard et al. 2005; Tsygankov et al. 2006; Kosourov et al. 2007; Degrenne et al. 2011). These reports concluded that although H_2 production is possible under autotrophic conditions, the production rates are low relative to heterotrophic conditions.

The addition of acetate to nutrient-replete Chlamydomonas cultures decreases the efficiency of PSII, the net O_2 production, the net CO_2 fixation and promotes transition to state 2 and mitochondrial respiration (Gans and Rebeille 1990; Endo and Asada 1996; Asada and Miyake 1999; Heifetz et al. 2000). All these factors help in the establishment of anoxia in sealed cultures under moderate/low illumination. Also, at the transcriptional level, acetate is a potent repressor of the synthesis of enzymes involved in CO_2 fixation and an inducer of the enzymes of the glyoxylate cycle (Martinezrivas and Vega 1993; Plancke et al. 2014).

During H_2 production conditions, carbon from acetate is simultaneously released as CO_2 and incorporated into carbohydrates and lipids. Under these conditions, if monofluoroacetic acid (MFA; an inhibitor of aconitase) is added to the medium, the production of H_2 is interrupted (Healey 1970; Gibbs et al. 1986). This suggests that acetate is assimilated via the TCA and glyoxylate cycles. The latter is coupled to the conversion of succinate to carbohydrates, and H_2 production is a metabolic byproduct linked to the photoassimilation of acetate through these two cycles.

Studies on enzyme localization and proteomics (Willeford and Gibbs 1989; Willeford et al. 1989; Atteia et al. 2009; Rolland et al. 2009) have helped in identifying the acetate photoassimilation pathway. The pathway requires the participation of the enzymes located in the cytosol, the mitochondria, and the chloroplast (Fig. 2). Illuminated sealed cultures behave similarly in both S-repleted and S-depleted conditions: there is an initial aerobic phase in which the cells

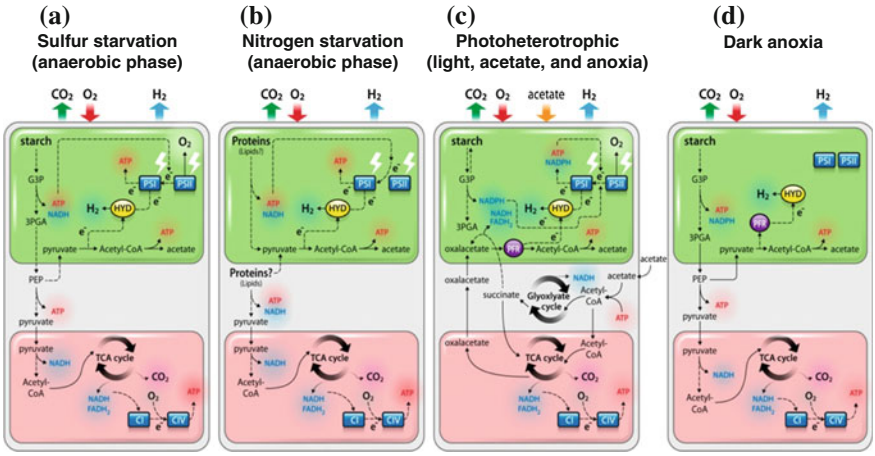

Fig. 2 Proposed pathways for hydrogen production under different conditions. *Panel A* Sulfur starvation (anaerobic phase). H_2 metabolism is characterized by low activity of PSII, a predominantly PSII-dependent photoproduction of H_2, and intense starch degradation. H_2 production linked to pyruvate fermentation is also possible. *Panel B* Nitrogen starvation (anaerobic phase). H_2 production is predominantly driven by PSII-independent photoproduction and fermentative pathways. There is no starch degradation. Protein (or lipids) degradation can provide the reductants for hydrogenase. *Panel C* Photoheterotrophic (light, acetate, and anoxia). H_2 metabolism is characterized by acetate assimilation via the tricarboxylic acid (TCA) cycle and the glyoxylate cycle. The resulting succinate and oxalacetate may provide reductants for the PSII-independent pathway via chloroplast-localized succinate dehydrogenase and malate dehydrogenase, respectively. Alternatively, oxaloacetate can act as substrate of PFR and support fermentative production of H_2. Once O_2 is completely depleted, acetate uptake ceases and starch degradation starts supplying reductants to the chloroplast. *Panel D* Dark anoxia. H_2 metabolism is characterized by fermentative degradation of starch. The resulting pyruvate can feed H_2 production via the PFR

actively consume acetate from the medium and accumulate starch as O_2 is gradually consumed. After the O_2 has been fully consumed, the acetate uptake ceases, starch is degraded, and H_2 is produced (Klein and Betz 1978; Bamberger et al. 1982; Gibbs et al. 1986; Melis et al. 2000; Antal et al. 2003). In the last stage, acetate accumulation is observed in the medium as a result of the simultaneous activation of several fermentative pathways (Klein and Betz 1978; Tsygankov et al. 2002; Hemschemeier and Happe 2005). Still, the role of acetate in the stimulation of H_2 production is not fully understood. It is largely assumed that acetate helps in H_2 production by both favoring the accumulation of starch and the establishment of anoxia in the cultures. Whether acetate has a more direct role in photoproduction of H_2 is not known. The PSII-independent pathway has been proposed as being responsible for the enhancement of H_2 production by acetate (Klein and Betz 1978; Bamberger et al. 1982; Gibbs et al. 1986; Melis et al. 2000; Antal et al. 2003). The chloroplast reductive equivalents required to feed the PQ pool may come from the degradation of the starch that has been previously accumulated. Alternatively, the presence of the two enzymes involved in acetate assimilation (i.e., succinic dehydrogenase and malate dehydrogenase) in the chloroplast may contribute in providing this organelle with the reductive equivalents needed to feed the PQ pool (Willeford and Gibbs 1989; Willeford et al. 1989).

In nutrient-replete media, it is still unresolved whether the presence of acetate favors the PSII-dependent H_2 production. Some studies reveal no impact of adding the herbicide DCMU on H_2 production (Healey 1970), but others have reported a substantial inhibition of both H_2 production and acetate uptake (Bamberger et al. 1982; Gibbs et al. 1986). As DCMU blocks both PSII activity and acetate uptake (and consequently starch accumulation) it is not easy to discriminate which H_2 photoproduction pathway is being affected. Similarly in S-depleted cultures, based on experiments using DCMU, it has been estimated that around 70 % of the H_2 production derives from the PSII-dependent pathway (Ghirardi et al. 2000; Antal et al. 2003; Fouchard et al. 2005; Antal et al. 2009). DCMU blocks acetate uptake and starch accumulation in both S-replete and S-deplete cultures (Bamberger et al. 1982; Gibbs et al. 1986; Fouchard et al. 2005) and this may result in an overestimation of the role of the PSII-dependent pathway. This may also explain the substantial reduction (around 70 %) of the net H_2 production observed using DCMU in starchless mutants (Posewitz et al. 2004a). Hence, estimating the contribution of the H_2 photoproduction pathways using DCMU in acetate-containing cultures requires caution.

Furthermore, a significant contribution of acetate to H_2 production via the fermentative pathway cannot be ruled out. For example, the PFR enzyme of Chlamydomonas has been shown to have an affinity for oxaloacetate (Noth et al. 2013) and this opens the possibility that acetate may be linked to fermentative production of H_2 in illuminated cultures via the glyoxylate cycle.

4 Molecular Strategies to Improve Hydrogen Production

Although the growth conditions can be manipulated to enhance H_2 production, the inherent biochemical and molecular limitations to H_2 production need to be addressed to greatly increase the production rate and yield. Strain improvement and selection are necessary to overcome many of the constraints. The barriers to be circumvented are summarized in Fig. 3. These include: (1) the light saturation of the PSs at low light levels; (2) the downregulation of photosynthetic electron transport due to the undissipated proton gradient; (3) establishment of CEF around PSI; (4) the competition for electrons by hydrogenase at the level of FDX; (5) the O_2 sensitivity, enzymatic reversibility, and low expression levels of hydrogenases; and (6) the nondissipation of the proton gradient (Dubini and Ghirardi 2014). These barriers and some of the methods for overcoming them are further discussed in this section.

Generation of suitable mutants and genetically modified algae is key to improving H_2 production, but often require the screening of thousands of clones. Large-scale screening methods for phenotypes that over/under produce H_2 have been developed. The earliest such screening methods used a chemochromic compound which turned blue when H_2 was produced (Seibert et al. 2001; Flynn et al. 2002; Posewitz et al. 2004a). Other water soluble indicators that change color on contact with H_2 have been used (Katsuda et al. 2006). A recently developed technique makes use of the H_2 sensing system of the photosynthetic bacterium *Rhodobacter capsulatus* for use in Chlamydomonas. In this screening method, an

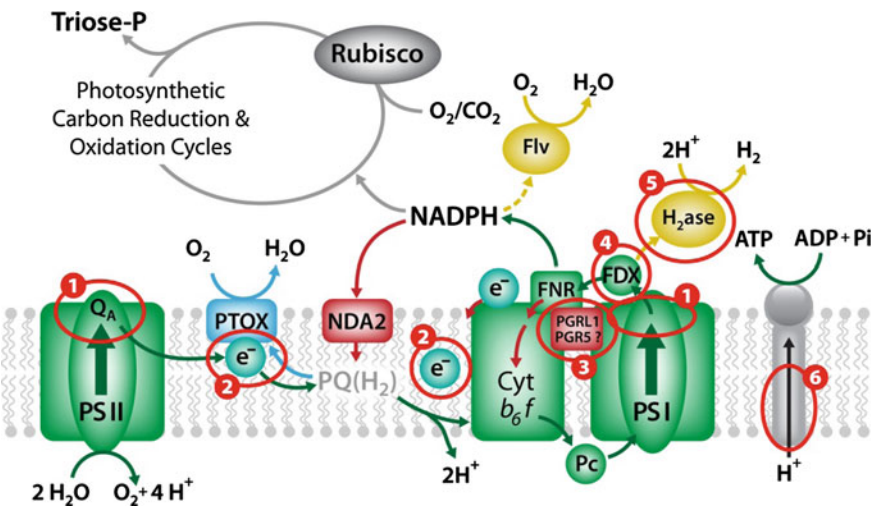

Fig. 3 Biological challenges that remain to be addressed to improve photoproduction of hydrogen. See text for details. The biological barriers to H_2 production are labeled 1–6. The ordering does not reflect the importance of a barrier. Adapted from Dubini and Ghirardi (2014)

emGFP reporter protein integrated behind the *Rhodobacter* hydrogenase uptake promoter fluoresces when the bacterial cells detect H_2 production from an underlay of algal cells (Elsen et al. 1993; Vignais et al. 1997; Dischert et al. 1999; Wecker et al. 2011). A combination of color indicators and robotic automation will likely provide rapid screening methods in the future.

4.1 Light Saturation

Photosynthetic organisms convert incident solar energy into chemical energy. This process typically involves two chlorophyll-containing protein complexes, PSI and PSII (Fig. 3, barrier 1). The total energy required to produce H_2 is governed by several parameters (Fig. 4). First, green algae can absorb sunlight only in the wavelength range from 400 to 700 nm, the photosynthetically active radiation (PAR). This part of the solar spectrum represents about 45 % of the total energy of sunlight. An average PAR photon has a bandgap (energy difference between the top of the valence band and the bottom of the conduction band) of around 2.25 eV, and is able to drive charge separation at PSII and PSI. Once excited, each PS stores energy in the charge-separated state. Of the energy of one photon, the PSII is able to store $\sim$1.9 eV whereas the PSI can store $\sim$1.6 eV from another photon (Ort and Yocum 1996). This energy capture represents 78 % of the energy of the two PAR photons, or 35 % of the incident light. The remaining energy is as lost as heat and fluorescence. The two redox pairs involved in generation of O_2 and H_2 are H_2O/1/2 O_2 and $2H^+/H_2$, respectively. The redox potential of these pairs is 0.82 eV and −0.42 eV, respectively, or an approximate total of 1.2 eV. This is the energy required per 2 photons (one for each PS) absorbed to produce O_2 and H_2 and it corresponds to 27 % of the energy of the two absorbed photons, or 12 % of the energy available from the incident sunlight (Fig. 4). This maximum theoretical energy is never achieved, as photosynthesis is limited by biochemical factors such as photoinhibition, low light saturation, a limited electron transport rate between PSII and PSI, and the intracellular mechanisms that sense and adapt to the ratio of the reductant generated (redox status) and ATP produced. As a consequence, the light conversion efficiency of photosynthetic processes declines at light intensities above the saturation point. This reduces the overall utilization of sunlight to about 2 % or less (Blankenship et al. 2011).

An obvious limitation to the light conversion efficiency of photosynthesis under high sunlight is the large antenna size of the PSs. Under high light fluxes, the photons absorbed by the light-harvesting antennae of PSI and PSII are underutilized and most of their energy is dissipated as fluorescence or heat. Thus, in a high-density mass culture, cells at the surface over-absorb and waste sunlight, whereas the cells deeper in the culture are deprived of light due to shading. The photosynthetic capacity of the cell is therefore not used to its maximum potential (Polle et al. 2002; Melis 2009).

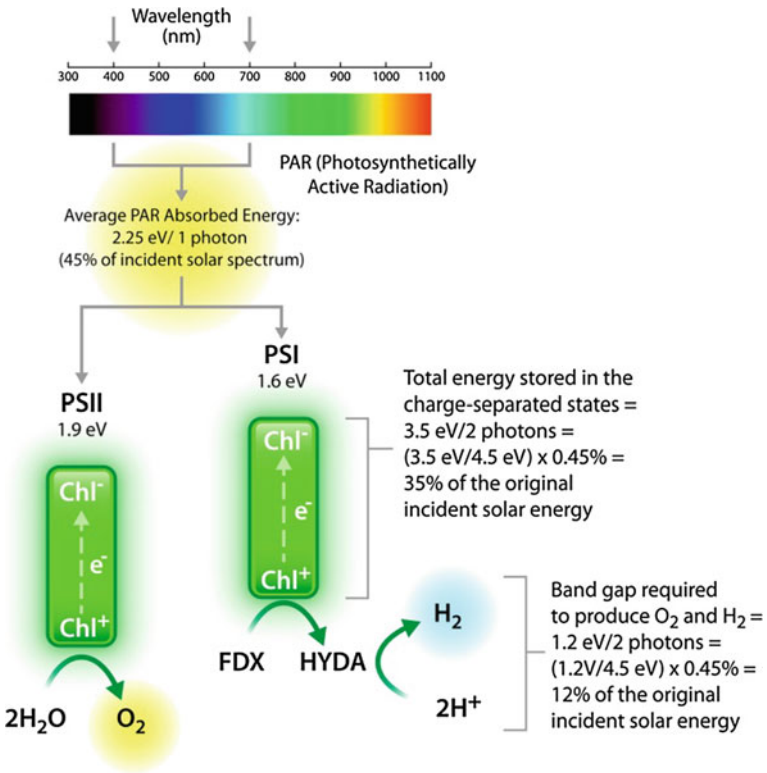

Fig. 4 Photosynthetic efficiency of hydrogen production in Chlamydomonas. A simplified schematic of the light absorption and utilization processes by PSI and PSII. The solar spectrum corresponds to the incident light that the PSs absorb. This energy is then used to produce O_2 and H_2. Each step involves energy loss. Adapted from Ghirardi et al. (2009b)

An obvious strategy to improve the efficiency of photosynthesis is to truncate the light-harvesting antennae of the PSs. Following this approach, Melis' group (Melis et al. 2000; Melis and Chen 2005) generated a series of truncated mutants, or *tla* (truncated light-harvesting Chl antenna). One particular mutant, *tla1*, showed increased H_2 production when immobilized *tla1* cells were exposed to high light under S deprivation conditions (Kosourov et al. 2011). RNAi knockdowns of the light-harvesting complexes LHCBM1, 2, and 3 have also been generated, as a means to reduce the antenna size and optimize light capture by Chlamydomonas. This triple mutant displayed a higher photosynthesis light saturation level and did not suffer photoinhibition under the saturating intensity. On S deprivation, the mutant strain showed an immediate onset of H_2 production, indicating that the intracellular O_2 levels were already poised to induce hydrogenase transcription. Furthermore, the rate of H_2 production observed in this strain was twice as high as for the wild-type strain (Oey et al. 2013).

4.2 *Effect of the Undissipated Proton Gradient on Electron Flow*

When algal cells transition to anaerobiosis, the PQ pool becomes over-reduced and this triggers a transition to state 2. To acclimate to this new condition some of the light-harvesting complex proteins of the PSII dissociate from it and become functionally attached to the PSI, increasing its light absorption capacity at the expenses of that of the PSII. This is accompanied by an increase in CEF over LET (Finazzi et al. 2002). CEF does not result in NADPH production but supports the formation of the proton gradient and ATP biosynthesis. H_2 production is therefore lower under these conditions because a large portion of the electrons are redirected toward CEF instead of toward FDX and hydrogenase (Fig. 3, barrier 3) (Tolleter et al. 2011). Furthermore, as a consequence of induced CEF the ratio of ATP/NADPH produced by photosynthesis increases (Forti et al. 2003). Subsequently, this triggers the reversible activity of the ATPase, which then degrades ATP to ADP and allows the proton (H^+) to reenter the lumen (Cruz et al. 2005). As photoproduction of H_2 does not consume ATP, it cannot help in dissipating the additional proton gradient caused by CEF activity when anaerobically induced cells are illuminated. Moreover, the nondissipated proton gradient induces NPQ mechanisms (qE) that lower the efficiency of photosynthetic electron utilization and therefore the efficiency of H_2 production (Fig. 3, barrier 6). A negative feedback loop is activated under these conditions and the photosynthetic electron transport from PSII to PSI is downregulated (Fig. 3, barrier 2) (Antal et al. 2003; Lee and Greenbaum 2003; de Vitry et al. 2004). This downregulatory process is mediated by the operation of the violoxanthin/zexanthin cycle. This cycle senses the acidification of the lumen and generates a quencher of absorbed light energy (Horton et al. 1991; Niyogi 1999; Muller et al. 2001). The role of the undissipated proton gradient in H_2 production has been confirmed by the effect of proton uncouplers that stimulated the rate of H_2 photoproduction in S-replete conditions (Happe et al. 1994).

Strategies are being developed to bypass the effect of electron transfer and the associated reaction. A mutant deleted in PGRL1 (Proton Gradient Regulation Like 1) protein that mediates CEF, was isolated and showed increased H_2 production yields under S deprivation, confirming the role of the CEF as a limiting factor for electron supply to hydrogenase (Tolleter et al. 2011). Similarly, a mutant blocked in state 1 and unable to transition to state 2 upon anaerobiosis showed no CEF but an increased H_2 production (Volgusheva et al. 2013).

4.3 *Competition for Photosynthetic Reductant*

FDXs directly donate electrons to hydrogenase. Although FDXs normally act as electron acceptors of the photosynthetic electron chain and of other reactions, they

are very promiscuous proteins that can donate electrons to diverse processes. Under aerobic photosynthetic conditions, FDXs transfer electrons preferably to FNR to generate the NADPH required for fixation of CO_2. Other important acceptors of electrons from FDXs include thioredoxins, nitrite and sulfite reductases, glutamate synthase, and α-ketoglutarate synthase. All of these pathways therefore have a negative impact on H_2 production as they compete for electrons at the level of FDX and reduce the electron flux to the HYDA (Fig. 3, barrier 4). In Chlamydomonas, only two of the six chloroplast-localized FDXs, i.e., FDX1 and FDX2, are functionally linked to the hydrogenase. These two FDXs share similar binding partners, but FDX1 serves as the primary electron donor to three important biological pathways: the $NADP^+$ reduction pathway, the photoproduction of H_2 as well as the fermentative pathway of H_2 production. In addition, FDX1 mediates CEF through the *cyt b6/f* complex by transferring electron to the PGRL1 protein. The FDX2 is also able to drive these reactions, at least in vitro, but at less than half the rate observed with FDX1 (Peden et al. 2013; Noth et al. 2013; van Lis et al. 2013). Hence, FDX1 and FDX2 are the key proteins that control the electron flow to the hydrogenase. In addition to the FDXs promiscuity, other processes can also contribute to the competition for electrons at the level of the photosynthetic electron chain as is the case of chlororespiration (Houille-Vernes et al. 2011).

Strategies for reducing the competition for electrons at the level of FDX are several. Under oxic conditions, most of the photosynthetic reductant is directed from FDX1 to FNR. In order to reduce this competition and bypass the dominating effect of FNR, an FDX–hydrogenase fusion protein was engineered and tested in vitro (Yacoby et al. 2011). It was shown that the H_2 photoproduction activity of the fusion protein was sixfold higher than the non-fused hydrogenase with the FDX added. The fusion product was able to overcome $NADP^+$ competitive inhibition and diverted more than 60 % of the photosynthetic electrons to H_2 production, compared to a less than 10 % diversion for the non-fused hydrogenase (Yacoby et al. 2011; Peden et al. 2013).

Another mutant strain (CC-2803), which was impaired in CO_2 fixation, also showed higher rates of H_2 production than its wild-type parent under S deprivation (Hemschemeier et al. 2008). This strain lacked the large subunit of Rubisco, an enzyme that has an important role in photorespiratory O_2 consumption. Similarly, an engineered Chlamydomonas strain harboring a mutation on tyrosine 67 of the Rubisco small subunit displayed 10- to 15-fold higher H_2 production rate than its wild-type parent under S deprivation (Pinto et al. 2013). This latter mutation was shown to impair the stability of Rubisco (Esquivel et al. 2006) and resulted in a decrease in PSII efficiency and the amount of PSII protein complexes in the cell (Pinto et al. 2013). The phenotype was explained by the feedback inhibitory effect of eliminating a major electron drain on the generation of reductant/protons by the PSII (Skillman 2008). Strategies are being developed to promote reductant flux to the hydrogenase. One of the approaches used was to engineer a hexose uptake protein from *Chlorella kesslerii* in Chlamydomonas to allow the host cells to use an external supply of glucose as an additional feedstock for H_2 production (Doebbe et al. 2007). This proved to be successful and H_2 production under S deprivation was improved.

4.4 Limitations Linked to the Hydrogenases

4.4.1 Oxygen Sensitivity of the Hydrogenases

Anaerobiosis is an indispensable prerequisite for H_2 production, as hydrogenases are highly sensitive to O_2. The hydrogenases transcripts, the maturation process of the protein, and hydrogenase's catalytic activity are all O_2 sensitive, preventing continuous production of H_2 (Erbes et al. 1979; Roessler and Lien 1984b; Forestier et al. 2003; Posewitz et al. 2004b; King et al. 2006; Mulder et al. 2010). O_2 inhibition is therefore one of the major issues that needs addressing in order to develop an efficient and stable system of algal H_2 production (Fig. 3, barrier 5).

Multiple strategies have been identified to generate an O_2 tolerant hydrogenase. Either an O_2-tolerant algal [FeFe]-hydrogenase may be engineered (Chang et al. 2007), or O_2-tolerant hydrogenases may be heterologously expressed in Chlamydomonas. Molecular dynamics simulations, solvent accessibility maps, and potential mean energy estimates have been used to identify gas diffusion pathways in model enzymes (Chang et al. 2007), followed by enzyme modification by site-directed mutagenesis (Long et al. 2009). However, these approaches have not been successful for Chlamydomonas hydrogenase due to the unexpected observation that the amino acid residues that comprise the area around the catalytic cluster are also involved in the formation of the gas channels. Thus, mutants affecting these residues are unable to properly fold the protein and produce a leaky, less O_2-tolerant enzyme. This observation explains the lower activity and higher O_2-sensitivity of mutants that were generated based on the information provided by the computational models (Liebgott et al. 2010).

Strategies based on removing the O_2 from the cell may also be helpful in improving the activity of hydorgenases. A decreased O_2 level in the cell may trigger a more rapid anaerobiosis. A PSII mutant strain affected in the D1 protein (mutant L1591-N230Y) was able to produce 20-fold more H_2 than the wild-type parent under S-deprived conditions (Scoma et al. 2012). Similarly, the use of a genetic switch that controlled the expression of the D2 protein was also successful in increasing the H_2 production rate by tenfold when compared to the wild-type (Surzycki et al. 2007). Finally, a mutant strain (APR1) exhibiting an attenuated photosynthetic activity/mitochondrial respiration ratio was shown to become anaerobic in the light and to produce more H_2 than the wild-type when glycolaldehyde was added to the cells (Ruehle et al. 2008).

Other approaches have been tested to scavenge O_2 from the cells. Introduction of O_2 sequesters in the chloroplast is one of these. Leghemoglobins (LbA), for example, sequester and transport O_2 in nitrogen-fixing Rhizobia. Chlamydomonas do not harbor genes encoding LbA proteins, but the relevant genes from Rhizobia may be transformed in the alga. Wu et al. (2010) demonstrated that the expression of LbA protein improved H_2 yield by decreasing the O_2 content in the medium. Alternative approaches to remove O_2 from the culture medium include the introduction of new pathways in Chlamydomonas that utilize O_2. The enzyme Pyruvate

Oxidase (PoX) catalyzes the decarboxylation of pyruvate to acetyl phosphate and carbon dioxide using O_2. The introduction of the *Escherichia coli poX* gene in Chlamydomonas reduced O_2 evolution without affecting the growth rate and increased H_2 production to twice the rate of the wild-type (Xu et al. 2011).

In another approach, purging of the culture medium and the bioreactor headspace with nitrogen or argon is widely used to attain anaerobiosis. In addition, chemicals such as sodium bisulfite ($NaHSO_3$) added to illuminated heterotrophic cultures have reduced O_2 levels and enhanced H_2 production. However, the addition of $NaHSO_3$ only supported H_2 production for a short period (Ma et al. 2011). Other approaches for decreasing the O_2 level have used genetic engineering (see Sect. 4.1).

4.4.2 Hydrogenases Reversibility and H_2 Partial Pressure

All of the hydrogenases identified so far have a reversible activity and are able to both produce H_2 and dissociate it into protons and electrons. The latter activity is commonly known as H_2 uptake. As in any chemical reaction, the equilibrium between the biosynthesis and uptake depends on the concentrations of the substrates and products. H_2 accumulation within a bioreactor increases the H_2 partial pressure to reduce the biosynthesis rate and eventually drive the uptake activity. This phenomenon has been demonstrated in cultures of Chlamydomonas (Cournac et al. 2002; Kosourov et al. 2012). Continuous purging of the anaerobic algal culture with an inert gas (argon or nitrogen) can reduce H_2 uptake (Greenbaum et al. 2001; Cournac et al. 2002) and sustain its production over long periods.

5 Conclusions

Green algae certainly have a potential for commercial production of biohydrogen. Multiple studies demonstrate the technical possibilities, but major issues remain to be addressed for an inexpensive production of biohydrogen to be achieved. Most of the factors preventing the large-scale production of H_2 are known and, potentially, can be addressed by genetic and metabolic engineering. The approaches proposed for stimulating H_2 production in S-deprived Chlamydomonas have been extensively reviewed (Ghirardi et al. 2007; Hankamer et al. 2007; Melis 2007; Melis et al. 2007; Rupprecht 2009). Improved H_2 production has been achieved through synchronization of cell division, optimization of chlorophyll content of the cell, formulation of the culture medium and protocols (Tsygankov et al. 2002; Kosourov et al. 2003; Laurinavichene et al. 2004; Kim et al. 2006; Giannelli et al. 2009). An improved understanding of the nutrient stress factors on H_2 production is needed. Some of the harmful effects of nutrient stress can be overcome using continuous or semi-continuous regimes of cultivation (Fedorov et al. 2005; Oncel and Vardar-Sukan 2009), or by a resupply of S to the medium (Kosourov et al. 2005).

This may be facilitated by immobilizing the cells on various matrices to allow easy cycling between S-repleted and S-depleted media (Laurinavichene et al. 2008; Kosourov and Seibert 2009).

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Biogas from Algae via Anaerobic Digestion

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Abstract Anaerobic digestion is a promising application of algal biomass for producing bioenergy while allowing recovery of inorganic nutrients (nitrogen and phosphorus) for reuse. Anaerobic digestion of algae requires pretreatment of the biomass and/or codigestion with carbon-rich cosubstrates, as discussed in this chapter. In the absence of pretreatment, the methane yield is reduced apparently because of the recalcitrance of the algal cell wall. Encouraging pretreatments and codigestion approaches have been developed but require validation at pilot-scale. Improved estimates of the energy demands of the various pretreatments are required to decide if a certain pretreatment is energetically worthwhile undertaking.

Keywords Microalgae · Macroalgae · Anaerobic digestion · Biogas · Biomass pretreatment · Methane

1 Introduction

Algae are regarded as a potential biomass feedstock for reducing our dependence on fossil fuels for transportation, electricity, and heat generation. In addition, algae have been widely investigated as a source of chemicals, cosmetics, healthcare products, animal feed, and human food. Anaerobic digestion is a promising application of algal biomass for producing methane. Anaerobic digestion has the potential for integration within an algae biorefinery to provide bioenergy as well as allow recovery of nutrients (nitrogen, phosphorus) for reuse in cultivation of microalgae.

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This chapter discusses the fundamentals of anaerobic digestion and biogas production from algae. The options to enhance methane yield are discussed. The environmental and economic aspects of anaerobic digestion of algal biomass are reviewed.

2 Anaerobic Digestion

2.1 Fundamentals

Anaerobic digestion is a natural process that occurs in a variety of anaerobic environments such as marine and fresh water sediments, sewage sludge, mud, and ruminant intestine. This is a biological process in which in the absence of oxygen, organic carbon is converted by subsequent oxidations and reductions to its most oxidized state (CO_2) and its most reduced state (CH_4). The mixture of gaseous products generated by anaerobic digestion is known as biogas and mainly consists of carbon dioxide and methane. Minor quantities of nitrogen, hydrogen, ammonia, and hydrogen sulfide (usually less than 1 % of the total gas volume) may be present.

The anaerobic digestion is performed by microorganisms that decompose the organic matter in four stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Fig. 1). In general, degradation of organic material (polymeric substances such as carbohydrates, protein, and fats) in anaerobic conditions involves principally four groups of bacteria (Fig. 1) which convert the organic

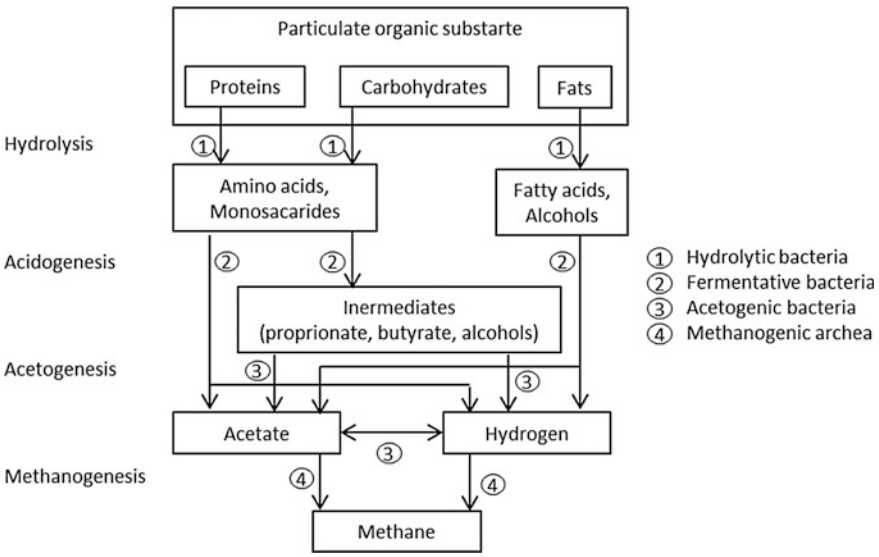

Fig. 1 Degradation steps of anaerobic digestion process

material to methane, carbon dioxide, and water. Hydrolytic and fermentative bacteria hydrolyze the organic polymers to soluble oligomers and monomers by the action of extracellular enzymes. Subsequently, the dissolved products are taken up by bacteria and fermented, forming acetate and other short-chain fatty acids, alcohols, hydrogen and carbon dioxide. These are released in the culture environment. Short-chain fatty acids larger than acetate and alcohols are oxidized by hydrogen producing acetogenic bacteria, resulting in the formation of hydrogen, acetate, formate, and carbon dioxide. The end products of the metabolism of fermentative and acetogenic bacteria are acetate, formate, and hydrogen. These are transformed into methane by the methanogenic bacteria.

The most important substrates in anaerobic digestion are the polymeric compounds such as carbohydrates, proteins and fats. Hydrolysis of these polymers to smaller units is the first step in anaerobic digestion. Different groups of fermentative bacteria are capable of degrading complex polymeric compounds in organic waste to oligomers and monomers through the action of extracellular hydrolytic enzymes. Proteolytic bacteria produce proteases which catalyze the hydrolysis of proteins into amino acids. Cellulolytic and xylanolytic bacteria produce cellulases and/or xylanases which degrade cellulose and xylan (carbohydrates) to glucose and xylose, respectively. The lipolytic bacteria produce lipases which degrade lipids (fat and oils) to glycerol and long-chain fatty acids.

A large fraction of the organic waste is composed of lignocellulose, a difficult-to-degrade complex polymer. The lignin associated with the cellulose hinders its microbial degradation and effective degradation of cellulose often requires a prior delignification step. The initial hydrolysis proceeds at various speeds depending on the nature of organic waste and in some cases this may be the rate limiting step for the entire process. If cellulose is the main component in the substrate, the hydrolysis step will be rate-limiting. The decomposition of acetate to methane is rate-limiting when the substrate consists of mainly the easily metabolized materials such as dissolved starch.

Simpler organic molecules produced via hydrolysis are absorbed by fermentative bacteria to produce compounds such as acetate, other fatty acids, alcohols, and hydrogen. In a well-functioning anaerobic digestion process, the main part of organic material is transformed directly by the fermentative bacteria to methanogenic substrates (hydrogen, carbon dioxide, and acetate). Nevertheless, a significant portion (approximately 30 %) is transformed into other fatty acids and alcohols. This portion is larger in an unbalanced process.

The second step of the anaerobic digestion is acidogenesis. Acidogenesis results in the conversion of hydrolyzed products into simple molecules such as short-chain fatty acids (or volatile fatty acids, VFA; e.g., acetic acid, propionic acid, and butyric acid), alcohols, aldehydes and gases such as CO_2, H_2 and NH_3. VFA and alcohols produced during acidogenesis are oxidized to acetate in a process that is coupled to a reduction of protons to hydrogen. A characteristic feature of the oxidation of VFA and alcohols is that the energy yield is low at standard conditions. In order to increase the energy yield of the oxidation of intermediates, the hydrogen partial pressure must remain low so that the equilibrium is shifted toward hydrogen formation.

The most important methane precursor is acetate, contributing nearly 70 % of the methane produced. The remaining 30 % of the methane is derived from H_2/CO_2 or formate. Methanogenic bacteria are divided into two main groups: aceticlastic methane bacteria (*Methanosarcina* sp. and *Methanosaeta* sp.) which degrade acetate; and the hydrogen consuming methanogens. A number of *Methanosarcina* species can transform hydrogen as well as acetate to methane. Substrates of less quantitative importance for methanogens are methanol, methylsulfides, methylamines and some higher alcohols. Methanogenesis is regarded as the motive force of the anaerobic degradation as it is an energy producing process under standard conditions, as opposed to some of the other processes involved in anaerobic degradation. Methanogenesis is the terminal step necessary for a complete mineralization of the organic matter.

2.2 *Factors Influencing Anaerobic Digestion*

As a complex biological process, anaerobic digestion is influenced by several environmental factors. Synergistic interaction among different bacteria is a key factor influencing the biogas production. Under unstable operation conditions, intermediates such as volatile fatty acids and alcohols accumulate at different rates depending on the substrate and the factors contributing to instability (Allison 1978). Thus, relative changes in the concentrations of intermediates are indicative of disturbance of the biogas production. The main environmental factors that can affect stability of production are temperature, pH, substrate composition, and the presence of toxic substances (Ahring et al. 1995).

2.2.1 Temperature

Temperature is one of the main factors affecting bacterial growth. Bacterial growth rates typically increase with increasing temperature up to a certain limit and beyond this limit the growth rate declines precipitously. In addition to growth rate, the temperature also influences physical properties of the culture broth such as viscosity, surface tension and mass transfer properties. Temperature stability is also important as even relatively small changes in temperature result in an efficiency drop until the microorganisms have adapted to the new temperature.

Anaerobic digesters operate either in the mesophilic range of 25–40 °C, or in the thermophilic range of >50 °C. The thermophilic process has a number of advantages relative to the mesophilic process. For example, the thermophilic process is faster and, therefore, requires a lower residence time of the waste in the digester. It is good at destroying pathogens, improves solubility and availability of substrates and achieves a more complete degradation of long-chain fatty acids. On the other hand, thermophilic process has a larger degree of instability, demands more energy

and involves a larger risk of ammonia inhibition. Methanogenesis is also possible under psychrophilic conditions (below 25 °C), but at lower process rates.

