Developments in Applied Phycology 5

Michael A. Borowitzka
Navid R. Moheimani
Editors

Algae for Biofuels and Energy

Springer

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Series Editor:
Michael A. Borowitzka
School of Biological Sciences and Biotechnology
Murdoch University, Murdoch, Western Australia, Australia

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Editors
Michael A. Borowitzka
Algae R&D Centre
School of Biological Sciences
and Biotechnology
Murdoch University
Murdoch, WA, Australia

Navid R. Moheimani
Algae R&D Centre
School of Biological Sciences
and Biotechnology
Murdoch University
Murdoch, WA, Australia

ISBN 978-94-007-5478-2 ISBN 978-94-007-5479-9 (eBook)
DOI 10.1007/978-94-007-5479-9
Springer Dordrecht Heidelberg New York London

Library of Congress Control Number: 2012954780

Cover design: Dr. Roberto de Philippis

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Preface

The concept of using algae as a source of renewable fuels and energy is quite an old one, dating back at least to 1931, but one which gained much attention during the 1990's oil crisis and then, once again, more recently interest in algae as a source of biofuels has risen dramatically. The potential attractive features of algae have often been listed, but as yet the high cost of producing algae biomass means that algal biofuels as an economical, renewable and sustainable source of biofuels and bioenergy is still somewhat off in the future. Microalgae are currently probably the most studied potential source of biofuels, and in the US alone there are some 30+ companies working in the area and total investment in R&D is in excess of several billion $US worldwide.

This book focuses on microalgae rather than seaweeds, as microalgae are the most attractive for renewable energy production, especially the production of biodiesel, although seaweed biomass can also be used. The aim of this book is to review in detail the most important aspects of the microalgae-to-bioenergy process, with an emphasis on microalgae as sources of lipids for the production of biodiesel and as potential sources of hydrogen. The book is meant as a guide and resource for both the experienced practitioners in the field and to those newer to this exciting field of research. However, no single book can cover all aspects of the production of bioenergy from algae; for example, we do not cover the fermentation of algal biomass to produce methane, nor the fermentation of algal sugars to ethanol or butanol.

This book begins (Chap. 1) with an introduction to the history and developments over the last 80 years or so in the area of large-scale and commercial-scale culture of microalgae and the extensive literature that is available. Much can be learned from the extensive research that has been carried out, and by knowing this history (some of which is not easily accessible) we can avoid repeating past mistakes.

One of the key attractions of microalgae is the high lipid content of some species and the lipid and fatty acid composition and metabolism is covered in Chap. 2 by Guschina and Harwood, and the production and properties of biodiesel from these algal oils is considered in detail by Knothe in Chap. 12, while Chap. 3 by Peters et al. considers hydrogenases, nitroginases and H_2 production by water-oxidizing phototrophs (i.e. algae and cyanobacteria). The first step in developing an algae bioenergy process is species and strain selection and this topic is considered in detail in Chap. 4. Chapter 5 by Beardall and Raven focuses on light and inorganic carbon supply as key limiting factors to growth in dense cultures and Chap. 6 by Rasala et al. looks at how genetic engineering may be used to improve and modify algae strains.

The systems for production of microalgae biomass are reviewed in Chaps. 7 (photobioreactors; Chini Zittelli et al.), 8 (open pond systems (Borowitzka & Moheimani) and 9 (systems utilizing waste waters; Craggs et al.). The key downstream processes of harvesting and dewatering and extraction of the lipids are covered in Chaps. 10 (Pahl et al.) and 11 (Molina Grima et al.). Finally, Chap. 13 (Jacobi and Posten) looks at the energy balances of closed photobioreactors and how these may be improved, Chap. 14 (Flesch et al.) looks at the greenhouse gas balance of algae based biodiesel using a range of models, and Chap. 15 (Borowitzka) describes the process of techno-economic modelling and how it can be used to guide R&D in the development of algae biofuels.

In our experience there is also often some confusion on the basic laboratory methods used in algae culture and for the analysis of their basic composition, and we have therefore included a chapter on these basic methods as used and verified in our laboratory over many years. We hope that, by providing this information in an easily accessible format, newer workers in the field will be able to produce more reliable results which can then be easily compared between different laboratories.

Michael Armin Borowitzka
Navid Reza Moheimani

Contents

Contributors

Peter J. Ashman School of Chemical Engineering, The University of Adelaide, Adelaide, SA, Australia

David Batten Low Cost Algal Fuels, CSIRO Energy Transformed Flagship, Aspendale, VIC, Australia

Niccoló Bassi Fotosintetica & Microbiologia S.r.l., Firenze, Italy

John Beardall School of Biological Sciences, Monash University, Clayton, VIC, Australia

Tom Beer Transport Biofuels Stream, CSIRO Energy Transformed Flagship, Aspendale, VIC, Australia

John R. Benemann Benemann and Associates, Walnut Creek, CA, USA

Natascia Biondi Dipartimento di Biotecnologie Agrarie, Università degli Studi di Firenze, Firenze, Italy

Michael A. Borowitzka Algae R&D Centre, School of Biological Sciences and Biotechnology, Murdoch University, Murdoch, WA, Australia

Eric S. Boyd Department of Chemistry and Biochemistry, and Astrobiology Biogeocatalysis Research Center, Montana State University, Bozeman, MT, USA

Peter K. Campbell University of Tasmania, Hobart, TAS, Australia

Rupert J. Craggs National Institute for Water and Atmospheric Research, Hamilton, New Zealand

Sarah D'Adamo Department of Chemistry and Geochemistry, Colorado School of Mines, Golden, CO, USA

Anne Flesch Veolia Environnement, Paris, France

Sophie Fon Sing Algae R&D Centre, School of Biological Sciences and Biotechnology, Murdoch University, Murdoch, WA, Australia

Antonio Giménez Giménez Department of Chemical Engineering, University of Almería, Almería, Spain

Javier A. Gimpel Division of Biological Sciences, University of California San Diego, San Diego, CA, USA

Tim Grant Life Cycle Strategies, Melbourne, VIC, Australia

Emilio Molina Grima Department of Chemical Engineering, University of Almería, Almería, Spain

Irina A. Guschina School of Biosciences, Cardiff University, Cardiff, Wales, UK

Mike J. Hannon Division of Biological Sciences, University of California San Diego, San Diego, CA, USA

John L. Harwood School of Biosciences, Cardiff University, Cardiff, Wales, UK

Maria Jose Ibáñez González Department of Chemical Engineering, University of Almería, Almería, Spain

Andreas Isdepsky Algae R&D Centre, School of Biological Sciences and Biotechnology, Murdoch University, Murdoch, WA, Australia

Anna Jacobi Karlsruhe Institute of Technology, Institute of Engineering in Life Science, Division of Bioprocess Engineering, Karlsruhe, Germany

Theo Kalaitzidis School of Chemical Engineering, The University of Adelaide, Adelaide, SA, Australia

Gerhard Knothe USDA/ARS/NCAUR, 1815 N. University St., Peoria, Peoria, IL, USA

Andrew K. Lee School of Chemical Engineering, The University of Adelaide, Adelaide, SA, Australia

David M. Lewis School of Chemical Engineering, The University of Adelaide, Adelaide, SA, Australia

Tryg J. Lundquist Civil and Environmental Engineering Department, California Polytechnic State University, San Luis Obispo, CA, USA

Stephen P. Mayfield Division of Biological Sciences, University of California San Diego, San Diego, CA, USA

Shigeki Joseph Miyake-Stoner Division of Biological Sciences, University of California San Diego, San Diego, CA, USA

Navid Reza Moheimani Algae R&D Centre, School of Biological Sciences & Biotechnology, Murdoch University, Murdoch, WA, Australia

David W. Mulder Department of Chemistry and Biochemistry, and Astrobiology Biogeocatalysis Research Center, Montana State University, Bozeman, MT, USA

Stephen L. Pahl School of Chemical Engineering, The University of Adelaide, Adelaide, SA, Australia

John W. Peters Department of Chemistry and Biochemistry, and Astrobiology Biogeocatalysis Research Center, Montana State University, Bozeman, MT, USA

Matthew C. Posewitz Department of Chemistry and Geochemistry, Colorado School of Mines, Golden, CO, USA

Clemens Posten Karlsruhe Institute of Technology, Institute of Engineering in Life Science, Division of Bioprocess Engineering, Karlsruhe, Germany

Beth A. Rasala Division of Biological Sciences, University of California San Diego, San Diego, CA, USA

John A. Raven Division of Plant Science, James Hutton Institute, University of Dundee at JHI, Dundee, UK

Liliana Rodolfi Dipartimento di Biotecnologie Agrarie, Università degli Studi di Firenze, Firenze, Italy

Suraj Sathe School of Chemical Engineering, The University of Adelaide, Adelaide, SA, Australia

Elizabeth A. Specht Division of Biological Sciences, University of California San Diego, San Diego, CA, USA

Jesse Therien Department of Chemistry and Biochemistry, and Astrobiology Biogeocatalysis Research Center, Montana State University, Bozeman, MT, USA

Miller Tran Division of Biological Sciences, University of California San Diego, San Diego, CA, USA

Mario R. Tredici Dipartimento di Biotecnologie Agrarie, Università degli Studi di Firenze, Firenze, Italy

Graziella Chini Zittelli Istituto per lo Studio degli Ecosistemi, CNR, Firenze, Italy

Energy from Microalgae: A Short History

1

Michael A. Borowitzka

We are like dwarfs sitting on the shoulders of giants. We see more, and things that are more distant, than they did, not because our sight is superior or because we are taller than they, but because they raise us up, and by their great stature add to ours.

John of Salisbury – Bishop of Chartres (1159) 'Metalogicon'.

1 Introduction

The current extensive research and development activities on microalgae as commercial sources of renewable fuels and energy rely on the basic and applied research on biology, physiology, culture methods, culture systems etc. undertaken in the past. This chapter provides a brief overview of some of the major steps in the development of R&D on the mass culture algae for practical applications and commercial products, with a particular focus on microalgae as sources of renewable energy. It is almost impossible to cover all of the advances made, both small and large, over the last 140 years or so, but this chapter attempts to highlight the development and evolution of many of the key concepts and research in the field. The reader is also referred to the excellent review of the history of applied phycology written by Carl Soeder (1986).

Much of the development of large-scale microalgae production can be traced through the chapters of a small number of key books. In 1952 an Algae Mass Culture Symposium was held at Stanford University, California, USA, bringing together most of the workers in the field at that time. One important outcome of this symposium what the publication of "*Algae Culture. From Laboratory to Pilot Plant*" edited by J.S. Burlew (1953a). This small, but very important, volume brings together almost all of the work done including the first larger scale outdoor trials made to date in the USA, Germany, Japan and Israel.

It took another 27 years until the publication of the next major book in the field, a compilation of papers presented at as Symposium on the production and use of micro-algae biomass held in Israel in 1978, and which brings together many of the major developments in the field since the Burlew book's publication (Shelef and Soeder 1980). The findings of research in India as part of a joint Germany-India research effort are summarised in the books by Becker and Venkataraman (1982) and Venkataraman and Becker (1985). There was also a German-Egyptian research project (El-Fouly 1980). Clearly the interest in the commercial uses of microalgae was rapidly growing and in 1988 the first book focusing on algal biotechnology edited by Amos Richmond (1986) was published, soon to be followed by the book edited by Michael and Lesley Borowitzka (1988b) and by Becker (1994). Since then other books on this topic have been published (e.g., Richmond 2004), and also several books on particular species of interest (e.g., Avron and Ben-Amotz 1992; Vonshak 1997; Ben-Amotz et al. 2009) have been published.

2 The Pioneers

The culturing of microalgae in the laboratory is only about 140 years old, and the commercial farming of microalgae less than 60 years. Compare this with the thousands of years history of farming other plants.

Early attempts at culturing microalgae include those of Cohn (1850) who cultivated the chlorophyte *Haematococcus pluvialis* in situ, and Famintzin (1871) who cultured the green algae *Chlorococcum infusionum* and *Protococcus viridis* (now known as *Desmococcus olivaceus*) in a simple inorganic medium. Modern microalgae culture started with the culture experiments of Beijerinck with *Chlorella vulgaris* (Beijerinck 1890) and the apparent axenic culture of diatoms by Miquel (1892). Once algae could be cultured in the laboratory

M.A. Borowitzka (✉)
Algae R&D Centre, School of Biological Sciences and Biotechnology, Murdoch University, Murdoch, WA 6150, Australia
e-mail: M.borowitzka@murdoch.edu.au

M.A. Borowitzka and N.R. Moheimani (eds.), *Algae for Biofuels and Energy*, Developments in Applied Phycology 5,
DOI 10.1007/978-94-007-5479-9_1,

reliably, study of their nutritional requirements and their physiology were possible (e.g., Warburg 1919). Further improvements in laboratory culture, including the culture of axenic strains can be found in the book by Pringsheim (1947), and continuous culture was developed by Ketchum, Redfield and others (Ketchum and Redfield 1938; Myers and Clark 1944; Ketchum et al. 1949). All work on microalgae whether in the laboratory or in the algae production plant owes a great debt to these pioneers of phycology.

3 The Early Years (1940s & 1950s)

The idea that microalgae could be a source of renewable fuels also has a long history. Harder and von Witsch were the first to propose that microalgae such as diatoms might be suitable sources of lipids which could be used as food or to produce fuels (Harder and von Witsch 1942a, b) and later Milner (1951) also considered the possibility of photosynthetic production of oils using algae. In a detailed study, Aach (1952) found that *Chlorella pyrenoidosa* could accumulate up to 70% of dry weight as lipids (mainly neutral lipids) in stationary phase when nitrogen limited (Fig. 1.1). This study is also the first use of an internally lit photobioreactor which allowed an estimation of photosynthetic efficiency.

It was recognized that although microalgae could accumulate very high levels of lipids, the actual lipid productivity was low. As the need for liquid fuel alternatives also was no longer a problem post World War II the focus of research for the application of microalgae turned to these algae as a potential protein and food source (Spoehr and Milner 1948, 1949; Geoghegan 1951).

Work on larger-scale culture and the engineering requirements for algae production systems began at the Stanford Research Institute, USA in 1948–1950 (Cook 1950; Burlew 1953a, b), in Essen, Germany, where the utilization of CO_2 in waste gases from industry was a possibility (Gummert et al. 1953), and in Tokyo, Japan (Mituya et al. 1953) (Fig. 1.2). Smaller scale studies were also carried out by Imperial Chemical Industries Ltd in England by Geoghean (Geoghegan 1953) and Israel (Evenari et al. 1953). All of these studies used strains of *Chlorella*. The first significant outdoor pilot plant studies on the production of *Chlorella* were carried out in 1951 at Arthur D. Little Inc. in Cambridge, Massachusetts, USA (Anon 1953). This was a seminal study and the details and findings are worthy of summary here. Two types of 'closed' microalgae culture systems, which are now usually called 'closed photobioreactors', were developed and tested (see Fig. 1.2). The first consisted of thin walled (4 mm) polyethylene tubes which, when laid flat had a width of 1.22 m. Two parallel tubes were laid flat with a length of about 21 m with a U-shaped connection between the two tubes at one

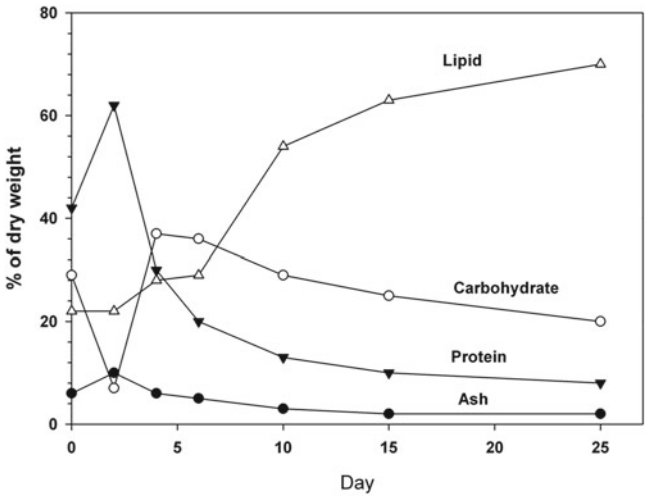

Fig. 1.1 Changes in proximate composition of *Chlorella pyrenoidosa* in batch culture showing accumulation of lipid as nitrogen is depleted (Redrawn from data in Aach 1952)

end. The total culture area was approximately 56 m^2 and the volume was 3,785–4,542 L. A heat exchanger was also installed. Circulation was by means of a centrifugal pump achieving flow rates of about 9 cm s^{-1} at a culture depth of 6.4 cm. The second unit designed to allow greater flow rates was straight, had no U-bend and an actual flow channel width of ~0.38 m and a length of 21.6 m, and achieved flow rates of about 30 cm s^{-1}. In both systems filtered air enriched with 5% CO_2 was provided and pH was maintained at about pH 6 by the periodic addition of dilute nitric acid. For inoculum production 10 vertical aerated (air + 5% CO_2) Pyrex columns (13.2 cm diameter × 1.8 m high) were used.

The first culture unit was operated for a total of 105 days in semi-continuous mode with daily harvests and with and without medium recycling after harvest between July and October (i.e. late summer). The average productivity over this period was about 6 g m^{-2} day^{-1} with productivities of up to 11 g m^{-2} day^{-1} achieved over shorter periods. The second culture system was operated from October to December, and daily productivities reached as high as 13 g m^{-2} day^{-1}. However, productivity was generally much lower due to declining weather conditions over this period (including snow!). This important study showed that reasonably long-term larger-scale outdoor culture of microalgae and recycling of the medium was possible, but it also highlighted the several, now well known, problems which can be experienced with 'closed' photobioreactors. For example, (i) cooling was necessary to maintain the culture below 27°C; (ii) contamination by other algae (especially *Chlorococcum*) and protozoa could not be eliminated; and (iii) adequate flow rates were essential to prevent the algae settling and sticking of the algae to the reactor walls could be a problem.

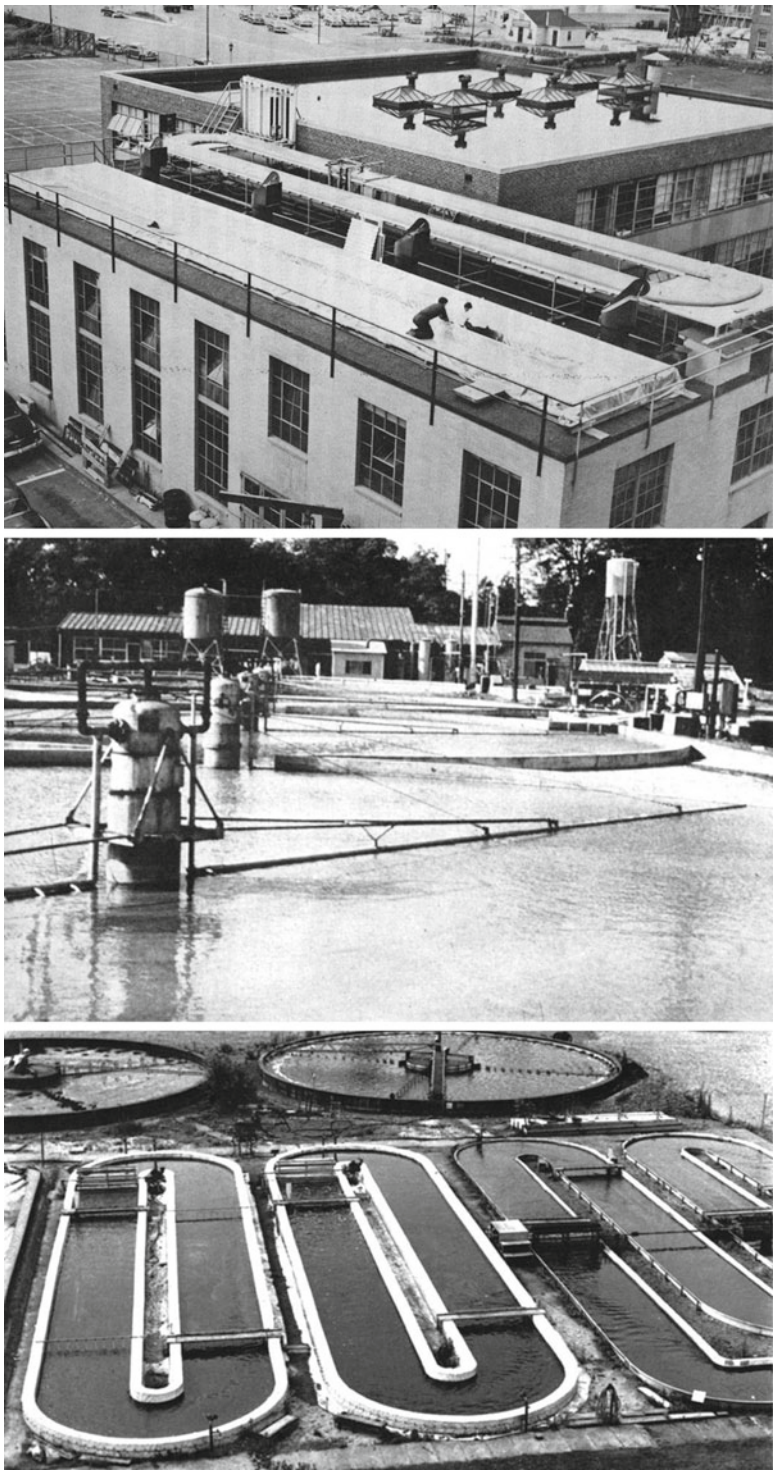

Fig. 1.2 Early large-scale algae culture systems. *Top*: Tube-type reactors on the roof of the building at Cambridge, Massachusetts, USA in 1951. The first unit in the *background* is in operation and the second unit in the *foreground* is under construction. The *glass columns* used to generate the inoculum can be seen at the *left* (From Anon 1953). *Middle*: Circular algae ponds at the Japanese Microalgae Research Institute at Kunitachi-machi, Tokyo (From Krauss 1962). *Bottom*: The outdoor algae ponds at the Gesellschaft für Strahlen- und Umweltforschung, Dortmund, Germany. The raceway ponds in the *foreground* are 20 m long and the circular ponds in the *background* have a diameter of 16 m (From Soeder 1976)

A German study (Gummert et al. 1953) carried out at the same time compared large-scale culture of *Chlorella pyrenoidosa* in 100 and 200 L tanks (15–21 cm deep) in a glasshouse with plastic lined, inclined trenches (9 m long, 70 cm wide, 20–24 cm deep at the low ends). The slope of the trenches was 6 mm m^{-1} and they were filled with 600 L culture with a culture depth of 9–15 cm. the tanks and the trenches were aerated with 1% CO_2 in air. The cultures were operated in semi-continuous mode for up to 10 days with the medium being recycled. As with the US pilot plant, contamination of the cultures with other algae and protozoa were issues at times, and it was recognized that the level of contamination

was greatly influence by climatic conditions as these affected the growth of the *Chlorella*. Some control of cyanobacterial contaminants was achieved by reducing the calcium concentration in the medium. It was also found that *Scenedesmus* (originally a contaminant) appeared to be more resistant to protozoan grazing, possibly because the *Scenedesmus* cells are larger than those of *Chlorella*.

In Wageningen, the Netherlands, larger-scale outdoor microalgae started in 1951 in 1 m^2 concrete tanks at a depth of 30 cm (Wassink et al. 1953), while in Russia large-scale outdoor cultivation of algae began in 1957 (see Gromov 1967 for review). Work in applied phycology began in Florence, Italy, in 1956, with a small pilot plant established in 1957 (Florenzano 1958).

These studies, as well as ongoing work in Japan (Sasa et al. 1955; Morimura et al. 1955; Kanizawa et al. 1958), using what we would now call both 'open' and 'closed' culture systems or photobioreactors, were the first steps from the laboratory towards eventual commercial microalgae production and identified most of the key issues still facing any attempts at commercial-scale microalgae production. Sasa et al. (1955) also were the first to do a detailed study of the seasonal variation in algae productivity over a whole 12 months period using a range of strains with different temperature tolerances. They demonstrated that, for year round culture, the species must have a wide temperature tolerance.

Another new application of microalgae, the use of algae in wastewater treatment, was proposed by Oswald and Gotaas (1957) following from the work of Oswald et al. (1953) on the oxygen-supplying role algal photosynthesis plays in sewage oxidation ponds. The option of generation energy from methane produced by fermenting the algal biomass obtained was also recognised (Golueke et al. 1957). Interestingly very little work has been done on microalgal biomass fermentation rather than methane production from seaweeds since then (c.f., Uziel 1978; Matsunaga and Izumida 1984; Chen 1987), and only now is this important topic again receiving attention.

At the same time as the above larger-scale culture experiments were taking place, important fundamental advances were being made in our understanding of algae light capture and photosynthesis. The study of photosynthesis and the efficiency of light utilization has been, and continues to be, central to attempts to optimize the productivity of algae cultures and is essential if the high-productivity cultures required for algae biofuels production are to be achieved. Kok (1948) demonstrated that about eight quanta are used per molecule of O_2 evolved although others (e.g., Pirt 1986) have suggested that fewer quanta are required. At Berkeley, USA, the pathway of carbon fixation in photosynthesis was also being elucidated by Calvin and Benson with the first paper of many published in 1948 (Calvin and Benson 1948) and including the discovery of the key role of ribulose 1,5-bisphosphate carboxylase (Quayale et al. 1954). The observation that alternating periods of light and dark enhanced the efficiency of light utilisation by algae (e.g., Emerson and Arnold 1932; Ricke and Gaffron 1943) – a phenomenon now generally known as the 'flashing-light effect' – led to further studies by Kok (1953, 1956) and Phillips and Myers (1954). The importance of the flashing-light effect and the duration of the light/dark cycles, and how these might be utilized in improving the productivity of algae cultures remains a topic of research and discussion (e.g., Laws 1986; Grobbelaar 1989, 1994; Grobbelaar et al. 1996; Nedbal et al. 1996; Janssen et al. 1999).

Another important discovery was made by Pratt who demonstrated that laboratory cultures of *Chlorella* could produce an autoinhibitor affecting both growth and photosynthesis (Pratt and Fong 1940; Pratt 1943). The problem of autoinhibition in high density cultures was later recognized (Javamardian and Palsson 1991) and the question of whether autoinhibitory substances reduce growth when culture medium is recycled is a current unresolved issue (Ikawa et al. 1997; Rodolfi et al. 2003).

Hydrogen production by algae in the light was also first demonstrated in 1942 (Gaffron and Rubin 1942), but the possibility of using microalgae hydrogen for energy was not considered until later.

Another important development at this time was the study of the sexuality and genetics of *Chlamydomonas* by Ralph Lewin (1949, 1951, 1953, 1954) which built on the earlier studies of Pascher (1916, 1918), Moewus (see excellent summary of Moewus' work by Gowans 1976), and Lerche (1937), paving the way for future genetic manipulation of microalgae (see review by Radakovits et al. 2010).

4 The 1960s and 1970s

By the beginning of the 1960s the understanding of many aspects of photosynthesis, microalgal biology, physiology and nutrition had come a long way (see for example: Hutner and Provasoli 1964).

Although the initial phase of work on microalgae mass culture in the USA had largely ceased by the mid-1950s it was revitalised at the beginning of the 1960s by William (Bill) Oswald and colleagues at the University of California, Berkeley who focused on the large-scale culture of algae for biomass production and for wastewater treatment (Oswald et al. 1957; Oswald and Golueke 1960). In the early 1960s a 2,700 m^2 (about 10^6 L capacity) meandering pond was constructed at Richmond, California (Oswald 1969a, b). The research carried out here eventually led to the construction of large-scale wastewater treatment ponds at several locations in California and which are still in operation (Oswald 1988). In 1971, John H. Ryther and colleagues at Woods Hole

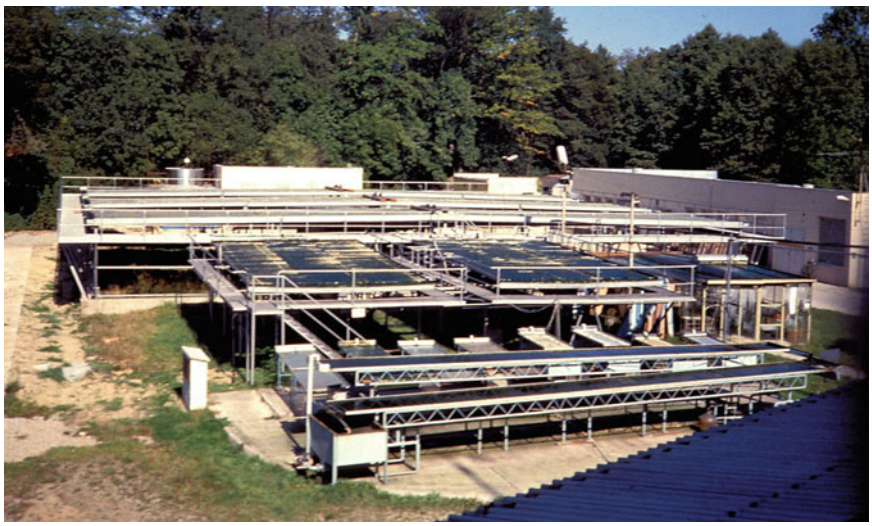

Fig. 1.3 The sloped cascade systems of the Academy of Sciences of the Czech Republic, Institute of Microbiology, Department of Phototrophic Microorganisms, at the Opatovicky mlyn near Trebon, Czech Republic

Oceanographic Institution, Massachusetts, USA, began work on the marine counterpart of Oswald's work starting with two small 4 m^2 (2,000 L) circular ponds (Goldman and Stanley 1974) and culminating in outdoor experiments with six 150 m^2 (35,000 L) ponds which were mixed by small pumps (Goldman and Ryther 1976; D'Elia et al. 1977; Goldman 1979). These studies, amongst other things, led to important advances in the understanding of nutrient requirements of the algae and limitations to growth, the effects of temperature and species succession in open ponds.

Work in Germany started in the 1950s continued at the Kohlenbiologische Forschungsstation in Dortmund where an extensive facility with four 80 m^2 paddle-wheel mixed raceway ponds and two 200 m^2 circular ponds similar to the Japanese design were constructed (Fig. 1.2) for studies of the freshwater algae *Scenedesmus* and *Coelastrum* (Stengel 1970; Soeder 1976, 1977). The Dortmund group also set up several algae research stations in collaboration with foreign governments in Thailand, Peru and India where the climate was better for algae culture than in Germany (Soeder 1976; Heussler 1980).

In 1960 the Laboratory for Microalgal Culture was established in Trebon, Czechoslovakia. In order to maximize productivity and yields, they developed a shallow sloping, extremely well mixed, culture system designed for the efficient utilization of light. The first unit built in 1960 had 12 m^2 of total surface area, and by the end of 1963 two 50 m^2 and one 900 m^2 units has been constructed (Setlik et al. 1967, 1970) and these, with some modifications, are still operational today (Doucha and Livansky 1995; Doucha et al. 2005) (Fig. 1.3). Similar 50 m^2 units were also built in Tyliez, Poland in 1966 and in Rupite, Rumania in 1968 (Vendlova 1969).

In Israel, the work of A.M. Mayer at the Hebrew University also continued in the 1960s with experiments in a 2,000 L, 1 m deep, tank which had a transparent side for better light supply to the algae (Mayer et al. 1964). Work on algae wastewater treatment was commenced by Shelef in the early 1970s with the construction of a 300 m^2 pond in Jerusalem modeled on Oswald's design (Shelef et al. 1973). In 1974 Amos Richmond commenced work on microalgae at the Beersheva campus of the Institute of Desert Research in 1974 with a number of 1 m^2 mini-ponds (Richmond 1976), later continuing the work at the Sede Boquer campus. Since then, the group a Sede Boquer has expanded and has made major contributions to the development of commercial scale algae culture, especially for *Spirulina, Porphyridium* and *Haematococcus* (e.g., Vonshak et al. 1982; Richmond 1988; Boussiba et al. 1997; Arad and Richmond 2004), and also towards our understanding of the limiting factors to outdoor microalgae production (e.g., Richmond et al. 1980; Richmond and Grobbelaar 1986; Hu et al. 1998b).

In France research on the mass culture of microalgae began at the Institute Petrole with research on culturing *Spirulina* (Clement et al. 1967; Clement 1975). At Oran in Tunisia and Antibes, France, several culture units between 5 and 700 m^2 were constructed. The culture units consisted of two adjacent horizontal channels, 10–20 cm deep, connected to a deep trough at each end. Circulation is by an airlift (using CO_2-enriched air) at opposing ends of the system so that the liquid is lifted up at one end of each through and flows down to the other end.

The first large-scale outdoor trials of growing *Dunaliella salina* were conducted in the Ukraine in the 1960s (Massyuk 1966, 1973; Massyuk and Abdula 1969)

Some work on large-scale outdoor tank culture of *Phaeodactylum tricornutum* was also undertaken in the UK (Ansell et al. 1963).

The state-of-the-art and a critical assessment of the possibility of using algae for energy towards the end of the 1970s was succinctly summarised by Oswald and Benemann (1977).

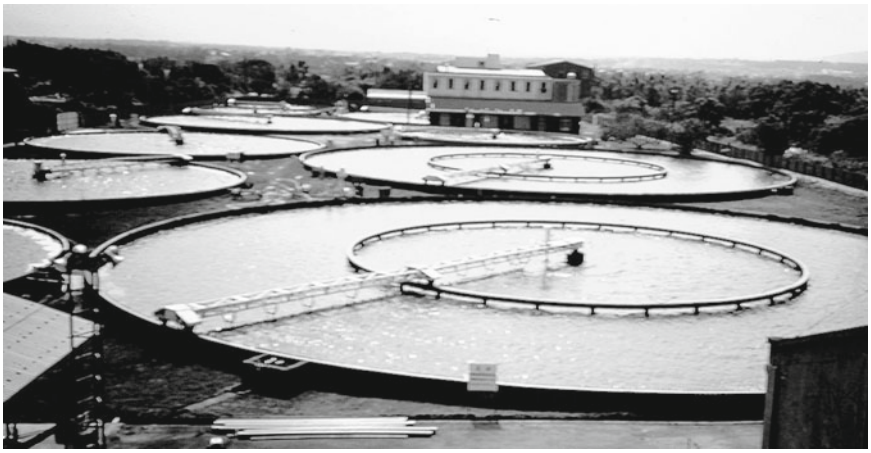

Fig. 1.4 Commercial *Chlorella* production farm near Taipei, Taiwan

Fig. 1.5 Early commercial *Spirulina* production ponds near Bangkok, Thailand

Importantly, the commercial production of microalgae, mainly for use as nutritional supplements and nutraceuticals, also started also in the 1960s (see below).

5 Commercial Production of Microalgae

Although microalgae have been harvested from natural populations (*Spirulina* and *Nostoc* spp) for food for hundreds of years in Mexico, Africa and Asia (Farrar 1966; Johnston 1970; Ciferri 1983), the 'farming' of microalgae was a very new development following from the early studies summarised above.

Unlike the early interest and studies on the mass culture of microalgae in the USA and Germany which had somewhat of a 'start-stop' pattern, those in Japan continued uninterrupted (Tamiya 1957; Krauss 1962) (Fig. 1.2) and eventually led to the development of a *Chlorella* industry in Japan and Taiwan in the early 1960s for use a heath food and nutritional supplements and expanded to China and other countries in Asia in the 1970s. Here the algae are grown in open pond systems, especially the circular centre-pivot systems (Fig. 1.4) or the closed circulation systems developed at the Tokugawa Institute for Biological Research, Tokyo, with mixotrophic culture using either acetate or glucose being common (Stengel 1970; Tsukuda et al. 1977; Soong 1980; Kawaguchi 1980). Harvesting is by centrifugation, followed by spray drying and breaking of the cells by bead mills or similar. This industry has been very successful and current annual production of *Chlorella* in Asia is about 5,000 t dry biomass, with a wholesale price of between US$20–30 kg^{-1}.

The first *Spirulina* (now known as *Arthrospira*) production plant was established in the early 1970s on Lake Texcoco near Mexico City, Mexico (Durand-Chastel 1980). This plant was however not really a controlled production system, but rather a managed harvest of the natural *Spirulina* population in the lake. This plant ceased operation in 1995. Other *Spirulina* production plants using raceway pond cultivation systems where developed in the early 1980s in the USA (e.g. Earthrise Nutritional LLC in California, and Cyanotech Corp in Kona, Hawaii) (Belay et al. 1994; Belay 1997). These two plants produced about 1,000 t $year^{-1}$ dry *Spirulina* biomass once fully operational. *Spirulina* plants were also established in Thailand (Tanticharoen et al. 1993; Bunnag et al. 1998; Shimamatsu 2004) (Fig. 1.5), and in the late 1990s *Spirulina*

production became established in China and rapidly grew with world production now estimated to be in excess of 5,000 t year^{-1} (Lee 1997; Li 1997; see also Fig. 1 in Borowitzka 1999).

The next microalgae to reach commercialization was the halophilic green alga, *Dunaliella salina,* as a source of β-carotene, with production plants being established in the early-mid 1980s in Israel, the USA and Australia. Much detail of the scientific journey from the laboratory to commercialization of *D. salina* in Australia has been published (Borowitzka and Borowitzka 1981, 1988a, b, 1989, 1990; Borowitzka et al. 1984, 1985; Moulton et al. 1987; Curtain et al. 1987; Schlipalius 1991; Borowitzka 1991, 1992, 1994). The two *D. salina* plants on Australia use extensive culture in very large (individual ponds up to 400 ha each and with a total pond area for each plant in excess of 700 ha), shallow unmixed ponds (Borowitzka 2005). Although this type of culture process means that productivity is much lower than in raceway ponds, low land costs, an extremely efficient low cost harvesting process, and an optimum climate for *D. salina* means that the Australian plants produce the algal biomass at a very low cost. On the other hand, the Israeli *D. salina* plant uses raceway ponds (Ben-Amotz and Avron 1990; Ben-Amotz 2004). Today production by the Australian and Israeli plants is estimated to be >1,000 t year^{-1} *Dunaliella* biomass and the extracted β-carotene sells for about US$600–3,000 kg^{-1} depending on formulation, mainly for use in the pharmaceutical and nutraceutical industries. Dried and stabilised whole algal biomass is also sold as use as a pigmenter in prawn feed (Boonyaratpalin et al. 2001).

In the late 1990s commercial production of the freshwater green alga *Haematococcus pluvialis* as a source of the carotenoid astaxanthin started at Cyanotech in Hawaii (Cysewski and Lorenz 2004). The culture system here is a combination of 'closed' tower reactors and raceway ponds. *Haematococcus* production by several other small producers commenced in Hawaii in subsequent years using a wide range of, mainly, 'closed' culture systems (e.g. Olaizola 2000). More recently, a large production plant in Israel, using a 2-stage culture process with combination of plate rectors and a large outdoor tubular photobioreactors has been established (Fig. 1.6). The astaxanthin from *Haematococcus* is mainly sold as a nutraceutical and antioxidant. The astaxanthin-containing algae are too expensive to use as a colouring agent in the farming of salmonids despite the algal biomass being a very effective pigmenter (Sommer et al. 1992). The heterotrophic production of *Crypthecodinium cohnii* as a source of eicosapentaenoic acid also commenced in the USA in the 1990s (Kyle et al. 1992; Barclay et al. 1994). In Germany, *Chlorella* is produced at Klötze in what is the world's largest tubular photobioreactor system (~700 m^3 volume, ~500 km of glass tube length) (Moore 2001).

The other important, and often overlooked, commercial production of microalgae is the production of microalgae as food for larval fish, mollusks and crustaceans and also in the grow-out diet of bivalve mollusks (Borowitzka 1997; Zmora and Richmond 2004; Neori 2011). Both in quantity and with respect to production cost, these algae are the most abundant and valuable produced. The high production cost is due to a combination of the species being grown and the relatively small-scale of the individual culture facilities.

Fig. 1.6 Commercial *Haematococcus pluvialis* production plant of Algatechnologies Ltd. in Israel (Courtesy Professor Sammy Boussiba)

Much can be learned from the experience of the commercial producers, however due to commercial sensitivity relatively little detailed information is publicly available.

6 The "Algae Species Programme" (USA)

The potential of algae as sources of energy was not completely forgotten and in 1960 Oswald and Golueke (1960) proposed the fermentation of microalgae biomass to produce methane as a source of energy. In 1980 the US Department of Energy began the 'Aquatic Species Programme (ASP)'. This initiative aimed to develop algae as sources of oils liquid fuels which would be able to compete with fossil fuels. Some earlier reports by Benemann and coworkers (Benemann et al. 1977, 1978) had suggested that this was possible.

The history of this programme and the main findings are summarised in detail by Sheehan et al. (1998) and a number of recommendations for future research are made. The reader is referred to this comprehensive report and only the major conclusions will be discussed here.

Sheehan et al. (1998) note in the conclusion to their report that 'perhaps the most significant observation is that the conditions that promote high productivity and rapid growth (nutrient sufficiency) and the conditions that induce lipid accumulation (nutrient limitation) are mutually exclusive. Further research will be needed to overcome this barrier, probably in the area of genetic manipulation of algal strains

to increase photosynthetic efficiency or to increase constitutive levels of lipid synthesis in algal strains'. With respect to photosynthetic efficiency they suggest that one approach is that photosynthetic productivity and light utilization could be maximized in microalgae by reducing the size of the light-harvesting antenna through mutation or genetic engineering as proposed by Neidhardt et al. (1998). This approach has been shown possible at the laboratory level (Melis et al. 1999).

They also point out that 'the ideal organism(s) for a biofuels production facility will likely be different for each location, particularly for growth in outdoor ponds. The best approach will likely be to screen for highly productive, oleaginous strains at selected sites, optimize growth conditions for large-scale culture, and optimize productivity and lipid production through genetic manipulation or biochemical manipulation of the timing of lipid accumulation in the selected strains. It is also likely that more than one strain will be used at a site, to maximize productivity at different times of the year.'

The ASP programme demonstrated that some species of microalgae could be cultivated reliably on a large scale for relatively long periods. These outdoor open pond studies showed that there were no fundamental engineering and economic issues that would limit the technical feasibility of microalgae culture, either in terms of net energy inputs, nutrient (e.g., CO_2) utilization, water requirements, harvesting technologies, or general system designs. However, although the productivities, in terms of total biomass and algal lipids (oils) achieved were high, they were still well below the theoretical potential, and (importantly) the requirements for economical viability. The authors of the report also note that the outdoor testing showed that most of the algae selected and tested in the laboratory could were not robust in the field and that, in fact, the best approach to successful cultivation of a consistent species of algae was to allow a contaminant native to the area to take over the ponds!.

Sheehan et al. (1998) also concluded that: 'the only plausible near- to mid-term application of microalgae biofuels production is integrated with wastewater treatment. In such cases the economic and resource constraints are relaxed, allowing for such processes to be considered with well below maximal productivities'. It remains to be seen whether this proves possible.

7 The RITE Biological CO_2 Fixation Programme (Japan)

In 1990 the Japanese Ministry of International Trade and Industry (MITI) through the New Energy and Industrial Technology Developments Organisation (NEDO) launched an innovative R&D programme including projects at the Research Institute of Innovative Technology for the Earth (RITE) to develop effective and clean methods of biological fixation of CO_2 based on the effective integration of photosynthesis functions of microorganisms (Michiki 1995). Although this initiative was not concerned with energy production, it is important within the context of the development of large-scale microalgae production. The RITE project had five major themes:

1. Highly-efficient photosynthesizing bacteria and microalgae with high CO_2 fixation capability;
2. Development of high-density, large-volume photosynthesis in culture systems (photobioreactors) for CO_2 fixation;
3. Development of technology to collect and utilise solar irradiance at the maximum efficiency;
4. The technology to produce useful substances and energy from microalgae;
5. The technology of the total system for CO_2 fixation and utilization at the maximum efficiency.

Unlike the US SERI programme there is no single source of information of the outcomes of the RITE programme and below I attempt to summarise some of the findings as published in the scientific literature.

The initial algae isolation and screening programme focused on high-CO_2–tolerant strains, strains which were acid and high temperature tolerant and strains with a high level of polysaccharide production (Hanagata et al. 1992; Kurano et al. 1995; Murakami and Inkenouchi 1997). The marine green alga *Chlorococcum littorale* was found to grow well at high CO_2 concentrations (Kodama et al. 1993; Chihara et al. 1994), whereas the rhodophyte *Galdieria partita* grew well at high temperature (50 °C) and acid pH (pH 1) and could tolerate 50 ppm SO_2 (Kurano et al. 1995; Uemura et al. 1997). The marine prasinophyte, *Prasinococcus capsulatus,* was the best strain isolated for extracellular polysaccharide production (Miyashita et al. 1993).

The focus of the culture systems was on closed photobioreactors with or without a solar collector to transmit light into the photobioreactor and included flat plate photobioreactors, internally lit stirred photobioreactors, and a dome-shaped photobioreactor (Usui and Ikenouchi 1997; Nanba and Kawata 1998; Zhang et al. 1999). Almost all of the studies were on a small lab-scale.

Using high cell density cultures (~80 g L^{-1}) in a flat panel photobioreactor high rates of CO_2 fixation of 200.4 g CO_2 m^{-2} day^{-1} could be achieved with *C. littorale* (Hu et al. 1998a). *C. littorale* could also produce ethanol by dark fermentation under anaerobic conditions (Ueno et al. 1998). Detailed studies on the physiology and biochemistry of this high-CO_2 tolerant alga were also carried out (e.g., Pesheva et al. 1994; Satoh et al. 2001) as were studies of some of the other species identified in the original screening programme (Suzuki et al. 1994; Uemura et al. 1997).

Some small-scale pond studies were also carried out near Sendai by Mitsubishi Heavy Industries and several electric utilities, in particular Tohoku Electric Co. Culture experiments were in small 2 m^2 raceway ponds using *Phaeodactylum tricornutum* and *Nannochloropsis salina* obtained from the NREL culture collection, and later with strains of *Tetraselmis* that spontaneously appeared and dominated the cultures (Negoro et al. 1993; Hamasaki et al. 1994; Matsumoto et al. 1995). The green alga, *Tetraselmis,* could be cultivated for the whole year with a annual mean productivity of about 11 g m^{-2} day^{-1}, whereas the cultures of the other two species were unstable.

These studies showed that microalgae could be grown on untreated CO_2-containing flue gas from power stations (Negoro et al. 1991, 1992), an important finding both for CO_2-bioremediation and for future work on growing microalgae for biofuels using power station flue gas as a CO_2 source.

8 Other Work

8.1 Botryococcus

While the studies in the USA focussed mainly of algae growing is saline water, the discovery in Australia that the green alga, *Botryococcus braunii*, produces long-chain hydrocarbons (Wake and Hillen 1980; Wake 1984) led to extensive studies of this species, especially in Europe. *Botryococcus braunii* is unusual in that it produces high levels of long-chain hydrocarbons (botryococcenes) and related ether lipids which have great similarity to fossil oils (Moldowan and Seifert 1980) and which could be a source of renewable fuels (Casadevall et al. 1985). These hydrocarbons are mainly accumulated in the extracellular matrix (Largeau et al. 1980; Bachofen 1982; Wake 1983) leading to the attractive concept of non-destructive extraction of the hydrocarbons. This alga has been studied extensively since the 1980s (see review by Metzger and Largeau 2005), but its slow growth has so far means that it is an unlikely candidate for commercial biofuels production.

8.2 Hydrogen

The discovery of Gaffron and coworkers (Gaffron 1939; Gaffron and Rubin 1942) that unicellular green algae were able to produce H_2 gas upon illumination was seen initially as a biological curiosity. In the 1970s biological production of H_2 became the subject of extensive applied research in the USA, Japan and Europe (Mitsui and Kumazawa 1977; Zaborski 1988), research which continues (Miyake et al. 2001). The concepts and developments have been extensively reviewed (Benemann 2000, 2009; Melis and Happe 2001).

8.3 Closed Photobioreactors

Work on closed photobioreactors, which has started with the work in the USA again gained impetus in the 1980s. In France Claude Gudin and Daniel Chaumont at the Centre d'Etudes Nucléares de Cararache constructed a tubular photobioreactor made of 64 mm diameter polyethylene tubes each 20 m long and with a total length of 1,500 m. They used a double layer of tubes with the culture in the upper layer of tubes. Temperature control was by placing the tubes in a pool of water and either floating or submerging the tubes by adjusting the amount of air in the lower tubes (Gudin 1976). The is pilot plant had five identical units, 20 m^2 in area with a total volume of 6.5 m^3 (Gudin and Chaumont 1983; Chaumont et al. 1988). This pilot plant operated from 1986 to 1989 and achieved productivities of 20–25 g m^{-2} day^{-1} with *Porphyridium cruentum*. In the UK at Kings College, London, Pirt and coworkers (Pirt et al. 1983) developed a tubular photobioreactor consisting of 52, 1 cm diameter, 1 m long glass tubes connected with silicone rubber U-bends to form a vertical loop, which they used to culture *Chlorella*.

Helical photobioreactors, consisting of flexible tubes wound around an upright cylindrical structure were first used in the laboratory by Davis et al. (1953) to grow *Chlorella*. A flattened version of this basic design and made of glass tubes was later used by Setlik et al. (1967), Jüttner (Jüttner et al. 1971; Jüttner 1982) and Krüger and Eloff (1981). Furthermore, Jüttner et al. (1971) developed an automated turbidostat system for continuous production of microalgae based on the principles outlined earlier by Myers and Clark (1944) and Senger and Wolf (1964). This basic tubular photobioreactor concept was developed further and improved for large-scale production and patented by Robinson and Morrison (1992). In their reactor, which they called the 'Biocoil', a number of bands of tubes were wrapped around an open cylinder for support and the bands of tubes are connected to a common manifold to equalise pressure within the tubes and reduce O_2 build-up due to shorter tube lengths, allowing the reactor to be scaled up to volumes of up to 2 m^3. At various times large (~ 1,000 L) reactors were operated in the UK (Luton and Dorking) (Fig. 1.7) and Melbourne, Australia, growing *Spirulina*, and in Perth, Australia, growing a range of microalgae including *Tetraselmis* and *Isochrysis* in continuous cultures for up to 12 months (Borowitzka, unpubl. results).

Flat plate-type photobioreactors also have a long history starting with the rocking tray of Milner (Davis et al. 1953) growing *Chlorella*, and later with designs by Anderson and Eakin (1985) growing *P. cruentum*, and Samson and LeDuy (1985) growing *Arthrospira* (*Spirulina*) *maxima.* Other, more sophisticated flat panel photobioreactors were developed in France (Ramos de Ortega and Roux 1986) and Italy (Tredici et al. 1991; Tredici and Materassi 1992) and later in Germany

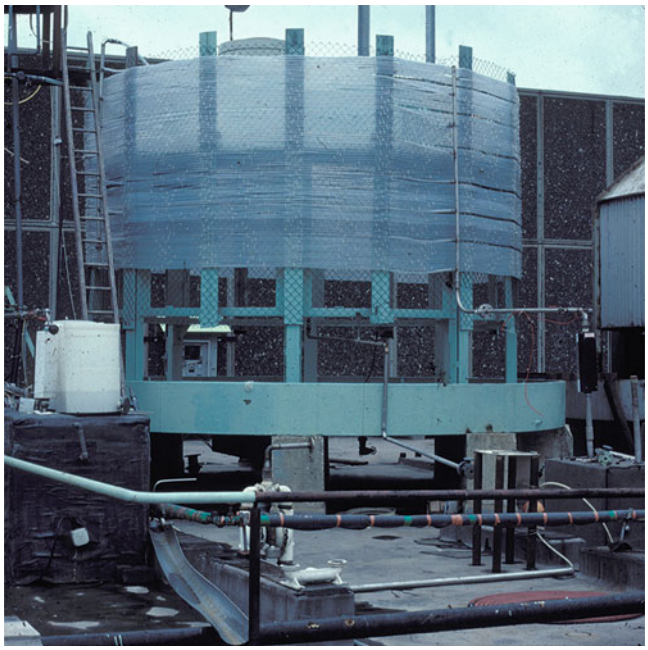

Fig. 1.7 The first pilot scale helical tubular photobioreactor (Biocoil) in Dorking, United Kingdom, located on the roof of a brewery in 1983. CO_2 from the brewing process was used to enhance the growth of *Spirulina*

(Pulz 2001) and Israel (Hu et al. 1996). Many versions of closed photobioreactors have been patented, especially in the last few decades (e.g., Ichimura and Ozono 1976; Selke 1976; Hills 1984; Huntley et al. 1991; Delente et al. 1992; Kobayashi 1997; Skill 1998; Buchholz 1999), and more details on the principal types of closed photobioreactors and their development can be found in the review by Tredici (2004) and Chapter 7 of this volume.

8.4 Downstream Processing

Although extensive work on the culture of microalgae was done up to the early 1980s relatively little work was done on the harvesting, dewatering and further processing of the algal biomass. Burlew (1953b) considered centrifugation or gravity settling, possibly followed by spray drying bur recognised that the costs of these processes at the large-scale had not yet been considered. Froth flotation as a method of harvesting was proposed by Levin et al. (1962), and the first studies of harvesting methods from sewage grown algae was that of Golueke and Oswald (1965) in the USA, and Caldwell, Connell Engineers (1976) in Australia, and the state-of-the art was reviewed by Benemann et al. (1980) and Shelef et al. (1984). It was recognised fairly early that the economics of commercial utilisation of microalgae was depended significantly on the cost of harvesting and dewatering (e.g., Soeder 1978). Of particular interest are also the detailed studies of the relative costs of different harvesting methods by Mohn and coworkers (Mohn 1980, 1988; Mohn and Cordero-Contreras 1990) based on detailed comparisons of different harvesting methods at Dortmund and Jülich in Germany.

9 Conclusion

It is impossible to do justice to all the work that has been done in the last 140 years on microalgae and the application of microalgae as sources of renewable energy. The above chapter is a short overview of the developments and highlights along the path of what we hope will be a commercially successful, renewable, and environmentally benign energy source for humanity in the future. Like most R&D efforts the path is full of bumps, interruptions, twists and turns and, at times, apparent dead ends. It is also the nature of applied research that much of the relevant information may not be found in the mainstream scientific literature, but in reports of contractors and consultants which may not be easily attainable. However, the effort to track down such reports can be very rewarding and very much can be learned from the extensive work that has been done.

The vagaries of research funding and political priorities have resulted in periodic intense bursts of research activity (e.g. the ASP programme in the USA and the RITE programme in Japan) and changes in the principal research focus (e.g. algae as sources of protein and nutrition, algae for high-value chemicals, algae for wastewater treatment, algae for biofuels, algae for CO_2 capture) and advances in basic biology and new research methods (e.g. studies on photosynthesis and the recent developments in molecular biology and the sequencing of algal genomes) have provided important new insights and opportunities. Much can be learnt from these past efforts, both the successes and the failures. Unfortunately recent papers on algae biofuels show that some people apparently have not learned from the experiences in the laboratory and in commercial microalgae production. However, most applied phycologists are very forward looking and inherently very optimistic, and they appreciate and recognise the important contributions made by their predecessors and build on these.

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Algal Lipids and Their Metabolism

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Irina A. Guschina and John L. Harwood

1 Introduction

Algal lipids can be divided into two main groups: the non-polar lipids (acylglycerols, sterols, free (non-esterified) fatty acids, hydrocarbons, wax and steryl esters) and polar lipids (phosphoglycerides, glycosylglycerides) (Gunstone et al. 2007). They are essential constituents of all living cells where they perform important functions.

Phosphoglycerides, glycosylglycerides and sterols are essential structural components of biological membranes. These lipids maintain specific membrane functions and provide the permeability barrier surrounding cells and between organelles within cells, as well as providing a matrix for various metabolic processes. Some polar lipids may act as key intermediates (or precursors of intermediates) in cell signalling pathways (e.g. inositol lipids, sphingolipids, oxidative products of polyunsaturated fatty acids). The non-polar lipids, mainly triacylglycerols (TAG), are abundant storage products which can be easily catabolised to provide metabolic energy (Gurr et al. 2002). Waxes commonly contribute to the extracellular surface layers covering different parts of higher plants. Moreover, they may act (in the form of wax esters) as energy stores especially in some organisms from cold water habitats (Guschina and Harwood 2007, 2008).

Algae comprise a large group of photosynthetic, heterotrophic organisms from different phylogenetic groups, representing many taxonomic divisions. They are distributed worldwide, inhabiting predominantly fresh- and seawater ecosystems. The ability of algae to adapt to environmental conditions is reflected in an exceptional variety of lipids as well as a number of unusual compounds. Many algae accumulate substantial amounts of non-polar lipids, mostly in the form of TAG or hydrocarbons, and these levels may reach up to 20–50% of dry cell weight. These oleaginous species have been considered as promising sources of oil for biofuels, such as surrogates of gasoline, kerosene and diesel, being both renewable and carbon neutral. The potential advantages of algae as a source of oil for biofuels include their ability to grow at high rates exhibiting a rapid biomass doubling time (usually 1–6 days) and producing 10–20 times more oil (ha^{-1} $year^{-1}$) than any oil crop plant. Algae can grow in saline, brackish and coastal seawater with little competition. They may utilize growth nutrients from wastewater sources and sequester carbon dioxide from emitted flue gases, thereby providing additional environmental benefits. Moreover, algae can produce valuable co- and by-products including carotenoids (β-carotene, astaxanthin, canthaxanthin and lutein), other pigments (phycocyanin and phycoerythrin), ω-3 fatty acids (eicosapentaenoic and docosahexaenoic acids), vitamins (tocopherols, vitamin B12 and provitamin A), polysaccarides and proteins. Thus, algae exhibit superior attributes to terrestrial crop plants as bioenergy sources. Moreover, in most cases algae will not compete for habitats used to produce food crops.

In spite of several technical limitations associated with existing technologies in the production of economically-viable algal oil, further research in this area is needed and such studies will clearly benefit from a better understanding of lipid metabolism and accumulation in algal cells. At present, relatively little information is available on lipid biosynthesis and its regulation in algae. Moreover, the lack of information about control mechanisms for lipid synthesis in different algal species limits our attempts to manipulate lipid metabolism in algae. However, some promising achievements in genetic and metabolic manipulations in higher plants are useful examples/directions to follow.

In the present chapter we will give an overview of lipid composition and lipid metabolism in algae with a special emphasis on the production of algal oils and/or their metabolism for biofuel applications. Previous useful reviews of algal lipids are Harwood and Jones (1989), Thompson (1996), Harwood (1998a) and Guschina and Harwood (2006a).

I.A. Guschina (✉) • J.L. Harwood
School of Biosciences, Cardiff University,
Museum Avenue, CF10 3AX Cardiff, Wales, UK
e-mail: guschinaia@cardiff.ac.uk; harwood@cardiff.ac.uk

M.A. Borowitzka and N.R. Moheimani (eds.), *Algae for Biofuels and Energy*, Developments in Applied Phycology 5,
DOI 10.1007/978-94-007-5479-9_2,

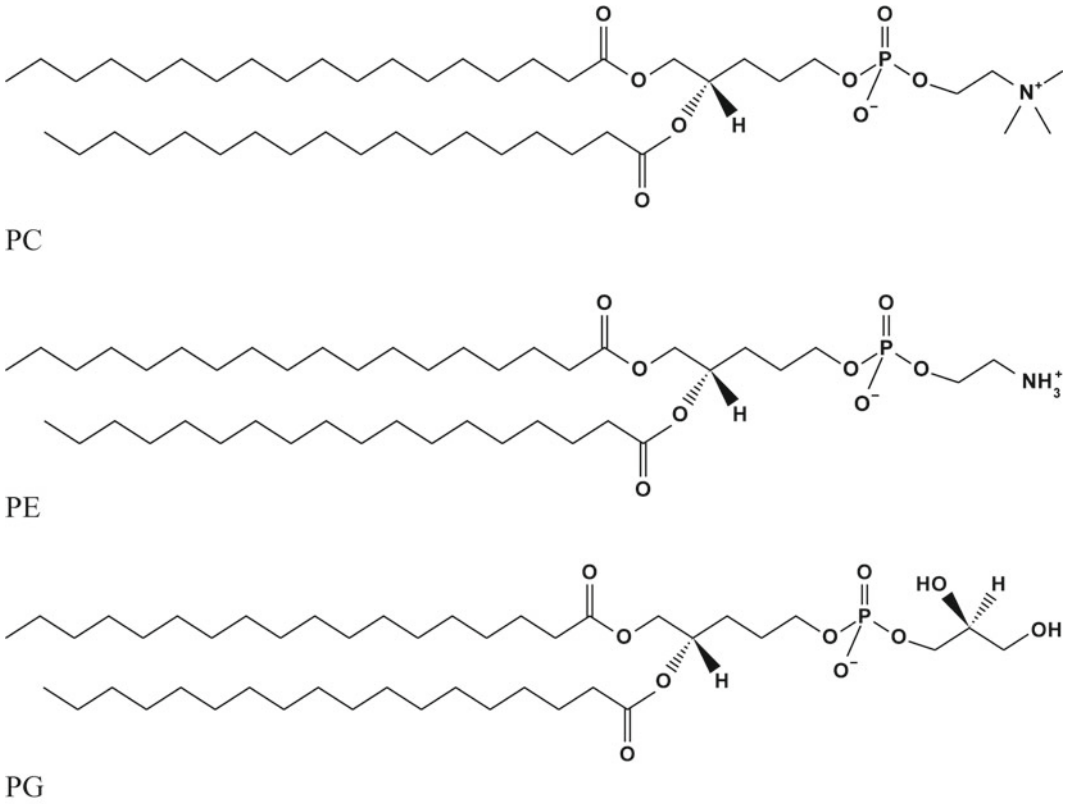

Fig. 2.1 Examples of the major phosphoglycerides of algae. *PC* phosphatidylcholine, *PE* phosphatidylethanolamine, *PG* phosphatidylglycerol

2 Algal Lipids

2.1 Polar Glycerolipids

2.1.1 Phosphoglycerides

The basic structure of phosphoglycerides (phospholipids) is a glycerol backbone metabolically derived from glycerol 3-phosphate to which are esterified hydrophobic acyl groups at the 1- and 2-positions, and phosphate is esterified to the *sn*-3 position with a further link to a hydrophilic base group. Three phospholipids, phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylglycerol (PG), are the major phosphoglycerides identified in most algae species (Fig. 2.1). In addition, phosphatidylserine (PS), phosphatidylinositol (PI) and diphosphatidylglycerol (DPG) (or cardiolipin) may also present in different algal cells in appreciable amounts. Phosphatidic acid is usually a minor component but is an important metabolic intermediate and may be a signalling compound.

The phospholipids are located in the extrachloroplast membranes with the exception of PG. This phospholipid is present in substantial quantities in thylakoid membranes. PG accounts for around 10 and 20% of the total polar glycerolipids in eukaryotic green algae. An unusual fatty acid, Δ^3-*trans*-hexadecenoic acid (16:1(3 t)), is present in all eukaryotic photosynthetic organisms, being highly enriched at the *sn*-2 position of PG (for review see Tremolieres and Siegenthaler 1998). The *trans*-configuration of the double bond and its Δ^3 position are both very unusual for naturally-occurring fatty acids (El Maanni et al. 1998). A possible role of PG-16:1(3 t) in photosynthetic membranes will be discussed below.

Several unusual phospholipids have been isolated from algae. A sulfonium analog of phosphatidylcholine has been identified in diatoms (Anderson et al. 1978a, b; Bisseret et al. 1984). In this lipid, a sulphur atom replaces the nitrogen atom of choline. This phosphatidylsulfocholine (PSC) completely replaces PC in a non-photosynthetic diatom, *Nitzschia alba*, whereas in four other diatom species both lipids were found with PSC at levels corresponding to 6–24% of the total PC + PSC fraction. Low levels of PSC (less than 2%) were also reported for the diatoms *Cyclotella nana* and *Navicula incerta* as well as for a *Euglena* spp. (Bisseret et al. 1984).

A novel lipid constituent was isolated from brown algae. It was identified as phosphatidyl-O-[N-(2-hydroxyethyl)glycine] with the glycine derivative as headgroup (PHEG) (Eichenberger et al. 1995). This lipid was present in all 30 brown algal species analysed in the range 8–25 mol% of total phospholipids. This common lipid of brown algae has been shown to be accumulated in the plasma membrane of gametes of the brown alga *Ectocarpus*. Arachidonic acid

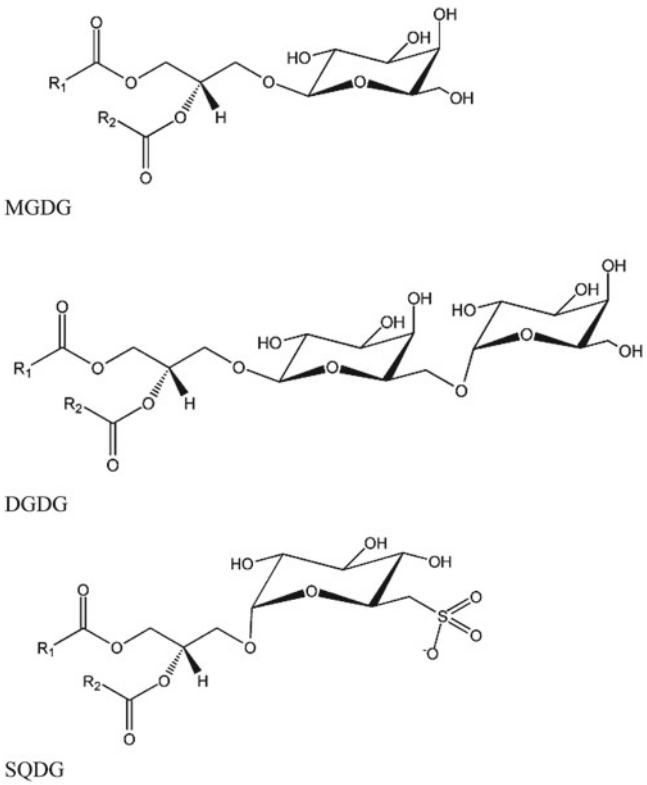

Fig. 2.2 The structures of the main glycosylglycerides of algae. *R1* and *R2* are the two fatty acyl chains. *MGDG* monogalactosyldiacylglycerol; *DGDG* digalactosyldiacylglycerol; *SQDG* sulfoquinovosyldiacylglycerol

(20:4n-3) and 20:5n-3 (eicosapentaenoic acid; EPA) were dominant in PHEG and represent 80 and 10%, respectively. Based on this finding, a special role of PHEG as an acyl donor for pheromone production and its possible participation in the fertilization of brown algae has been proposed (Eichenberger et al. 1995).

2.1.2 Glycosylglycerides

Glycosylglycerides (glycolipids) are characterized by a 1,2-diacyl-*sn*-glycerol moiety with a mono- or oligosaccharide attached at the *sn*-3 position of the glycerol backbone. The major plastid lipids, galactosylglycerides, are uncharged, polar lipids. They contain one or two galactose molecules linked to the *sn*-3 position of the glycerol corresponding to 1,2-diacyl-3-O-(β-D-galactopyranosyl)-*sn*-glycerol (or monogalactosyldiacylglycerol, MGDG) and 1,2-diacyl-3-O-(α-D-galactopyranosyl)-(1 → 6)-O-β-D-galactopyranosyl-*sn*-glycerol (or digalactosyldiacylglycerol, DGDG) (Fig. 2.2). In plants, MGDG and DGDG account for 40–55% and 15–35% of the total lipids in thylakoid membranes, respectively Harwood (1998a). Another class of glycosylglyceride is a sulfolipid, sulfoquinovosyldiacylglycerol, or 1,2-diacyl-3-O-(6-deoxy-6-sulfo-α-D-glucopyranosyl)-*sn*-glycerol (SQDG) (Fig. 2.2). It is present in both photosynthetic and in non-photosynthetic membranes of algae and may reach up to 30% of total lipids as found in the raphidophycean alga *Chattonella antiqua* (Harwood and Jones 1989). SQDG is unusual because of its sulfonic acid linkage. The sulfonoglucosidic moiety (6-deoxy-6-sulfono-glucoside) is described as sulfoquinovosyl and its sulfonic residue carries a full negative charge at physiological pH (see review by Harwood and Okanenko 2003).

Plastid galactolipids are characterised by a very high content of polyunsaturated fatty acids (Harwood 1998a). Thus, MGDG in fresh water algae contains α-linolenic (C18:3n-3) as the major fatty acid, and C18:3n-3 and palmitic acid (C16:0) are dominant in DGDG and SQDG. The glycolipids from some algal species, e.g. green algae *Trebouxia* spp., *Coccomyxa* spp., *Chlamydomonas* spp., *Scenedesmus* spp., may also be esterified with unsaturated C16 acids, such as hexadecatrienoic (C16:3n-3/C16:3n-2) and hexadecatetraenoic (C16:4) (Guschina et al. 2003; Arisz et al. 2000). In contrast, the plastidial glycosylglycerolipids of marine algae contain, in addition to C18:3n-3 and C16:0, some very-long-chain polyunsaturated fatty acids, e.g. arachidonic (C20:4n-6), eicosapentaenoic acid (C20:5n-3), docosahexaenoic (C22:6n-3) as well as octadecatetraenoic acid (18:4n-3) (Harwood and Jones 1989).

In an extract of the marine chloromonad *Heterosigma carterae* (Raphidophyceae), a complex mixture of SQDGs with C16:0, C16:1n-7, C16:1n-5, C16:1n-3 and C20:5n-3 as the main fatty acids has been identified (Keusgen et al. 1997). MGDG from the marine diatom *Skeletonema costatum* contains another unusual fatty acid, C18:3n-1, at a relatively high amount (about 25%) (D'Ippolito et al. 2004).

In some species of algae, a few unusual glycolipids have been identified in addition to MGDG, DGDG and SQDG. Trigalactosylglycerol has been found in *Chlorella* spp. (Harwood and Jones 1989). It has also been shown that glycolipids may contain sugars other than galactose (e.g., mannose and rhamnose) as reported for some red algae (Harwood and Jones 1989). An unusual glycolipid, sulfoquinovosylmonogalactosylglycerol (SQMG) was isolated from the marine red alga, *Gracilaria verrucosa* (Son 1990).

A carboxylated glycoglycerolipid, diacylglyceryl glucuronide (DGGA) has been described in *Ochromonas danica* (Chrysophyceae) and in *Pavlova lutheri* (Haptophyceae) (Eichenberger and Gribi 1994, 1997). This glycolipid accounts for about 3% of the glycerolipids in *O. danica*. Its predominant molecular species contained a C20:4/C22:5-combination of fatty acids. C22:5n-6 (44.4% of total FA) and C22:6n-3 acids (18.9%) were present in DGGA from the haptophyte *Pavlova lutheri* (Pavlovophyceae) (Eichenberger and Gribi 1997).

A new glycoglycerolipid with a rare 6-deoxy-6-aminoglucose moiety, avrainvilloside, has been reported for the marine green alga *Avrainvillea nigricans* (Andersen and Taglialatela-Scafati 2005). Three minor new glycolipids were also found

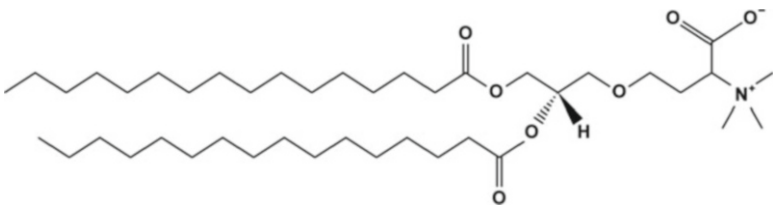

Fig. 2.3 The main betaine lipid of algae, 1,2-diacylglyceryl-3-*O*-4′-(*N,N,N*-trimethyl)-homoserine (DGTS)

in crude methanolic extracts of the red alga, *Chondria armata* (Al-Fadhli et al. 2006). They were identified as 1,2-di-O-acyl-3-O-(acyl-6′-galactosyl)-glycerol (GL_{1a}), the sulfonoglycolipid2-O-palmitoyl-3-O-(6′-sulfoquinovopyranosyl)-glycerol and its ethyl ether derivative. GL_{1a} has been mentioned as the first example of a glycolipid acylated at the 6’ position of galactose which occurred naturally (Al-Fadhli et al. 2006).

In algae (as in higher plants and cyanobacteria), glycolipids are located predominantly in photosynthetic membranes and their role in photosynthesis is discussed below.

2.1.3 Betaine Lipids

Betaine lipids have a betaine moiety as a polar group which is linked to the *sn*-3 position of glycerol by an ether bond. Betaine lipids contain neither phosphorus nor carbohydrate groups. 1,2-diacylglyceryl-3-*O*-4′-(*N,N,N*-trimethyl)-homoserine (DGTS), 1,2-diacylglyceryl-3-*O*-2′-(hydroxymethyl)-(*N,N,N*-trimethyl)-β-alanine (DGTA) and 1,2-diacylglyceryl-3-*O*-carboxy-(hydroxymethyl)-choline (DGCC) are three types of betaine lipids identified in algae (Dembitsky 1996) (Fig. 2.3). They are all zwitterionic at neutral pH since their molecules have a positively charged trimethylammonium group and a negatively charged carboxyl group (Fig. 2.3).

Betaine lipids are common components of algae (as well as ferns, bryophytes, lichens, some fungi and protozoans), but they are not found in higher plants, either gymnosperms or angiosperms. The taxonomic distribution of betaine lipids in various groups of algae has been reviewed in detail by Dembitsky (1996) and Kato et al. (1996).

The fatty acid composition of DGTS varies significantly between freshwater and marine species. So, in freshwater algae mainly saturated fatty acids (C14:0 and C16:0) were found at the *sn*-1 position of the glycerol backbone and C18 acids (predominantly C18:2n-6 and C18:3n-3) at the *sn*-2 position. DGTS in marine algae can contain very long chain polyunsaturated fatty acids at both the *sn*-1 and *sn*-2 positions. For example, in the marine eustigmatophyte UTEX 2341 (previously identified as *Chlorella minutissima*) which produced DGTS at unusually high levels (up to 44% of total lipids), DGTS was exceptionally rich in EPA. The latter’s level constituted over 90% of total fatty acids of DGTS in this alga (Gladu et al. 1995; Haigh et al. 1996).

A structural similarity between betaine lipids and phosphatidylcholine (as well as their taxonomical distribution with a reciprocal relationship between PC and betaine lipids in many algal species) has led to the suggestion that betaine lipids, especially DGTS, are more evolutionarily primitive lipids which, in lower plants, play the same functions in membranes that PC does in higher plants and animals (Dembitsky 1996).

2.1.3.1 Role of Polar Glycerolipids and Their Fatty Acids in Photosynthesis

Photosynthesis is a key process of converting atmospheric carbon dioxide into numerous metabolites, and it is pivotal for many metabolic pathways involved in the production of new biomass. To harness the potential of algae to grow rapidly and to accumulate lipids in large amounts, a deeper understanding of photosynthetic metabolism and especially its regulation in algae may be useful. In this part of our chapter, we would like to give a brief review of the role of lipids as important structural and regulatory compounds of chloroplast membranes. For more detailed information on the role of lipids in photosynthesis refer to Jones (2007) and Wada and Murata (2010).

The unique lipid composition in chloroplast membranes (e.g. high level of fatty acid unsaturation, the presence of PG-16:1(3 t) as well as the galactosylglycerides which are mainly located in these cell organelles) has been suggested to be important for normal photosynthetic function (Murata and Siegenthaler 1998). Investigation of a series of *Chlamydomonas* mutants with specific alterations in lipid composition has been shown to be a powerful tool to study structure-function relationships. To examine the role of SQDG in thylakoid membranes, Sato and co-workers (Sato et al. 2003a) compared the structural and functional properties of photosystem II (PSII) between a mutant of *Chlamydomonas reinhardtii* defective in SQDG (*hf-2*) and the wild type. Through characterization of the photosynthetic apparatus of an SQDG-defective mutant, it has been suggested that SQDG is involved in maintenance of the normal properties of PSII (Sato et al. 2003a). Selected mutants of *C. reinhardtii* lacking Δ^3-*trans*-hexadecenoic acid-containing phosphatidylglycerol (PG-16:1(3 t)) have been used to study a possible role of this lipid in the biogenesis and trimerization of the main light-harvesting chlorophyll-protein complex, the LHCII (El Maanni et al. 1998; Dubertret et al. 2002; Pineau et al. 2004). From a number of experiments where PG-16:1(3 t) was reincorporated into the photosynthetic membranes of the living mutants, it has been concluded that PG plays a crucial role in the LHCII

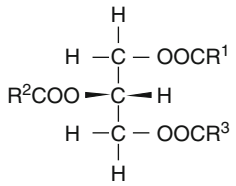

Fig. 2.4 Triacylglycerol structure. R^1, R^2 and R^3 are (usually different) fatty acyl chains

trimerization process. Moreover, 16:1(3 t) confers special properties to the PG molecule allowing high affinity interactions with some specific sites in the chlorophyll-protein complex (Dubertret et al. 2002; Pineau et al. 2004). An excellent review providing information on possible functions for PG in photosynthesis is that by Domonkos et al. (2008).

The role and contribution of lowered unsaturation of chloroplast lipids to adaptation and tolerance of photosynthesis to high temperature has been shown when studying a mutant of *C. reinhardtii* (*hf-9*) with impaired fatty acid desaturation of its chloroplast lipids (Sato et al. 1996).

2.2 Non-polar Storage Lipids

2.2.1 Triacylglycerols

Triacylglycerols (Fig. 2.4) are accumulated in many algae species as storage products. The level of TAG accumulation is very variable (Fig. 2.5) and may be stimulated by a number of environmental factors (see below). When algal growth slows down and there is no requirement for the synthesis of new membrane compounds, the cells divert fatty acids into TAG synthesis before conditions improve and there is a need for further growth.

It has been shown that, in general, TAG synthesis is favoured in the light period when TAG is stored in cytosolic lipid bodies and then reutilized for polar lipid synthesis in the dark (Thompson 1996). Nitrogen deprivation seems to be a major factor which is important for the stimulation of TAG synthesis. Many algae sustain a two- to three-fold increase in lipid content, predominantly TAG, under nitrogen limitation (Thompson 1996). Algal TAG are generally characterized by saturated and monounsaturated fatty acids. However, some oleaginous species may contain high levels of long chain polyunsaturated fatty acids in TAG (Table 2.1). The dynamics of arachidonic acid accumulation in TAG has been studied in the green alga *Parietochloris incisa* (Bigogno et al. 2002a). They found that arachidonyl moieties were mobilised from storage TAG into chloroplast lipids when recovering from nitrogen starvation (Bigogno et al. 2002a; Khozin-Goldberg et al. 2000, 2005). In this alga, PUFA-rich TAG have been hypothesised to be metabolically active in serving as a reservoir for specific fatty acids. During adaptation to sudden changes in environmental conditions, when the de novo synthesis of PUFA would be slow, PUFA-rich TAG may provide specific acyl groups for polar lipids thus enabling a rapid adaptive reorganisation of the membranes (Khozin-Goldberg et al. 2005; Makewicz et al. 1997).

The biosynthesis of TAG in algae is discussed in a later section.

2.2.2 Hydrocarbons

Some algae are known and characterised by their capacity to synthesise and accumulate a significant amount of hydrocarbons and have, therefore, excellent capability for biodiesel production. One of the most promising species in this algal group is *Botryococcus braunii*. This green colonial fresh water microalga has been recognised for some time as having good potential as a renewable resource for the production of liquid hydrocarbons (Metzger and Casadevall 1991; Metzger and Largeau 2005). It is of interest, that geochemical analysis of petroleum has shown that botryococcene- and methylated squalene-type hydrocarbons, presumably generated by microalgae ancestral to *B. braunii*, may be the source of today's petroleum deposits (Eroglu and Melis 2010).

The structure of hydrocarbons from *B. braunii* varies depending on the race, and *B. braunii* has been classified into A, B, and L races depending on the type of hydrocarbons synthesised. Thus, the A race produces up to 61% (on a dry biomass basis) of non-isoprenoid dienic and trienic hydrocarbons, odd numbered n-alkadienes, mono-, tri, tetra-, and pentaenes, from C25 to C31, which are derived from fatty acids. Race B yields C30–C37 highly unsaturated isoprenoid hydrocarbons, termed botryococcenes and small amounts of methyl branched squalenes. Race L produces a single tetraterpenoid hydrocarbon known as lycopadiene (Rao et al. 2007a, b). Botryococcenes are extracted from total lipids in the hexane-soluble fraction and can be converted into useful fuels by catalytic cracking (Raja et al. 2008). It has been reported that on hydrocracking, the distillate yields 67% gasoline, 15% aviation turbine fuel, 15% diesel fuel, and 3% residual oil. The unit area yield of oil is estimated to be from 5000 to 20,000 gal acre^{-1} year^{-1} (7,700–30,600 L ha^{-1} year^{-1}). This is 7–30 times greater that the best oil crop, palm oil (63 5 gal acre^{-1} year^{-1} = 973 L ha^{-1} year^{-1}) (Raja et al. 2008).

In general, the hydrocarbon content in *B. braunii* varies between 20 and 50% of dry weight depending upon the environmental conditions. In natural populations, the content of botryococcenes varies from 27–86% of dry cell mass and may be affected by various growth conditions. Nitrogen limitation has been shown to lead to a 1.6-fold increase in lipid content in this species (Singh and Kumar 1992). Anaerobiosis under nitrogen-deficient conditions also led to a greater lipid production in comparison to anaerobiosis in nitrogen-sufficient medium. Growth of *B. braunii* (race A) and production of hydrocarbons has been shown to be influenced by

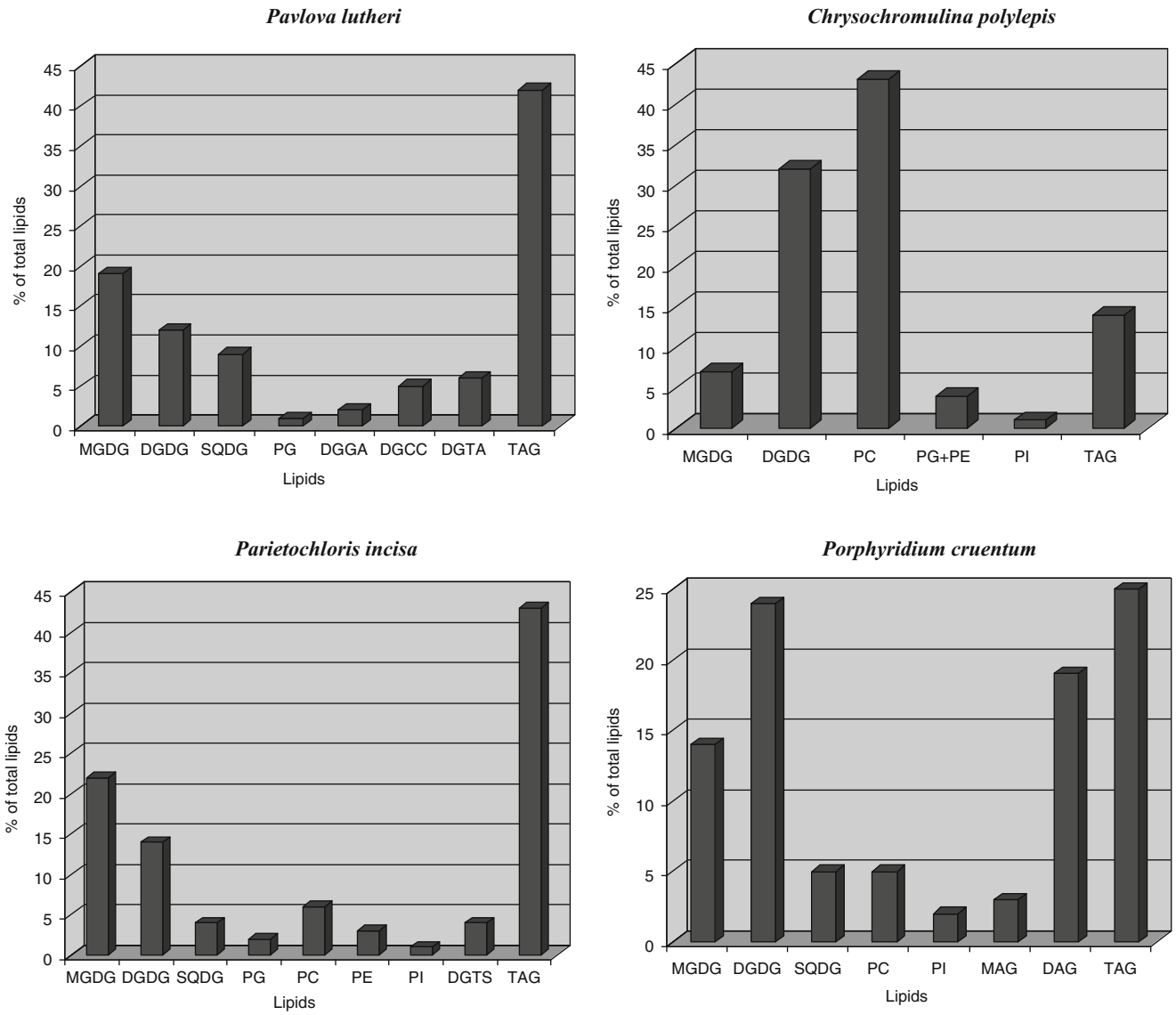

Fig. 2.5 Glycerolipid composition of selected species of algae. *Pavlova lutheri* (Eichenberger and Gribi 1997); *Chrysochromulina polylepis* (John et al. 2002); *Parietochloris incise* (Bigogno et al. 2002a); *Porphyridium cruentum,* (Alonso et al. 1998). Abbreviations: *MGDG* monogalactosyldiacylglycerol, *DGDG* digalactosyldiacylglycerol, *SQDG* sulfoquinovosyldiacylglycerol, *PG* phosphatidylglycerol, *PC* phosphatidylcholine, *PE* phosphatidylethanolamine, *PI* phosphatidylinositol, *DGTS* diacylglyceryltrimethylhomoserine,*DGTA* diacylglycerylhydroxymethyltrimethylalanine, *DGGA* diacylglycerylglucuronide, *DGCC* diacylglycerylcarboxyhydroxymethylcholine, *MAG* monoacylglycerol, *DAG* diacylglycerol, *TAG* triacylglycerol. The lipids were quantified on the basis of their fatty acid contents

different levels of salinity and CO_2 (Vazquez-Duhalt and Arredondo-Vega 1991; Rao et al. 2007a, b).

The biomass was found to increase with increasing concentrations (from 17 to 85 mM) of NaCl and the maximum biomass yield was achieved in 17 and 34 mM salinity (Rao et al. 2007a). Maximum hydrocarbon contents (28%, wt/wt) were observed in 68 mM salinity. The total lipid content of this alga was also affected by salinity varying from 24 to 28% (wt/wt) whereas in control it was 20% (Rao et al. 2007a). Stearic and linoleic acids were dominant in control cultures while palmitoleic and oleic acids were in higher proportions in algae grown at two different salinities (34 and 85 mM NaCl) (Rao et al. 2007a). The biomass production and hydrocarbon yield have been shown to be also increased with increasing concentrations of CO_2 in cultures (from 0.5 to 2%) (Rao et al. 2007b). Maximum hydrocarbon content was found at 2% CO_2 (Rao et al. 2007b).

The growth of *B. braunii* B70 and the size of oil granules in cells can be significantly increased by an addition of low concentrations of glucose (2–10 mM) to the culture medium (Tanoi et al. 2011). The possibility of using wastewater from a soybean curd (SCW) manufacturing plant as a growth

Table 2.1 Fatty acid distribution reported in TAG from selected algae species

	14:0	16:0	16:1	16:2	16:3	16:4	18:0	18:1	18:1	18:2	18:3	18:4	20:2	20:3	20:4	20:5	22:5	22:6
Algae				n-4	n-4	n-1		n-9	n-7	n-6	n-3	n-3		n-6	n-6	n-3		n-3
Eustigmatophyceae																		
Nannochloropsis sp.	18.8	41.6	33.7	–	–	–	1.0	3.8	–	1.1	–	–	–	–	–	–	–	–
Chlorophyceae																		
Parietochloris Incisa	–	8.4	0.4[a]	–	–	–	3.1	18.0	4.0	14.1	0.4[b]	–	–	1.1	47.1	0.7	–	–
Rhodophyceae																		
Porphyridium cruentum	1.6	21.1	1.5	–	–	–	3.7	4.0	0.9	12.2	–	–	1.0	1.1	24.2	15.9	–	–
Bacillariophyceae																		
Phaedactylum tricornutum	4.3	13.3	17.4	4.8	2.1	0.5	2.5	1.4	0.1	1.0	–	–	–	–	3.7	35.5	–	1.2
Prymnesiophyceae																		
Isochrysis galbana	4.8	12.3	21.7	–	–	–	1.2	4.9	1.2	2.2	1.5	7.2	–	–	–	25.6	1.2	8.1
Haptophyceae																		
Pavlova lutheri	8.6	39.2	32.9	–	–	–	tr.	2.1[c]	–	3.7	–	–	4.3	–	–	7.0	–	1.1

The positions of double bonds were assigned following capillary gas-liquid chromatography but were not confirmed by other methods. Dashes mean none detected, tr. = trace. Only the major fatty acids present are shown

Nannochloropsis sp. (grown under low light conditions) (Sukenik et al. 1993); *Parietochloris incisa* (stationary phase culture analysed) (Bigogno et al. 2002a); (Eichenberger and Gribi 1997); *Isochrysis galbana, Porphyridium cruentum, Phaeodactylum tricornutum* (Alonso et al. 1998)

[a]0.4 – 16:1 represents C16:1n-11 isomer

[b]0.4 – 0.7% of C18:3n-6 also present

[c]2.1 – sum of two isomers present. 16:1 is a mixture if isomers

promoter of *B. braunii* strain BOT-22 has been evaluated (Yonezawa et al. 2012). The growth and hydrocarbon accumulation were significantly higher in the cultures with 1 and 2% SCW. An addition of SCW also caused a shift in the hydrocarbon profile from $C_{34}H_{58}$ to $C_{32}H_{54}$ (Yonezawa et al. 2012). In addition, higher production of hydrocarbons in *B. braunii* Bot-144 (race B) has been achieved when it is grown under red light (Baba et al. 2012).

Although *B. braunii* can be found in all climatic zones, its habitats are restricted to freshwater or brackish water. Recently, a marine microalga, *Scenedesmus* sp. (strain JPCC GA0024, tentatively identified as *S. rubescens*), has been characterised for biofuel production (Matsunaga et al. 2009). It has been shown that the maximum biomass of 0.79 $g.L^{-1}$ could be obtained in 100% artificial seawater without additional nutrients for 11 days. The lipid content reached 73% of dry biomass under starvation conditions (no nutrient addition), which is equivalent to that of *B. braunii* (Matsunaga et al. 2009). Among non-polar lipids, aliphatic hydrocarbons were estimated as 0.6% of dry biomass in nutrient-rich medium. This value was higher than other hydrocarbon-producing cyanobacterial species (0.025–0.12%) but significantly lower than that of *B. braunii* (Matsunaga et al. 2009).

An understanding of hydrocarbon biosynthetic pathways and their regulation may provide an important tool for metabolic manipulation and increasing the yield of hydrocarbons in potential algal species. In this direction, some achievements have been demonstrated when studying hydrocarbon biosynthesis in *B. braunii*. From a number of radiolabelling experiments, it has been shown that oleic acid (but not palmitic or stearic acids) was a precursor (through chain elongation-decarboxylation reactions) for non-isoprenoid hydrocarbon production in the A race of *B. braunii* (Templier et al. 1984; Laureillard et al. 1988). The suggested mechanism of biosynthesis was also confirmed by experiments where thiols were used as known inhibitors of hydrocarbon formation in various higher plants (Templier et al. 1984).

The production of triterpenoid hydrocarbons isolated from race B of *B. braunii*, botryococcene and squalene, both of which are putative condensation products of farnesyl diphosphate, has also been studied (Okada et al. 2000). In order to understand better the regulation involved in the formation of these hydrocarbons, a squalene synthase (SS) gene was isolated and characterised from *B. braunii* (Okada et al. 2000). Comparison of the *Botryococcus* SS (BSS) with SS from different organisms showed 52% identity with *Nicotiana tabacum*, 51% with *Arabidopsis thaliana*, 48% with *Zea mays*, 40% with rat, 39% with yeast and 26% with *Zymomonas mobilis*. Expression of full-length and carboxy-terminus truncated BSS cDNA in *Escherichia coli* resulted in significant levels of bacterial SS enzyme activity but no botryococcene synthase activity (Okada et al. 2000). Later, botryococcene synthase (BS) enzyme activity was reported for *B. braunii* (Okada et al. 2004). It was shown that BS enzyme activity was correlated with the accumulation of botryococcenes during a *B. braunii* culture growth cycle, which was different from the profile of SS enzyme activity (Okada et al. 2004). Recently, high yields of squalene production have been achieved and measured in plants engineered for trichome specific expression of a soluble form of squalene synthase targeted to the chloroplast (Chappell 2009). Thus, it has been demonstrated that the unique biochemistry of *Botryococcus* can be engineered into other organisms thereby providing new tools for the manipulation of algal oil production. Recently, some additional studies to define the botryococcene biosynthetic pathway and to identify the genes coding for these unique enzymological transformations have been conducted (Niehaus et al. 2011). Three squalene synthase-like (SSL) genes have been identified, and it has been shown that the successive action of two distinct SSL enzymes was required for botryococcene biosynthesis (Niehaus et al. 2011).

3 Biosynthesis of Glycerolipids

3.1 Fatty Acid and Polar Glycerolipid Biosynthesis

Detailed discussions of plant/algal glycerolipid biosynthesis are available from a number of detailed reviews to which the reader is referred (Roughan and Slack 1982; Harwood et al. 1988; Harwood and Jones 1989; Browse and Somerville 1991; Dörmann 2005; Hu et al. 2008). In plants, biosynthesis of fatty acids and glycerolipids involves cooperation of two subcellular organelles, plastids and the endoplasmic reticulum (ER) (Fig. 2.6) and for eukaryotic algae this is probably also the case.

Higher plants synthesise palmitate, stearate and oleate through a pathway located in the plastid. This is one of the primary pathways of lipid metabolism and the main *de novo* source of the acyl chains of complex lipids. It begins with acetyl-CoA and then uses malonyl-acyl carrier protein (ACP) as the two-carbon donor (Fig. 2.7).

The acetyl-CoA needed for this synthesis comes ultimately from photosynthesis. The actual process of de novo synthesis to produce long-chain saturated fatty acids involves the participation of two enzymes, acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS). In most plants, the chloroplastic ACC is a multiprotein complex containing several functional proteins (a biotin carboxyl carrier protein, biotin carboxylase and two different subunits of the carboxyltransferase).

FAS is the second major enzyme complex involved in de novo fatty acid formation. The plant FAS is a Type II dissociable multiprotein complex (Harwood 1996) (like the *E. coli* system and unlike that of animals). Thus, the individual proteins that make up FAS can be isolated and their function

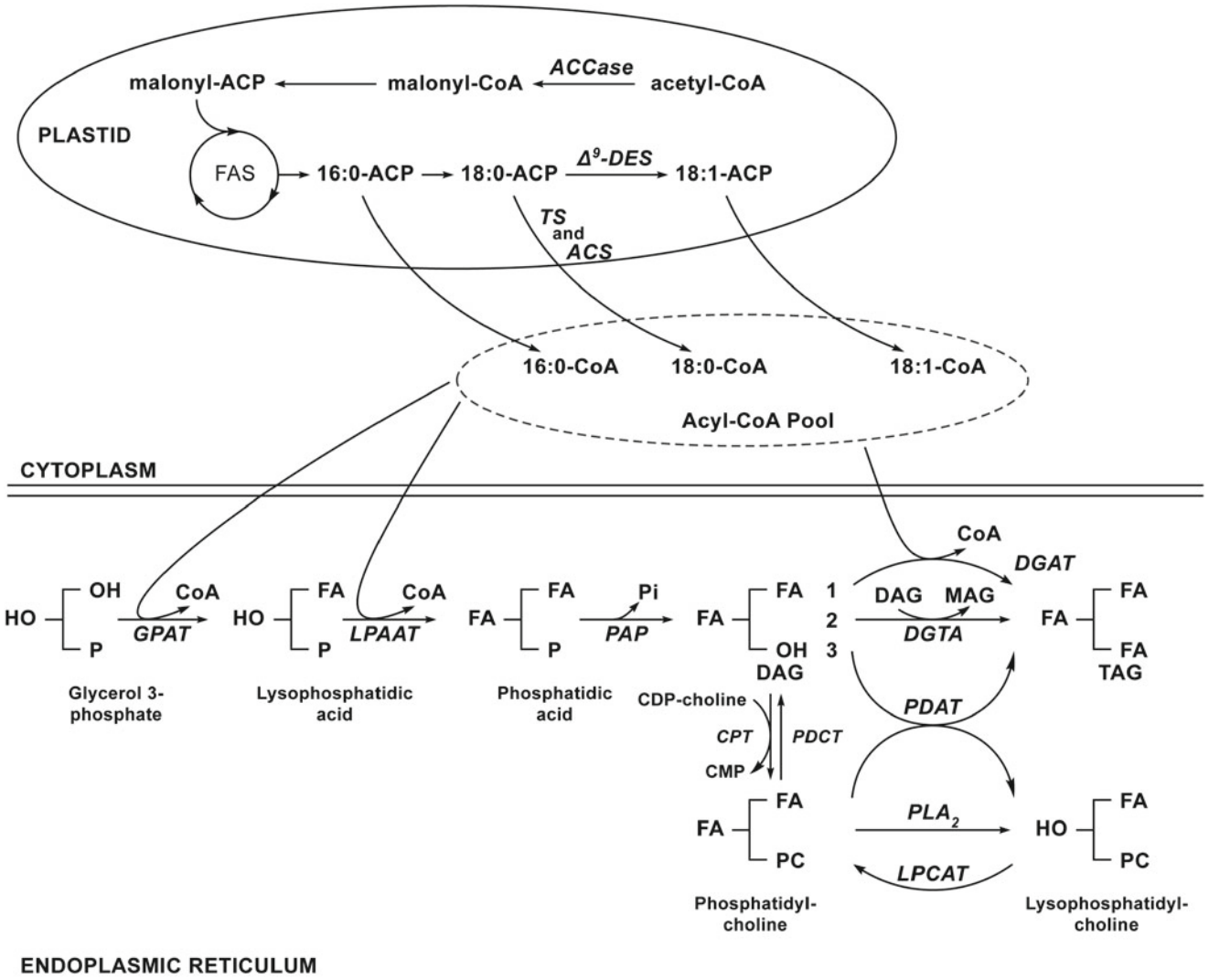

Fig. 2.6 Simplified scheme of TAG biosynthesis in plants. *ACCase* acetyl-CoA carboxylase, *ACP* acyl carrier protein, *ACS* acyl-CoA synthase, *CPT* CDP-choline:1,2-diacylglycerol cholinephosphotransferase, *Δ9-DES* Δ^9-desaturase, *DGAT* DAG acyltransferase, *DGTA* diacylglycerol:diacylglycerol transacylase, *FAS* fatty acid synthase, *GPAT* glycerol 3-phosphate acyltransferase, *LPAAT* lysophosphatidate acyltransferase, *LPCAP* lysophosphatidylcholine acyltransferase, *PAP* phosphatidate phosphohydrolase, *PDAT* phospholipid:diacylglycerol acyltransferase, *PLA_2* phospholipase A_2, *TE* acyl-ACP thioesterase, *PDCT* phosphatidylcholine:diacylglycerol cholinephosphotransferase

demonstrated separately. The first condensation reaction in fatty acid synthesis is catalysed by β-ketoacyl-ACP synthase III (KAS III) that uses acetyl-CoA and malonyl-ACP substrates to give a 4C-keto-intermediate. Successive reduction, dehydration, and a second reduction then produce a 4C fatty acid, butyrate, with all reactions taking place while esterified to acyl carrier protein (ACP). The next six condensations are catalysed by KAS I to produce 6-16C fatty acids. The final reaction between palmitoyl-ACP and malonyl-ACP uses KAS II and results in synthesis of stearate. The remaining enzymes of FAS are β-ketoacyl-ACP reductase, β-hydroxylacyl-ACP dehydrase and enoyl-ACP reductase (Fig. 2.7).