2.2.2 pH

The anaerobic digestion process is limited to a relatively narrow pH interval from 6.0 to 8.5. Each of the microbial groups involved in anaerobic degradation has a specific pH optimum and can grow in a specific pH range. Methanogens and acetogens have a pH optimum at 7. Acidogens have lower pH optimum of around 6. If the pH is less than 6.6, the growth of methanogens is slowed a lot. In an anaerobic reactor, instability leads to accumulation of VFA with a consequent drop in pH, in a process known as acidification. An accumulation of VFA does not always cause a decline in pH in view of the buffering capacity of some wastes. In such cases, when a drop in pH is eventually observed, the acid concentration may be already too high and the process may have been fatally affected. In addition to influencing microbial growth, the pH can affect other factors of importance in the anaerobic digestion process. For example, the dissociation of important compounds (ammonia, sulfide, and organic acids) is affected by pH.

2.2.3 Carbon/Nitrogen Ratio

The carbon/nitrogen (C/N) ratio is important for process stability. A C/N ratio of 25–32 is reported to beneficially affect the methane yield (Kayhanian and Tchobanoglous 1992). At lower C/N ratios, the excess nitrogen not used in biomass synthesis may become inhibitory. Too high a C/N ratio results in N-deficiency and insufficient production of microbial biomass. Wastes with a very high chemical oxygen demand and a low content of nitrogen may not be digested unless the C/N ratio is suitably adjusted. Olive mill effluents are examples of such wastes (Angelidaki and Ahring 1997).

2.2.4 Toxic Compounds

Certain chemicals are toxic to the microorganisms involved in anaerobic digestion. The nature of the toxic compounds present depends mainly on the composition of the substrate. Ammonia is a common inhibitor of anaerobic digestion (Rajagopal et al. 2013). Ammonia originates from soluble ammonium produced as a result of degradation of proteins and compounds such as urea. Often the substrates encountered in anaerobic treatment contain ammonia at toxic concentrations. Such substrates include pig and poultry manure, slaughterhouse waste, highly proteinaceous sludge, and wastewater from shale oil and coal liquefaction processes. What constitutes an inhibitory level of ammonia depends on other factors such as pH, temperature and the adaptation history of the inoculum. Methanogenic bacteria are

especially sensitive to inhibition by ammonia. However, the cultures can be acclimatized to withstand higher concentrations of a toxicant compared to the nonadapted culture. Inhibition tends to be stronger under thermophilic conditions than under mesophilic conditions. Increasing pH and temperature increase the level of free ammonia and therefore may be inhibitory. In an ammonia inhibited process, an increase in the concentration of VFA causes a decrease in pH and this reduces the concentration of the free ammonia to partly counteract the inhibitory effect.

Anaerobic treatment of wastewater containing high sulfate concentrations results in the formation of hydrogen sulfide which is inhibitory. A total hydrogen sulfide concentration in the range of 100–300 mg/L, or free hydrogen sulfide concentration in the range of 50–150 mg/L, cause severe inhibition resulting in a complete cessation of biogas production (Imai et al. 1998).

Long-chain fatty acids such as oleate and stearate, have been found to be toxic in anaerobic digestion (Palatsi et al. 2010). Other toxic compounds include heavy metals. These disrupt enzyme function and structure by binding to thiol and other functional groups on the protein molecules, or by displacing the naturally occurring metal ion of a metalloprotein (Vallee and Ulner 1972).

In addition to any toxic compounds present in the waste, some readily degraded substrate components can also inhibit the digestion process. Therefore, the levels of lipids and proteins in the feed stream entering the biogas plant require careful control. A sudden spike in the concentration of lipids fed to a biogas plant can inhibit anaerobic degradation: hydrolytic, acidogenic and methanogenic bacteria are inhibited by accumulation of long-chain fatty acids resulting from the hydrolysis of lipids. The toxicity of lipids therefore depends on how fast they are hydrolyzed compared to the rate of consumption of the resulting fatty acids via the downstream fermentation processes. Degradation of proteins results in the formation of ammonia which can be inhibitory. A long recovery period may be required if a large amount of protein is added to a biogas digester not adapted to a high level of ammonia.

3 Algae Anaerobic Digestion

3.1 Macroalgae

Macroalgae, or seaweeds, have received attention as a biofuel feedstock as a consequence of their prolific growth in eutrophic coastal waters fouling beaches and waterways. Anaerobic digestion has been used to produce biogas from macroalgal biomass (Habig et al. 1984; Vergara-Fernandez et al. 2008). Unlike biomass of higher plants, the macroalgal biomass is generally easily hydrolyzed to sugars and proteins. The biomass has a low level of lignin (Nkemka and Murto 2010) and high proportion of hemicellulose.

Methane potential of macroalgal species such as *Laminaria* sp., *Sargassum* sp., *Macrocystis* sp., *Macrocystis pyrifera* and *Durvillea antarctica* has been reported (Chynoweth et al. 1993; Bird et al. 1990; Singh and Gu 2010; Vergara-Fernandez et al. 2008). The reported methane yields from various macroalgae are summarized in Table 1. The yields depend on the species and the other operational parameters. Methane yield values of up to 400 L/kg volatile solids (VS) have been reported (Table 1).

The effect of supplementing the biomass of the macroalgae *Gracilaria tikvahiae* and *Ulva* sp. with nitrogen (NH_4Cl) on biogas production has been discussed by Habig et al. (1984). A decreasing nitrogen concentration increased the biogas yield from 410 L/kg VS (33.7 % CH_4) to 560 L/kg VS (59.5 % CH_4). Methane production was higher under nitrogen deficient conditions.

The net biogas production has been observed to depend on the operating temperature (25, 35, and 55 °C) and the nature of the inoculum used (Migliore et al. 2012). The best operating temperature was 35 °C. The use of anaerobic sediments as inoculum significantly improved the biogas yield depending on the temperature: the yield increased from 53 to 283 L/kg VS at 25 °C, the increase was from 175 to 375 L/kg VS at 35 °C, and from 2 to 104 L/kg VS at 55 °C.

The effect on biogas production, of pretreating the macroalgal biomass has been reported. For example, Grala et al. (2012) used hydrothermal depolymerization and enzymatic hydrolysis of a mixture of *Pilayella*, *Ectocarpus* and *Enteromarpha* biomass. The biogas yield improved from 33 to 54 L/kg VS as a result of the pretreatment (see Sect. 4 for further discussion of the effects of pretreatments).

The barriers to anaerobic digestion of marine macroalgae include recalcitrance of materials such as polyphenols, cellulosic fibers and lignin type components found in some species. Presence of such materials reduces biodegradability of the biomass hence limiting gas production (Bird et al. 1990; Briand and Morand 1997). Other limitations include seasonality of availability and variability of the feedstock.

Table 1 Methane yield from different macroalgae species under mesophilic conditions (<40 °C)

Algal species	Methane yield (L/kg VS)	References
Gracilaria sp.	280–430	Bird et al. (1990), Habig et al. (1984)
Laminaria sp.	350	Chynoweth et al. (1993)
Macrocystis	390–410	Chynoweth et al. (1993)
Palmaria palmate	320	Jard et al. (2012)
Saccharina latissimi	270	Jard et al. (2012)
Sargassum sp.	120–190	Bird et al. (1990)
Ulva sp.	170–330	Habig et al. (1984)
Mixture of *Gracilariopsis longissima* and *Chaetomorpha linum*	53–157	Migliore et al. (2012)

3.2 Microalgae

Both freshwater and marine microalgae have drawn attention as substrates for anaerobic digestion. A wide range of microalgal species, digester configurations and operational parameters have been evaluated for methane production through anaerobic digestion (González-Fernández et al. 2012a). The composition (contents of protein, lipids and carbohydrates) of the feed, the digester design, the hydraulic retention time and the temperature influence methane production (Sialve et al. 2009). The composition of the biomass is in turn influenced by the conditions used in growing it.

Anaerobic digestion of a mixed culture of *Chlorella* sp. and *Scenedesmus* sp. was reported nearly a half-century ago (Golueke et al. 1957). The productivity and quality of biogas were found to be comparable to production from sewage sludge. Biogas production was 986 L/kg VS at 35 °C and 1020 L/kg VS at 55 °C and the methane content in the gas ranged from 61 to 63 %. The production of biogas was observed to depend on the algal species, in particular on the structure and composition of the cell wall.

Data on biogas production from various microalgae are summarized in Table 2. Production values of up to 390 L CH_4/kg VS have been reported for *Chlamydomonas reinhardtii*. Production was lower (about 100 L CH_4/kg VS) from the biomass residue of *Scenedesmus* left after the lipids had been extracted. Biogas production potential of an alga depends on its composition and the cell wall structure, but has no relationship to the taxonomic group of a species, or its habitat (marine or freshwater) (Mussgnug et al. 2010; Zamalloa et al. 2012). Susceptibility to anaerobic digestion of individual species is related to the structure of their cell walls.

Species such as *Chlamydomonas reinhardtii, Arthrospira platensis* and *Epicrates gracilis* have a protein-based cell wall free of cellulose and hemicellulose

Table 2 Methane yield from different microalgae species under mesophilic conditions (<40 °C)

Microalgae species	Methane yield (L/kg VS)	References
Chlamydomonas reinhardtii	390	Rodolfi et al. (2008)
Dunaliella sp.	320–440	Metting (1996), Rodolfi et al. (2008)
Chlorella sp.	130–150	Dismuken et al. (2008)
Microcystis sp.	196–340	Zeng et al. (2010)
Phaeodactylum tricornutum	400–420	Zamalloa et al. (2012)
Scenedesmus sp.	148–214	Rodolfi et al. (2008), Zamalloa et al. (2012)
Scenedesmus residue after lipid extraction	10–140	Ghirardi et al. (2000)
Spirulina sp.	260–320	Metting (1996), Spolaore et al. (2006)

(Mussgnug et al. 2010) and are easy to digest. In contrast, species such as *Chlorela kessleri* and *Scenedesmus obliquus* are characterized by carbohydrate-based cell walls containing hemicellulose and are more difficult to digest. The cell wall of *S. obliquus* has been described as being particularly rigid because of the presence of a sporopollenin-like biopolymer (Burczyk and Dworzanskib 1988). The complex silica-based cell wall of diatoms has been suggested as the main reason for their low biogas yield (Hildebrand et al. 2012). An easily degraded cell wall, or the total absence of a cell wall, does not necessarily imply that an alga is a good substrate for anaerobic digestion. Other factors, such as the presence of inhibitory substances, also influence degradability (Hildebrand et al. 2012).

The feasibility of digesting the microalgal biomass remaining after the lipids have been extracted for biodiesel production has been demonstrated. Anaerobic digestion of the residual biomass is an important option for its disposal as the residue may be as much as 65 % of the initial biomass. The carbohydrates and proteins in the residual biomass can be used to produce biogas. For example, Yang et al. (2011) obtained a methane yield of 390 L CH_4/kg VS by digesting residual *Scenedesmus* biomass of oil extraction processes. Ramos-Suárez et al. (2014) reported a methane yield of 177 L/kg VS (79.1 % CH_4) from whole biomass of *Scenedesmus* sp. The yield from the residual biomass after amino acids extraction was 364 L/kg VS (58.3 % CH_4) and it was 401 L/kg VS (68.0 % CH_4) after lipid extraction. The significant increase in biogas yield from the biomass residue remaining after amino acid extraction was attributed to the disruption of the microalgal cell walls during the extraction process and an increase in the C/N ratio of the biomass through loss of protein. The slightly lower methane yield after lipid extraction was attributed to the loss of C in the lipids which otherwise would contribute the most to methane production.

4 Pretreatment of Algal Biomass

Both macroalgae and microalgae can be resistant to anaerobic digestion and this may limit biogas production. Pretreatment of the biomass may be required to make it more digestible and improve methane yield. Several pretreatment techniques have proven useful in improving digestibility of organic substrates such as sewage sludge and lignocellulosic biomass (Carrère et al. 2010; Hendriks and Zeeman 2009). Similar methods can be used with algal biomass. Various pretreatments have improved methane yield from algal biomass by up to 70 % (220 % in one case) relative to control (Table 3). The effectiveness of a pretreatment depends mainly on the characteristics of the alga, i.e., on recalcitrance of the cell wall and the macromolecular composition of cells.

Pretreatment methods can be divided into three types: physical, chemical and biological. Physical treatment may be exclusively mechanical or it may involve a thermal process. For macroalgae, physical pretreatments often consist of washing, drying and maceration, or chopping. Physical pretreatments alone or in combination

Table 3 Impact of various thermal pretreatments of algae on biogas production

Algae species	Pretreatment conditions	Methane yield increment	References
Thermal pretreatment			
Scenedesmus sp.	70 and 90 °C, 3 h	12 and 220 % (from 76 to 85–243 L CH_4/kg VS)	González-Fernández et al. (2012a)
Scenedesmus sp.	70 and 80 °C, 25 min	9 and 57 % (from 82 to 89–129 L CH_4/kg VS)	González-Fernández et al. (2012b)
Chlorella sp., *Scenedesmus* sp.	80 °C, 30 min	14 % (from 340 to 380 L CH_4/kg VS)	Cho et al. (2013)
Mixed culture[a] of *Stigeoclonium* sp., *Monoraphidium* sp. and *Nitzschia* sp.	75 and 95 °C, 10 h	67 and 72 % (from 180 to 300 and 310 L CH_4/kg VS)	Passos and Ferrer (2014)
Mixed culture[a] of *Scenedesmus* sp. and *Chlorella* sp.	100 °C, 8 h	33 % (from 270 to 360 L biogas/kg VS)	Chen and Oswald (1998)
Hydrothermal pretreatment			
Chlorella sp. and *Scenedesmus* sp.	120 °C, 30 min	20 % (from 340 to 400 L CH_4/kg VS)	Cho et al. (2013)
Nannocloropsis salina[a]	100–120 °C, 2 h	108 % (from 130 to 270 L CH_4/kg VS)	Schwede et al. (2013)
Mixed culture of *Chlamydomonas* sp., *Scenedesmus* sp. and *Nannocloropsis* sp.	110 and 140 °C, 15 min	19 and 33 % (from 272 to 323 and 362 L CH_4/kg VS)	Alzate et al. (2012)
Mixed culture of *Acutodesmus* sp. and *Oocystis* sp.	110 and 140 °C, 15 min	11 and 31 % (from 198 to 219 and 260 L CH_4/kg VS)	Alzate et al. (2012)
Mixed culture of *Microspora* sp.	110 and 140 °C, 15 min	62 and 50 % (from 255 to 413 and 382 L CH_4/kg VS)	Alzate et al. (2012)
Thermal pretreatment with steam explosion			
Scenedesmus sp.	170 °C, 8 bar, 30 min	81 % (from 180 to 330 L CH_4/kg VS)	Keymer et al. (2013)
Chlorella vulgaris	140–180 °C, 20 min	64 % (from to 160 to 260 L CH_4/kg COD)[b]	Mendez et al. (2014)
Mixed culture of *Chlamydomonas* sp., *Scenedesmus* sp. and *Nannocloropsis* sp.	170 °C, 6 bar, 15 min	46 % (from 272 to 398 L CH_4/kg VS)	Alzate et al. (2012)

(continued)

Table 3 (continued)

Algae species	Pretreatment conditions	Methane yield increment	References
Mixed culture of *Acutodesmus* sp. and *Oocystis* sp.	170 °C, 6 bar, 15 min	55 % (from 198 to 307 L CH_4/kg VS)	Alzate et al. (2012)
Mixed culture of *Microspora* sp.	170 °C, 6 bar, 15 min	41 % (from 255 to 359 L CH_4/kg VS)	Alzate et al. (2012)
Saccharina latissimi	130 and 160 °C, 10 min	20 % (from 220 to 270 L CH_4/kg COD)[b]	Vivekanand et al. (2012)

[a]Studies carried out with continuous reactors
[b]*COD* Chemical oxygen demand

with heat are regarded as most effective in breaking or disrupting cells of microalgae. Among the chemical pretreatments, thermochemical ones have been successfully used in particular with macroalgae.

4.1 Thermal Pretreatments

In thermal pretreatments biomass is solubilized by applying heat. Thermal pretreatments at temperature ranging from 50 to 270 °C have been commonly used prior to anaerobic digestion to enhance the disintegration of particulate organic matter (Carrère et al. 2010; Hendriks and Zeeman 2009). The optimal treatment temperature and time depend on the characteristics of the substrate. Thermal pretreatments may be purely thermal, or may involve the action of water and heat (i.e., hydrothermal treatment) or the action of heat may be combined with other physical forces as, for example, in steam explosion of biomass.

A purely thermal pretreatment is usually regarded as one carried out at a temperature of <100 °C. This type of pretreatment has enhanced the methane yield in both mesophilic and thermophilic anaerobic digestion processes. A relatively low-temperature thermal pretreatment has the advantage of being less energy intensive compared to the more severe treatments. Energy balances based on laboratory-scale digesters with a hydraulic retention time of 20 days have shown that the net energy recovery shifts from neutral (i.e., no net gain) to positive after a thermal pretreatment at 75 °C (Passos and Ferrer 2014). In effect, a suitable thermal pretreatment can enhance the overall energy recovery from an anaerobic digestion process. Increase in methane yields after a pretreatment at 50–80 °C appears to be quite variable (Table 3), but after a pretreatment at 85–100 °C, the methane yield shows a consistent increase ranging from 20 to 108 % relative to the untreated control. The variable performance of the lower temperature pretreatment is likely a result of the differences in the characteristics of different microalgae species and the pretreatment conditions.

A hydrothermal pretreatment by definition involves the use of a temperature of >100 °C. Heating must be carried out under pressure. The effects of a hydrothermal pretreatment on biogas production are similar to those of a lower temperature pretreatment, but a hydrothermal treatment requires much shorter treatment time compared to a thermal pretreatment. For example, in one case the methane yield was increased by 60 % after pretreatment at 70–95 °C for 3–10 h (González-Fernández et al. 2012a; Passos et al. 2013a). In comparison to this, the yield increased by 60–120 % after pretreatment at 120–140 °C for 15–30 min (Alzate et al. 2012; Cho et al. 2013) (Table 3). For *Chlorella* sp. and *Scenedesmus* sp., the methane yield was maximum after a pretreatment at 120 °C. This pretreatment increased methane yield from 0.34 to 0.40 L CH_4/g VS (Cho et al. 2013).

The rapid release of pressure after a high temperature pretreatment also has a dramatic effect on cells. This effect is similar to that of a steam explosion treatment. In the latter, the biomass held in a pressure vessel may be heated to something like 160 °C at around 6 bars for a few minutes (10–30 min) and afterwards, the pressure is rapidly released to flash off steam (Keymar et al. 2013). Steam explosion technology is commercially used in treating lignocellulosic biomass prior to enzymatic digestion and is available for pretreatment of sewage sludge (Kepp et al. 2000). Biogas production from sewage sludge may be increased by 50–100 % after the steam explosion pretreatment (Kepp et al. 2000).

Steam explosion pretreatment has been tested for biogas production from microalgae in anaerobic batch tests. Steam explosion pretreatment has been reported to be more effective than the use of ultrasonic pretreatment (Alzate et al. 2012). With steam exploded algal biomass, the methane yield was increase by 41–55 % relative to control (Alzate et al. 2012). In another study, similar treatments increased the methane yield from microalgae biomass and the residual lipid-extracted biomass by 81 and 58 %, respectively (Keymar et al. 2013). The extent of anaerobic biodegradability was enhanced by pretreatment more than the rate of anaerobic digestion (Keymar et al. 2013).

4.2 Mechanical Pretreatments

Mechanical pretreatments directly break cells by the use of a physical force. For lipid extraction from microalgae, mechanical methods are usually preferred as they are less affected by the choice of the algal species and are less likely to contaminate the lipid product with chemical additives (Lee et al. 2012). As their main disadvantage, physical pretreatments are highly energy intensive. The most commonly used physical pretreatments for microalgae are sonication with ultrasound, irradiation with microwaves, grinding in bead mills, and disruption in high-pressure homogenizers.

Ultrasonication involves exposing the cells to rapid compression and decompression cycles of sonic waves. Intense turbulence is generated in the cell slurry. This and the shockwaves produced by imploding cavitation bubbles damage cells

directly, or indirectly (Kim et al. 2013). Ultrasonication is an effective method of cell disruption (Kim et al. 2013). As with other pretreatments, the results depend on the microalgal species and the pretreatment conditions. The sonication frequency is generally fixed and the controllable treatment parameters are mainly the sonication power and the duration. Different combinations of sonication power input (e.g., 600–1000 W) and duration (e.g., 20–100 min) may be tested to identify the best treatment conditions. In one case, treatment at 800 W for 80 min released the highest level of glucose (0.37 g glucose/g dry weight) from the biomass (Zhao et al. 2013). As seen in Table 4, increase in the methane yield did not exceed 30 % if the sonication energy input was <75 MJ/kg TS (Alzate et al. 2012; Gonzalez-Fernandez et al. 2012b). The yield increased by 75–90 % if the treatment used sonication energy levels of 100–200 MJ/kg TS (Gonzalez-Fernandez et al. 2012b; Park et al. 2013). Results indicate that a high sonication energy input (>100 MJ/kg TS) is necessary for improving methane yield from microalgal biomass. Ultrasonication pretreatment appears to affect the methane yield more than it affects the rate of hydrolysis (Passos et al. 2014a, b).

Microwaves are electromagnetic waves in the frequency range of 300 MHz to 300 GHz. Microwaves are absorbed by water molecules and increase their vibrational frequency. The resulting heating effect causes the temperature to rise. The microwave energy is incapable of breaking covalent bonds, but hydrogen bonds can be broken. Heat generated by vibration of water molecules denatures proteins (Kaatze 1995). As with sonication, the main controllable parameters of microwave pretreatment are the output power and the exposure time (Passos et al. 2013b). Microwaves penetrate the biomass and therefore the treatment can be rapid (Kim et al. 2013), but the electric power consumption tends to be high and depends on the biomass concentration. Optimal pretreatment conditions are those with an energy input to biomass of about 65.4 MJ/kg TS, regardless of the output power and the duration of exposure (Passos et al. 2013b). Irradiation at optimal energy input may lead to an eight-fold increase in biomass solubilization (Passos et al. 2013b).

For macroalgae, mechanical pretreatments such as maceration and grinding are quite effective in increasing the methane yield by as much as 70 % relative to the untreated biomass (Table 4).

4.3 Chemical Pretreatments

Chemical pretreatments are relatively far less used compared to thermal and mechanical pretreatments. Of the many possible chemical methods, only the acid and alkali pretreatments appear to have been used with microalgae. Acid and alkali pretreatments act by solubilizing polymers to improve the availability of readily digestible small molecules (Bohutskyi and Bouwer 2013). The small amount of residual alkali remaining in the pretreated biomass may be useful in countering the pH decline during the subsequent acidogenesis step. Notwithstanding their efficacy,

Table 4 Imapct of mechanical pretreatments of algae on biogas production

Algae species	Pretreatment	Methane yield increment	References
Ultrasound			
Scenedesmus sp.	100–130 MJ/kg TS	75–90 % (from 80 to 140–150 L CH_4/kg COD)	González-Fernández et al. (2012b)
Chlorella sp. and *Scenedesmus* sp.	130 W; 30, 90, and 180 s	6, 10 and 15 % (from 340 to 360–380 L CH_4/kg VS)	Cho et al. (2013)
Chlorella vulgaris and sludge	200 J/mL	90 %	Park et al. (2013)
Mixed cultures of *Chlamydomonas* sp., *Scenedesmus* sp. and *Nannocloropsis* sp.	10–60 MJ/kg TS	12–14 % (from 272 to 305–310 L CH_4/kg VS)	Alzate et al. (2012)
Acutodesmus sp. and *Oocystis* sp.	10–60 MJ/kg TS	6–13 % (from 198 to 209–223 L CH_4/kg VS)	Alzate et al. (2012)
Microspora sp.	10–60 MJ/kg TS	18–23 % (from 255 to 301–314 L CH_4/kg VS)	Alzate et al. (2012)
Mixed culture of *Stigeoclonium* sp., *Monoraphidium* sp. and *Nitzschia* sp.	16–67.2 MJ/kg TS	6–33 % (from 148 to 156–196 L CH_4/kg VS)	Passos et al. (2014)
Others			
Microalgae[a] *Stigeoclonium* sp., *Monoraphidium* sp. and *Nitzschia* sp.	Microwave (900 W; 3 min 70 MJ/kg VS; 26 g TS/L)	60 % (from 170 to 270 L CH_4/kg VS)	Passos et al. (2014)
Macroalgae	Holander beater	69–71 %	Tedesco et al. (2013)
Macroalgae *Gracilaria vermiculophylla*	Washing, drying and maceration	5–10 %	Oliveira et al. (2014)
Macroalgae[a] *Ulva lactuca*	Chopping and mechanical maceration	68 %	Nielsen and Heiker (2011)

[a]Studies carried out with continuous reactors

some acid and alkali treatments result in the formation of byproducts that can be inhibitory to the microorganisms involved in anaerobic digestion.

Alkaline pretreatments involving sodium hydroxide, calcium hydroxide, and ammonium hydroxide have been extensively investigated for use with solid organic wastes. Thermochemical pretreatment of microalgae with alkali and acids has been reported by Mendez et al. (2014). *Chlorella vulgaris* was first heated at 120 °C for 40 min and then NaOH or H_2SO_4 was added. All the tested pretreatments improved the algal biodegradability, but the highest increase in methane yield of 93 % was achieved after the thermal pretreatment without any chemicals being added. Nonetheless, the thermo-alkaline pretreatment caused a higher solubilization of

carbohydrates and proteins compared to the thermal pretreatment. With the macroalgae *Palmaria palmate* (Jard et al. 2013) and *Gracilaria vermiculophylla* (Oliveira et al. 2014), the pretreatments improved biomass solubilization, but not the methane yield. This was explained as being a consequence of the unidentified inhibitory byproducts of the pretreatment processes limiting the anaerobic digestion (Mendez et al. 2014). In general, chemical pretreatments have not been extensively investigated and the results are sometimes contradictory. Nevertheless, a combination of thermal and chemical pretreatments can be promising.

4.4 Biological Pretreatments

Biological pretreatment involves the use of enzymes to damage the wall of the algal cells and hydrolyze biopolymers. Biological pretreatments are promising alternatives to the other pretreatments (Bohutskyi and Bouwer 2013). In view of the mild conditions used, the enzymatic pretreatment does not normally produce inhibitory compounds. Hydrolytic enzymes breakdown the polymers making up the algal cell wall to make the cells permeable and more amenable to anaerobic digestion. Enzymatic pretreatment is influenced by the enzyme dose, the temperature, the pH and the duration of the treatment. Pretreatment temperature and pH are set to the optimal activity range of the specific enzyme used. Some of the drawbacks of this method are the high cost of the enzymes and the fact that there may be no universally good enzyme considering the enormous diversity of composition and structure of the cell walls of different microalgae.

The literature on enzymatic pretreatment is quite scarce. The enzyme endo-β-1,4-glucanase from *Cellulomonas* sp. YJ5 could hydrolyze the cell walls of *Chlorella sorokiniana* to cause cell lysis after 60–180 min of treatment (Fu et al. 2010). A combined treatment involving mechanical blending and an enzyme cocktail (α-amylase, protease, lipase, xylanase) has been applied to *Rhizoclonium* sp. biomass. This pretreatment improved the methane yield by >20 % as compared to the use of mechanical pretreatment alone. Among single enzyme pretreatments, cellulase showed the best results, but pretreatment with the mixed enzymes was better (Ehimen et al. 2010). In a mixed enzyme system the hydrolytic action of one enzyme allows the others a better access to hydrolyze their respective substrates and this explains the efficacy of the enzyme cocktail relative to any one enzyme. Using macroalgae, Grala et al. (2012) observed an increase in biogas yield from 33 to 54 L/g VS as a consequence of pretreatment with an enzyme mixture of two cellulases (Celluclast, 1,4-(1,3:1,4)-β-D-glucan 4-glucano-hydrolase; Novozym 188, β-glucosidase) and a hemicellulase.

5 Codigestion

Codigestion is about mixing substrates being fed to an anaerobic digester so that the C/N ratio of the mixture is close to optimal. The performance of anaerobic digestion is strongly determined by the C/N ratio of the substrate. Algal biomass is generally high in nitrogen, with a C/N ratio of around 6 (Yen and Brune 2007). This is much lower than the desired range of 25–32. A high protein content of the biomass may lead to generation of large levels of ammonia and a consequent accumulation of the toxic volatile fatty acids. Therefore, a N-rich biomass requires supplementing with carbon-rich cosubstrates to ensure good conversion to methane.

Although initially the codigestion approach focused on mixing substrates to achieve synergistic interactions during digestion, it is now believed that the methane production is mainly a function of the total organic load rate rather than of synergism of different substrates (Mata-Alvarez et al. 2014). Therefore, a wide range of carbon-rich substrates are suitable for codigestion with the algal biomass.

Table 5 summarizes the results of codigestion of algae with different cosubstrates. In most cases, the methane yield increased significantly (e.g., from 50 to 200 %) (Table 5) as a consequence of codigestion. This was mainly due to the carbon contribution of the cosubstrate which increased the C/N to 20 (Yen and

Table 5 Results of codigestion of algae on biogas production

Algae species	Cosubstrates	C/N ratio	Methane yield or methane production increase	References
Chlorella sp.	Waste activated sludge (89–96 % in mass)	–	73–79 %	Wang et al. (2013)
Mainly *Chlorella* sp.	Primary sludge (75 % on VS basis)	–	220 % (from 90 to 291 L CH_4/kg VS)	Solé et al. (2014)
Mainly *Scenedesmus* sp. and *Chlorella* sp.	Waste paper (50 % on VS basis)	18	Twofold (1170 L CH_4/kg VS)	Yen and Brune (2007)
Scenedesmus sp.	*Opuntia maxima* (75 % on VS basis)	15.6	66.4 % (233.6 L CH_4/kg VS)	Ramos-Suárez et al. (2014)
Cyanobacterium *Spirulina maxima*	Primary sludge (50 % on VS basis)		2.1-fold	Samson and Leudy (1983)
Residual microalgal biomass	Lipid waste (50 % of the organic loading in mass)	–	Up to 540 L CH_4/kg VS	Park and Li (2012)
Macroalgae from lagoons	Sewage sludge (70 %)		Same methane yield as 100 % sludge	Cecchi et al. (1996)
Macroalgae *Ulva* sp., *Graciliaria* sp.	Waste activated sludge (85 % on TS basis)	–	51 % (296 L CH_4/kg VS)	Costa et al. (2012)
Macroalgal sludge	Corn straw	20	61.7 % (325 L CH_4/kg VS)	Zhong et al. (2012)

Brune 2007; Zhong et al. 2012; Ramos-Suárez et al. 2014). Sewage sludge (primary sludge, waste activated sludge, or a mixture of both) has generally been used as the cosubstrate. In wastewater treatment processes, microalgal biomass is produced as a byproduct of the process and mixing this biomass with sewage sludge generated in the same process for use in the anaerobic digester may effectively optimize waste management (Solé et al. 2014).

Other carbon-rich cosubstrates that have been tested are paper waste (Yen and Brune 2007), crop and plant waste (Zhong et al. 2012; Ramos-Suárez et al. 2014) and waste lipids (Park and Li 2012). For example, the addition of paper waste to a mixture of *Scenedesmus* sp. and *Chlorella* sp. nearly doubled methane production from 140 to 230 L CH_4/kg VS (Yen and Brune 2007). Similarly, the methane yield was increased by 8–74 % when microalgal biomass was codigested with different quantities of swine manure (González-Fernández et al. 2011). Codigestion of these substrates allowed operation with higher organic loading rates than would have been possible with either of the substrates singly. In addition to improving the methane yield, codigestion often also improves the kinetics of the anaerobic digestion process (Costa et al. 2012; Ramos-Suárez et al. 2014; Solé et al. 2014).

Codigestion of the microalgae biomass residue of a lipid extraction process, with lipid-rich waste (fat, oil, and grease) increased the methane yield to 540 L CH_4/kg VS compared to a yield of 150 L CH_4/kg VS when the algal residual biomass was digested alone (Park and Li 2012). Similarly, codigestion of the oil-extracted *Chlorella* biomass residue with waste glycerol from a biodiesel production process resulted in the methane yield increasing by 4–7 % (Ehimen and Connaughton 2009). It has been pointed out that some solvents used in extracting the oil from the algal biomass can adversely affect subsequent methane production by anaerobic digestion (Ehimen and Connaughton 2009). Chloroform is one such solvent.

6 Economic and Environmental Aspects

A consideration of economics and environmental impact of anaerobic digestion is essential prior to commercial implementation. There is a general lack of information in these areas, although theoretical assessments exist. According to Benemann et al. (2012), oil production from microalgae coupled with anaerobic digestion of the residual biomass eliminates the need for fossil energy input and does not result in net emissions of greenhouse gases. With regards to macroalgae, the sustainability of seaweed (*Gracilaria chilensis* and *Macrocystis pyrifera*) production as a source of biogas depends on the cultivation and the subsequent processing methods used (Aitken et al. 2014). A similar conclusion was reached by Alvarado-Morales et al. (2013).

Delrue et al. (2012) assessed production of biodiesel from microalgae in combination with the use of the residual biomass for anaerobic digestion. Methane produced from residual biomass accounted for 33 % of the total energy output of the overall (biodiesel and biogas) process.

7 Conclusions

Anaerobic digestion of algae is certainly promising, but requires pretreatment of the biomass and/or codigestion with carbon-rich cosubstrates. In the absence of pretreatment, methane yield is reduced apparently because of the recalcitrance of the algal cell wall. Encouraging pretreatments and codigestion approaches have been developed but require validation at pilot-scale. Improved estimates of the energy demands of the various pretreatments are required to decide if a certain pretreatment is energetically worthwhile undertaking.

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Food and Feed Applications of Algae

Michael A. Packer, Graham C. Harris and Serean L. Adams

Abstract Microalgae and seaweeds have a long history and increasingly important applications as both food ingredients and animal feed. The vast majority of algal species have yet to be evaluated for these applications. However, due to their extensive diversity, it is likely that they will lead to the discovery of many new algal products and processes in the future. This chapter covers algae as food, feed, nutraceuticals, functional food and food ingredients as well as production systems for food from algae.

Keywords Microalgae · Aquaculture · Shellfish · Functional food · Feed

1 Introduction

Algae, including microalgae and seaweeds, have a long history and increasingly important applications as both food ingredients and animal feed. This is well recognized in some countries such as Japan where today, seaweed forms a significant part of many meals (especially as wakame, nori, kombu and hijiki) and accounts for as much as 10 % of the overall nutritional intake (Mouritsen 2013). Japan also observes an annual Seaweed Day (February 6), which commemorates the day in 701 AD that the emperor of the time began to accept seaweed as payment of tax, recognizing its historical and cultural importance. Algae have also been used historically as food in several Asian countries, such as China, Indonesia, the Philippines, North Korea, South Korea and Malaysia (Fig. 1). In China, approximately 4.2 million tonnes of kelp, *Laminaria japonica*, is now cultivated annually, mostly for food (Lüning and Pang 2003). In many countries, there are detailed regulations for harvesting seaweed, highlighting the economic importance of algae as a crop (Chopin and Neish 2014; Mouritsen 2013).

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Fig. 1 *Porphyra* production on the Fujian's mudflats in China. The mudflats also produce oysters in addition to this edible algae, which is used to make nori (seaweed sheets). China is one of the world's biggest producers of seaweed—with seaweed farming being a vital component of China's fishing sector. The algae are farmed in a very similar way on a large scale in Japan where it is used mainly for making sushi. Credit: Wong Chi Keung (Hong Kong)

As most of the world's arable land is already in use or otherwise unavailable, the demand for algae and its importance as a food source is likely to increase markedly over the coming decades to help fulfil the world's food production requirements for projected population growth. Approximately 70 % of the earth's surface is oceanic, representing a relatively untapped resource of potential space to farm seaweed, not only as edible food, food ingredients and animal feed but also for other raw materials such constituents of cosmetics, agrochemicals, biomaterials and as renewable bioenergy feedstocks. The vast majority of algal species have yet to be evaluated for these applications. However, due to their extensive diversity, it is likely that they will lead to the discovery of many new algal products and processes in the future (Chopin and Neish 2014).

2 Algae and Their Constituents

Algae have many characteristics that distinguish them from terrestrial crops. In addition to the information supplied in Chap. 1, this chapter emphasizes the food and feed focus which follows; the simplest description is that algae are aquatic plants. However, this is not entirely accurate, as some microscopic varieties show

microbial versatility and can opportunistically live in damp terrestrial environments (e.g., damp soil, mountainous areas and ice-fields). Algae are found in a range of salinities from freshwater to marine and some grow optimally at intermediate saline levels. There are some 350,000 species of algae but only a few species are currently domesticated. The unexploited potential for use as food or in feeds is therefore large. The composition of different types of microalgae and seaweed reflect their different uses as food, food ingredients and feeds.