Many enzymes involved in fatty acid synthesis (β-ketoacyl-ACP reductase, β-ketoacyl-ACP synthase, acyl-ACP thioesterase, β-ketoacyl-CoA synthase and β-ketoacyl-CoA reductase) have been either up- or down-regulated in higher plants (Guschina and Harwood 2008). From these studies, it has been concluded that malonyl-CoA is a potential limiting factor affecting the final oil content and, thus, ACCase is a key enzyme in the complex reactions of fatty acid synthesis. Indeed, the enzyme shows high flux control for lipid synthesis in the light (Page et al. 1994). ACC is a soluble Class 1 biotin-containing enzyme that catalyses the ATP-dependent formation of malonyl-CoA from bicarbonate and acetyl-CoA. The product, malonyl-CoA, is used for de novo synthesis of fatty acids inside plastids. In addition, malonyl-CoA is needed for elongation of fatty acids on the endoplasmic reticulum as well as for synthesis of various secondary

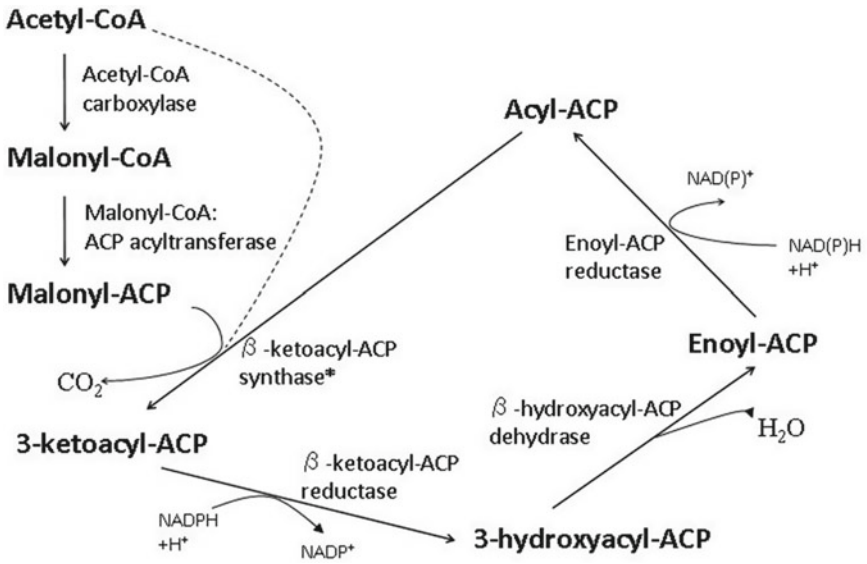

Fig. 2.7 Simplified scheme of de novo fatty acid synthesis in plants. *β-Ketoacyl-ACP synthase (KAS III) catalyses the first reaction of condensation using acetyl-CoA and malonyl-ACP as substrates. The next six condensation reactions are catalysed by KAS I. The final condensation between palmitoyl-ACP and malonyl-ACP is catalysed by KAS II

metabolites in the cytosol. As expected from such requirements, two isoforms of ACC are found in plants, the second of which is extra-chloroplastic (presumed to be cytosolic) and is a multifunctional protein. These isoforms have distinct properties which give rise to their different susceptibility to herbicides (Alban et al. 1994; Harwood 1996). Some success has been achieved in increasing ACCase activity and an associated increase of oil yield by 5% as a result of targeting of a cytosolic version of the enzyme to rapeseed plastids (Roesler et al. 1997).

In algae, ACCase has been purified and characterised from the diatom *Cyclotella cryptica* and it showed a high similarity to higher plant ACCase (Roessler 1990). ACCase from this alga was not inhibited by cyclohexanedione or aryloxyphenoxypropionic acid herbicides as strongly as monocotyledon ACCase but was strongly inhibited by palmitoyl-CoA. In this respect, the diatom enzyme more closely resembled ACCase from dicotyledonous plants than the enzyme from monocotyledonous plants (Roessler 1990). In *Isochrysis galbana,* grown under various environmental conditions, lipid synthesis and accumulation were related to the *in vitro* activity and cellular abundance of ACCase (Sukenik and Livne 1991). Later, the gene encoding ACCase in *C. cryptica* was cloned and characterized (Roessler and Ohlrogge 1993), and some attempts to over-express the ACCase gene have been reported (Hu et al. 2008). Although the experiments did not lead to increased oil production, this still remains one of the possible engineering approaches towards increasing algal oil production.

The fatty acids produced in plastids can be incorporated into the plastid pool of phosphatidate which can be subsequently converted into chloroplast lipids, MGDG, DGDG, SQDG and PG. Similar to cyanobacteria, algal glycerolipids synthesised through this pathway in plastids, have C16 fatty acids esterified at the *sn*-2 position of glycerol and either C16 or C18 fatty acids at the *sn*-1 position of their glycerol skeleton. Such lipids and the pathway responsible for their biosynthesis are called "prokaryotic". Within the ER, glycerolipids are synthesized by the core glycerol 3-phosphate ("Kennedy") pathway with TAG (see below) and phosphoglycerides as products (Gurr et al. 2002) (Fig. 2.6). Diacylglycerol (DAG) originating from a pool of endoplasmic reticulum PC, may be transferred from ER to plastids and be used there as a substrate for synthesis of chloroplast lipids. The *sn*-2 position of glycerolipids from this pathway is esterified with C18 fatty acids. These lipids and the pathway are designated as "eukaryotic". The distinct character of the esterification of the *sn*-2 position of glycerolipids in plastids and the ER, respectively, can be explained by the substrate specificities of lysophosphatidate acyltransferases (Gurr et al. 2002).

According to the above, the fatty acid composition of MGDG allows higher plants to be divided into two groups: 16:3 and 18:3 plants. MGDG from 16:3-plants is esterified with both C16 and C18 acids, and produced through both prokaryotic and eukaryotic pathways, whereas MGDG from 18:3 plants is esterified mainly with C18 acids and synthesised almost exclusively using the eukaryotic pathway (Roughan and Slack 1982).

It is believed that green algae and algae which contain PUFA of no more than 18 carbon atoms are similar to higher plants in so far as their metabolism is generally concerned (Khozin et al. 1997). So, green algae such as *C. vulgaris* and *Chlorella kessleri* have been shown to contain both prokaryotic and eukaryotic types of MGDG (with C16 and C18 acids at the sn-2 position) (see Sato et al. 2003b). Moreover, the existence of a eukaryotic pathway in *C. kessleri* has been proven by a number of radiolabelling experiments (Sato et al.

2003b). The authors suggested that the physiological function of the eukaryotic pathway in this alga is to supply chloroplast membranes with 18:3/18:3-MGDG which may improve their functioning and, hence, be favoured during evolution into land plants (Sato et al. 2003b).

However, algae species with C20 PUFA as well as algae where PC is substituted with betaine lipids have been show to possess differences from higher plants and more complex pathways (Giroud et al. 1988; Cho and Thompson 1987; Khozin et al. 1997; Eichenberger and Gribi 1997). Based on results from the betaine lipid-containing *Pavlova lutheri*, it has been concluded that extraplastid DGCC was involved in the transfer of fatty acids from the cytoplasm and, thus, in the biosynthesis of MGDG (Eichenberger and Gribi 1997). Moreover, these authors suggested that individual fatty acids rather than DAGs were transferred from the cytoplasm to the chloroplast and were incorporated into MGDG by an exchange mechanism (Eichenberger and Gribi 1997).

In the red microalga *Porphyridium cruentum,* EPA-containing galactolipids have been shown to be both eukaryotic and prokaryotic types (Khozin et al. 1997). The analysis revealed the presence of EPA and AA at the *sn*-1 position and C16 fatty acids, mainly C16:0, at the *sn*-2 position in prokaryotic molecular species. In the eukaryotic molecular species both positions were esterified by EPA or arachidonic acid. However, based on studies using radiolabelled precursors, the authors suggested that both prokaryotic and eukaryotic molecular species were formed in two pathways, ω6 and ω3, which involved cytoplasmic and chloroplastic lipids (Khozin et al. 1997). In the ω6 pathway, cytoplasmic C18:2-PC was converted to 20:4ω6-PC whereas in the minor ω3 pathway, C18:2-PC was first desaturated to 18:3ω3 and then converted into 20:5ω3-PC using the same desaturases and elongases as the ω6 pathway. The diacylglycerol moieties of the products were exported to the chloroplast to be galactosylated into their respective MGDG molecular species (Khozin et al. 1997).

Biosynthesis of the betaine lipid, DGTS, has been studied in *C. reinhardtii* using [^{14}C-carboxyl]-*S*-adenosyl-*L*-methionine (Moore et al. 2001). It has been shown that S-adenosylmethionine was the precursor used for both the homoserine moiety and the methyl groups. The activity was associated with the microsomal fraction and did not occur in the plastid (Moore et al. 2001). The discovery of the betaine synthase gene ($BTA1_{Cr}$) has been also recently reported for this alga (Riekhof et al. 2005).

The synthesis of phosphatidylinositol was also studied in *C. reinhardtii* (Blouin et al. 2003). Their data provided evidence for the operation of both of the biosynthetic pathways which had been described in plant and animal tissues previously. One reaction involved CDP-diacylglycerol and was catalyzed by PI synthase (CDP-diacylglycerol: *myo*-inositol 3-phosphatidyltransferase). In the second reaction (which did not in fact result in net PI formation), a free inositol was exchanged for an existing inositol headgroup. The major site of PI biosynthesis in *C. reinhardtii* was the microsomal (containing endoplasmic reticulum (ER)) fraction (Blouin et al. 2003).

3.2 Biosynthesis of TAG

As mentioned above, glycerolipids are synthesized within the ER by the core glycerol 3-phosphate pathway with TAG, phosphoglycerides and glycosylglycerides as major products (Gurr et al. 2002). The first two reactions in this Kornberg-Price pathway to TAG are the formation of phosphatidic acid by the stepwise acylation of glycerol 3-phosphate (Fig. 2.6). These reactions are catalysed by two distinct acyltransferases which are specific for positions sn-1 and sn-2. Membrane-bound glycerol 3-phosphate acyltransferase (GPAT) initiates the process by transferring the acyl chain from acyl-CoA to the sn-1 position of glycerol 3-phosphate with the formation of lysophosphatidic acid (monoacylglycerol 3-phosphate) (Eccleston and Harwood 1995; Manaf and Harwood 2000). One report has been published on the gene for the membrane-bound form of GPAT (Weselake et al. 2009), which is believed to have a low selectivity for different acyl chains. (The soluble chloroplast form of GPAT, which uses acyl-ACP substrates, has, however, been well studied). The transfer of acyl chains from acyl-CoAs to the sn-2 position to form phosphatidic acid, is catalyzed by lysophosphatidic acid acyltransferase (LPAAT) which, in plants, prefers unsaturated acyl chains (Voelker and Kinney 2001). The phosphatidic acid is then dephosphorylated to produce diacylglycerol (DAG). The final step in the pathway is the addition of a final fatty-acyl group to the sn-3 position of DAG to produce TAG. It is catalyzed by diacylglycerol acyltransferase (DGAT), an enzyme unique to TAG biosynthesis. In plants, two unrelated genes have been shown to encode DGAT enzymes. One form (DGAT 1) is related to acyl-CoA:cholesterol acyltransferase, whereas a second form (DGAT 2) does not resemble any other known genes.

Recent studies in plants provide evidence for alternative reactions for TAG synthesis in plants. In one of these reactions, a fatty acid residue is directly transferred from the sn-2 position of PC to DAG forming lyso-PC and TAG. This is referred to a phospholipid:diacylglycerol acyltransferase (PDAT). There is also a reaction involving acyl transfer between two molecules of DAG (i.e. DAG:DAG transacylase) (Stobart et al. 1997). Another enzyme which probably plays a key role in exchanging the diacylglycerol from phosphatidylcholine for the bulk pool and, hence, allowing entry of polyunsaturated fatty acids into TAG synthesis is phosphatidylch oline:diacylglycerol cholinephosphotransferase (PDCT) (Lu

et al. 2009). It remains to be seen whether all these enzymes exist in algae and, in addition, how important they are for TAG formation.

Since DGAT is a key mediator of plant TAG biosynthesis, over-expression of DGAT genes has been suggested as a promising strategy to boost TAG yield. It is known that DGAT is an integral endoplasmic reticulum protein presented in oil bodies and plastids. As mentioned above, two classes of DGAT have been isolated: DGAT1 protein consists of nine to ten putative transmembrane domains whereas DGAT2 only contains two such domains (Lung and Weselake 2006). DGAT2 has been shown to be important for the production of TAG in several cases where unusual fatty acids are involved. In contrast, DGAT1 seems to be quantitatively most important for TAG synthesis when common fatty acids are esterified (see Weselake et al. 2009; Li et al. 2010a).

The potential benefit of acyl-CoA:diacylglycerol acyltransferase-transformed plants has been demonstrated. Thus, over-expression of *AtDGAT1* in tobacco leaves produced up to seven-fold increase in TAG content of the tissue. Seed-specific expression of *AtDGAT1* in *Arabidopsis* led to an increase of 11–28% in seed oil content and similar studies have been made with oilseed rape (Weselake et al. 2008, 2009). These studies have confirmed the important role of DGAT in regulating the quantity of seed TAGs.

As to TAG biosynthesis in algae, one GPAT and two DGATs have been identified and characterized (Xu et al. 2009; Wagner et al. 2010; Guihéneuf et al. 2011). A membrane-bound GPAT was isolated from the marine diatom *Thalassiosira pseudonana*. This enzyme has been shown to prefer saturated C16 fatty acid as a substrate and to play a significant role in determining the fatty acid profile in glycerolipids (Xu et al. 2009). Three putative DGAT two genes were identified from the green alga *Osteococcus tauri* by a database search in its genome (Wagner et al. 2010). For two of the cDNA sequences (OtDGAT2A and B), enzyme activity has been determined by heterologous expression in *Saccharomyces cerevisiae* mutant strains which had impaired TAG metabolism (Wagner et al. 2010). DGAT1 isolated from the diatom microalga *Phaeodactylum tricornutum* (*PtDGAT1*) showed a high homology to several functionally characterised higher plant DGAT1 proteins, and functional expression of *PtDGAT1* was achieved in *S. cerevisiae* (Guihéneuf et al. 2011).

The recent advances in the identification of genes involved in algal lipid metabolism have been thoroughly reviewed by Khozin-Goldberg and Cohen (2011).

It has been recently demonstrated that *C. reinhardtii* may employ a distinct pathway that uses DAG derived almost exclusively from the chloroplast to produce TAG (Fan et al. 2011).

4 Factors Affecting Lipid Composition and Lipid Productivity of Algae

Knowledge of how various cultivation conditions affect the lipid composition and productivity of algae is important for choosing optimal growth conditions for better growth rates, biomass production and the level of TAG accumulation as well as for the modulation of oil fatty acid composition. The latter should not be ignored for at least two reasons. Firstly, the fatty acid profile determines the optimal biofuel characteristics. Thus, it has been concluded from various studies that biodiesel with high levels of methyl oleate or palmitoleate will have excellent characteristics with regard to ignition quality and fuel stability. In many oleaginous algae, TAG and other lipids contain very high levels of polyunsaturated fatty acids which are excellent for the cold stability of the fuel but not desirable for its other characteristics. The second reason is that valuable n-3 fatty acids are co-products of the biofuel technological process and their increased yield, which may be manipulated by growth conditions, can be highly desirable for high value nutraceuticals.

4.1 General Growth Conditions

4.1.1 Temperature

Light and temperature are probably most important and well-studied factors influencing the lipid and fatty acid composition of algae. Changes to the lipids of photosynthetic tissues (and other organisms) as a response to different temperatures and/or light conditions have been recently reviewed (Harwood 1998b; Guschina and Harwood 2006a, b; Morgan-Kiss et al. 2006; Guschina and Harwood 2009a, b). It is believed that many of the lipid changes alter the physical properties of membranes which allows their unimpaired functioning in important physiological processes including photosynthesis, respiration and membrane transport.

For alterations in environmental temperature, changes in fatty acid unsaturation are the most common modification in membrane lipids observed (Harwood 1998b). Low temperature modification of lipid composition has been extensively analysed in the green alga, *Dunaliella salina* (Thompson 1996). A temperature shift from 30 to 12°C increased the level of lipid unsaturation in this alga significantly (Thompson 1996). Retailoring the molecular species of pre-existing PE and PG (especially an increase in molecular species with two unsaturated fatty acids) was noted as a quick response to the temperature shift (Thompson 1996). In addition, a rise in C18:3/C16:1-PG from 48 to 57% and a concomitant decrease in C18:2/C16:1-PG from 34 to 26% of total chloroplast PG was correlated with a significant alteration in the threshold temperature of thermal denaturation of the photosynthetic

apparatus (Thompson 1996). No effect of temperature shift on the content of the acidic lipids, SQDG and PG, has been noticed in *C. reinhardtii* (Sato et al. 2000). However, in the marine haptophyte alga *P. lutheri,* significant changes in lipid class content and fatty acid composition have been reported for cultures grown at 15°C compared to 25°C (Tatsuzawa and Takizawa 1995). Lower temperatures resulted in increased relative amounts of the polyunsaturated fatty acids, EPA and DHA. In addition, the relative percentage of betaine lipids, PG and SQDG increased when algae were cultivated at 15°C with a concomitant decrease in the levels of TAG and MGDG.

Lowering of the growth temperature increased the proportion of the eukaryotic molecular species of MGDG, especially C20:5/C20:5 MGDG, in the red microalga *Porphyridium cruentum* (Adlerstein et al. 1997). A special role for these molecular species in adaptation of PUFA-rich algae to low growth temperatures has been suggested (Adlerstein et al. 1997). Such algae (as well as *Parietochloris incisa*) accumulate high levels of arachidonic acid (AA) in the storage TAG and the reported transfer of this acid from TAG to membrane lipids has been suggested as an adaptive mechanism to low temperature stress (Khozin-Goldberg et al. 2000; Bigogno et al. 2002b).

In two green microalgae, *C. vulgaris* and *B. braunii*, increased growth temperatures led to a decrease in the relative content of more unsaturated intracellular fatty acids, especially trienoic species, while the composition of fatty acids secreted into a medium was unchanged (Sushchik et al. 2003). A decrease in cultivation temperature from 25 to 10°C resulted in an elevation of the relative proportion of oleate in the green alga *Selenastrum capricornutum* (McLarnon-Riches et al. 1998). In contrast to the general expected increase in the proportion of fatty acid unsaturation levels, a decrease in linoleate and stearidonate (C18:4) at lower temperatures has been also shown in this alga (McLarnon-Riches et al. 1998).

In cultures of the haptophyte *I. galbana* grown at 15 and 30°C, lipids and fatty acids were analysed and compared (Zhu et al. 1997). At 30°C, total lipids accumulated at a higher rate with a slight decrease in the proportion of non-polar lipids, an increase in the proportion of glycosylglycerides but no change in the proportion of phospholipids. Higher levels of α-linolenate and DHA with a corresponding decrease in linoleate, monounsaturated and saturated fatty acids were found in the cells grown at 15°C.

Four tropical Australian microalgal species, a diatom *Chaetoceros* sp., two cryptomonads, *Rhodomonas* sp. and *Cryptomonas* sp., and an unidentified prymnesiophyte, cultured at five different temperatures showed decreased levels of EPA and DHA at higher cultivation temperatures (Renaud et al. 2002). Similarly, the content of EPA and PUFAs in marine diatom *Phaeodactylum tricornutum* have been shown to be higher at lower temperatures when comparing cultures grown at 10, 15, 20, or 25°C (Jiang and Gao 2004).

In general, lower growth temperatures lead to increased levels of unsaturated fatty acids in algae although the details of these alterations may vary from species to species. Moreover, some additional subtle alterations may be often seen in many algae rather than a simple correlation of increased unsaturation with lower temperatures. The general alterations in fatty acid unsaturation in algae mirror similar changes observed in other eukaryotes as well as cyanobacteria (Harwood 1998b).

4.1.2 Light

Light intensity also influences algal lipid metabolism and, therefore, lipid composition (Harwood 1998b). In general, high light intensities usually lead to oxidative damage of polyunsaturated fatty acids. In *Nannochloropsis* sp., the degree of unsaturation of fatty acids decreased with increasing irradiance, especially the percentage of total n-3 fatty acids (from 29 to 8% of total fatty acids) mainly due to a decrease of EPA (Fabregas et al. 2004). In other EPA-producing algae (*P. tricornutum* and *Monodus subterraneus*) this tendency had been noticed previously when increasing light intensity had caused a reduction in EPA accumulation (cited by Adlerstein et al. 1997). High light exposure (300 μmol photons.m^{-2}.s^{-1}) decreased the total phospholipid content and increased the level of non-polar lipids, namely TAG, in the filamentous green alga *Chladophora* sp. (Napolitano 1994).

Variations in lipid composition were studied in the marine red alga *Tichocarpus crinitus* exposed to different levels of photon irradiance (Khotimchenko and Yakovleva 2005). Light intensity caused significant alterations in both storage and structural lipids. Exposure of this alga to low light intensity (8–10% of the incident photosynthetically active radiation (PAR)) resulted in increased levels of some cell membrane lipids, especially SQDG, PG and PC, whereas cultivation of algae at higher light intensities (70–80% of PAR) increased the level of storage TAG. The light conditions used did not change the total fatty acid composition in *T. crinitus*, although there were a few changes noticed in the fatty acid composition of individual lipids (Khotimchenko and Yakovleva 2005).

In the green alga *Ulva fenestrata*, the relative amounts of MGDG, SQDG and PG increased 2–3.5 times when grown at 24% of the incident photosynthetically active radiation (PAR) compared to algae cultured at 80% PAR (Khotimchenko and Yakovleva 2004). In contrast, the relative contents of DGDG and betaine lipid, as well as the relative proportions of fatty acids in TAG, MGDG and SQDG, were not affected by light intensity (Khotimchenko and Yakovleva 2004).

Light:dark cycles also have a significant effect on algal lipid composition. As an example, a detailed study of various

light regimes on lipids of the diatom, *Thalassiosira pseudonana,* may be cited (Brown et al. 1996). The light regimes used were 100 µmol photons.m^{-2}.s^{-1} on a 12:12 h light:dark (L:D) cycle; 50 µmol photons.m^{-2}.s^{-1} on a 24:0 h L:D cycle and 100 µmol photons.m^{-2}.s^{-1} on a 24:0 h L:D cycle. An increased percentage of TAG and a reduced percentage of the total polar lipids were found for the cells grown under 100 µmol photons.m^{-2}.s^{-1} continuous light. The fatty acid composition of algae in the logarithmic growth stage under the two continuous light regimes showed no differences, whereas the cell grown under 100 µmol photons.m^{-2}.s^{-1} and 12:12 h L:D conditions contained a higher proportion of PUFAs and a lower proportion of saturated and monounsaturated fatty acids (Brown et al. 1996).

Low light decreased the relative percentage of palmitate and increased in those of palmitoleate and α-linolenate in two green freshwater algae *Cladophora* sp. and *Spirogyra* sp. (Napolitano 1994). Dark treatment caused a decrease in the relative proportion of oleate and an increase in that of linoleate in the green alga *S. capricornutum* (McLarnon-Riches et al. 1998). In the dinoflagellate *Prorocentrum minimum*, dark exposure led to a reduced content of TAG and galactosylglycerides, while the total content of phospholipids changed little with decreased PC, PE and PG and increased PS, PA and PI levels. The decrease of TAG and galactosylglycerides was in parallel to an increase in the activity of β-oxidation and isocitrate lyase indicating that TAG and galactosylglycerides were utilized as alternative carbon sources by the cells under non-photosynthetic growth conditions (McLarnon-Riches et al. 1998).

Lipid production in *P. cruentum* has been studied under different culture conditions (Oh et al. 2009). A higher lipid accumulation (19.3%, w.w^{-1}) was demonstrated using a 12:12 h light:dark cycle and a 25°C growth temperature compared to 35°C (Oh et al. 2009).

In general, light will stimulate the algal biomass production. Stimulation of fatty acid and membrane lipid, mainly chloroplast, synthesis are normally expected as a result of an increase in light intensity.

4.1.3 Salt Concentrations

Some algae exhibit an excellent ability to tolerate high salt concentrations. The genus *Dunaliella,* growing in the wide range of salinities, is a good example as well as an useful model in studying mechanisms of such resistance (Azachi et al. 2002). It has been shown that the expression of β-ketoacyl-coenzyme A (CoA) synthase (KCS) (which catalyzes the first step in fatty acid elongation) was induced in the cells of *D. salina* transferred from 0.5 to 3.5 M NaCl (Azachi et al. 2002). In these cells, a considerably higher ratio of 18C (mostly unsaturated) to 16C (mostly saturated) fatty acids was also noted. The authors suggested that the salt-induced KCS, together with fatty acid desaturases, may play a role in adapting intracellular membranes to function in the high internal glycerol concentrations used to balance the external osmotic pressure created by high salt (Azachi et al. 2002). (However, it must be noted that such a proposal assumes that KCS is responsible for 18C fatty acid production rather than fatty acid synthase).

In *D. salina* cells, the lipid content was manipulated by salt stress and nitrogen limitation, and it reached a value of about 38% in cells grown at 16% NaCl combined with 2.5 mM unspecified nitrogen salts (Abd El-Baky et al. 2004). These conditions also increased the relative proportion of PUFAs, in particular the C18:3n-3 and C16:4n-3 fatty acids (Abd El-Baky et al. 2004).

An increase in the initial salt concentration from 0.5 M NaCl to 1.0 M resulted in an increase (from 60 to 67%) of intracellular lipid content in *Dunaliella tertiolecta* (Takagi et al. 2006). The further increase in lipid content up to 70% has been achieved when 0.5 or 1.0 M NaCl were added at mid-log phase or the end of log phase during cultivation with the initial NaCl concentration of 1.0 M (Takagi et al. 2006).

4.1.4 pH

Lipids are also affected during growth of algae at extreme pH. Thus, it has been shown that alkaline pH stress increased the TAG percentage accumulation and decreased the relative level of membrane lipids in *Chlorella* sp. (Guckert and Cooksey 1990). The effects of pH on the lipid and fatty acid composition of a *Chlamydomonas* sp., isolated from a volcanic acidic lake and *C. reinhardtii*, obtained from an algal collection (Institute of Applied Microbiology, Tokyo) have been studied and compared (Tatsuzawa et al. 1996). Fatty acids in the polar lipids were more saturated in the unidentified *Chlamydomonas* sp. than those in *C. reinhardtii* when grown under the same conditions. The TAG content (as % of total lipids) was also higher in *Chlamydomonas* sp. grown at pH 1 than that in the cells cultivated at higher pH. The increase in saturation of fatty acids in membrane lipids of *Chlamydomonas* was proposed to be an adaptive reaction to low pH in order to decrease membrane lipid fluidity (Tatsuzawa et al. 1996).

4.1.5 Nutrients

Nutrient availability affects significantly the lipid composition of algae, and a number of broad effects of nutrient limitation have been reported as important modulators of algal lipid biosynthesis. It is accepted, that when algal growth slows down as a result of nutrient deficiency, and there is no requirement for the synthesis of new membrane compounds, the cells can transfer fatty acids into their storage lipids before conditions improve. There are many examples showing that algal species deficient in nutrients can more than double their lipid and TAG content (see Thompson 1996; Guschina and Harwood 2006a).

Nutrient-deficiency has been shown to affect markedly the lipid composition of the freshwater diatom *Stephanodiscus minutulus* when grown under silicon, nitrogen, or phosphorus limitation (Lynn et al. 2000). An increase in TAG accumulation and a decrease of polar lipids (as % of total lipids) was noticed in all of the nutrient-limited cultures (Lynn et al. 2000). An increase in TAG percentages (from 69 to 75% of total lipids) together with phospholipids (from 6 to 8%) was reported for the microalga *P. tricornutum* as a result of reduced nitrogen concentration. In contrast, the proportion of galactolipids decreased from 21 to 12% in nitrogen-starved cells (Alonso et al. 2000).

In *Chlamydomonas moewusii*, nutrient-limitation resulted in alterations in the fatty acid composition of the chloroplast lipids, PG and MGDG (Arisz et al. 2000). The PUFAs, C16:3, C16:4 and C18:3, which were present in the plastidic galactolipids, and C16:1(Δ3-*trans*), specific for plastidic PG, decreased under nutrient-limited conditions. This may, possibly, be due to a reduction in the intracellular content of chloroplasts although that was not specifically examined. The synthesis of storage lipids has been suggested to be stimulated by depletion of nutrients and this was consistent with the rise in the levels of C16:1 and C18:1 which were prominent in storage lipids (Arisz et al. 2000).

Euglena gracilis has been cultivated under various conditions of autotrophy and photoheterotrophy in order to estimate the contribution of lactate (a carbon source) and ammonium phosphate (a nitrogen source) to its metabolism (Regnault et al. 1995). Effects of increasing ammonium phosphate concentration on lipid composition were noticed only when lactate was depleted. Such conditions increased the content of galactolipids rich in polyunsaturated 16C and 18C fatty acids as well as the ratio of MGDG/DGDG. Excess of nitrogen did not change the content of medium chain (12-14C) acids but induced a reduction of 22C acids. When ammonium phosphate was absent in the cultural medium, increasing the lactate concentration led to a decrease in all plastid lipids, whereas the accumulation of storage lipids (enriched with myristate and palmitate) increased. Biosynthesis of 18C PUFAs was reduced as indicated by the accumulation of oleate (Regnault et al. 1995).

The effects of sodium nitrate as a nitrogen source on cell growth and lipid accumulation has been studied in a green alga, *Neochloris oleoabundans*, one of the most promising oil-rich microalgal species (Li et al. 2008). The highest lipid cell content (0.40 $g.g^{-1}$ dry weight) was obtained at the lowest sodium nitrate concentration (3 mM), whereas a higher lipid productivity of 0.133 $g.L^{-1}.day^{-1}$ was achieved at 5 mM with a lipid cell content of 0.34 $g.g^{-1}$ (Li et al. 2008).

Other studies with *N. oleoabundans* showed that the best growth was obtained when the algae were cultivated at 30°C under conditions of nitrogen-sufficiency and CO_2 supplementation (Gouveia et al. 2009). However, the maximum lipid content (56% of dry weight) was shown after 6 days of nitrogen depletion without CO_2 supplementation (Gouveia et al. 2009).

Similar results for nitrogen limitation were also found during cultivation of microalgae *Chlorella* sp. with urea (Hsieh and Wu 2009). Initial urea concentrations of 0.025, 0.050, 0.100, 0.150 and 0.200 $g.L^{-1}$ were used to investigate its effect on cell growth and lipid productivity in batch cultures. The microalgae showed the highest total lipid content (0.661 $g.g^{-1}$ dry weight) when cultured with the lowest concentration of urea. However, the maximum lipid productivity of 0.124 $g.L^{-1}.day^{-1}$ was shown for cells in media containing 0.100 $g.L^{-1}$ urea (Hsieh and Wu 2009).

In experiments with *Scenedesmus obliquus*, lipid accumulation has been studied under various culture conditions including nitrate, phosphate, sodium thiosulfate and glucose supplementation (Mandal and Mallick 2009). Lipid accumulation was found to be more affected by the concentrations of nitrate, phosphate and sodium thiosulphate than glucose supplementation to the growth media (Mandal and Mallick 2009). The most significant accumulation of lipids (43% of dry cell weight) was recorded under N-deficiency (against 2.7% of lipids of dry cell weight under control conditions). Under P-deficiency and thiosulphate supplementation lipid accumulation also increased (up to 30% of dry cell weight) (Mandal and Mallick 2009).

The lipid composition of seven species of marine algae has been studied when cultured in phosphorus-limiting conditions (Reitan et al. 1994). Such conditions caused an increase in total lipid content in *P. tricornutum, Chaetoceros* sp., and in *P. lutheri*, but a decrease of that in the green flagellates, *Nannochloris atomus* and *Tetraselmis* sp. A higher relative content of palmitate and oleate and lower levels of C18:4*n*-3, EPA and DHA have been shown for the more severe nutrient-limited conditions of cultivation (Reitan et al. 1994). In contrast, for phosphorus-starved cells of the green alga *Chlorella kessleri*, an elevated level of unsaturated fatty acids has been reported in all the individual lipids identified, namely PC, PG, DGDG, MGDG and SQDG (El-Sheek and Rady 1995). These studies reveal considerable variation in the way that individual algal species react to nutrient limitation.

In *C. reinhardtii*, the acidic lipids in thylakoid membranes have been studied under sulfur- and phosphorus-starved cultivation (Sato et al. 2000). Sulfur-limited cells lost the most of their SQDG as compared with normal conditions. In this organism, PG content increased by two-fold, representing a compensatory mechanism for the reduced level of the other anionic lipid, SQDG. In agreement, *C. reinhardtii* grown in a media with limited phosphorus, showed a 40% decrease in PG and a concomitant increase in the SQDG content. In general, the replacement of membrane phospholipids by non-phosphorus glycolipids and betaine lipids under phosphate limitation has

been demonstrated in many organisms, including higher plants, photosynthetic bacteria, and algae (e.g., Benning et al. 1995; Härtel et al. 2000; Andersson et al. 2003; Jouhet et al. 2007).

The effect of phosphate starvation on the lipid and fatty acid composition has been studied in some detail in the fresh water eustigmatophyte *Monodus subterraneus* (Khozin-Goldberg and Cohen 2006). Incubation of this alga in media with decreasing phosphate concentrations (175, 52.5, 17.5 and 0 μM) resulted in a gradual decrease in the relative EPA concentration whereas the cellular total lipid content increased, mainly due to TAG accumulation. In phosphate-depleted cells, the proportion of phospholipids reduced from 8.3 to 1.4% of total lipids (Khozin-Goldberg and Cohen 2006).

A general reduction in the degree of fatty acid unsaturation as a response to elevated CO_2 concentration has been reported for several species of green algae (Thompson 1996). In *C. kessleri*, cells grown under low CO_2 (0.04% CO_2 compared to 2% CO_2) showed elevated contents of α-linolenate, especially at both *sn*-1 and *sn*-2 positions of MGDG and DGDG, and also at the *sn*-2 position of PC and PE (Sato et al. 2003c). CO_2 has also been shown to change the content and composition of fatty acids and chloroplast lipids in the unicellular halophilic green alga *Dunaliella salina* (resistant to CO_2 stress) (Muradyan et al. 2004). The response was seen after a one day-long increase in CO_2 concentration from 2 to 10% and resulted in an increase in the total amount of fatty acids on a dry weight basis by 30% (Muradyan et al. 2004).

Effects of CO_2 concentration on the biomass production and lipid accumulation of *Nannochloropsis oculata* has been investigated in a semicontinuous culture (Chiu et al. 2009). Based on the results, this microalga was thought to be best grown in a semicontinuous system aerated with 2% CO_2 and operated by 1-day replacement for long-term biomass production and the higher lipid yield (Chiu et al. 2009). A highly CO_2 tolerant alga, *Chloroccoccum littorale*, has a good potential for aquacultural fatty acid production, and photoautotrophic fatty acid accumulation was investigated in this alga in the presence of inorganic carbon and nitrate at a light intensity of 170 μmol photons.m^{-2}.s^{-1} (Ota et al. 2009). The results of this study showed that fatty acid synthesis was increased at low CO_2 concentrations after nitrate depletion with a controlled HCO_3^-/CO_2 ratio. The relative FA content was 34 wt.% on a dry weight basis under the conditions of 22°C, light intensity of 170 μmol photons.m^{-2}.s^{-1} and CO_2 concentration of 5% with O_2-free gas and this content was comparable with plant seed oils (Ota et al. 2009).

Biomass and lipid productivities of *C. vulgaris* were studied and compared under autotrophic, heterotrophic and mixotrophic growth conditions (Liang et al. 2009). While autotrophic growth did provide a higher cellular lipid content (38% of dry cell weight), the lipid productivity was much lower compared with that during heterotrophic growth with acetate, glucose, or glycerol. Optimal cell growth (2 g.L^{-1}) and lipid productivity (54 mg.L^{-1}.day^{-1}) were obtained using glucose or glycerol at 1% (wt.v^{-1}) whereas higher concentrations were inhibitory (Liang et al. 2009).

To increase the biomass, corn powder hydrolysate (CPH) was supplied as substrate for heterotrophic growth of microalgae *Chlorella photothecoides* (Xu et al. 2006). Growth of *C. photothecoides* and its lipid accumulation using glucose or cassava starch hydrolysate (CSH) as carbon sources have also been compared (Wei et al. 2009). Their data demonstrated that the highest biomass (15.8 g.L^{-1}) and the maximum total lipid yield (4.19 g.L^{-1}) were obtained when CSH was used as a carbon source. In addition, glucose, but not glycerol, has been shown to be a suitable carbon source for the heterotrophic growth of *Porphyridium cruentum* (Oh et al. 2009). The lipid class and fatty acid composition of the green microalga *Chlorella zofingiensis* have been compared under photoautotrophic and heterotrophic cultivation conditions (Liu et al. 2011). Heterotrophic cells fed with 30 g.L^{-1} of glucose were shown to increase lipid yield by 900% and the content of oleic acid (from 17.9 to 35.2% of total fatty acids) in comparison to photoautotrophic cells. Thus, it was concluded that oils from heterotrophic *C. zofingiensis* appeared to be more suitable for biodiesel production (Liu et al. 2011).

For *Scenedesmus obliquus*, lipid accumulation was boosted up to 2.16 g.L^{-1}, to give a value about 40-fold higher in comparison to control conditions, when the cells were pregrown in the optimised medium supplemented with 1.5% glucose (Mandal and Mallick 2009). It should also be noted that the presence of palmitate and oleate as its major fatty acids makes *S. obliquus* biomass a very suitable feedstock for algal-based biodiesel (Mandal and Mallick 2009). To induce the lipid accumulation in *Scenedesmus* sp LX1, algal cells were treated with an antialgal allelochemical, ethyl-2-methyl acetoacetate (EMA) (Xin et al. 2010). Under EMA concentrations of 1.0–2.0 mg.L^{-1} the relative TAG content (about 20 wt.% in control cultures) and TAG productivity (about 23 mg.L^{-1} in control cells) were increased by 79 and 40%, respectively (Xin et al. 2010).

Another efficient approach to increase the cellular TAG content is to shift carbon flux from energy-rich storage compounds, such as starch, to TAG biosynthesis. This has been supported by a number of studies including the inhibition of starch biosynthesis in *C. reinhardtii* and using stress induction of TAG accumulation in *C. reinhardtii* starchless mutants (Wang et al. 2009; Li et al. 2010a, b).

5 Conclusion and Future Directions

Algae provide many potential advantages as feedstocks for biofuel production in comparison to oil crop plants. Although many advances have been made in studying lipid metabolism

in algae, a more detailed understanding of lipid (especially TAG) formation as well as regulation of the carbon flux in general is necessary in order to optimize TAG biosynthesis in algae. For this purpose, the identification of all genes encoding the key enzymes (ACCases, DGAT etc.) controlling TAG synthesis is an important priority. Mutagenesis and enzyme modulators may be useful tools in understanding the primary regulatory mechanisms for lipid pathways in different algae species. These approaches may lead to some additional strategies for enhancing algal oil content. In addition, more attention should be paid to the fatty acid profiles in TAG in relation to optimal biofuel characteristics.

Finally, many results on oil improvement and the regulation of lipid biosynthesis in oil-crop plants (as mentioned in this chapter) can be useful for comparative purposes and to provide clues as to useful potential strategies for algal lipid accumulation.

Acknowledgement Recent research in the authors' laboratory has been supported financially by grants from the BBSRC, DuPont, NERC, the Royal Society and Syngenta for which we are grateful.

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Hydrogenases, Nitrogenases, Anoxia, and H_2 Production in Water-Oxidizing Phototrophs

3

John W. Peters, Eric S. Boyd, Sarah D'Adamo, David W. Mulder, Jesse Therien, and Matthew C. Posewitz

1 Introduction

The production of H_2 for use as a renewable energy carrier using solar energy and electrons derived from water is widely regarded as one of the most environmentally benign and sustainable energy solutions. Several water-oxidizing, phototrophic algae and cyanobacteria have the remarkable ability to use low potential electrons from the photosynthetic electron transport chain, or from sugar oxidation, to reduce protons to H_2. In nature, H_2-producing phototrophs are components of complex microbial ecosystems. In these ecosystems biological H_2 production occurs predominately at night, when oxygenic photosynthesis ceases and regions of microbial mat communities and the water column of aquatic ecosystems rapidly become anoxic as a result of heterotrophic respiration (Fig. 3.1). Under anoxic and dark conditions, phototrophs ferment carbon stores to maintain metabolic activity and use H_2 production, via hydrogenases, as a means to recycle reduced electron carriers that accumulate during the anaerobic oxidation of carbohydrates. Many hydrogenases are O_2 sensitive, and thus are incompatible with active oxygenic photosynthesis. In addition to hydrogenase-catalyzed H_2 production, a significant amount of H_2 is also produced as a product of biological N_2 fixation. The enzymatic apparatus responsible for biological N_2 fixation, termed nitrogenase, produces a minimum of 1 mol of H_2 for each mole of N_2 reduced. Nitrogen fixation is restricted to prokaryotes and therefore does not occur in algae, but is widespread among cyanobacteria where N_2 fixation is either spatially separated from oxygenic photosynthesis in heterocysts or is temporally separated in a day-night cycle. In most cases, the complex microbial consortia that harbor H_2-producing organisms are highly efficient at sequestering any H_2 produced and recycling the reducing potential to drive energy yielding processes. Thus, hydrogen does not generally accumulate in natural systems and the production of bioH_2 will require either the manipulation of natural consortia or the controlled culture of individual organisms.

When H_2 is used as an energy carrier in fuel cell technologies only water vapor is emitted, and significant environmental benefits can be realized if sufficient quantities of H_2 are sustainably produced in a cost effective manner. Consequently, major research efforts are aimed at developing a more informed understanding of the physiological parameters dictating H_2 production in water-oxidizing, phototrophic microorganisms, with the ultimate goal of improving H_2 yields. Currently, the yields of H_2 production are far below those required for the economically-viable production of H_2 and substantial improvements are required to generate the quantities of H_2 necessary to replace a meaningful portion of our current energy portfolio. Nevertheless, recent biological H_2 production research efforts are rapidly elucidating (a) the metabolic pathways that supply reductant to H_2-producing enzymes, (b) the metabolic and mechanistic requirements for maturation of the metallo-enzyme centers required in H_2-producing enzymes, (c) novel hydrogenase enzymes, and (d) the genetic techniques required for manipulating metabolism in H_2-producing organisms.

J.W. Peters (✉) • E.S. Boyd • D.W. Mulder • J. Therien
Department of Chemistry and Biochemistry, and Astrobiology Biogeocatalysis Research Center, Montana State University, Bozeman, MT 59715, USA
e-mail: john.peters@chemistry.montana.edu

S. D'Adamo • M.C. Posewitz (✉)
Department of Chemistry and Geochemistry, Colorado School of Mines, Golden, CO 80401, USA

2 Structure, Function and Maturation of H_2-Producing Enzymes

Biological H_2 production in photosynthetic microorganisms is catalyzed by either the hydrogenase or nitrogenase enzymes (Boichenko and Hoffmann 1994; Tamagnini et al. 2002; Boichenko et al. 2004; Kruse et al. 2005b). Hydrogenases are widely distributed in diverse microorganisms

M.A. Borowitzka and N.R. Moheimani (eds.), *Algae for Biofuels and Energy*, Developments in Applied Phycology 5,
DOI 10.1007/978-94-007-5479-9_3,

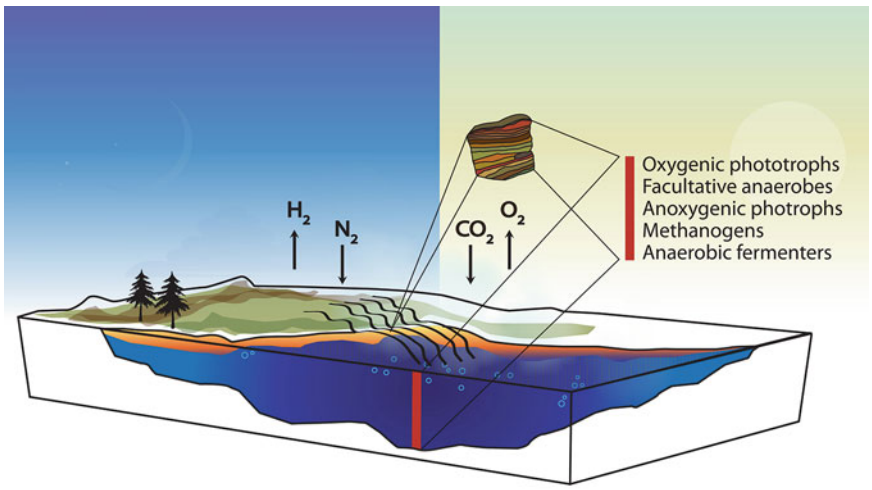

Fig. 3.1 Hydrogen production is a ubiquitous occurrence in aquatic microbial ecosystems, where metabolic capabilities enabling photosynthesis, carbon fixation, nitrogen fixation and hydrogen production/uptake are found to co-exist. During the day, sunlight and photosynthesis are able to produce reduced carbon compounds with the co-evolution of O_2. During the night, cellular respiration reduces O_2 to water. As a result, microbial communities and mats rapidly enter anoxia resulting in the induction of nitrogenase and hydrogenase activity, both of which can produce H_2. In addition to the temporal separation of anoxic metabolism, certain organisms are able to spatially separate anoxia intracellulary by precluding O_2 transport into the cell

(Adams 1990; Wu and Mandrand 1993; Vignais et al. 2001; Vignais and Colbeau 2004; Posewitz et al. 2008), and two distinct classes of hydrogenase efficiently catalyze the reversible oxidation of H_2 ($H_2 \leftrightarrows 2H^+ + 2e^-$) in phototrophs. These enzymes are termed the [NiFe]- and [FeFe]-hydrogenases to reflect the composition of their metal-containing active sites (Wu and Mandrand 1993; Vignais et al. 2001; Vignais and Colbeau 2004). The [NiFe]- and [FeFe]-hydrogenases do not share sequence similarity and clearly have different evolutionary origins (Vignais et al. 2001). There are only a subset of organisms where [NiFe]- and [FeFe]-hydrogenases co-occur (most notably a number of sulfate reducing bacteria) and to date there appears to be a strict segregation with respect to the occurrence of hydrogenases in water-oxidizing phototrophs. Only the [FeFe]-hydrogenases are found in eukaryotic green algae, and only [NiFe]-hydrogenases are found in cyanobacteria (Ludwig et al. 2006). A [NiFe]-hydrogenase was proposed to be present in the eukaryotic alga *Scenedesmus obliquus* (Zinn et al. 1994), as well as a marine *Tetraselmis* species (Bhosale et al. 2009); however, these findings have yet to be confirmed and no [NiFe]-hydrogenase sequences have been revealed in any known eukaryotic genomes (Florin et al. 2001). This strict segregation in occurrence of [NiFe]- and [FeFe]-hydrogenases amongst phototrophic microorganisms together with the observation that neither class of hydrogenase has been found to be associated with any higher plants cannot be intuitively explained but clearly has broader evolutionary implications. Hopefully the rapid advances in the tools for phylogenetic analysis coupled with the rapidly growing databases of available genome sequences will help to provide some insight into this intriguing question.

Although [NiFe]- and [FeFe]-hydrogenases are evolutionarily unrelated, the enzyme classes are unified in that (a) their active sites contain the biologically novel ligands CO and CN^- coordinated to Fe; and (b) each active site contains a binuclear metal center (Fig. 3.2). Carbon monoxide ligands bound to iron are also associated with a third type of hydrogenase termed the [Fe]-hydrogenase or Hmd-hydrogenase. Hmd-hydrogenases do not catalyze reversible H_2 oxidation formally through the aforementioned reaction ($H_2 \leftrightarrows 2H^+ + 2e^-$) but rather catalyze the dehydrogenation of methylene-tetrahydromethanopterin to form H_2 and methenyl-tetrahydromethanopterin. These enzymes are only found in methanogenenic archaea and do not show any sequence similarity to either the [NiFe]- or the [FeFe]-hydrogenases. The diatomic ligands such as cyanide and/or carbon monoxide are a unifying feature of the three hydrogenase enzymes but appear to be found nowhere else in nature. That these unique features evolved separately as components of only hydrogenase enzymes of different ancestries is likely to be one of the most profound examples of convergent evolution.