The term microalgae strictly refer to eukaryotic photosynthetic microorganisms. Eukaryotes are cells that have a high degree of internal organization, including a membrane-bound nucleus (containing genetic material) and several other internal organelles that are also surrounded by membranes. There are many different types of microalgae including dinoflagellates, green algae (Chlorophyta or Chlorophyceae), golden algae (Chryosophyceae) and diatoms (Bacillariophyceae). Most species of microalgae are unicellular and can be motile or non-motile depending on the presence of flagella. Where multicellular or colonial conglomerations exist, very little specialization of cell types occurs, which distinguishes them from seaweeds.

There are about 8000 species of green algae, including micro- and macroscopic varieties. Like most other groups, they contain complex long-chain sugars (polysaccharides) in their cell walls as opposed to cellulose, hemicellulose and lignin which are found in the cell walls of terrestrial plants. These carbohydrate cell walls account for a large proportion of the carbon contained in algae, though many species also contain high levels of various lipids (lipids are fats or oils—the term 'natural oil' distinguishes these oils produced by biochemical processes from petroleum oils that are produced through fossilization). In some species, under certain conditions, the level of lipids may be as high as 80 % by wet weight (Chap. 6) (Chisti 2007; Miller et al. 2009).

Diatoms, which are mostly unicellular marine species, can also accumulate very high levels of lipids. As well as the common triacylglycerol lipids (TAGs), diatoms also store energy in phospholipids and chrysolaminarin, a β-(1-3)-linked glucan molecule. There are estimated to be around 100,000 species of diatoms, which dominate the marine phytoplankton and in contrast to other algal species, have a silicate cell wall.

Often the term microalgae is also used to include cyanobacteria (blue-green algae), which are prokaryotes (cells that lack a distinct nucleus). Some species of cyanobacteria have important food and feed applications (e.g., Spirulina (*Arthrospira* spp.) and *Aphanizomenon flos-aquae*). There are also many species of photosynthetic bacteria, such as purple-sulphur bacteria and green-sulphur bacteria, that are not normally included in the general term microalgae and as currently none of these are used as food or in feed, they are not discussed further here.

Seaweeds (macroalgae or macrophytes), are multicellular algal plants that usually have some specialization of cells into tissues. The degree of specialization, although higher than that of colonial microalgae, is much less than for terrestrial vascular plants and it is partly because of this that the productivity of some species is very high, generally much higher than terrestrial crops (Sect. 4). Around 200 species are used worldwide as food or food ingredients (Carlsson et al. 2007).

Seaweeds are divided into three broad groups, based on secondary photosynthetic pigments contained within them and are referred to as the green (Chlorophyta), red (Rhodophyta) and brown (Phaeophyta) seaweeds. These are not strictly taxonomic groups but are convenient conventions.

Green seaweeds, like their microalgal relatives have a diverse range of polysaccharides in their cell walls. Red seaweeds contain non-fibrillar and sulphated polysaccharides in their cell walls such as carrageenans, agars and complex sulphated galactans. These polysaccharides are well known for their gelling and thickening properties and are used as ingredients in many food products. The main species containing carrageenans include *Chondrus crispus*, *Gigartina* spp., *Eucheuma* spp. and *Hypnea* spp. Brown seaweeds are rich in other sulphated polysaccharides (Chattopadhyay et al. 2008, 2010), such as laminaran, alginic acid and fucoidan, which have been proven to show a wide range of biological activities important to human health (Sect. 7.1) (Cumashi et al. 2007; Teas et al. 2013).

About 200 species of seaweeds used worldwide as food or food ingredients, but only a few are intensively cultivated on a large scale including, the red seaweeds *Porphyra*, *Eucheuma*, *Kappaphycus* and *Gracilaria*, and the brown seaweeds *Laminaria japonica* and *Undaria pinnatifida* (Lüning and Pang 2003). Microalgal species that are commercially produced include *Chlorella* spp., *Dunaliella spp.*, *Haematococcus* spp., *Phaeodactylum* spp., *Schizochytrium* spp. and the cyanbacteria Spirulina (*Arthrospira* spp.) and *Aphanizomenon flos-aquae* (Chisti 2007; Guccione et al. 2014). Microalgae species important for feed applications are described in Sect. 6.

3 What Drives the Interest in Algae as Food, Food Ingredients and Animal Feed?

The use of algae as food ingredients and animal feed can be traced back several hundred years in some countries. More recently, the interest in algae for food and feed products has been sparked by the appeal of producing algae as renewable energy (such as for biodiesel) in response to global economic drivers such as the fossil fuel shortages in the late 1970s and the mid-1990s. The nascent peak after 2000 was blunted by the onset of the global financial crisis. Over the last decade, many surviving enterprises are exploring higher value products from algae including biotechnology products and foodstuffs or food ingredients. This has allowed momentum in the field to continue to the point where both food and fuel crops are seen as having large-scale commercial development potential.

Algae have several characteristics that distinguish them from terrestrial crops as sources of food or feed. Microalgae often combine the flexible metabolic repertoire of microorganisms with higher level eukaryotic sophistication, such as post-translational modification and partitioning ‘products’ within or exporting them out of the cell. This results in their actual and potential utility as self-replicating biochemical processing factories with high efficiency (Briggs 2004; Packer 2009).

The high productivity of microalgae compared with terrestrial crops is a feature shared with many seaweeds; this is further discussed in Sect. 4.

In addition, other characteristics of algae, or attributes of their use that are desirable for commodity-scale production include (Benemann 1997; Chopin 2012; Mouritsen 2013; Pulz and Gross 2004; Spolaore et al. 2006):

- Simple growth requirements; including nutrients—a nitrogen and phosphate source; trace metals; water; CO_2 and sunlight.
- High efficient productivity with comparatively low water use (Brown and Zeiler 1993; Chelf et al. 1994). Note that leaching from farmland, soil erosion and sewage contribute eutrophying phosphates, nitrates and other limiting nutrients to rivers, lakes and the sea where algae consume them thus enhancing growth in these areas.
- It is easy to provide optimal nutrient levels in a well-mixed aqueous environment of algal growth medium as compared with complex matrices such as soil.
- Absence of non-photosynthetic supporting structures (roots, stems, fruit): Single-celled and colonial microalgae are self-contained, meaning they do not have to spend energy moving storage molecules like starch around between tissues, almost the entire biomass is productive and no other tissues have to be supported. Seaweeds too are predominantly all productive tissues compared with terrestrial crops that require extensive above and below-ground structures such as roots and stems to support their productive components (leaves). Algae have few leaf-like structures, and are relatively efficient producing little inedible matter.
- Ability to be grown continuously: Although many species of microalgae undergo sexual cycles under certain conditions, most often they reproduce vegetatively through simple cell fission. Continuous harvest rates can be adjusted to maintain optimal culture density. This is especially so with continuous culture systems such as raceway ponds and bioreactors, where harvesting efforts can be modulated to match productivity. Continuous production also enables constant processing, unlike terrestrial crops that have seasonal cycles of planting, growing, harvesting and processing.
- Ease of moving and handling and automating harvest. One can simply pump microalgae whereas the handling of terrestrial crops is more difficult often requiring expensive crop specific equipment. These characteristics result in the industrial suitability of microalgal crops for a variety of uses, particularly for food and feed products (Chap. 4).
- Suitability as an aquaculture feed. Algal culture may support more valuable organisms like shellfish and fish. This can be achieved directly (lab cultures) or in-direct (bulk culture or natural blooms), e.g. in integrated multi-trophic aquaculture (IMTA), algae form part of the diet of cultured fish and/or shellfish, by absorbing and growing from animal waste products. The result is less expensive fish and shellfish, with less pollutive waste from farming them intensively as monocultures (Sect. 4).
- Algae can grow in environments like non-arable land, lakes and offshore oceanic waters that are unsuitable for other crops.

- The high reproduction rate of microalgae allows research and development to proceeds several orders of magnitude faster than with terrestrial crops. Furthermore, there is substantial evidence that the results of small-scale, cost-effective experiments can be translated effectively to commodity scale for food production.
- Learning and leveraging microalgal research and development from other fields including wastewater treatment and aquaculture.
- There is a niche societal aspect of food production from algae. Traditional farming and harvesting of microalgae and seaweeds has benefited communities and specific groups within those communities such as the coastal fisher women described in (Periyasamy et al. 2014).

Chopin coins the term "aquanomy" (in Greek, "the laws of the aquatic fields"), and suggests that seaweed production can be an ecosystem-responsible aquaculture. Seaweed farming has been identified as a "responsible aquaculture practice" by the UN Food and Agriculture Organization (FAO), providing high levels of employment with very low environmental impact (FAO 2012). The same can be applied to the production of microalgae. In the future, as large amounts of algae are grown as crops so must our understanding of how to efficiently grow them and manage this new farming activity. This includes its environmental, economic and societal impacts (Chopin 2012). Policies, scale-up trajectories and technological advances will be important for the future of the growing world population (Chopin and Neish 2014).

4 Production of Food from Microalgae and Seaweeds

As described in Chaps. 1, 2, 3 and 4, algae can be grown in a variety of different ways each with various trade-offs and suitability as fit for purpose (Fig. 2). In early 2000, Pulz and Gross indicated the largest closed commercial system for microalgal culture was a 700 m^3 tubular photobioreactor operated by Roquette Klötze GmbH & Co. KG (Klötze, Germany), producing about 100 tonnes of high-quality *Chlorella* biomass annually for the health food market (Pulz and Gross 2004).

In addition to microalgal culture in dedicated raceways and closed photobioreactor systems, open ocean systems, especially for seaweeds, are also important. These systems have developed from traditional wild harvest of seaweed, via family or craft-scale seaweed farming (Hurtado and Agbayani 2002). Several red seaweeds such as *Eucheuma* sp. are grown in tropical regions, and *Porphyra* sp. in temperate shallow sheltered sea areas (Fig. 3). These favourable natural locations support development of craft-level industries with simple equipment. The nori industry has grown to be approximately one third of the total seaweed market by value, extending well past its traditional craft basis. This has notably occurred via gaining an understanding in the 1940s of the complex life cycle of *Porphyra* sp., a genus that grows in cold shallow sea water, and undergoes an alternation of generations

Fig. 2 Raceways ponds for microalgal food production. Raceways are a pond style for suitable for microalgal growth that optimizes light utilization in a given unit area, in a cost-effective manner. Open ponds are managed for continuous production of microalgae. The technology has been developed over the last 40 years resulting in a well-understood industrialized production method for many species. The commodity-scale is demonstrated by the size of the figure walking between raceways in the centre of the image. Credit Cyanotech Corporation, Hawaii, USA

Fig. 3 Seaweed farming ropes for *Eucheuma* sp. in Bantaeng South Sulawesi Indonesia. Systems for growing seaweeds have developed from family or craft-scale practices and subsistence farming to a wide-spread industry. Over 95 % of world seaweed supply now comes from aquaculture sources rather than wild harvest. Credit Saipul Rapi, PT Mars Symbioscience Indonesia on the seaweed farm owned by Daeng Karim

with microscopic and macrophyte life stages (Drew 1949). Though cultivated since the seventeenth century, this new scientific understanding has allowed the industry to manage all aspects of production and move from a subsistence farming practice to an investable industry (Fig. 1). The importance of seaweed farming as opposed to wild harvest is demonstrated by the fact that 95.5 % of world seaweed supply now comes from aquaculture sources (FAO 2012). Since 2004, seaweed has constituted the largest group of organisms cultured at sea, representing 50.9 % of the total world mariculture production by mass (Chopin 2013).

Where conditions are favourable, microalgae too can be farmed inexpensively. For example, Spirulina (*Arthrospira* spp.) is grown in a relatively hands-off manner in natural soda lakes in Africa (it thrives at high pH) and there are several large ponds (~50 ha total area) in Australia for mass culture of this cyanobacterium. These are essentially very shallow (~20 cm deep) artificial lagoons constructed either on the bed of a hypersaline coastal lagoon, or formed by artificially expanding a lagoon (Belay 1997). The green microalga *Dunaliella salina* is often a by-product of large-scale salt producing evaporative ponds and is used as a health food and to produce β-carotene extract (Beuzenberg et al. 2014; Borowitzka and Borowitzka 1990; Oren 2005). To cultivate algae other than in these precious natural localities requires considerably more investment. This is currently only economic for higher value products, but costs are decreasing rapidly as new technologies develop.

Another possibility is the development of intermediate-scale freshwater microalgal production as an integrated waste water treatment system in sheltered marine bays and estuaries, but these, such as the OMEGA project of floating photobioreactors (Trent 2012) are unlikely to be used for food and feed production in the near or intermediate term because of the potential for contamination (Sect. 8 and Chap. 13).

Integrated food-waste systems have gained acceptance in India and throughout South East Asia. In the simplest of these systems, vegetarian fish such as tilapia are grown in ponds where algal growth is encouraged with nutrients derived from terrestrial animal or human waste with various levels of prior treatment. Such traditional systems have developed into the modern concept of IMTA which can be either marine or freshwater and can involve microalgae, seaweeds and animal species such as molluscs in addition to finfish (Holdt and Edwards 2014). In an increasingly resource-constrained world and with good understanding of contamination control, acceptability for this kind of process is likely to grow. Some aquaponics systems, a term used to describe the freshwater variation of IMTA (Chopin and Neish 2014), rely entirely on algae within the system to capture energy as sunlight using this to fix carbon into biomass and are thus not dependent on external food sources or waste utilization. Here, the animals in the system, consume the algae or seaweed. Bacteria present convert their nitrogenous waste to nitrate, along with other waste such as carbon dioxide, for use by the algae. Therefore, an efficiency is gained with greater quantities of algae, fish and/or shellfish produced.

As mentioned previously, an attractive feature of algae as food crops is their comparatively simple structure and biochemical efficiency which results in greater

productivity than that of the terrestrial crops. There is a wealth of information on the productivity of microalgae in the literature (Banerjee et al. 2002; Benemann 2008; Carlsson et al. 2007; Chisti 2007; Huntley and Redalje 2007; Murakami and Ikenouchi 1997; Sheehan et al. 1998; Usui and Masahiro 1997; Weissman and Goebel 1987).

Generally, biological systems double in activity with a 10 °C rise in temperature, up to different limits for individual organisms/species. Microalgae are a broad group with varied requirements and limits. Some species can cope with temperatures approaching 40 °C. Thermophilic species thrive at even higher temperatures with some able to grow at temperatures approaching boiling water. Likewise, there are cold water adapted (cryophilic) species that can be equally productive at very low temperatures. Most of the species suitable for food and feed are grown at temperate through to tropical temperatures.

The photosynthetic efficiency of algae results in approximately 9–12 % of incident solar energy being converted to biomass. This is about 25 % of photosynthetically active radiation (PAR) (the band of wavelengths of visible light that can be utilized by plants) which is about 45 % of total full spectrum of sunlight (Carlsson et al. 2007; Melis 2009; Stephens et al. 2010).

Under optimal laboratory conditions, many microalgal species can double their biomass in a few hours. Maximum biomass productivity occurs under nutrient-sufficient conditions including the availability of light. Actual growth rates vary greatly depending on the growth method employed. Year round productivity in large-scale open ponds is generally accepted to be somewhere between 25 and 300 t ha^{-1} $year^{-1}$ of biomass (6.85–82.2 g m^{-2} day^{-1}) (Benemann and Oswald 1996; Heubeck and Craggs 2007; Ratledge and Cohen 2008).

For seaweeds, there is more variation in productivity data and less data available. This variation reflects not only different methods and units of reporting growth but also the variation in the ways it is being farmed, which differ in intensity. It is also important to consider that the growth and environmental conditions can significantly alter algal composition and performance of the crop as a feedstock (Chynoweth 1987).

Kappaphycus sp., (or *Eucheuma cottonii*, there has been some confusion over its taxonomy due to morphological plasticity), may be the fastest growing seaweed with values of 173 dry weight t ha^{-1} $year^{-1}$ reported (Hurtado and Agbayani 2002; Zuccarello et al. 2006) (Fig. 4). It is a red seaweed farmed for carrageenan as food additive. The brown seaweed *Macrocystis pyrifera*, a kelp, has been recorded growing at 15 g $frond^{-1}$ day^{-1} (wet weight) in California (Zimmerman and Robertson 1985) and at 1.3 kg carbon m^{-1} $year^{-1}$ in Canada (Wheeler and Druehl 1986). This equates to about 7 t ha^{-1} $year^{-1}$ if one assumes a value 2 times the amount of carbon needed per unit of biomass (values between 1.8 and 2.88 are commonly used for various algae). For *Gracilaria* spp., a red seaweed grown for relatively high value ingredients including carrageenan, productivity has been estimated at 10–12 t ha^{-1} $year^{-1}$ (Carlsson et al. 2007; Taw 1993) a value that is comparable with the 12 t ha^{-1} $year^{-1}$ for the same species farmed in experimental farming trials in the Philippines, where each plant was measured growing between

Fig. 4 *Eucheuma* sp. attachment on seaweed farming ropes. Also commonly referred to as *Kappaphycus sp.*, this is one of the fastest growing species of seaweed and is grown on a large scale for food products in many tropical and temperate parts of the world. Credit Southeast Asian Fisheries Development Center Aquaculture Department, Creative commons license 2.0, via Wikimedia Commons

6.0 and 11.2 % per day (Taw 1993). The carrageenan industry is large, with the Philippines having a capacity to process 120,000 tonnes of raw seaweed a year. Most producers are small and the economics are marginal for the vast majority of them. The industry structure is one of monopsony, where there are few food ingredient manufacturers buying from multiple tiers of aggregators, meaning that the producers may have little leverage.

The current annual global seaweed harvest has been estimated at between 12 and 19 million tonnes (Chopin 2013; Cleland et al. 1990; Mouritsen 2013) with an estimated value of US$5–7 billion (Chopin and Neish 2014; FAO 2009). Of this, approximately 80 % is used for human food (Mouritsen 2013). A large proportion of this is, around US$2 billion, is for a single genus, *Porphyra* that is farmed as nori, mostly for sushi.

The costs of production of algae for food are given in Table 1. The costs are dominated by harvesting expenditure, especially for microalgae (Pulz and Gross 2004). Some of the developing technologies for this are incompatible with food uses of the harvested microalgae (Chap. 5). Regardless, microalgal production for food is commercially viable. Martek Biosciences, Maryland USA, produces algae

Table 1 Example costs of production of algae

Organism	Purpose	Cost range/kg	Basis	Date	Reference
Seaweed	Biomass	$0.28–0.73[a]	Estimate	1987	Chynoweth (1987)
Seaweed	Food	$12–20	Wholesale price	2009	Irish Sea Fisheries Board Seaweed
Microalgae	Biomass	$30–70	Estimate	2007	Carlsson et al. (2007)
Microalgae	Biomass	$5–7.30	Actual	2011	Norsker et al. (2011)
Microalgae	Supplement	$210	Actual	2013	Nguyen (2013)

[a]US$

oil for inclusion in infant formula via a heterotrophic fermentation of sugars, which is a US$200 million per year business. β-Carotene from *Dunaliella* spp. in the health food market sells for approximately US$4000 per kilogram with a total sale of US$100 million per year in Japan & Far East markets.

5 Algae as Food

Algae already provide food and food ingredients for human consumption at both the top and bottom ends of the market. Few seaweeds are inedible; *Desmarestia* species can contain vacuoles of sulfuric acid with a pH as low as 0.44, making it inedible, but this is unusual. As mentioned previously, in many Asian countries algal products form a significant part of the overall nutritional intake and have done so historically. *Chondrus crispus*, commonly called Irish moss or carrageen moss ('carraigeen' is gaelic for 'moss of the rock' and 'carraigín' means 'little rock'), is a relatively small species of red seaweed which grows abundantly along the rocky parts of the Atlantic coast of Europe and North America. It was most famously used by the Irish during the famine of the nineteenth century. *Caulerpa racemosa*, commonly called sea grapes are found in many shallow sea areas throughout the world and are consumed as part of traditional diet in Pacific Island nations including Fiji, Samoa and Tonga (Morris et al. 2014). In contrast with seaweeds, the difficulty of gathering or growing microalgae makes them an unlikely subsistence food.

Algae have food qualities that are otherwise hard to achieve, and also a potential for commodity-scale production that is almost impossible to realize with terrestrial crops.

5.1 Algal Nutritional Content

There is a multitude of the literature on the nutritional qualities of many microalgae species (Doucha et al. 2009; Fleurence 1999; Garcia-Malea et al. 2005; Pulz and

Gross 2004; Spolaore et al. 2006) and a good deal of quality information on seaweed species such as *Macrocystis pyrifera*, *Durvillaea antarctica*, *Kappaphycus* sp., *Pyropia columbina*, *Caulerpa* sp. and *Callophyllis* (Astorga-Espana and Mansilla 2014; Fleurence 1999; Nagappan and Vairappan 2014; Sjamsiah et al. 2014). The carbohydrates in seaweeds are not readily digestible, but their protein and fat content can provide energy. Despite the diversity there are some themes that can be summarized;

Lipids Algae can be considered masters of lipid metabolism. As mentioned previously, many microalgal species contain high levels of various lipids and this has driven recent interest in them as sources for renewable energy feedstocks for biodiesel and other biofuels (Chaps. 6 and 7), however, many of the fatty acids and lipids synthesized in algae are important for human health (Farzaneh-Far et al. 2010; Wen and Chen 2001a, b). Fatty acids are carboxylic acids with 4–28 carbon atoms in a straight (aliphatic) chain and although humans and other mammals use various biosynthetic pathways to both break down and synthesize lipids, most vertebrates do not possess enzymes to form double bonds at the n-3 and n-6 positions of the fatty acid carbon chain. Therefore, humans must obtain the essential fatty acids linoleic acid (C18:2n-6) and alpha linolenic acid (ALA, C18:3n-3) from dietary sources. Alpha linolenic acid can be extended to eicosapentaenoic acid (EPA C20:5n-3) and docosahexaenoic acid (DHA C22:6n-3) through elongation and desaturation (Kromhout et al. 2012). Most seaweeds, in contrast to most microalgae, contain relatively little total lipid, approximately 1–2 % in dulse (the red seaweed *Palmaria palmata*) and kombu (the brown kelp *Laminaria* spp.) and about 4–5 % in wakame (the brown seaweed *Undaria pinnatifida*). Often a high proportion of those, however, are health-giving polyunsaturated fatty acids (Mouritsen 2013). There is incomplete understanding of the roles of fatty acids in the diet, but it seems likely that there needs to be a balance between the dietary amounts of omega-6 and omega-3 fatty acids. The ratio of beneficial omega-3 fatty acids like EPA and DHA to omega-6 fatty acids is thought to provide health benefits for a variety of human conditions, and the ratio in microalgae and seaweeds is very favourable compared to terrestrial foods. Together with the low overall total fat this makes seaweeds healthy as a food, and microalgae as high producers of 'good oils' for food ingredients and supplements.

Algal-derived n−3 PUFAs, EPA and DHA, possess a range of anti-inflammatory bioactivity and have the potential to be added to foods as functional ingredients (Sect. 7) (Burr 2000; Kromhout et al. 2012; Yates et al. 2014). Currently, most EPA and DHA for human and animal consumption are fish or krill oil derived in unsustainable practices. Wild stocks of the fish supplying these healthy oils are rapidly declining meaning that algae will become increasingly important as a source.

As with other nutrients, the exact levels vary depending on how the algae is grown and, therefore, for wild harvested seaweed, the time of year and the place where the crops is grown can have an influence (Martineau et al. 2013; Miller et al. 2012; Mouritsen 2013; Nalder et al. 2015).

Proteins and amino acids Some algae have a high proportion of proteins and peptides as compared to other basic macromolecules. Many species, again depending on the exact conditions of growth, often include all of the amino acids that are essential to humans or domesticated animals. The protein content does vary considerably, reflecting nitrate levels in the culture water/media and the season in which they are harvested (Beattie and Beattie 2014). Amino acids are amine (containing nitrogen) and carboxylic acid containing small organic molecules that are the building blocks of peptides and proteins. Nine of the 21 amino acids most commonly used in higher organisms are classified as essential amino acids (EAAs) to humans because they cannot be synthesized from other amino acids. They must therefore be consumed in the diet. The ratio of amino acids varies widely between species and marine algae can be relatively low in the sulphur-containing amino acids, leucine and lysine. Seaweed protein is a source of all amino acids and is an especially good source of glycine, alanine, arginine, proline, glutamic, and aspartic acids. Almost a half of total amino acids in algae are EEAs and their protein profile is close to that of egg protein (Černá 2011).

Polysaccharides Being plants, algae have complex carbohydrate cell walls. Algal cell wall polysaccharides are more diverse than terrestrial plants. These long-chain sugar molecules are difficult to break down and therefore also to digest. As a result, they have little nutritional value. This means that they are low in food calories but still curb hunger. It is thought that some of these polysaccharides may have prebiotic effects (potentiating gut bacteria as opposed to probiotic which is the inoculation or seeding of gut bacteria), and may bind with cholesterol, causing it to be excreted. The polysaccharides contribute to other characteristics of food products, such as texture and many are bioactive—those that have a function, useful or otherwise, over and above being strictly nutritional (Sect. 5.3 and Chap. 14).

Minerals Marine algae in particular often contain a wide range of minerals (magnesium, selenium, chromium, zinc), some which may be in short supply in places ashore (Mouritsen 2013; Sugimura et al. 1976; Thomson et al. 1996). There is often as much potassium as sodium, which may help balance the higher sodium content of terrestrial food to a ratio that is better for human health. Some minerals may be excessively concentrated by algae. Iodine is often found in algae at levels greater than recommended for human consumption causing thyroid problems in regular consumers (Phaneuf et al. 1999). Caesium is also concentrated in seaweeds, which is of concern only when the water is contaminated with radioactive caesium.

Vitamins Some algal species are good sources of vitamins A, C, E, and some of the B-vitamins—thiamine, B_2 (riboflavin), B_3 (nicotinamide), B_6 (pyridoxine) and B_9 (folic acid).

Pigments Algae produce a range of photosynthetic pigments. Carotenoids are common in algae and cannot be synthesized by humans but are effective antioxidants and may have health benefits including bioactivity (Sect. 7).

Nucleic acids Most microalgae species, being single cells, contain high proportions of nucleic acids. This proportion is even greater when one considers prokaryotic species such as the cyanobacteria *Arthrospira* sp. (Spirulina) and *Aphanizomenon flos-aquae*. Nucleic acids are converted by humans into uric acid, which if present at sufficient levels precipitates into limb joints causing gout. This together with the levels of minerals mentioned above, make microalgae and cyanobacteria unsuitable as whole foods, but they are rarely considered in this way, whereas seaweeds have been used as subsistence whole foods.

5.2 Bio-Available Nutrients

Minerals are normally poorly absorbed in their elemental state. In marine algae, however, they are often chelated and thereby better absorbed. Most species have a substantial fraction of their carbohydrate in the form of indigestible fibre (polysaccharides). Dietary fibre is sometimes thought to reduce the absorption of some minerals and vitamins, but that may be true only where purified fibre is consumed (FAO 1998).

5.3 Attractiveness—Building on Nutrition

Wider than palatability—the attractiveness of a food to the eater—is an important contributor to the value of food and feed. It can be broken down to the following characteristics; (1) **Flavour**; Umami is a word used in Japan from the beginning of the twentieth century to mean a pleasant savoury flavour distinguished from the other four basic tastes (sweet, sour, salt and bitter). Umami is found in a wide range of foods, but is particularly associated with some brown algae because of their high content of the salts of the amino acid glutamic acid—up to 3 % dry weight. (2) **Texture**; Algal extracts are very commonly used to improve the texture of food products. Carrageenans, linear sulphated polysaccharides extracted from edible red seaweed, are often used in prepared foods such as yoghurt and ice cream. It has the EU additive E-number E407 or E407a. In the presence of potassium ions carrageenans bind dairy proteins into a firm aqueous gel, which both thickens the product, making it feel more substantial, and helps to prevent the components separating. One example of this is to prevent water drops forming as ice cream melts slightly during transport from supermarkets and then re-freezing as ice crystals in home freezers. There are many other uses of carrageenan. A *Kappaphycus* extract, containing carrageenan can be added to bread dough to increase moisture content, decrease stickiness of the dough and produce acceptably firmer bread (Mamat et al. 2014). (3) **Colour**; Algae have a wide range of colour, which is derived from the combination of chlorophyll and accessory pigments. The combination and therefore resulting colour is mostly attributable to the latitude,

Fig. 5 Nori seaweed sheets from *Porphyra* sp. for Sushi in Japan. A variety of sushi styles, including this Maki Sushi, rely on nori to provide not only nutrition but also texture and structure aiding presentation. Credit Janet Hudson, Creative commons license 2.0, via Wikimedia Commons

depth and turbidity of the water in which they grow. Some seaweeds are also translucent which when combined with their colour can make them very attractive on the plate. *Chondrus crispus* is a very palatable seaweed which is translucent and can be cultured in green, yellow, pink and purple shades, as a premium accompaniment to dishes in the East and West. (4) **Structure and Aesthetics**; *Porphyra* is attractive as food in several cultures. As previously mentioned it provides the nori in sushi but it is also used as gim in Korea and laver on the Atlantic coast of Europe. Nori provides nutritional qualities and a structural support to sushi and its Korean counterpart gimbap, but the aesthetics are a vital part of the dishes' appeal (Fig. 5). Nori provides a fine glossy coat and a well-defined edge, both of which help make sushi suitable as finger food. The production technique turns a naturally variable raw material into a consistent product with acceptable shelf life that is suitable for commercial kitchen techniques.

6 Algae as Feed

Many of the same characteristics of algae for human food apply with regards to use as animal feed. Algae feed production is important for many aquaculture species and is well established, large-scale and industrialized. This is because algae are often naturally in the diet, or a part of the diet, of many of the species farmed and

also because practices have developed from traditional or craft approaches. Many aquaculture species rely on heterotrophic protists, microscopic and near-microscopic organisms such as rotifers and brine shrimp as food which in turn feed on microalgae. For terrestrial animal consumption, the focus is mostly on the ability of algae to supply nutrients in a form that the animal can digest. Algae are rarely a whole food for animals for the same reasons they are not for humans, and are usually a component of a diet created from several sources, which can be balanced by adding components. There are some exceptions though (Sect. 6.3) and algae can also constitute a higher proportion of the diet in animal feed if complete nutrition is met because other factors such as variety and palatability characteristics are reduced to a minor concern (Evans and Critchley 2014).

6.1 Finfish

The most obvious group of animals where algae are used for feed are with various fish species. The IMTA and aquaponics concepts described earlier have evolved from historical practices (Holdt and Edwards 2014). Several fish species widely used in these systems, such as freshwater or brackish tilapia, can feed directly on microalgae growing in, or added to the water. Herbivorous fishes such as the marine butterfish (*Odax pullus* or *Odax cyanoallix*) graze directly on seaweeds (Mouritsen 2013). Such fishes contain a complement of gut enzymes and bacteria to digest a larger proportion of the algal biomass than can other animals, so they can degrade the otherwise indigestible cell wall polysaccharides (Johnson et al. 2012). An advantage of the use of vegetarian fishes versus the carnivorous varieties, which are often used in aquaculture, is that they are a lower trophic level meaning more availability of available energy. This trophic level efficiency arises because there is an energy loss of some 10–25 % at each rise in the trophic level (e.g., primary plant producer to herbivore or herbivore to first carnivore). Thus, overall food conversion ratios tend to be better if vegetarian fishes are farmed rather than carnivorous species (Chopin and Neish 2014).

6.2 Non-finfish Aquatic Species

By far the largest group of animals farmed that depend on algae as feed, is the enormous variety of mollusc species that are used in aquaculture worldwide. These include the New Zealand greenshell™ mussel (GSM, *Perna canaliculus*), the blue mussel (*Mytilus edulis*), Pacific oyster (*Crassostrea gigas*), flat oysters (*Ostrea edulis*, *Ostrea chilensis* and others), geoduck, scallop and various species of clam, abalone and snail. Shellfish hatcheries always have dedicated algal production systems to generate feed, especially microalgae (Fig. 6). The Cawthron Institute's aquaculture park in Nelson, New Zealand, has two independent microalgal

Fig. 6 Microalgae production for aquaculture species feed. The microalgae production facility at the Cawthron Institute in Nelson, New Zealand. Hanging plastic bags are used as photobioreactors for the growth of several monospecies of microalgae, which are used to provide high nutritional quality food for both research use and commercial scale shellfish production. At this facility much of the production is continuous where harvested, algae collects in 200 L white bins (*centre*). Algae from these bins are supplied to juvenile shellfish species, such as oysters and New Zealand Greenshell™ mussel, by a computer-control pneumatic pumping system. Some facilities rely on batch cultures of algae, which are less efficient on an industrial scale. The Cawthron Aquaculture Park also has ~1 ha of outdoor open ponds for mixed-species microalgal production as bulk food for developing shellfish species. Credit Cawthron Institute, Nelson, New Zealand

production systems for feeding juvenile shellfish. A plastic bag-based continuous photobioreactor system is used commonly to produce single-species mono-cultures of algae for this purpose—*Isochrysis* affinis *galbana* (T-Iso), *Pavlova lutheri* (Droop), *Chaetoceros calcitrans*, *Chaetoceros muelleri*, *Skeletonema* sp. and *Tetraselmis suecica*. These microalgal species—suitable because of their nutritional content, size and palatability—are grown to feed the smallest most recently hatched shellfish. After a period of development the shellfish are fed mixed algal cultures that are produced in ~1 ha of onshore marine open ponds that are managed to encourage algal blooms representing bulk food. The Cawthron Institute's facility incorporates both commercial and research work, so the algal production systems are flexible and sophisticated. Fully commercial hatcheries employ a range of different methods for their algal production with varying degrees of sophistication. The biggest variation is whether continuous or batch culture is performed for specific species of microalgae and the style of photobioreactor used. Pond styles and management vary also.

Most shellfish can feed by filtering the microalgae out of the water they grow in. Other species such as abalone and some snails graze on benthic algal mats and seaweeds (Fig. 7). A wide range of seaweeds are used depending on the region,

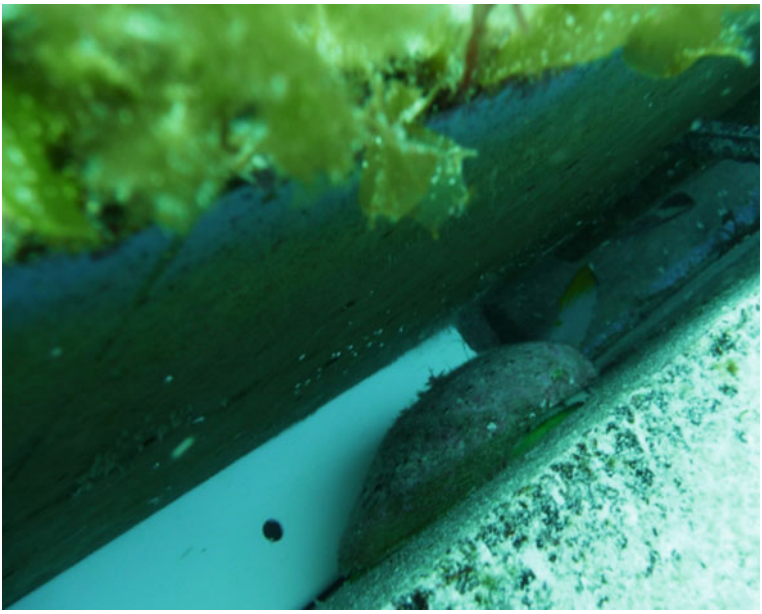

Fig. 7 The green alga *Ulva* sp. used as abalone feed. Many species of seaweeds are either harvested as feed for aquaculture species feed or co-produced in a variety of integrated multitrophic aquaculture systems. Irfan Amas from Curtin University, Australia

season and availability and are rarely grown for this purpose, more often being wild harvested. *Macrocystis* spp, *Ecklonia* spp., *Undariopsis peterseniana* and *Undaria pinnatifida*, *Eisenia arborea* and *Champia parvula*, have all been used for abalone feed in IMTA and commercial mariculture facilities around the world (Bangoura et al. 2014; Hwang et al. 2014; Zertuche-Gonzalez et al. 2014).

Non-mollusc invertebrate aquaculture species include various crustaceans such as crabs and shrimp (Sanchez et al. 2014), and echinoderms (mostly sea cucumbers) that have differing requirements of feed, many, feeding on benthic or planktonic microalgae or seaweeds.