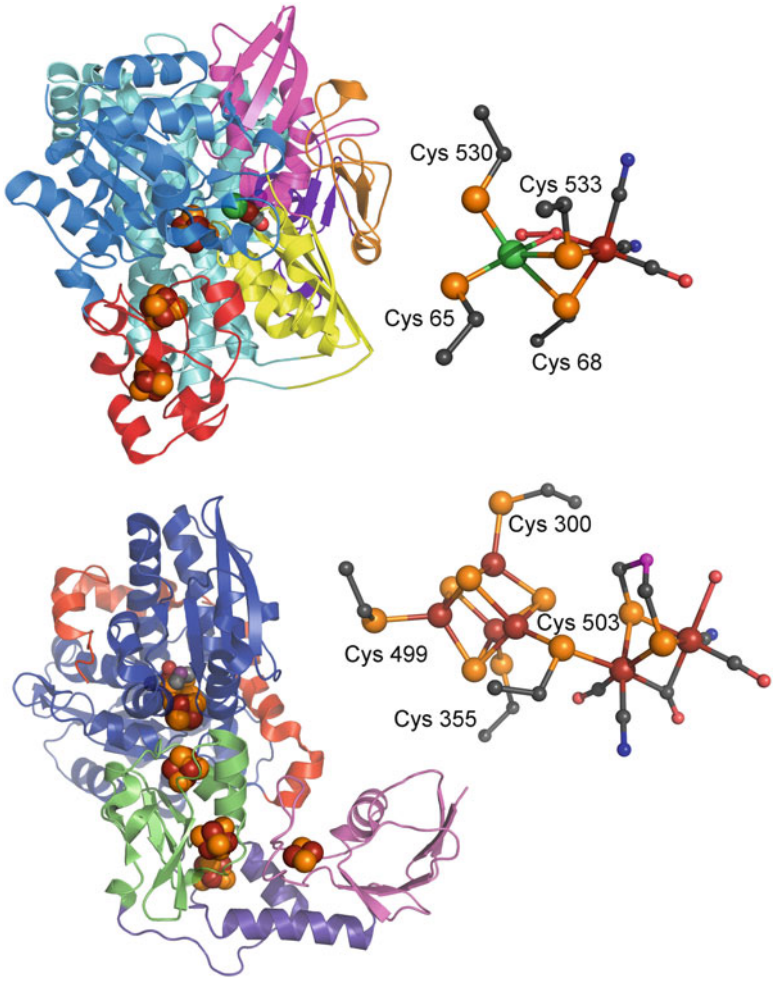

Fig. 3.2 (**Top**) Ribbon/space filling representation of the [NiFe]-hydrogenase from *D. gigas* (PDB code 1YQ9) and active site (*right*) in ball and stick representation. CO and CN^- ligands are present at the [NiFe] active site in addition to a bridging species depicted as a peroxide ligand for the oxidized "unready" state of the enzyme. (**Bottom**) Ribbon/space filling representation of the [FeFe]-hydrogenase CpI (PDB code 3C8Y) and active site H-cluster (*right*) in ball and stick representation. Like the [NiFe] active site, CO and CN^- ligands are present at H-cluster in addition to a dithiolate ligand. Also, for the presumed oxidized state, a water molecule is present at the distal Fe atom of the 2Fe subcluster. Coloring scheme: protein domains represented with different colors; Fe, *dark red*; S, *orange*; O, *red*; N, *blue*; C, *dark grey*; central atom of the dithiolate ligand, magenta. All protein ribbon representations and atomic models were generated in PyMOL (DeLano 2002)

3 [FeFe]-Hydrogenase Occurrence and Diversity

The ever-expanding number of available genome sequences has greatly extended our understanding of the genetic and putative functional diversity of [FeFe]-hydrogenase-encoding genes (*hydA*) in biology. HydA-encoding genes have been identified in 171 of the nearly 1,800 fully sequenced or draft bacterial genomes and 16 of the nearly 200 sequenced or draft eukaryl genomes, with the latter primarily detected in lower order eukaryotes. Importantly, while the majority of bacterial genomes that encode for HydA also encode for the active site cofactor biosynthesis proteins HydEFG, only 3 of the eukaryl genomes encode for HydEFG, which includes the green alga *Chlamydomonas reinhardtii* and the protozoan *Trichomonas vaginalis*. Thus, it is unclear if HydA in eukaryotes which lack HydEFG are functional. In contrast to bacterial and eukaryl genomes, *hydA* has yet to be detected in the archaeal domain, despite the presence of nearly 80 fully sequenced or draft archaeal genome sequences.

The distribution of *hydA* among bacterial phyla is uneven and is generally restricted to phyla that characteristically inhabit anoxic environments. HydA is common among genome sequences from the Firmicutes where 109 of the 218 sequenced genomes contain at least one copy of *hydA* (Table 3.1). Similarly, 9 of the 12 sequenced genomes from the Thermotogae contain at least one copy of *hydA*. The tendency for bacterial genomes to contain multiple copies of

Table 3.1 Distribution of *hydA* homologs among sequenced bacterial genomes

Phylum	Sequenced genomes	Genomes with *hydA*	% of sequenced genomes with *hydA*	*hydA* copies per Genome[a]
Firmicutes	218	109	50.0	1.8
Actinobacteria	128	1	0.8	1.0
Bacteroidetes	89	11	12.4	1.1
Chloroflexi	12	4	33.3	1.0
Dictyoglomi	2	2	100.0	2.0
Elusimicrobia	1	1	100.0	1.0
Lentisphaerae	1	1	100.0	2.0
Nitrospirae	1	1	100.0	2.0
Proteobacteria	495	29	5.9	1.3
Spirochaetales	19	3	15.8	1.0
Thermotogae	12	9	75.0	2.9
Verrucomicrobia	5	1	20.0	1.0

[a]Only genomes with *hydA* considered

hydA is high, particularly in the Thermotogae where the majority of sequenced genomes contain three copies of *hydA* (average copy number per genome is 2.8). Among the eukarya, *hydA* has been identified in the genomes of several lower order taxa including the phototrophic and unicellular algae *Chlamydomonas reinhardtii* and *Scenedesmus obliquus*. In addition, *hydA* has been identified in the protozoan *Trichomonas vaginalis*. Like the Thermotogae within the bacteria, the tendency for eukaryotic genomes to contain multiple copies of *hydA* is high with the genome of *T. vaginalis* encoding for five copies of *hydA* and the genome of *C. reinhardtii* encoding for two copies, which suggests a diversity of enzyme functions within these taxa. Importantly, many of the copies of HydA in these taxa, including those from *T. vaginalis*, *Halothermothrix orenii*, and others are not monophyletic, suggesting that these sequences do not necessarily derive from gene duplication within a given lineage. Rather, these observations suggest a role for lateral gene transfer in the acquisition of multiple HydA-encoding ORFs in these taxa and the presence of a strong evolutionary pressure to maintain this functional enzyme diversity. An intriguing example comes from the genome sequence of *H. orenii*, a thermophilic halophile isolated from a Tunisian salt lake (Cayol et al. 1994). *H. orenii* ferments a variety of sugars into ethanol, acetate and H_2. The *H. orenii* genome contains [NiFe]-hydrogenases, as well as five ORFs with homology to [FeFe]-hydrogenases that have been biochemically characterized, and three additional ORFs with significant homology to the H-cluster binding domain of HydA. The latter homologs have yet to be examined biochemically; however, it is clear from the primary sequence that several members of this class of homologs contain additional domains that are associated with redox sensing, signal transduction, and gene transcription. Like *H. orenii*, the genome of the firmicute *Candidatus Desulforudis audaxviator* encodes for five phylogenetically distinct HydA as well as several group 1 [NiFe]-hydrogenases that have been phylogenetically classified as H_2 uptake hydrogenases.

In addition to isolate genome sequences, recent environmental genome sequencing projects and targeted PCR-based approaches have revealed the widespread distribution and undersampled diversity of *hydA* in natural environments. For example, a recent metagenomic sequencing project of a 1 μL volume termite hindgut microenvironment revealed 159 phylogenetically distinct *hydA* sequences (Warnecke et al. 2007), underscoring the complexity of anaerobic niches even in the small spatial scale of a termite hindgut. Metagenomic sequencing of DNA obtained from a 2.8-km deep gold mine in South Africa revealed the presence of a single species (*Candidatus D. audaxviator*) affiliated with the Firmicutes (Chivian et al. 2008). As previously mentioned, the genome of *Candidatus D. audaxviator* encodes for five distinct HydA in addition to metabolic machinery necessary to reduce inorganic carbon, N_2, and sulfate. In addition to metagenomics, targeted studies of the distribution and diversity of *hydA* using degenerate primers has identified patterns in the primary sequence of HydA that reflect adaptation to local environmental conditions. For example, HydA deduced amino acid sequences recovered from Guerrero Negro (GN), Baja, Mexico salterns were significantly enriched with hydrophilic amino acids when compared to HydA from non-halotolerant taxa (Boyd et al. 2009). The enrichment of residues with hydrophilic character is hypothesized to reduce the effects of salt-driven protein misfolding and/or aggregation and is a commonly observed adaptation at the protein-level in phylogenetically-diverse and halotolerant taxa (Reistad 1970; Lanyi 1974).

All HydA characterized to date contain a highly conserved ~500 residue core, termed the-H cluster binding domain. HydA from eukaryotic green algae such as *C. reinhardtii* and from several bacteria within the Firmicutes consist of only the H-cluster binding domain and represent the simplest [FeFe]-hydrogenase architecture observed to date

(Florin et al. 2001; Wunschiers et al. 2001; Happe et al. 2002; Happe and Kaminski 2002; Winkler et al. 2002a, b; Forestier et al. 2003). Residues associated with H-cluster ligation have been outlined in detail previously by Vignais et al. (2001). Three distinct binding motifs termed L1 (TSCCPxW), L2 (MPCxxKxxE) and L3 (ExMACxxGCxxGGGxP) are typically observed in the [FeFe]-hydrogenase primary sequence (Vignais et al. 2001). However, sequence alignments of newly identified HydA from both genome sequencing projects and PCR-based amplifications have uncovered several deviations from these prototype H-cluster motifs. A notable deviation observed in approximately 15% of HydA is the presence of serine in the place of the second cysteine in the L1 motif. This cysteine is terminally coordinated to the [4Fe-4S] cubane that is linked to the active site 2Fe subcluster in [FeFe]-hydrogenase structures from *Clostridium pasteurianum* and *Desulfovibrio desulfuricans* (Peters et al. 1998; Nicolet et al. 1999). Substitution of this cysteine by serine would be expected to have implications in the redox properties of the H-cluster and potentially influence whether these enzymes are involved in H_2 production or uptake. Interestingly, the [FeFe]-hydrogenase from *Thermoanaerobacter tengcongensis* also contains a serine in place of the second cysteine in the L1 binding motif and has been shown to have hydrogenase activity (Soboh et al. 2004). In vitro, this enzyme exhibits greater rates of H_2 oxidation relative to H_2 production. Additional biochemical and biophysical examination, comparing ligation of the [4Fe-4S] cubane of the H-cluster by serine relative to cysteine, will be required to better understand the enzymatic implications of this substitution on hydrogenase activity.

HydA sequences recovered from the salterns at GN commonly exhibit additional deviations in the L1 sequence motif, including the presence of a threonine in place of serine (TTCCPxW) (Boyd et al. 2009), a substitution which is also observed in one of the five HydA in the genome of *T. vaginalis*. In addition, proline is often observed in the place of serine (TSCSSxW) in deduced amino acid sequences recovered from GN (Beer et al. 2009; Boyd et al. 2009). It is unlikely that the Ser → Thr substitution will a have significant effect on the properties of the hydrogenase, although the Pro → Ser substitution might have a significant effect on the cluster binding properties of this motif. A single example of this substitution has been identified in one of the two HydA present in the genome of the firmicute *Clostridium novii*, although this enzyme has not been biochemically characterized. In addition, while the L2 H-cluster binding motif is highly conserved in representative sequences identified from microbial genome sequencing projects, surveys of HydA diversity in GN reveal an additional and common Pro → Cys substitution (MCCxxKxxE) (Fig. 3.3). Additional characterization of the diversity of HydA from natural environments that span a range of chemical and physical conditions promises to continue to identify enzymes with potentially novel biochemical properties.

The sequence structure of [FeFe]-hydrogenases is diverse, consisting of proteins comprising hardly more than the conserved H-cluster binding domain to those which contain up to six additional FeS cluster binding domains. Meyer recently surveyed the available genome sequences and examined the structural diversity of then available HydA (Meyer 2007). Biophysical characterization of a number of these structurally diverse and catalytically active [FeFe]-hydrogenases demonstrates that several diverse enzymes have evolved in a variety of organisms, the sequences of which are often compared to the two structurally characterized enzymes DdH and CpI. DdH coordinates two [4Fe-4S] clusters in addition to the H-cluster (Nicolet et al. 1999). CpI has an H-cluster binding domain, a two [4Fe-4S] cluster binding domain similar to bacterial ferredoxins, another domain that binds a unique [4Fe-4S] cluster that is ligated by three cysteines and a histidine, and an N-terminal [2Fe-2S] plant-type ferredoxin binding domain (Peters et al. 1998).

Enzymes consisting of only the H-domain (M1 architecture) and those having the H-cluster domain in addition to two (M3 architecture), three (M4 architecture) and four (M5 architecture) additional FeS cluster binding domains were identified. In addition, two phylogenetically-related HydA from the recently sequenced genome of *Desulfitobacterium hafniense* have six additional FeS cluster binding motifs (M7 architecture). All HydA from the genomes of green algae (e.g., *C. reinhardtii*, *Chlorella fusca, Scenedesmus obliquus*) consist of just the H-domain (Florin et al. 2001; Wunschiers et al. 2001; Happe et al. 2002; Happe and Kaminski 2002; Winkler et al. 2002a, b; Forestier et al. 2003). Proteins with M1 architecture have also been identified in the genomes of several lower order eukaryotes including the fungus *Neocallimastix frontalis* and in the firmicutes *Clostridium leptum* and *Clostridium methylpenosum*. Proteins consisting of only the H-domain are of biochemical and biotechnological interest as they lack the additional FeS-clusters observed in most native [FeFe]-hydrogenases (see below) which can complicate the direct biophysical examination of the H-cluster Fe atoms. Continued characterization of the algal enzymes (Posewitz et al. 2005; McGlynn et al. 2008; Mulder et al. 2009), and truncated versions of bacterial [FeFe]-hydrogenases that have been cloned and expressed as active enzymes with only the H-cluster binding domain (King et al. 2006a, b) are beginning to shed light on the biosynthesis of the H-cluster and of the mechanism for H_2 activation. The M3 architecture is common among lower order eukaryotes as well as sulfate reducing bacteria including DdH and a number of *Clostridium* and *Thermotoga* strains. The M4 and M5 architectures are the most commonly observed forms of HydA in bacterial genomes (see Meyer 2007 for review).

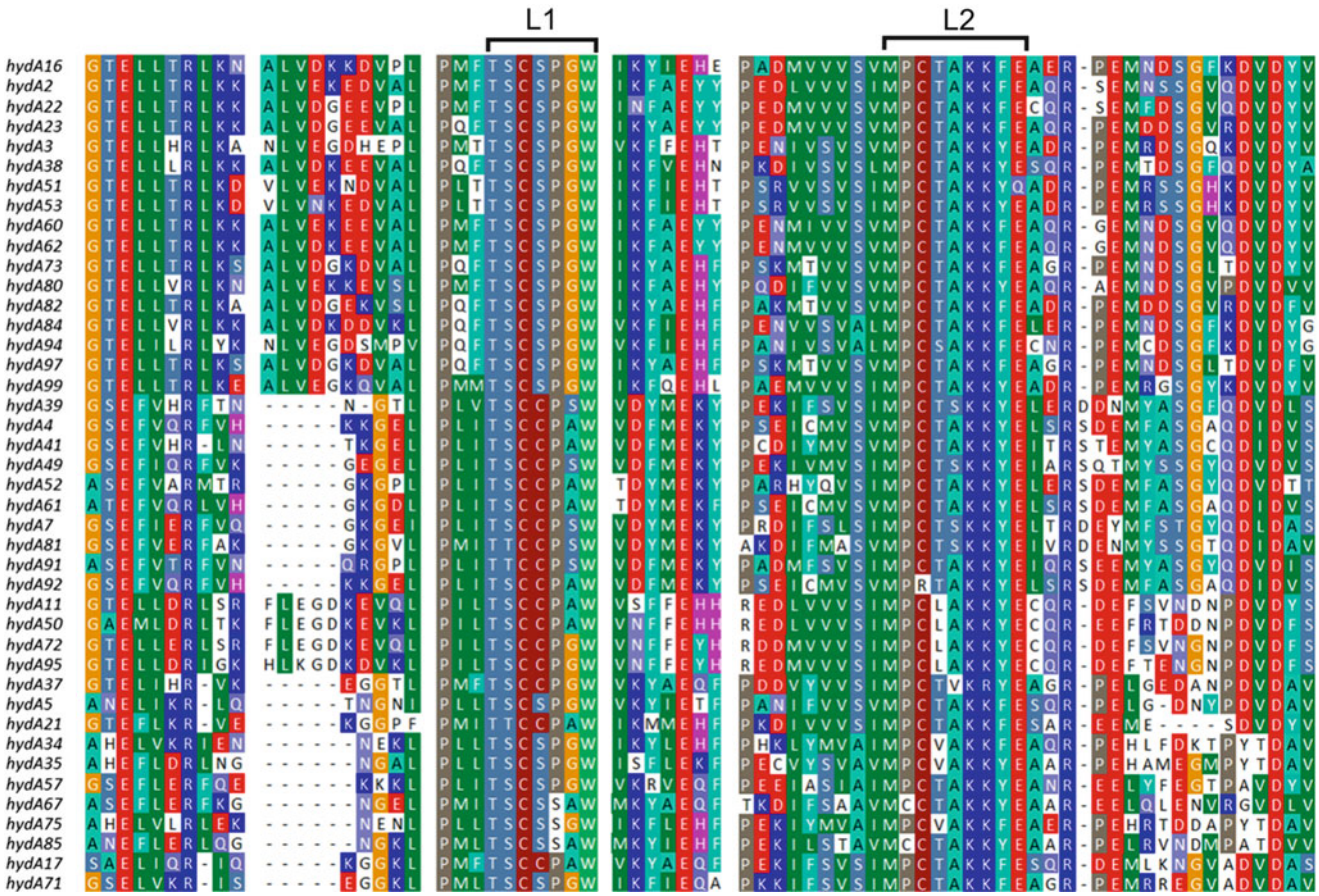

Fig. 3.3 Unique amino acid substitutions in the H-cluster L1 and L2 seqeunce motifs from deduced amino acid sequences recovered from salterns in Guerrero Negro, Mexico. Sequence notation corresponds to those presented in Boyd et al. (2009)

4 [FeFe]-Hydrogenase Structure and Function

The [FeFe]-hydrogenases exist as monomers or in heteromeric complexes, and X-ray crystallographic structural data are available for two representative bacterial enzymes (Peters et al. 1998; Nicolet et al. 1999; Peters 1999). The [FeFe]-hydrogenase catalytic site is known as the H-cluster, and consists of a [4Fe-4S] cluster coordinated to four cysteines and connected via a single bridging cysteine thiolate to a unique binuclear 2Fe center (Fig. 3.2). Terminal CO and CN^- ligands are bound to each Fe atom of the 2Fe center, and a third CO ligand bridges both of the Fe atoms in the enzyme's resting state. A unique non-protein dithiolate ligand, proposed to be either di(thiomethyl)amine, di(thiomethyl)ether or dithiopropane, also bridges the 2Fe atoms of the active site in a bidentate fashion (Nicolet et al. 1999, 2001; Pandey et al. 2008). This unusual ligand complement putatively functions to tune the unique chemistry of the H-cluster, stabilizing lower redox states of Fe that facilitate reversible H_2-oxidation chemistry. The nature or composition of this non protein dithiolate ligand has been of significant interest since the central or sometimes termed bridgehead group of the ligand is located in close proximity to the distal site and it has been suggested that if the bridgehead group were an amine, it could cycle between different protonation states and serve as a proton donor/acceptor group during catalysis (Fan and Hall 2001; Nicolet et al. 2001; Liu and Hu 2002). The chemical composition of this group to date has still not been determined but recently a spectroscopic study supports that an amine group is located at this position (Silakov et al. 2009), however recent computational work questions whether an amine group at this position could act as a proton/acceptor group in catalysis (Pandey et al. 2008). Although all the details concerning how reversible H_2 oxidation occurs at this active site metal center are not clear, the structural differences observed for the enzyme in different oxidation states as well as the structural analysis of the carbon monoxide inhibited state of the [FeFe]-hydrogenase from

Clostridium pasteurianum indicate that H_2 binding and H_2 production occur at the Fe of the 2Fe subcluster distal from the [4Fe-4S] subcluster (Lemon and Peters 1999; Bennett et al. 2000; Lemon and Peters 2000; Nicolet et al. 2000, 2001; Chen et al. 2002).

All [FeFe]-hydrogenases are presumed to possess what is thought to be an analogous active site; however, detailed biochemical studies have only been conducted on enzymes from a limited number of sources, most notably *Clostridium pasteurianum, Desulfovibrio desulfuricans, Desulfovibrio vulgaris,* and more recently *Clostridium acetobutylicum* and *Chlamydomonas reinhardtii.* The [FeFe]-hydrogenases have one-to-two orders of magnitude higher catalytic activities than the [NiFe]-hydrogenases but are generally more O_2 sensitive and are irreversibly inactivated by O_2 (Adams 1990). Their high specific activities, and the relationship between the hydrogenase active site structure and well-characterized Fe carbonyl complexes, have made the H-cluster active site the target of many inorganic and organometallic synthetic chemists (for review see (Gloaguen and Rauchfuss 2009; Tard and Pickett 2009)). Although numerous cluster mimics have been synthesized incorporating many ligand variations, none of the cluster mimics have been effective H_2-production catalysts.

The key structural and functional differences in the [FeFe]-hydrogenases are related to cellular location and electron donor/acceptor partners. These differences are reflected in the architecture of the enzymes from different sources. The [FeFe]-hydrogenase from *C. pasteurianum* functions to recycle reductants generated during fermentation in the cytoplasm and exists as a monomer, which in addition to the active site H-cluster has four additional iron-sulfur clusters that are presumably present to help shuttle electrons from external electron donors (Peters et al. 1998; Peters 1999). In contrast, the [FeFe]-hydrogenases from the sulfate reducing bacteria *D. desulfuricans,* and *D. vulgaris,* and the facultative anaerobe *Shewanella oneidensis* exist as dimers with a small subunit that has some sequence conservation to the C-terminal portion of the monomeric enzymes and occupies the same structural space (Voordouw and Brenner 1985; Nicolet et al. 1999; Heidelberg et al. 2002). In addition to this structurally analogous region, these small subunits carry the canonical twin arginine transporter sequence that signals transport and localization of these enzymes to the periplasmic space. These enzymes also exist with only two accessory clusters, putatively reflecting different metabolic roles and different electron transfer partners. Regardless of the complement of accessory clusters, the quaternary structure, or the cellular location of these enzymes, the unifying feature is the presence of the H-cluster active site domain.

5 [FeFe]-Hydrogenase Maturation

As mentioned above, the [FeFe]-hydrogenase active site H-cluster exists as a regular [4Fe-4S] cluster bridged to a 2Fe subcluster which harbors the unique non-protein ligands (carbon monoxide, cyanide, and dithiolate). The nonprotein ligands are synthesized and inserted into the metal cluster through the activity of maturation enzymes. The direct involvement of three gene products in hydrogenase maturation was confirmed by their requirement for the heterologous expression of active hydrogenase in *E. coli* (Posewitz et al. 2004a, b, 2005; King et al. 2006a, b). The three genes, *hydE, hydF,* and *hydG,* appear to be the only hydrogenase-specific genes common to all organisms possessing [FeFe]-hydrogenases, suggesting that these are the only genes required for synthesis and insertion of the H-cluster. Of the three gene products, two (HydE and HydG) have been shown to be members of the radical SAM superfamily, which use [4Fe-4S] clusters and S-adenosylmethionine (SAM) to initiate radical-mediated chemistry (Posewitz et al. 2005; Rubach et al. 2005).

We recently put forth a proposal regarding the roles of HydE and HydG, and radical-SAM chemistry, in the biosynthesis of the H-cluster of hydrogenase (Fig. 3.4) (Peters et al. 2006). We suggested that in the first biosynthetic step, HydE or HydG serves to convert a standard [2Fe-2S] cluster (bound to HydE or HydG or to the scaffold protein HydF) to a dithiolate-bridged [2Fe-2S] cluster; the dithiolate ligand is generated by alkylation of the two bridging sulfides of the cluster, the alkylating group likely arising from compounds available in the cell such as intermediates in central metabolism (e.g., amino acids, nucleotides). This type of reaction is precedented in the radical SAM superfamily by the enzymes LipA (lipoate synthase) and BioB (biotin synthase), both of which catalyze insertion of a bridging cluster sulfide into an alkane C-H bond to generate a thiolate (Wang and Frey 2007; Frey et al. 2008). In the case of HydE/HydG, this insertion would occur twice to yield a dithiolate coordinated to the [2Fe-2S] cluster; this modification would result in a cluster with altered properties, which we propose allows the subsequent radical SAM reaction to take place as described below.

According to our original hypothesis, the modified, dithiolate-bridged [2Fe] cluster would then be transferred from the first radical-SAM enzyme (HydE or HydG) to the second (HydE or HydG), or would remain bound to the scaffold HydF, for the next step in cluster assembly, the generation of the carbonyl and cyanide ligands. These distinctly non-biological ligands are a unique characteristic feature of the active site metal clusters of hydrogenases. Based on the involvement of radical SAM enzymes in H-cluster biosynthesis, we proposed that the CO and CN^- ligands resulted from radical-mediated decomposition of a common

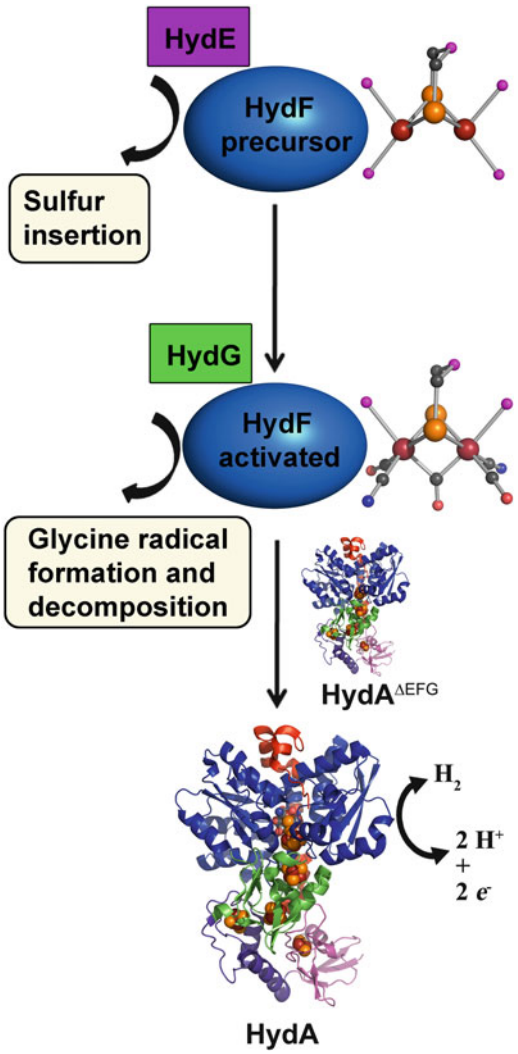

Fig. 3.4 H-cluster biosynthesis process for [FeFe]-hydrogenases. The 2Fe subcluster is proposed to be assembled on HydF which serves as a chemical scaffold for the process. Radical SAM enzyme HydE is proposed to modify a basic [2Fe-2S] cluster with a dithiolate ligand in the first step, followed by further cluster modification with addition of CO and CN^- ligands by the radical SAM enzyme HydG from the substrate tyrosine. In the final step, activation and maturation is achieved by transfer of the ligand modified 2Fe subcluster to the immature structural protein ($HydA^{\Delta EFG}$), which already contains the [4Fe-4S] cluster of the H-cluster

metabolite such as glycine. Radical-based chemistry at glycyl residues in proteins is also precedented in the radical SAM enzymes (e.g. in the activating enzymes for pyruvate formate-lyase, anaerobic ribonucleotide reductase, and benzylsuccinate synthase), and we proposed a reasonable chemical mechanism for the generation of CO and CN^- via breakdown of a glycine radical (Peters et al. 2006). The scaffold protein HydF was proposed to serve as the final site of H-cluster precursor assembly, and the site from which this precursor is transferred to the hydrogenase structural protein (HydA) to effect activation. This overall hypothetical scheme has provided an ideal fundamental basis on which to design experiments aimed at probing the overall process of H-cluster assembly and considerable progress has been made.

We have developed an *in vitro* system by which to study HydA maturation, and used it to demonstrate that an *E. coli* extract in which HydE, HydF, and HydG are expressed together is able to generate an active hydrogenase when combined with HydA expressed in *E. coli* (McGlynn et al. 2007). Activation was complete in the 5 min required to perform the assay and proceeded without addition of small molecule reagents, suggesting that the H-cluster precursor was already assembled in the HydE/HydF/HydG extract and was readily transferred to the HydA apoenzyme to generate active [FeFe]-hydrogenase. It was also demonstrated that the activation component was protein-associated and not a freely diffusing small molecule. Together, our observations were consistent with a model for H-cluster biosynthesis in which one or more of the accessory proteins, and not the hydrogenase structural protein, served as the physical scaffold for the assembly of an H-cluster precursor. We subsequently demonstrated that when His-tagged HydF was purified from *E. coli* cell extracts, in which all three accessory proteins were expressed, this purified HydF expressed in a genetic background in which HydE and HydG are coexpressed ($HydF^{EG}$) could affect activation of HydA expressed heterologously in *E. coli* lacking HydE, HydF, and HydG ($HydA^{\Delta EFG}$) (McGlynn et al. 2008). The characteristics of this activation by purified $HydF^{EG}$ were consistent with a role for HydF as a scaffold in H-cluster assembly. The identification of HydF as a scaffold led us to a refined hypothesis in which the AdoMet proteins HydE and HydG enact chemistry upon HydF to produce an H-cluster intermediate, which is then transferred to HydA to accomplish activation. In addition to serving as a scaffold for assembly of an H-cluster precursor, HydF is a GTPase (Posewitz et al. 2004a, b; Brazzolotto et al. 2006). This functionality was initially predicted based on the amino acid sequence of HydF, which includes a Walker P-loop as well as other motifs characteristic of GTPases, and GTPase activity has been confirmed in our lab as well as others (Posewitz et al. 2004a, b; Brazzolotto et al. 2006; Shepard et al. 2010). Evidence to date indicates that this GTPase activity is *not* associated with transfer of the subcluster from loaded HydF to HydA, and therefore it is likely associated with the interactions between HydF and HydE and/or HydG (McGlynn et al. 2008; Shepard et al. 2010).

Further insight has been gained into the nature of the species transferred from $HydF^{EG}$ to $HydA^{\Delta EFG}$ to effect activation by detailed spectroscopic and structural studies of $HydA^{\Delta EFG}$. EPR, Mössbauer, and X-ray absorption spectroscopic studies demonstrated that $HydA^{\Delta EFG}$ purified from *E. coli* contains a [4Fe-4S] cluster (Mulder et al. 2009). The observation that this protein can be activated by $HydF^{EG}$ supports the hypothesis that the activation process involves transfer of

only a 2Fe subcluster from HydF to HydA to produce the 6Fe active site H-cluster. Further, when the [4Fe-4S] cluster is removed from $HydA^{\Delta EFG}$ by chelation, $HydF^{EG}$ can no longer effect activation but the ability to activate can be restored when the [4Fe-4S] cluster is reconstituted into HydA. In support of these spectroscopic and functional studies is our recent crystal structure of $HydA^{\Delta EFG}$, which shows the presence of a single [4Fe-4S] cluster. The structure shows definitively that the [4Fe-4S] subcluster of the H-cluster is synthesized intact in the absence of the HydE, HydF, and HydG and is structurally poised to accommodate insertion of an intact 2Fe-subcluster. These new structural results strongly support a mechanism of H cluster synthesis that is stepwise with [4Fe-4S] subcluster preceding the insertion of the 2Fe subluster and reveals very interesting parallels to the mechanism of nitrogenase maturation.

The radical SAM enzymes HydE and HydG are most likely responsible for synthesis of the nonprotein ligands to the H-cluster. In terms of primary amino acid sequence, HydE most closely aligns with radical SAM proteins that catalyze sulfur insertion, such as biotin synthase, suggesting that HydE serves to synthesize the dithiolate ligand of the H-cluster. The substrate for HydE has yet to be identified; however the crystal structure of HydE from *Thermotoga maritima* has been solved. Although its function is not yet understood it has been shown to have an affinity for thiocyanate in the active site in silico leading to the proposal that HydE may be involved in synthesis of the cyanide ligands (Nicolet et al. 2008). HydG shows significant homology to ThiH, a radical SAM enzyme that catalyzes tyrosine cleavage. In fact, both our recent work (Driesener et al. 2010), and a recent report from the Fontecave lab (Pilet et al. 2009), demonstrate that HydG catalyzes the cleavage of tyrosine. Fontecave and coworkers have proposed that this tyrosine cleavage results in the formation of dehydroglycine with serves as a precursor for the synthesis of the dithiolate ligand (Pilet et al. 2009). The production of dehydroglycine was not measured as a product of Tyr cleavage in this work (Pilet et al. 2009) and we have maintained that these results may point toward the generation of carbon monoxide and cyanide ligands by amino acid (Tyr/dehydroglycine) decomposition mechanism along the lines of that previously suggested (Peters et al. 2006). Very recently, we have observed that HydG-catalyzed tyrosine cleavage results in the production of cyanide in equivalent stoichiometry to *p*-cresol (Driesener et al. 2010). These results clearly indicate a role for HydG in the production of the diatomic ligands and are among the key observations that lay the groundwork for the next generation of experiments probing the mechanism of H-cluster biosynthesis (Fig. 3.4).

Another key set of observations from our group that help to define our hypothetical scheme and shape the next generation of experiments relates to the function of HydF as a scaffold. We have been able to show that HydF (expressed in the absence HydE and HydG) exhibits characteristic EPR spectra consistent with having both [4Fe-4S] clusters and [2Fe-2S] clusters. Interestingly, when HydF is expressed in a genetic background in which HydE and HydG are coexpressed ($HydF^{EG}$), the [2Fe-2S] cluster is not observed by EPR (Shepard et al. 2010). Examination of the $HydF^{EG}$ by Fourier transform infrared (FTIR) spectroscopy indicates that instead of a canonical [2Fe-2S] cluster, $HydF^{EG}$ harbors a cluster biosynthesis intermediate that resembles that of the 2Fe subcluster of the H-cluster (Shepard et al. 2010). Recent spectroscopic characterization of HydF from *Clostridium acetobutylicum* also suggests the presence of a 2Fe cluster with CO and CN^- ligands on HydF (Czech et al. 2009). Such insights have allowed us to refine our hypothetical scheme for H cluster biosynthesis.

6 [NiFe]-Hydrogenase Phylogeny

[NiFe]-hydrogenases are widely distributed among the bacteria and the archaea, but have yet to be identified in the genomes of eukaryotes. Phylogenetic studies of these enzymes have been used to group these enzymes into several classes and subclasses (Wu and Mandrand 1993; Vignais et al. 2001; Cournac et al. 2004), which generally correspond to the physiological role of the respective enzymes. These classes and subclasses include the (1) membrane bound H_2 uptake, (2a) cyanobacterial uptake, (2b) H_2 sensing, (3a) F_{420} reducing, (3b) bifunctional hyperthermophilic, (3c) MV-reducing, (3d) bidirectional NAD-linked and (4) membrane bound H_2 evolving [NiFe]-hydrogenases (Vignais et al. 2001). Accordingly, these enzymes are involved in a variety of metabolic functions and exhibit a broad range of biochemical characteristics; features which are reflected in the distribution of each class of enzyme across the bacteria and archaea (see Vignais and Billoud 2007 for detailed review).

The respiratory uptake [NiFe]-hydrogenases (Group 1 or membrane-bound H_2 uptake) are the most common of the classes of [NiFe]-hydrogenase. These enzymes typically oxidize H_2 to supply reducing equivalents for cellular growth and for metabolic uses. The group 1 [NiFe]-hydrogenases function in a variety of metabolisms such as lithotrophic CO_2 fixation and the respiration of O_2, NO_3, SO_4 or metal ions. These hydrogenases are generally present in the periplasm or are membrane bound and can couple electron transfer with trans-membrane proton translocation (Sargent et al. 1998; Dubini and Sargent 2003). The respiratory uptake enzymes are distributed across a diversity of bacteria including the Bacteroidetes, Chlorobi, Proteobacteria, Firmicutes, and Aquificae (Vignais and Billoud 2007). Similarly, membrane-bound H_2 uptake hydrogenases are common among the Crenarchaeota and many lineages of Euryarchaeota,

including the Methanosarcinales and Archaeoglobi. Among the bacteria, the only major phylogenetic group that lacks homologs of the group 1 [NiFe]-hydrogenase are the Cyanobacteria and the Planctomycetes, although only a single genome of the latter has been sequenced to date. Among the Archaea, homologs of group 1 [NiFe]-hydrogenase have yet to be identified in the genomes of the Methanomicrobia, Methanococci, Methanomicrobia, and Thermococci.

Group 2 [NiFe]-hydrogenases are generally located in the cytoplasm and include the H_2-sensing enzymes (Group 2b) that are involved in the regulation of hydrogenase transcription (Kleihues et al. 2000; Vignais et al. 2000) as well as the cyanobacterial uptake enzymes (Group 2a), which function to recycle H_2 produced by nitrogenase during N_2 fixation in N_2-fixing organisms (Tamagnini et al. 2000). The group 2 [NiFe]-hydrogenases have limited distribution when compared to Group 1 [NiFe]-hydrogenase. Homologs of the Group 2b [NiFe]-hydrogenase (H_2-sensing) have only been identified in the genomes of the Proteobacteria, and are predominantly found in the genomes of members of the α-, β-, γ-, and ε-Proteobacteria and have yet to be identified in the δ-Proteobacteria. The group 2b H_2-sensing enzymes interact with a two-component kinase system in response to H_2 levels, resulting in the regulation of additional hydrogenases at the transcriptional level. H_2-sensing enzymes exhibit very low levels of hydrogenase activity and the H/D exchange reaction catalyzed by the *Rhodobacter capsulatus* HupUV hydrogenase is not inhibited by O_2 (Vignais et al. 2000). The low catalytic rates and O_2 insensitivity of the H_2-sensing enzymes may be a consequence of a smaller hydrophobic channel leading to the active site, which restricts gas access (Vignais et al. 2000; Buhrke et al. 2005; Duche et al. 2005). Homologs of the cyanobacterial uptake [NiFe]-hydrogenases (Group 2a) are highly similar (~90% sequence identities) and have been identified in the genome sequences of most orders of cyanobacteria, some of which are not capable of fixing nitrogen. Group 2a homologs have not been identified among the Prochlorales (*Prochlorothrix hollandica*) and the genomes of *Synechococcus* spp. and *Synechocystis* spp. within the Chroococcales. Group 2a [NiFe]-hydrogenase homologs have also been identified in the genomes of several orders of Proteobacteria, including a single genome affiliated with the order Oceanospiralles (*Oceanospirillum* sp.) and several orders within the α-Proteobacteria including the Rhizobiales, Rhodospirillales, Sphingomonadales and the *β*-proteobacterium *Dechloromonas aromatica* (Order Rhodocyclales). The cyanobacterial uptake hydrogenases contain the appropriate [NiFe]-hydrogenase amino acid motifs; however active enzymes have yet to be purified and characterized. In some heterocystous cyanobacteria, excision of a DNA element by site-specific recombination occurs within the gene encoding the large subunit during heterocyst differentiation, resulting in the expression of the uptake hydrogenase only in N_2 fixing cells (Carrasco et al. 1995, 2005).

The subclasses a-d comprising group 3 [NiFe]-hydrogenases are uniquely distributed, with representatives of classes 3a (F_{420}-reducing) and 3c (MV-reducing) present only in the genomes of methanogenic archaea and representatives of class 3b (bifunctional hyperthermophilic) present only in the genomes of Thermococci (Euryarchaeota) and several bacterial phyla including the Proteobacteria, Actinobacteria, and Chlorobi. Homologs of class 3d (bidirectional NAD-linked), [NiFe]-hydrogenase, have been identified among the Cyanobacteria and several orders of Proteobacteria, Chloroflexi, and Actinobacteria. These enzymes are generally multimeric, reversible enzymes that contain additional subunits which vary among the different group 3 enzymes. These enzymes commonly interact with soluble cellular redox components such as the pyridines NAD^+, $NADP^+$ or the flavin F_{420}. The F_{420} reducing [NiFe]-hydrogenases are trimeric enzymes that are involved in the reduction of CO_2 to methane in methanogenic archaea (Vaupel and Thauer 1998; Vignais et al. 2000); these enzymes are present in all orders of methanogens. The thermophilic bifunctional [NiFe]-hydrogenases are a unique group of tetrameric enzymes that are predominant among sulfur reducing archaea and are involved in peptide or sugar fermentation. The group 3b enzymes from *Pyrococcus furiosus* couple with NADP(H) (Ma et al. 2000), which is unique relative to most group 3 [NiFe]-hydrogenases, that show a strong preference for NAD(H) relative to NADP(H). Presently, the MV-reducing [NiFe]-hydrogenases have not been extensively characterized and additional examination is required to better define the biochemical properties and physiological function of these enzymes. Homologs of the group 3c enzymes are present in the Methanomicrobiales, Methanobacteriales, Methanopyrales, and Methanococcales, but not in the Methanosarcinales. The group 3d NAD-linked [NiFe]-hydrogenases reversibly reduce NAD^+ at the expense of H_2. These enzymes are common among representative lineages of cyanobacteria and in the genomes of several additional bacterial phyla including Bacteriodetes, Proteobacteria, Acidobacteria, Actinobacteria and Chloroflexi, but have yet to be identified in an archaeal genome. Cyanobacterial enzymes are currently being investigated as a means to produce H_2 from phototrophic organisms (Schutz et al. 2004; Rupprecht et al. 2006; Hankamer et al. 2007). As shown in Fig. 3.5, the group 3d [NiFe]-hydrogenases that have been characterized are heteropentameric, consisting of a large and small subunit (HoxYH) as well as a diaphorase component (HoxEFU) (Tamagnini et al. 2000; Schutz et al. 2004). In *Synechocystis* sp. PCC 6803, Hox has been proposed to function as an electron valve (Appel et al. 2000). The catalytic subunits of the diaphorase moiety (HoxFU) contain NAD, FMN and FeS binding sites and function to couple NAD(H) oxidation or reduction with hydrogenase activity. HoxE is thought to coordinate an FeS cluster and may be involved in

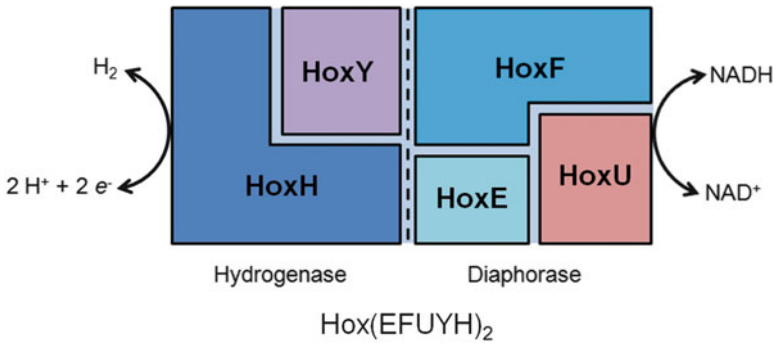

Fig. 3.5 Cartoon representation of the hox encoded NAD(P) reducing/NAD(P)H oxidizing hydrogenase

membrane attachment and/or in electron transport (Appel and Schulz 1996; Boison et al. 2000; Schmitz et al. 2001, 2002).

The class 4 [NiFe]-hydrogenases (H_2 evolving) are unique in that, unlike the other subclasses of [NiFe]-hydrogenase, they are thought to function most often in H_2 evolution *in vivo*. Homologs of H_2-evolving hydrogenases are common among the Euryarchaeota and been identified in the genomes of the Firmicutes, Planctomycetes, Chloroflexi, and Proteobacteria. Characterized members of this enzyme class include the energy conserving hydrogenase (Ech) from *T. tengcongensis*, the CooH enzyme from *Rhodospirillum rubrum* and hydrogenase 3 in *E. coli* (Hedderich and Forzi 2005), the latter of which couples formate oxidation to proton reduction (Bohm et al. 1990; Hedderich and Forzi 2005). The group 4 Ech enzymes contain four additional subunits and are typically able to accept electrons from reduced ferredoxin (Hedderich and Forzi 2005). Although the majority of [NiFe]-hydrogenases do not efficiently couple to ferredoxin, several group 4 [NiFe]-hydrogenases are able to oxidize reduced ferredoxin (Maness et al. 2002; Soboh et al. 2004; Hedderich and Forzi 2005). In *P. furiosus*, it has been demonstrated that this activity is coupled to an ion gradient used for ATP synthesis (Sapra et al. 2003).

Like the NAD(P)(H)-linked [FeFe]-hydrogenases, the subunits of [NiFe]-hydrogenases and their respective subunits are homologous to proteins comprising complex I involved in cellular respiration (Vignais et al. 2001; Vignais and Colbeau 2004; Hedderich and Forzi 2005). Unlike [FeFe]-hydrogenases, [NiFe]-hydrogenases are generally less sensitive to O_2 (Adams 1990; Bleijlevens et al. 2004; Burgdorf et al. 2005; Cohen et al. 2005a, b; Vincent et al. 2005a, b). While the majority of [FeFe]-hydrogenases are irreversibly inhibited by O_2, several [NiFe]-hydrogenases can be reactivated using a variety of reductants and reducing conditions after exposure to O_2. Thus, [NiFe]-hydrogenases, especially the NAD-linked bidirectional subclass, are actively being pursued for the efficient photoproduction of H_2.

7 [NiFe]-Hydrogenase Structure and Function

The structure of [NiFe]-hydrogenases is well-studied and the x-ray crystal structures of [NiFe]-hydrogenases from sulfate reducing bacteria *Desulfovibrio gigas* (Volbeda et al. 1995, 1996) (Fig. 3.2), *D. vulgaris* Miyzaki F (Higuchi et al. 1997, 1999), *D. fructosovorans* (Montet et al. 1997; Volbeda et al. 2002, 2005) and *D. desulfuricans* (Matias et al. 2001) have been determined as well as [NiFe(Se)]-hydrogenases, a subclass to the [NiFe]-hydrogenases, from *Desulfomicrobium baculatum* (Garcin et al. 1999) and *D. vulgaris* Hildenborough (Marques et al. 2009). In addition, preliminary x-ray analysis for the photosynthetic bacterium *Allochromatium vinosum* has been reported (Kellers et al. 2008). In their simplest and most characterized form, membrane-bound H_2 uptake [NiFe]-hydrogenases are heterodimeric and composed of a large (~60 kDa) and small subunit (~30 kDa) (Vignais et al. 2001; Vignais and Colbeau 2004; Vignais and Billoud 2007). For other classes of [NiFe]-hydrogenases, additional subunits can be present. Hydrogen catalysis takes place at the [NiFe] active site present in the large subunit. The small subunit contains at least one [4Fe-4S] cluster proximal to the large subunit domain in addition to other accessory FeS clusters which presumably function to assist electron transport to and from the catalytic site that is buried well within the protein. For the membrane-bound H_2 uptake [NiFe]-hydrogenases, the small subunit contains three FeS clusters spaced ~12 Å apart. The clusters include a proximal [4Fe-4S] cluster, medial [3Fe-4S] cluster, and distal [4Fe-4S] cluster in relation to the large subunit. They play a critical role for hydrogen oxidation and respiratory uptake. Their special arrangement across the enzyme makes possible electron transport from the active site to the protein surface where electrons can be delivered to cytochrome c_3, one example of a physiological electron acceptor.

The heterobimetallic active site is comprised of a Ni atom bridged by two cysteine thiolate ligands to an Fe atom (Fig. 3.2). The Ni atom is coordinated to the protein by two cysteine thiolate ligands. [NiFeSe]-hydrogenases contain a

selenocysteine ligand substituted for one of the cysteine ligands at the Ni atom (He et al. 1989; Sorgenfrei et al. 1993). Similar to the [FeFe]-hydrogenase active site, is the presence of non-protein, diatomic CO and CN^- ligands, and the Fe atom in [NiFe]-hydrogenases is coordinated by one terminal CO and two terminal CN^- ligands, which were detected using IR-spectroscopy (Volbeda et al. 1996; Happe et al. 1997; Pierik et al. 1999). Also present in the as-isolated oxidized form is a species believed to be either a peroxide molecule or a sulfenate from the oxidation of the bridging cysteine thiolate ligands (Lamle et al. 2004; Ogata et al. 2005; Volbeda et al. 2005; van Gastel et al. 2006). [NiFe]- and [FeFe]-hydrogenases differ significantly in O_2 sensitivity and O_2 inhibition can be reversible for [NiFe]-hydrogenases (Liebgott et al. (2010); Adams 1990; Burgdorf et al. 2005; Cohen et al. 2005a, b; Vincent et al. 2005a, b; Cracknell et al. 2009). [NiFe]-hydrogenases can be isolated aerobically and later activated by reduction with H_2. Reactivation following exposure to O_2 can be achieved by reducing conditions and the presence of H_2 involves the loss of the bridging oxygen species (Garcin et al. 1999; Higuchi et al. 1999). Hydrogen activation is believed to take place at the Ni atom by nucleophilic addition and heterolytic bond cleavage (Niu and Hall 2001; Fichtner et al. 2006; Pardo et al. 2006). Also, hydrophobic channels allowing for H_2 diffusion to the active site have been identified (Montet et al. 1997; Fontecilla-Camps et al. 2007).

During hydrogen catalysis, the [NiFe] active site passes through multiple redox and structural states which have been the targets of extensive spectroscopic and structural studies (reviewed in Armstrong 2004; De Lacey et al. 2005, 2007; Ogata et al. 2009). The Ni atom goes through three different oxidation states (Ni^{1+}, Ni^{2+}, Ni^{3+}) while the Fe atom remains as Fe^{2+} throughout the cycle (Huyett et al. 1997; Trofanchuk et al. 2000; Fan and Hall 2002; Foerster et al. 2003; Gu et al. 2003). The variable redox state of the Ni atom provides further support that it is the site for H_2 activation and heterolytic bond cleavage. In the as-isolated oxidized state, the active site can exist in two forms: Ni-A ("unready") and Ni-B ("ready") (De Lacey et al. 2007; Fontecilla-Camps et al. 2007). While, the Ni-B state can be activated by the reduction of H_2 in seconds, activation of the Ni-A state is much slower and is on the time scale of hours (Fernandez et al. 1985; Lamle et al. 2004). Activation of the two oxidized states involves the loss of the bridging ligand between Ni and Fe atoms (Garcin et al. 1999; Higuchi et al. 1999) and it has been determined that this ligand is different for the two states and thus can logically be attributed to the difference in time required for activation. For the Ni-B state it is likely a single oxygen species (i.e. hydroxide) and for the Ni-A state likely a multiple oxygen species (i.e. peroxide) (Lamle et al. 2004; Ogata et al. 2005; Volbeda et al. 2005; van Gastel et al. 2006). Other redox detected states of the [NiFe]-active site are created by the reduction of the Ni-A and Ni-B states. One electron reduction and loss of the bridging species yields the EPR active Ni-C state, which is light sensitive and can give way to the photoproduct Ni-L at cryogenic temperatures. The fully reduced state is termed Ni-R. Also, similar to [FeFe]-hydrogenases, the [NiFe] active site is reversibly inhibited by CO and binding has been shown to take place at the Ni atom (van der Zwaan et al. 1990; Bagley et al. 1994; Ogata et al. 2002; Pandelia et al. 2009).

[NiFe]-hydrogenases display higher levels of O_2 tolerance than [FeFe]-hydrogenases (Liebgott et al. (2010); Adams 1990; Burgdorf et al. 2005; Cohen et al. 2005a, b; Vincent et al. 2005a, b; Cracknell et al. 2009) and three hydrogenases from *Ralstonia eutropha* have even been shown to catalyze hydrogenase oxidation in the presence of O_2, although at significantly slower rates (Burgdorf et al. 2005; Vincent et al. 2005a, b; Goldet et al. 2008). Hydrogenases from these organisms are the target for biotechnological applications. Also, For both [NiFe]- and [FeFe]-hydrogenases, O_2 diffusion pathways have been identified by analysis of the crystal structures and by molecular dynamics calculations giving way to experiments designed to engineer hydrogenases that are O_2 tolerant and/or have a decreased level of oxygen sensitivity (Liebgott et al. (2010); Montet et al. 1997; Nicolet et al. 2000; Cohen et al. 2005a, b). Experiments aimed at mutating amino acid residues along identified hydrophobic channels to decrease O_2 diffusion, O_2 access to the active site, and ultimately lower the O_2 sensitivity have been conducted and this approach has been demonstrated to be successful for a [NiFe]-hydrogenase (Leroux et al. 2008; Dementin et al. 2009).

8 [NiFe]-Hydrogenase Maturation

The [NiFe]-hydrogenases also require the activity of specialized maturation enzymes for the synthesis and insertion of the unique non-protein ligands present at the active site. The maturation of the active site of [NiFe]-hydrogenases has been studied in detail using a number of different organisms, and a model for the individual steps in the process has been developed in detail for hydrogenase-3 in *E. coli* (Fig. 3.6) (reviewed in Bock et al. 2006). Interestingly, there is no cross-over between the maturation enzymes required for [NiFe]- and [FeFe]-hydrogenase maturation and both classes of hydrogenases require unique maturation enzymes (Vignais et al. 2001; Vignais and Billoud 2007). In contrast to only three maturation enzymes required for [FeFe]-hydrogenases, at least six maturation enzymes are required for the synthesis of the active site of [NiFe]-hydrogenases (Bock et al. 2006). This in part is to be expected given the characteristics of nickel and its toxicity and often additional proteins are involved in the biosynthesis process for activation, transport, insertion and binding of nickel. Taking into account the

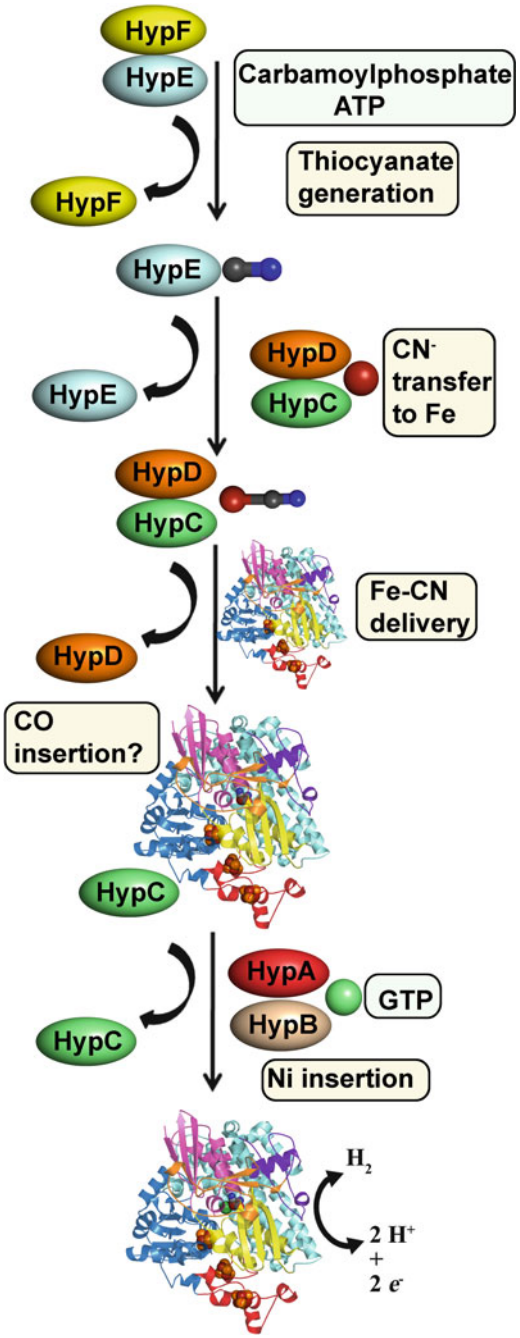

Fig. 3.6 Scheme of the biosynthesis of the active site of [NiFe]-hydrogenases based on hydrogenase-3 in *E. coli*. In the first step, HypE and HypF form a complex to allow synthesis of thiocyanate from the precursor carbamoyl phosphate and ATP forming thiocyanate bound by HypE. HypE is transferred to the Fe coordinating HypCD complex, which transfers the $Fe(CN)_2CO$ moiety into the apo-hydrogenase large subunit before Ni insertion. The precursor for the CO ligand is unknown. Ni is inserted by HypA and the GTPase HypB. In most organisms, the maturation process is completed by cleavage of an extended C-terminal peptide by an endopeptidase

diversity of [NiFe]-hydrogenases, it is not surprising that unique maturases are required for organisms in certain cases (Bleijlevens et al. 2004; Lenz et al. 2005; Bock et al. 2006); however, the focus here will be directed at the six essential maturation enzymes HypA, B, C, D, E, and F (encoded by *hyp* genes) using hydrogenase-3 in *E. coli* as a model for the maturation process.

The overall process of maturation requires the synthesis and insertion of the Fe atom bound to non protein ligands CO and CN^- and the activation, transport and insertion of Ni for incorporation into the active site. This process takes place stepwise with the synthesis and insertion of the $Fe(CN)_2CO$ moiety preceding the incorporation of Ni (Maier and Bock 1996; Blokesch et al. 2002; Bock et al. 2006). In the first step, HypE and HypF form a complex to allow synthesis of thiocyanate from carbamoyl phosphate, the biological precursor for the CN^- ligands (Paschos et al. 2001, 2002; Reissmann et al. 2003; Blokesch et al. 2004a, b). By hydrolysis of carbamoyl phosphate on HypF and subsequent transfer of the carbomyl group to HypE followed by hydrolysis, a thiocyanate is attached to the sulfur of the C-terminal residue on HypE. HypC and HypD also complex together and HypE transfers the thiocyanate to the complex where it is able to bind Fe, although the exact mechanism is not known (Blokesch et al. 2004a, b; Blokesch and Bock 2006; Watanabe et al. 2007). At this point, CO must be incorporated into the precursor, however the origin of CO and detail for this step are not yet clear (Roseboom et al. 2005; Forzi et al. 2007; Lenz et al. 2007). Ultimately, the HypCD complex transfers the $Fe(CN)_2CO$ moiety into the apo-hydrogenase large subunit before Ni insertion. HypA and HypB are involved in the insertion of Ni (Waugh and Boxer 1986; Maier et al. 1993) and recent crystal and solution structures of HypA have been reported (Watanabe et al. 2009; Xia et al. 2009). In addition, the SlyD protein can be involved in this step for some organisms (Zhang et al. 2005; Leach and Zamble 2007). HypA and HypB interact with each other to form a complex, although the exact mechanism for Ni insertion is not known (Maier et al. 1993). HypB is a GTPase and the insertion of Ni involves GTP hydrolysis by HypB (Maier et al. 1995). HypA may act as a metallochaperone between HypB and the large hydrogenase subunit in the insertion process (Atanassova and Zamble 2005; Leach and Zamble 2007). Following complete maturation of the active site is proteolysis of an extended C-terminus present in some [NiFe]-hydrogenases by an endopeptidase (Menon et al. 1993; Menon and Robson 1994; Rossmann et al. 1994, 1995).

9 Nitrogenase Genetic Diversity

The reduction of N_2 to ammonia is catalyzed in a small number of bacteria and archaea by three nitrogenase metalloenzyme complexes that contain chemically distinct active site cofactors (Burgess and Lowe 1996; Eady 1996; Ribbe et al. 1997). The majority of present-day N_2

reduction is catalyzed by the molybdenum-dependent nitrogenase (Mo-nitrogenase or *nif*) which is present in all known diazotrophs (Burgess and Lowe 1996). Alternative forms of nitrogenase, which contains vanadium and iron (V-nitrogenase or *vnf*) or iron alone (Fe-nitrogenase or *anf*) in the active site, are found in a limited subset of diazotrophs, and their expression and activity in cultivated organisms is regulated by the availability of Mo (Bishop et al. 1980; Eady 1996; Masepohl et al. 2002; Bishop and Joerger 2003). In addition to *nif*, *vnf*, and *anf* forms of nitrogenase, several nitrogenase homologs with uncharacterized active site cluster metal content have been identified (Ribbe et al. 1997).

Mo nitrogenase exists as a two component enzyme system in which one component (Fe protein) acts as the unique electron donor to the second component (MoFe protein) which contains a heterometallic site containing the FeMo-cofactor (FeMo-co), the site of dinitrogen binding and reduction (Bulen and LeComte 1966) (Fig. 3.7). FeMo-co is comprised of 7 Fe atoms, 9 S atoms, 1 Mo atom, 1 unidentified light atom (C, N or O) and a molecule of *R*-homocitrate (Shah and Brill 1977; Hoover et al. 1987; Chan et al. 1993; Einsle et al. 2002). The ~230 kDa MoFe protein is an $\alpha_2\beta_2$ tetramer of the *nifD* and *nifK* gene products that, in addition to two FeMo-cos, contains two P clusters (Kim and Rees 1992a, b; Peters et al. 1997; Mayer et al. 1999; Einsle et al. 2002). The ~60 kDa Fe protein exists as a homodimer which contains a single [4Fe-4S] cubane and functions in MgATP hydrolysis and electron transfer to the FeMoco of the MoFe protein (Georgiadis et al. 1992; Howard and Rees 1994; Seefeldt and Dean 1997). The active site FeMo-co cluster is synthesized on a scaffold complex (NifEN, VnfEN) that is homologous to the Nif/VnfDK complex (Roll et al. 1995; Eady 1996; Fani et al. 2000; Dos Santos et al. 2004; Raymond et al. 2004; Rubio and Ludden 2005). *nif* regulons always encode for NifEN and *vnf* regulons generally encode for VnfEN. In contrast, *anf* regulons and regulons encoding for homologs with uncharacterized active site metal clusters do not encode for EN scaffold proteins (Eady 1996; Fani et al. 2000; Raymond et al. 2004; Rubio and Ludden 2005).

nif regulons have been identified in 119 bacterial genomes and 8 archaeal genomes. Whereas *nif* is widely distributed in bacteria, the distribution of *nif* in archaea is constrained to several lineages of methanogens, namely the Methanococcales, Methanobacteriales, and the Methanosarcinales (Raymond et al. 2004). Nitrogenase has yet to be identified in a eukaryotic taxon. Within the bacteria, *nif* is commonly found in the genomes of aerobes, facultative anaerobes, and strict anaerobes. For example, *nif* has been identified in a number of aerobic soil bacteria including *Azotobacter vinelandii* and a variety of cyanobacteria. Nif has been identified in the

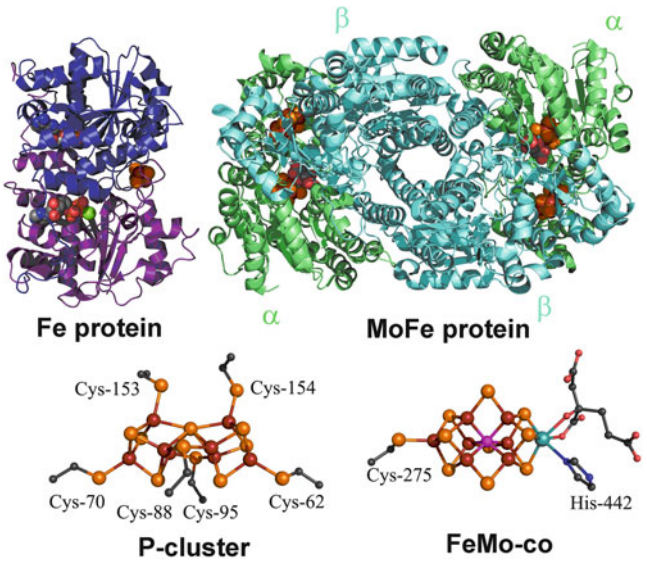

Fig. 3.7 Ribbon/space filling representation of the nitrogenase Fe (PDB code: 1FP6) and MoFe proteins (PDB code: 1M1N). The proteins are colored by subunit. The Fe protein, a dimer, is shown associated with one αβ unit of the MoFe protein which is an $\alpha_2\beta_2$ dimer. The Fe protein, shown in the MgADP bound state, contains a [4Fe-4S] cluster bridged between the two subunits and nucleotides. The αβ unit of the MoFe protein contains two complex FeS clusters, the P-cluster ([8Fe-7S]) and FeMoCo (7Fe-Mo-9S-homocitrate-X) (*shown below*). The atomic coloring scheme is identical to Fig. 3.2 with the unknown atom colored magenta

genomes of 21 of the 44 sequenced cyanobacterial genomes, including those that inhabit terrestrial environments (e.g. *Cyanothece* and *Synechococcus* strains) and in marine strains (*Crocosphaera watsonii*). In addition, *nif* regulons are common in the Firmicutes, Chloroflexi, Chlorobi, and Bacteroidetes and in several lineages of Actinobacteria and Proteobacteria.

Alternative forms of nitrogenase (*anf*/*vnf*) are also found in both bacteria and archaea, but have yet to be identified in eukaryotes. Both *anf* and *vnf* nitrogenase are present in the genomes of several members of the *Methanosarcinales*, but have yet to be identified in a genome from a member of the *Methanococcales* or *Methanobacteriales*. Anf regulons are also present in the several phylogenetically disparate bacterial genomes including a single cyanobacterial genome (*Anabaena variabilis*), a single chlorobi genome (*Chloroherpeton thalassium*), several firmicute genomes, and several proteobacterial genomes. The distribution of *vnf* regulons in bacteria is constrained to several proteobacterial and firmicute genomes, and *A. variabilis*. Nitrogenase homologs with uncharacterized metal content have been identified in the genomes of the firmicutes *Candidatus Desulforudis audaxviator* MP104C and *Caldicellulosiruptor saccharolyticus*, several unclassified methanogen genomes, and two strains of *Roseiflexus* (Chloroflexi) (Soboh et al. 2010).

10 Nitrogenase Structure and Function

Nitrogenase, as mentioned above, is a two component enzyme that functions to reduce N_2 to ammonia in a process that, under most conditions, requires the hydrolysis of 2 mol of ATP per electron transferred to substrate. The observed stoichiometry for the reaction also includes the production of 1 mol of H_2 gas for each mole of N_2 reduced to 2 mol of ammonia. All nitrogenases function as two component enzymes with one component being the Fe protein that donates electrons to a second component, which houses the sites for N_2 binding and reduction. The first component is analogous for all three systems (Mo, V, and Fe-only) and is termed the Fe protein. The Fe protein is homodimeric with a single [4Fe-4S] cluster bridging the subunits and two sites for MgATP binding and hydrolysis (Georgiadis et al. 1992). For the most prevalent and most well-characterized nitrogenases, the Mo nitrogenases, the second component, termed the MoFe protein, is heterotetrameric and contains two complex Fe sulfur clusters termed the P clusters and the FeMo-cofactors (FeMo-cos) (Kim and Rees 1992a, b; Peters et al. 1997; Mayer et al. 1999; Einsle et al. 2002). The P-clusters are located at the interface of the MoFe protein α- and β-subunits and exist as two [4Fe-4S] clusters that are fused by virtue of a shared cluster sulfide (Kim and Rees 1992a, b; Peters et al. 1997; Mayer et al. 1999). The clusters reside near the docking interface of the Fe protein and during the catalysis the two-fold symmetric axis of the Fe protein pairs with a pseudo two-fold symmetric axis that relate the MoFe protein α- and β-subunits for intramolecular electron transfer such that the P clusters function as intermediate acceptors of electron on route to the FeMo-co N_2 reduction sites. As mentioned previously, the FeMo-cos are Mo-7Fe-9S centers with an organic constituent homocitrate that binds as a chelate to Mo (Shah and Brill 1977; Hoover et al. 1987; Chan et al. 1993; Einsle et al. 2002). The structure of the FeMo-co can also be explained as an assemblage of modified [4Fe-4S] cubanes. Each FeMo-co is made up of a [Mo3Fe-3S] partial cubane bridged to a [4Fe-3S] partial cubane by three shared sulfides. The P cluster and the FeMo-co as well as the hydrogenase active site clusters have been termed "bridged biological metal assemblies" and have been intriguing challenging targets for inorganic synthesis (Lee and Holm 2004).

During catalysis the Fe protein and MoFe protein repeats a cycle of association and dissociation in a process that couples ATP hydrolysis to the transfer of individual electrons from the Fe protein to the MoFe protein (Howard and Rees 1994; Seefeldt and Dean 1997). A minimum of 8 electron transfer cycles are required for turnover and the reduction of one molecule of N_2 to produce two molecules of ammonia and 1 mol of hydrogen. The requirement for ATP is a large drain on cellular energy and the high demand for ATP required for this process is not fully understood. It is presumed that the role of ATP in nitrogenase catalysis is in the coupling of ATP binding and hydrolysis to conformational changes in the Fe protein that modulate component protein interactions and electron transfer (Howard and Rees 1994; Seefeldt and Dean 1997). The requirement for this manner of electron transfer is thought to be effective gating of electron transfer that maintains electron flow toward substrate reduction; this enables the MoFe protein to accumulate electrons and to acquire the multiple electron reduced states known to be required for the binding of N_2 (Lowe and Thorneley 1984; Thorneley and Lowe 1984; Barney et al. 2005; Howard and Rees 2006; Peters and Szilagyi 2006).

Since the first structures of the nitrogenase components were determined nearly 20 years ago (Georgiadis et al. 1992; Kim and Rees 1992a, b; Chan et al. 1993), much attention has been placed on how N_2 is bound and reduced at the active site FeMo-co metal center. Even prior to the structure determination there was some debate in the community concerning whether we should anticipate the relevant N_2 binding and reduction site to be associated more specifically with the numerous Fe sites or the Mo site. From the stand point of the many biologists that were involved in the long history of N_2 fixation research, the idea that Mo could be the important catalytic center seemed unlikely since nitrogenase is known to exist in alternative forms without Mo as a component of the active site cluster. On the other hand, some inorganic chemists championed an alternative view because a number of Mo based inorganic compounds have been synthesized that can bind and activate nitrogen (Laplaza and Cummins 1995; Yandulov and Schrock 2003; Spencer et al. 2006). Another camp of inorganic chemists was particularly intrigued, however, by the six apparent, coordinately unsaturated, trigonal Fe sites making up a prismatic structure in the core of the FeMo-co present in the first generation of crystal structures of nitrogenase (Holland 2008). Although the next generation of high resolution crystal structures reveals a central atom in the FeMo-co that serves as a fourth ligand to all six Fe atoms of the FeMo-co prismatic core (Einsle et al. 2002), there is still considerable evidence emerging that substrate binding occurs at an Fe atom (Seefeldt et al. 2004; Dos Santos et al. 2005; Barney et al. 2006). Recent detailed characterization of active site mutants, generated in the vicinity of the FeMo-co core Fe sites, have revealed that substrates are likely to bind to one of the three equivalent four Fe faces of the FeMo-co core (Kim et al. 1995; Mayer et al. 2002; Barney et al. 2004, 2005; Seefeldt et al. 2009). In addition, theoretical studies probing gas transfer channels coupled with mutagenesis and structural work indicate that substrates access this site specifically (Deeth and Field 1994; Siegbahn et al. 1998; Rod and Norskov 2000; Reiher and Hess 2002; Dance 2003).

Analysis of intermediates of natural and artificial substrates of nitrogenase by electron paramagnetic spectroscopy and electron-nuclear double resonance spectroscopy, provide strong support for substrate reduction at Fe sites and provide significant insights into potential mechanisms for N_2 activation at these sites (Hwang et al. 1973; Davis et al. 1979; Christie et al. 1996; Lee et al. 1997, 2000, 2004, 2005; Sorlie et al. 1999; Ryle et al. 2000; Barney et al. 2009; Hoffman et al. 2009).

Hydrogen evolution associated with nitrogenase activity has always been of interest and has considerable impact on the rationalization of any potential chemical mechanisms for N_2 reduction. It was recognized very early on in the biochemical characterization of the nitrogenase reaction that H_2 could be produced in the absence of other reducible substrates and hydrogen production was diminished but not eliminated in the presence of most reducible substrates (Hadfield and Bulen 1969; Winter and Burris 1976). Interestingly, acetylene as a substrate can eliminate hydrogen production but nitrogen cannot (Rivera-Ortiz and Burris 1975). Since hydrogen production in contrast to the reduction of N_2 and other substrates is not inhibited by carbon monoxide (Rivera-Ortiz and Burris 1975) it is thought that hydrogen evolution must occur at a site either physically or electronically distinct from the site of N_2 reduction. Initially, it was thought that this was potentially not an integral component of the biochemical mechanism of nitrogenase catalysis but could be attributed to a defect or leaky nature of the enzyme in directing electron flux solely to N_2 reduction. In 1984, this was probed directly by conducting nitrogenase assays under a 50 atm overpressure of nitrogenase and it was clearly shown that stoichiometry of one H_2 produced for each N_2 converted to ammonia was observed even under these conditions thus concluding this was indeed part of the overall mechanism (Simpson and Burris 1984).

The potential for photobiological hydrogen production via nitrogenase is of active interest and a number of key barriers in practical implementation have been realized (Schutz et al. 2004). In nature, the reducing power potentially lost in the form of H_2 in the nitrogenase reaction is typically recaptured by H_2 oxidation catalyzed by *hup* encoded membrane –bound uptake hydrogenases (Houchins and Hind 1984). Analysis of hydrogen uptake (*hup*) deficient mutants supports these enzymes having a specific role in recapturing reducing equivalents from the hydrogen produced by nitrogenase (Happe et al. 2000; Lindberg et al. 2002; Lindblad et al. 2002; Masukawa et al. 2002a, b). Like many hydrogenases, nitrogenase is very sensitive to O_2; however, it is associated with anaerobic, facultative anaerobic, and obligate anaerobic microorganisms. Biological N_2 fixation has been demonstrated to be associated with a number of filamentous and unicellular cyanobacteria but not in Eukaryotes (Haselkorn and Buikema 1992). The incompatibility between the O_2 sensitivity of nitrogenase and oxygenic photosynthesis is dealt with in different ways in filamentous and unicellular cyanobacteria by either spatial or temporal separation of nitrogenase catalysis from oxygenic photosynthesis. For unicellular cyanobacteria, N_2 fixation occurs only at night in microbial communities characterized by dense biomass (Omoregie et al. 2004; Steppe and Paerl 2005; Steunou et al. 2006). At night, in the absence of photosynthetic oxygen production, the high respiratory rate of these communities quickly consumes oxygen and the environment goes anoxic. Under these conditions, N_2 fixation is constrained energetically since the ATP to drive the process has to be derived from a fermentative metabolism. In contrast, the heterocyst forming cyanobacteria provide a physical barrier, or oxygen free niche much like what is provided by nodule forming legumes (Colebatch et al. 2002), as a favorable environment for N_2 fixation (Fay 1992). This compartmentalization allows the process of oxygenic photosynthesis to occur simultaneously with N_2 fixation and is the only known natural method of directly coupling reducing equivalents derived from photosynthesis to H_2 production. Because this direct coupling is possible, the H_2 production produced as a byproduct of the energy intensive process of N_2 fixation is still the most efficient mechanism for solar linked H_2 production (Hansel and Lindblad 1998).

11 Nitrogenase Maturation and FeMo-Cofactor Biosynthesis

Like the hydrogenases, nitrogenases have complex FeS cofactors and require complicated maturation machinery. Studies on nitrogenases maturation are perhaps more established than those on hydrogenase maturation and have been ongoing for approximately 30 years. Early studies involving the biochemical and spectroscopic examination of the MoFe protein revealed the presence of two types of complex FeS clusters with differing properties (for review see Burgess and Lowe 1996). Further studies revealed that one of the metal centers could be extracted intact using a combination of organic solvents and one of the metal clusters was destroyed (Shah and Brill 1977). The metal center that was extractable was found to possess Mo, Fe, and S and was initially termed the "M center" (later termed the FeMo-co) and the other FeS cluster that was destroyed during the extraction process was thereby inferred to be more protein associated and termed the "P center" which evolved to what is commonly referred to as the P cluster today.

The P-cluster, as mentioned above, is a simple assemblage of fused [4Fe-4S] clusters that is coordinated by Cys residues. In the case of this more complex fused assembly, the [4Fe-4S] clusters share a common sulfide and also share common bridging thiolate ligands such that the entire 8Fe

assembly is coordinated to the protein through six cysteine residues. Thiolates bridging Fe atoms are rare in biology and the only other example known is that observed in the [FeFe]-hydrogenases described above. The P-clusters are synthesized by the activity of NifU and NifS which are N_2 fixation specific homologs of the generalized housekeeping enzymes for FeS cluster biosynthesis IscU and IscS (Frazzon et al. 2002; Frazzon and Dean 2003; Johnson et al. 2005). These enzymes use a scaffolding process to build FeS clusters from Fe and S derived from Cys in a desulfurase reaction catalyzed by IscS and NifS. Recent work on P-cluster biosynthesis suggest that the P clusters first form as two independent [4Fe-4S] clusters and are later reductively coupled in a reaction involving the Fe protein (Hu et al. 2008; Lee et al. 2009). These results revealed an additional function for the Fe protein, which in addition to nitrogenase catalysis, is also involved in FeMo-co biosynthesis.

FeMo-cofactor biosynthesis is a complicated process and over the past 30 years there have been many insights gleaned. In many respects FeMo-co biosynthesis serves as the paradigm for complex FeS cluster and has served as a model system for experimental design in probing other systems, especially the H-cluster biosynthesis. Recent studies by the groups of Rubio and Ribbe have resulted in a fairly clear picture of the major players and the steps involved in FeMo-co biosynthesis (see reviews Hu et al. 2008; Rubio and Ludden 2008). FeMo-cofactor assembly occurs on a scaffold that has significant homology to the MoFe protein (Goodwin et al. 1998; Hu et al. 2005, 2006). The scaffold is encoded by the NifEN complex where the NifE gene product has significant homology to NifD or the α-subunit of the MoFe protein and the NifN gene product is homologous to NifK or the β-subunit (Roberts and Brill 1980; Brigle et al. 1987). Although there are now structural data available for NifEN, it can be inferred by primary sequence conservation and homology modeling, in addition to the biochemical characterization, that the NiFEN complex adopts a structure similar to NifDK and possesses what appear to be [4Fe-4S] clusters in the place of P clusters and sites for the assembly of FeMo-co in the analogous sites where FeMo-co is covalently bound in the MoFe protein (Roberts and Brill 1980; Goodwin et al. 1998; Hu et al. 2005, 2006; Corbett et al. 2006; Soboh et al. 2006; George et al. 2007). Additional accessory proteins serve to deliver various components of the FeMo-co to the NifEN scaffold for assembly and the completed FeMo-co is then transferred to a FeMo-co deficient MoFe protein for final insertion and maturation of active nitrogenase by a protein termed NafY (Rubio and Ludden 2002). The role of many of the other accessory proteins is now known and a hypothetical scheme for the entire process has been developed. In addition to the role NifS and NifU that play in P cluster biosynthesis, they are also necessary for FeMo-co biosynthesis and the current evidence points to a role for these proteins in having a role in building the FeS core of the cofactor on NifB, a radical SAM enzyme (Curatti et al. 2006; Zhao et al. 2007). Although the specific role of NifB and radical chemistry has not been revealed, it is attractive to envision NifB in having a role in generating and inserting the central interstitial atom in a FeS core intermediate. What is known is that the cofactor intermediate produced by NifB, NifB-co, exists as the 6Fe-9S core of FeMo-co lacking Mo and homocitrate (Shah et al. 1994; George et al. 2008). For cofactor assembly, NifBco is transferred to the NifEN complex by NifX (Hernandez et al. 2007). The homocitrate and Mo are provided by the activities of NifV and the nitrogenase Fe protein where NifV functions in homocitrate synthesis and the Fe protein acts in Mo insertion (Hoover et al. 1988; Rangaraj and Ludden 2002).

12 Hydrogen Production in Phototrophic Organisms

Hydrogen production in cyanobacteria was first reported over 100 years ago (Jackson and Ellms 1896), and over 70 years ago seminal experiments performed by Hans Gaffron and co-workers demonstrated hydrogenase activity in the eukaryotic alga *Scenedesmus obliquus* (Gaffron and Rubin 1942; Homann 2003; Melis and Happe 2004). Subsequently, several diverse phototrophic microorganisms have been shown to produce H_2 using low-potential electrons provided by either organic substrate fermentation or light-driven photosynthetic pathways, as illustrated for eukaryotic algae in Fig. 3.8 (Healey 1970; Kessler 1974; Weaver et al. 1980; Brand et al. 1989; Happe and Kaminski 2002; Boichenko et al. 2004; Ghirardi et al. 2007).

13 Hydrogenase Activity in Eukaryotic Phototrophs

In eukaryotic algae, *Chlamydomonas reinhardtii* has emerged as the model organism for investigating H_2 metabolism, and substantial progress has been made in elucidating the mechanisms of H_2 production, as well as in improving H_2-photoproduction activity (Melis et al. 2000; Kruse et al. 2005a, b; Kosourov and Seibert 2009). Detailed genetic and physiological studies describing several aspects of algal H_2-production are available, and several fascinating mutants linked to H_2 metabolism have been isolated. These contributions demonstrate that *C. reinhardtii* has an unprecedented repertoire of metabolic capabilities that allow adaptation to rapidly changing environmental conditions, including anoxia, which is necessary for H_2-production activity.

The metabolic flexibility of *C. reinhardtii* and other phototrophic microorganisms likely facilitates acclimation to

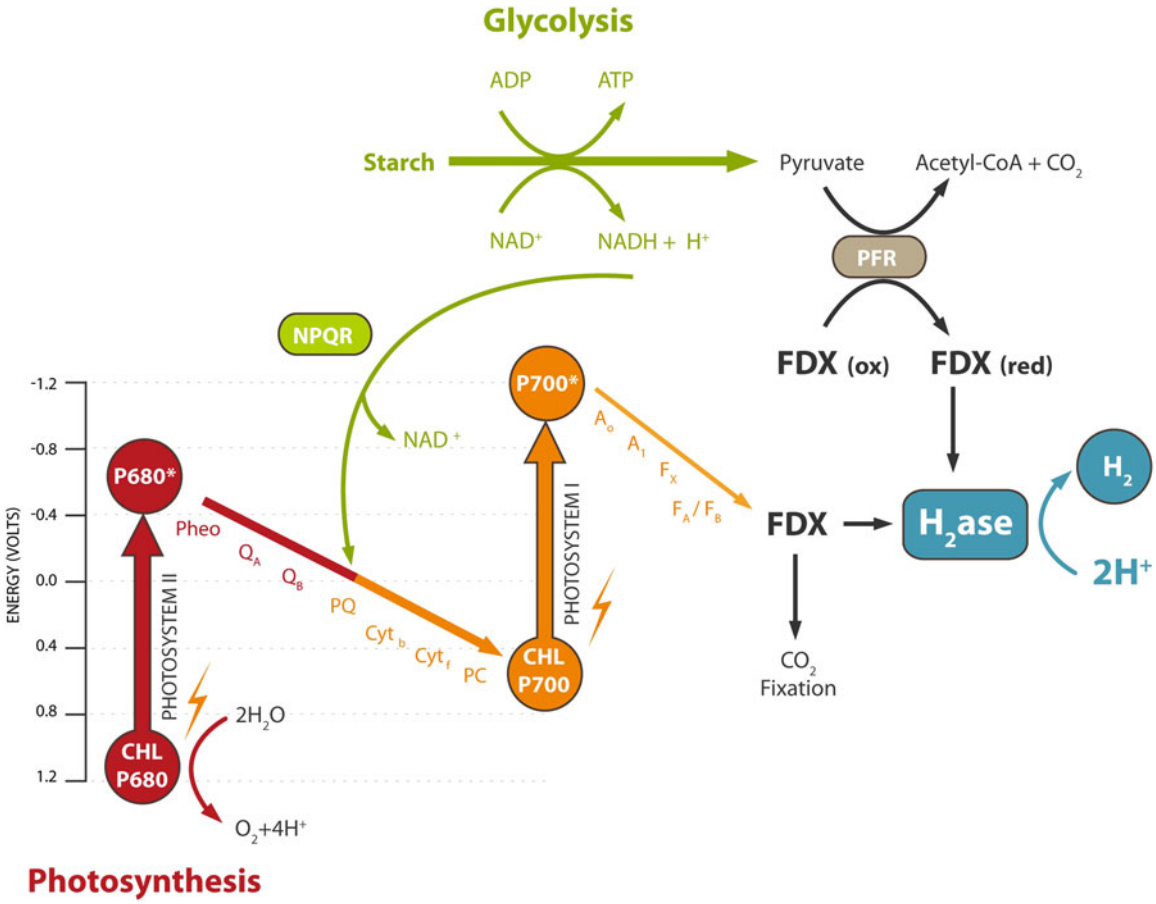

Fig. 3.8 Metabolic pathways required for the production of H_2 in algae. Two independent H_2-photoproducion pathways are known. The first relies on water oxidation by PSII and electron transfer through PSI to the hydogenase enzyme. The second relies on the non-photochemical reduction of PQ by the oxidation of organic substrates, and electron transfer mediated by PSI to hydrogenase. This later pathway can operate anaerobically under certain physiological conditions. A dark, anaerobic pathway also results in H_2 production via the oxidation of pyruvate and the reduction of hydrogenase mediated by ferredoxin

natural energetic fluxes arising from environmental conditions and diurnal light-dark cycles, allowing these organisms to readily adjust their metabolic flux in response to diverse challenges. Several proteins, including the hydrogenases, are typically found in strictly anaerobic organisms and are used in anoxic metabolism. Although the presence of fermentation pathways typically observed in anaerobic chemotrophs in an oxygenic phototroph was initially considered somewhat paradoxical, it is now apparent that photosynthetic microbes frequently experience extended periods of limited O_2 availability (Quinn et al. 2002; Steunou et al. 2006; Mus et al. 2007). The distinguishing feature in algae is that the hydrogenases can interact with the photosynthetic electron transport chain under the appropriate conditions. As nitrogenases have never been observed in eukaryotic organisms, H_2 production in eukaryotic algae relies solely on hydrogenase activity.

[FeFe]-hydrogenase gene sequences derived from green algae (Florin et al. 2001; Wunschiers et al. 2001; Happe and Kaminski 2002; Winkler et al. 2002a, b, 2004; Forestier et al. 2003) indicate that the majority of algal [FeFe]-hydrogenase genes encode small, monomeric proteins (approximately 45–50 kDa) containing only the H-cluster binding domain. However, it was recently observed that *Chlorella* NC64A possesses an [FeFe]-hydrogenase with F-cluster domains, and has both fermentative and H_2-photoproduction activities. These additional FeS F-clusters are found in most bacterial [FeFe]-hydrogenase enzymes and are putatively required for electron transport from/to electron mediators. Why the majority of algal [FeFe]-hydrogenases characterized to date lack these additional F-clusters, and the functional rationale for the evolution of these truncated [FeFe]-hydrogenases, which are currently only found only in green algae, is unresolved. Interestingly, green algae having hydrogenase activity often contain two isoforms of the [FeFe]-hydrogenase enzyme, denoted HYDA1 and HYDA2 (Winkler et al. 2004). The precise contribution of each hydrogenase enzyme in H_2 metabolism is currently unknown. A third protein with [FeFe]-hydrogenase homology is also present

in the *C. reinhardtii* genome (NCBI accession number EDP03395). However, this protein has similarity to the family of Narf-like proteins that are proposed to play a role in FeS cluster assembly in some eukaryotes, and is not believed to have hydrogenase activity (Balk et al. 2004).

The algal [FeFe]-hydrogenases contain transit peptides of variable length at the N-terminus, which are required for translocation to the chloroplast. The majority of algal enzymes sequenced to date also contain an insertion of 15–54 amino acids at the C-terminus, and a smaller insertion, approximately nine amino acids to the N-terminal side of the L1 motif. The physiological significance of these insertions is currently unknown; however, they may have roles in regulating enzyme activity, protein interactions, or cellular localization.

Anaerobiosis is required to induce hydrogenase activity in *C. reinhardtii*, which is achieved in the laboratory in a variety of ways including: (1) purging with inert gas, (2) providing exogenous reductant, and/or (3) allowing cellular respiration to metabolize dissolved O_2. Following the establishment of anaerobiosis, cultures are sealed to prevent the introduction of O_2 from the atmosphere. Cultures grown in nutrient-replete media must be maintained in the dark or at very low light levels to prevent O_2 accumulation from endogenous photosynthetic activity. Hydrogen photoproduction is observed at high initial rates after illumination of dark, anaerobically adapted cells. However, in cultures grown in a nutrient-replete medium, these initial rates of H_2 photoproduction rapidly diminish as O_2 levels from photosynthesis increase and cells adapt to an aerobic metabolism and CO_2-fixation.

13.1 Hydrogen Production Pathways

Hydrogenase activity is not observed in all genera of green algae (Brand et al. 1989; Boichenko et al. 2004; Melis and Happe 2004), and the precise role of the hydrogenases and H_2 metabolism in eukaryotic algae is not clearly resolved, but likely have diverse functions in redox balancing, photoprotection, and fermentative metabolism.

Figure 3.8 illustrates the three distinct H_2 production pathways in *C. reinhardtii*. These include two photoproduction pathways, and a dark, fermentative pathway. The direct biophotolysis H_2-photoproduction pathway is dependent on both algal photosystems (PSII and PSI), and relies on light-induced water oxidation at PSII, electron transfer from PSII to PSI, and finally light-dependent excitation of electrons by PSI to reduce ferredoxin (PETF or FDX), the physiological electron donor to *C. reinhardtii* hydrogenases (Roessler and Lien 1984; Happe and Naber 1993). The indirect biophotolysis pathway involves the non-photochemical reduction of PQ using electrons from NAD(P)H, generated primarily from the oxidation of organic substrates, followed by the light-dependent reduction of ferredoxin by PSI. This pathway is independent of PSII; however, it is dependent on NAD(P)H-plastoquinone oxidoreductase (NPQR) activity (Godde and Trebst 1980; Maione and Gibbs 1986a; Bennoun 1998; Cournac et al. 2000; Mus et al. 2005).

The PSII-independent H_2-photoproduction is not affected by 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU), which blocks electron transfer from PSII to PQ (Ben-Amotz and Gibbs 1975), but is completely inhibited by 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB), which blocks PQ oxidation by the cytb_6f complex. Inhibitor studies and genomic evidence indicate the involvement of a type II NADH dehydrogenase in non-photochemical reduction of the PQ pool in *C. reinhardtii* (Mus et al. 2005; Bernard et al. 2006). The *C. reinhardtii* genome encodes seven proteins homologous to known NDH-2 proteins (Mus et al. 2005; Jans et al. 2008) and it is likely that the recently characterized NDA2 protein is responsible for PQ pool reduction via NADH oxidation under H_2 producing conditions (Desplats et al. 2009).

The direct biophotolysis pathway contributes between 50 and 90% of the total electron flux used in H_2 photoproduction, as determined by DCMU inhibition, which has a variable impact depending on the algal strain, culturing and assay conditions, and the method of anaerobic induction (Laurinavichene et al. 2004). Both of the H_2 photoproduction pathways minimally require PSI activity (Redding et al. 1999), and are inhibited by DBMIB.

Fermentative H_2 production in the dark is coupled to starch catabolism (Gfeller and Gibbs 1984; Kreuzberg 1984; Ohta et al. 1987), and is likely analogous to H_2-producing-heterofermentation pathways described in anaerobic bacteria (Nath and Das 2004; Hallenbeck 2009), or in amitochondriate eukaryotes (Dyall et al. 2004). Pyruvate oxidation is coupled to the reduction of ferredoxin in these organisms via the enzyme pyruvate-ferredoxin-oxidoreductase (PFR), as illustrated in Fig. 3.9.

Chlamydomonas reinhardtii encodes six [2Fe-2S] ferredoxin homologs, and recent studies have begun to elucidate the physiological roles of these distinct proteins (Jacobs et al. 2009; Terauchi et al. 2009; Winkler et al. 2009). These studies indicate that PETF is the likely physiological donor to the *C. reinhardtii* hydrogenases (Winkler et al. 2009). However, it remains unclear whether dark- or light-mediated H_2-production and H_2-uptake pathways (see below) can interact with distinct ferredoxins or other physiological electron donors. These studies also demonstrate that the abundance of *FDX5* transcripts increases dramatically during anaerobic acclimation; however, its physiological role is unclear, and it is unable to reduce hydrogenase in vitro (Jacobs et al. 2009). Although no evidence exists at this point, the oxidation of

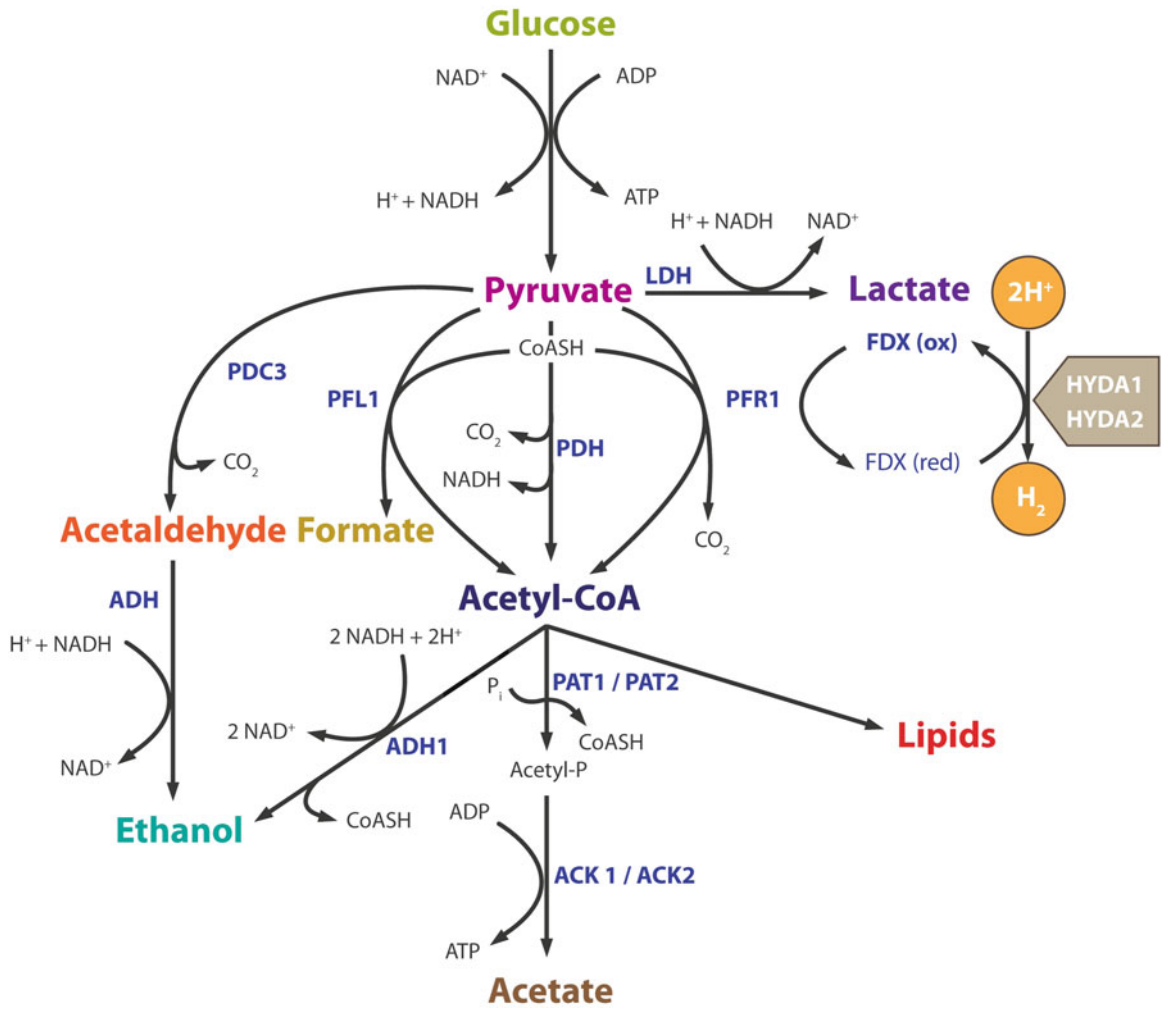

Fig. 3.9 Proposed metabolic pathways leading to the secretion of the indicated metabolites during anoxia. Pyruvate can be metabolized to acetyl-CoA via pyruvate ferredoxin oxidoreductase (PRF) yielding acetyl-CoA, reduced ferredoxin and CO_2. Alternatively, pyruvate can be cleaved by pyruvate formate lyase (PFL) yielding acetyl-CoA and formate. The [FeFe]-hydrogenases (HYDA1, HYDA2) can then be reduced by FDX, catalyzing H_2 production. Pyruvate may also be decarboxylated by the pyruvate decarboxylase pathway (PDC) resulting in the production of acetaldehyde. Ethanol may be produced from either acetyl-CoA or acetaldehyde, as indicated, oxidizing NADH for the continuation of glycolysis. Oxidation of NADH may also be acheived by the D-lactate dehydrogenase (LDH) pathway. The sequential action of acetate kinase (ACK) and phosphotransacetylase (PAT) results in the conversion of acetyl-CoA to acetate, CoA and ATP. For clarity, this illustration does not include other aspects of metabolism that could potentially influence product formation

NADH generated during glycolysis could also potentially provide reductant to hydrogenase indirectly (e.g., via NAD(P)H ferredoxin oxidoreductase activity or interacting diaphorase proteins), as proposed at high NADH concentrations in species of Clostridia (Saint-Amans et al. 2001) and amitochondriate eukaryotes (Dyall et al. 2004).

13.2 Hydrogen Utilization Pathways

Hydrogen-uptake activity is also observed in *C. reinhardtii* and two distinct pathways have been described (Gaffron and Rubin 1942; Gaffron 1944; Kessler 1974; Maione and Gibbs 1986a, b; Chen and Gibbs 1992). The first pathway, anaerobic CO_2 photoreduction, couples H_2 oxidation and cyclic PSI activity to Rubisco-mediated CO_2 fixation. This light-dependent pathway functions anaerobically in the absence of PSII mediated O_2 evolution. The oxidation of H_2 reduces ferredoxin, which then reduces ferredoxin-NADP oxidoreductase (FNR) leading to the production of the NADPH necessary for the Calvin-Benson cycle. Cyclic electron flow by PSI provides the ATP necessary for CO_2 fixation. Photoreduction can be disrupted by either DBMIB or cyanide-*p*-trifluoromethoxyphenyl hydrazone (FCCP), which uncouples electron transport from photophosphorylation.

The second H_2 uptake pathway is the oxy-hydrogen reaction, which can also be coupled to CO_2 fixation (Gaffron 1942, 1944; Kessler 1974). At O_2 levels below the inhibitory

threshold of *in vivo* hydrogenase activity, the concomitant uptake of both H_2 and O_2 is observed in the dark (Russell and Gibbs 1968; Kessler 1974; Maione and Gibbs 1986a, b; Chen and Gibbs 1992). Maione and Gibbs proposed that ferredoxin is reduced by hydrogenase after H_2 oxidation. Reduced ferredoxin then mediates reduction of the PQ pool, either directly or by the formation of NADPH followed by NADPH/PQ oxidoreductase activity (Maione and Gibbs 1986b). The chlororespiratory pathway would then reduce O_2, oxidizing the PQ pool and leading to the observed uptake of both H_2 and O_2. The coupling of the oxy-hydrogen reaction to dark CO_2 reduction has also been reported (Gaffron 1942, 1944; Russell and Gibbs 1968; Maione and Gibbs 1986b; Chen and Gibbs 1992). The source of ATP required for CO_2 reduction under these conditions is likely linked to mitochondrial respiration (Maione and Gibbs 1986a, b; Chen and Gibbs 1992), which would require the exchange of reducing power between mitochondria and the chloroplast.

13.3 Hydrogen Production During Sulfur Deprivation

To date, the most successful method for the production of volumetric quantities of H_2, is the use of sulfur deprivation. Sulfate-deprived cultures have significantly attenuated rates of photosynthetic O_2 evolution (Wykoff et al. 1998), but maintain levels of respiration that exceed the rates of photosynthetic O_2 evolution (Ghirardi et al. 2000; Melis et al. 2000). Consequently, sealed cultures become anaerobic, and sustained H_2 production is observed for several days from batch cultures (Ghirardi et al. 2000; Melis et al. 2000). The establishment of anaerobiosis induces *HYDA1* and *HYDA2* expression and the respective functional proteins can be detected after about 3–4 h (Forestier et al. 2003). Hydrogen production is detected after another 5 h, which is accompanied by a concomitant loss of Rubisco activity (Zhang et al. 2002).

As a response to sulfur stress, cellular starch levels increase approximately 8–20-fold in *C. reinhardtii* (Tsygankov et al. 2002; Zhang et al. 2002). The initial accumulation of starch appears to stimulate H_2-photoproduction yields under some conditions. However, recent studies using *C. reinhardtii* starchless mutants have demonstrated that starch is not essential for high levels of H_2-photoprodution activity (Chochois et al. 2009). Interestingly, these mutants demonstrate increased acetate utilization, which could potentially offset the use of starch to sustain the respiratory activity required to maintain anaerobiosis (Chochois et al. 2009). Although acetate is typically in the medium during sulfur deprivation experiments, it was recently shown that photoautotrophic *C. reinhardtii* cultures can produce significant quantities of H_2 under sulfur-deprived conditions. This approach required a higher pH (7.7) than normally used (7.3), and a specialized light regime that allowed high levels of starch accumulation (Tolstygina et al. 2009). Therefore, it appears that distinct carbon oxidation mechanisms are available to maintain respiratory activity at the levels necessary to maintain anaerobiosis during sulfur deprivation.

In batch cultures, H_2 production under sulfur-deprivation eventually ceases, likely due to the more generalized effect of sulfur deprivation on cell metabolism. Longer periods of H_2 can be sustained by operating algal cell suspensions in cycles of +S/−S (Ghirardi et al. 2000), or by physically separating the O_2 from the H_2-evolution stages in separate photobioreactors (Fedorov et al. 2005), which produces H_2 at low rates for approximately 6 months.