6.3 Terrestrial Livestock

As in the case of human consumption, the high mineral content of marine algae can limit its use for terrestrial animal feed as a whole food. In domestic animals, as little as 10 % seaweed in the diet has been shown to reduce growth (Evans and Critchley 2014). There are some exceptions though. The sheep of North Ronaldsay in the Orkney Islands off Scotland live almost exclusively on a variety of seaweed species as they are confined to graze only at the shoreline for most of the year (NRSF 2014). In parts of Ireland cattle are sometimes taken to graze on wild *Chondrus*

crispus. Sheep and cattle generally find some seaweed palatable as part of their diet and have been shown to self-regulate intake (Beattie and Beattie 2014).

Algae may be valuable not only as a high-protein feed but can also reduce greenhouse gas emissions because of its low structural cellulose content. Cattle fed on algae emit less methane than those fed with terrestrial feeds as it is the rumen gut microflora digestion of cellulan, the polysaccharide predominant in land plants that leads to methane production. In Australia, James Cook University scientists have evaluated the green seaweed *Ulva* spp. as a low greenhouse gas output high-quality feed for cattle (Cawood 2009).

There are many reports of algae being used for other terrestrial livestock including chickens, ducks, horses and pigs (Moore 2001).

7 Algae as Nutraceuticals, Functional Food and Food Ingredients

Nutraceuticals are natural health products (NHPs) including foods and food products which "reasonable clinical evidence has shown to have a medical benefit that its manufacturer cannot claim to the public or the physician under present regulatory policy" (DeFelice 2014). Nutraceuticals are supplements often taken without prescription (Plaza et al. 2008, 2009; Siró et al. 2008). A functional food is "a food that beneficially affects one or more target functions in the body beyond adequate nutritional effects in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease. It is consumed as part of a normal food pattern. It is not a pill, a capsule or any form of dietary supplement" (Plaza et al. 2008, 2009; Siró et al. 2008).

Algae can be well suited to NHP use because they contain constituents that are well known to have health benefits with few if any side-effects, and are uncommon in other foods. These include the Omega-3 fatty acids and some pigments, such as β-carotene and astaxanthin (Sect. 7.1).

Governments around the world have regulated NHPs differently and so there is considerable overlap and variability about what constitutes a functional food and what constitutes a nutraceutical. China and Japan have long traditions of NHPs being consumed, with a resulting regime that is supportive of both suppliers and consumers. The USA regime has no such tradition, and seeks to minimize harm much as pharmaceutical regulation does. These differences in outlook result in regimes with little similarity, so what qualifies in one country may be excluded in another.

- **USA**

NHPs are called dietary supplements under US regulation. They are easiest to market if they consist of substances documented as Generally recognized as safe (GRAS), which is based on evidence that the substance has been part of the human diet over a long term with little or no adverse effect. However, this produces a

dilemma for suppliers—GRAS marks them as ordinary, so how can they be marketed as having particular health functions?

Any claim made on the package is regulated by the Food and Drug Administration (FDA) and must be supported by data; but if data are generated from clinical studies, the FDA is inclined to classify the product as a drug and the burden for proof of efficacy is extremely high and expensive. Any claim that a product can treat a disease likewise makes the product a drug, not a food.

On the other hand, claims made in advertising (not on the package) are regulated by the FTC (Federal Trade Commission), not the FDA. The FDA has the power to pre-empt sale, whereas the FTC can only order the producer to cease and desist, and can be challenged. A product that can avoid the FDA tests can thus have a substantial market lifetime before FTC can successfully intervene.

Under FDA regulations, claims are permitted provided they are expressed as nutrient content, health, qualified health or structure/function claims. The content of a specific nutrient can be listed, and compared with other foods. Health claims may suggest that the product might reduce the risk of a disease, and if the data are incomplete or inconclusive must be qualified by saying so. The Significant Scientific Agreement (SSA) standard puts the burden of proof on the producer to show that the claims are supported by published studies and opinions from qualified professionals. Structure/function claims can propose an effect of the nutrient on specific body structures or functions, such as joints or eyes, if there is supporting evidence, and the claim does not suggest that it can treat a disease. Such constraints on claims make it particularly difficult for producers to distinguish their product from competitors, except by the amount of a specific nutrient they contain.

- **The European Union**

The EU has a category of Foods for Particular Nutritional Use (PARNUTS) regulated between the categories of Food and Medicine (Coppens et al. 2006). Generally, the test is special preparation to meet the "particular nutritional requirements of certain categories of persons whose digestive processes or metabolism are disturbed; or of certain categories of persons who are in a special physiological condition and who are therefore able to obtain special benefit from controlled consumption of certain substances in foodstuffs." The supplier must notify the competent authority of their intent to market as PARNUTS. Within PARNUTS, the category Foods for special medical purposes requires medical supervision. EU member nations vary in how they interpret the regulations. Suppliers' claims for their product will determine whether it is classified as a Food, a Medicine or as a PARNUTS product.

- **Japan**

Japan has a category of Food with Health Claims (FHC), which is legally distinct from either medicine or food. Most functional food is treated simply as food. Food with health claims may rely on generic claims of nutrient content, or may use the regulated category Foods for Specified Health Uses (FOSHU) which comprises of foods officially approved to claim their physiological effects on the human body.

Positive approval of the claim by the Minister of Health, Labour and Welfare (MHLW), as well as evidence of the absence of harm and of quality is required by FOSHU. Evidence must include human efficacy and safety data generated by trials in Japan, perhaps alongside overseas data. A FOSHU claim can be granted with any of levels levels of compliance, depending upon the quality of evidence provided.

- **Australia and New Zealand**

Food Standards Australia New Zealand (FSANZ) has recently brought in a new food standard (Standard 1.2.7) to regulate nutrition content claims and health claims on food labels and in advertisements. Food businesses must comply with the new standard from January 2016. Nutrition content claims and health claims are voluntary statements made by food businesses on labels and in advertising about a food. Nutrition content claims are claims about the content of certain nutrients or substances in a food, such as 'low in fat' or 'a good source of calcium'. These claims need to meet certain criteria set out in the Standard. For example, with a 'good source of calcium' claim, the food will need to contain more than the amount of calcium specified in the Standard.

Health claims refer to a relationship between a food and health rather than a statement of content. For Australia and New Zealand, there are two types of health claims:

1. General level health claims which refer to a nutrient or substance in a food and its effect on a health attribute. For example, 'calcium is good for bones and teeth'. The claims must not refer to a serious disease or to a biomarker of a serious disease.
2. High level health claims which refer to a nutrient or substance in a food and its relationship to a serious disease or to a biomarker of a serious disease. For example, 'diets high in calcium may reduce the risk of osteoporosis in people 65 years and over'. An example of a biomarker health claim is 'phytosterols may reduce blood cholesterol'.

7.1 Bioactivity of Functional Foods

Some of the constituents in algae that make them good food for people and animals can also be extracted and incorporated as functional food ingredients utilizing their bioactivity in other products. Algal extracts are fully described in Chap. 14, but because functional food ingredients are a rapidly growing area, they are worth mentioning here, especially when considering algal-based ingredients as part of food.

Some of the beneficial properties of algae in food are multiple. For instance, carrageenans though used widely as thickening agents in a range of food products also possess bioactivities that have been ascribed to a variety of health benefits (Chattopadhyay et al. 2008, 2010; Fenoradosoa et al. 2009; Mehta et al. 2010;

Nagappan and Vairappan 2014). As mentioned previously carrageenans are sulphated polysaccharides and marine algae are particularly rich in them (Renn 1997). Carrageenans are found especially in red algal species, such as *Chondrus crispus*, *Eucheuma* spp., *Gigartina* spp. and *Hypnea* spp., but other classes of the sulphated polysaccharides such as alginic acid, laminaran and fucoidan are found in all algal groups (Rocha de Souza et al. 2007; Wang et al. 2008). There is a continuum from the consumption of algae as food for nutrients through to the specific bioactive effects of an extracted molecule from algae. NHPs fall within these two extremes and sometimes the bioactivity is due to the complex matrix of the food consumed itself. For example the Pathway 27 project, a pan-European interdisciplinary group of life and social scientists and high technology food processing SMEs, are assessing the role and mechanisms of action of three bioactives (docosahexaenoic acid (DHA), β-glucan and anthocyanins) as fortifying ingredients in widely consumed food matrices (dairy, bakery and egg products). They are aiming to critically "evaluate the bioactive-food matrix interaction" (Bordoni and Ricciardiello 2014).

When algae are used as foods or food ingredients the dose of a particular moiety may not be sufficient for effective bioactivity and this is when extracts from algae play a role. Bioactivity is important to human and animal health and this bioactivity in algal constituents for food products are wide-ranging and include anti-inflammatory, antioxidant antiviral, anti-tumoural, anti-angiogenic, anti-adhesive, antithrombotic and anticoagulant activity (Cumashi et al. 2007) and many of these are potentially beneficial at the low levels ingested in foods incorporating algae.

- **Antioxidant activity**. There are both lipids and polysaccharides from algae with antioxidant activity. Various carotenes are ascribed many health benefits attributed to their antioxidant activity (Lee et al. 2013) (Boussiba and Vonshak 1991; Chou et al. 2010; Lavy et al. 2003; Yang et al. 2013). β-carotene, produced on an industrial scale from halotolerant *Dunaliella* microalgae, is a carotenoid, a relatively small molecular weight lipid with a strong colour. Astaxanthin, another strongly coloured carotenoid that has potent antioxidant activity, is produced commercially from the freshwater green algae *Haematococcus pluvialis* (Boussiba and Vonshak 1991). Astaxanthin has also been shown to be immunomodulatory (Jyonouchi et al. 1991) and a protectant against UV-induced skin damage (Yamashita 2006). These effects are most likely via decreased oxidative stress, bearing in mind the important role of oxidants in inflammation and the immune response (Andersen et al. 2007; Bennedsen et al. 1999; Jyonouchi et al. 1991; Park et al. 2010). The requirement of astaxanthin for healthy growth in finfish, such as for salmon, is an example of dietary intake of effective levels of astaxanthin through consumption of the feed of this important aquaculture species. Another structurally related carotenoid, fucoxanthin that is found in diatoms such as *Nitzchia* spp., *Phaeodactylum tricornutum* (Kim et al. 2012) and other species and the haptophyte *Isochrysis* affinis *galbana* (Kim and Pan 2012) is also a powerful antioxidant. It is thought that it has bioactive effects independent of its antioxidant activity though, such

as for controlling diabetes, obesity and in decreasing dietary cholesterol assimilation (Beppu et al. 2012; Sho et al. 2012) and may have anti-cancer health benefits (Irwandi et al. 2011; Nakazawa et al. 2009). Structurally dissimilar from carotenoids, furan fatty acids (F-acids) are potent free radical scavengers due to an electron-rich furan ring contained within the fatty acid carbon backbone (Spiteller 2005) that have gained a great deal of recent interest. They have been implicated as powerful anti-inflammatory agents (Spiteller 2005; Wakimoto et al. 2011). F-acids also occur in *Isochrysis* sp. and *Phaeodactylum tricornutum* microalgae, but may be in many more species but have not been detected yet because of their low stability.

Fucoidan is another class of sulphated fucan polysacharide that is present especially in brown algae such as the seaweeds *Undaria pinnatifida* (Miyeok), *Laminaria japonica*, *Turbinaria conoides* and *Fucus vesiculosus*, which has been demonstrated to have useful antioxidant effects (Chattopadhyay et al. 2010; Cui et al. 2010).

- **Anti-inflammatory activity**. The lipids n−3 PUFAs, EPA and DHA from algae have a range of anti-inflammatory bioactivity in mammals, which includes reducing eicosanoid, cytokine and adhesion molecule production, enhanced resolving production and decreased leukocyte-EC adhesive interactions (Yates et al. 2014). As described above, in addition or possibly related to, its antioxidant bioactivity, fucoidan also has anti-inflammatory actions (Cui et al. 2010; Cumashi et al. 2007), suppresses pro-inflammatory cytokines (Yoon et al. 2009) and may be useful to prevent neurodegenerative disorders (Kim et al. 2010; Synytsya et al. 2010).

  The consumption of various algae as food and as ingredients in food, likely results in significant antioxidant and anti-inflammatory action. There are other bioactivities ascribed to algae, including antiviral (Damontea et al. 2004), anti-cancer (Synytsya et al. 2010) and antibiotic activities (Manivannan et al. 2011) that may not be realized when consumed as foods because of the levels obtained when dietarily consumed. These bioactive products from algae are more fully described in Chap. 14.

8 Algae Food Contaminants

Countries where algal food consumption has been traditional, notably Japan and China, have experienced concern over marine and freshwater environmental pollution. Both ocean-grown seaweeds and microalgae grown ashore in different ways are subject to contamination of various kinds, which can render them less suitable for consumption as food or feed. Spread of infectious disease, contamination with heavy metals, personal care products and hormones are all problematic.

Contamination issues are greater still when recycling of nutrients is used in production such as in ITMA and aquaponics systems. Modern wastewater treatment solutions are able to render recycled nutrients suitable for use, but at a cost. As demand for algal food production grows this will become increasingly important and necessary.

Biological contamination can also occur when other species grow in the same environment as the crop species. As described in Chaps. 2 and 3, one of the trade-offs of open production systems is the risk of contamination. Target species may be contaminated either with non-target algal species or with predators such as zooplankton that consume the algae. Current systems, processes and growth management regimes are designed to provide selective pressure to maximize target species production and discourage and decrease the risk of biological contamination. For instance, two of the most widely grown domesticated species of microalgae are grown under conditions that suit them but few others. Halotolerant *Dunaliella* spp. thrives in high salt and Spirulina in very alkaline water, restricting the growth of competitors and predators (Fig. 8).

Seaweeds are often contaminated with epiphytes—other algae, growing on the surface of their thalli—or with animals, particularly encrusting types such as

Fig. 8 Large-scale food production from microalgae. Spirulina produced as a whole cell food and nutritional supplement from the cyanobacterium *Arthrospira* sp. at a 90-acre facility in Kona, Hawaii by Cyanotech Corporation. This company also grows the microalgae *Haematococcus pluvialis* microalgae in open raceway ponds to produce nutraceuticals. Credit Cyanotech Corporation, Hawaii, USA

bryophytes and sponges. Depending on the ultimate use of the seaweed this can have a big effect on its value as food as commodity-scale processing to remove encrusting non-target species is rarely cost-effect.

Chemical contamination results from the water in which algae grows is being contaminated with substances that may be harmful in excessive concentration.

Seaweeds concentrate certain chemicals; for example, some kelps may concentrate iodine as much as 100,000 times compared with the surrounding water (Mouritsen 2013; Phaneuf et al. 1999). Hijiki (*Sargassum fusiforme*) concentrates arsenic in its tissues, and is poisonous if it is not boiled in large volumes of seawater. As a result, its sale is prohibited in several countries. Nuclear contamination is now also a concern in Japan and surrounding countries since the Fukushima accident. When the March 2011 tsunami damaged the Fukushima 1 Nuclear Power Plant, radionuclides leaked into the sea. Radioactive caesium was taken up by seaweeds. At sites 50 km from the reactor, 2 months after the accident, radioactivity in *Undaria* sp., used for food as wakame) was about 100,000 times higher than before, and after 2 years was still at least 100 times elevated (Kawai et al. 2014).

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Microalgae Applications in Wastewater Treatment

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Abstract Algal wastewater treatment is effective in the removal of nutrients (C, N and P), coliform bacteria, heavy metals and the reduction of chemical and biological oxygen demand, removal and/or degradation of xenobiotic compounds and other contaminants. Microalgae wastewater treatment technologies have long been in existence; however, uptake of the technology to date has been limited mainly due to considerations of land requirements and volumes of wastewater to be treated. This chapter gives an overview of algal applications in wastewater treatment with specific reference to nutrient removal, phycoremediation of heavy metals, high-rate algal ponds, symbiosis of algae with bacteria for wastewater treatment, and utilisation of wastewater-grown microalgae.

Keywords Microalgae · High-rate algal ponds · Wastewater treatment · Nutrient removal · Heavy metals

1 Introduction: Conventional WW Treatment Plants and Limitations

The conventional activated sludge biological nutrient removal (CAS-BNR) process is globally, one of the most applied biologically driven treatment methods for both industrial and domestic wastewaters. Although there are many variations of BNR configurations, most processes consist of steps such as raw sewage screening, primary treatment, secondary treatment, tertiary treatment, disinfection, and solids handling. In most cases, secondary treatment is achieved by manipulating three types of biochemical reactions (anaerobic, anoxic and aerobic) under which the microorganisms (mostly bacteria) can be favoured to perform the respective nutrient

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(COD, phosphorus and nitrogen) removal. Different aerobic technologies are used to supply oxygen to the microorganisms in conventional treatment processes which are usually quite energy intensive. Following the aeration step, microorganisms are separated from the liquid by sedimentation and the clarified liquid is the secondary effluent. A portion of the biological sludge (microorganisms) is recycled to the aeration basin and the remainder is removed from the process and is normally combined with primary sludge for sludge processing. The secondary effluent is subsequently directed to the disinfection tank(s).

Although conventional treatment technologies are simple and are important for wastewater treatment, they are expensive processes and require high energy input. Moreover, management of these sewage treatment plants in rural areas is also challenging due to the lack of local technical expertise. Most of these microorganisms are very sensitive to changes in operational and environmental conditions and therefore malfunctions occur readily under unfavourable conditions. Loss of nitrification (due to the decrease in activity of nitrifiers), bulking and foaming (due to the excess growth of filamentous bacteria) are some of the challenges that are often faced by the WWTP (Khin and Annachhatre 2004; de-Bashan and Bashan 2004). Moreover, the conventional treatment process generates large amounts of sludge, and handling and disposal of this waste sludge is one of the largest bottlenecks to the technology. The high operational and maintenance requirements of wastewater treatment plants including solid waste material handling make it economically unfeasible. Furthermore, tertiary treatment for total removal of organic ions via chemical treatment is prohibitively expensive and has the potential to generate additional pollution. Biological tertiary treatment of wastewater although effective, costs up to four times that of primary treatment (Abdel-Raouf et al. 2012). Algae-mediated wastewater treatment is a potential solution for treatment of wastewater in peri-urban and rural settings due to the low operational costs and lesser requirement for technical skill in operation of the system. This chapter provides a broad overview of the applicability of algae-mediated wastewater treatment for nutrient removal; algae-based wastewater treatment systems and remediation of heavy metal containing wastewaters.

2 Phycoremediation

Phycoremediation is broadly defined as the utilisation of algae for the removal of contaminants from water. Algal wastewater treatment is effective in the removal of nutrients (C, N and P), coliform bacteria, heavy metals and the reduction of chemical and biological oxygen demand, removal and/or degradation of xenobiotic compounds and other contaminants (Abdel-Raouf et al. 2012; Cai et al. 2013, Rawat et al. 2011; Olguín 2003). It is applicable to various types of wastewater including: human sewage, livestock wastes, agro-industrial wastes, industrial wastes, piggery effluent, food processing waste and other agricultural waste substrates (Abdel-Raouf et al. 2012; Cai et al. 2013). Wastewater treatment using algae

offers several advantages over conventional techniques. These include lesser sludge formation, lower energy requirements, reduction in greenhouse gases, lower costs and concurrent production of energy-rich algal biomass which can be processed for a number of uses including biofuels, bio-fertilizers, biopolymers, bio-plastics, lubricants, paints, dyes and colourants (Batista et al. 2014; Cai et al. 2013).

The production of sludge, as compared to conventional activated sludge wastewater, is reduced as there is no need for the utilisation of flocculants for the removal of P. This indirectly reduces the sludge-handling costs and the requirement of land required for drying. Reduction in greenhouse gases is accomplished by the biological binding of carbon dioxide obtained from organic carbon (which would have been volatilised by bacterial respiration) or carbon dioxide from the atmosphere. Energy demand is lowered as there is no need to oxygenate the reactor (Rawat et al. 2011). Microalgae produce oxygen which is used by heterotrophic bacteria in the system which further reduce nutrient concentrations (Abdel-Raouf et al. 2012). Algal systems are easy to operate and require limited skilled labour (Aziz and Ng 1992). One of the major challenges to the technology is the substantially larger footprint as compared to CAS-BNR (Abdel-Raouf et al. 2012). This is mainly due to the requirement for shallow ponds. Algal treatment systems are favoured over conventional treatment processes for decentralised wastewater treatment due to the significantly lower cost of construction and operation and ease of operation without the requirement for skilled labour.

2.1 Nutrient Removal

Uncontrolled growth of algae in aquatic bodies receiving nutrient rich wastewater streams results in eutrophication and its associated complexities (Conley et al. 2009), which include very low oxygen levels with increased depth in water bodies and the formation of dead zones (Diaz and Rosenberg 2008). Since conventional wastewater treatment technologies are largely designed for organic carbon removal; they are not always efficient in removing other nutrients, thus giving the need for tertiary treatment (Arceivala and Asolekar 2007). The controlled growth of algae in semi-engineered systems such as; oxidation ponds, facultative ponds, or lagoons etc., are effective tertiary treatment strategies for removal of residual nutrients from wastewater and have been practiced globally with varying degrees of success (Brockett 1977; Saqqar and Pescod 1996; Mahapatra et al. 2013). Renewed interest in the production of biofuels from microalgae has resulted in search for cheaper and more readily available cultivation media for growing algae with high productivities. Cultivation of algae on nutrient-rich wastewater presents a unique opportunity to achieve both objectives of nutrient removal and production of algal biomass for producing biofuels or other high-value products (Han et al. 2014; Rawat et al. 2011).

Microalgae are able to take up nutrients from surrounding environments in excess to their immediate requirements and store them within their cell for future utilisation in cell synthesis (Droop 1974). This ability allows microalgae to remove

nutrients at high rates during their exponential growth phase. Droop observed this property of microalgae while working with vitamin B12 and introduced the concept of cell quota to explain such storage of essential nutrients (Droop 1968). Subsequently many researchers have found evidence of luxury uptake and storage of nitrogen and phosphorus by microalgae (Nambiar and Bokil 1981; Powell et al. 2009). It could be postulated that microalgae have developed the ability of storing nutrients in the cell to deal with the variable levels of nutrients in the natural environment (Leadbeater 2006). Earlier research postulated that luxury uptake of nitrogen and phosphorus was a mechanism to maintain the average stoichiometry in the cell as close to Redfield ratio (16N: 1P) as possible (Redfield 1958). Recent literature provides evidence of deviations from this ratio and establishes vast variations in the uptake rates of nitrogen and phosphorus in accordance to their levels in the surrounding environments (Klausmeier et al. 2004). This flexibility in nutrient uptake allows microalgae to effectively tolerate the variability in different wastewaters to a certain level without having detrimental effects on the culture. For example, *Chlorella vulgaris* has been reported to treat wastewater having N/P molar ratios from 1.80 (Travieso et al. 2006) to 2017 (Ryu et al. 2014) effectively. The potential to take up nutrients varies between different algal species (Boelee et al. 2011; Zhang et al. 2008) and also depends on environmental factors such as temperature and light (Richmond 1992). Species belonging to various divisions such as chlorophytes, cyanobacteria, and diatoms have been widely utilised for nutrient removal effectively (Cai et al. 2013). Removal of algae from the system is vital to avoid recycling of nutrients back to the receiving waters.

2.1.1 Nitrogen Removal

The presence of nitrogen in wastewater is due to various anthropogenic activities. Nitrogen is primarily in the form of ammonia, but can also be present in other forms such as nitrate, nitrite, or organic nitrogen (Metcalf & Eddy et al. 1998). High levels of unionised ammonia or nitrate/nitrite are toxic to aquatic life and humans (Bryan et al. 2012; Braissant 2010). In addition, their presence has great potential to lead to eutrophication. Hence, nitrogen removal from wastewater is essential before it can be safely discharged.

Microalgae show a great potential to effectively remove nitrogen as it is an essential macronutrient for their growth. Nitrogen is required for synthesis of peptides, proteins, ribonucleic acid (RNA), and deoxyribonucleic acid (DNA), etc. (Conley et al. 2009; Cai et al. 2013). Microalgae assimilate the inorganic nitrogen present in wastewater, such as ammonium, nitrate and nitrite; and convert them to various organic nitrogen species required for cell synthesis. Microalgae prefer ammonium over other inorganic nitrogen species due to the fact that it can easily be converted to the amino acid glutamine without any redox reaction and thus utilises less cellular energy (Cai et al. 2013; Flynn et al. 1997). Nitrate and nitrite are also assimilated by microalgae depending on their availability in the wastewater, and are reduced to ammonium inside the cell. Such reduction is mediated by various

enzymes and involves many intermediate products during such reduction pathways (Dortch et al. 1984). Pathways usually involve reduction of nitrate to nitrite mediated by an enzyme nitrate reductase, and then nitrite to ammonium by nitrite reductase (Flynn et al. 1997; Cai et al. 2013). In addition to assimilation of nitrogen in the cell, indirect removal in the form of ammonia stripping also occurs due to increased pH with algal cultivation (García et al. 2000).

2.1.2 Phosphorus Removal

The presence of phosphorus in wastewater streams is also predominantly due to human activities, particularly the application of phosphorus fertilizers in agriculture. Phosphorus is mainly present as phosphates, such as orthophosphate, polyphosphate, or organic phosphate. The bio-availability of phosphorus varies with the chemical speciation (Dueñas et al. 2003). The presence of phosphorus in water has potential to lead to eutrophication (Abdel-Raouf et al. 2012). Hence phosphorus removal from wastewater is essential.

Microalgae have the ability to take up phosphorus, mainly orthophosphate as HPO_4^{2-} and $H_2PO_4^{-}$, and utilise it as an essential macro nutrient in the synthesis of various compounds such as nucleic acids, phospholipids, and proteins, etc. via phosphorylation. It is also vital for energy transfer during various metabolic activities within the cell since it forms a primary part of ATPs and ADPs (Conley et al. 2009). Excess amounts of orthophosphate taken up by microalgae during luxury uptake are stored as polyphosphate granules in the cell for future utilisation (Rasoul-Amini et al. 2014). While acid-soluble polyphosphate can be used for production of protein, nucleic acids, and various metabolic activities; acid insoluble polyphosphates are considered as a storage pool for future use when external phosphorus is exhausted or limiting (Powell et al. 2009). Indirect phosphorus removal also occurs due to precipitation of phosphate at high pH often observed with algal cultures (Nurdogan and Oswald 1995).

3 Phycoremediation of Heavy Metals

The metals in the aquatic environments come from both natural as well as anthropogenic sources; however, the release of significant amounts of toxic metals to the aquatic ecosystems due to extended industrial activities such as mining, agriculture, metal plating and paint industries, is of serious concern. Once, the metals are released to the aquatic environment, they remain in the water column for long durations and get deposited to the sediment systems. Therefore, the sediment systems acts as sinks for metals and significant amounts of metals are released to the aqueous medium from the sediment due to prevailing environmental factors. Thus the sediment system itself becomes a secondary source for the metals under certain environmental conditions. The major concern regarding these types of

pollutants is that they get accumulated in the biological systems and aquatic food chain over a period of time and once beyond the thresholds, they have deleterious effects on the aquatic organisms and ultimately to the human beings through aquatic food chains (Gupta and Rastogi 2008; Babu and Gupta 2008).

Conventional methods such as precipitation, flotation, ion exchange, electrochemical and biological processes have long been practiced for metal removal from wastewater; however, most of these methods are often ineffective and/or are energy-intensive, thus too expensive for removal of low-level metal contaminants from water and wastewater. The conventional and physico-chemical treatments for the removal of heavy metals from the metal-contaminated water or sediment matrix is typical, therefore the need of the hour is the economically sound and environmentally sustainable technologies. Microalgal and cyanobacteria-based phycoremediation technologies have gained much attention recently as alternative bioremediation techniques over traditional methods for eco-friendly clean-up of metal-contaminated wastewater, industrial effluents and soil matrix (De Philippis et al. 2011; Sandau et al. 1996).

Of the physico-chemical or electrochemical treatments, phycoremediation through biosorption of toxic metals on live or dead algal biomass has emerged as an eco-friendly technology. Thus, phycoremediation is one of the most prominent technologies which can be used for metal removal/recovery from aqueous phase and eco-friendly disposal. Some of the microalgal and cyanobacterial species which are metal stress tolerant possess high resistivity towards metal toxicity, high surface binding and intracellular uptake due to inherent abilities. Such species, therefore, could serve as suitable and attractive alternatives for phycoremediation purposes (Terry and Stone 2002).

Various species of microalgae and cyanobacteria have been used for metal removal from water. It has been observed that significant proportions of toxic metals can be removed by algae and cyanobacteria. Earlier studies have demonstrated the potential application of cyanobacteria in *in situ* phycoremediation of heavy metals without external input of materials and energies (De Philippis et al. 2011; Doshi et al. 2007). The major proportion of the metals that are removed by algal species are mainly mediated through extracellular adsorption/diffusion or surface binding which is facilitated by the inherent properties of the algal cell walls. The extracellular surface binding of metal ions with the algal cell wall is rapid and better known as passive uptake followed by slow intracellular active uptake (Gupta et al. 2006). The algal cell wall exhibits chemical affinity to the metal ions due to the presence of various functional groups such as carboxyl, carbonyl, amino, amido, hydroxyl, sulfhydryl, sulphonate and phosphorus, etc. which confer negative charge to the cell surface and therefore facilitate surface binding with positively charged metal ions through adsorption, ion exchange, coordination, surface complexation, chelation and microprecipitation, etc. (Fourest and Volesky 1997). However, charge transfer between metal cations and carboxyl groups of algae plays a significant role (Sandau et al. 1996; Gupta and Rastogi 2008).

This is also an important mechanism of algal and cyanobacterial cells to tolerate elevated metal concentrations (De Philippis et al. 2011). The rapid extracellular

adsorption/diffusion of metals is followed by additional slow uptake of metal ions by irreversible surface binding through surface precipitation, covalent bonding, crystallisation on the cell surface or redox reactions (Wilde and Benemann 1993). In some cases, intracellular organelles and cell constituents such as protein and other materials also facilitate the diffusion and binding of metal ions into the algal cells. Past experience advocates that due to efficient membrane binding, the live algal biomass possess comparatively higher metal biosorption potential than the dead biomass. Moreover, the active metabolism also plays significant role in intracellular uptake (Terry and Stone 2002; Doshi et al. 2007). However, some of the studies have also demonstrated that even the dead algal biomass possesses similar or better adsorption capacity (Fourest and Volesky 1997). The passive metal uptake by algae is mainly facilitated by the physico-chemical properties of the surrounding water matrix whereas the intracellular uptake is mediated by cell metabolism (Raungsomboon et al. 2008). Schiewer and Volesky (1995) reported that metal ions bind first to the surface ligands with higher affinity, then to those with lower affinity. Studies of Ke et al. (1994) revealed that for binding of Ag+, pH-independent binding sites displayed a greater affinity than pH-dependent sites.

3.1 Factors Influencing Algal Sequestration of Metallic Ions

The chemical composition and functional groups and therefore ionic charges of algal cell wall vary from species to species; thus, the adsorption capacity also varies from species to species. The metal removal potential of algae is dependent on various physico-chemical factors, mainly pH, salinity and hardness of the water. The ionic charges of metal ions and chemical composition of the aqueous media also play significant roles (Wilde and Benemann 1993; Romero-González et al. 2001). In a study, Fourest and Volesky (1997) reported that the metal binding capacities of the seaweeds are directly proportional to their respective total carboxyl group content and the electromagnetivity of elements. Studies have demonstrated that pH of the medium is one of the important limiting factors in that biosorption of metal ions increases with increasing pH. Studies on surface charges showed that increasing pH increases the number of negative sites which facilitate binding of more metallic cations (Schiewer and Volesky 1995). Ionic strength also plays a significant role in metal adsorption/uptake. Metal removal is inversely proportional to the ionic strength, therefore, it increases with the decrease of ionic strength (Cho et al. 1994; Sandau et al. 1996).

3.2 *Significance of Phycoremediation of Heavy Metals*

The inherent properties of survival on radiant energy and rapid metabolism in the absence of organic carbon makes various algal species seem superior sequesters for metals than any other microorganisms such as bacteria and fungi. Moreover, various algal biomasses are low cost biosorption materials and possess comparatively high adsorption capacity and can be produced with the lowest energy consumption.

4 High-Rate Algal Ponds

The concept of high-rate algal ponds (HRAP) or raceway ponds (RWP) was first proposed by Prof. Oswald in late 1950s and 1960s as an improvement over existing oxidation ponds by providing paddlewheels for efficient mixing of algal biomass in the pond and by reducing the depth to 0.2–1 m (Oswald et al. 1957; Goldman 1979; Arbib et al. 2013). These improved designs provide much better conditions for algal growth and increased productivities in these ponds, which are usually designed as raceways, due to better light utilisation which in turn results in higher efficiencies of wastewater treatment with lower retention times of 4–10 days (García et al. 2000). A properly designed HRAP could efficiently remove nutrients as well as organic carbon from the wastewater. Nutrient removal is achieved directly due to algal growth, and also by indirect effects of such cultivation, namely ammonia stripping and phosphate precipitation due to increase in pH. Both direct and indirect removals are important in ponds. In addition, organic carbon removal is also achieved due to the presence of aerobic bacteria having symbiotic relationship with microalgae (García et al. 2000). Mixing is provided by paddle wheels which are designed to maintain horizontal flow velocities of 0.15–0.30 cm s^{-1} to overcome frictional losses while ensuring continuous flow (Chiaramonti et al. 2013).

Such ponds are the most commonly used systems for large-scale production of algal biomass (Chiaramonti et al. 2013). Raceway ponds could achieve productivities of up to 50 t ha^{-1} $year^{-1}$ (Rawat et al. 2011). However, their application for wastewater treatment as a primary objective rather than biomass production is considered to be more economically viable since large parts of capital and operational costs are recovered due to wastewater treatment itself. In addition, their water and energy footprints are lower (Park et al. 2011). HRAPs are also utilized in treating industrial or hazardous wastewaters such as effluents from tannery, mines, or zinc refinery (Rawat et al. 2011).

Despite these advantages, two critical limitations of raceway ponds are large land requirement and risk of culture contamination (Chiaramonti et al. 2013). Since these ponds are restricted in depth due to the need of efficient light penetration, the footprint is huge and may become a limiting factor for the applicability of this technology. In addition, the presence of various zooplanktons and protozoa that graze on microalgae may reduce the production and hence the treatment. Since

these are open systems, variability in light and temperature affects the treatment efficiencies of these ponds. Other challenges associated with pond operation are evaporative losses and difficulty in maintaining carbon dioxide in the system required for algal growth (Singh and Sharma 2012). Another challenge in operating HRAPs is the entrainment of microalgae with the pond effluent due to poor settling properties, and which requires effective harvesting protocols.

5 Advanced Integrated Wastewater Pond Systems

The concept of natural wastewater treatment in ponds was explored in detail and an advanced pond system design was proposed by Oswald (1990) and his group at Berkeley to achieve comprehensive wastewater treatment with lower energy input. Such advanced integrated wastewater pond systems (AIWPS) are potentially applicable in developing nations where cost-effective treatment processes are in great demand to achieve sanitation goals effectively (Oswald 1990). Such advanced treatment ponds aim to maximise the applicability of conventional ponds while minimising their drawbacks. In comparison to conventional ponds, AIWPS require much less energy and resources including land. In addition, the issues of odour and sludge build-up of other treatment processes are also minimised (Oswald 1990).

Integrated ponds consist of at least four basic ponds which are designed to achieve different objectives. These objectives are similar to those in conventional wastewater treatment, namely, primary sedimentation, flotation, fermentation, aeration, secondary sedimentation, nutrient removal, storage and final disposal. These ponds include a facultative pond which consists of a deeper pit inside for anaerobic fermentation, a high rate algal pond, an algae settling pond, and finally a maturation pond.

5.1 Facultative Pond with Internal Fermentation Pit

The primary treatment of wastewater occurs in this facultative pond. A deep anoxic pit is provided within this pond to achieve fermentation of various solids, and eventually degrade them so as to minimise sludge build-up. Such pits are designed to avoid the intrusion of oxygen-rich water which might compromise the fermentation process. Raw sewage is allowed to enter at the bottom of this fermentation pit. The overflow velocities are kept low (<1.5 m d^{-1}) which helps in settling of the solids. Since these velocities are less than settling velocities of helminth ova and parasite cysts, these are also trapped within this pit. As the solids settle and accumulate at the bottom of the pit, they create an anoxic layer which results in the fermentation of such solid biomass. The resulting biogas bubbles due to fermentation entrap some of the solids and carry them upwards in the pit. Such bubbles get bigger and finally detach from such solids which again settle to the bottom. This

process results in a long sludge age within the pit, which eventually results in almost complete sludge digestion, and sludge removal is required over decades of operation. Carbon dioxide in the produced biogas reaches the upper layers within the pond where it helps in algal photosynthesis and eventual oxygen production. Photosynthetic oxygen results in oxygenic conditions in the upper water layers in the pond and aids in odour removal by aerobic degradation of causative compounds. The effluent from this pond is removed from the upper layers (at least 1 m below the water level to avoid passing of floating objects) with oxygen rich conditions. A properly designed pond of this nature could remove almost 100 % suspended solids and 60–70 % of the biochemical oxygen demand (BOD) from the influent wastewater (Oswald 1990).