The Melis group has successfully attenuated expression of the *SULP* gene, which encodes a protein regulating sulfate uptake into the chloroplasts (Chen and Melis 2004; Chen et al. 2003). The rates of photosynthetic O_2 production and respiratory O_2 consumption (P/R ratio) (Melis and Melnicki 2006) was close to one, which allows cultures to become anaerobic even in the presence of sulfate, and to photoproduce H_2 (Chen et al. 2005).

The sulfur deprivation method was also applied to photosynthetic mutants that exhibited lower rates of O_2 evolution. D1 mutants with different levels of attenuated PSII activity were shown to undergo a faster transition to anaerobiosis upon sulfur deprivation; however, this was accompanied by low starch accumulation and reduced H_2 photoproduction (Makarova et al. 2007).

Another D1 mutant (L159I; N230Y), whose phenotype showed reduced chlorophyll content, and higher photosynthesis and respiration rates, demonstrated improved H_2 production yields relative to the wild type. Under standard conditions of sulfur deprivation, the mutant produced more than one order of magnitude more H_2 than its parental strain, and approximately five-times more H_2 than the CC124 wild-type strain under similar conditions (Torzillo et al. 2009). The better performance of the mutant was mainly the result of a longer H_2-production period, putatively a consequence of the very high conversion of violaxanthin to zeaxanthin during H_2 production, which is known to be involved in energy dissipation within the light-harvesting antenna (DemmigAdams and Adams 1996). The ability of the D1 mutant to produce large amounts of zeaxanthin may be a prerequisite for protecting PSII from rapid degradation and thus for prolonging the PSII-based H_2-production period. Moreover, the biogas produced by the mutant was almost composed almost entirely of H_2 (99.5%), while the level of CO_2 was extremely low (0.5%), indicating that in this mutant the role of PSII was primarily responsible for the providing reductant for H_2 production relative to the contribution of carbohydrate oxidation (Torzillo et al. 2009).

Immobilization of *C. reinhardtii* cells in alginate films under conditions of phosphate/sulfur deprivation demonstrate

light conversion efficiencies of approximately 1% under laboratory conditions and intriguingly, demonstrate the capacity to produce significant quantities of H_2 even in the presence of atmospheric levels of O_2 (Kosourov and Seibert 2009). Immobilized cells also demonstrate the capacity to produce H_2 for prolonged periods of time (Laurinavichene et al. 2008), and this technique shows great promise as a mechanism to manipulate culture media and prevent cell division in bioenergy strategies.

Other algal species with hydrogenase activity do not necessarily exhibit H_2-photoproduction during sulfur deprivation (Winkler et al. 2002b). Recent surveys of algae for novel strains capable of robust H_2 photoproduction during sulfur deprivation have demonstrated a strong correlation between acetate utilization and H_2 accumulation (Skjanes et al. 2008; Meuser et al. 2009). In *C. reinhardtii*, the incorporation of a glucose transporter has been shown to further stimulate H_2-photoproduction (Doebbe et al. 2007). These results continue to underscore the important linkage between carbon metabolism, and likely cellular respiration, with sustained H_2-photoproduction under conditions of sulfur deprivation.

13.4 Hydrogenase Transcriptional Regulation

Anaerobiosis induces significant increases in the transcript level of *HYDA1* and *HYDA2*, as well as numerous other fermentation genes (Posewitz et al. 2004a, b, 2005; Mus et al. 2007; Dubini et al. 2009). Although the precise regulatory mechanisms are currently unknown, anoxia has long been established as necessary for hydrogenase activity, and real-time PCR data demonstrate that the levels of hydrogenase transcripts increase over 100-fold during cellular acclimation to anoxia (Mus et al. 2007; Dubini et al. 2009). Metabolic mutants have been isolated that exhibit attenuated hydrogenase gene transcription under anaerobic conditions (Posewitz et al. 2004a, b). For example, two *C. reinhardtii* starchless mutants, *sta6* (Zabawinski et al. 2001) and *sta7* (Posewitz et al. 2004a, b), have significantly attenuated levels of hydrogenase activity, as well as decreased levels of hydrogenase transcripts under dark, anaerobic conditions (Posewitz et al. 2004a, b). This is likely a consequence of cellular redox status or intermediates of starch catabolism influencing signal-transduction events controlling the expression of the hydrogenase genes during anaerobiosis. The degradation of starch, which occurs under anaerobic conditions, can influence intracellular levels of NAD(P)H and/or the oxidation state of the plastoquinone pool, both of which have been demonstrated to regulate transcriptional processes (Escoubas et al. 1995; Rutter et al. 2001; Pfannschmidt et al. 2003). Recently, Chochois et al. (2009), demonstrated that the *C. reinhardtii* starchless mutants *sta6* and *sta7* have near wild-type levels of hydrogenase activity when anaerobically induced in the light under conditions of sulfur deprivation. This is in contrast to the situation where hydrogenase activity and transcript abundance is attenuated during dark, anaerobic acclimation (Posewitz et al. 2004a, b, 2005). Anaerobic induction in the light under conditions of sulfur deprivation allows reductant from the photosynthetic electron transport chain to alter cellular redox status, which likely accounts for the differential regulation of hydrogenase expression in the starchless mutants during anaerobiosis in the light or dark. Inhibition of PQ reduction by DCMU in the starchless mutants under sulphur deprived conditions resulted in severe attenuation of H_2 photoproduction, despite hydrogenase activity that was similar to uninhibited cells (Chochois et al. 2009). This led the authors to conclude that although reductant supply to the hydrogenase was attenuated, hydrogenase expression was unaffected and that the proton gradient established by cyclic photosynthesis may influence hydrogenase expression levels. Although the precise regulatory elements controlling hydrogenase expression in *C. reinhardtii* remain undefined, it is clear that factors other than O_2 regulate transcript abundance at some level.

13.5 Hydrogenase Oxygen Sensitivity

Algal hydrogenases characterized to date are particularly sensitive to O_2 and are irreversibly inactivated within minutes after exposure to atmospheric levels of O_2 (Abeles 1964; Erbes et al. 1978, 1979; Happe and Naber 1993; Ghirardi et al. 1997; Cohen et al. 2005a; King et al. 2006b). To maximize H_2-production yields in algae it is likely necessary to incorporate an O_2-tolerant enzyme and configure electron transfer pathways to deliver reductant to the hydrogenase during aerobic metabolism. The *C. reinhardtii* HYDA enzymes are among the most O_2-sensitive hydrogenases examined to date, and are more rapidly inactivated by O_2 than clostridal [FeFe]-hydrogenases tested under similar conditions (Cohen et al. 2005a, b; King et al. 2006a, b). It is conceivable that hydrogenases in oxygenic green algae evolved hypersensitivity to O_2 to attenuate hydrogenase activity in the presence of O_2, when the cell preferentially uses higher energy-yielding metabolic processes that require O_2 as the terminal electron acceptor.

Significant research efforts are currently aimed at engineering an [FeFe]-hydrogenase with amino acid substitutions that sterically inhibit O_2 diffusion, via putative gas channels within the enzyme structure, to the enzyme active site (Cohen et al. 2005a, b; King et al. 2006a, b). The smaller diameter of H_2 allows diffusion from the protein catalytic site through alternative paths that are not as accessible to O_2. These results suggest the possibility of preferentially restricting O_2 migration to the [FeFe]-hydrogenase active site, without significantly affecting the ability of H_2 to diffuse from the

enzyme active site. Recent studies suggest that O_2 inhibits *C. reinhardtii* HYDA1 enzyme activity by first binding to the 2Fe center of the H cluster, followed by destruction of the 4Fe4S cluster, and indicate that steric factors near the active site are likely to significantly affect the ability of O_2 to reach the H-cluster (Stripp et al. 2009). A restricted gas channel has been proposed as a possible mechanism for the observed O_2 tolerance exhibited by some H_2-sensing [NiFe]-hydrogenases, which have relatively low catalytic activity (Buhrke et al. 2005; Duche et al. 2005), and recent studies have shown that the replacement of small amino acids with methionine within a gas channel can decrease the O_2-sensitivity of a [NiFe]-hydrogenase (Dementin et al. 2009).

In addition to enzyme O_2 sensitivity, Lee and Greenbaum (2003) proposed that O_2 levels below those required to inhibit hydrogenase activity *in vivo* (ca. 0.1%) activate an undefined metabolic pathway, likely related to CO_2 fixation, that effectively competes with hydrogenase for photosynthetic reductant contributing to the observed attenuation of H_2-photoproduction activity in the presence of O_2. These data indicate that both metabolic and enzymatic O_2 sensitivities need to be overcome to maximize H_2 yields.

13.6 Hydrogenase Activity Modulation

Metabolites including acetate, CO_2, nitrate, and nitrite significantly influence H_2-photoproduction activity. Hydrogen photoproduction is clearly enhanced when *C. reinhardtii* is cultured in the presence of acetate (Lien and Pietro 1981; Bamberger et al. 1982; Gfeller and Gibbs 1984; Gibbs et al. 1986; Happe et al. 1994), or in elevated CO_2 (Semin et al. 2003). This is likely due to the increased accumulation of cellular carbohydrates that can be oxidized to provide electrons to the photosynthetic electron transport chain at the level of PQ. CO_2 fixation is able to very effectively compete with H_2 photoproduction and H_2-photoproduction activity is significantly attenuated when assayed *in vivo* in the presence of CO_2 (Cinco et al. 1993).

The reductive utilization of nitrate and nitrite also suppresses H_2 photoproduction (Aparicio et al. 1985). The mechanism by which hydrogenase activity is attenuated is unknown, but is likely the result of the ability of nitrite reductase, which reduces nitrite to ammonia, to oxidize ferredoxin (Aparicio et al. 1985). Sulfate likely provides a similar competition at the level of ferredoxin-sulfite reductase; however, detailed experiments examining this process have not been reported.

Hydrogenase activity in *C. reinhardtii* is dramatically affected by uncouplers of electrogenic phosphorylation, such as carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) or FCCP, and inhibitors of ATP synthesis, such as $NaAsO_4$. The addition of uncouplers to anaerobically adapted cells stimulates H_2 photoproduction by removal of the inhibitory effect of the proton gradient on electron transport (Lien and Pietro 1981; Bamberger et al. 1982; Happe et al. 1994; Lee and Greenbaum 2003). In the absence of uncouplers, and under conditions where ATP utilization by carbon fixation is limited, proton translocation is inhibited. This results in the acidification of the lumen, causing decreased photosynthetic activity (Lee and Greenbaum 2003). Proton ionophores are able to collapse this proton gradient, which results in increased electron flux to hydrogenase and an increase in H_2 photoproduction. Lee and Greenbaum (2003) have proposed uncoupling proton translocation from ATP synthesis as a possible mechanism to enhance H_2 photoproduction. This approach requires introducing anaerobically inducible proton channels that span the thylakoid membrane, and that are independent of ATPase activity. Hydrogen photoproduction via the PSI only pathway is also prolonged by the addition of FCCP, which indicates that this pathway is likewise limited by an electrochemical gradient across the thylakoid membranes (Cournac et al. 2002). In sum, these data clearly indicate that cellular electrochemical gradients (and therefore ATP levels) significantly influence H_2 metabolism in green algae.

It is also evident that numerous metabolic pathways, requiring activities in both the mitochondria and the chloroplast, are able to influence hydrogenase activities. The ability of these integrated processes to modulate H_2 production was highlighted by characterization of the *C. reinhardtii stm6* mutant (Schonfeld et al. 2004; Kruse et al. 2005a, b). The *stm6* mutant is inhibited in cyclic electron transport during anaerobiosis, over-accumulates starch, has a decreased number of active PSII centers, exhibits increased rates of respiration, and is capable of photoproducing H_2 at higher rates and for longer time periods than its parental background strain under sulfur-deprived conditions (Kruse et al. 2005a, b; Rupprecht et al. 2006). The *stm6* mutant contains a disruption in the nuclear-encoded Moc1 protein, which is targeted to the mitochondrion, and is involved in the assembly of the mitochondrial respiratory chain.

Hydrogen production in *stm6* was initially investigated under the hypothesis that cyclic electron transport effectively competes with hydrogenase for electrons, and that mutants locked in linear electron transport may have increased H_2-production rates. Although this is observed in the *stm6* mutant, other state transition mutants do not show enhanced levels of H_2 photoproduction (Kruse et al. 2005a, b). Therefore, the dramatic increase in hydrogenase activity in *stm6* is likely a consequence of the synergistic effects of attenuated cyclic electron transport, starch overaccumulation and reduced PSII O_2 evolution (Kruse et al. 2005a, b).

13.7 Fermentative Metabolism in *C. reinhardtii*

Based on available genome sequences and existing data, it is clear that *C. reinhardtii* and several other diverse, water-oxidizing phototrophs encode a variety of metabolic pathways that allow acclimation to hypoxia and anoxia (Hemschemeier and Happe 2005; Atteia et al. 2006; Mus et al. 2007; Dubini et al. 2009). Pyruvate is the central metabolite of glycolysis and *C. reinhardtii* encodes each of the enzymes required for the predominant pathways used in anaerobic pyruvate metabolism (Fig. 3.9). Many of these pathways are also observed in other algae and cyanobacteria and these phototrophs secrete organic acids and alcohols, as well as H_2 during anoxia, providing a rich source of organic carbon and reduced redox carriers to microbial communities.

The primary fermentation products secreted by *C. reinhardtii* during anoxia include formate, actetate, and ethanol, typically in a 2:1:1 ratio, with traces of H_2 and CO_2 also being produced (Gfeller and Gibbs 1984; Kreuzberg 1984); however, culturing and induction conditions, and the use of different laboratory strains, appear to influence subtle deviations in the product ratios (Ohta et al. 1987; Mus et al. 2007; Dubini et al. 2009). Glycerol and lactate have also been reported under certain culture conditions. The accumulation of ethanol and formate and acidification of the cellular environment by organic acids can be toxic to the cells, and product ratios are likely to reflect the need to balance ATP formation, NADH oxidation, and suppress the accumulation of potentially toxic fermentation products.

Pyruvate can be converted to acetyl-CoA by either the PFR1 or PFL1 pathways, and PFL1 is dually targeted to both the chloroplast and mitochondrion (Atteia et al. 2006); whereas the localization of PFR1 is currently unknown. Acetyl-CoA can be converted to acetate via the phosphotransacetylase (PAT) and acetate kinase (ACK) pathway. Two genes encoding each of these proteins are present in *C. reinhardtii*, with PAT1 and ACK2 being found in the mitochondrion, whereas PAT2 and ACK1, whose genes are adjacent to *HYDA2*, are putatively localized in the chloroplast (Atteia et al. 2006). The production of acetate results in substrate-level formation of ATP; however, NADH needs to be reoxidized to sustain glycolysis. Conversion of acetyl-CoA to ethanol via acetaldehyde oxidizes two NADH molecules and requires aldehyde and alcohol dehydrogenase activities. Both of these activities are likely catalyzed by the alcohol/aldedhyde dehydrogenase (ADH1) homolog (Hemschemeier and Happe 2005; Atteia et al. 2006; Mus et al. 2007; Dubini et al. 2009). Ethanol may also be formed via acetaldehyde by the pyruvate decarboxylase pathway, which oxidizes one NADH.

13.8 Genomics and Systems Biology in *C. reinhardtii*

The nearly complete *C. reinhardtii* genome sequence has provided the groundwork for experimental examination of the transcriptome, proteome, metabolome, and localisome, as well as for computational systems biology efforts and many 'omics' studies have been recently undertaken (Stauber and Hippler 2004; Bolling and Fiehn 2005; Naumann et al. 2007; May et al. 2008; Nguyen et al. 2008; Simon et al. 2008; Dubini et al. 2009; Matthew et al. 2009; Rolland et al. 2009). A better understanding of anaerobic metabolism in *C. reinhardtii* and metabolic fluxes associated with diurnal periods of light and dark will facilitate the development of a more comprehensive model of anoxic algal physiology. These studies will be required to comprehensively integrate the complex signalling, regulatory and metabolic networks that affect H_2 production and to better understand the contributions of sub-cellular compartments in overall cellular energetics.

High-density, oligonucleotide-based microarrays have provided unique insights into the genome-wide responses initiated during dark, anoxic acclimation (Mus et al. 2007; Dubini et al. 2009). As expected, several of these transcripts were associated with fermentative metabolism, and analysis of a H_2 production mutant indicates a remarkable metabolic flexibility that activates fermentative pathways not utilized in wildtype cells in the absence of hydrogenase activity (Dubini et al. 2009). Interestingly, the majority (>70%) of differentially expressed genes identified in these studies encode putative proteins of unknown function, indicating that we are just beginning to elucidate the mechanisms by which anaerobic metabolism is integrated into water-oxidizing, phototrophic microorganisms.

A more precise knowledge of the metabolic and regulatory context required for H_2 production will be necessary to understand and improve current limitations in H_2 yields. Accurate models of overall cellular metabolism will have to account for the diversity of metabolic processes available in water-oxidizing phototrophs, as well as consider metabolite fluxes in multiple cellular compartments and cross talk between organelles. Additional examination of the proteins involved in anoxic adaptation, as well as accurate localization of the proteins involved, will provide a foundation for metabolic engineering and modelling, as well as facilitate a better understanding of the metabolic fluxes occurring.

14 Hydrogen Production in Cyanobacteria

Cyanobacteria have also gained considerable attention as a means to generate biological H_2. In contrast to eukaryotic algae, cyanobacteria utilize [NiFe]-hydrogenase and/or

nitrogenase for the production of H_2. Cyanobacteria are eubacterial, oxygenic phototrophs found in almost every conceivable habitat on earth, and have distinct morphologies including unicellular, colonial, and filamentous forms. Similar to some eukaryotic algae, many species have fermentative metabolisms that are activated during anoxia/hypoxia. All cyanobacteria carry out oxygenic photosynthesis, but some species are also able to perform anoxygenic photosynthesis using sulfide as an electron donor. Moreover, as mentioned above, many cyanobacteria are able to form heterocysts and use non-photochemical PQ reduction and PSI for N_2 fixation/H_2 production (Abed et al. 2009).

The overwhelming knowledge on the diversity and physiology of cyanobacteria serves as an excellent base for exploring their applications in biotechnology. More than 500 genera have been classified, and tens of thousands of metabolically distinct strains are in culture collections, and only a comparatively small number of strains have been studied in detail. Many but not all cyanobacteria have the genes necessary for H_2 production. Available cyanobacteria genome sequences indicate that approximately half of these organisms have bidirectional hydrogenase genes (Ananyev et al. 2008). More than 14 cyanobacteria genera including *Anabaena*, *Calothrix*, *Oscillatoria*, *Cyanothece*, *Nostoc*, *Synechococcus*, *Microcystis*, *Gloeobacter*, *Aphanocapsa*, *Chroococcidiopsis* and *Microcoleus* have been shown to produce H_2 (Angermayr et al. 2009). Although numerous cyanobacteria that have been explicitly studied for their ability to make H_2, relatively little is known about the various metabolic pathways that influence H_2 rates and yields.

Cyanobacteria produce H_2 either as a by-product of N_2 fixation using nitrogenase or by a reversible NAD(P)H-dependent [NiFe]-hydrogenase. Cyanobacteria fix N_2 anaerobically in heterocysts, or by temporal separation of nitrogenase activity during light/dark cycles. As mentioned above, the reduction of N_2 to NH_4^+ leads to the formation of H_2 as a byproduct, with different stoichiometries depending on the type of nitrogenase (Prince and Kheshgi 2005). Because nitrogenase requires the input of at least two molecules of ATP per electron, the overall efficiency for H_2-production is rather low, and in the case of H_2-photoproduction, requires additional photon inputs to generate the necessary ATP (Prince and Kheshgi 2005). In most N_2-fixing cells, evolved H_2 is rapidly consumed by an uptake [NiFe]-hydrogenase. As most of the H_2 produced via nitrogenase is reoxidized by the uptake hydrogenase, little net production is detected, and as expected, the deletion of the uptake hydrogenase dramatically improves H_2-photoproduction yields.

Most H_2-producing cyanobacteria contain the NAD(P)H-dependent [NiFe]-bidirectional hydrogenase which exists in both N_2- and non-N_2-fixing strains. Since the activity of the bidirectional hydrogenase is not directly dependent on ATP, use of this enzyme may be energetically more efficient than the use of nitrogenase. Under anaerobic conditions, it can either produce and/or oxidize significant amounts of H_2. The bidirectional hydrogenase is encoded by the *hoxEFUYH* operon, forming a hydrogenase heterodimer (HoxYH) and an electron-transfer partner protein (HoxEFU) used in NADH oxidation/reduction. Unlike nitrogenase, the bidirectional hydrogenase is not specifically localized in an anoxic environment, and operates close to chemical equilibrium, which inhibits H_2 production above a certain H_2 partial pressure. Therefore, continuous removal of both O_2 and H_2 is necessary for sustained activity (Magnuson et al. 2009).

Similar to the case in eukaryotic algae, environmental conditions such as light, temperature, salinity, nutrients and their availability, gaseous atmosphere play significant roles in H_2 production. In particular, light energy supply, CO_2 enrichment, nutrient availability and supply, mixing, temperature control, pH, and shear stress play significant roles in the accumulation of H_2 (Yoon et al. 2008). Consequently, detailed studies of the pathways and regulation of H_2 metabolism are required to accomplish efficient genetic and metabolic modifications to improve H_2 production.

Several studies have focused on species amenable to large-scale cultivation including *Spirulina* and *Anabaena* spp., which have been cultured in a variety of bioreactors that included vertical column reactor, tubular type and flat panel photobioreactor. In particular, the *Arthrospira* spp. are robust cyanobacteria that grow to high cell densities at high rates.

Arthrospira (Spirulina) maxima is a species of filamentous cyanobacterium that thrives under extreme environmental conditions in alkaline soda lakes (pH 9.5–11) at high concentrations of sodium (0.4–1.4 M). Noninvasive fluorescence measurements on intact filaments show that *A. maxima* possesses high quantum efficiency of PSII and the fastest *in vivo* water-oxidizing complex of all oxygenic phototrophs examined to date (five-fold faster than algae and plants and even some other cyanobacteria) (Ananyev and Dismukes 2005). *A. maxima* filaments have no heterocysts and do not fix N_2 and are able to produce H_2 only by the reversible [NiFe]-hydrogenase. *Arthrospira maxima* is an attractive candidate to serve as a cell factory for large-scale H_2 production because (a) *Arthrospira* species have high biomass productivity and technology has been developed for mass production; (b) it propagates in the wild with minimal parasitic microbial contamination, due to its ecological niche in concentrated carbonate alkaline media; and (c) its long filaments are buoyant and amenable to easy harvesting and media transfer.

By growing cells in nickel-sufficient medium via a two-stage illumination process involving rapid growth in high light (actually ca. 25% of full solar intensity) followed by subsequent low-light conditions, it is possible to obtain cultures with significantly higher H_2-production yields (Ananyev et al. 2008). Conditioned cells concentrated to densities of 15.1 g (dry weight) L^{-1} and placed under dark anaerobic

conditions produced as much as 36 mL H_2 g^{-1} (initial dry weight) (or 550 mL of $H_2 L^{-1}$) in 10 days, corresponding to 10% H_2 in the headspace. In some experiments concentrated cell suspensions produce as much as 18% headspace H_2.

Overall, the observations obtained in this study lead the authors to four main conclusions: (a) anaerobic H_2 production in the dark by nondiazotrophic cyanobacteria is linked primarily to the fermentative production of NADH; (b) H_2 evolution is a major pathway for energy production during fermentation, and responds to environmental and nutritional perturbations in a predictable manner that is consistent with the role of hydrogenase as a terminal electron acceptor during anoxia enabling hydrogenase to regenerate the NAD^+ necessary to maintain glycolysis; (c) nitrate is a significant electron sink for intracellular reducing equivalents that compete with H_2 evolution, and the removal of nitrate from fermenting cells leads to a large increase in H_2 evolution and; (d) physiological and nutritional conditions that are optimal for photoautotrophic growth are often non-optimal for H_2 evolution; notably, factors that increase ATP production (sodium extrusion to maintain osmotic balance) and elevate intracellular reduction potential (NADH/NAD ratio) favor H_2 evolution (Ananyev et al. 2008). The accumulation of stored carbohydrates for fermentation is clearly the main approach for optimizing dark H_2 yields, together with conditions that optimize cell growth and then conditioning for reliance on fermentation, which are equally important for cyanobacterial H_2 production.

Another organism that is able to produce significant amounts of H_2 is *Anabaena variabilis*, which also has a completed genome sequence. Several mutants of interest are available and H_2 production has been assayed under different irradiances and CO_2 concentrations. In a recent study, several parameters, including illumination, gas exchanges, pH, and nutrient sources, necessary for optimizing H_2 production were elucidated. In particular, the results indicate that a low or high intensity of light inhibited cell growth, and the optimum light intensity of 100–110 μmol photons m^{-2} s^{-1} PAR was derived. Interestingly, cell productivity increases by adjusting the light intensity rather than using constant intensity illumination.

In regards to gas exchange, high superficial gas velocities are desirable in cyanobacterial cultures to create a high degree of turbulence, allowing a fast circulation of the cells from the dark zone to the light zone of the photobioreactor. Because the average shear rate in a bubble column is proportional to the superficial gas velocity, cyanobacterial cells could be damaged and optimizing superficial gas velocity is necessary. High superficial gas velocities were shown to inhibit cell growth; whereas, maximal cell densities (0.79 g L^{-1}) and productivities (0.26 g L^{-1} day^{-1}) were obtained at intermediate superficial gas velocities.

Culture pH is also critical for the cell growth, and varies rapidly during cell culturing. It was shown that specific doubling times in the exponential growth phase were 1.14, 1.25, 1.27, and 1.22 day at pH 6.0, 6.5, 7.0, and 7.3, respectively. In this system, CO_2 was used as a carbon source, which can act as both a weak acid and buffer. Therefore, by appropriately titrating CO_2, pH is controlled and the required carbon source is supplied.

14.1 Intrinsic Factors Studies

In conjunction with examination of the nutritional and media components necessary to optimize H_2 production, several studies have focused on cyanobacterial hydrogenase and nitrogenase enzymes to understand the unique factors controlling expression, maturation and regulation in cyanobacteria. In the last few years, transcription factors directly involved in the regulation of cyanobacterial hydrogenases have been identified with *ntc*A and *lex*A being implicated in some species in the transcriptional regulation of the uptake and the bidirectional enzyme, respectively (Tamagnini et al. 2007). In cyanobacteria, the pleiotropic (*hyp*) [NiFe]-hydrogenase maturases, as well as genes encoding specific endopeptidases (*hup*W and *hox*W) have also been identified.

Cyanobacterial H_2 production can be significantly stimulated by modifying selected pathways or genes. An obvious step in the design of a H_2 evolving strain is to engineer a mutant without the capacity to recycle H_2, and disruption of the genes coding uptake hydrogenase has been shown to improve H_2 production in strains encoding an uptake hydrogenase. This was first achieved using chemical mutagenesis and later, by the targeted knock-out of structural or maturation genes. Uptake hydrogenase-deficient mutants of *A. variabilis* have been shown to be significantly better H_2 producers (from two to six fold) compared with their respective wild types (Happe et al. 2000; Lindblad et al. 2002). Uptake and bidirectional hydrogenase knockouts in N_2-fixing cyanobacteria also produced H_2 at a rate four to seven times that of wildtype in short-term assays (Masukawa et al. 2002a, b). In another study, *Nostoc PCC* 7422 was identified as a promising H_2 production strain with high *in vivo* nitrogenase activity in the presence of O_2 when the hydrogenase uptake gene was disrupted (Yoshino et al. 2007).

Attempts have been made to introduce less O_2-sensitive hydrogenases into cyanobacteria, including a hydrogenase from the purple-sulfur photosynthetic bacterium *T. roseopersicina* into a *Synechococcus* strain (Kovacs et al. 2005). In addition, several other putative O_2-tolerant [NiFe]-hydrogenases have been identified from the marine environment that could be alternative candidates to be introduced into a cyanobacterial background (Maroti et al. 2009). Moreover,

genes encoding the more O_2-tolerant [NiFe]-hydrogenase of the purple non-sulfur photosynthetic bacterium *Rubrivivax gelatinosus* CBS and its accessory proteins, are being introduced into *Synechocystis* (Ghirardi et al. 2005).

An elegant strategy for the creation of more efficient H_2 producers would be the expression of a more efficient non-cyanobacterial hydrogenases, such as an [FeFe]-hydrogenases with higher turnover rates, in the anaerobic environment of the heterocysts of a filamentous cyanobacterium, and this strategy is likely to be realized in the near future.

15 Engineering Approaches for Improved H_2 Production

Several avenues exist to improve renewable H_2 yields from algae, and theoretical yield calculations relative to current H_2 yields demonstrate that significant improvements are required. Direct biophotolysis is one of the most aggressively pursued approaches; however, yield improvements will require (a) an O_2-tolerant enzyme, (b) engineering photosynthetic pathways that override competition with CO_2 fixation, and (c) increased light utilization. Simplistic direct biophotolysis calculations in which 800 μmol m^{-2} s^{-1} of photosynthetically active radiation (PAR) can be harvested by algae and used to provide electrons to hydrogenase during 12 h of sunlight each day indicate that, at a maximum, 12,614 mol of PAR and therefore 6,307 mol of reduced ferredoxin at the acceptor side of PSI could be produced per square meter each year, resulting in 3,154 mol of H_2. This represents 6.4 kg of H_2, with 1 kg of H_2 being equivalent to approximately 1 gal (3.785 L) of gasoline. As the H_2 produced is a gas this maximum yield represents 77,125 L of H_2 gas m^{-2} $year^{-1}$ (211 l of H_2 gas m^{-2} day^{-1}) at atmospheric pressure, far below current yields. Moreover, effective techniques for separating O_2 and H_2 must be developed to prevent explosive gas mixtures from accumulating.

Current efforts to incorporate an O_2-tolerant enzyme in water-oxidizing phototrophs are focused on manipulating gas channels in hydrogenases as mentioned above, or incorporating hydrogenases that are inherently more O_2 tolerant. Although significantly improved H_2 yields resulting from the heterologous expression and metabolic integration of these enzymes in algae or cyanobacteria have not emerged, significant progress has been made in characterizing the mechanisms of O_2 sensitivity and in the discovery of less O_2-sensitive enzymes.

Some hydrogenases are intrinsically less O_2-sensitive. These include the soluble NAD-dependent hydrogenase of *R. eutropha* (Buhrke et al. 2005; Burgdorf et al. 2005), and the group 4 *Rubrivivax gelatinosus* [NiFe]-hydrogenase (Maness et al. 2002).

Recent reports indicate an unusual O_2 tolerance in *Thermotoga neapolitana* for H_2 production catalyzed by an [FeFe]-hydrogenase compared with other microorganisms belonging to the same order, among which is *Thermotoga maritima,* which contains a well characterized trimeric [FeFe]-hydrogenase (Verhagen et al. 1999; Vignais et al. 2001). In these studies, *T. neapolitana* showed H_2 production (25–30% v/v) in microaerobic conditions (i.e., 6–12% of O_2 in the gaseous phase of the batch reactor) (Van Ooteghem et al. 2002, 2004). Curiously, both *T. neapolitana* and *T. maritima* share a very similar gene operon structure and hydrogenase gene similarity (85–91%). In an *in silico* study, it was shown that the subtle differences between these two enzymes may be responsible for the increased O_2 resistance in *T. neapolitana*. This could be represented by two substitutions (E475S and T539L), both placed on the two main entrances to the hydrophobic channel, which confer less charged and more hydrophobic channel entrances. This may represent a mechanism to potentially increase O_2 tolerance and facilitate engineering other [FeFe]-hydrogenases to achieve more efficient bio-H_2 production (Tosatto et al. 2008).

Lastly, techniques for generating random hydrogenase libraries (Nagy et al. 2007) and highly specific PCR primers for amplifying hydrogenase genes from environmental samples have been developed (Xing et al. 2008; Beer et al. 2009; Boyd et al. 2009), and these approaches can be used to screen and isolate novel enzymes.

Experiments have also been undertaken to diminish reductant competition with CO_2 fixation. Hemschemeier et al. (2008) demonstrated that a Rubisco deficient mutant could produce H_2 when anaerobically adapted in the light without S deprivation, illustrating a proof of concept that when CO_2 fixation pathways are abolished, electrons can be effectively diverted to hydrogenase. It should be noted that these results were not demonstrated in the analysis of another Rubisco mutant (White and Melis 2006), but this strain contained only a partial gene disruption. Although the mutant used by Happe and co-workers is capable of H_2 photoproduction without sulfur deprivation, heterotrophic growth is required. The starchless mutants represent another approach in which biomass can be generated photosynthetically, but a deletion in genes essential for starch, the main carbon storage product in *C. reinhardtii*, should therefore restrict reductant flux to CO_2 fixation pathways, and perhaps improve H_2 production.

Attempts are also underway to directly covalently tether hydrogenase to components of PSI as a potential mechanism of increasing electron flow to the hydrogenase (Ihara et al. 2006; Lubner et al. 2010). It is also conceivable that a similar approach may work at PSII, which would then require a single photosystem for H_2 production potentially improving the photon requirement for H_2 production two-fold, as excitation of PSI would no longer be required.

Another critical aspect diminishing H_2-photoproduction yields is light utilization by the photosystems of algae and cyanobacteria. Light intensities reach 2,500 μmol photons m^{-2} s^{-1} during midday and water-oxidizing phototrophs with natural antenna systems are unable to effectively utilize this density of photons, significantly diminishing H_2-photoproduction yields (Melis 2009). Attempts to improve photosynthetic efficiency by reducing chlorophyll antenna size or by lowering the number of light harvesting complexes have been realized (Nakajima and Ueda 2000; Nakajima et al. 2001; Lee et al. 2002; Polle et al. 2002, 2003; Mussgnug et al. 2007; Tetali et al. 2007; Beckmann et al. 2009), and an excellent review on the topic by Melis is available (Melis 2009).

In cyanobacteria, a phycocyanin-deficient mutant PD1 of *Synechocystis* PCC 6714, generated by chemical mutagenesis, showed up to 50% higher maximal photosynthesis activity under high light conditions compared with the wild type. The combination of appropriate antenna mutants and optimized environmental conditions (growth media composition, light quality, and quantity) considerably upregulates linear electron transport, which in turn is a prerequisite for achieving high photosynthesis-based H_2-production yields (Bernat et al. 2009).

16 Glucose Oxidation for H_2 Production

Water-oxidizing phototrophs may also be used to produce saccharides that can be used in a variety of oxidative strategies to produce H_2. This could theoretically be achieved via endogenous fermentation or by using algal sugars as substrates in classical bacterial-type fermentations, which are capable of yielding over 2 L of H_2 per liter of culture per hour (Ghirardi et al. 2009). As previously described, anaerobic fermentation produces H_2 as well as organic acids and alcohols. The maximum theoretical stoichiometric H_2 yield from endogenous microbial fermentation pathways is four per glucose (two from pyruvate-ferredoxin oxidoreductase and two from glycolytic NADH), termed the Thauer limit (Thauer et al. 1977). However, recent developments have shown that acetate can be used in microbial fuel cells (MFCs) to generate four additional H_2 molecules per acetate which could potentially push yields closer to the theoretical maximum of 12 H_2/glucose (Woodward et al. 2000; Zhang et al. 2007).

With the help of fermentative microorganisms, H_2 can be obtained from virtually any source of biodegradable organic matter, including human, animal and food-processing wastewaters (Logan 2009) and corn stover waste (Ren et al. 2009). Some species known to produce H_2 come from the genus *Enterobacter*, *Clostridium* (Kim et al. 2008), and *Bacillus* (Kalia et al. 1994). However, mixed cultures isolated from natural environments are more feasible (Hawkes et al. 2002) because they are able to use a complex mixture of organic matter and are less likely to become contaminated with various H_2 consumers such as methane producing bacteria and sulfate-reducing bacteria (Noike and Mizuno 2000).

Fermentation of hexose to acetate or butyrate produces H_2 and CO_2 (Eqs. 3.1 and 3.2) while fermentation to propionate or lactate does not produce H_2 (Hawkes et al. 2002). Therefore, metabolisms resulting in acetate, producing 4 H_2, or butyrate, producing 2 H_2, are desired (Hawkes et al. 2002).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 4H_2 + 2CO_2 + 2C_2H_4O_2 \tag{3.1}$$

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2CO_2 + C_4H_8O_2 \tag{3.2}$$

Current fermentation techniques produce a maximum of 2–3 mol H_2/mol glucose (Kalia and Joshi 1995; Liu et al. 2005). Additionally, H_2 can be produced by the secondary fermentation of the initial fermentation products like acetate. This acetate can be used by exoelectrogenic bacteria as a source of electrons that can then be used to reduce protons to H_2 (Eqs. 3.3 and 3.4) with the help of an MFC.

$$C_2H_4O_2 + 2\,H_2O \rightarrow 2\,CO_2 + 8\,e^- + 8\,H^+ \tag{3.3}$$

$$8\,e^- + 8\,H^+ \rightarrow 4\,H_2 \tag{3.4}$$

In an MFC organic matter is oxidized in an anaerobic anode chamber by microorganisms that can transfer their electrons to the anode while releasing protons into solution (Liu et al. 2005; Logan 2009). The anode and cathode are separated by a proton exchange membrane (PEM), which allows protons to cross into the cathode chamber. There are two possible outcomes depending on the setup of the cathode chamber. Firstly, in an aerobic cathode chamber, electrons and protons can combine with O_2 to form water (Liu et al. 2005). This, however, limits the production of H_2 to 4 mol for each mole of glucose (Liu et al. 2005). There is also an abundance of the fermentation product acetate left over. Acetate can theoretically yield 4 mol of H_2 but instead is degraded to CO_2, protons and electrons by the microorganisms in an MFC. However, if the cathode chamber is made anaerobic, the second outcome is the combination of electrons and protons to produce additional H_2. In order to reduce the protons to H_2, an added potential of 410 mV is needed while a typical MFC can only produce around 300 mV (Liu et al. 2004; Logan and Liu 2004). Because of this, a small current is required in addition to that produced by the microbes in the MFC. Theoretically, 12 H_2 can then be produced from the complete oxidation of glucose; however, this has yet to be achieved. This modified microbial fuel cell is then called a microbial electrolysis cell (MEC). Despite recent increases in MFC power production of six orders of magnitude between

1999 and 2006 (Logan 2009), we have yet to reach a financially feasible system for production of bio-hydrogen. However, this technology may soon become feasible for wastewater treatment with the added bonus of H_2 and electrical current generation (Du et al. 2007).

Efforts have also demonstrated that an oxidative pentose phosphate pathway (PPP) can be constructed *in vitro* for the complete oxidation of glucose to produce nearly 12 H_2 (Woodward et al. 1996; Zhang et al. 2007; Ye et al. 2009). Lastly, it has been proposed that NADH from citric acid cycle (CAC) reactions could be oxidized by hydrogenase enzymes allowing the theoretical maximum to be approached (Nath and Das 2004). Although extensive utilization of the PPP or CAC pathways for H_2 production has not been demonstrated within an organism, it is conceivable that metabolic engineering, or *in vitro* systems could be configured to generate H_2 at stoichiometries above the Thauer limit.

17 Outlook

The primary barriers to optimizing H_2 photoproduction from water-oxidizing phototrophic organisms include (a) photon utilization efficiencies, (b) competition for reductant by CO_2 fixation pathways, (c) a hydrogenase enzyme that can efficiently couple to the photosynthetic electron transport chain and remain active under high concentrations of O_2, and (e) the accumulation of ATP during H_2 photoproduction; whilst the primary barrier to fermentative H_2 production from glucose is the ability to approach the theoretical maximum of $12H_2$ per glucose in an economically viable manner.

As described in this review, progress is being achieved in each of these areas and it is likely that continued research efforts with provide breakthroughs in the current bottlenecks. The development of heterologous expression systems for hydrogenases, hydrogenase specific PCR primers, a mechanistic understanding of enzyme structure/function, and effective screening techniques will dramatically accelerate the pace at which suitable enzymes can be discovered and evolved into H_2-production applications. Advanced 'omics' efforts and rapidly evolving genetic techniques will allow pathway engineering to progress at an unprecedented rate, and an advanced understanding of photosynthetic processes will allow rational approaches to be developed for improved H_2-production yields. Lastly, novel fermentation approaches that can be applied *in vivo* or *in vitro* are already improving H_2 yields from glucose and these efforts will likely make continued advances. Sustained worldwide research efforts are making effective progress in all of the current bottlenecks limiting the development of cost-effective biological H_2 production, and ongoing efforts will likely continue to sustain this momentum.

Acknowledgments The authors would like to thank the U.S. Air Force Office of Scientific Research (MURI award FA9550-05-01-0365); the U.S. Department of Energy's Office of Science BES and BER programs, the Hydrogen, Fuel Cell and Infrastructure Technologies Program; and the NSF (award 0328187) for support.

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Species and Strain Selection

4

Michael A. Borowitzka

1 Introduction

An estimated 35,000 species of microalgae have been described to date, and it is estimated that the actual number of species is significantly higher. Almost all algal Phyla have microalgal representatives and microalgae can be found in most environments on earth. This evolutionary extremely diverse assemblage of (predominantly) photoautotrophic microorganisms represents an exceptionally rich resource for bioprospecting for species with particular biochemistries and a range of commercially exploitable organic metabolites (Borowitzka 1992a) and bioactive compounds (Patterson et al. 1994), as well as other applications such as wastewater treatment (Oswald 1988) and metabolic conversions (e.g., Pollio et al. 1994). For example, the halophilic green alga *Dunaliella salina* is the main source of natural β-carotene (Borowitzka 2010b), and the freshwater alga *Haematococcus pluvialis* is cultured for the production of astaxanthin for use in the nutraceutical and pharmaceutical industries (Cysewski and Lorenz 2004). Microalgae are also cultured as commercial sources of some long-chain polyunsaturated fatty acids (Sukenik 1999), phycobilin pigments for use a fluorescent markers in research and medicine, and for use in cosmetics and cosmaceuticals (Kronick and Grossman 1983), etc.

Microalgae are of particular interest for the production of renewable biofuels as they contain lipids (mainly triacylglycerols) and/or a range of sugars as storage compounds. Potential biofuels from algae include biodiesel from algal lipids, bioethanol or biobutanol from algal sugars, long-chain hydrocarbons, hydrogen, methane, and crude oils from the pyrolysis of algal biomass (Benemann 2000; Demirbas 2006; Chisti 2007; Lee 2008; Torzillo et al. 2009; Borowitzka 2010a).

The first, and critical, step in developing a reliable and commercially viable process of producing microalgae for the production of biofuels, or any other product, is the selection of the algae species and strain to be grown (see Table 4.1 for definitions). Although culture collections are an easily accessible source of these algae only a relatively small proportion of the total number of microalgae species occurring in nature can be found in algal culture collections (see Appendix for a list of major culture collections), and many more species and strains remain to be isolated from nature.

The isolation and screening of new microalgae strains with potential for biofuels production is not new. For example, the Solar Energy Research Laboratory (SERI) in the USA commenced an extensive isolation and screening programme of microalgae from saline habitats in 1983 and carried out extensive and detailed studies of many of the strains isolated (Barclay et al. 1985; Sheehan et al. 1998). They found that significant improvements in productivity can be achieved by selection of the correct strain (e.g., Barclay et al. 1987). Similarly, the importance of selecting the best strains(s) also has been demonstrated in commercial algae production (e.g., Belay 1997).

There are many methods isolating microalgae into unialgal culture and these methods include single cell picking, plating on solid media, sequential dilutions, and flow cytometry. Multiparametric flow cytometry looks like a new and particularly powerful method (Sinigalliano et al. 2009). Details of traditional methods for isolating microalgae into culture form field samples can be found in Andersen and Kawachi (2005) and the newer automated methods in Sieracki et al. (2005).

2 Species and Strain Characteristics

For any commercial microalgae process the selected species and strain must demonstrate high productivities of the target product when grown at commercial scale in the proposed culture system. For commercial-scale biofuels production this means that high productivity of the desired biofuel feedstock such as lipid (Griffiths and Harrison 2009) is an

M.A. Borowitzka (✉)
Algae R&D Centre, School of Biological Sciences,
Murdoch University, Murdoch, WA 6150, Australia
e-mail: M.borowitzka@murdoch.edu.au

M.A. Borowitzka and N.R. Moheimani (eds.), *Algae for Biofuels and Energy*, Developments in Applied Phycology 5,
DOI 10.1007/978-94-007-5479-9_4,

Table 4.1 Definition of terms used in this chapter

Term	Definition
Species	The lowest taxonomic rank of algae having common characteristics and (usually) capable of mating with one another and named according to the International Code of Botanical Nomenclature (now named The International Code of Nomenclature for Algae, Fungi, and Plants) Knapp et al. (2011).
Strain	A unialgal culture of defined origin which is kept as a distinct 'lineage' by serial transfer or continuous culture
Isolate	A strain which arises from a single individual (cell)
Clone	An isolate which is propagated exclusively through asexual reproduction

essential characteristic; lipid productivity (usually expressed as g lipid·L^{-1}·day^{-1} or g·lipid m^{-2}·day^{-1}) is defined as:

$$Lipid\ Productivity = \mu Q \qquad (4.1)$$

where μ is the specific growth rate (day^{-1}), Q is the quantity of the algal product per unit volume (L) or pond area (m^2).

This equation shows that for high productivity a high cell content of the desired product, such as triglycerides for biodiesel is, in itself not sufficient unless it is also accompanied by an adequate growth rate. For example, it has been known for a long time that many algae may have a high lipid content in stationary phase when nutrient limited; i.e. especially N limitation for most algae and Si limitation for diatoms (Aach 1952; Taguchi et al. 1987; Roessler 1988, 1990; Li et al. 2008); but the actual lipid productivity (g lipid·L^{-1}·day^{-1}) is low because of a slow growth rate. Therefore, the algae to be selected should have a high growth rate and a high lipid content (= high lipid productivity – see Eq. 4.1) during active (exponential) growth. Screening for algae for a high lipid content while in stationary phase is not appropriate, and screening for lipid content alone is not sufficient. The paper by Griffiths and Harrison (2009) discusses this important concept in some detail and reviews the recent literature on lipid productivity in microalgae. Unfortunately there still are some papers which confuse lipid content or yield with lipid productivity (e.g., Abou-Shanab et al. 2011).

The screening of the algae strains for lipid productivity not only requires the algae to be grown under the light, temperature, and nutrient conditions that would be experienced in the large-scale production culture system, but also requires the lipid content (in the case of algae for biodiesel) to be determined. The standard method for total lipid determination is gravimetric using solvent extraction and weighing according to the Bligh and Dyer method (Bligh and Dyer 1959; Kates and Volcani 1966) or the Folch method (Folch et al. 1951).[1] These methods require at least 10–15 mg of wet algal biomass for extraction and are relatively labour and time intensive. The Bligh & Dyer and Folch methods also not only extract neutral lipids such as triacylglycerides, but also extract all lipids including glycolipids, phospholipids and sterols, which are generally not suitable for biodiesel production using standard transesterification methods. For more rapid screening of the neutral lipid content the Nile Red fluorescence method can be used (Cooksey et al. 1987). However, this method may underestimate the neutral lipid content because of limited penetration of the Nile Red dye into the cells in many species, or due to high background fluorescence from chlorophyll. Several improvements to the Nile Red staining method have been made to overcome these difficulties (Chen et al. 2009; Doan and Obbard 2011). Recently new rapid and potentially high throughput methods for determining lipid content using Nile Red have been developed (Chen et al. 2009; Huang et al. 2009), but it remains to be determined how effective these methods are with a much wider range of species as the staining ability of Nile Red is affected by a wide range of factors, especially by the ability of the stain to penetrate into the cell and this is, to some degree, species-specific. An alternative to Nile Red is the lipophilic fluorescent dye BIODIPY 505/515 (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene) (Cooper et al. 2010; Mou et al. 2012) which stains lipid bodies green and chloroplasts red.

Another possible method for lipid detection is the colorimetric sulpho-phospho-vanillin method (SPV) originally developed by Chabrol and Charonnet (1937) and refined by Johnson et al. (1977). Cheng et al. (2011) have developed a rapid variant of this method using microplates, which worked well to determine the lipid content of *Chlorella* and could potentially be used for high-through-put screening. A quantitative high-throughput column-based method for extraction of triacylglycerols (TAGs) has also been developed by Tonon et al. (2002).

Raman spectroscopy also can be used to rapid screening method for the composition of microalgae (Heraud et al. 2007) and has been used to determine the degree of unsaturation of the lipids *in vivo* (Samek et al. 2010; Wu et al. 2011). Fourier Transform Infrared Spectroscopy (FTIR) (Stehfest et al. 2005; Dean et al. 2010). and Near Infra Red (NIR) (Laurens and Wolfrum 2011) methods for rapid screening of lipid content and high-resolution magic angle spinning

[1] Detailes of basic analytical methods can be found in Chapter 16 of this book.

(HR-MAS) magnetic resonance spectroscopy for whole cell profiling (Chauton et al. 2003; Merkley and Syvitski 2012) are also being used.

Although a high lipid productivity is an important property, it is not in itself sufficient for the alga to be attractive for potential commercial production, and there are many other characteristics of the algae strain which are necessary and/or desirable to improve the reliability of the cultures and the economics of the whole production process. These characteristics are discussed below.

2.1 Optimum Temperature and Temperature Tolerance

The culture system to be used for large-scale culture and the geographical location will define the maximum temperature the alga may be exposed to and which it must be able to survive. For example, outdoor ponds located in regions of high insolation which are preferred to maximise productivity, can reach temperatures of about 35–40°C, and closed photobioreactors outdoors can reach even higher temperatures unless cooled. Thus it is essential that the algae strain selected is able to tolerate these maximum temperatures to avoid loss of the culture. A characteristic of algae is that the lethal temperature is usually only a little higher than the optimum temperature (Borowitzka 1998).

Similarly, as temperature control of very large-scale cultures is virtually impossible to do economically and, as temperature will vary over the day and with season, the selected algae strain should have a broad temperature optimum in the range of the average temperatures expected in the culture system during daylight hours. Already in 1955, Sasa et al. (1955) demonstrated the importance of temperature tolerance for successful year-round outdoor culture of microalgae. Some algae have a quite narrow temperature optimum, whereas others can show good growth over a wide temperature range (Goldman 1977; Borowitzka 1998). Since algae grow best near their optimum temperature and utilise light more efficiently at these temperatures (Borowitzka 1998), the selection of species and strains whose optimum growth temperatures are in the temperature range they will experience in the large-scale cultures is therefore extremely important. However, it must be recognised that temperature changes not only over the day and from day-to-day, but also with season. For example, during outdoor trials in raceway ponds in Roswell, New Mexico, USA, Weissman et al. (1989) found that although an *Amphora* sp had high productivities in summer this species could not survive in winter. *Monoraphidium minutum*, on the other hand, could be grown at all times of the year, even in winter when the ponds sometimes froze over, although the productivity was markedly lower in winter.

Since respiration is an important metabolic process reducing net productivity (see Sect. 2.3), the importance of night-time temperature and its effect on respiration should also not be underestimated. Respiration during the night leads to biomass loss reducing productivity, and the rate of respiration is affected by temperature (Grobbelaar and Soeder 1985). Furthermore, algae growing during the day at suboptimal temperatures have a higher night-time respiration rate and biomass loss (Torzillo et al. 1991a, b; Molina Grima et al. 1996).

As commercial-scale cultures are too expensive to heat in winter, the alga also should be able to grow during the coldest months of the year. Considering the high capital costs of culture systems – be they open or closed systems – as well as the associated downstream processing plant, the ability to produce algal biomass over the whole year ensures best use of these resources and reduces overall algae biomass and biofuel production costs.

2.2 Carbon Supply, pH and Oxygen Tolerance

For high rates of photosynthesis efficient uptake of inorganic C by the cell is very important. All algae can take up CO_2 and some, but not all, can also take up HCO_3^- (Korb et al. 1997; Giordano et al. 2005). In water inorganic C exists in four forms: CO_2, H_2CO_3, HCO_3^- and CO_3^{2-}. These inorganic carbon species are the main buffering system in water and, in algal cultures where the pH is unregulated, photosynthetic CO_2-uptake results in a rise in the pH of the medium, which in turn this leads to a reduction in the proportion of the inorganic C which is in the form of CO_2 and an increase in the proportion of HCO_3^- and CO_3^{2-}. If the alga can only take up CO_2 and not HCO_3^- then, once a pH of about pH 9.0 is reached (depends upon salinity and temperature), photosynthesis will effectively cease as no free CO_2 is available in the medium for the algae to take up. If the alga can also utilize HCO_3^-, either by having external carbonic anhydrases or an active HCO_3^- uptake system (Giordano et al. 2005), then the alga will be able to continue to photosynthesise until a pH of <pH 9.5 is reached when HCO_3^- is also now no longer available as almost all of the inorganic C is now in the form of CO_3^{2-}.

As CO_2 uptake requires less energy than HCO_3^- uptake, maintaining a high CO_2 concentration in the medium will enhance growth. In most commercial-scale cultures the algae are C-limited. This limitation can be reduced and the CO_2 supply to the algae cells can be optimized by controlling the culture pH by adding CO_2 to the medium using a pH-stat system. If the pH is uncontrolled then algae species which are highly efficient HCO_3^- users such as the coccolithophorid, *Pleurochrysis carterae* (Moheimani and Borowitzka 2007), are less likely to be C-limited during most of the day. The ability to continue to photosynthesise at high pH will also give the algae

a potential competitive advantage in large-scale cultures (Goldman et al. 1982a, b). It should also be noted that CO_2 diffusion from air into the medium is also greater at more alkaline pH (Hoover and Berkshire 1969; Lee and Pirt 1984), and this may be advantageous in a large-scale culture system as it increases the CO_2 supply from the atmosphere; i.e. 'free' CO_2. High concentrations of CO_2 are potentially toxic to many algae (e.g., Lee and Tay 1991), although there are some algae tolerant of very high CO_2 concentrations such as the green alga *Chlorococcum littorale* (Kodama et al. 1993; Ota et al. 2009) and the red alga *Cyanidium caldarium* (Seckbach et al. 1970).

Oxygen (O_2) is a product of photosynthesis and competes with CO_2 as a substrate for ribulose *bis*-phosphate carboxylase/oxygenase (Rubisco) (Badger et al. 1998). The medium in actively photosynthesizing dense algae cultures rapidly reaches oxygen supersaturation where photosynthesis is significantly inhibited by the high O_2 concentration (Vonshak et al. 1994; Raven 1997) especially at non-optimal temperatures (Ugwu et al. 2007). High [O_2] in open ponds and photobioreactors is a major limiting factor of algal productivity (Ogawa et al. 1980; Kliphuis et al. 2011) and it is therefore desirable to have strains whose Rubisco is less sensitive to oxygen.

Excessive light also leads to reduced photosynthesis, increased respiration and reduction in productivity. There is also the build-up of reactive oxygen species such as the photochemically generated singlet Oxygen (^1O) as well as $^\bullet O_2^-$. H_2O_2 and $^\bullet OH$ (Demmig-Adams and Adams 1992) which can lead to damage to Photosystem II. High irradiance will also be damage components of the Photosystem II reaction center, in particular the D1 and D2 proteins (Ohad et al. 1994; Jansen et al. 1999). Together these processes are known as photoinhibition. Aside from inhibiting photosynthesis, photodamage during photoinhibition needs to be repaired and this has a metabolic cost to the alga (Raven 2011) reducing net productivity. The level of photoinhibition in an algal culture outdoors can be reduced to some degree by managing the cell density (i.e. the average irradiance received by the cells), but some degree of photoinhibition during the day is inevitable (Lu and Vonshak 1999; Richmond 2004; Kromkamp et al. 2009). Differences in how algae avoid or deal with photoinhibition such as photochemical and non-photochemical quenching (Raven 2011) and their photosynthetic pigment systems, including the 'packaging effect' (Berner et al. 1989; Key et al. 2010), can be important to the productivity observed in outdoor cultivation systems. Selection of species showing less photoinhibition can therefore be of advantage.

2.3 Respiration Rate

The respiration rate of algae is strongly affected by growth conditions (light, temperature, N source etc. – Falkowski and Owens 1978; Grobbelaar and Soeder 1985; Turpin et al. 1988; Flameling and Kromkamp 1994) as well as cell size (Banse 1976; Tang and Peters 1995) – however, there is also evidence of species and strain variations in the intrinsic (basal) respiration rate (Humphrey 1975; Geider and Osborne 1989). Considering that respiratory losses may reduce net productivity by up to 50%, even a small change in respiration can have an important effect on net productivity. Screening for strains with a reduced intrinsic respiratory rate may therefore be useful.

2.4 Salinity

Saline water such as seawater or saline groundwater is the preferred basic water source for sustainable commercial-scale culture of algae for biofuels as it does not compete with the need for freshwater for land based agricultural production of food crops (Borowitzka and Moheimani 2010). In open ponds there is the need to replace water lost by evaporation which represents the largest water requirement for these cultures. There are two options: (1) use freshwater to make up for evaporative losses while maintaining a constant salinity, or (2) use saline water to make up for evaporative losses leading to a slow increase over time in the pond salinity. Thus, if option (2) is used, algae with a wide salinity tolerance will be preferred so that cultures can be continued for a long time.

An elevated salinity optimum and a high salinity tolerance potentially also make it easier to manage contaminating algae and other organisms in long-term, large-scale cultures.

2.5 Morphology

The morphology of the algae affects several aspects of the production process such as harvesting and dewatering, and extraction of the product. For example, larger, filamentous or colonial species such as *Spirulina/Arthrospira*, *Skeletonema, Chaetoceros* or *Botryococcus* potentially can be harvested by filtration, and species with little or no cell wall such as *Dunaliella* or the motile cells of *Haematococcus* are easier to extract compared to thick-walled coccoid species such as *Nannochloropsis, Chlorella. Scenedesmus* or the aplanospores of *Haematococcus*, where the cell walls need to be ruptured to achieve efficient extraction of lipids. However, smaller algae grow faster (Lewin 1988; Tang 1995) and are therefore more likely to have a higher lipid productivity.

Planktonic species grow faster than species whose normal growth mode is to be attached to surfaces. This is because cells in suspension, rather than those growing on surfaces, have a thinner boundary layer surrounding them which means that diffusion of nutrients and CO_2 from the bulk medium to the cell surface is more rapid. Algae in suspension also receive light from all directions rather than just

from one side. Therefore, 'sticky' species such as most pennate diatoms for example, are unsuitable candidates for high productivity large-scale culture. Similarly, free-living cells grow faster than species with a colonial morphology such as *Botryococcus* or chain formers such as *Chaetoceros.*

Morphology, especially the type of cell covering of the cell, also has an impact on shear tolerance which becomes especially important when they are to be grown in closed photobioreactor systems where the pumps or airlifts used to circulate the culture create more shear (Gudin and Chaumont 1991; Barbosa et al. 2003). Small, non-flagellated cells such as those of *Nannochloropsis* or *Chlorella* with robust cell walls are very shear resistant, whereas larger cells and flagellated cells such as those of *Dunaliella* and especially dinoflagellates and coccolithophorids are more shear sensitive (Thomas and Gibson 1990; Mitsuhashi et al. 1995; Sánchez Mirón et al. 2003) and therefore are more likely to be damaged by the method used to circulate the culture medium. For example, the coccolithophorid *P. carterae* grows extremely well in raceway ponds, but is very difficult to impossible to grow in photobioreactors because of its shear sensitivity (Moheimani and Borowitzka 2006, 2007; Moheimani et al. 2011).

The nature of the cell covering also affects many aspects of downstream processing including harvesting and extraction. For example, the silica valves of diatoms or the coccoliths of coccolithophorid algae, make the cells heavier and they settle easily potentially allowing pre-concentration of the cells by settling possibly thus simplifying the harvesting of these algae. However, this also means that more energy is required to keep the cells suspended during culture. The cell covering also affects the extraction step. For example lipid extraction from cells with thick resistant cell walls such as *Chlorella* or *Nannochloropsis* is much more difficult and may necessitate rupture of the cells in order to achieve efficient extraction. Methods such as mechanical breakage using bead mills, sonication and microwave treatment can be used (Chisti and Moo-Young 1986; Lee et al. 2010) but this adds to the costs of the lipid production process. On the other hand, species such as *Dunaliella* which do not have a cell wall are easy to extract. Similarly in some diatoms the valves separate easily, whereas in others separation of the valves to access the cell contents can be very difficult. The easy of valve separation may therefore be an important characteristic when screening diatoms.

The green alga *Botryococcus braunii* presents a special case. This alga actually excretes its lipids and hydrocarbons which then accumulate within the extracellular colony matrix (Metzger and Largeau 2005) and the lipid droplets can be extracted from this extracellular matrix by applying pressure. As the hydrocarbons are extracellular they are also easier to extract using solvents (e.g., Frenz et al. 1989). Unfortunately this colonial alga is slow growing and the lipid and hydrocarbon productivity therefore is low.

2.6 'Competitive' Strains

For reliable outdoor culture in open culture systems as well as in 'closed' photobioreactors, the species/strain selected must be able to outcompete other contaminating algae species and protozoa. Often such competitively superior strains grow in highly selective environments: e.g. *D. salina* grows at high salinities, *Chlorella* at high nutrient concentrations and *Arthrospira* (*Spirulina*) in high bicarbonate/pH conditions. Some species can outcompete contaminating organisms by being able to grow at a very high pH and/or also produce allelopathic compounds such as dimethyl-sulphoxide (DMSO) which breaks down to acrylic acid (Sieburth 1959) which acts as an antibiotic. The latter appears to be a major reason for the successful long-term outdoor culture of *Pleurochrysis* (Moheimani and Borowitzka 2006).

In general, any algal species growing under optimal conditions will better be able to outcompete other species for which these conditions are not optimal. Therefore, selection of strains with a temperature range and salinity optimum which matches the conditions which will be found in the large-scale culture system is critical.

2.7 Lipid Composition and Quality

For the production of biodiesel or other liquid fuels from algae the suitability of the lipids and their fatty acid composition for conversion to a liquid fuel meeting the required standards is also important. This requires analysis of the lipid classes and their fatty acid composition (see Chapters 11 & 12).

2.8 Co-products

Since lipids for biodiesel and sugars for bioethanol are very low value products and as algae production and processing is fundamentally expensive as shown in many techno-economic analyses (e.g., Stephens et al. 2010; Davis et al. 2011), many studies have proposed that there is a need to also be able to produce other, more high value products in order to achieve the production of economically viable algae biofuels. It is therefore desirable to also screen the algae for the presence and content of potential high value products such as carotenoids, long-chain polyunsaturated fatty acids, bioactives etc. (Borowitzka 1995, 2010b) These must be present in sufficient quantity to make their extraction and purification worthwhile (Borowitzka 1999).

3 Species or Strain?

Differences between species and higher taxa are important in the selection process for they determine the important general characteristics such as morphology, gross chemical composition, and broad salinity range tolerated. At the strain level we find smaller variations and these variations are often very important to the optimization of large-scale cultures. The degree of variation between strains isolated from nature differs between species. In some species there is great variation in the composition of the cells and in their response to growth conditions between strains (Vonshak 1987; Shaw et al. 1989; Borowitzka 1992b; Rodolfi et al. 2009). For example, strains of the coccolithophorid *Emilianea huxley* differ in their response to elevated CO_2 (Langer et al. 2009). Similarly, differences in physiology such as in growth in the presence of heavy metals and in the excretion of different sugars have been observed between strains of *C. vulgaris* (Kessler 1986; Kessler et al. 1991) and in the production of allelochemicals in the dinoflagellate *Alexandrium tamarense* (Tillmann et al. 2009). Significant strain differences in the optimum temperature for growth have also been observed (Li and Qin 2005; Kobiyama et al. 2010; Teoh et al. 2012), and this will effect how well the different strains grow in different environments.

These strain differences in physiology can be exploited by careful strain selection to increase the productivity of the algae cultures or increase the yield of a desired product. For example, Vonshak et al. (1996) found marked variation in the response and acclimation capability to high photon flux densities between three strains of the cyanobacterium *Spirulina* (*Arthrospira*) *platensis* and Belay (1997) reports increased annual production by using a strain able to grow at lower temperatures at the Earthrise *Spirulina* plant in California allowing a longer growth season over the year and reducing costs. The composition and content of the various hydrocarbons also varies between strains of the various 'races' (possibly different species?) of the green alga, *Botryococcus braunii* (Metzger and Largeau 1999).

It is therefore not surprising that several studies have shown that potential gains in productivity and lipid quality, at least at the small scale, may be achieved by careful strain selection.

The algal cells in a culture of a particular strain may not all be identical, genetically or physiologically. This can be due to several reasons: (a) during isolation the original culture was not derived from a single cell, but a number of cells, or (b) genetic variation has arisen over time in the culture. Just as strain variation has arisen in nature, similar evolutionary processes occur in cultures and variations between individual cells can also arise over time in cultures (Lakeman et al. 2009).

Thus, re-isolating new strains by selecting single cells from existing cultures can result in new strains (clones) with improved characteristics. For example, when 60 clones of the diatom *Cylindrotheca fusiformis*, isolated from a single culture, were screened it was observed that under identical growth conditions the total lipid content ranged from 7.3 to 23.4% of dry weight and with a similar variation in the fatty acid distribution of the lipids (Liang et al. 2005). Similarly, Alonso et al. (1992a, b) also found marked variation in the lipids and fatty acids of *Isochrysis galbana*. Molina Grima et al. (1995) used phenotypic selection to improve the eicosapentaenoic acid (EPA) content of an isolate of *I. galbana*. They isolated 59 clonal cultures from the original culture and the used the clonal culture with the highest EPA content to isolate a further 42 clones. Using this approach they were able to select a strain with an EPA content with double the original EPA content. What is not known is whether these genetic differences have arisen by mutation or genetic drift during the long period in culture of the original strain, or because the original culture was not derived from a single cell but from a number of cells (Alonso et al. 1994). Doan and Obbard (2011) used fluorescence-enhanced cell sorting to select for strains with significantly enhanced lipid content in *Nannochloropsis* sp.

This approach does not work for all algae. For example, Alonso et al. (1994) did not observe any variation in the EPA content of five different strains of *Phaeodactylum tricornutum* from the UTEX culture collection.

Spontaneous mutants have been shown to arise regularly in cultures (Reboud and Bell 1997; Collins and Bell 2004; Lakeman and Cattolico 2007). Most of these mutations are not expected to affect the phenotype, because of the high cell densities and high growth rates in cultures rare mutations that affect the phenotype are unlikely to occur. Mutations arising from DNA replication errors will not only be point mutations or small scale insertions/deletions, but also by the movement of mobile genetic elements (transposons). Analyses of the genomes of the diatoms *P. tricornutum* and *Thalassiosira pseudonana*, and the prasinophyte *Ostreococcus tauri*, have shown that about 2–6% of the genome contains various types of transposons (Armbrust et al. 2004; Derelle et al. 2006). Whole and partial genome duplication (polyploidy and aneuploidy) has also been shown to occur in cultures. The possibility of mutation in culture not only presents an opportunity to isolate new, improved strains, but also can present an issue for the maintenance of the desired characteristics in selected elite strains.

However, long-term maintenance in culture does not always lead to genetic variation. For example, Müller et al. (2005) found no genomic differences in pairs and groups of duplicate strains of *Chlorella vulgaris* from the SAG, UTEX and CCAP culture collections, despite the fact that the SAG and UTEX strains have been maintained by routine serial subculturing and had the opportunity to evolve, whereas the CCAP strains have been cryopreserved at −196°C since the 1970s.

4 Strain Selection *In Situ*

In large scale cultures, whether in open ponds or photobioreactors, *in situ* natural strain selection goes on, especially if the cultures are grown in continuous or semi-continuous culture. This selection process is the same as occurs in laboratory cultures but, in addition, in open ponds there also is a high likelihood of new 'wild-type' strains being introduced which can possibly become to dominate the culture over time. The challenge in large-scale culture therefore is the maintenance of the strain in the culture which has the desirable characteristics.

Two main options are available to the algaecultureist: (1) regular re-establishment of the large-scale culture with fresh inoculum derived from pure laboratory stock cultures; or (2) maintaining a selective environment in the large-scale cultures which favors the desired strain. In fact, the latter strategy can be used as an alternative to laboratory-based strain selection. For example, the commercial culture of *D. salina* in Australia in extensive open ponds at Hutt Lagoon, Western Australia, and Whyalla, South Australia, uses exactly this strategy. Both of these algae production plants grow the autochthonous *Dunaliella* flora occurring at these sites and use selective growth conditions which favor the highly carotenogenic species *D. salina* over the other non-carotenogenic *Dunaliella* species such as *D. viridis* and *D. bioculata* which co-occur in the ponds (Moulton et al. 1987; Borowitzka and Borowitzka 1988). The highly selective hypersaline environment in which these algae grow, as well as the greater tolerance of high irradiance of *D. salina* compared to the other, non-carotenogenic *Dunaliella* species, simplifies this approach.

5 A Rapid Screening Approach

The source of algae for screening may be either environmentally collected water or soil samples, or existing unialgal cultures from culture collections. Samples collected from environments similar to those where the large-scale production is envisaged are preferred.

The initial screening step before isolation of species is quite simple and involves enriching the field collected samples with nutrients (i.e., N and P at the level found in standard media such as F medium (Guillard and Ryther 1962) for marine species and BG11 medium (Allen 1968) or Bold's Basal medium (Bischoff and Bold 1963) for freshwater species), and setting up enrichment cultures at the temperature at which the algae will have to grow in the production system to be used. If the algae do not grow or grow slowly at this temperature they clearly are not suitable for large-scale production. The enrichment with nutrients will select for those species able to grow at high nutrient concentrations; algae which do not grow at high nutrients concentrations also have a low biomass productivity and are therefore not suitable for commercial production. The use of simple light-temperature gradient systems such as those developed by Halldal and French (1958), Thomas et al. (1963), and Siver (1983) can speed up the process of determining temperature optima of strains.

Where algae with a tolerance of salinity are desired the above enrichment cultures may also be set up at a range of salinities by adding NaCl to the enrichment to select for species able to grow over a range of salinities.

A further pre-selection step using the mixed species culture derived from the enrichment cultures now may be carried out (see also Sect. 4.4 above). This is especially useful if open pond culture is planned to be used for producing the algal biomass. Here the enrichment cultures are scaled up and eventually inoculated into outdoor mini-ponds of about 1–2 m^2 area. These cultures are then grown in the ponds for extended periods of up to several months allowing natural selection for those species and strains best able to grow and compete under outdoor conditions in the environment under which the final commercial-scale algae will be grown. The species which come to dominate these pond cultures will have the key properties of competitive vigour and environmental tolerance, both of which are essential characteristics for strains required for reliable large-scale production outdoors.

Following this initial selection of strains by enrichment under the conditions selecting for strains with the appropriate temperature and nutrient requirements and salinity conditions (and by outdoor mixed culture, if carried out) the algae now need to be isolated into unialgal culture using standard methods (Andersen and Kawachi 2005; Sieracki et al. 2005), followed by further characterisation and selection for desirable traits. At this stage it is desirable to isolate as many strains as possible from single cells (i.e. clonal cultures) to explore the phenotypic diversity (especially with respect to lipid productivity) in the culture. The strains now need to be screened for their lipid content. This can be done in several ways. For example, flow cytometry and cell sorting methods can be used to select clones with a high lipid content for isolation into unialgal culture. Alternatively, clonal cultures can be established and then screened for lipid content. Clearly, clones with a high lipid content while in the exponential phase of growth are more desirable as they will have higher lipid productivity.

Once a suite of clonal cultures has been established, they can be further screened for traits such as the range of salinity tolerance and performance over a wide temperature range, as would be expected in the commercial-scale conditions and the most productive strains can be selected. If possible, this screening should be carried out at irradiances which would be experienced in the outdoor culture system.

The suite of species and strains selected by the above processes can now be ranked on their overall characteristics and suitability for large-scale production and downstream processing. The various characteristics (see Table 4.2) should be assigned a relative value based on an assessment of their

Table 4.2 Summary of desirable properties of microalgae for large scale culture for energy production

Property	Reason	Comments	Selected references
Rapid Growth	Required for high productivity (also reduces risk of contamination by other algae and/or predators and pathogens)	Smaller cells generally grow faster. Single, planktonic cells also generally grow faster than cell 'clumps', colonial or filamentous forms, or cells growing on surfaces	Tang (1995), Kagami and Urabe (2001)
High lipid content (for biodiesel production)	Required for high lipid productivity	For maximum lipid productivity the lipid content should be high in actively growing cells rather than in stationary phase	Griffiths and Harrison (2009)
Temperature optimum	A high temperature optimum and tolerance (at least 30–35°C) is very important.	Temperature in open raceway ponds can reach 30–35°C in summer, and closed photobioreactors can reach even higher temperatures unless cooled.	Belay (1997)
	A broad temperature optimum is desirable.	A broad temperature optimum means a higher productivity over the whole year.	
Maximum temperature tolerated	Avoid possible culture 'crash'	In the case of a breakdown of the culture system temperatures may rise and the ability to withstand high temperatures means that the culture will not die.	
High photosynthetic efficiency	Increases productivity		Richmond (1999)
Ability to tolerate high irradiances	Reduces photoinhibition and photodamage at high irradiances and can increase productivity	The irradiances reached in ponds or photobioreactors at noon can cause photoinhibition and damage to the photosynthetic apparatus	Vonshak et al. (1988), Vonshak and Guy (1992), Raven (2011)
Salinity	For sustainability saline water will have to be used to make up for evaporative losses, especially in open ponds. This will result in increasing salinity in the culture over time	Algae with an ability to grow over a wide salinity range are desirable	Borowitzka and Moheimani (2010)
Shear tolerance	Must tolerate the shear created by the circulation system	For high productivities mixing is essential. The degree of shear depends on the culture system	Hondzo and Lyn (1999), Barbosa et al. (2003)
Non-'sticky' cells	For rapid growth cells must remain suspended in the water column		
Grows in a 'selective' environment	Makes management of contamination easier		Borowitzka et al. (1985)
Reduced sensitivity of photosynthesis (Rubisco) to high O_2 concentration	Higher productivity		Ogawa et al. (1980), Marquez et al. (1995)
Lower intrinsic respiratory rate	Higher productivity	Strains with lower intrinsic respiration rates will have higher new productivities	Geider and Osborne (1989)
Large cell size or colonial or filamentous morphology	Ease of harvesting	Large cells (or colonies or filaments) are easier and cheaper to harvest. However, larger cells generally grow slower.	
High specific gravity	Heavier cells are easier to harvest	However, heavier cells are harder to keep in suspension in culture system.	
Weak cell covering or no cell wall	Easier to extract product	However, cells with no or weak cell covering may be more sensitive to shear.	
No production of autoinhibitors	Reduces potential problems with medium recycling and allows productive high density culture		Mandalam and Palsson (1995), Rodolfi et al. (2003)
Lipid composition (for biodiesel production)	Affects yield, efficiency of transesterification and the quality of the biodiesel produced	Aside from taxonomic affiliation lipid composition is affected by growth conditions (light, N-source, nutrient limitation, salinity, temperature etc.)	Lipids for biodiesel: Knothe (2005), Meher et al. (2006), Chapter 12
			Effect of environmental conditions on algae lipid composition Borowitzka (1988), Guschina and Harwood (2006), Harwood and Guschina (2009), Chapter 2

Based, in part, on Borowitzka (1992a) and Grobbelaar (2000)
These are desirable properties and it is unlikely that any one strain will meet all of these. The relative importance of each property will depend, in part, on the culture system being used and the location

impact on the final economic outcomes of the process and the properties of each strain can then be assessed. This process allows ranking of the strains based on characteristics relevant to large-scale outdoor culture.

Those strains ranked highest should then be grown in the culture system proposed for large-scale production (i.e., in mini-ponds for example) to see how they perform under 'real life' conditions. In most cases this will need to be done over a whole annual climate cycle to allow assessment of performance over the range of environmental conditions experienced to provide actual annual average productivity data. This will also allow assessment of how easy it is to manage contamination by other algae and protozoa and other grazers. If the strain continues to perform well under these conditions optimisation of the culture conditions to maximise productivity can now be carried out.

6 Strain Improvement

One option for overcoming some of the limitations of 'wild'-type algae is to modify the strains by either mutagenesis or genetic engineering. One of the earliest suggestions for improving the productivity of algae cultures was by producing algae strains with a low antenna pigment content (Benemann 1989). A low antenna pigment content would allow more light to pass into the culture, diluting the light (Kok 1973). This was demonstrated by Nakajima and Ueda (1997, 1999, 2000) using mutants of the cyanobacterium *Synechocystis* PCC6714 and the green alga *Chlamydomonas perigranulata*, and by Melis et al. with *D. salina* (Melis et al. 1999). Whether strains with reduced antenna size can be successfully and reliably grown in large-scale systems, especially outdoor 'open' systems, remains to be shown. Algae have evolved large antenna size and large light harvesting pigment complexes so as to be able to efficiently capture light in low light environments such as in the mornings and evening (low sun angle), deeper water and in turbid waters and to minimise photoinhibition (c.f. Raven 2011).

Early attempts to improve lipid productivity by genetic engineering have been carried out with the diatom *Cyclotella cryptica* (Roessler and Ohlrogge 1993; Dunahay et al. 1995, 1996) but were unable to increase lipid production. Homologous recombination has recently been used with *Nannochloropsis* sp to insert transformation constructs (Kilian et al. 2011) and this may prove a very useful technique for genetically modifying microalgae.

7 Maintenance of Cultures

The maintenance of stock cultures is important as it allows the large-scale cultures to be restarted with fresh inoculum if these large-scale cultures have become contaminated or have lost some of their desirable characteristics and of course also when these cultures have 'crashed'. In culture collections the stock cultures are maintained either in liquid culture or on agar slopes which are periodically sub-cultured. However, these methods can result in the cultures loosing their original characteristics (Table 4.3). Where possible the stock cultures should be cryopreserved (Taylor and Fletcher 1998; Brand 2004; Harding et al. 2008). Cryopreservation also has the advantage of being less labour intensive than regular sub-culturing.

8 Conclusion

Selecting the best strain with a high productivity of the desired product suited to the environmental conditions under which it will be grown in large-scale culture is a critical step

Table 4.3 Evolutionary processes in algal cultures and approaches that can be taken to minimise the potential impacts of these processes in laboratory cultures and large-scale cultures

		How to minimize impact	
Process	Impact	In laboratory	In large-scale cultures
Mutation	Input of genetic variation	Cryopreservation (if possible)	Cannot be eliminated. Requires periodic re-start of cultures from stock cultures
Recombination	Novel combination of alleles. Increased rate of genetic adaptation	Avoid sexual reproduction where possible	Avoid sexual reproduction where possible
Genetic drift	Stochastic population dynamics. Fixation of maladaptive alleles. Mutational meltdown.	Avoid small population bottlenecks; frequent large-inoculum transfers	Use selective environment and culture management practices to maintain strain with desired characteristics
Inbreeding depression	Lowered fitness	Avoid sexual reproduction where possible. Alternatively, establish defined parallel mating lines	Avoid sexual reproduction where possible
Selection	Genetic adaptation to culture conditions. Correlated changes in linked, but unselected traits.	Mimic isolates 'natural' conditions as closely as possible.	Maintain selective environments for strain with desired characteristics

Adapted from Lakeman et al. (2009)

for achieving reliable production at the lowest cost possible. However, other considerations such as the harvestability and extractability of the desired product (i.e. lipids) of the strain are important selection criteria. Relatively rapid screening can be carried in the laboratory, but finally the strains selected must be tested under outdoor conditions, preferably over a whole annual cycle to assess performance under the highly variable (diurnal and seasonal) conditions of irradiance and temperature.

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Limits to Phototrophic Growth in Dense Culture: CO_2 Supply and Light

5

John Beardall and John A. Raven

1 Introduction

In recent years there have been tremendous advances in algal biotechnology. While earlier work has concentrated on the possibilities of using algae to produce a host of high-value compounds such as β-carotene and astaxanthin, recent interest has focussed on the possibility of using algal cultures as a source of lipids for second-generation biofuels such as biodiesel (see Chap. 12 by Knothe, this volume).

While a range of technologies for algal growth have been put forward, including open ponds (Borowitzka and Mohemani, Chap. 8 this volume) and a range of photobioreactor designs (Tredici, Chap. 7 this volume), there remain however some fundamental constraints on algal growth that potentially limit the growth rates and/or yield of algal and cyanobacterial cultures, irrespective of the technological approach. Some of these represent intrinsic features of the biology of autotrophic cells while others relate to extrinsic (environmental) factors that potentially limit performance.

In this chapter we briefly assess some of the intrinsic constraints on photoautotrophic growth. We then go on to specifically consider the two most important environmental factors that can potentially limit performance of photoautotrophic cells in dense culture – light and CO_2 supply.

J. Beardall (✉)
School of Biological Sciences, Monash University,
Clayton, VIC 3800, Australia
e-mail: john.beardall@monash.edu

J.A. Raven
Division of Plant Sciences, The James Hutton Institute, University of Dundee at the JHI, Invergowrie, Dundee, DD2 5DA, UK
e-mail: j.a.raven@dundee.ac.uk

2 Intrinsic Limitations to Growth

There appear to be inherent constraints to the efficiency by which organisms can use external resources in growth. There are also fundamental limitations to the rate at which organisms can grow. To some extent these questions are linked to issues such as cell size (Raven 1988; Beardall et al. 2009) and to genomics, through e.g. rRNA gene copy number (Flynn et al. 2010).

Compared to heterotrophic organisms, photolithotrophic (and chemolithotrophic) species generally have slower growth rates (Raven 1988, 1994, Table 5.1). Bacteria generally have higher maximal specific growth rates (μ_{max}) than eukaryotes with the same trophic mode, although the smallest difference between bacteria and eukaryotes is found for oxygenic photolithotrophic organisms.

Rather than these differences between growth capacity of species with differing trophic modes being related to cell size and the impact of increasing cell size on decreasing μ_{max}, they are, in part at least, related to the fraction of resources in the cell that are allocated to acquisition of organic carbon from the environment (organotrophy) or to the production of organic carbon from inorganic substrates (lithotrophy).

Certainly a larger fraction of resources is allocated to organic carbon production in lithotrophs than to organic carbon acquisition in organotrophs, at least in microorganisms (Raven 1995). There is roughly half as much protein per unit cell volume in catalysts processing organic carbon delivered from photosynthesis in photolithotrophs as in the proteins processing the organic carbon delivered from external organic substrates in chemo-organotrophs. In many photolithotrophs there are large numbers of copies of certain proteins per cell volume. Ribulose-1,5-BisPhosphate Carboxylase Oxygenase (Rubisco) can thus account for about 10% of total protein in cells of chlorophytes and euglenophytes grown at high CO_2 (Lord and Brown 1975; Rabinowitz et al. 1975; Freyssinett et al.

M.A. Borowitzka and N.R. Moheimani (eds.), *Algae for Biofuels and Energy*, Developments in Applied Phycology 5,
DOI 10.1007/978-94-007-5479-9_5,

Table 5.1 Maximum growth rate (μ_{max}) of micro-organisms with different trophic modes (After Raven 1988, 1994)

Organism	Trophic mode	μ_{max}, s^{-1}
Bacteria	Heterotrophs	$\leq 280 \times 10^{-6}$
Bacteria	Chemolithotrophs	$\leq 40 \times 10^{-6}$
Bacteria	Photo-heterotrophs	$\leq 50 \times 10^{-6}$
Bacteria	Anoxygenic photolithotrophs	$\leq 27 \times 10^{-6}$
Bacteria	Oxygenic photolithotrophs	$\leq 24 \times 10^{-6}$
Eukarya	Heterotrophs	$\leq 170 \times 10^{-6}$
Eukarya	Phagotrophs	$\leq 70 \times 10^{-6}$
Eukarya	Photo-heterotrophs	$\leq 28 \times 10^{-6}$
Eukarya	Oxygenic photolithotrophs	$\leq 26 \times 10^{-6}$

1984). If the chlorophyll:total protein ratio is unaltered by the CO_2 concentration for growth (cf. Palmqvist et al. 1990), Rubisco can be as little as 6% of total protein in air-grown cells of chlorophytes and euglenophytes, using the Rubisco:chlorophyll data of Yokota and Canvin (1985) for high CO_2-grown and air-grown cultures. Taking the probably unrealistically low apoprotein per unit chlorophyll quotient of 2 kg mol^{-1} chlorophyll (Raven 1984a, b), the data on Rubisco as a percentage of cell protein and or Rubisco per unit chlorophyll suggest a lower limit of 10–16% of total protein in the apoproteins of thylakoid pigment-protein complexes of chlorophyte and euglenophyte algae. Using more recent apoprotein per unit chlorophyll values, i.e. 2.8 kg protein per mol chlorophyll (Evans and Seeman 1989, using a protein N to protein conversion of 5.8), the pigment apoproteins in the algae comprise 14–22% of cell protein. These values can be compared to those for the mature leaf of a high light grown herbaceous flowering plant, where Rubisco and photosynthetic pigment-binding apoprotein account for 23 and 28% of total proteins respectively (Evans and Seeman 1989). The higher values in the flowering plant can be related to delegation of activities such as growth and uptake of elements other than carbon from the medium to other parts of the organism: the algae have a single cell which does everything.

Clearly a greater range of catalysts is needed for photolithotrophy than is the case for organotrophy, and consequently fewer genes are needed for organotrophy than for photolithotrophy. For instance, the smallest known genome of a free-living chemo-heterotrophic bacterium is that of a member of the SAR 11 clade, the marine species *Pelagibacter ubique* HTCC1062 (Giovannoni et al. 2005). This has 1,354 genes in a 1.31 Mbp genome. In contrast, the smallest known genome of a photo-lithotrophic cyanobacterium is that of the marine cyanobacterium *Prochlorococcus marinus* MED 4, with 1,716 genes in a 1.66 Mbp genome (Rocap et al. 2003).

3 The End Product of Metabolism and the Production of Secondary Metabolites Can Impact on Growth Rate

For organisms with a given trophic mode, the end products of metabolism will also have an influence on growth rate. Given the interest in using microalgae for production of useful products, many of which are considered secondary metabolites rather than being directly associated with growth, it is instructive to consider the case of lipid producing microalgae that are targeted for, among other things, biodiesel production. Oil-producing algae tend to have lower specific growth rates than similar-sized cells with no intracellular oil, in part because there is less room for catalysts necessary for metabolism and growth. Even in cases where oil is produced extracellularly, cells are still likely to have a lower specific growth rate because photosynthetic products are being diverted into an end-product rather than producing more catalysts used for growth. This is an inevitable consequence of producing secondary metabolites rather than growth-related materials. Much has been made of controlling growth rates to increase production of, for instance, lipid, where nutrient limitation of growth frequently leads to accumulation of lipid. However, simple modelling (Fig. 5.1) indicates that striving for maximum lipid (in this case) production should not concentrate on maximising cellular lipid concentration, but rather there will be some sub-optimal growth rate at which lipid production (but not lipid/cell) will be optimal and there will be trade-offs between growth and secondary product formation.

A more detailed discussion of intrinsic limitations to algal growth can be found in Raven (1988). We now concentrate here on the role of external factors that impact particularly on dense algal cultures, namely light availability to cells and CO_2 supply.

4 Light Is Attenuated Exponentially in Cultures, So Is Potentially Growth Limiting

Full sunlight at noon has a flux of photosynthetically active radiation (PAR; 400–700 nm) of approximately 2,000 μmol photons m^{-2} s^{-1}. If all incident PAR is absorbed and fully utilised, and assuming growth on nitrate (1 mol C assimilated into cell material per 12 mol photons absorbed) and a 12:12 light–dark cycle, the theoretical net achievable primary productivity would be ≤167 μmol C m^{-2} s^{-1} (≤86 g C m^{-2} day^{-1}). Somewhat higher yields would be achievable if the nitrogen source is ammonium rather than nitrate.

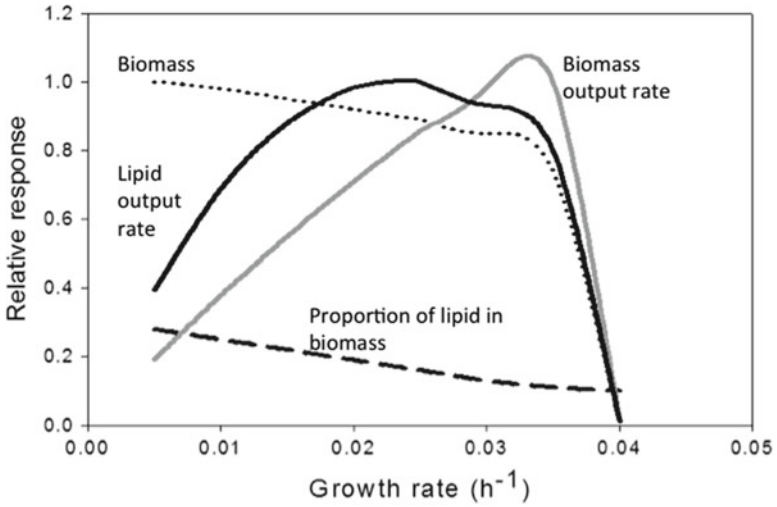

Fig. 5.1 Greater overall product formation may be linked to faster growth rather than to cellular product concentration

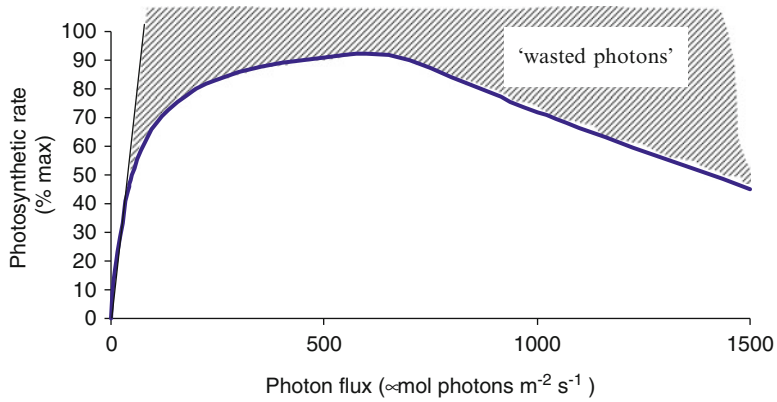

Fig. 5.2 A representation of the photosynthesis vs. irradiance response. The *stippled area* represents photons that are essentially wasted as large increases in photon flux produce diminishing increases in photosynthetic output. This can be exacerbated at even higher photon fluxes where photoinhibition may occur

Mass algal production involves optically dense cultures (biomasses of ~1 kg m^{-3}, giving light attenuation coefficients of ~200 m^{-1} are not uncommon). If grown outside, such cultures are frequently challenged by supra-optimal incident irradiances. In these cultures, approximately 90% of the photons would be absorbed in the first 10 mm by algae suffering severe light inhibition, and so using photons very inefficiently. The remaining 90% or more of the culture is using photons very efficiently, but cells here are severely light limited and exist in virtual darkness (see Fig. 5.2).

Maximising the efficiency of light utilization and minimising light limitation of growth is thus one of the major challenges in mass culture of algae. There are a number of approaches to this issue.

In the simplest form, cultures can be optically configured to minimise self-shading. However, such an approach still leads some cells open to photoinhibition, but perhaps more importantly for biotechnological application, the yield of biomass and useful product per unit area of culture would be much reduced by such an approach. However, Richmond et al. (2003) have achieved high density and production (260 mg L^{-1} h^{-1}) with *Nannochloropsis*, in culture systems in which the optical pathway was reduced to as little as 1 cm.

A second approach is to utilise the plasticity of algal physiological responses. High light adapted algal cells will reduce the size of the light harvesting antennae, which in turn will decrease the collection rate of photons incident on a culture. This reduction in chlorophyll content would thus allow more light to penetrate to depth and be utilised. This would therefore increase the number of photons delivered to the deeper parts of the culture and increase the efficiency of use of incident photons and areal productivity. This decreased cellular light interception would also decrease photoinhibition at high irradiance. A number of groups have reported success using this approach for *Chlamydomonas* cells. For instance, a 35–50% decrease in the antenna size of PSII

doubled the photosynthetic capacity and growth of cultures at high irradiance (Mussgnug et al. 2007; Polle et al. 2003; Beckmann et al. 2009). Similar data have also been presented for *Dunaliella* where a 60% reduction in antenna size of PSII resulted in a doubling of maximum photosynthetic capacity (Polle et al. 2002).

The third approach to improving photon use efficiency relates to the time scales in photosynthesis. Events associated with photon capture operate on time scales of the order of microseconds, while CO_2 fixation occurs about 10^6 times slower. Acclimation processes where cells optimise their performance to the incident photon flux take about 10^3 times longer again (on the time scale of tens of minutes to hours). Since energy dissipation through CO_2 fixation processes occurs on a time scale much slower than photon capture and primary charge separation, under high photon fluxes electron flow can be saturated and much of the incident photon energy is wasted. However, cells in dense cultures are potentially exposed to very high light and then darkness on scales much shorter than acclimation responses. Optimising turbulence in cultures, so cells cycle between periods of high and low photon flux, can be used to 'fine-tune' the efficiency of photon use so, in essence, CO_2 fixation can catch up with photon capture.

The efficacy of this 'flashing effect' has been demonstrated in a number of cases. For instance, Laws et al. (1983, 1986) achieved a more than doubling of areal productivity with mixing frequencies 0.5–1 Hz. With very dense cultures, frequencies up to 10 Hz have been shown to be effective in stimulating growth and productivity (Grobbelaar et al. 1996; Nedbal et al. 1996; Grobbelaar 2009).

Even with improvements in the technology associated with algal ponds and bioreactors, sustainable yields of >40 g m^{-2} day^{-1} are unusual (Williams and Laurens 2010). Values above this are in general associated with short path length (<5 cm) bioreactors. In the short path length culture system of Richmond et al. (2003), described earlier, high density and production (260 mg L^{-1} h^{-1}) of *Nannochloropsis* could be achieved by reducing the optical pathway to as short as 1 cm so the light–dark cycling regime was more closely aligned (33 ms at 1 cm optical path length) with the photosynthetic unit turnover time, providing the culture medium was frequently renewed. Similar data were achieved with *Spirulina* cultures in which areal productivities of up to ~50 g m^{-2} day^{-1} were recorded (Hu and Richmond 1996; Hu et al. 1996).

For outdoor cultures, even with efficient design, changes in light availability over the seasons and with latitude may restrict sustainable, year-long, high yields to lower latitudes (<35°, Williams and Laurens 2010). Assuming 50% carbon in cells and thus a yearly average of 20 g C m^{-2} day^{-1} assimilation in microalgal cultures, drawdown of 6×10^9 g km^{-2} $year^{-1}$ may be possible (with 300 days per year at this productivity). This is still small compared with annual anthropogenic CO_2 emissions – for instance China's emissions (2006 figures) are ~1.37 Pg C $year^{-1}$, so would require $1.37 \times 10^{15}/(6 \times 10^9) = 0.23 \times 10^6$ km^2 devoted to microalgal culture. This is, though, is still less than 2.5% of China's land area.

5 Diffusive Entry of CO_2 Is Not Necessarily Limiting Under Air-Equilibrated Conditions, But Can Rapidly Become Limiting in Dense Cultures

In aqueous environments, inorganic carbon is present in the form of both CO_2 and HCO_3^-. CO_2 in water is (sometimes) in equilibrium with that in the atmosphere. CO_2 can enter the cells by diffusion across the plasmalemma. The most reliable, to date, measurement of the CO_2 permeability coefficient for an algal membrane is still that by Sültemeyer and Rinast (1996) for the plasmalemma of *Chlamydomonas reinhardtii* where values of between 1.5 x 10^{-5} and 1.8 x 10^{-5} ms^{-1} were obtained. In contrast, cell membranes are essentially impermeable to HCO_3^-.

Although diffusion of dissolved gases such as CO_2 in water is 10^{-4} times the diffusion rate in air, Raven (1991a) has shown that in water well-equilibrated with the atmosphere, the rate of diffusive transport of CO_2 into small cells is unlikely to limit photosynthesis, at least for small single cells without a large boundary layer. In larger cells, or in macroalgae, constraints on diffusion in an aqueous environment plus increased diffusive resistance from deeper boundary layers can limit the availability of CO_2 to cells.

However, in dense cultures, intense photosynthetic activity will decrease the DIC concentration significantly. In poorly buffered systems, as algae take up CO_2 from the water, so the pH will rise. This will lead to a decrease in the CO_2 to bicarbonate ratio as well as in reduced absolute CO_2 concentration. As a result of this photosynthetic activity, DIC levels, even in quite well aerated cultures, are rarely as high as might be expected from air-equilibrium. Thus Johnston and Raven (1992) reported a ten-fold drop in DIC over a 6.5-day growth period for cultures of *Phaeodactylum tricornutum*. Williams and Colman (1996) showed that, with low aeration rates, the DIC concentration in cultures of *Chlorella saccharophila* dropped from 450 μM (air equilibrium) to below 30 μM over 3 days, and Young and Beardall (2005) reported that, despite being buffered and aerated at one culture volume per minute, semi-continuous cultures of *Dunaliella tertiolecta* still exhibited a 50% decrease in DIC (and a 0.4 unit rise in pH) compared to fully air-equilibrated conditions. This decrease in DIC below air-equilibrium can impose limitations on diffusive supply of CO_2, even for small cells.

6 The Kinetic Characteristics of Rubisco Potentially Limit Inorganic Carbon Acquisition Under Air-Equilibrated Conditions

The basis of C assimilation in algae and cyanobacteria is fixation by Rubisco and the Calvin Benson Bassham cycle. All known Rubiscos have competitive carboxylase and oxygenase functions and have relatively low substrate-saturated carboxylase activities on a protein mass basis. The selectivity factor, defining the relative rates of carboxylase and oxygenase reactions, is given by Eq. 5.1:

$$S_{rel} = \frac{K_{1/2}(O_2)\cdot k_{cat}(CO_2)}{K_{1/2}(CO_2)\cdot k_{cat}(O_2)} \qquad (5.1)$$

where $k_{cat}(CO_2)$ = CO_2 saturated specific rate of carboxylase activity of Rubisco (mol CO_2 mol^{-1} active site s^{-1}), $K_{½}(CO_2)$ = concentration of CO_2 at which the CO_2 fixation rate is half of $k_{cat}(CO_2)$, $k_{cat}(O_2)$ = O_2 saturated specific rate of oxygenase activity of Rubisco (mol O_2 mol^{-1} active site s^{-1}) and $K_{½}(O_2)$ = concentration of O_2 at which the O_2 fixation rate is half of $k_{cat}(O_2)$.

The half-saturation concentration of substrate ($K_{½}$) for, and the substrate-saturated rates (k_{cat}) of, carboxylation and oxygenation both show significant phylogenetic variability (Badger et al. 1998; Raven 1997, 2000; Tortell 2000; Raven et al. 2000; Raven and Beardall 2003; Giordano et al. 2005; Tcherkez et al. 2006). The general correlation is that a low $K_{½}(CO_2)$, and a high S_{rel} are correlated with a low $k_{cat}(CO_2)$, and *vice versa.* For instance some red algae have very high S_{rel} but relatively low $k_{cat}(CO_2)$, whereas cyanobacteria and dinoflagellate Rubiscos have low S_{rel} values but a high $k_{cat}(CO_2)$.

Rubisco evolved at a geological time in which CO_2 levels were very much higher than at the present day. However, the long-term decline in CO_2 levels and rise in oxygen has given rise to a situation where competition between O_2 and CO_2 has become restrictive to net carbon fixation. For instance for green algae the kinetics of Rubisco are such that half saturation of photosynthesis occurs at ~30 μM CO_2, cf ~12 μM CO_2 in water. As a result, algae relying on diffusive CO_2 entry and fixation by Rubisco would be, therefore, C-limited under present day CO_2 levels.

However, many algae possess an active transport system for dissolved inorganic carbon (DIC). This CO_2 concentrating mechanism (CCM) increases CO_2 levels, and CO_2:O_2 ratios, at the active site of Rubisco to levels many times the external concentration. This improves the carboxylation, and decreases oxygenase, activity of Rubisco. CCMs are based on active transport of HCO_3^- and/or CO_2. Indeed, more than one type of transporter can be present simultaneously (see Giordano et al. 2005 for a recent review of CCMs in algae). Expression of a CCM increases the affinity of cells for CO_2 such that C fixation is essentially saturated at present day atmospheric equilibrium levels.