5.2 High-Rate Algal Pond (HRAP)

The second pond in this system is a high-rate algal pond where algal growth is maximised with the application of paddlewheels for proper mixing and shallower depths for better light utilisation. High productivity of algae is achieved in these ponds with sufficient removal of nutrients at a shorter retention time. Oxygen production due to algal photosynthesis is utilised by aerobic bacteria to remove the remaining BOD in the wastewater. In addition, pH increases above 9–10 due to algal photosynthesis and helps in disinfection from most pathogenic bacteria. Part of this high pH and oxygen-rich effluent from HRAP is recycled to the top of first facultative pond to help with odour and pathogen removal.

5.3 Algae Settling Pond

The use of paddle wheels in HRAP promotes the dominance of algal species with better settling characteristics in the HRAP, and this property is utilized for removing algae from effluent in algae settling ponds (Oswald 1990). The effluent from HRAP is sent to settling ponds, where algae settle to the bottom and clarified effluent is achieved. The water in this pond is required to be in quiescent condition to accelerate algal settling. Settled algae are rich in nutrients and can be utilized for further applications. At least two such settling ponds are operated in parallel to achieve periodic harvesting of settled algae without affecting the plant operation. The effluents from algal settling ponds are low in BOD and nutrients. However, enhanced disinfection might be necessary if effluent from such ponds is used for purposes where prolonged human contact is expected. In addition, additional algae harvesting processes might be necessary to achieve complete algae removal from the effluent.

5.4 Maturation Pond

Last of the ponds is a maturation pond where effluents from the settling pond are stored for additional 10–15 days to achieve enhanced levels of disinfection especially of faecal coliforms, while also acting as storage pond for irrigation applications. Additional BOD and nutrient removal is also achieved in this pond. In addition, these maturation ponds also act as a habitat for aquatic life. The effluents from such ponds are low in BOD, nutrients, and pathogens; and hence suitable for application in agriculture or other such objectives. In addition to domestic wastewater, such AIWPS technology has also been applied for treating industrial wastewaters such as tannery effluents (Tadesse et al. 2004). Such ponds have been also found effective in removing chromium (Tadesse et al. 2006), selenium (Green et al. 2003), etc. from wastewaters.

6 Symbiosis of Algae with Bacteria for Wastewater Treatment

Microalgal photosynthesis provides a unique opportunity for maintaining a mutually beneficial relationship with aerobic bacteria during wastewater treatment. The oxygen produced by microalgae fulfils the requirement of bacteria during degradation of organic carbon, and the carbon dioxide produced as a result of this degradation as well as bacterial respiration is an important substrate for photosynthesis. In addition, the excretion of organic carbon from microalgae also provides substrates for bacterial growth (Munoz and Guieysse 2006). However, such relationships are very complex in nature and include mutually beneficial or harmful effects.

Many researchers have observed such relationships to be species specific. Growth of microalga *Asterionella glacialis* was promoted with the addition of bacterial strain Pseudomonas sp. 022, while no effect on growth was shown with *Vibrio* sp. 05. Further study showed that *Pseudomonas* sp. 022 produced a glycoprotein that acted as a growth factor for *A. glacialis* (Riquelme et al. 1987). Similarly, bacterial strain *Spirillum* 7697 exhibited a 40-fold difference in taking up the extracellular products of algae *S. costatum* when compared to strain *Pseudomonas* HNY (Bell et al. 1974). Watanabe et al. (2008) studied the composition of metabolites from Chlorella sorokiniana and their relative uptake by its many bacterial symbionts. The growth of *Chlorella vulgaris* was found to increase when co-immobilized and co-cultured with *Azospirillum brasilense* (Gonzalez and Bashan 2000). In addition to mutual growth promotion, microalgae and bacteria also exhibit bactericidal and algicidal effects, respectively towards certain species. For example, the presence of bacterial strain *Flavobacterium* sp. 5N3 resulted in suppression of red tide plankton, *Gymnodinium mikimotoi* (Fukami et al. 1997).

These complex interactions between microalgae and bacteria also result in their changing behaviour within phycosphere in comparison to the surrounding milieu (Bell et al. 1974). For example, *Chlorella sorokiniana* IAM C-212 produces a polysaccharide gel, termed as sheath, under photoautotrophic conditions which is a suitable habitat for several symbiotic microorganisms as it ensures close proximity (Imase et al. 2008).

These symbiotic relations have been utilized in open ponds or closed photo-bioreactors for achieving enhanced removal of nutrients and organic carbon (Olguín 2012). In addition, various hazardous elements such as acetonitrile (Muñoz et al. 2005), salicylate (Muñoz et al. 2003b), phenanthrene (Muñoz et al. 2003a), etc. have also been successfully degraded with combined applications of algae and bacteria (Munoz and Guieysse 2006).

7 Utilisation of Wastewater-Grown Microalgae

Microalgal biomass (except those used for heavy metal remediation) can be utilized for biofuel production viz. biodiesel, bioethanol, biomethane, etc., as a feed in aquaculture and poultry, in fertilizers, pharmaceuticals, and nutraceuticals industry (Singh et al. 2014; Vanthoor-Koopmans et al. 2013). Recently, there has been renewed interest in microalgae as commercial sources of bioenergy and high-value products such as β-carotene, astaxanthin, docosahexaenoic acid, eicosahexaenoic acid, and phycobilin. However, high production cost is still a bottleneck for commercial scale production of lower value products (Singh et al. 2015). Integration of wastewater treatment with algae biomass production is one of the methods to reduce costs of microalgae mass cultivation (Ramanna et al. 2014).

Several studies have been conducted on the utilisation of wastewater as growth medium for microalgal cultivation and the biomass produced can be utilized in several ways. Most of these studies are conducted on utilisation of wastewater as growth media for microalgal biomass production that can be used as biodiesel feedstock. Cho et al. (2013) studied the feasibility of wastewater for cultivation of *Chlorella* sp. in order to achieve high biomass production with low cost input. In their study the highest biomass (3.2 g L^{-1}) was obtained from the wastewater grown culture which was 1.72 higher than BG 11 medium. High lipid accumulation is a key factor for algal biodiesel production. Available nutrients in wastewater may not be sufficient for growth of microalgae. For production of commercial products such as biodiesel, bioethanol, pigments, fertilizers, wastewater may require supplementation with some nutrients.

Ramanna et al. (2014) studied the effect of different nitrogen sources on lipid accumulation of *Chlorella sorokiniana* while using domestic wastewater as growth medium. In their study they obtained maximum lipid accumulation of 61.7 % in the experiment with urea supplemented domestic wastewater. The fatty acid profile also confirms the suitability of their strain for biodiesel production. *Chlorella vulgaris* and *Botrycococcus teribillis* grown in domestic wastewater supplemented with

glycerol as carbon source were reported as potential candidates for biofuel production. On the basis of biochemical compositions obtained, biomass was found suitable for production of biodiesel, bioethanol, biomethane and its utilisation as bio fertilizer (Cabanelas et al. 2013) (Table 1).

Microalgae can accumulate large amount of carbohydrates in the form of starch, glucose, cellulose, hemicelluloses, and various kinds of polysaccharides (Borowitzka 2013). These microalgal carbohydrates are conventionally used for biofuel production, especially for bioethanol (Vanthoor-Koopmans et al. 2013) and hydrogen (Beer et al. 2009). Recently these microalgal polysaccharides have been discovered as source of bioactive compounds. Specifically, algal polysaccharides contain sulphate esters called sulphated polysaccharides (e.g., fucoidan, carrageenans and agarans) (Spolaore et al. 2006; Yen et al. 2013). These sulphated polysaccharides have been shown to possess numerous medicinal activities, such as antioxidant, antitumor, anticoagulant, anti-inflammatory, and antiviral (Pulz and Gross 2004; Skjanes et al. 2013; Yen et al. 2013). A high-weight polysaccharide from *Chlorella pyrenoidosa* has very high immunostimulatory and antitumor effects with potential use in cancer therapy (Shi et al. 2007). Colourful appearance of the microalgae is because of the pigments which capture light and initiate photosynthesis (Spolaore et al. 2006). Carotenoids are pigments present in all classes of algae and serve as photo-protectors against the photo-oxidative damage resulting from excess energy captured by light-harvesting antenna (Cardozo et al. 2007). Astaxanthin is an oxidised form of carotenoid with high oxidation capacity (Qin et al. 2008). Astaxanthin has many applications in healthcare industry as it can be used for prevention and treatment of various conditions, such as chronic inflammatory diseases, eye diseases, skin diseases, cardiovascular diseases, cancers, neurodegenerative diseases, liver diseases, metabolic syndrome, diabetes, diabetic nephropathy and gastrointestinal diseases (Wayama et al. 2013). *Chlorella zofingiensis* was grown on waste molasses and examined for oil accumulation and astaxanthin production. A lipid productivity of 710 mg L^{-1} d^{-1} and astaxanthin 1.7 mg L^{-1} d^{-1} was obtained (Liu et al. 2012).

Arthospira is a well-known source for commercial products such as phycocyanin pigment and poly unsaturated fatty acids. Phycocyanin is a blue pigment with anti-oxidative property and known as immunity promoter in human and animals (Sarada et al. 1999). Arthospira was evaluated for its nutrient removal capacity and biomass productivity while growing on piggery wastewater. High biomass production (11.8 g L^{-1} d^{-1}) and high protein (48.9 %) content was obtained for Arthospira. The microalgal biomass was also evaluated for its suitability for fish feed and extraction of other valuable chemicals (Olguín 2003).

Microalgae can synthesise numerous compounds that have nutraceutical value. Microalgae have become more ubiquitous sources of nutraceuticals due to the capability of producing necessary vitamins, essential elements and essential amino acids and Omega 6 (Arachidonic acid) and Omega 3 (Docosahexaenoic acid, eicosapentaenoic acid) fatty acids (Spolaore et al. 2006). *Chlorella* (lutein, vitamin B12), *Spirulina* (single cell protein), *Haematococcus* (antioxidant) and *Dunaliella* (β-carotene) are the most popular nutraceutical sources (Vanthoor-Koopmans et al. 2013).

Table 1 Utilisation of microalgal biomass grown in wastewater

Microalgal strain	Wastewater type	Biomass productivity	Lipid productivity or lipid content	Carbohydrate productivity	Other	Application	References
Scenedesmus sp.	Carpet mill	126.54 mg L^{-1} d^{-1}	16.2 mg L^{-1} d^{-1}				Chinnasamy et al. (2010)
Chlamydomonas reinhardtii	Municipal centrae	2000 mg L^{-1} d^{-1}	505 mg L^{-1} d^{-1}	–	–	Biodiesel	Singh et al. (2014)
Chlorella pyrenoidosa	Soyabeen process		400 mg L^{-1} d^{-1}	–	–	Biodiesel	Hongyang et al. (2011)
Dunaliella tertiolecta	Carpet mill untreated	28.3 mg L^{-1} d^{-1}	4.3 mg L^{-1} d^{-1}	–	–	Bioremediation, biodiesel	Chinnasamy et al. (2010)
Chlorella sorokiniana	Domestic wastewater with urea supplementation	200 mg L^{-1} d^{-1}	61.52 % lipid content	–	–	Biodiesel	Ramanna et al. (2014)
Chlorella vulgaris	Domestic waste water + glycerol (50 mM)	5.69 ton $year^{-1}$	894.2 kg $year^{-1}$	415.0 kg $year^{-1}$	–	Biodiesel, bioethanol, biomethane, fertilizer	Cabanelas et al. (2013)
Botrycococcus teribillis	Domestic waste water + glycerol (50 mM)	13.58 ton $year^{-1}$	1683.4 kg $year^{-1}$	1072.6 kg $year^{-1}$	–	Biodiesel, bioethanol, biomethane, fertilizer	Cabanelas et al. (2013)
Chlorella zofingiensis	Waste cane molasses	1550 mg L^{-1} d^{-1}	710 mg L^{-1} d^{-1}	–	1.7 mg L^{-1} d^{-1} astaxanthin	Bio-oil, pigments	Liu et al. (2012)
Arthospira	Piggery waste	11.8 g m^{-2} d^{-1}	–	–	48.9 % protein	Aquaculture, pigments	Olguín et al. (2003)

However, utilisation of wastewater grown biomass for human consumption, animal and fish feed and healthcare applications could face ethical and biosafety issues. Chemical and physical characterisation as well as microbiological assessment for pathogens of the products is important for safety considerations. The products must be examined to determine the potential for toxicity, the possibility for naturally occurring toxins (from the source organism), heavy metals, and hazardous levels of pathogenic microorganisms, as well as potential hazardous by-products formed from the degradation of certain macromolecules. Wastewater-grown microalgae may contain some heavy metals and pathogenic microorganisms which are critical in evaluating the toxicity of the products.

Microalgae components are valuable, with a wide range of applications. The carbohydrates present in microalgae are considered as an appropriate feedstock for various energy sources (bioethanol, biomethane, etc.) and source of various polysaccharides. The high lipid content in algal biomass makes it promising feedstock for biodiesel production, while the long-chain fatty acids, pigments and proteins have their nutraceutical and pharmaceutical applications (Table 1).

Integration of wastewater for generation of microalgal biomass reduces the production cost. Therefore, wastewater-grown microalgae deserve further investigations in particular for commercial viability, large scale cultivation, assessment of environmental and safety risk, ethical issues of converting the components of microalgae into biofuels and other valuable products.

8 Conclusion

With increased environmental awareness, the world has moved towards a zero waste strategy and valorisation of waste substrates. Phycoremediation of wastewater offers a significant avenue towards achieving this outcome. Despite the challenges associated with the technology, the lower cost and ease of operation make this technology attractive. The sheer versatility offered by algal wastewater treatment with regard to substrate and ability to derive value in terms of nutrient recycling and the potential for energy generation make phycoremediation essential for environmental protection.

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Major Commercial Products from Micro- and Macroalgae

Melinda Griffiths, Susan T.L. Harrison, Monique Smit and Dheepak Maharajh

Abstract Macro- and microalgae are used in a variety of commercial products with many more in development. This chapter outlines the major products, species used, methods of production, extraction, and processing as well as market sizes and trends. Foods, nutraceuticals, and feeds are the major commercial products from algae. Well-known culinary products include Nori, Wakame, Kombu and Dulse, from whole macroalgal biomass. The microalgae *Spirulina* and *Chlorella* have been widely marketed as nutritional supplements for both humans and animals. Several microalgae with a high nutritional value and energy content are grown commercially as aquaculture feed. The major processed products from macroalgae are the hydrocolloids, including carrageenan, agars, and alginates, used as gelling agents in a variety of foods and healthcare products. Pigments extracted from algae include β-carotene, astaxanthin, and phycobiliproteins. These are generally used as food colorants, as additives in animal feed or as nutraceuticals for their antioxidant properties (Radmer in Bioscience 46:263–270, 1996; Pulz and Gross in Applied Microbiology and Biotechnology 65:635–648, 2004). Polyunsaturated fatty acids (PUFAs) are another high value product derived from microalgae. Other potential products include fertilizers, fuels, cosmetics and chemicals. Algae also have application in bioremediation and CO_2 sequestration, as well as producing many interesting bioactive compounds. Algae have great potential to produce a wide range of valuable compounds, beyond their current exploitation. To date, commercialization of new products has been slow (Milledge in Reviews in Environmental Science and Biotechnology 10:31–41, 2011; Wijffels in Trends in Biotechnology 26:26–31, 2007; Radmer in Bioscience 46:263–270, 1996; Pulz and Gross in Applied Microbiology and Biotechnology 65:635–648, 2004; Spolaore

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et al. in Journal of Bioscience and Bioengineering 101(2):87–96, 2006), however, microalgal biotechnology is a relatively new industry, and therefore, it is unsurprising that significant challenges remain to be solved. The advantages associated with algal production are likely to ensure that efforts continue.

Keywords Microalgae · Macroalgae · Pigments · High value products · Bioactive compounds

1 Introduction

The term algae encompasses an extremely diverse taxonomic group, including both prokaryotic and eukaryotic organisms, with a wide genetic and metabolic range (Apt and Behrens 1999; Radmer 1996). Structurally, species range from the unicellular, colonial and filamentous through to complex multicellular structures resembling higher plants (e.g., kelp). The most general grouping divides these into the microalgae (those that are microscopic) and the macroalgae (those that can be seen with the naked eye). They are efficient cell factories, the majority operating photosynthetically to turn light energy and simple, inorganic nutrients into a range of complex biomolecules. Some species are heterotrophic, or able to grow in the dark on organic carbon sources such as sugar, similarly to yeast, fungi and bacteria. Occasionally species can switch between these modes (mixotrophy), although often grow better in one mode than the other. Common products of algal metabolism include proteins, carbohydrates, pigments, lipids, toxins and bioactive compounds (Radmer 1996).

Foods, food additives, nutraceuticals and feeds are the major commercial products from algae. Well-known culinary products from macroalgae include Nori, Wakame, Kombu and Dulse. The microalgae *Spirulina* and *Chlorella* have been widely marketed as nutritional supplements for both humans and animals. Several microalgae with a high nutritional value and energy content are grown commercially as aquaculture feed. The major processed products from algae are the hydrocolloids, including carrageenan, agars and alginates. These polysaccharides are extracted from algal biomass and used as gelling agents in a variety of foods and health-care products. Pigments extracted from algae include β-carotene, astaxanthin and phycobiliproteins. These are generally used as food colorants, as additives in animal feed or as nutraceuticals for their antioxidant properties (Radmer 1996; Pulz and Gross 2004).

Production of algae for human consumption and nutraceuticals together make up a multi-billion dollar industry with the majority of production and consumption in the Far East (Radmer 1996). Macroalgae, mostly certain species of red (*Rhodophyta*) and brown (*Phaeophyta*), have an established history of harvest and cultivation for the production of food and hydrocolloids. The annual world market for these products is approx. $600 billion, with over 7.5 million tons harvested per

Table 1 Major commercial algal products, species and market value

Product	Species	Application	Market value (million US$)	Approx. price (US$)
Nori	*Porphyra*	Food (sushi)	2000[a]	66–166/kg sheets[i] 70–100/kg sheets[k]
Wakame	*Undaria pinnatifida*	Food	600[a] 150[j]	169/kg dried[k]
Kombu	*Laminaria japonica*	Food	600[a]	50–200/kg dried[k]
Health food	*Spirulina*	Nutraceutical	80[a] 20–25[f] 40[h]	10–20/kg bulk powder[d] 35/kg[k] powder 68–112/kg[k] tablets
Health food	*Chorella*	Nutraceutical	100[a]	100/kg[a] 100–120/kg[k] tablets
Alginates	*Laminaria*, *Macrocystis* and *Ascophyllum*	Thickening, gelling, water retention	230[a]	3–190/kg[k,n]
Carrageenans	*Eucheuma cottonii*, *E. spinosum* and *Chondrus crispus*	Gelling, thickening, stabilizing	100[a]	5–140/kg[k,n] powder
Agars	*Gracilaria*, *Gelidium* and *Pterocladia*	Gelling: food and biotechnology	160[a]	5–100/kg[k,n] powder
Agarose	From agar	Biotechnology	50[a]	Up to $25 000/kg[a] 750–2600/kg[k]
Phycobiliproteins	*Arthrospira platensis*	Food colorant, nutraceutical	2[a] 12[b] 50[c]	370/kg[d] (food grade) 5000/g[a] (reagent grade)
Aquaculture feed	*Isochrysis galbana*, *Phaeodactylum tricornutum*, etc. (Table 2)	Feed	700[b]	50–300/kg[m]
β-carotene	*Dunaliella salina*	Pigment, feed, health supplement	280[b] 44–133[d]	1400/kg[a] 300–3000/kg[c] 444/kg[d]

(continued)

Table 1 (continued)

Product	Species	Application	Market value (million US$)	Approx. price (US$)
Astaxanthin	*Haematococcus pluvialis*	Pigment, feed additive, pharmaceuticals, health supplement	150[b] 22–44[d] <5[g] (nutraceuticals)	2500/kg[c] 2220/kg[d] 2000/kg[g] (feed) > $100,000/kg[g] (nutraceutical)
Fatty acids, DHA, EPA, PUFA	*Odontella aurita*	Baby food, pharmaceuticals, cosmetics	1530[b] 15[o] (DHA in the USA)	650/kg[l]
Isotopes		Biotechnology	5[b]; 13[c]	>100/g[a] 260–5900/g[c]

[a]Radmer (1996), [b]Pulz and Gross (2004), [c]Spolaore et al. (2006), [d]Borowitzka (1992), assuming 1AU$ = 0.74US$ [1992 average]; [f]Metting (1996), [g]Olaizola (2003), [h]Singh et al. (2005), [i]Merril (1993), [j]Watanabe and Nisizawa (1984), [k]www.amazon.com, [l]Belarbi et al. (1999), [m]Muller-Feuga (2000), [n]www.made-in-china.com, [o]Bux (2013)

year (Pulz and Gross 2004). Microalgal biotechnology is a much newer industry. *Spirulina*, possibly the most well-known microalgal product, has been collected and eaten by the Aztecs and people in Central Africa for centuries, however, commercial production in man-made ponds only began in 1978 (Belay 1997). Microalgae are cultured mainly as health food, as the source of pigments such as β-carotene and as a feed or feed additive in aquaculture (Borowitzka 1997). In 2004, the market for microalgal biomass was estimated to be about 5000 tons per year with a value of about $1.25 billion (Pulz and Gross 2004; Spolaore et al. 2006).

The production of commercial products from algae ranges from low-tech ocean farming (e.g., harvesting seaweed from the wild for production of food, feed or fertilizer) to high-tech bioprocess engineering (e.g., intensive culture of specific strains under sterile, controlled conditions in fermentation systems for the production of isotopically labeled compounds) (Radmer 1996). With the expansion of the biotechnology industry has come an increase in the variety of tubes, columns, panels and hanging bags (collectively known as photobioreactors) in which microalgae are cultivated, however, the majority of commercially successful algal processes today continue to use open ponds. The high cost of closed photobioreactors can only be offset by a high value product, for example a pharmaceutical (Wijffels 2007).

This chapter provides an overview of the major current commercial products from algae (summarized in Table 1) and those in development. It outlines the major species used, methods of production, extraction and processing as well as market sizes and trends.

2 Food and Nutraceuticals

Algae are rich in protein, lipids, carbohydrates, vitamins, minerals and essential nutrients, making them an excellent source of nutrition for humans and animals. Macroalgal food products, produced and sold primarily in the Far East, have the largest market value of any algal product (Radmer 1996). Despite high hopes for the use of microalgae for food in the mid twentieth century, this has not materialized to date, likely due to the high cost of production. While basic cost of production of microalgal biomass in raceway systems lies in the range US$0.23–0.6 per kg dry mass, in photobioreactors average predicted costs lie in the range US$3.2–9.5 per kg with data reported across the range US$0.42–30.4 per kg (Harrison et al. 2013). Nutraceuticals and health foods, where algal biomass can command premium prices, are the dominant markets for microalgae.

2.1 Macroalgae

The major algal foods are derived from macroalgae, generally harvested from wild, or from managed or cultivated populations. Indonesia, the Philippines, Malaysia and China are among the highest macroalgae producing nations in the world (Hurtado 2014). Whole algal biomass is often sun-, spray- or oven-dried, and sold as a sheet, powder, tablet or capsule, or incorporated into other foods. The only processing involved is the sorting, cleaning or preserving of the biomass (Radmer 1996). Many types of macroalgae are eaten as foods. Those with the major commercial markets are listed here.

2.1.1 Nori

Produced from the blade or 'leaf' of the red algae *Porphyra*, Nori has been collected since the year 530, and actively cultivated since 1640 (Pulz and Gross 2004). It is the dominant algal product on the market today, with an annual turnover of more than $1 billion (Pulz and Gross 2004). In 1993, Nori production was 40,000 tons/annum (Jense 1993). Its principle use is as a component of sushi. Toasted nori sheets (Fig. 1) are the most popular product. Nori cultivation, similarly to other macroalgal food products and hydrocolloid species, is essentially underwater farming. Conchospores (seed-like propagules) are seeded onto nets and hung in sheltered ocean areas, strung between poles or attached to surface buoys. More recently, devices designed to be raised out of the water have been developed, allowing more mechanized harvesting and processing (Radmer 1996).

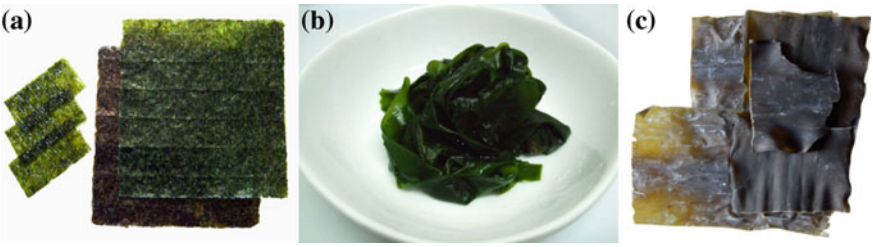

Fig. 1 **a** Dried Nori sheets, **b** boiled Wakame and **c** Kombu from *Laminaria japonica* (**a** and **c** Alice Wiegand, http://en.wikipedia.org/wiki/Nori#mediaviewer/File:Nori.jpg and http://en.wikipedia.org/wiki/Kombu#mediaviewer/File:Kombu.jpg; **b** えむかと一 http://en.wikipedia.org/wiki/Wakame#mediaviewer/File:Boiled_wakame.jpg)

2.1.2 Wakame

Wakame is derived from the brown algae *Undaria pinnatifida*. It has been cultivated, primarily in Japan, Korea and China, since the 1950s. It is sold in many forms, the most popular product being boiled and salted. It is used as an ingredient in noodles, soups and salads. In 1990, approximately 20,000 tons were produced annually with a market value of $600 million (Radmer 1996).

2.1.3 Kombu

Harvested from *Laminaria japonica* and related brown algal species, Kombu is another popular culinary alga, served with meat, fish, in soups or as a vegetable. Algae are collected, dried and boiled. The annual market is estimated at $600 million (Radmer 1996).

Other major macroalgae used for human consumption include Dulse, derived from the macroalgae *Palmaria palmata* in Europe and *Rhodymenia* sp. in North America, and *Nostoc*. Dulse has been consumed by people living on the coast of Europe for centuries (Radmer 1996). *Nostoc* is a filamentous cyanobacteria that is cultivated and consumed for its high protein and vitamin content, mostly in Asia. *N. flagelliform* and *N. commune* are the most popular varieties in China and Japan, and *N. ellipsosporum* in Central Asia.

2.2 Microalgae

In the early 1950s, the rise in world population and concerns over a future shortage of food led to a search for alternative protein sources. Microalgae were identified as promising candidates due to their high protein content. Studies have shown that the

quality of algal-derived protein to be superior to that from many conventional plants (Spolaore et al. 2006). Algae provide the full spectrum of amino acids. Algae are the original source of polyunsaturated fatty acids (PUFA) in the food chain, and thus provide essential fatty acids. Microalgae also represent a source of nearly all essential vitamins, and contain pigments such as chlorophyll, carotenoids and phycobiliproteins, which have been shown to have nutraceutical effects (Spolaore et al. 2006). Although microalgae and their extracts are used for food in certain niche applications, the large-scale production of algae to solve the world's food crisis and shortage of protein has not materialized (Milledge 2011). This could be due in part to the relatively high cost of microalgal production in artificial containers, the undesirable taste, odor and strong color of many dried algal powders, and the public perception of algae as 'slimy pond-scum' to be avoided.

Historically, nutritional supplements have been the largest commercial microalgal product. *Chlorella* and *Spirulina* (*Arthrospira platensis*) dominate the market and are predominantly used in health food products (Pulz and Gross 2004). Either whole cell biomass or extracts are sold as nutraceuticals or functional foods, to be consumed on their own as a nutritional supplement, or combined into other products (Apt and Behrens 1999).

2.2.1 Spirulina

The filamentous cyanobacteria *Arthrospira platensis* and other species of *Arthrospira*, commonly known as *Spirulina*, have a long history of human consumption. It was originally harvested from natural alkaline lakes in Chad and Mexico (Radmer 1996). The first commercial facility (Sosa Texcoco in Mexico) was established to harvest and enhance natural production in Lake Texcoco (Spolaore et al. 2006). Several large-scale commercial producers exist today, e.g., Earthrise, Cyanotech and Dainappon Ink & Chemicals, each producing hundreds of tons of dry product per year.

Spirulina is a relatively easy microalga to cultivate due to its filamentous morphology (meaning it can be readily harvested by filtration) and the alkaline, saline conditions for cultivation prevent the growth of the majority of other algal species and contaminants (Radmer 1996). *Spirulina* is commercially produced in large open raceway ponds (Fig. 2), harvested by filtration and dried by spray drying, solar drying or oven drying. Products include powder, tablets and capsules and are sold mainly in Europe, North America and Asia (Spolaore et al. 2006; Metting 1996). Prior to their consumption, algal biomass must be tested for safety in terms of heavy metal content, bacterial load and presence of toxins from contaminating microalgae (Spolaore et al. 2006).

It is sold primarily as a health food, or as an additive in animal feed. There is a large body of literature examining the nutritional value of *Spirulina* in human and animal diets (Radmer 1996). As a source of usable protein, *Spirulina* contains in

Fig. 2 *Spirulina* being cultivated in a raceway pond

excess of 50 % protein. This is superior to plants, comparable to meat and dairy products, but inferior to poultry and fish. It contains the full range of amino acids, but has low levels of sulfur-containing essential amino acids (Metting 1996). It also contains some essential fatty acids, unusually high levels of the vitamins A and B_{12}, and many useful minerals. *Spirulina* biomass has been suggested to have various health-promoting effects, e.g., immune boosting, antiviral, promotion of growth of healthy intestinal flora (Spolaore et al. 2006), however, most of these have not yet been validated in clinical studies.

2.2.2 Chlorella

Chlorella is a green microalga marketed primarily as a health food product. It was first cultivated commercially in the early 1960s by Nihon Chlorella (Taiwan). Today more than 70 companies produce it commercially (Spolaore et al. 2006). Success in its cultivation in open reactors relies on its fast growth rate to out-compete contaminants, as it grows under moderate conditions. It can also be grown heterotrophically on glucose or acetate, usually for the production of high value compounds, e.g., pharmaceuticals. Biomass is harvested by centrifugation and dried to a powder (Radmer 1996). It is purported to have a variety of health-promoting effects, e.g., immune-stimulation, reduction in blood lipids, antioxidant properties, efficacy on gastric ulcers, wounds, and constipation (Spolaore et al. 2006). However, these investigations are often initiated by the *Chlorella* producing company itself and have not been conclusively demonstrated.

3 Feed

3.1 Aquaculture

The growth in the production of farmed fish and shellfish has been strong, increasing in one decade from 10 to 29 Mt in 1997. Nutrition of these farmed fish has been reliant on by-products of wild fish and terrestrial agriculture. Enhancing availability of algae for fish feed enables independence from wild fish by-products to be approached in a sustainable manner (Naylor et al. 2000). Algae are an important food and feed additive in the aquaculture of a variety of animals, including fish, molluscs (clams and oysters), crustaceans (shrimp) and zooplankton (e.g., rotifers and *Artemia*), which serve as live food for fish and shellfish (Borowitzka 1997; Metting 1996). Microalgae are required during a brief but vital period in larval nutrition, either for direct consumption by molluscs or shrimp, or as food for the live prey fed to fish larvae (Spolaore et al. 2006). Algae are the preferred food source as they are the natural food of these animals, although alternatives are available (e.g., yeast and artificial feeds) (Borowitzka 1997).

There are three slightly differentiated markets for algae in aquaculture (Pulz and Gross 2004):

- the unialgal cultivation of specific microalgal species as a starting food for larvae and young vertebrates
- the addition of small amounts of algae, e.g., *Spirulina*, *Chlorella*, and *Dunaliella* into fish feed, similarly to animal feed, to enhance health and color
- the use of macroalgae as whole grazing fodder, or incorporated into feed mixtures/pellets for feeding to larger aquaculture stocks.

Microalgae represent the largest market for aquaculture feed. More than 40 microalgal species are used in aquaculture worldwide, depending on the local requirements (Pulz and Gross 2004). Cultures of specific algal strains are necessary in the production of a defined food with characterized properties to ensure the survival and high productivity of larvae. Those commonly used are listed in Table 2. The nutritional content of microalgae can be altered through adjusting the culture conditions. This can be exploited to enhance the nutritional content of the algae in order to improve larval survival rates. For example, the content and ratio of the long-chain fatty acids docosahexanoic acid (DHA), eicosapentanoic acid (EPA) and arachidonic acid (AA) in feed have been recognized to affect the larval development. This can have a large impact on the economics of the aquaculture facility (Apt and Behrens 1999).

Macroalgae used as grazing fodder for animals such as urchins and abalone include *Ecklonia maxima*, *Ulva lactuca* and *Gracilaria gracilis* (Naidoo et al. 2006).

Of the 1000 tons of microalgae produced for aquaculture in 1999, 62 % was used for molluscs, 21 % for shrimps and 16 % for fish (Spolaore et al. 2006). Microalgae are often grown on-site in aerated, hanging plastic bags or tanks, and

Table 2 Algae commonly used as aquaculture feed

Species	Aquaculture crop
Amphora	Shrimp
Chaetoceros	Molluscs, Crustaceans
Chlorella	Fish
Cyclotella	Molluscs
Dunaliella	Crustaceans
Haematococcus	Fish, mainly salmon
Isochrysis	Molluscs
Nannochloropsis	Rotifers
Navicula	Abalone, Shrimp
Nitzschia	Abalone
Pavlova	Molluscs
Phaeodactylum	Fish
Skeletonema	Molluscs, Crustaceans
Spirulina	Fish, e.g., koi, tilapia
Tetraselmis	Crustaceans

Apt and Behrens (1999), Borowitzka (1997), Khatoon et al. (2009), Pulz and Gross (2004), Spolaore et al. (2006)

are fed fresh or dried, alone or combined with other nutrients. To be a good aquaculture feed, algae must contain the correct nutrients for the target organism at the required growth stage. Protein and fatty acid content are of particular importance in larval nutrition, as well as vitamins. In addition, they must be the correct size and shape for the consumer to feed on, have a digestible cell wall, be nontoxic and be cost-effective to cultivate at a medium scale (Borowitzka 1997; Spolaore et al. 2006).

Microalgal production is costly and can contribute 30–40 % of the aquaculture production cost (Borowitzka 1997). Bivalve and shrimp hatcheries require substantial amounts of algae on a continuous basis. Photosynthetic production costs are reported to be as high as >$160/kg dry weight, representing a substantial part of the production cost (Radmer 1996), suggesting the need for focus on improved algal productivity and efficiency of production of the required scale to improve competitiveness, as their value in aquaculture is well recognized. The reliability of feed supply is critical, as an interruption could lead to the death of the aquaculture crop (Borowitzka 1997).

The addition of microalgae to the tanks of aquaculture organisms has been found to have unexpected additional positive benefits, e.g., algae helps to improve the quality of the water, leading to higher survival and growth rates. This may be due to uptake of nutrients, oxygen formation, pH stabilization, regulation of bacterial population and/or probiotic and immune boosting effects (Spolaore et al. 2006).

The color of aquaculture organisms can be an important factor in their desirability by consumers and hence impact their market price (Apt and Behrens 1999). In the natural food chain, algae are the primary source of pigments that impart this

coloration, e.g., the characteristic orange–pink color of salmon, trout and shrimps. Artificial aquaculture feeds generally lack these pigments, and hence artificial supplements such as canthaxanthin are often added. The pigment astaxanthin is most commonly used. Astaxanthin is sourced from the microalga *Haematococcu*s (see also the astaxanthin section under pigments). Several companies have successfully produced and marketed natural astaxanthin from *Haematococcu*s for aquaculture feeds (Apt and Behrens 1999).

3.2 Pets and Farm Animals

Microalgae can be incorporated into the feed of a variety of animals, including fish, pets and farm animals with beneficial outcomes. In 2006, approximately 30 % of world microalgal production (including 50 % of *Spirulina* production) was sold as feed supplements (Spolaore et al. 2006). Feed quality has a large influence on the survival, development, growth and fertility of animals. There is evidence that small amounts of *Spirulina*, *Chlorella* and *Scenedesmus* incorporated into the feed can have a positive effect, particularly in boosting the immune response (Pulz and Gross 2004). Poultry can be fed up to 5–10 % *Spirulina* and this has been shown to enhance the yellow color of the skin and egg yolks due to the carotenoid content (Milledge 2011). *Spirulina* is easily digested due to its soft cell wall, however, some other algal species show poor digestibility due to the high content of cellulosic cell wall material. Ruminates such as sheep and cattle are capable of digesting cellulosic material, but algae as a major part of the feed has not gained much commercial favor yet (Milledge 2011).

Another promising application for microalgal biomass or extracts is in the pet food market. Studies on minks and rabbits have shown evidence of health-promoting effects (Pulz and Gross 2004). *Spirulina* has been included in the diets of cats, dogs, aquarium fish, ornamental birds, poultry, horses, cows and pigs, with positive effects on physiology, e.g., improved immune response and fertility, as well as improved appearance of coats and skin (Spolaore et al. 2006).

4 Hydrocolloids

Several useful polysaccharides, collectively known as hydrocolloids, are extracted from macroalgae. Together they make up the largest market ($500 million) for algal extracts (Pulz and Gross 2004). Hydrocolloids are produced by species of red and brown macroalgae, either harvested from the wild or cultivated in stands, similarly to those for food. The hydrocolloids are extracted through a series of steps involving hot solvent extraction and purification. Agarose is derived from agar by further separation and purification (Radmer 1996). The hydrocolloids are used for

their thickening and gelling properties in a variety of food and industrial applications (Pulz and Gross 2004).

4.1 Alginates

Alginates, polymers of D-mannuronic acid and L-gluronic acid, are salts of alginic acid. The exact composition varies in different sources. Alginates are used as thickeners, to form gels and for their water-retaining properties in the food, paper, biomedical and biotechnology fields. In 1990, the market for alginates was approximately $230 million (27,000 tons). The major sources of alginates are brown macroalgae, particularly *Laminaria*, *Macrocystis* and *Ascophyllum*. A typical process involves extraction of the alginic acids in hot sodium bicarbonate, followed by filtration and purification (Radmer 1996).

4.2 Carrageenans

Carrageenans are extracted primarily from the red macroalgae *Eucheuma cottonii*, *E. spinosum* and *Chondrus crispus*. Carrageenans are complex polysaccharides, made of sulfonated galactose polymers, typically extracted with hot water. They are used to gel, thicken, suspend and stabilize foods, cosmetics, pharmaceuticals and other products. Approximately 15,500 tons of carrageenans, with a market value of $100 million, were sold annually (data from 1990, Radmer 1996).

4.3 Agars and Agarose

The first reports of agar production date back to 1658 in Japan (Pulz and Gross 2004). Agars are a mixture of polysaccharides extracted from red algae. Similarly to carrageenans, they are also composed of galactose-related monomers, with varying amounts of sulfate, pyruvate and methoxy groups. The content varies with the source species and processing procedure. Primary sources include *Gracilaria*, *Gelidium*, *Pterocladia*, *Acanthopeltis* and *Ahnfeltia*. Agars are usually extracted with hot water, concentrated, and dried before being milled for packaging. Agar forms a gel that is stable under a range of temperatures, humidities, and chemical conditions. Agars are used in food and to produce solid growth media for microorganisms in laboratory studies. Agars command a significantly higher price than alginates and carrageenan. Annual sales are in the range of $160 million, with a volume of 11,000 tons (Radmer 1996). Agaroses are refined from agar by isolating the less ionic fractions of agar. The main applications of agarose are in the field of biotechnology (Radmer 1996).

5 Pigments

Chlorophyll, carotenoids and phycobiliproteins are the major pigments produced by algae (Metting 1996). The types and ratios present are dependent on species, light, nutrition and other environmental factors (Metting 1996). In addition to chlorophyll, algae contain various other pigments in order to increase the efficiency of light usage (e.g., phycobiliproteins) and to protect against solar radiation and free radicals (e.g., carotenoids) (Pulz and Gross 2004).

There is increasing demand for natural colorants from sustainable sources for use in the food, cosmetics, pharmaceuticals, textile, and printing industries (Dufosse et al. 2005). Few natural colorants have the tinctorial values and persistence required for industrial use (Sekar and Chandramohan 2008). Carotenoids and phycobiliproteins are among these. The major pigments of commercial interest are β-carotene from *Dunaliella*, astaxanthin from *Haematococcus* and the phycobiliproteins from cyanobacteria and some red algae.

5.1 Carotenoids

Carotenoids are tetraterpene, lipid soluble pigment molecules, most often in the brown–red–orange–yellow color range. The coloration of many brown and golden algae is due to their carotenoid content. The variety found in algae is much greater than in land plants (Metting 1996). Only very few of the 40+ known carotenoids are produced commercially (Metting 1996). These are mainly β-carotene and astaxanthin, and, to a lesser extent, lutein, zeaxanthin and lycopene (Spolaore et al. 2006).

5.1.1 β-carotene

β-carotene is produced naturally by the halotolerant green alga *Dunaliella*. Species of the *Dunaliella* are generally grown in hypersaline, open ponds (Fig. 3). Once the biomass has accumulated, it is subjected to high salt, high light environments. These stress conditions cause accumulation of β-carotene up to 14 % dry weight (Radmer 1996; Spolaore et al. 2006). The extreme growth conditions assist in limiting the growth of other algae and microorganisms. Harvesting of the dilute cells from a large volume of media represents a major expense in the process (Radmer 1996). The biomass is processed by a variety of techniques to produce products ranging from high-carotene content biomass to β-carotene in oil (Radmer 1996).

β-carotene is used as a natural food colorant and feed additive. It is also marketed as a health food and finds application in cosmetics for its putative health benefits. Carotenoids are precursors to Vitamin A and have antioxidant and

Fig. 3 Aerial view of open raceway ponds used for the production of β-carotene near Uppington, South Africa

anti-inflammatory properties (Radmer 1996; Pulz and Gross 2004). β-carotene occurs in many higher plants as well as algae and can be produced synthetically. As the synthetic form can be produced at a lower cost, the market for the natural form of β-carotene relies on the consumer perception that the natural form is superior. The natural form supplies cis-isomers in the natural ratio, which is generally accepted to be superior to the all-trans synthetic form (Spolaore et al. 2006).

5.1.2 Astaxanthin

Astaxanthin is produced commercially from *Haematococcus pluvialis*. This alga can accumulate astaxanthin up to 3 % dry weight (Pulz and Gross 2004). It is primarily sold as an additive in aquaculture feed for coloration purposes (Pulz and Gross 2004; Spolaore et al. 2006). The largest astaxanthin consumer is the salmon feed industry (Olaizola 2003). Human nutraceuticals has also expanded as a new market for astaxanthin since the 1990s. The annual aquaculture market of this pigment is estimated at $200 million, with an average price of $2500/kg (Spolaore et al. 2006).

Much research has been done on the production of *Haematococcus* for astaxanthin. In Hawaii and China, it is produced in open ponds, while other manufacturers, e.g., in Israel, have opted for closed systems (Pulz and Gross 2004). A combination of closed and open systems is also possible, the first to generate

unialgal biomass and the second to provide stress conditions in the final pigment accumulation stages of cultivation. *Haematococcus* is produced in a two-stage culture process: an initial algal growth stage under conditions favorable for biomass productivity, where the algae are green and flagellated, followed by a pigment accumulation stage where a stress condition or combination of stressors, usually high light and low nutrient conditions, are applied to trigger accumulation of pigment to high levels (1.5–3 % dry weight), yielding red, nonmotile cells (Spolaore et al. 2006).

Astaxanthin can also be manufactured synthetically. Natural production cannot compete with the synthetic form on price, but still finds a market due to consumer preference for natural products in the nutraceutical and cosmetics markets. There is little consumer influence in the aquaculture market as consumers remain uninformed about rearing practices, however, natural astaxanthin does offer enhanced performance in some applications, e.g., the natural form leads to enhanced deposition in the tissues of carp, chicken and red sea bream (Olaizola 2003; Spolaore et al. 2006).

5.2 *Phycobiliproteins*

Phycobiliproteins are unique to algae. They are a group of water-soluble, light harvesting proteins found in cyanobacteria, rhodophytes, cryptomonads, and glaucophytes (Sekar and Chandramohan 2008; Erikson 2008). They 'close the gap' in light absorption by absorbing light in the range (495–650 nm) that chlorophyll and carotenoids do not (Apt and Behrens 1999). The three major groups of phycobiliproteins, classified according to their spectral properties, are phycocyanin (PC), phycoerythrin (PE), and allophycocyanin (APC) (Table 3). The different phycobiliproteins are organized into complexes called phycobilisomes on the outer surface of thylakoid membranes (Fig. 4) (Sekar and Chandramohan 2008).

Phycobiliproteins are some of the most abundant proteins in many cyanobacteria (Eriksen 2008). The nature of the culture conditions, particularly light and nutrients, can influence the cellular content of phycobiliproteins. Glucose and acetate have been found to enhance cell growth and PC production in *Spirulina platensis* (Chen et al. 1997). PE production in *Porphyridium* was shown to depend on the concentration of chloride and nitrate, and to a lesser extent, sulfate and phosphate in the culture medium (Kathiresan et al. 2007).

The extraction of phycocyanin as a food colorant is fairly straightforward. Water-soluble components are extracted, generally from dry biomass, into buffer and the solids removed. Preparation of pure phycobiliproteins such as PE for use in fluorescent applications requires further separation and purification, generally with chromatographic techniques. The high cost of this process is offset by the high price of the purified product (up to $5000/g). The market size for the reagent is estimated to be $2 million (Radmer 1996).

Phycobiliproteins are used as food colorants and natural dyes: phycocyanin mostly in the food industry and phycoerythrin mainly in cosmetics. Phycocyanin

Table 3 Characteristics and origin of the major phycobiliproteins

Phycobiliprotein	Color	Max absorption (nm)	Max emission (nm)	Common sources	Commercial applications
Phycocyanin (PC)	Blue	610–620	650	*Spirulina*, *Anabaena*, *Synechococcus*, *Nostoc*, *Galdieria*	Food colorant, Nutraceutical
Phycoerythrin (PE)	Red	540–570	577	*Porphyridium*, *Porphyra*	Fluorescence
Allophycocyanin (APC)	Blue–green	650–655	660	*Spirulina*, *Microcystis*	Fluorescence

Eriksen (2008), Sekar and Chandramohan (2008), Abalde et al. (1998), Bermejo et al. (2006)

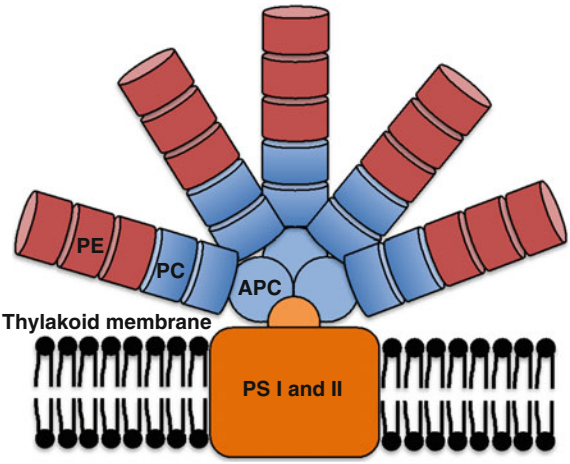

Fig. 4 Typical structure of a phycobilisome. *PS* photosystem, *PC* phycocyanin, *PE* phycoerythrin, *APC* allophycocyanin

has a bright blue color (Fig. 5) and is considered more versatile than alternative natural blue colorants, such as gardenia and indigo, although it has a lower stability to heat and light (Sekar and Chandramohan 2008). It is used in ice cream, soft drinks, candies, chewing gum, desserts, cake decorations, icings and frostings, milk shakes as well as lipsticks and eyeliners (Sekar and Chandramohan 2008; Eriksen 2008; Sarada et al. 1999).

Phycobiliproteins also have fluorescent properties that make them useful as fluorescent dyes and markers. PE and APC are most commonly used, although other phycobiliproteins also fluoresce at different wavelengths, yielding a toolbox of colors for multicolor detection systems. They can be conjugated with molecules with specificity to certain substrates, e.g., antibodies, receptions, strepavidin, and biotin, yielding fluorescent tags or probes that will bind to a specific protein, tissue or cell type. Fluorescent phycobiliproteins are used in fluorescent microscopy, flow cytometry, fluorescence-activated cell sorting, diagnostics, immunolabeling and

Fig. 5 Phycocyanin extracted from *Spirulina platensis* dissolving in water

immunohistochemistry (Eriksen 2008; Sekar and Chandramohan 2008). They have also been proposed for use as a light sensitive agent in cancer tumor photodynamic therapy (Niu et al. 2007).

As with many pigments, phycobiliproteins also exhibit pharmaceutical activity. Studies have indicated them to have the following properties: antioxidant, anti-inflammatory, neuroprotective, hepatoprotective, immunomodulatory, antiviral, and antitumor (Eriksen 2008; Sekar and Chandramohan 2008). Applications are expanding into the cosmetics, nutrition, and pharmacy markets (Pulz and Gross 2004).

6 Polyunsaturated Fatty Acids (PUFAs)

PUFAs, including the omega-3 fatty acids eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) as well as arachidonic acid (AA), are well known for their nutritional importance. They confer flexibility and selective permeability properties to cellular membranes, which have been shown to be vital for brain development, are beneficial to the cardiovascular system and form important nutraceutical and pharmaceutical targets in both human and animal subjects (Yongmanitchai and Ward 1989; Agostoni et al. 1995; Ward and Singh 2005). Health benefits associated with these fatty acids include brain health and development, prevention of cardiovascular disease, stroke, asthma, as well as rheumatoid arthritis prevention (Agostoni et al. 1995; Kris-Etherton et al. 2003; Ward and Singh 2005; Judé et al. 2006). Ultimately, the beneficial effects of omega-3 fatty acids result from both the competitive inhibition of compounds produced from

omega-6 fatty acids, and the direct production of beneficial compounds (eicosanoids) from the omega-3 fatty acids (Simopoulos 2002).

PUFA's are almost exclusively synthesized de novo by plants, microalgae and some bacteria. Animals can convert one form of PUFA to another through elongation and desaturation, but very few can synthesize these fatty acids (Brett and Müller-Navarra 1997). Longer chained PUFAs, with 20 or more carbon atoms, occur commonly in the lipids of microalgae, such as phytoflagellates and dinoflagellates, green and red microalgae, and in diatoms (Radwan 1991). PUFA's such as EPA and DHA are commonly found in deep-sea cold water microalgae. This is largely due to the fact that EPA and DHA increase the cell membrane fluidity, as they have very low melting points compared to other biolipids, in effect acting as membrane lipid anti-freeze for fish and algae living in cold environments (Brett and Müller-Navarra 1997; Thompson 1996).

The advantages of using algal biotechnology to produce PUFA's include easier extraction and purification (lower operational costs) as well as an increase in product content, quality and safety. Fungi, especially of the order *Mucorales*, and bacteria of the genera *Shewanella*, *Alteromonas*, *Flexibacter*, and *Vibrio* can accumulate relatively large amounts of EPA (Yongmanitchai and Ward 1989), however, the ability of bacterial and fungal fermentations to compete economically with traditional sources of omega-3 fatty acids is limited by low productivities and excessively long fermentation times (Barclay et al. 1994). Additionally, long-chain omega-3 fatty acid productivities reported for the microalgal fermentation systems are 1–2 orders of magnitude greater than productivities reported for fungal or bacterial systems (Table 4).

The current market price of EPA and DHA ethyl ester (95 % pure) in bulk quantities is about $650/kg and any new source would need to compete with that price (Belarbi et al. 1999). This article goes on to state that for microalgal EPA to be competitive with fish oil derived material, the price of microalgal biomass (dry basis) must not exceed about $5/kg. The estimated market size for DHA is estimated at US$15 million in the USA (Bux 2013).

6.1 PUFA Production

Microalgal species such as *Skeletonema costatum* and *Chlorella minutissima* can accumulate up to 45 % of their total fatty acid as EPA or DHA (Radwan 1991). Triacylglycerides (TAG's) fall under the saturated storage or neutral lipid group. This is of interest to the biofuel industry, whereas PUFA's tend to consist of membrane structural 'polar' lipids (Berge et al. 1995). Average cell age also has a profound effect on lipid classes, producing changes in the amounts of TAGs and polar lipids at different cultivation stages. In general, the content of polar lipids (which generally make up PUFA's) tends to decrease with culture age (Wen and Chen 2003). This does not however, avoid the complication of various EPA and DHA levels in different environments and in different species of microalgae.

Table 4 Polyunsaturated C20-fatty acid content of representative fungi, microalgae, macroalgae, and mosses (Radwan 1991)

Species	Percentage of total fatty acids[a]		
	20:3	20:4	20:5
Fungi (lower Phyomycetes)			
Allamyces javonicus		10.3 (0.3)	
Blastocladiella emersonii	3.2 (0.2)	16.4 (1.1)	
Synchytrium endobioticum		4.8	5.9
Achlya americana		9.9	7.1
Sparolegnia ferax	8.9 (0.6)	16.6 (1.2)	
Pythium debaryanum	11.9 (1.3)		
Phytophthora erythroseptica	12.1 (0.5)		
Microalgae			
Euglena gracilis		8.0	9.0
Peridinium trochoideum		1.0	13.0
Chlorella minutissima			45.0 (22.5)
Dunaliella tertiolecta		4.0	10.0
Porphyridium cruentum	2.0	36.0	17.0
Skeletonema costatum	2.0	2.0	30.0
Lauderia borealis		1.0	30.0
Navicula pelliculosa			26.0
Macroalgae (brown and red)			
Fucus platicarpus (brown)		11.0	8.0
F. serratus (brown)		10.0	8.0
F. versiculosus (brown)		10.0	8.0
Sargassum salicifolium (brown)		24.9 (4.3)	
S. boveanum (brown)		12.6 (0.6)	13.3 (0.7)
Layengaria stellata (brown)		6.9 (0.4)	10.0 (0.5)
Colpomenia sinousa (brown)		8.5 (0.7)	10.2 (0.8)
Plocamium coccinium (red)		12.0	22.0
Rhodomella subfusa (red)		14.0	24.0
Gelidium latifolium (red)		10.1 (0.9)	24.6 (2.1)
Eolysiphonia coacta (red)		6.4 (0.5)	4.4 (0.4)
P. lanosa (red)		10.2 (0.1)	38.6 (0.4)
Chondrus crispus (red)		25.0	26.9
Mosses			
Pogonatum urnigerum	75.7 (3.0)	2.8 (0.1)	0.4
Ctenidium molluscum	10.0 (0.4)	22.9 (1.0)	5.3 (0.2)

[a]Values in parentheses are expressed in g/100 g biomass, and were calculated considering total lipid contents

The lipid and fatty acid contents of microalgae also vary depending on culture conditions and strain. It has been found that, in some cases, lipid accumulation and composition can be enhanced by various growth conditions such as nitrogen starvation, silicon deficiency, phosphate limitations, high salinity, and heavy metal stress (Guschina and Harwood 1996). In one study, supplementation of media with 100 ng of vitamin B12 per liter produced a 65 % increase in yield of EPA from *Phaeodactylum tricornutum* compared to the control (Yongmanichai and Ward 1990). Changes in the lipid composition, lipid production rate, and growth of algae often occur as a result of variations in environmental or culture conditions. This, in turn, is complicated by different algal species responding uniquely to these environmental fluctuations.

7 Fertilizers and Soil Conditioners

Macroalgae have historically been used as soil fertilizer in coastal regions throughout the world. The carbohydrate polymers in macroalgae assist in particle adherence and water retention, as well as enhancing the mineral composition of the soil (Pulz and Gross 2004). They can be applied in the form of seaweed meal, liquid fertilizer, or concentrated plant supplements. The nitrogen and phosphates in the biomass act as fertilizer, and, perhaps most beneficially, the high concentration of plant growth hormones (e.g., auxins and cytokinins) in algal biomass can have a stimulating effect on plant growth (pers. comm. Kelpak 2014). Extracts from macro and microalgae have been shown to promote germination, growth or flowering (Pulz and Gross 2004). Liquid extracts from algae are used to aid plant establishment and growth, particularly in the remediation of mining areas (Pulz and Gross 2004). Polysaccharide producing species of *Chlamydomonas* have been used as soil-conditioning agents to prevent erosion (Metting 1996).

Nitrogen (N)-fixing microalgae are able to absorb and transform N from the atmosphere into a form accessible to higher plants (Pulz and Gross 2004). Several species of N-fixing algae (e.g., *Anaebena*, *Nostoc*, *Aulosira*, *Tolypothrix*, and *Scytonema*) are regularly used in rice cultivation in China, India, and Asia. They can provide in excess of 20 kg N/Ha/year, or up to a third of the requirements of traditional rice cultivars (Metting 1996). Despite the demonstration of algae as useful plant growth and soil enhancers, there has been limited research effort in this area to date.

8 Fuels

Aside from foods to feeds, the other commodity industry where algae have received much focus is in the production of fuel. World energy demand continues to rise and the necessity to reduce fossil fuel use is becoming more urgent, and hence, there is

currently great focus on the production of sustainable energy. Algae are a potential source of a variety of renewable fuels (Harun et al. 2010). Algal biomass could be combusted directly or converted via liquefaction, gasification or pyrolysis into 'green crude,' syngas, heat, electricity, and liquid fuels. Algae can also produce significant quantities of lipids and carbohydrates that could be converted into biodiesel or bioethanol, respectively. There has also been some research into the use of algae for biological hydrogen production (Benemann 2000). In addition, algal biomass could form the substrate for anaerobic digestion to produce methane (Chisti 2013).

Despite considerable academic and commercial interest, algal fuels are not currently widely available on the market, largely due to the practical and economic challenges of producing a low value, commodity product from algae on a very large scale. Over 50 algal biofuel companies have existed and as yet none are producing commercial-scale product at competitive prices (Milledge 2011). Much of the initial work on production of fuel from algae was conducted by the Solar Energy Research Institute under the Aquatic Species Program (Sheehan et al. 1998). Although the technical feasibility of algal biofuels has been demonstrated (Miao and Wu 2006; Mata et al. 2010), at present, the process appears to be uneconomic. This is largely due to the artificially low cost of energy derived from fossil fuels, which relies on a large historical storage reserve of energy, accumulated from biomass, such as microalgae, over millennia, and hence does not take into account the cost of producing the fuel, nor that of CO_2 emissions. It is essential that the net energy balance of algal fuel production be positive (Walker 2009). The reduction in process energy input will be vital here.

To date, commercially successful algal companies have focused on products with a high sales price. For commodity algal products such as fuels to be successful, the cost of algal production needs to be dramatically reduced from current costs. To produce a reasonable quantity of fuel, the scale of production would also need to be orders of magnitude greater than current production (Wijffels 2007).

9 Bioactive Compounds

The intricacy of microalgal chemical composition and range of biochemical products has led to the interest and potential use of these compounds in the food, feed, pharmaceutical, and research industries (Pulz and Gross 2004). Bioactive compounds are defined by Bhatnagar (2010) as *secondary metabolites produced by certain microalgae displaying a degree of bioactivity either against another microorganism, or acting against certain physiological states of a diseased body*. Algae produce a variety of secondary metabolites with anticancer, antifungal, antibiotic as well as antiviral properties, as well as a variety of toxins.

9.1 Anticancer Compounds

Anticancer bioactive compounds include PUFAs (specifically EPA), carotenoids, and lipopeptides. PUFAs have been used in cancer treatments as an adjuvant for chemotherapy, as compounds with direct anticancer effects, or as supplements to alleviate the effects of radiation and chemotherapy (Vaughan 2013). The anti-inflammatory nature of EPA is likely the source of the anticancer effects seen, as it reduces damage caused by oxidative stress (Vaughan 2013). Furthermore, the direct anticancer effects of these compounds have been suggested to work specifically against tumors through the inhibition of angiogenesis and metastasis (Baracos 2004). Carotenoids also act as antioxidants, reducing stress from oxidative damage. *Dunaliella salina* is a good natural source of β-carotene, which has been shown to reduce the risk of cancer and degenerative diseases in humans (Ben-Amotz 1999).

Nitrogen-containing compounds (lipopeptides), occurring in cyanobacteria, target tubulin or actin filaments in eukaryotic cells, making them an attractive source of anticancer agents (Jordan and Wilson 2004). An example of these antimicrotubule agents are curacin-A and dolastatin 10, currently in preclinical and/or clinical trials as potential anticancer drugs (Gerwick et al. 2001). The majority of these biomolecules are found in *Nostocales*, and members belonging to the genera *Lyngbya, Oscillatoria,* and *Symploca* (Tan 2007).

9.2 Antifungal and Antibiotic Compounds

Bioactive compounds with antifungal and antibiotic activity are currently investigated for use as components in antifouling paints for maritime industries around the world (Bhadury 2004). Microalgal species tested for antibiotic activity included *Rhodophyta*, *Chlorophyta*, and *Phaeophyta* and exhibit inhibition against the gram-positive bacteria *Bacillus subtilus* and *Staphylococcus aureus* (Falch 1992).

9.3 Antiviral Compounds

The antiviral bioactive compound cyanovirin-N (CV-N) was discovered as a constituent of the cultured cyanobacterium, *Nostoc ellipsosporum*, which irreversibly inactivates diverse primary strains of HIV-1 during sexual transmission of HIV. CV-N also blocks the cell-to-cell transmission of HIV infection (Burja 2001; Yang 1997). Another bioactive compound, shown to protect human lymphoblastoid T-cells from the cytopathic effect of HIV infection, was extracted from blue-green algae *Lyngbya lagerheimeii* and *Pormidium tenue* (Gustafson et al. 1989).

9.4 Toxins

Marine cyanobacteria are also a source of potent neurotoxins that target the eukaryotic voltage gated sodium channel (VGSC) and may act as either blockers (lalkitoxin and jamaicamide A) or as activators (antillatoxin) of the eukaryotic VGSC. Hepatotoxin includes the cyclic peptides microcystin and nodularin. *Microcystis aeruginosa* and *Nodularia spumigena* synthesize these toxins that are able to destroy liver cells by the inhibition of protein phosphatases (Burja 2001). To date, over 50 different variants of microcystins have been isolated from the species *Anabaena*, *Hapalasiphon*, *Microcystis*, *Nostoc*, and *Oscillatoria* (Singh 2005).

The potential contribution of microalgae to the discovery of new bioactive compounds is large. Natural products have been isolated from a wide variety of organisms and tested for various biological activities. Cyanobacteria are regarded as good candidates for drug discovery, with applications in the pharmaceuticals and cosmetic sector. Despite the vast diversity of microalgae and the plethora of bioactivity displayed, no drug of microalgal origin is on the market as of yet, although several compounds are currently in clinical trials (Table 5, *). With novel research investigating the effects of these (and new) compounds, more can be expected to enter clinical trials, which may eventually lead to the market entry of these bioactive compounds.

Table 5 Consolidated group of bioactive compounds applicable to the pharmaceutical sector: bioactive compounds and microalgal producers

Bioactive type	Bioactive compound	Microalgal producer	References
Anticancer Antimicrotubule Antitumor	Dolastatin-10* Curacin-A* Dolastatin-15*	Cyanobacteria	Tan (2007), Gerwick (2001), Tan (2007), Mita (2006), Burja (2001)
Antifungal	Toyocamycin Ciguatoxin Okadaic acid	Cyanobacteria *Gambierdiscus toxicus* *Prorocenrum lima*	Bhatnagar (2010)
Antiparasitic Antimalaria	Calothrixin A	Cyanobacteria	Rickards (1999)
Anti-protozoal	Viridamides A Crude extracts	Cyanobacteria Green-algae (*Cladophora, Codium* and *Ulva*)	Simmons (2008), Allmendinger (2010)
Antiviral	Cyanovirin-N Extract	*Nostoc ellipsosporum* *Lyngbya lagerheimeii* *Phormidium tenue*	Burja (2001) Yang (1997) Gustafson et al. (1989)

*Bioactive compounds in various stages of clinical trials

Table 6 Cosmetics incorporating algal extracts: products and microalgal producers (adapted from Spolaore et al. 2006; Kim et al. 2008)

Cosmetic effect	Market product	Microalgal producer
Anti-aging	Protulines®	*Arthospira (Spirulina)*
Stimulates collagen synthesis	Dermochlorella®	*Chlorella vulgaris*
Skin tightening	Pepha®-Tight	*Nannochloropsis oculata*
Stimulates cell proliferation	Pepha®-Ctive Blue Retinol™	*Dunaliella salina*
Repairs UV damaged skin	Remergent™	*Anacystis nidulans*

10 Cosmetics

In addition to the use of algal-derived hydrocolloids as thickening and water-retaining agents in cosmetics, macro- and microalgal extracts and bioactive compounds, such as antioxidants, pigments, and essential fatty acids are incorporated into a variety of cosmetic products ranging from antiaging creams and anti-UV products to hair care products and anti-irritant skin peels (Spolaore et al. 2006). Other cosmetics incorporating algae include microalgal whole cell shampoo and conditioner, oil and salt scrubs, clay masks, and soaps (Oilgae.com). Current market products (Table 6) usually do not specify the exact formulation of each product, however, research investigating compounds and possible cosmeceutical effects suggest they are likely microsporines and microsporine-like amino acids, tocopherols, phenolic compounds, and terpenoids that aid in skin protection against UV radiation that behave as antioxidants, emollients, and diuretics (Kim et al. 2008). *Chlorella* is most commonly used in cosmetics production but other microalgae currently investigated for their biochemical properties include *Parachlorella*, *Neochloris*, *Bracteacoccus*, *Scenedesmus*, *Anabaena*, *Ankistrodesmus*, *Chlorococcum*, *Schizochytrium*, *Spirulina*, *Crypthecodinium*, *Cryptomonas*, *Isochrysis*, *Rhodococcus*, and *Nannochloropsis* (oilgae.com). The global cosmetics industry is worth more than $40 billion—and ever increasing. Prominent companies currently involved are Heliae, Solazyme, Indeed Laboratories, Biotherm, and Algalscientific.

11 Chemicals

The adaptation of classical fermenters to photosynthetic organisms and the use of heterotrophic algae have allowed the production of biomass of consistent quality under controlled conditions. This allows for the potential production of high quality, higher value pharmaceuticals, and specialty biomolecules from algae (Apt and Behrens 1999). Algae grow more slowly than other microorganisms, such as bacteria and yeast, and reactor design is complicated by the requirement for efficient

light distribution throughout the culture. Therefore, there must be a compelling reason to use algae for the production of biologics that could be produced in other microorganisms. Such reasons could include:

- reduced cost and improved sustainability through the use of light energy to power production
- reduced risk of infection and contamination by production of animal proteins in plant cells.

11.1 Stable Isotopically Labeled Compounds

Microalgae are ideally suited to the production of stable, isotopically labeled compounds. Cultivated under strictly controlled conditions, algae can be used to convert ^{13}C, ^{15}N and ^{2}H from simple inorganic compounds ($^{13}CO_2$, $^{15}NO_3$, and $^{2}H_2O$) into more complex labeled organic substrates (e.g., amino acids, sugars, lipids, and nucleic acids). These labeled compounds have application as substrates for investigating the metabolism of other microorganisms, in elucidating the structure of proteins, and for noninvasive diagnostics. They can be used to investigate the metabolism of microorganisms, e.g., bacteria, yeast, and mammalian cells, by tracing the labeled carbon through metabolic pathways and evaluating its incorporation into macromolecules, e.g., lipids, carbohydrates, and proteins. NMR technology, together with stable-isotope-editing techniques can also be used to elucidate the structure of proteins into which isotopes have been incorporated. Another application of microalgal-produced isotopes is breath tests for medical diagnosis. A substrate labeled with ^{13}C is ingested, and the rate at which it is absorbed, metabolized and expelled as $^{13}CO_2$ is measured. The rate and amount of appearance of $^{13}CO_2$ in the breath is indicative of the patient's physiological state (Apt and Behrens 1999; Radmer 1996). The market for labeled compounds is approx. $5 million, with prices in excess of $100/g (Radmer 1996).

11.2 Biomanufacturing and Specialty Chemicals

Green microalgae have attracted interest recently as a potential host for the production of recombinant proteins. Algae can be used as cell factories to produce enzymes, vaccines, biologics, and bioactive molecules, such as antioxidants, toxins, and molecules with pharmaceutical properties, such as anticancer or antimicrobial activity. The advantages of using photosynthetic plant cells include safety, low cost of substrates, and ease of genetic transformation (Rasala and Mayfield 2014). *Chlamydomonas reinhardtii* is the most widely used species due to well-established transformation methods and genetic tools, as well as a fully sequenced genome, but

other species such as *Chlorella* and *Dunaliella* are also potential candidates (Rasala and Mayfield 2014).

Microalgae have been investigated for the production of vitamins and vitamin precursors, e.g., ascorbic acid, riboflavin, and tocopherol, for food, cosmetics, and aquaculture (Metting 1996). Heterotrophic production of ascorbic acid (vitamin C) by *Chlorella* was demonstrated by Running et al. (1994). Microalgae have also been investigated as a source of polysaccharides, lipids and hydrocarbons for use in food, cosmetics, and as lubricants. For example, the green alga *Botryococcus braunii*, although a slow grower, produces and excretes a range of interesting long-chain hydrocarbons. Investigations of nondisruptive harvesting by 'milking' are currently underway (Moheimani et al. 2013).

11.3 Feedstock for Industrial Bioprocesses

Microalgae could potentially be used as a nutrient source for other microorganisms in the production of industrial chemicals. The photosynthetic production of algal biomass, particularly if produced for environmental reasons, e.g., CO_2 sequestration, could provide a cheap and sustainable source of nutrients, e.g., carbohydrates, sugars, proteins, lipids, for fermentation by other microorganisms to produce valuable products (Harun et al. 2010). For example, Nakas et al. (1983) found the yield of solvents (propanediol, butanol, and ethanol) from *Clostridium pasteurianum* was improved when fed on *Dunaliella* sp. A yield of 14–16 g L^{-1} of mixed solvents was achieved on the microalgal substrate.

12 Environmental Applications

12.1 CO_2 Sequestration

Microalgal biomass contains about 50 % carbon, usually obtained photosythetically from CO_2. This, coupled with their ability to grow on non-arable land, utilizing waste nutrients, have made them an attractive potential vehicle for carbon sequestration (Milledge 2011). The global rise in CO_2 levels is an international problem demanding feasible solutions. The ultimate goal should be to drastically curb CO_2 emissions through decreasing dependency on fossil fuels, as well as investigating options for CO_2 sequestration. Microalgae, due to their high growth rates and the fact that they do not require arable land, appear to be a promising option for fixation of CO_2 photosynthetically from point source emissions (Harun et al. 2010). Several companies and research groups have investigated this process. It has been shown to be technically viable but not economically feasible and has yet to be implemented on a large scale (Pulz and Gross 2004). Significant challenges include the large land areas required (and in close proximity to the power station),

due to the low biomass concentrations achieved in the currently most economically feasible cultivation option: open ponds, and the large requirement for water and fertilizers such as nitrogen and phosphates. The area remains promising, as several valuable products could potentially be produced from the algal biomass, and one of the limitations to algal cultivation generally is CO_2 provision. Nutrients may need to be recycled, e.g., through anaerobic digestion of the residual biomass after product extraction, and hypersaline species sought which do not require a supply of fresh water (Fon Sing et al. 2014).

12.2 Wastewater Treatment and Bioremediation

Algae are important components of biological treatment methods for municipal and industrial effluents. They contribute oxygen from photosynthesis, e.g., in facultative ponds and high rate oxidation ponds, to aerobic microorganisms. These algal communities are commonly dominated by green microalgae (e.g., *Chlorella*, *Scenedesmus*, *Ankistrodesmus*) (Metting 1996). Algae have been investigated for the final polishing of municipal wastewater, or the direct treatment of a variety of industrial wastewaters. Algae are very efficient at removing nutrients such as nitrogen and phosphates, as well as environmental contaminants, e.g., heavy metals and toxins, from water, (Harun et al. 2010). However, microalgae have a much slower growth rate than bacteria, which limits the speed of their bioremediation. The biosorption and removal of metals, such as chromium, cadmium, nickel, and zinc is an important function in the bioremediation of industrial effluents such as mining or ash dam waters (Murphy et al. 2008).

The use of immobilized algae, particularly as biofilms, e.g., in an algal turf scrubber, appears to be a promising strategy for water treatment and bioremediation. The quality of large amounts of relatively dilute wastewater can be improved, while the biomass is retained. Depending on the nature of the wastewater, the resulting algal biomass could be used for animal feed, or anaerobically digested to produce methane (Spolaore et al. 2006). Cyanobacteria were reported to be effective in the treatment of wastewater from pulp to paper processing. Treatment of wastewater containing phenols with bacteria requires addition of an organic carbon source, whereas algal cells can efficiently remove phenols from wastewater using CO_2 as the carbon source. *Scenedesmus* has also been investigated for degrading cyanide from mining process waters (Harun et al. 2010).

13 Conclusions

Algae are responsible for over 50 % of the photosynthesis on earth. They are the primary producers in the oceans and are efficient and adaptable sunlight factories, producing a range of useful products. It has been estimated that at least 200,000

algal species exist worldwide, with roughly 36,000 algal species known. The enormous diversity of algal species remains commercially underutilized. Current commercial production relies on only 10–20 species, most of them macroalgae (Radmer 1996; Pulz and Gross 2004; Milledge 2011). While humans have taken advantage, to a limited extent, of naturally harvested algae, such as seaweeds and *Spirulina*, as a food source for centuries, it is only relatively recently that microalgal biotechnology has offered the potential to produce a wide array of products from industrial chemicals to pharmaceuticals (Olaizola 2003). Dozens of useful products from microalgae have been identified, but there have been few successes in scale-up and marketing (Olaizola 2003). Major commercial products are limited to the food and feed industries, despite the wide range of potentially useful metabolites produced, beyond the current exploitation (Milledge 2011).

General limitations to the commercialization of algal products include their low growth rates compared to other microorganisms, such as bacteria or yeast, and low maximum biomass densities in outdoor algal culture (Apt and Behrens 1999). As a result, large culture volumes are required per unit product, which implies large capital and running costs, particularly in terms of pumping, mixing, and harvesting, as well as large volumes of fresh water. Much research is being directed toward enhancing the biology of the algae as well as engineering cheaper and more efficient photobioreactors. At present, however, a financially viable algal production process requires an easily harvestable species (e.g., filamentous *Spirulina*), a high value product that can cover the relatively high cost of production (e.g., pigments, PUFAs, and other fine chemicals), a suite of products derived from a unit of biomass which collectively cover the costs (the biorefinery concept), or very low costs of production, such as in natural lagoons (e.g., β-carotene production in Hutt lagoon, Australia). Another challenge to commercialization of algae as foods, food additives or in the production of pharmaceuticals is the regulatory environment, and customer acceptance. Successful authorization of a new species, product, or application can take several years (Pulz and Gross 2004).

None of the challenges associated with large-scale production of algal products appear insurmountable, however, a concerted, coordinated effort across disciplines is required for commercial success. Algae have great promise as the next generation of biological factories, with the potential to produce a wide range of valuable compounds for food, feed, fuel, pharmaceutical, and research use (Milledge 2011; Wijffels 2007; Radmer 1996; Pulz and Gross 2004; Spolaore 2006). With the ever-increasing world population, the requirement for agricultural land is becoming a global challenge. In addition to photosynthetic production, algae have the advantage of not requiring arable land, which could help to displace some nonfood-related production processes to arid and semi-arid regions. The industry focus should be on those products with a large potential market, where production using microalgae leads to clear competitive advantages (Milledge 2011). It is also imperative that the research focus around algae perseveres to address the technical hurdles that currently prevent a broader commercial exploitation of this valuable natural resource.

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Harmful Algae and Their Commercial Implications

Lesley Rhodes and Rex Munday

Abstract Microalgae are an important source of primary production in the oceans' food webs and are therefore beneficial to humankind. Of the huge diversity of algae only 6 % are estimated to multiply to form harmful algae blooms (HABs) and less than 2 % of the described species produce potent biotoxins. This small number of algae can, however, have great economic impacts in terms of public health, commercial fisheries and recreation and tourism.

Keywords Microalgae · Harmful algae blooms · Toxins · Economic impact · Public health

1 Introduction

Microalgae are an important source of primary production in the oceans' food webs and are therefore beneficial to humankind. Of the approximately 5000 living microalgae species known, 6 % multiply to form harmful algae blooms (HABs). Less than 2 % of the described species produce potent biotoxins (Hallegraeff 2014), which may be directly toxic to shellfish or fish, or toxic to animals or humans consuming contaminated seafood.

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The key economic impacts of HABs are:

- public health
- commercial fisheries
- recreational and tourism
- monitoring and management costs.

In an effort to assess the impact of HABs in U.S. waters, and to link research with policy, a "National assessment of harmful algal blooms in U.S. waters" has been published (CENR 2000). At that time, the average total impact was calculated as between US$34 M and $82 M over the 6 years data interrogated, with public health and commercial fisheries having experienced the greatest economic impacts. The cost of monitoring programmes, while giving protection to the industries, is in itself expensive, with its own economic burden for the industries (Anderson et al. 2000).

In the case of shellfish aquaculture, if shellfish become contaminated with biotoxins, and harvesting is delayed until toxin depuration is confirmed, there is the potential risk of shellfish spawning and losing condition. For the finfish aquaculture industry, fish cages may be towed to non-HAB areas and the stock saved. In the wild, fish will generally escape HAB impacts unless trapped in bays or harbours. In the latter case, deoxygenation of the water or clogging of gills by microalgae, rather than the biotoxins themselves, may be the cause of death (Jones and Rhodes 1994).

Human illnesses associated with HABs do occur, for example respiratory illness from breathing aerosolised, toxic microalgal particles. Such particles are created when coastal waters are agitated during storms. The costs to public health services and from lost work hours from such events are unknown (Backer et al. 2003).

As the scale of microalgae production increases, bioprocess engineering challenges and safety issues will also increase (Garcia Camacho et al. 2007). Advances continue in methods of detection and enumeration of HABs. New molecular tools (Rhodes et al. 2013; Wood et al. 2013) and chemical tests (McNabb 2014) support public health and marine farm and biotechnology industry management strategies. In fact, HAB toxins may bring economic rewards when mass produced and processed for such uses as standards for chemical testing. Patents and patent applications for dinoflagellate toxins are documented in the literature; these are mostly for the production and extraction/purification of toxins rather than for new products for medicine or industry (Gallardo-Rodríguez et al. 2012).

2 Public Health: Direct Impacts of HABs on Microalgae Products

Many and varied products are produced directly from microalgae. These include aquaculture and animal feeds, renewable fuels, pharmaceuticals and health supplements. Microalgae feedstocks were worth US$700 M one decade ago (Pulz and Gross 2004) and production continues to increase globally. The health food market

is valued at many millions of dollars and includes production of carotenoids, polyunsaturated oils and antioxidants to name a few.

Many thousands of metric tonnes of *Arthrospira platensis* (previously known as *Spirulina platensis* and commonly marketed as 'spirulina') are produced every year. Spirulina is produced in at least 23 countries, with key producers being the USA, China, Thailand and India (Takenaka and Yamaguchi 2014). There are reports suggesting producers can earn approximately US$15 M per year from an annual production of >900 tonnes. The main use is as a dietary supplement for human consumption, but it is also used as a supplement in animal feeds, in particular for farmed fish and poultry.

Public concern regarding cyanobacteria and their toxins has increased in recent years, due to an increase in the prevalence of blooms and a greater awareness of the health risks associated with toxic species. Cyanotoxins can cause various health issues from gastrointestinal disturbances to liver cancer and neurological impairment. Spirulina itself is non-toxic and safe for consumption. However, because photobioreactors and closed systems are costly to operate, open pond raceway systems are commonly used for the cultivation of spirulina. This means that there could be some risk of contamination of cultivated spirulina by toxic species. Cyanobacterial poisonings are well documented (Woodhouse et al. 2014), but there are no reported illnesses related to cyanotoxins in spirulina products, probably due to well-managed monocultures of *A. platensis*. However, to remove the perceived risk of contamination, the "Technical Booklet for the Microalgae Biomass Industry" was produced as a guide for the use of assays for detection of two cyanotoxins (microcystins and nodularins) in human food supplements (Gershwin and Belay 2009).

Anatoxin-a is an unregulated cyanotoxin that has been detected in dietary supplements. In one study, 7.7 % of supplements tested were positive for anatoxin-a. This included one contamination each of fish, bird and human cyanobacterial food products (Rellán et al. 2009).

A potential positive economic benefit from cyanobacterial toxins is the utilisation of their ecological role as allelochemicals, which could have uses as algaecides, herbicides or insecticides (e.g. against mosquito larvae) (Berry et al. 2008).

3 Impacts of HABs on Aquaculture

3.1 Shellfish

Microalgae are also the basis of farmed fish and shellfish industries. The market is rapidly expanding as the demand for seafood increases and the wild catch declines. The global aquaculture market in 2012 was estimated at US$135 B and growing (Sheela 2013). The small percentage of microalgae species that do produce toxins can impact on the seafood industry (Fig. 1). However, the impacts of HABs on the global economies are difficult to assess as management 'around' HAB events is

Fig. 1 Marine farms in the Marlborough Sounds, New Zealand. Greenshell mussels™ (*Perna canaliculus*) growing on ropes (*left*) and caged Chinook salmon (*Oncorhynchus tshawytscha*; *right*) (*Images* Cawthron Institute, Nelson, New Zealand.)

often practised. Losses may then be avoided, although there may be a break in the continuity of supply, with associated costs.

In New Zealand, from late 1992 to early 1993, a bloom of the dinoflagellate genus *Karenia* (predominantly *K. mikimotoi*) led to a neurotoxic shellfish poisoning (NSP) event (Jasperse 1993; Todd 2002). This caught the industry and government agencies by surprise. Before this event, New Zealand's biotoxin programme was minimal, and because of the event a 3-month closure of the entire New Zealand coastline was instigated, with all shellfish industry operations suspended until the cause was determined. Subsequently, ongoing monitoring programmes were established.

Ireland experienced a similar unexpected setback with the emergence of a toxin in mussels harvested from Killary Harbour in 1995 (Twiner et al. 2008; Tillmann et al. 2014). The causative organism was then unknown, but contaminated shellfish was causing human illness (Furey et al. 2010). The Irish shellfish aquaculture industry, with a value of >US$65 M per annum, suffered economic losses from closed markets until the issue was resolved. As a result, a new biotoxin management plan and monitoring regime was implemented. The toxins responsible were identified as azaspiracids (AZAs), although the organism responsible for their production was not identified until more than a decade later. The chemical-based technology of liquid chromatography coupled with mass spectrometry (LC–MS/MS) enabled the fast and reliable detection of AZAs. The small dinoflagellate producer was classified as *Azadinium spinosum* (Tillmann et al. 2009). Many other *Azadinium* species, both toxic and non-toxic, have been identified since 2007 (Tillmann et al. 2014).

Australia's Tasmanian oyster industry provides direct employment for >300 people with approximately 4 million dozen oysters harvested annually at an estimated value to the farmer of >US$20 M. The mussel industry continues to expand in Tasmania with production during 2010–2011 of 717 tonnes, valued at US$2.3 M. In late October 2012, Japanese import authorities identified paralytic

shellfish toxins (PSTs) at levels above the regulatory limit in a shipment of fresh mussels (*Mytilus galloprovincialis*) from the east coast of Tasmania. Recalls from export markets were instigated for >100 tonnes of mussels harvested between September and October. The cause of the biotoxin contamination of the mussels proved to be an undetected bloom of the PST-producing dinoflagellate *Alexandrium tamarense*. The bloom was later shown to have impacted several other commercial seafood species including oysters, rock lobsters, clams and scallops (Campbell et al. 2013). Oyster producers reported that, due to the harvesting closures which ensued, about 540,000 dozen oysters valued at US$3.4 M were withheld from the Australian markets. Losses flowed on to service providers. For example, freight operators had estimated losses of US$583,000. On a direct economic basis, the cost of the bloom event was estimated at US$8 M, representing impacts on revenue and expenditure across all stakeholders. Since then, monitoring procedures have been reviewed and exports are again being accepted into overseas markets (Campbell et al. 2013).

The Japanese pearl oyster industry was hit hard in the 1990s by another dinoflagellate, *Heterocapsa circularisquama*, which causes bivalve mortalities. The industry has lost millions of dollars since the first event in 1998 (GEOHAB 2010).

3.2 *Finfish*

Fish mortalities may occur because of hypoxia or anoxia, or from mucilage production by some microalgae species that can clog fish gills.

Asia, particularly China, has the highest production of aquaculture fish and shellfish globally (89 % of global marine aquaculture production in 2006), and this is at a time when HABs are reported to be increasing (GEOHAB 2010). A key species of concern is the ichthyotoxic dinoflagellate, *Cochlodinium polykrikoides*, which caused losses of US$0.8 M in western Japan in 1979 (GEOHAB 2010). Since that first report the losses have been even greater, for example US$330 M in the Yatsushiro Sea in 2000.

In Korea, *C. polykrikoides* caused economic losses of US$95.5 M in 1995. This loss was borne by fish farmers. Losses have continued over the last two decades and intensive efforts have gone into the early prediction and warning of blooms (Kim 2012). Korea now has a national monitoring programme for both shellfish and finfish HAB risks with the aim of early detection; monitoring even includes aerial helicopter surveys. Mitigation is a research focus at national research institutes and universities and a novel mitigation method has been developed which uses the settling of yellow clay particles to drag blooms down to the sediments and at the same time adsorb toxins (Fig. 2; Kim 2012; Choi and Lee 2012).

Sea cage Chinook salmon (*Oncorhynchus tshawytscha*) mortalities have caused serious financial losses for the New Zealand finfish aquaculture industry (Fig. 1). There have been two major events, one in Big Glory Bay (Stewart Island) in 1989, and another in the Marlborough Sounds in 2010. The causative organisms were the

Fig. 2 Clay application to remove *Cochlodinium polykrikoides* blooms and their toxins from fish farms in South Korea (*Image* L. Rhodes)

raphidophyte *Heterosigma akashiwo* and the dictyophyte *Pseudochattonella verruculosa* (Fig. 3), respectively (MacKenzie 1991; MacKenzie et al. 2011). The salmon deaths in 1989 (>600 tonnes) were estimated to cost the industry approximately US$15 M (Chang et al. 1990) and the 2010 event (losses of 200 tonnes) was only tempered by the removal of the sea cages to an area free of the bloom.

P. verruculosa blooms have also been responsible for losses of Atlantic salmon (*Salmo salar*) in Scandinavian coastal waters (1100 tonnes in 2001; Riisberg and Edvardsen 2008) and have caused losses of a variety of fish species in Japan (Baba et al. 1995; Yamamoto and Tanaka 1990).

In the northern summer of 1997, fish kills associated with the heterotrophic dinoflagellate, *Pfiesteria piscicida*, occurred in Maryland; complaints of health effects were also reported by local fishers. The physical impacts were localised, but the economic ramifications affected the whole state. Consumers panicked and sales of seafood went down with a calculated loss of US$43 M. The 'halo effect' also led to the avoidance of Maryland for boat charters with the recreational fishing industry also losing several million dollars. An overall economic loss of US$50 M was estimated for just a four-month period (CENR 2000).

Ciguatera fish poisoning (CFP) is considered a neglected disease globally but its incidence appears to be increasing (Chinain et al. 2010) with economic impacts through loss of sales of reef fish. No Polynesian archipelago in the Pacific is considered safe from CFP. There has been a >6-fold increase in incidences in the Australes, French Polynesia, over a 2-decade period (Chateau-Degat et al. 2007; Chinain et al. 2010). Effects of CFP are mainly from the consumption of reef fish that have grazed on macroalgae harbouring the causative organisms, which are cells of toxic species in the benthic/epiphytic dinoflagellate genus, *Gambierdiscus*

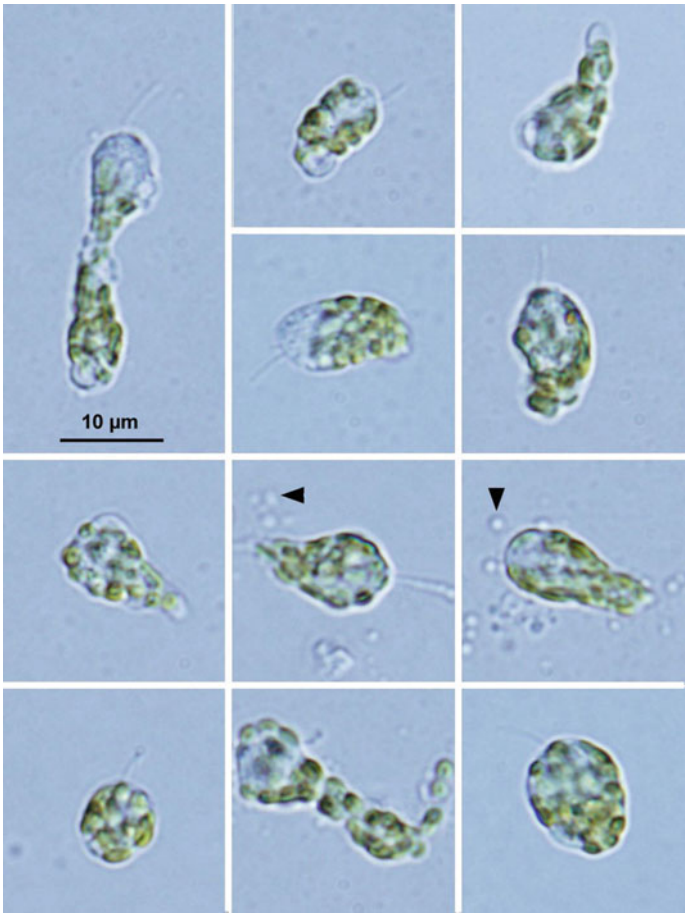

Fig. 3 Light micrographs of *Pseudochattonella veruculosa* (*Image* L. MacKenzie, Cawthron Institute, NZ.)

(Litaker et al. 2009, 2010). *Gambierdiscus*, and the associated CFP incidences, is expanding its range, even into temperate regions (Kohli et al. 2014). CFP is common in many Pacific Islands, with the greatest incidence being reported from the Cook Islands (Rongo and van Woesik 2012). According to Fleming et al. (1998) the estimated global number of poisonings was 50,000–500,000 per year nearly two decades ago. A later report estimated 5–600 cases per 10,000 people annually, depending on the geographic region (Lange 1994). In Australia, OzFoodNet data (2001–2010) shows 283 ciguatera poisonings during that decade (Braidotti 2014). The economic impacts of CFP are largely unreported, but are likely to increase in the future with the double impact of rising seawater temperatures and the acclimatisation of the dinoflagellates to cooler waters.

Lyngbya majuscula is a benthic filamentous marine cyanobacterium that occurs throughout the tropics and subtropics to depths of 30 m. This species impacts on commercial fisheries because fish will actively avoid bloom areas. Local fish stocks are affected when toxins and/or low oxygen levels lead to mortalities of fish and other aquatic organisms.

Freshwater fisheries can also be impacted by cyanobacteria HABs. For example, there was an estimated loss of approximately US$200 K per annum for commercial fisheries in the UK due to damages induced by cyanobacterial blooms (Pretty et al. 2003; Hamilton et al. 2014).

4 Impacts of Coastal HABs on Tourism

Karenia brevis has been cited as the cause of respiratory distress in beach-goers in Florida, and has been responsible for the deaths of iconic sea mammals such as the manatee. This has resulted in a loss of tourism dollars, estimated as US420 M annually (CENR 2000), although the actual losses to the region are difficult to determine. The average losses by beachfront restaurants in Southwest Florida were statistically determined over a 7-year period as 13.7–15.3 % when 'red tides' were present (Morgan et al. 2009).

Blooms of the benthic/epiphytic dinoflagellate genus *Ostreopsis* occur throughout the Mediterranean. These have caused human health issues and hospitalisations both from beach-goers inhaling the aerosolised toxins (palytoxin-like compounds) produced by the dinoflagellate, and from skin irritation from direct contact with the toxins. These events have the potential for similar economic impacts on tourism as occurred in Florida. The aerosol route is the main cause of concern in this regard, although invertebrate taxa may accumulate low concentrations of the toxin with a risk of illness from consumption of these animals (Ciminiello et al. 2006, 2008; Munday 2008; Shears and Ross 2009; Rhodes 2011; Rhodes et al. 2002, 2008a, b). It is noteworthy that human deaths have been linked to the eating of palytoxin-contaminated crabs in the Philippines (Gonzales and Alcala 1977; Yasumoto et al. 1986).

Ciguatera fish poisoning does not appear to impact on tourism at present, although the true impacts are unknown. Many tourists, having eaten contaminated reef fish in tropical regions, show symptoms of CFP after returning to their country of origin, which may discourage future visits. The symptoms range from relatively minor (diarrhoea) to serious and long-lasting neurological symptoms and even death. If such problems become more widely known, a negative impact on tourism in the Pacific is to be expected.

Cyanobacterial blooms are a common occurrence globally. In Australia, the toxins produced by *L. majuscula* can cause skin, eye and respiratory irritation (Osborne et al. 2001). These blooms have increased in Moreton Bay, Queensland, in recent years. The Moreton Bay Regional Council has developed a '*Lyngbya* Management Strategy' and a response plan to reduce impacts of the blooms on

human health and the economy. The South East Queensland's recreational fisheries have been valued at more approximately US$180 K and the blooms, which occur throughout the state, can lead to a decrease in visitor numbers and thus to a significant economic impact on the tourism industry. Shore clean-ups by the former Caboolture Shire Council to remove *Lyngbya* washed onto its local beaches reached approximately US$700 K over four summers.

The Tasmanian PST event of 2012 had a negative impact on the tourist industry in that state. The economic impacts on accommodation and restaurants/cafes ranged from US$14 K to $90 K per month (Campbell et al. 2013).

5 Costs of Managing HABs

The initial cost of monitoring programmes to the industry and New Zealand government was approximately US$3.6 M per annum, although this figure has since been scaled back substantially (Rhodes et al. 2013). More cost-effective biotoxin testing (e.g. the replacement of the mouse bioassay by chemical analyses) and greater reliance on phytoplankton testing and calculated biotoxin risk levels have allowed this cost saving trend to continue (McNabb et al. 2005; McNabb 2008, 2014). The success of the monitoring programmes is exemplified by the excellent management of blooms of the paralytic shellfish toxin (PST) producing HAB species *Gymnodinium catenatum*, *Alexandrium minutum* and *A. catenella* in recent years (Fig. 4; MacKenzie 2014).

The application of LC-MS has allowed better advice on food safety and in many cases has enabled commercial enterprises to continue to operate. Where the LC-MS has not been so readily accepted as a replacement tool, problems have arisen over the validity of the mouse bioassay. For example, in Arcachon, France, oyster harvesting was halted between 2006 and 2009 based on mouse bioassay results, although no known microalgal toxin was found (McNabb 2014). Similarly, closures to oyster harvesting was halted in northern New Zealand and South Australia in 2006 and 2009 due to positive mouse biosassays; the toxins were later shown to be the unregulated pinnatoxins (Rhodes et al. 2010, 2011).

Spirulina is considered a dietary supplement in the U.S., so there is no active industry-wide regulation of its production and no enforced safety standards for its production or purity. The U.S. National Institute of Health describes spirulina supplements as 'possibly safe', provided they are free of microcystin contamination, but 'likely unsafe' (especially for children) if contaminated. The fear of toxic contaminants entering cultured blue-green algae (BGA) health supplements, and the detection of microcystins at low levels in some products (Heussner et al. 2012) has led to the setting of regulations by the Oregon Health Division and the Oregon Department of Agriculture. They established a regulatory limit of 1 μg/g for microcystins in BGA-containing products (Gilroy et al. 2000). Many producers

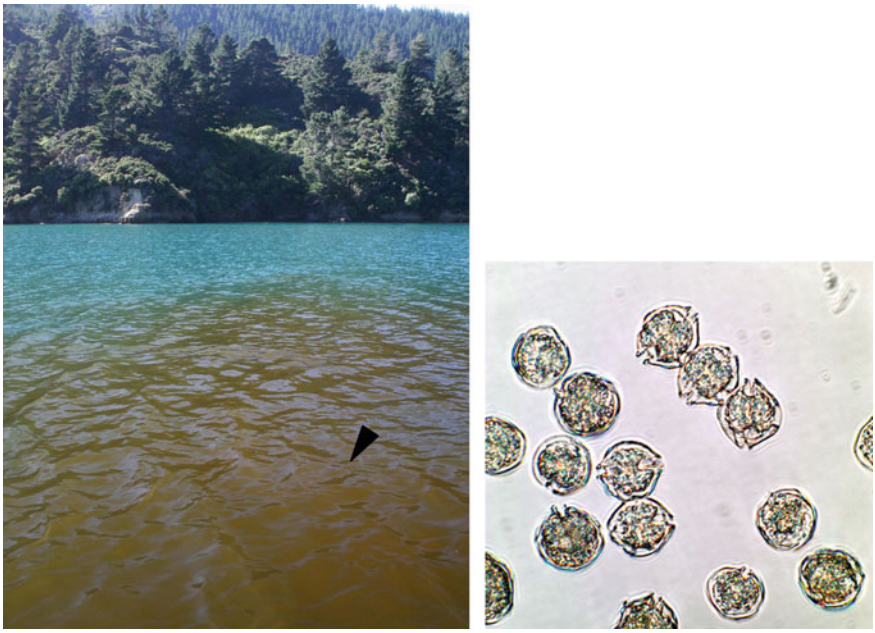

Fig. 4 (*L*, *arrowed*) A bloom of *Alexandrium catenella* in the Marlborough Sounds, New Zealand and (*R*) light micrographs of motile cells of *A. catenella* (*Images* L. MacKenzie, Cawthron Institute, NZ.)

choose to attain certification (e.g. USDA NOP,[1] Ecocert,[2] OCIA–IFOAM[3]) and if the producer is approved (e.g. USP,[4] HACCP,[5] BVQI ISO 9001, ISO 14001[6]) the risk of toxins is virtually eliminated.

In conclusion, the impact of HABs can be devastating, particularly for those countries that have a high dependence on seafood. As coastal waters continue to be overfished, aquaculture is seen as the obvious alternative. It is expected that the

[1]United States Department of Agriculture National Organic Program;

[2]Ecocert is an inspection and certification body established in France in 1991 by agronomists aware of the need to develop environmentally friendly agriculture and of the importance of offering some form of recognition to those commited to this method of production;

[3]Organic Crop Improvement Association;

[4]The U.S. Pharmacopeial Convention (USP) is a scientific nonprofit organisation that sets standards for the identity, strength, quality and purity of medicines, food ingredients, and dietary supplements manufactured, distributed and consumed worldwide;

[5]Hazard analysis and critical control points (HACCP) is a systematic preventative approach to food safety from biological, chemical and physical hazards in production processes that can cause the finished product to be unsafe, and designs measurements to reduce these risks to a safe level;

[6]Bureau Veritas Certification assesses management systems and provides certification to the particular standard.

Fig. 5 Semi-continuous bag cultures of microalgae for production of valuable compounds (*Image* G. Stirling)

value of world aquaculture production will soon outperform the economic returns from the total catch of wild fish and shellfish (Hallegraeff 2003). As more of the coastal 'space' is given over to marine farms it is likely HABs that were of no consequence previously will begin to pose threats. The implementation of monitoring programmes and the advent of faster, cheaper test methods are reducing marine farm closures and minimising product recall from markets. This will be critical for a world dependent on seafood through aquaculture.

A continuing trend is the development of valuable products from algae (Fig. 5). Commercial developments based on microalgae require certified materials and in this regard culture collections are critical to ensure that strains are kept pure. Where possible, cryopreservation in curated collections also ensures physiological processes (e.g. toxin production) remain unaltered (Wood et al. 2008).

Changes in the climate globally and the increasing acidification of our oceans are likely to impose unexpected impacts with unforeseen economic stresses and these impacts can be expected on pond production as well as coastal aquaculture. Prediction of adverse events is a challenge but will be even more critical in the future as global populations grow and reliance on farmed seafood and products from cultured microalgae increases.

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Genetic and Metabolic Engineering of Microalgae

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Abstract Microalgae are promising producers of many valuable compounds serving the food, feed, healthcare, and pharmaceutical industries. Microalgae grow rapidly and generally tolerate a wide range of environments. They can serve as cell factories for economical and sustainable production of diverse products. Microalgae can be transformed through genetic and metabolic engineering methods to overproduce the desired chemicals. Advancements in the "omics" technologies are generating information to allow design and creation of super algal strains for producing biofuels and other products.

Keywords Microalgae · Genomics · Metabolomics · Genetic engineering · Metabolic engineering · Strain improvement · Biofuel

1 Introduction

The unicellular microalgae have been recognized as natural sources of valuable compounds such as carotenoids, long-chain fatty acids, hydrocarbons, and pharmaceuticals. Being eukaryotes and photoautotrophs, they may serve as attractive bioreactor systems that require no organic carbon source and allow cost-effective large-scale production of high value compounds including heterologous recombinant products. In addition, microalgae have high growth rates, are easy to culture, and are amenable to manipulation of their physiological, biochemical, and genetic processes. The feasibility of genetic modification and expression of heterologous

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genes in microalgae has widened the scope of products from traditional food and feed additives to new bioactive compounds for industrial and healthcare applications. Among the new products are human antibodies, hormones (Mayfield et al. 2003; Hempel et al. 2011), insecticidal proteins (Borovsky 2003), and vaccines (Geng et al. 2003). The high photosynthetic efficiency and broad environmental tolerance of microalgae, coupled with their high lipid productivity, have brought attention to them as biofuel feedstocks of great potential (Kleinováa et al. 2012; Blatti et al. 2013).

The domestication of microalgae, similar to other crops, is driven by the need to produce uniform, high-yielding organisms (Gressel et al. 2013). While nonrecombinant techniques such as breeding, sexual hybridization, and strain selection, together with biochemical engineering (BE), have less risk issues, developments in genetic engineering (GE), metabolic engineering (ME), and transcription factor engineering (TFE) with a focus on the regulation of metabolic pathways in the whole cell may prove to be more promising in the long term (Courchesne et al. 2009), as they provide good control and predictability of the system (Blatti et al. 2013). The success of the latter technologies is made possible by contributions from characterization of algal genomes (genomics) and other "omics" technologies (proteomics, metabolomics) allowing trait mining via high-throughput computational systems, for use with advanced genetic engineering tools (Yang and Cao 2012).

2 Microalgal Genomics

To date, more than 20 algal whole genomes have been sequenced, and together with nuclear, mitochondrial, and chloroplast genomes and expressed sequence tags (ESTs) provide the sequence information required for genetic and metabolic engineering of microalgae (Table 1). The National Centre for Biotechnology Information (NCBI) indicates that many more sequencing projects are on-going. The genomics databank provides the fundamental information on the key genes, metabolites, enzymes, proteins, and the biosynthetic pathways, for facilitating both genetic and metabolic manipulation.

The green microalga *Chlamydomonas reinhardtii* has been considered as the model for photosynthesis and chloroplast biogenesis for nearly 50 years (Rochaix 2002). The first set of expressed sequence data (EST) of *Chlamydomonas* was from *C. reinhardtii* growing under photoautotrophy, and was generated by the Kazusa Institute (Asamizu et al. 1999). In this study 3433 independent ESTs were generated of which 817 showed significant similarity to sequences registered at public protein databases, and 140 were matched with previously identified *C. reinhardtii* genes. The remaining ESTs were novel sequences. Over 60 % of ESTs had full-length protein coding regions. The subsequent launch of the *Chlamydomonas* Genome Project by Arthur Grossman was followed by generation of more than 40,000 cDNA data (Shrager et al. 2003). The cDNA was derived from cells

Table 1 List of selected microalgal genome sequences which have been completed

Phylum	Species	Strain	Description	GenBank	Length (nt)	References
Completed (*nuclear genomes sequenced*)						
Chlorophyta						
	Chlamydomonas reinhardtii	CC-503 cw92 mt+	Model species, freshwater	ABCN00000000	120,405,000	Merchant et al. (2007)
	Chlorella variabilis	NC64A	*Paramecium* symbiont, model for viral–algal interactions	ADIC01000000	46,200,000	Blanc et al. (2010)
	Micromonas pusilla	CCMP1545	Marine picoeukaryote	ACCP00000000	21,706,984	Worden et al. (2009)
	Ostreococcus lucimarinus	CCE9901	Marine picoeukaryote	CP000581-CP000601	13,200,000	Palenik et al. (2007)
	Ostreococcus tauri	OTH95	Marine picoeukaryote	CR954201-CR954220	12,578,000	Derelle et al. (2006)
	Volvox carteri f. nagariensis	Eve	Simple multicellular relative of *C. reinhardtii*	ACJH00000000	125,467,762	Prochnik et al. (2010)
Heterokontophyta						
	Nannochloropsis gaditana	CCMP526	Euryhaline eustigmatophyte, rich in PUFA		29,000,000	Radakovits et al. (2012)
	Nannochloropsis oceanica	CCMP1779	Marine eustigmatophyte, rich in PUFA		30,000,000	Pan et al. (2011)
Ochrophyta						
	Thalassiosira oceanica	CCMP1005	Marine diatom as model for tolerance to low-iron conditions		81,600,000	Lommer et al. (2012)

(continued)

Table 1 (continued)

Phylum	Species	Strain	Description	GenBank	Length (nt)	References
Rhodophyta						
	Cyanidioschyzon merolae	10D	Thermo-acidophile	AP006483(DDBJ)	16,520,000	Matsuzaki et al. (2004)
	Galdieria sulphuraria		Thermo-acidophile		11,400,000	Muravenko et al. (2001), Barbier et al. (2005)
Haptophyta						
	Emiliania huxleyi	CCMP1516	Bloom-forming marine coccolithophore	AHAL00000000	167,700,000	Read et al. (2013)
Completed (*mitochondrion genomes sequenced*)						
Chlorophyta						
	Chlamydomonas reinhardtii	*CC-503 cw92 mt+*	–	U03843	15,758	Maul et al. (2002)
	Dunaliella salina	*CCAP19/18*	–	GQ250045	28,331	Smith et al. (2010)
Completed (*chloroplast genomes sequenced*)						
Bacillariophyta						
	Phaeodactylum tricornutum	*CCAP1055/1*	–	EF067920	117,369	Oudot-Le Secq et al. (2007)
Chlorophyta						
	Chlamydomonas reinhardtii	*CC3269*	–	BK000554	203,828	Maul et al. (2002)
	Chlorella vulgaris	*C-27*	–	AB001684	150,613	Wakasugi et al. (1997)
	Parachlorella kessleri	*SAG 211-11g*	–	FJ968741	123,994	Turmel et al. (2009)

(continued)

Table 1 (continued)

Phylum	Species	Strain	Description	GenBank	Length (nt)	References
Cryptophyta						
	Cryptomonas paramecium	*CCAP977/2a*	–	GQ358203	77,717	Donaher et al. (2009)
	Rhodomonas salina	*CCMP1319*	–	EF508371	135,854	Khan et al. (2007)
Haptophyta						
	Emiliania huxleyi	*1516*	–	AY741371	105,309	Sanchez Puerta et al. (2005)

exposed to various stress conditions such as nutrient deprivation. The other recombinant libraries were from other stress conditions such as anaerobiosis, oxidative stress, high light conditions, high-activity osmotic conditions, heavy-metal exposure, and iron and copper deprivation. These results allowed the identification of genes that are potentially activated under different stress conditions. In addition, it demonstrated the protocols for establishing high-quality unigene sets and microarray data (Eberhard et al. 2006; Jain et al. 2007) which can facilitate the investigation of gene function, structure, and regulation for *Chlamydomonas* and potentially other microalgae. The cDNA-based microarrays were used to study gene expression in various conditions such as understanding changing gene expression during the deprivation of sulfur (S) and phosphorus (P) (Zhang et al. 2004; Moseley et al. 2006). These studies not only focused on genes related to S and P metabolism but also on those involved in photosynthesis; carbon metabolism; respiration alternative electron transfer pathways, ATPases, transporter; oxidative stress, chaperones, proteolysis; and other metabolic and biosynthetic processes.

Phosphorus and sulfur are micronutrients that are essential for sustaining life. Studies have shown that the limitation of S and P will affect growth and reduce photosynthesis efficiency (Wykoff et al. 1998; Shimogawara et al. 1999; Zhang et al. 2002). Knowledge on the genes involved and level of expression are important for further application in genetic and metabolomic engineering where the expression of metabolites can be manipulated to produce desired bioproducts. Lohr et al. (2005) used comparative genome analyze to understand genes that are involved in the chlorophyll and carotenoid biosynthesis and to examine their phylogenetic relationships with the deduced sequence of the protein from vascular plants, other algae, and cyanobacteria. Results showed that there are additional conserved domains in the algal and plant proteins but not in cyanobacteria, which may directly influence protein activity, assembly, or regulation. This study was based on phylogenetic studies, theoretical evaluation of gene expression through analysis of expressed sequence tag data, and codon bias of each gene. It provided the hypotheses concerning the function and regulation of the individual genes, and proposed targets for future research. Quantitative polymerase chain reaction (PCR) was used to examine the effect of very low fluence light (VLFL) on the level of expression of key genes which are critical for chlorophyll and carotenoid biosynthesis. The expressions of some of the genes in chlorophyll synthesis such as Glu-1-semialdehyde aminotransferase (*GSA*) and ALA dehydratase (*ALAD*) were found to increase significantly by 15-fold and sevenfold, respectively, after 2 h of exposure to VLFL. Other genes that showed increased expressions were uroporphyrinogen III decarboxylase (*UROD1*), and protoporphyrin IX Mg-chelatase subunit H (*CHLH1* and *CHLH2*). Only the phytoene desaturase (*PDS)* gene was observed to be involved in carotenoid biosynthesis. These provided information on the specific photoreceptors in the biosynthesis of specific pigments.

RNA silencing or RNA interference (RNAi) has emerged as the tool for knocking down gene expression in eukaryotes since the 1990s (Hannon 2002). This technology has been used on several strains of *Chlamydomonas* in which 30

different genes have been reported to be down regulated by antisense or inverted approaches but the efficiency in silencing is often variable for different constructs (Schroda 2006). MicroRNAs (miRNAs) can also be used to down regulate the expression of endogenous genes through mRNA cleavage (Zhao et al. 2009). Zhao et al. (2009) developed two artificial miRNAs (amiRNAs) targeting the *MAA7* and *RBCS1/2* genes, respectively, in *Chlamydomonas*, and when overexpressed, they could cleave their respective targets precisely at the predicted sites, resulting in greatly decreased accumulation of *MAA7* and *RBCS1/2* transcripts and showed expected mutant phenotypes. This study showed that the miRNA technique gives much higher efficiency in silencing the same set of genes as compared to study by Rohr et al. (2004) which used the RNAi approach.

The publication of the *Chlamydomonas reinhardtii* nuclear genome (Merchant et al. 2007) followed and pushed forward microalgal metabolic engineering, including engineering of fatty acid biosynthesis for biofuel (Blatti et al. 2013). The smallest known eukaryotes of about 1 μm diameter which have been subjected for full genome sequencing are the *Ostreococcus*: *O. tauri* (Derelle et al. 2006) and *O. lucimarinus* (Palenik et al. 2007). The genome sequence of a multicellular green volvocine alga *Volvox carteri* with a genome size of 138 MB, approximately 17 % larger than the *Chlamydomonas*, was published in 2010 (Prochnik et al. 2010). The larger size is mainly due to larger repetitive DNA in *V. carteri*. Another commercially relevant species, *Chlorella variabilis* NC64A, of 46 Mb genome size was sequenced in 2010 (Blanc et al. 2010). The eustigmatophytes *Nannochloropsis gaditana* (Radakovits et al. 2012) and *N. oceanica* (Pan et al. 2011) with high productivity of polyunsaturated fatty acids provided the data necessary for enhancing lipid productivity in these oleaginous species. Of the cyanobacteria (or Cyanophyta, as referred to by phycologists), more than 30 species have been sequenced, including *Synechococcus elongatus* (2.7 Mb), *Synechocystis* sp. (3.6 Mb), and *Prochlorococcus marinus* (1.7–2.4 Mb) (Hallmann 2007). Much of the genome data are directed to understand gene structure, composition, arrangement, and evolutionary relationships. Efforts to quickly annotate and characterize genes and proteins, for example, those involved in fatty acid biosynthesis and lipid metabolism (Blatti et al. 2013), have to be made before genetic and metabolomic engineering approaches and can sustainably increase biomass and lipid productivity of oleaginous microalgae.

3 Genetic Engineering of Microalgae

The creation of new strain varieties with the incorporation of desired traits using recombinant DNA technology is the long-term objective of biotechnology. The development of genetic engineering and manipulation has provided opportunities to genetically imbue strains with beneficial properties, to produce heterologous recombinant proteins, and to create new applications of natural resources. The development of an efficient transformation system includes processes for

introducing transgenes into the desired host, identifying or selecting transformants, enhancing the expression of desired genes, and maintaining stable expression. The recent advances in the field of algal genomics may serve as a powerful catalyst for progressing genetic manipulation and engineering. The establishment of genome information for model microalgae such as *Chlamydomonas reinhardtii* (Shrager et al. 2003; Merchant et al. 2007), *Dunaliella salina* (Smith et al. 2010; Zhao et al. 2011), and *Phaeodactylum tricornutum* (Bowler et al. 2008) would facilitate significant progress in the field of algal genetic engineering. The availability of genome data has enhanced understanding of the metabolic pathways, enabled identification of possible DNA elements or endogenous genes that could be modified or utilized for genetic improvement which includes regulatory elements such as promoters to achieve efficient expression of genes.

3.1 Construction of Transformation and Expression Vectors

The design and construction of efficient expression vectors play a critical role in the development of genetically engineered microalgae. The vector design involves the choice of selectable marker for efficient detection or selection of transformed strains of the initial bacteria host and the final algal host; as well as the utilization of an efficient expression system such as the use of efficient endogenous regulatory elements and codon optimization.

Various antibiotic resistance genes have been applied as selectable markers in microalgae transformation. These include the gene for resistance to kanamycin (Bateman and Purton 2000), G418 (Dunahay et al. 1995; Hawkins and Nakamura 1999; Cheng et al. 2012), spectinomycin (Cerutti et al. 1997; Mayfield et al. 2003), phleomycin (Stevens et al. 1996; Kim et al. 2002), hygromycin (Berthold et al. 2002; Kathiresan et al. 2009), chloramphenicol (Toyomizu et al. 2001; Niu et al. 2011; Guo et al. 2013), and paromomycin (Jakobiak et al. 2004; Sizova et al. 2011). The use of a mutant gene encoding acetohydroxyacid synthase (AHAS) that confers resistance to the herbicide sulfometuron methyl was reported for the selection of transformed *Porphyridium* species (Lapidot et al. 2002). The application of antibiotic resistance genes as selectable markers is limited in microalgae transformation as most microalgae are resistant to antibiotics naturally. Moreover, the use of antibiotic resistance genes as selectable markers raises concerns about transfer of such resistance to other species (Manimaran et al. 2011). Other alternatives include the use of reporter genes such as β-galactosidase (lacZ), β-glucuronidase (GUS), green fluorescent protein (GFP), and luciferase (Luc) (Falciatore et al. 1999; Gan et al. 2003; Jiang et al. 2003; Tolonen et al. 2006; Kathiresan et al. 2009; Cheng et al. 2012; Guo et al. 2013). Synthetic versions of codon-optimized green fluorescent protein (GFP) and luciferase reporter genes have been designed for expression in the nucleus or chloroplast of *Chlamydomonas* (Fuhrmann et al. 1999,

2004; Franklin et al. 2002). The *C. reinhardtii* gene *ARG7* encoding the enzyme argininosuccinate lyase (ASL) is a popular selectable shuttle marker used for this alga. Its utilization resulted in the rescue of arginine-requiring arg7 mutants to prototrophy (Debuchy et al. 1989). Other *C. reinhardtii* genes used as transformation markers were the nitrate reductase gene (*NIT1*) (Fernández et al. 1989; Kindle et al. 1989) and the arylsulfatase gene (*ARS*) (de Hostos et al. 1989).

An efficient promoter plays a crucial role in obtaining high expression of the inserted gene. Various promoters have been utilized to drive transgene expression in microalgae, namely the CaMV35S promoter from plant virus (Kim et al. 2002; Tan et al. 2005), promoter of the maize ubiquitin-Ω (Chen et al. 2001; Geng et al. 2003), and endogenous promoters from specific microalgae. Successful application of the diatom fucoxanthin–chlorophyll a/c binding protein gene (*FCP*) promoter was reported in marine diatoms (Falciatore et al. 1999; Zaslavskaia et al. 2000). The ribulose bisphosphate carboxylase/oxygenase small subunit (RBCS2) promoter from *C. reinhardtii* showed higher efficiency than the CaMV 35 S promoter in transformed *Dunaliella salina* (Sun et al. 2005). Niu et al. (2011) used the promoter and terminator of the nitrate reductase gene from *Phaeodactylum tricornutum* for expression of the transgene in *Chlorella vulgaris.* Poulsen and Kroger (2005) observed transgene expression driven by the promoter (Pnr)/terminator (Tnr) cassette derived from the nitrate reductase gene, and would be switched on when cells were grown in the presence of nitrate. Expression of *ARS* gene driven by the NIT1 promoter (Ohresser et al. 1997) and β2-tubulin promoter (Davies et al. 1992) was also observed in transformed *C. reinhardtii*. In addition, fusion of the *Chlamydomonas* heat shock protein 70A (HSPp70A) promoter to the other promoters acted as a transcriptional enhancer of the existing *Chlamydomonas* promoters, namely, RBCS2, β2-tubulin, and HSP70B resulting in higher level of expression (Schroda et al. 2000). Stable nuclear transformation of *D. salina* was reported with the endogenous salt-inducible promoter of duplicated carbonic anhydrase 1 (DCY1) (Li et al. 2010a; Lu et al. 2011). It was observed that the salt-inducible expression of transgenes was regulated by highly repeated GT sequences of the promoter (Li et al. 2010a).

In vector design, inclusion of species-specific 5′,3′-untranslated regions (UTRs) would enhance expression of transgenes. The 5′ region of chloroplast genes has been reported to play a role in RNAs stability (Salvador et al. 1993) and its *cis*-acting elements regulate translation (Nickelsen et al. 1999). Nickelsen (1999) has utilized the 5′ regions of the spinach psbB and the wheat psbA genes including their promoters to drive the expression of a reporter gene in the chloroplast of *Chlamydomonas*. He reported that the resulting transcripts were unstable although the plant promoters were still active indicating different molecular mechanisms governing the posttranscriptional regulation in plants and *Chlamydomonas*. Stevens et al. (1996) observed the expression of the phleomycin-resistance gene in *Chlamydomonas* when they fused the *ble* gene which has similar codon usage as *C. reinhardtii* to the 5′ and 3′ untranslated regions of *C. reinhardtii* rbcS2. It was also

reported that the use of endogenous intron enhanced the expression of the transgene in *Chlamydomonas* (Lumbreras et al. 1998). Although endogenous sequence elements were reported to be species-specific and would enhance the expression of foreign genes, it was also demonstrated that selenocysteine insertion sequence (SECIS) element from either human Sep15 or *C. reinhardtii* selenoprotein W1 could drive the expression of a heterogeneous protein, human selenoprotein (Sep15) in *C. reinhardtii* (Hou et al. 2013). Three types of human Sep15 gene fragments were constructed, namely, Sep15ORF-hSECIS, Sep15ORF-chSECIS, and wtSep15 and upon transformation to *C. reinhardtii*, these transgenes integrated into its genome. Expression of the human Sep15 was detected at both mRNA and protein levels.

3.2 Methods of Gene Introduction

Several methods of gene introduction have been successfully used to achieve expression of transgenes in either the nucleus or the plastid of microalgae, although, in most cases, transgenes were transiently expressed. These methods include trans-conjugation, agitation in the presence of glass beads or silicon carbide whiskers, electroporation, biolistic microparticle bombardment, and *Agrobacterium tumefaciens*-mediated transformation (Table 2). Stable transformation has been achieved for both nuclear and chloroplast transformations of microalgae (Dunahay et al. 1995; Kim et al. 2002; Lapidot et al. 2002). The first report on the stable transformation of *Chlorella* via microparticle bombardment resulted in the rescue of nitrate reductase-deficient *C. sorokiniana* mutants using a homologous selectable marker, the nitrate reductase gene from *C. vulgaris* (Dawson et al. 1997). The use of microparticle bombardment and this homologous marker has also enabled stable transformation of *Volvox carteri* (Schiedlmeier et al. 1994). In microparticle bombardment high velocity microprojectiles are used to deliver transgenes into the host cells (Klein et al. 1987). In electroporation, or electropermeabilization, a high-intensity electric pulse is used to create transient pores in the cell membrane to facilitate the entry of transgenes (Rae and Levis 2002). The removal of the external field results in the resealing of the membrane electropores. This method has been successfully used for the introduction of transgene into *Chlamydomonas* (Brown et al. 1991). The optimal parameters for efficient transformation by electroporation vary among strains. These parameters include electric field strength, temperature, osmolarity, and the concentration of transgene (Shimogawara et al. 1998).

Agrobacterium, a gram-negative plant pathogen, has been widely used in the genetic engineering of plants. *Agrobacterium*-mediated transformation is a low-cost method that offers a high transformation efficiency. The presence of acetosyringone was reported to enhance the transformation efficiency of *Agrobacterium*-mediated transformation by activating the *vir* genes of *Agrobacterium* (Stachel et al. 1985; Men et al. 2003; Kumar et al. 2004). *Agrobacterium*-mediated transformation has

Table 2 Gene introduction methods applied to genetically engineered microalgae

Methods	Transformed microalgae
Conjugation	*Prochlorococcus* (Tolonen et al. 2006) *Pseudanabaena* (Sode et al. 1992) *Synechococcus* (Sode et al. 1992; Brahamsha 1996) *Synechocystis* (Sode et al. 1992)
Agitation with glass beads	*Chlamydomonas reinhardtii* (Kindle 1990; Purton and Rochaix 1995; Ohresser et al. 1997; León et al. 2007) *Dunaliella salina* (Feng et al. 2009)
Agitation with silicon carbon whiskers	*Amphidinium* sp. (Lohuis et al. 1998) *C. reinhardtii* (Dunahay 1993) *Symbiodinium microadriaticum* (Lohuis et al. 1998)
Electroporation	*C. reinhardtii* (Brown et al. 1991) *Chlorella vulgaris* (Niu et al. 2011) *C. zofingiensis* (Liu et al. 2014) *Nannochloropsis* (Kilian et al. 2011) *Scenedesmus obliquus* (Guo et al. 2013) *Spirulina platensis* (Toyomizu et al. 2001) *Synechococcus* (Matsunaga et al. 1990)
Biolistic microparticle bombardment	*Chaetoceros* sp. (Miyagawa et al. 2011) *C. reinhardtii* (Bateman and Purton 2000; Mayfield et al. 2003; Sun et al. 2003) *C. zofingiensis* (Liu et al. 2014) *D. salina* (Tan et al. 2005) *Navicula saprophila* (Dunahay et al. 1995) *Phaeodactylum tricornutum* (Falciatore et al. 1999; Miyagawa et al. 2009) *Porphyridium* sp. (Lapidot et al. 2002) *Thalassiosira pseudonana* (Poulsen et al. 2006)
Protoplast transformation with polyethylene glycol and dimethyl sulfoxide	*Chlorella* (Hawkins and Nakamura 1999) *C. ellipsoidea* (Kim et al. 2002)
Agrobacterium tumefaciens-mediated transformation	*C. reinhardtii* (Kumar et al. 2004) *D. bardawil* (Anila et al. 2011) *Haematococcus pluvialis* (Kathiresan et al. 2009) *Nannochloropsis* sp. (Cha et al. 2011) *Schizochytrium* (Cheng et al. 2012)

also been successfully applied to microalgae. Kathiresan et al. (2009) reported successful *Agrobacterium*-mediated transformation of *Haematococcus pluvialis* cells without the presence of acetosyringone or induced injury. They have used continuous light which plays an important role in the *Agrobacterium*-mediated transformation process and enhances transformation frequency (Zambre et al. 2003). In addition, the cultures were maintained at 22 °C during co-cultivation which has been reported to be the optimal temperature for the *Agrobacterium*-mediated transformation of plants (Dillen et al. 1997).

3.3 Potential Applications of Genetic Transformation in Microalgae

The green microalga *Chlamydomonas* is widely used as a single-celled photosynthetic model organism for various biological studies. The establishments of various transformation methods for nuclear and plastid transformation of *C. reinhardtii* (Rochaix 1995; Kindle 1990) together with the characterization of genes from *C. reinhardtii,* namely, the *ARG7* (Debuchy et al. 1989; Purton and Rochaix 1995), *NITI* (Fernández et al. 1989), and *NIC7* (Ferris 1995), have enabled the genetic manipulation of this microalga. The achievement of random integration at nonhomologous sites through nuclear transformation of *C. reinhardtii* has been exploited to generate new mutants by tagging mutagenesis (Adam et al. 1993; Davies et al. 1994; Prieto et al. 1996) and to isolate genes of unknown products (Tam and Lefebvre 1993). Cagnon et al. (2013) screened transformants generated by random insertional mutagenesis and isolated oil mutants of *C. reinhardtii* with enhanced oil accumulation under the nitrogen-replete condition as well as mutants with altered oil content under nitrogen depletion. Adam and Loppes (1998) reported the cloning of a gene from *C. reinhardtii* which is required for derepressible neutral phosphatase activity, PHON24 using ARG7 as an insertional mutagen. Auchincloss et al. (1999) described the cDNA sequence of ARG7 and successfully generated vectors with smaller size and reduced repetitive DNA compared to the genomic DNA initially used for gene tagging.

Microalgae have been demonstrated as useful bioreactors for the expression of valuable proteins such as vaccines, antibodies, and hormones. In the first such report, a monoclonal antibody directed against the herpes simplex virus glycoprotein D was produced in the chloroplast of engineered *C. reinhardtii* (Mayfield et al. 2003). The fusion proteins of foot-and-mouth disease virus (VP1) and cholera toxin B subunit have also been expressed in the chloroplast of *C. reinhardtii* (Sun et al. 2003) and have shown potential application as a mucosal vaccine source. Transient expression of the hepatitis B surface antigen was reported in *Dunaliella salina* (Geng et al. 2003). A vector was also designed with an extracellular secretion signal sequence inserted between a promoter region and the human growth hormone (hGH) gene for the expression of this heterologous protein in *Chlorella* although no stable transformation was observed (Hawkins and Nakamura 1999). In addition, transgenic microalgae can be used to improve animal feed. Flounder fry fed with the transformed *Chlorella* expressing the flounder growth hormone gene showed enhanced growth (Kim et al. 2002). Similarly, a transgenic cyanobacterium expressing the *cryIVD* gene of *Bacillus thuringiensis* var. *israelensis* and a transgenic *Chlorella* expressing trypsin-modulating oostatic factor exhibited larvicidal activity for mosquito control (Stevens et al. 1994; Borovsky 2003). Microalgal strains were also engineered to be imbued with additional useful features. León et al. (2007) genetically engineered *Chlamydomonas reinhardtii* to express the β-carotene ketolase gene from *Haematococcus pluvialis* via the use of the *Chlamydomonas* constitutive promoter of the rubisco small subunit (*RbcS2*).

They utilized the *Chlamydomonas* transit peptide sequences of Rubisco small subunit (*RbcS2*) or ferredoxin (*Fd*) to direct the product to the chloroplast. Transgenic *Phaeodactylum tricornutum* that accumulated the high value omega-3 long-chain polyunsaturated fatty acid docosahexaenoic acid (DHA) was produced by expressing the Δ5-elongase gene from *Ostreococcus tauri* (Hamilton et al. 2014).

The instability of petroleum price and an uncertain long-term availability have focused attention on finding new sustainable biofuel resources. Globally, there are intensive efforts to enhance the accumulation of lipids, hydrocarbons, and other energy compounds in various organisms including microalgae through genetic and metabolic engineering. Nitrogen stress or starvation has been reported to decrease growth rate, protein content, photosynthetic activity, and cell size, but increases the lipid and carbohydrate content of algae (Li et al. 2012; Simionato et al. 2013; Pancha et al. 2014).

Studies were conducted to elucidate the role of genes and their products in the regulation of algal lipid accumulation. Inhibiting cell cycle progression has been observed to result in the accumulation of lipids in microalgae (Guckert and Cooksey 1990). Transcriptomic and proteomic analysis showed that under nitrogen stress, mRNAs and proteins associated with fatty acid and lipid biosynthesis were up-regulated (Miller et al. 2010; Guarnieri et al. 2011). In addition, a number of potential gene targets which are involved in cell signaling, transcriptional regulation, lipid biosynthesis, and cell cycle control were identified. These discoveries would facilitate further studies such as knockdown or inducible repression of these genes as novel approaches to mimic nitrogen stress-induced cell cycle arrest and hence, enhance the accumulation of lipid. Reports strongly suggested that abolishment of the starch synthesis pathway would switch photosynthetic carbon flux toward triacylglyceride (TAG) synthesis as an alternative carbon reserve for cells under stress (Wang et al. 2009; Li et al. 2010b). Hence, this has become an effective strategy rather than direct manipulation of the lipid synthesis pathway to obtain high accumulation of TAG. Direct manipulation and over-expression of the acetyl-CoA carboxylase gene in the microalgae *Cyclotella cryptica* and *Navicula saprophila* did not successfully enhance the lipid content (Dunahay et al. 1996). In another attempt, over-expression of three type-2 acyl-CoA, diacylglycerol acyltransferase genes (DGATs) in *Chlamydomonas reinhardtii,* also did not increase TAG accumulation (Russa et al. 2012).

4 Metabolic Engineering

Metabolic engineering is defined as "directed improvement of product formation or cellular properties through the modification of specific biochemical reactions or the introduction of new genes with the use of recombinant DNA technology" (Stephanopoulos et al. 1998). Metabolic engineering is different from genetic engineering: the main focus of the latter is on enzymes and genes while the former is concerned with the modification of biochemical pathways or the integrated

metabolic pathways rather than the individual reaction (Stephanopoulos and Sinskey 1993). The metabolic engineering approach in combination with advances in high-throughput computing enables efficient investigation of cellular metabolism and physiology at the systems level, leading to enhancement of multiple traits like product concentrations, yield, productivity, and tolerance (Jang et al. 2012). This new approach is termed systems metabolic engineering.

Investigations into cellular functions have been made possible by efficient comparative genome sequence analysis to identify genes for system manipulation toward desired metabolic phenotypes through transcriptomics, proteomics, and metabolomics profiling. Transcriptome profiling uses DNA microarrays and allows for identifying target genes through differential expression profiling under various environmental conditions (Sindelar and Wendisch 2007; Jang et al. 2012). Proteomics provide protein profiling. In metabolomics, an array of advanced tools like mass spectrometry–chromatography and nuclear magnetic resonance identify metabolites including substrates, products, and intermediates associated with different metabolic states of the cell (Jang et al. 2012). All such information can then be incorporated into in silico metabolic models (Kim et al. 2008; Schellenberger et al. 2010) and algorithms which point the way toward metabolic engineering (Park et al. 2009, 2010; Choi et al. 2010).

4.1 Metabolic Engineering of Lipid Metabolism

Microalgae are a promising feedstock for biofuels such as biodiesel, biohydrogen, and bioethanol. Many microalgae achieve maximal lipid yields under stress conditions (Hu et al. 2008) that hinder growth and result in cell compositions which are not ideal for biofuel applications (Courchesne et al. 2009). Metabolic engineering through genetic manipulation presents a promising strategy for the over-production of algal oils. The available approaches may include random and targeted mutagenesis and gene transformation. In this review, we will focus on some of the examples on how metabolic engineering can be used to enhance algal biofuel production.

The lipid metabolism of microalgae is highly complex but an understanding of the biosynthetic pathways is essential before the creation of the best strain for biodiesel production can take place. Recent work on the introduction of the genes for enzymes related to lipid biosynthesis, such as acetyl-CoA carboxylase (ACC), KAS III, and ACL into higher plants like *Arabidopsis, Brassica napus*, and tobacco, has shown increased production of lipid (Courchesne et al. 2009). A similar approach can be applied for microalgae. ACC is the first enzyme in the lipid biosynthesis pathway of triacylglycerol and its over-expression may enhance lipid yield as shown in *Arabidopsis* (Roessler et al. 1997). The ACC gene from *Cyclotella cryptica* was introduced into two species of diatoms, *C. cryptica* and *Navicula saprophila*, but disappointingly there was no increase of oils in the cells (Dunahay et al. 1995; Sheehan et al. 1998). The lipid increase in plants, as

compared to rather low increase in microalgae, suggested there is a mechanism or regulatory control that needs further study. Recently, the plastidic acetyl-CoA carboxylase (ACCase) was shown to be the rate-limiting enzyme in the fatty acid synthesis in *Brassica napus* (Andre et al. 2012).

The cloning of single genes related to fatty acid synthesis did not increase the fatty acid contents, as shown above. In plants, a multi-gene approach has successfully enhanced lipid production (Courchesne et al. 2009). A similar approach of cloning multiple genes related to fatty acid synthesis in *Haematococcus pluvialis* under different stress conditions showed the expression of the key genes correlated with fatty acid synthesis (Lei et al. 2012). Lei et al. (2012) cloned seven key genes of fatty acid synthesis: 3-keto acyl-acyl carrier protein synthase gene (*KAS*), acyl-acyl carrier protein thioesterase (*FATA*), ω-3 fatty acid desaturase (*FAD*), acyl carrier protein (*ACP*), malonyl-CoA:ACP transacylase (*MCTK*), biotin carboxylase (*BC*), and stearoyl-ACP-desaturase (*SAD*) into *H. pluvialis*. The clones were grown in various stress conditions: nitrogen depletion, salinity, and high or low temperature. In general, the results showed that high temperature, high salinity, and nitrogen depletion favored fatty acid (FA) synthesis and the FA quality was not affected much. At the same time, the cells were also harvested for RNA in order to quantify the expressions of the seven key genes. The correlations between different fatty acid syntheses and gene expressions were different. ACP, KAS, and FATA shared close correlations with fatty acid synthesis, while the other enzymes did not. The ACP, important in both fatty acid and polyketide biosynthesis, had its gene expression up-regulated to 8.7 times with high temperature compared to only 2.6 times at low temperature. The expression of KAS (catalyzes the initial condensing reaction in FA biosynthesis) was increased by Fe + AC (acetate) supplementation approximately 3.5 times, while individual treatments with Fe and AC promoted its gene expression to 81 and 42 %, respectively, in comparison to the control. The FATA functions as a chain-length-determining enzyme in de novo biosynthesis of plant fatty acid synthesis, and the FATA mRNA levels were up-regulated significantly under all treatments in this study: 2.0 times under nitrogen depletion; 2.9 times with Fe + AC combined; 3 times under low temperature; 9.9 times with Fe; 13.8 times under high temperature; and 18.8 times with AC. The information derived from this research was significant in identifying the potential candidate genes for use in metabolic engineering to enhance the production of FA in terms of quantity and quality.

Fatty acids derived from microalgae need to be of the correct chain length for use in production of biodiesel. Thioesterases (TE) are key enzymes in fatty acid biosynthesis that determine fatty acid carbon chain length in most plant tissues. TEs have been engineered into a variety of plant species to successfully alter the fatty acid profiles (Thelen and Ohlrogge 2002). This same approach has been applied to microalgae. The thioesterase PtTE was overexpressed in *Phaeodactylum tricornutum*, resulting in an increase of 72 % in the total fatty acids, although it did not change the fatty acid composition (Gong et al. 2011). In 2012, Blatti et al. (2012) manipulated the fatty acid biosynthesis of *C. reinhardtii* through interactions between the fatty acid acyl carrier protein (ACP) and thioesterase (TE) that regulate

fatty acid hydrolysis within the chloroplast of *C. reinhardtii.* The results showed that TE functionally interacts with CrACP to release fatty acids. In this case, increased levels of short-chain fatty acids in *C. reinhardtii* chloroplast were observed and the fatty acid profile remained unaltered. This shows that in order to engineer microalgae for the desired composition of fatty acids, the alteration of fatty acid biosynthesis can be done through protein–protein interactions.

In addition to the traditional genetic engineering (GE) approach of inserting single or multiple key genes relating to lipid production in the microalgae to increase the FA production, the TFE approach has been proposed as an alternative method. The transcription factors (TFs) may regulate or increase the activity of multiple enzymes controlling the production of microalgal lipids (Courchesne et al. 2009). This metabolic engineering approach has successfully increased the production of valuable metabolites in plants and animals (Segal et al. 2003; Broun et al. 2004; Reik et al. 2007). In order to use the TF strategy for improving lipid production, the TFs for microalgae have to be identified. For plants and animals, several TFs that are related to regulation of lipid biosynthesis have been identified. For instance, the sterol regulatory element-binding protein (SREBP) has been well known as a regulator of lipid homeostasis in mammals (Hitoshi 2005; Porstmann et al. 2005; Goldstein et al. 2006; Todd et al. 2006; Espenshade and Hughes 2007; Kotzka et al. 2010). In *Arabidopsis* a few transcription factors such as *LEC1*, *LEC2*, and *WRI1* have been found to regulate the seed oil content (Cernac and Benning 2004; Baud et al. 2007, 2009; Santos-Mendoza et al. 2008).

The manipulation of TFs can enhance the production of fatty acids (Mu et al. 2008; Tan et al. 2011). Wang et al. (2007) discovered 28 DNA-binding-with-one-finger (*Dof*) type transcription factors (*GmDof1*-21 and *β*-Tubilin) in soybean, an important oil crop. These TFs were found to affect the gene expressions of various organs. Among the 28 types of *Dof*, two genes, *GmDof*4 and *GmDof*11, were found to increase the total fatty acids and lipids contents in *GmDof*4 and *GmDof*11 transgenic *Arabidopsis* seeds. The *Dof* transcription factor family genes are found in various groups of organisms, including the green unicellular alga *C. reinhardtii* (Moreno-Risueno et al. 2007).

Riaño-Pachón et al. (2008) identified 234 genes encoding 147 TFs and 87 TRs (transcription regulators) in *C. reinhardtii*; however, there is not much information on their functions. The first study in making use of TFs to over express algal lipid production was carried out by Ibăñez-Salazar et al. (2014). Only one *Dof* gene in *C. reinhardtii* was found that was located in chromosome 12 at position 4426.865–4427.015 bp. This was discovered after a thorough blast analysis of the conserved domain in plants and comparison with the genome of *C. reinhardtii*. The Phytozome database (http://www.phytozome.net/cgi-bin/gbrowse/chlamy/) was used. Phylogenetic tree analysis further revealed the *Dof* sequence from *C. reinhardtii* to have a close relationship with *Volvox carteri*. Although the function of *Dof* in *C. reinhardtii* is unknown, the authors suggested it may have a possible role in increasing the fatty acid and lipid production. Based on the *Dof* sequences from *C. reinhardtii*, a synthetic *Dof*-type transcription factor gene was designed and plasmid constructed. The plasmid was transformed into the *C. reinhardtii* nucleus

using *Agrobacterium tumefaciens.* The transformation successfully yielded two transgenic lines (*Dof* 9 and *Dof* 11). In order to verify the possible function of the introduced *Dof* transcription factor, a transcription profile of 14 genes [eight genes involved in fatty acid biosynthesis: β-carboxyltransferase (*BCX1*), biotin carboxylase (*BCR1*), acyl carrier protein (*ACP1*), 3-ketoacyl-ACP synthase 2 (*KAS2*), 3-ketoacyl-ACP synthase 3 (*KAS3*), 3-ketoacyl-ACP reductase (*KAR1*), enoyl-ACP-reductase (*ENR1*), and acyl-ACP thiolase (*FAT1*); and six genes involved in glycerolipid biosynthesis: UDP-sulfoquinovose synthase (*SQD1*), the sulfolipid synthase (*SQD2*), monogalactosyldiacylglycerol synthase (*MGD1*), digalactosyldiacylglycerol synthase (*DGD1*), CDP-DAG-synthetase (*CDS1*), and phosphatidylglycerophosphate synthase (*PGP1*)] was examined. Among these enzymes, enoyl-ACP-reductase (ENR1) [fatty acid biosynthesis], phosphatidyl-glycerophosphate synthase (PGP1), monogalactosyldiacylglycerol synthase (MGD1), and sulfolipid synthase (SQD2) (glycerolipid biosynthesis) were over expressed in the two transgenic strains in comparison to the wild strain. The transgenic lines showed increased production in total lipids as well as fatty acids in comparison to the wild strain. The fatty acid composition of both transgenic lines and wild strain were similar, being dominated by palmitic acid (C16:0), γ-linolenic acid (C18:3, n3), and stearidonic acid (C18:4). This study showed the potential future application of TFs for increasing total lipid and fatty acid production in microalgae.

There have been several successes in manipulation of the prokaryotic cyanobacteria (or Cyanophyta) that make them good alternative biofactories for biofuels. Liu et al. (2010) successfully inserted the genes related to lipid biosynthesis from plants and *Escherichia coli* into mutant strains of *Synechocystis* sp. PCC 6803. They constructed five strains of PCC 6803 in which the inserted genes replaced the genes imparting properties that either competed with or inhibited the production of free fatty acids. The genetically modified strains successfully over-produced fatty acids (C10–C18) and secreted them into the medium at levels of up to 133 ± 12 mg/L of culture per day at a cell density of 1.5×10^8 cells/mL (0.23 g of dry weight/L). According to the authors, the genetically constructed strains could theoretically produce 6500 gallons of biodiesel per acre per year in a cost-effective system that eliminated the extraction costs. Subsequently, a sixth generation was constructed by Liu et al. (2011) which was genetically enabled to grow in high light and a maximum fatty acid secretion level of 197 ± 14 mg/L of culture at a cell density of 1.0×10^9 cells/mL was obtained.

4.2 Metabolic Engineering of Biohydrogen Production

Photosynthetic microalgae have the ability to produce hydrogen, another potential biofuel. Under the normal aerobic growing conditions, microalgae will not produce hydrogen. However, if anaerobiosis can be induced with low oxygen levels at night, a hydrogenase enzyme is expressed in the chloroplast for light-mediated generation of hydrogen (Melis et al. 2007). The presence of oxygen inhibits the transcription

and activity of hydrogenase(s), but production may be continued with the addition of the herbicide DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea; a PSII electron chain uncoupler) (Esquivel et al. 2011). Several approaches have been used to increase hydrogen production of microalgae that grow photosynthetically but under anoxic conditions. Sulfur depletion can enhance hydrogen production in *C. reinhardtii* through induction of anoxia and consequent expression of hydrogenase. The Leghemoglobin (*LBS*) genes from the soybean root nodules that have high affinity to O_2 were genetically engineered into *C. reinhardtii* and successfully increased the hydrogen production by 22 % (Wu et al. 2011). Scoma et al. (2012) introduced the high hydrogen producer D1 protein mutant strain of L159I-N230Y of *C. reinhardtii* where leucine residue L159 was replaced by isoleucine, and the N230 asparagine was replaced by tyrosine to improve hydrogen production. The mutant strain had higher carbohydrate and hydrogen production capacity compared to the control.

5 Limitations and Risks in Genetic and Metabolic Engineering of Microalgae

Microalgal biotechnology has entered an exciting era in which advances in analytical and computational tools allow redirecting metabolic processes toward desired outcomes. The intense search for the cost-effective lipid producers and production system has pushed emerging technologies like the "omics," genetic and metabolic engineering to new limits. Integration of information derived from genomics, proteomics, and metabolomics continues to improve and fundamental eco-physiological responses of algae to a changing environment are becoming better understood.

While genetic engineering appears to be a powerful tool in algal biotechnology, there are hurdles to overcome. Availability of cost-effective and efficient tools for gene delivery and detection of expression is one limitation (Qin et al. 2012). Potential ecological impact of the engineered strains is another concern. Biosafety entails both elimination of harm to humans and the natural ecosystem (Qin et al. 2012). At present, the most pressing issue is that of the efficiency of the transformation systems. For example, a low success rate in gene expression and poor stability of the transformants are persistent issues. Transgenic algal clones exhibit suppression of exogenous genes when cultured under nonselective conditions (Leon-Banares et al. 2004). Better understanding of regulation of gene expression including transgene silencing, as well as cellular responses to the vector or gene construct, is required (Wu-Scharf et al. 2000; Leon-Banares et al. 2004; Hallmann 2007). Anila et al. (2011) showed stable transgene integration in *Dunaliella bardawil* after 18 months of continuous culture in the absence of selection pressure. The transformant had been produced via *Agrobacterium*-mediated transformation.

Risks from transgenic algae relate to human health and the environment (Hallmann 2007). Transgenics may introduce toxic compounds and allergens

causing dietary problems, while transfer of novel genes to non-target species may occur through the use of transgenics. Escaped transgenics may outcompete indigenous species and become dominant, resulting in major ecological upsets (Henley et al. 2013). Use of enclosed photobioreactors offers some protection against escape but additional mechanisms are needed to prevent survival of escaped cells in nature. Use of "completely algae-derived vectors" may have some benefits (Qin et al. 2012). Henley et al. (2013) recommended that multiple biocontainment strategies could be implemented through simultaneous introduction of traits or mutations into the transgenics to reduce risks. These include reduced growth fitness especially in relation to the wild type (Gressel et al. 2014) and conditioned lethality in the wild, as well as impaired reproduction, both asexual and sexual (Henley et al. 2013). Finally, risk assessments based on actual experimental data (Gressel et al. 2013) would allow the development of regulatory guidelines for monitoring and management of algal transgenics.

6 Concluding Remarks

Advances in the "omics" technologies have accelerated the development of more refined genetic and metabolic engineering tools to transform algal cells into bio-factories for producing valuable chemicals. The earlier and perhaps the most successful approaches of physiologically stressing the cells to produce desired compounds had the limitation of reducing the product yields. Genetic and metabolic engineering are more promising in the long run and potentially allow better controlled and predictable bioprocesses enhanced with the use of regulation of multiple enzymes to control metabolism (Courchesne et al. 2009). A thorough analysis of potential risks of using transgenics is required and robust methods of managing such risks need to be developed.

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