In addition to their role in improving inorganic carbon supply to Rubisco, CCMs may improve resource use efficiency when nutrients such as N, P, Fe, S etc. are in short supply (Raven 1991b; Raven and Johnston 1991; Raven and Beardall 2003) and may also play a role in excess energy dissipation (Sukenik et al. 1997; Beardall and Giordano 2002; Tchernov et al. 1998).

Different species show differences in affinity for inorganic carbon (brought about by a combination of S_{rel} for Rubisco and CCM activity), which are reflected in different responses of photosynthesis and growth to changing CO_2 and/or DIC levels. Thus species such as diatoms, with S_{rel} values with high carboxylase vs. oxygenase ratios, show relatively low CCM capacity. Species such as cyanobacteria, on the other hand, have Rubiscos with lower carboxylase:oxygenase ratios and these show the highest CCM capacity of oxygenic photoautotrophs.

As a consequence of the differing affinities of algal species for CO_2 in photosynthesis, alterations in external CO_2 levels can impinge differently on differing species (see Giordano et al. 2005), and in natural populations impacted by increasing CO_2 from global change, this can lead to shifts in species composition (see Beardall et al. 2009 for a recent review).

Light, as established earlier, is potentially growth limiting to cells in dense culture. Light and CO_2 metabolism interact in defining the lower limits of light availability for growth of algae. This in turn will affect the capacity of cells to grow in dense, self-shaded, cultures. Operation of the CCM is energetically expensive. Energy for inorganic carbon transport and accumulation is generally believed to be derived from cyclic electron flow associated with PSI (Ogawa and Ogren 1985; Ogawa et al. 1985; Palmqvist et al. 1990; Spalding et al. 1984). It might thus be argued that species such as diatoms with high S_{rel} Rubiscos and relatively low CCM activity might be expected to have an ecological advantage under low light (Tortell 2000). However, despite the energy costs incurred in running a CCM, cost-benefit analyses suggest that algae with a CCM can perform quite favourably compared with species reliant on diffusive CO_2 entry and fixation via Rubisco, and subsequently incurring the energetic costs of photorespiration and glycolate metabolism (Beardall 1991; Raven 1991b; Raven et al. 2000). It should also be noted that the different pathways for processing glycolate from Rubisco oxygenase activity also have implications for the minimum light requirements for growth and hence for the limits to density of cells in mass culture (see Raven et al. 2000).

Light also impinges on CCM activity and efficiency of C fixation. Beardall (1991) has shown that light will directly affect inorganic carbon transport **activity** in the cyanobacterium *Anabaena variabilis* and a number of studies

(Beardall 1991; Young and Beardall 2005; Shiraiwa and Miyachi 1983) have reported that cells grown at lower light exhibit a lower affinity for CO_2 in photosynthesis, implying a down-regulation of CCM **capacity**. Clearly then it would be expected that cells in dense, light-limited culture might exhibit less active CCMs than those exposed to high light, but there have been no studies on CCM activity in dense cultures where cells might be alternately exposed to very high (saturating) photon flux, then extremely light-limiting conditions.

CCM activity is also down-regulated by external CO_2 concentrations in the gas phase. In cyanobacteria, CCMs appear to be controlled by the subsequent HCO_3^- concentration in the medium (Mayo et al. 1986), while in many eukaryotes it has been shown that dissolved CO_2 is the DIC species controlling expression of the CCM (Berman-Frank et al. 1995; Matsuda and Colman 1995, 1996; Bozzo et al. 2000; Matsuda et al. 2001; Fukuzawa et al. 2001; Beardall and Giordano 2002). Clearly in mass cultures which are bubbled with gases containing high CO_2 to avoid DIC limitation of growth, CCMs would potentially be down-regulated, with possible implications for cells' capacity to show improved growth rates at lower light levels. An interesting example of the interplay between light and CO_2 in cultures comes for the data of Young (1999) in which batch cultures of *Dunaliella tertiolecta* showed increasing affinity for CO_2 as nutrient replete cultures grew and depleted the DIC to 10 μM. Affinity for CO_2 in older cultures however decreased as the cell density increased and light-limitation increased. Clearly there are complex interactions in regulation of cell growth and productivity of mass cultures by light and inorganic carbon supply.

Acknowle dgements The University of Dundee is a registered Scottish charity, No SC015096. John Beardall's work on microalgal physiology and inorganic carbon acquisition is supported by the Australian Research Council.

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Genetic Engineering to Improve Algal Biofuels Production

6

Beth A. Rasala, Javier A. Gimpel, Miller Tran, Mike J. Hannon, Shigeki Joseph Miyake-Stoner, Elizabeth A. Specht, and Stephen P. Mayfield

1 Introduction

Due to a limited supply of fossil fuels and the detrimental effect of CO_2 release on the environment, the development of dependable, environmentally sustainable, and economically viable sources of alternative fuels is a major goal for the United States and the world. Uncertainties in supply and fluctuating costs of foreign oil underlie significant economic and security concerns. Likewise, exacerbation of global climate change associated with the use of fossil fuels is a challenge that may be addressed in part by the development of new sources of cleaner alternative fuels. Lipid-rich algae are increasingly regarded as promising, renewable sources of high quality oils that can serve as feedstocks for transportation and other fuels (Beer et al. 2009; Chisti 2007; Courchesne et al. 2009; Dismukes et al. 2008; Greenwell et al. 2009; Hannon et al. 2010; Rittmann 2008; Rosenberg et al. 2008; Schenk et al. 2008).

The characteristics of an ideal algal biofuel-production strain include fast growth rate, high growth density, high oil content, ease of harvesting, and low susceptibility to invasive species. Although there are more than 500,000 known species of algae, to date only a few species have been investigated as potential sources of biofuels, and none have been shown to be ideal. Thus, while current and future prospecting is important, it is likely that breeding and genetic engineering of algae will be required to develop strains of algae that are economically viable.

Below we will discuss the recent advances made in algal genetic engineering, including genetic transformation, stable heterologous gene expression, as well as other current and potential ways to use molecular genetics to advance algal biofuel production.

2 Tools and Techniques for *Chlamydomonas reinhardtii* Genetic Engineering

One of the most studied microalgae is the freshwater green alga, *Chlamydomonas reinhardtii. C. reinhardtii* is a popular model organism for physiological, molecular, biochemical and genetic studies. It is a relatively simple, single-celled organism that survives in both a haploid and diploid state. It has sexual and asexual lifecycles that can be controlled by nutrient deprivation and light cycles. *C. reinhardtii* is easily cultivated photoautotrophically, mixotrophically (with light and a reduced carbon source), or heterotrophically (relying on reduced carbon only). It has a doubling rate of 5–8 h and can grow to densities above 10^7 cells mL^{-1}. All three genomes – nuclear, chloroplast, and mitochondria – have been sequenced (Maul et al. 2002; Merchant et al. 2007; Popescu and Lee 2007). *C. reinhardtii* has been the model alga for studies of photosynthesis, flagella function, protein translation and organization, and function of basal bodies. As such, the molecular toolkit for this microorganism is highly developed. Indeed, methods for genetic transformation of all three genomes are well established. Below we will describe the recent advances in the genetic engineering of *C. reinhardtii*, and their potential to enable biofuel production.

2.1 Chloroplast Engineering

2.1.1 The Chloroplast

The chloroplast is the organelle in plants and eukaryotic algae that is the site of photosynthesis, as well as the site for production of starch, fatty acids, and pigments. *Chlamydomonas reinhardtii* has a single, cup-shaped chloroplast that occupies

B.A. Rasala • J.A. Gimpel • M. Tran • M.J. Hannon • S.J. Miyake-Stoner • E.A. Specht • S.P. Mayfield (✉)
Division of Biological Sciences, University of California, San Diego, 2150C Bonner Hall, MC: 0368, 9500 Gilman Dr., La Jolla, CA 92093-0368, USA
e-mail: brasala@ucsd.edu; jagimpel@ucsd.edu; mit005@ucsd.edu; mjhannon@ucsd.edu; smiyakes@ucsd.edu; especht@ucsd.edu; smayfield@ucsd.edu

M.A. Borowitzka and N.R. Moheimani (eds.), *Algae for Biofuels and Energy*, Developments in Applied Phycology 5,
DOI 10.1007/978-94-007-5479-9_6,

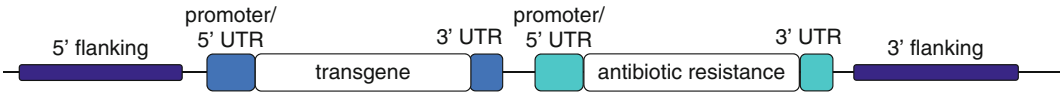

Fig. 6.1 A chloroplast transformation vector. Schematic diagram of a *C. reinhardtii* chloroplast transformation vector, consisting of the transgene under the control of an endogenous promoter and 5′ and 3′ untranslated regions (UTRs), a dominant selection marker gene in *cis* under the control of different endogenous promoter and UTR elements, and flanking sequence homologous to the site of integration (*dark blue*)

approximately one third the volume of the cell. The chloroplast has a 203 kb circular genome encoding for ~100 genes (Maul et al. 2002). The majority of these genes encode for either components of the transcription-translation machinery, or proteins involved in photosynthesis (Maul et al. 2002). Furthermore, the chloroplast genome is highly polyploid, with 50–80 copies present in each chloroplast.

2.1.2 Chloroplast Transformation

The first stable transformation of the chloroplast genome of *C. reinhardtii* was reported in 1988 using a microparticle bombardment or biolistics method (Boynton et al. 1988), and still remains the most efficient and reliable method for chloroplast transformation. In brief, a lawn of algal cells are spread onto an agar plate and placed into a vacuum chamber. The plate is bombarded with DNA-coated gold or tungsten particles, some of which penetrate the cell wall, plasma membrane, and chloroplast membranes, depositing the transforming DNA, which then integrates into the plastid genome.

Genetic transformation of the chloroplast genome proceeds through homologous recombination between the vector sequence and the corresponding genomic sequences (Boynton et al. 1988). Thus, stable integration of heterologous DNA into the chloroplast genome requires sufficient flanking endogenous sequences that are homologous to the targeted insertion site (Fig. 6.1). A good rule-of-thumb is to include ~1 kb of sequence on either side of the recombinant gene to ensure efficient integration. Because chloroplast transformation occurs via homologous recombination, transformation events can be precisely targeted to any region in the genome, and these are generally targeted to regions that contain a so-called "silent site", a region lacking any known coding sequences. Recombinant genes can also be used for targeted disruption of endogenous genes. For example, we frequently target heterologous gene insertion to the silent site at the 3HB locus, or as a replacement of the *psbA* locus.

2.1.3 Translational and Transcriptional Control of Transgenes

A recombinant gene construct usually consists of the transgene under control of a promoter, and 5′ and 3′ untranslated regions (UTRs) (Fig. 6.1). Promoter regions are responsible for initiating transcription, while 5′ UTRs function to regulate ribosome association and translation rates (Barnes et al. 2004; Marin-Navarro et al. 2007). The 3′ UTRs appear to influence mRNA stability (Herrin and Nickelsen 2004; Lee et al. 1996; Monde et al. 2000; Stern et al. 1991) and may interact with 5′ UTRs (Katz and Danon 2002). In general, the regulation of chloroplast gene expression is primarily controlled at the translational level (Eberhard et al. 2002; Nickelsen 2003; Zerges 2000).

Despite the relative ease of introducing new DNA into the chloroplast genome, *Chlamydomonas* chloroplasts maintain stringent control of gene expression. By far the most successful regulatory elements identified to date have been endogenous ones. Little to no protein accumulation has been measured from synthetic or heterologous promoters/UTRs, and no known virus infects the chloroplasts of *C. reinhardtii*, so the use of viral elements for heterologous gene expression is likely not an option. Thus much work has been done to identify endogenous transcriptional and translational regulatory elements for maximum heterologous gene expression (reviewed by Harris et al. 2009; Marin-Navarro et al. 2007; Purton 2007). And, similar to endogenous chloroplast gene expression, heterologous gene expression from the chloroplast genome seems to be primarily regulated at the level of translation (Barnes et al. 2005; Rasala et al. 2010, 2011). The highest levels of heterologous protein accumulation in *C. reinhardtii* thus far have been achieved using the *psbA* promoter and 5′ and 3′ UTRs in a *psbA*-deficient strain (Manuell et al. 2007; Surzycki et al. 2009). Other promoter and 5′ UTRs in use for transgene expression include those from the endogenous genes *atpA*, *atpB*, *petD*, *rbcL*, *psbD* and *psaA* (Fletcher et al. 2007; Purton 2007; Michelet et al. 2010). Each of these have been used with some success, though it is unclear why certain regulatory elements engender high expression levels with some transgenes but not others (Marin-Navarro et al. 2007). Recently, we demonstrated that the use of the *16S* promoter fused to the *atpA* 5′ UTR supported high levels of heterologous gene expression in a photosynthetically-competent genetic background (Rasala et al. 2011).

An inducible system for chloroplast transgene expression has been developed by taking advantage of the copper-sensitive cytochrome c(6) nuclear promoter and the nuclear-encoded, chloroplast-targeted Nac2 protein (Surzycki et al. 2007). Nac2 is specifically required for *psbD* mRNA stability and acts on its 5′ UTR. By transforming a copper induced cytochrome c6 promoter fused to the *Nac2* coding sequence into a *Nac2* deficient strain, proteins encoded with the *psbD* regulatory region only accumulate in the presence of copper (Surzycki et al. 2007).

Table 6.1 Selectable markers available for *Chlamydomonas reinhardtii* genetic transformation

Gene	Source	Compartment	Antibiotic	References
Heterologous genes				
aphA6	*Acinetobacter baumannii*	Chloroplast	Kanamycin	Bateman and Purton (2000)
			Amikacin	
aadA	*E. coli*	Nuclear/chloroplast	Spectinomycin	Goldschmidt-Clermont (1991)
			Streptomycin	
ble	*Streptoalloteichus hindustanus*	Nuclear	Zeocin phleomycin	Stevens et al. (1996)
aphVIII	*Streptomyces rimosus*	Nuclear	Paromycin	Sizova et al. (2001)
			Kanamycin	
			Neomycin	
aph7	*Streptomyces hygroscopicus*	Nuclear	hygromycin B	Berthold et al. (2002)
Point mutations				
16S rRNA	*C. reinhardtii*	Chloroplast	Streptomycin	Harris et al. (1989)
			Neamine	Harris et al. (1989)
			Kanamycin	
			Spectinomycin	Harris et al. (1989)
			Spectinomycin	Guhamajumdar and Sears (2005)
23S rRNA	*C. reinhardtii*	Chloroplast	Erythromycin	Harris et al. (1989)
			Lincomycin	Harris et al. (1989)
			Chloramphenicol	Harris et al. (1989)
rps12	*C. reinhardtii*	Chloroplast	Streptomycin	Liu et al. (1989)
psbA	*C. reinhardtii*	Chloroplast	Atrazine	Erickson et al. (1984)

2.1.4 Selection

Selection is required for the identification of transformants, and can be supplied in *cis* on the same vector as the transgene (Fig. 6.1), or in *trans* on a second plasmid. One can select for transformants by rescue of nonphotosynthetic mutants (i.e. atpB), by expression of a heterologous gene that confers antibiotic resistance, or by antibiotic resistance through introduced point mutations in endogenous genes (Table 6.1). Commonly used heterologous genes for antibiotic resistance include aphA6 from *Acinetobacter baumannii* for kanamycin resistance (Bateman and Purton 2000) and aadA from *E. coli* for spectinomycin/streptomycin resistance (Goldschmidt-Clermont 1991). Antibiotic resistance through point mutations in the 16S and 23S ribosomal RNA genes has been well characterized since the late 1980s (Harris et al. 1989; Newman et al. 1990). Furthermore, combinations of these point mutations can confer varying levels of resistance to spectinomycin or streptomycin (Newman et al. 1990). These ribosomal RNA mutations can also confer resistance to neamine/kanamycin and erythromycin (Harris et al. 1989). Similarly, *Chlamydomonas* (as well as higher plants) can be screened for resistance to certain herbicides. A class of herbicides including atrazine act by inhibiting the function of photosystem II (Erickson et al. 1984), and point mutations in the psbA gene encoding the 32kD photosystem II protein D1 can confer resistance to atrazine (Erickson et al. 1984).

For the purposes of producing molecules such as vaccines or protein therapeutics, the addition of selectable marker genes that require antibiotics is not desirable. After the transformation, screening, and selection process is completed, these selectable markers can be removed though a technique called gene excision. By placing direct repeats on either side of the resistance gene, the gene will be spontaneously "looped out" through homologous recombination upon removal of the selection pressure, i.e. by switching to antibiotic-free media (Fischer et al. 1996).

2.1.5 Generating Stable Homoplasmic Lines

There are 50–80 copies of the genome within each *Chlamydomonas* chloroplast, not all of which are initially transformed. In order for the transgene to be stable, all copies of the genome must contain the transgene, a state described as homoplasmic. Homoplasmy is achieved through multiple rounds of subcloning under selection and the state of homplasmy can be screened for by Southern blot or PCR. In general homoplasmic lines are identified within three or four rounds of subcloning.

2.1.6 Chloroplasts as Protein Factories

Chlamydomonas reinhardtii has been engineered to express a wide range of recombinant proteins, including reporters such as GUS (Sakamoto et al. 1993), luciferase (Mayfield and Schultz 2004; Minko et al. 1999), or GFP (Franklin et al. 2002), protein therapeutics such as antibodies (Franklin and Mayfield 2005; Mayfield and Franklin 2005; Mayfield et al. 2003; Tran et al. 2009), hormones, growth factors (Manuell et al. 2007; Rasala et al. 2010), vaccines (Surzycki et al. 2009), and industrial enzymes (our unpublished data). Many

proteins that have been quantified have been shown to accumulate as high as 10% of total soluble protein (TSP) (Manuell et al. 2007). Many have also been shown to be properly folded and bioactive (Franklin et al. 2002; Manuell et al. 2007; Mayfield and Franklin 2005; Mayfield et al. 2003; Mayfield and Schultz 2004; Rasala et al. 2010; Tran et al. 2009). Of the many proteins we have tried to express, we estimate that we have successfully expressed about 50 % of them. This success rate is similar to those of yeast and mammalian cells (Aricescu et al. 2006; Banci et al. 2006), and much better than the rate of soluble protein expression achieved in *E. coli* (Alzari et al. 2006).

While we still do not understand why some heterologous proteins fail to express, we do know that bias in codon usage is an important consideration for the expression of heterologous genes. The chloroplast genome is AT-rich (Maul et al. 2002), and transgenes synthesized in the chloroplast codon bias generally express at higher levels than the native genes (Franklin et al. 2002).

2.2 Nuclear Engineering

2.2.1 The Nuclear Genome

Techniques for transformation of the nuclear genome of *C. reinhardtii* are also well established. The nuclear genome sequence was completed in 2007 (Merchant et al. 2007). The genome has high GC content and frequent repeat regions. Unlike the chloroplast, nuclear gene expression is primarily regulated at the transcriptional level. Transgene expression from the nuclear genome offers several advantages, including inducible expression, heterologous protein-targeting to various compartments within the cell as well as secretion, and post-translational modifications (Leon-Banares et al. 2004). However, protein accumulation is often much lower compared to expression in the chloroplast. In addition, nuclear-expressed transgenes are often silenced (see below).

2.2.2 Nuclear Transformation

The first reported nuclear transformation of *C. reinhardtii* was with the yeast *arg4* locus (Rochaix and van Dillewijn 1982). Since then, many techniques, markers, and reporters have been developed for integrating heterologous DNA into the nuclear genome. Methods used for nuclear transformation include particle bombardment (Debuchy et al. 1989; Kindle et al. 1989; Mayfield and Kindle 1990b), agitation of cell wall deficient strains with glass beads (Kindle 1990), electroporation (Shimogawara et al. 1998), agitation with silicon-carbide whiskers (Dunahay 1993), and biologically mediated gene transfer by *Agrobacterium tumefaciens* (Kumar et al. 2004).

Transformation into the nuclear genome of *C. reinhardtii* occurs primarily by random insertion through non-homologous end joining (Tam and Lefebvre 1993). Transformation with linear DNA promotes insertion of multiple copies in one locus, and leads to fewer large deletions or rearrangements (Cerutti et al. 1997a; Kindle 1998). Because transgene insertion is, for the most part, random, nuclear transformation may lead to undesirable off-target effects of disrupted genes or regulatory elements. This can be resolved by phenotypic and genetic screening of transformants. However there are arguable benefits to this phenomenon as well. Strategies such as insertional mutagenesis are being employed on a high-throughput scale to knock-out and characterize genes of unknown function (Dent et al. 2005).

Another consequence of random insertion is position effect: the level of transgene expression can be influenced by the surrounding genomic regions (Leon and Fernandez 2007). Thus, it may be necessary to screen large numbers of transformants to identify genetic individuals that demonstrate the desired level of protein expression.

Targeted gene integration through homologous recombination (HR) has been shown to occur at a low, but measurable frequency (Gumpel et al. 1994; Nelson and Lefebvre 1995; Sodeinde and Kindle 1993; Zorin et al. 2005). The use of single-stranded transforming DNA has been shown to improve the ratio of homologous recombination to nonhomologous integration events, thereby facilitating the isolation of homologous recombinants (Zorin et al. 2005, 2009). There is continued effort to understand and direct homologous recombination for the goal of directed gene replacement and mutagenesis in *C. reinhardtii* (Zorin et al. 2009).

A significant roadblock in maintaining *C. reinhardtii* transformants is that of transgene silencing, especially those with non-essential or cytotoxic products. After a number of generations, if there is no selective pressure to maintain the expression of an exogenous protein, gene silencing is frequently seen. It is thought that methylation or other mechanisms including RNA-mediated silencing are responsible (Cerutti et al. 1997a, b; De Wilde et al. 2000). However, Berthold et al. noted that *aph7* transformants retained resistance to hygromycin B 7 months and beyond without selective pressure, which was thought to be attributed to the similar codon usage and GC content of *aph7* to nuclear DNA versus non-codon optimized transgenes (Berthold et al. 2002).

2.2.3 Translational and Transcriptional Control of Transgenes

Effective promoters found to work in *C. reinhardtii* are derived from endogenous or chimeric variants of endogenous promoters. The most effective promoters are β_2*TUB* (β_2-tubulin) (Davies et al. 1992), *NIT1* (*NIA1*), inducible by ammonium starvation (Ohresser et al. 1997), *LHCB1* (a protein of the light-harvesting complex of photosystem II) (Fuhrmann et al. 2004; Hahn and Kuck 1999), *HSP70A* (heat-shock protein 70A) (Schroda et al. 2000), *RBCS2* (Rubisco small subunit)

(Goldschmidt-Clermont and Rahire 1986; Kozminski et al. 1993; Nelson et al. 1994), and *PSAD* (subunit of photosystem I) (Fischer and Rochaix 2001). Heterologous gene expression from the *RBCS2* promoter/5′ UTR was shown to be improved by the addition of the first intron of the *RBCS2* gene as an enhancer element (Lumbreras et al. 1998). Expression from this promoter/5′ UTR was further improved by fusing it to the HSP70A promoter, which seems to act as an activator (Lodha et al. 2008; Schroda et al. 2002, 2000; Wu et al. 2008).

Similar to other heterologous gene expression from the chloroplast genome, transgene expression from the nuclear genome is enhanced by codon-optimization to reflect high frequency nuclear codon usage (Fuhrmann et al. 2004, 1999; Heitzer and Zschoernig 2007; Mayfield and Kindle 1990a).

2.2.4 Selection

There are now a variety of different selection methods for isolating nuclear transformants. The first selection markers rescued auxotrophic mutants, such as the classic *ARG7* for arginine biosynthesis (Debuchy et al. 1989; Rochaix and van Dillewijn 1982), *NIT1* encoding nitrate reductase (Kindle et al. 1989), *NIC7* for nicotinamide biosynthesis and *THI10* for thiamine biosynthesis (Ferris 1995), *atpC* or *OEE1* to restore photosynthetic capability (Mayfield and Kindle 1990a; Smart and Selman 1993), and *oda6* or *pf-14* to restore flagellar motility (Diener et al. 1990; Mitchell and Kang 1991).

The first selectable marker for the antibiotic kanamycin was reported in 1985, by using aminoglycoside 3′-phosphotransferase (APH) (Hasnain et al. 1985). In the following years, other markers for resistance genes were developed to overcome the need for strains with specific mutant backgrounds, and the need for time-consuming selection and screening for hard to identify phenotypes (Table 6.1). Antibiotic resistance genes include *CRY1* for cryptopleurine and emetine (Nelson et al. 1994), *sh-ble* for zeocin and phleomycin (Stevens et al. 1996), *aadA* for spectinomycin (Cerutti et al. 1997a), *aphVIII* for kanamycin, paromomycin and neomycin (Sizova et al. 2001), and *aph7* for hygromycin B (Berthold et al. 2002). Resistance to zeocin and phleomycin, which induce DNA double-strand breaks, occurs through the binding of the *ble* gene product to the antibiotic in a 1:1 ratio. Thus high level of expression is necessary for stringent selection, whereas the *aph7* gene product enzymatically modifies the antibiotic hygromycin B, and can survive at much higher concentrations with lower protein accumulation.

2.2.5 Expression of Foreign Genes

Reporter genes offer another quantitative readout of protein expression, localization, and accumulation. The first reporter successfully used *in vivo* was *ars*, encoding an arylsulfatase that can cleave a chromogenic substrate for rapid read-out (Davies et al. 1992). Notable others that have followed are *cgfp* encoding a codon-optimized green fluorescent protein (Fuhrmann et al. 1999), *YFP* (Neupert et al. 2009), *uidA* encoding beta-glucuronidase, a commonly used plant reporter (Kumar et al. 2004), Renilla luciferase (Fuhrmann et al. 2004) and Gaussia luciferase (Shao and Bock 2008). An example of the current yields of protein expression in the nucleus is the accumulation of GFP to 0.2% total soluble protein (Neupert et al. 2009). This was accomplished by alternating antibiotic selection and UV light-induced mutagenesis, then transforming *cgfp* into the individuals with the highest antibiotic resistance.

2.2.6 Gene Silencing in the Green Alga *Chlamydomonas reinhardtii*

Post-transcriptional gene silencing can be accomplished through RNA interference (RNAi) in *C. reinhardtii*, and efforts have been made to optimize this method for gene function discovery and metabolic engineering (Fuhrmann et al. 2001; Molnar et al. 2009; Rohr et al. 2004; Schroda et al. 1999; Zhao et al. 2008). Because direct gene knock out via homologous recombination is rare (Nelson and Lefebvre 1995), knock down approaches like RNA-mediated silencing has become a valuable reverse genetics tool to study gene function and could potentially be used for algal genetic engineering for biofuels production (Grossman 2000; Wilson and Lefebvre 2004; Cerutti et al. 2011).

Chlamydomonas reinhardtii contains Dicer and Argonaute (AGO) proteins, the necessary machinery to perform RNAi (Casas-Mollano et al. 2008; Cerutti and Casas-Mollano 2006) thus allowing for the development of strategies to knock down the expression of specific genes. The first gene to be successfully silenced in *C. reinhardtii* was *HSP70B*, a chloroplast localized chaperone (Schroda et al. 1999). The authors used the *HSP70-RBCS2* promoter fused to an antisense copy of *HSP70B* to knock down expression. Improvements were made to the antisense technique through the expression of inverted repeat RNAs (Fuhrmann et al. 2001). An alternative approach was taken by Rohr et al. (2004), who developed a construct with a tandem inverted repeat consisting of the 3′ UTR of *MAA7* and the 3′ UTR of the target gene fused to the antisense sequences by a short linker. *MAA7* encodes the tryptophan synthase β-subunit, which can metabolize 5-fluoroindole into a toxic analogue. Thus, transformants selected on 5-fluoroindole in the presence of tryptophan will have most likely silenced *MAA7* and the target gene as well. This strategy enables better identification of transgenic lines showing effective gene silencing and limits the epigenetic silencing of RNAi constructs (Rohr et al. 2004).

Recently, *C. reinhardtii* was shown to contain microRNAs (Molnar et al. 2007; Zhao et al. 2007). MicroRNAs (miRNAs) are naturally occurring small double stranded RNAs (dsRNAs) that function to down-regulate endogenous genes by mRNA cleavage (Bartel 2004). Previously, it was believed

that unicellular organisms did not produce miRNAs and that this characteristic evolved along with the development of multicellular organisms (Allen et al. 2004). However, it was recently demonstrated that *C. reinhardtii* contains miRNAs that are similar to those from land plants (Molnar et al. 2007; Zhao et al. 2007). The endogenous miRNAs in land plants differ from those of animals in that they generally do not have many offsite targets and cleave only a small number of mRNAs. In addition, the miRNA precursors only yield one miRNA in *C. reinhardtii* and are also not normally associated with transcriptional silencing (Molnar et al. 2009). These characteristics allow miRNAs to be highly specific for their mRNA target making an artificial miRNA system a desirable genetic tool.

Recently, multiple laboratories have developed artificial miRNA (amiRNA) gene silencing systems for *C. reinhardtii* by generating amiRNAs based on endogenous miRNA precursors (Molnar et al. 2009; Zhao et al. 2008). The Baulcombe laboratory generated amiRNA precursors that were used to silence *COX90*, a nuclear encoded gene involved in oxidative phosphorylation that is targeted to the mitochondria, and *phytoene synthase*, which catalyzes the first step in the carotenoid biosynthetic pathway (Molnar et al. 2009). In the Qi laboratory, a similar strategy was employed to silence the *RBCS1/2* gene (Zhao et al. 2008). This construct also combined the amiRNA technique with a similar one developed in 2004 by Rohr et al. and used a dimeric RNAi precursor to simultaneously silence the *MAA7* gene allowing for additional negative selection (Rohr et al. 2004). This strategy decreased the message accumulation of the *RBCS1/2* gene to 10% and the *MAA7* message to 0.5% of wild-type levels, as opposed to 15–40% *RBCS1/2* mRNA when not using the dimeric amiRNA precursor (Zhao et al. 2008).

These continuous improvements to gene silencing have given rise to a new era of genetics in *C. reinhardtii.* The ability to now perform reverse genetics will undoubtedly prove helpful in our understanding of microalgae. These advances allow us to knock down the expression of targeted genes to levels where we can study their biological functions, as well as enable more sophisticated genetic and metabolic engineering.

3 Genetic Engineering of Other Algae

While the molecular toolkit of *C. reinhardtii* is highly developed, it seems unlikely that this freshwater green alga will be a commercial biofuel producer. Prior to the recent investigation of microalgae for biofuels, only a few species have been genetically modified outside of *Chlamydomonas*. These include other green algae, diatoms, dinoflagellates, and red algae (see (Walker et al. 2005) for a comprehensive review) (Table 6.2). As with *C. reinhardtii*, successful transformation strategies include microparticle bombardment, electroporation, and agitation with silicon whiskers. For the majority of species that have been transformed, the initial experiments involved complementation of a known mutation or introduction of a resistance marker. These methods and vectors, discussed below, are valuable tools that should enable further genetic and metabolic engineering of these species.

3.1 Green Algae

In addition to *C. reinhardtii*, nuclear transformations of several species of green algae have been reported (Table 6.2). *Volvox carteri* is a spheroid multicellular colonial green alga that has been transformed with *nitA* to rescue an auxotroph mutant (Schiedlmeier et al. 1994), the bleomycin resistance gene *ble* (Hallmann and Rappel 1999), the paromycin resistance gene *aphH* (Jakobiak et al. 2004) and the hexose/H^+ symporter gene *HUP1* from *Chlorella kessleri* (Hallmann and Sumper 1996). The primary strategy for transformation of *V. carteri* is through particle bombardment. *Haematococcus pluvialis* is an important producer of the keto-carotenoid astaxanthin and has been transiently and stably transformed by particle bombardment (Steinbrenner and Sandmann 2006b; Teng et al. 2004). The halotolerant green microalga *Dunaliella salina*, which is used to produce β-carotene, has been successfully transformed by particle bombardment (Tan et al. 2005), electroporation (Geng et al. 2004; Sun et al. 2005), and agitation with glass beads (Feng et al. 2009). Reports of successful nuclear transformation of several *Chlorella* species, including *C. ellipsoidea*, *C. saccharophila*, *C. sorokiniana* and *C. vulgaris* have also been published (Walker et al. 2005). Particle bombardment and protoplast electroporation are the primary methods for transformation of most *Chlorella* species, however initial attempts only generated transient transformants. Furthermore, evidence suggests that nuclear transformation of *C. sorokiniana* proceeds through homologous recombination (Dawson et al. 1997). Finally, stable nuclear transformation of the volvocine alga *Gonium pectorale* was achieved by microparticle bombardment (Lerche and Hallmann 2009). The heterologous genes *aphVIII* and luciferase were expressed under the control of *C. reinhardtii* or *V. carteri* promoters and UTRs.

3.2 Diatoms

Diatoms are a large and diverse group of unicellular eukaryotic algae characterized by their unique cell walls made of silica called a frustule (for a comprehensive review see Bozarth et al. 2009). Diatoms play a vital role in the oceans as an important source of biomass as well as CO_2 fixation and O_2 production. They are also important commercial sources for feed for aquaculture and specialty oils such as omega-3 fatty acids (Apt and Behrens 1999). In addition, there has been recent interest in developing uses for diatoms in nanotechnology (Hildebrand 2005).

Table 6.2 Strategies for genetic transformation of algae

Species/variety	Class	Method	References
Nuclear transformation			
Chlamydomonas reinhardtii	Chlorophyceae	Bombardment, Si Carbide Whisker, Electroporation, Glass Bead, Agrobacterium	Debuchy et al. (1989), Dunahay (1993), Kindle et al. (1989), Kindle (1990), Kumar et al. (2004), Mayfield and Kindle (1990b), Rochaix and van Dillewijn (1982), Shimogawara et al. (1998)
Dunaliella salina	Chlorophyceae	Electroporation, Bombardment, Glass Beads	Feng et al. (2009), Geng et al. (2004), Sun et al. (2005), Tan et al. (2005)
Gonium pectorale	Chlorophyceae	Bombardment	Lerche and Hallmann (2009)
Haematococcus pluvialis	Chlorophyceae	Bombardment	Steinbrenner and Sandmann (2006b), Teng et al. (2004)
Volvox carteri	Chlorophyceae	Bombardment	Hallmann and Rappel (1999), Hallmann and Sumper (1996), Jakobiak et al. (2004), Schiedlmeier et al. (1994)
Cyclotella cryptica	Diatom	Bombardment	Dunahay et al. (1995)
Cylindrotheca fusiformis	Diatom	Bombardment	Fischer et al. (1999), Poulsen and Kroger (2005)
Navicula saprophila	Diatom	Bombardment	Dunahay et al. (1995)
Phaeodactylum tricornutum	Diatom	Bombardment	Apt et al. (1996), Falciatore et al. (1999), Zaslavskaia et al. (2000)
Thalassiosira weissflogii	Diatom	Bombardment	Falciatore et al. (1999)
Thalassiosira pseudonana	Diatom	Bombardment	Poulsen et al. (2006)
Amphidinium spp.	Dinoflagellate	Si Carbide Whisker	ten Lohuis and Miller (1998)
Symbiodinium microadriaticum	Dinoflagellate	Si Carbide Whisker	ten Lohuis and Miller (1998)
Chlorella ellipsoidea	Trebouxiophyceae	Protoplast Transformation	Jarvis and Brown (1991)
Chlorella saccharophila	Trebouxiophyceae	Electroporation	Maruyama et al. (1994)
Chlorella vulgaris	Trebouxiophyceae	Protoplast Transformation	Hawkins and Nakamura (1999)
Chlorella sorokiniana	Trebouxiophyceae	Bombardment	Dawson et al. (1997)
Porphyra yezoensis	Rhodophyta	Bombardment	Cheney et al. (2001)
Chloroplast transformation			
Euglena gracilis	Euglenaceae (Kingdom: Excavata)	Bombardment (transient)	Doetsch et al. (2001)
Chlamydomonas reinhardtii	Chlorophyceae	Bombardment	Boynton et al. (1988)
Porphyridium spp.	Porphyridiophyceae	Bombardment	Lapidot et al. (2002)

To date, several species of diatoms have been transformed (Table 6.2), but *Phaeodactylum tricornutum* has the most developed molecular toolkit (Bozarth et al. 2009). In each case, nuclear transformation was achieved with microparticle bombardment. A common promoter for heterologous gene expression in *P. tricornutum* is the *fcpA* (fucoxanthin chlorophyll binding protein) promoter and markers such as *ble*, *cat*, and *nptII* have been used (Apt et al. 1996; Dunahay et al. 1995; Falciatore et al. 1999; Zaslavskaia et al. 2000). Importantly, a recent report demonstrated successful RNA silencing in *P. tricornutum*, opening the way for reverse genetics in diatoms (De Riso et al. 2009). As new genomes are sequenced, RNAi should be applicable to many algae species.

3.3 Dinoflagellates

Dinoflagellates are a diverse group of eukaryotic algae that are found in both marine and freshwater habitats. Most are unicellular and about half are photosynthetic. To date, there has only been a single report of nuclear transformation of dinoflagellates (ten Lohuis and Miller 1998). Three different bacterial genes were introduced, *nptII* (G418 resistance), *hpt* (hygromycin B resistance), and the reporter gene *β-glucuronidase* (GUS) into two dinoflagellates, *Amphidinium* sp. and *Symbiodinium microadriaticum*, by agitation with silicon whiskers (ten Lohuis and Miller 1998). Each gene was fused to a heterologous promoter and appeared to be stably integrated into the nuclear genome.

3.4 Red Algae

There have been several reports on the transformation of red algae: plastid transformation of *Porphyridium* spp. by microparticle bombardment (Lapidot et al. 2002) (see below), nuclear transformation of the red macroalgae *Por-phyra*

yezoensis by *Agrobacterium*-mediated gene transfer (Cheney et al. 2001), and nuclear transformation of *Cyanidioschyzon merolae* (Minoda et al. 2004).

3.5 Plastid Transformation

While many tools exist for nuclear genetic engineering in algae, plastid genetic engineering has a more limited set. Indeed, only *C. reinhardtii* has well established protocols for plastid transformation, and only two other algae species have been successfully transformed, *Euglena gracilis* (Doetsch et al. 2001) and *Porphyridium* spp. (Lapidot et al. 2002) (Table 6.2). *E. gracilis* was transformed with *aadA* (streptomycin/spectinomycin resistance) under the control of endogenous promoter and UTR elements. *Porphyridium* was transformed with a mutant version of the endogenous gene encoding for acetohydroxyacid synthase that confers resistance to the herbicide sulfometuron methyl. In both cases, microparticle bombardment was used to deliver the transforming DNA, which stably integrated by homologous recombination (Doetsch et al. 2001; Lapidot et al. 2002).

3.6 Inducible Systems

Many algae respond to different environmental or developmental factors by upregulating or downregulating a variety of genes. In the diatoms the fucoxanthin, chlorophyll a/c-binding protein gene (FCP) promoter of *P. tricornutum* has been shown to regulate a firefly luciferase protein in a light inducible manner (Falciatore et al. 1999). In addition a nitrogen inducible system has been developed using the nitrate reductase promoter and terminator for both *Cylindrotheca fusiformis* and *Thalassiosira pseudonana* (Poulsen et al. 2006; Poulsen and Kroger 2005). In *Volvox* reports of arylsulfatase and the ISG glycoprotein promoters have both been demonstrated as potential inducible systems (Hallmann and Sumper 1994).

3.7 Riboswitches

In addition to regulation of the transcript, it has been found that a number of genes in algae are regulated at the translational level through riboswitches. In *Chlamydomonas reinhardtii* the thiamine pyrophosphate (TPP) riboswitch has been shown to be functional for regulating a luciferase reporter gene in the presence or absence of thiamine and similar sequences have been identified in *Volvox carteri* suggesting that this mechanism may be usable in other algae species (Croft et al. 2007).

4 Metabolic Engineering of Microalgae

Despite the increasing popularity of algae as a biofuel production platform, few reports have directly demonstrated genetic engineering for improving strains for biofuel production. However, considerable genetic engineering efforts for strain improvement, biohydrogen production, and metabolic engineering for carotenoid production have been undertaken, as discussed below. Furthermore, it is conceivable that most of the ongoing research in the lipid-based biofuel field has yet to be published due to its potential for commercial applications.

4.1 Enhanced Lipid Production

At the time of this writing, there is only one report that directly attempts to increase lipid production in microalgae. For this purpose the acetyl-CoA carboxylase (ACCase) gene, which is the enzyme that catalyzes the first committed step for fatty acid synthesis, was cloned from the diatom *Cyclotella cryptica* (Roessler and Ohlrogge 1993). Expression vectors were made and transformation protocols were developed for *C. cryptica* and the diatom *Navicula saprophila*. The authors reported a two to threefold increase in the level of ACCase activity for the transformed diatoms but no increase in fatty acid accumulation was detected. However, no experimental data was presented for the increase of ACCase activity (Dunahay et al. 1996). Furthermore, metabolic engineering of *E. coli* and plants has shown that overexpression of ACCase alone is not a good strategy, probably because it might not be a limiting step or there is an additional bottleneck in the pathway (Courchesne et al. 2009). Although this approach did not have the desired results, it was an excellent proof of concept that a metabolic engineering system could be developed from scratch for a target strain of microalgae.

4.2 Enhanced Hydrogen Production

Hydrogen has also been studied as an alternative for algae-derived fuels (Rupprecht 2009). *C. reinhardtii*, *Chlamydomonas moewusii*, *Scenedesmus obliquus*, *Lobochlamys culleus* and *Chlorella fusca* have been shown to produce hydrogen under anaerobic conditions in the presence of light. However, this process is not very efficient and difficult to reproduce at a large scale (Ghirardi et al. 2000; Meuser et al. 2009). Oxygen sensitivity of hydrogenase, anaerobiosis induction, avoiding competition for electrons from other pathways and increasing the sources of electrons are all steps that could be improved through genetic engineering (Beer et al. 2009; Melis et al. 2007). Using a classical genetic approach a mutant strain of *C. reinhardtii* (Stm6) has been selected that has a block in

cyclic electron flow through PSI (less competition for electrons), has increased accumulation of starch (source of H^+ and e^-) and has lower intracellular concentrations of oxygen (inhibitor of hydrogenase) (Kruse et al. 2005). Furthermore this strain has been engineered with a hexose transporter for supplying an additional source of protons and electrons (Doebbe et al. 2007) (see Trophic Conversion section below). The standard method for inducing anaerobiosis has been sulfur deprivation (Melis et al. 2000), but genetic engineering has also been used for this purpose. *C. reinhardtii* has been transformed with an antisense construct against a sulfate transporter gene (SulP), leading to the accumulation of hydrogen even in the presence of 100 μM sulfate. By contrast, wild-type cells could not reach an anaerobic state under those conditions and therefore could not photoproduce hydrogen (Chen et al. 2005). Another system for achieving anaerobiosis is based on a copper responsive nuclear transgene that is necessary for the expression of protein D2 of PSII. Upon the addition of copper, the transgene is repressed, along with D2, thus stopping evolution of oxygen through PSII. A brief period of anaerobiosis and hydrogen production is achieved, but this is much shorter than the standard method through sulfur deprivation (Surzycki et al. 2007).

4.3 Light-Harvesting Antennae Engineering

Microalgae have evolved large light-harvesting antennae for maximizing light absorption under low-light conditions. Unfortunately, this implies an excess of light absorption under artificial culture conditions (high light), which is dissipated through heat and fluorescence quenching. Furthermore, the excess energy that cannot be dissipated results in photodamage. Additionally, the large size of the light-harvesting complexes also limits light penetration into the culturing media, therefore lowering the maximum cell density that can be achieved for a given bioreactor (Mussgnug et al. 2007). To overcome this, a single RNAi construct has been effectively used for silencing all 20 light-harvesting complex (LHC) protein isoforms of *C. reinhardtii*. These cells have lower mRNA (0.1–26% relative to wild-type) and protein accumulation for all LHC genes and 68% less chlorophyll than the parental strain, resulting in 290% higher light transmittance in the culture. Furthermore they present less fluorescence quenching, which leads to an increase in photosynthetic quantum yield. Under high light conditions, transformed cells were less susceptible to photoinhibition (measured as the decrease in oxygen evolution) and grew at a faster rate (Mussgnug et al. 2007). However, transformed cells did not reach a higher cell density, which is considered a highly desirable trait for biofuel production. The same group achieved similar results by downregulating LHC expression at the translational level. NAB1 is a repressor of LHCBM translation (Mussgnug et al. 2005) and its activation is redox dependent (Wobbe et al. 2009). A constitutively activated version of NAB1 (2 amino acid mutations) was overexpressed in *C. reinhardtii*, resulting in a similar phenotype to the one obtained through RNAi. However, the effects were less dramatic, having a chlorophyll/cell reduction of 20% compared to that of 68% using RNAi (Beckmann et al. 2009). Nonetheless, over-expression of a single repressor is easier to reproduce than the silencing of 20 LHC isoforms.

4.4 Trophic Conversion

Growing microalgae under heterotrophic or mixotrophic conditions via the addition of a reduced carbon source has several advantages. Fermentation systems have been widely studied and successfully applied in industry. The culture conditions are highly controlled and reproducible. Moreover, heterotrophic cultures of microalgae achieve higher cell densities, thus resulting in lower harvesting costs (Chen and Chen 2006). Furthermore, heterotrophic cultivation of *Chlorella protothecoides* for biofuel production resulted in four-fold higher lipid accumulation than autotrophic cultures (Miao and Wu 2006). However, many microalgae species are strict autotrophs, a problem that could be circumvented by genetic engineering. *V. carteri* has been transformed with a hexose transporter (*HUP1*) from *Chlorella*, resulting in a strain that can survive on glucose under dark conditions (Hallmann and Sumper 1996). *P. tricornutum*, *Cylindrotheca fusiformis* (diatoms) and *C. reinhardtii* have also been successfully transformed with *HUP1*, resulting in glucose transport into the cells (Doebbe et al. 2007; Fischer et al. 1999; Zaslavskaia et al. 2001). It is worth noting that despite glucose incorporation, the extent of conversion to full heterotrophy is variable between these four algae. It is also particularly interesting that transformed *C. reinhardtii* cells produce 150% more hydrogen than the parental strain upon induction in the presence of glucose (Doebbe et al. 2007).

Trophic conversion is a good proof of concept for microalgae metabolic engineering, but despite the advantages of heterotrophic culture, it might not to be optimal for biofuel production. Additional costs for adding a carbon source (which could be directly used for producing ethanol) and the requirement for enclosed bioreactors (given the higher risk of contamination) are major drawbacks compared to phototrophic systems.

4.5 Metabolic Engineering of Carotenoids

Dunaliella salina and *Haematococcus pluvialis* are the main sources for commercial production of beta-carotene and astaxanthin, respectively. Other microalgae also show great

potential for producing other relevant carotenoids such as lutein (Dufosse et al. 2005). Despite this, there are few reports on metabolic engineering for improved carotenoid production in algae. Wong attempted to produce keto-carotenoids (e.g. astaxanthin) in *C. reinhardtii* by overexpressing beta-carotene ketolases from *H. pluvialis* (*bkt3*) and *C. reinhardtii* itself (*CRBKT*) (Wong 2006). After extensive efforts using different expression vectors, no keto-carotenoids were obtained. Leon et al. (2007) followed a similar approach but instead used the *bkt1* gene from *H. pluvialis* (Leon et al. 2007). They could detect small accumulation of 4-keto-lutein, which was not present in the parental strain. However, no astaxanthin was detected. *H. pluvialis* has been stably transformed with an herbicide-resistant version of its phytoene desaturase (*PDS*) gene for enhanced astaxanthin accumulation. This approach led to 26% higher astaxanthin accumulation 48 h after induction with high-light when compared to the wild-type strain (Steinbrenner and Sandmann 2006a).

Isoprenoids, such as carotenoids, can also be regarded as potential fuel molecules. Moreover, highly valuable carotenoids could be recovered as co-products along with storage lipids, thus subsidizing the production costs for microalgae biofuels. Fukusaki et al. (2003) successfully expressed an archeal gene encoding a thermostable version of the geranylgeranyl-pyrophosphate (GGPP) synthase enzyme involved in isoprenoid biosynthesis in the chloroplast of *C. reinhardtii*, although there was no effect on isoprenoid biosynthesis (Fukusaki et al. 2003). It is expected that metabolic engineering of carotenoids in algae will be an increasingly growing research field, as it is in higher plants.

5 Other Areas Appropriate for Genetic Engineering for Algal Biofuels Production

5.1 Crop Protection

Perhaps no area of large-scale algal production has so little information as crop protection. In terrestrial agriculture, crop protection is one of the most important aspects of a successful harvest, and clearly this will also be the case for large-scale algal production. A number of strategies have been employed to protect terrestrial crops from insects, fungi, bacteria and viruses. The most notable of these protection strategies are the introduction of resistance genes from one species or race to another. In algae, it is not yet even known if resistance genes exist, let alone how they might function. This is one area of algal biofuel production where years of basic research will be required before a coherent strategy for crop protection emerges.

One advantage that algae may have over terrestrial crops in this area is an ability to screen large populations of cells to identify potential resistance genes. Once these genes are identified it should also be possible to quickly introduce them into production species using molecular techniques, rather than the traditional breeding schemes generally used in terrestrial crops, which can take years to introduce a new resistance trait.

5.2 Co-Products

For biofuel production the optimal yield of oil is likely to be in the 30–40% range. This leaves 60–70% of the biomass as residual biomass, and this residual biomass must be monetized in order for biofuels to be economically viable. One way to realize economic return from this biomass is to generate valuable co-products from the remaining protein component. Depending upon the algae used for production this protein could be in the form of animal feed, in which case the entire residual biomass would have value. The protein co-product could also be a specific protein or industrial enzyme, which might require purification, but in theory could have sufficient value on its own to produce a significant economic return. Other co-products might include biopolymers or other high value small molecules.

5.3 Altered Metabolic Profiles

Clearly an ability to produce greater quantities of oil, or to produce higher quality oils will be important factors in developing economically viable biofuels from algae. Algae naturally produce storage lipids, and tend to increase accumulation when they are placed under light, nutrient or temperature stress. As growing algae under stress is unlikely to produce acceptable amounts of biomass we will need to understand, and then engineer, an ability to increase lipid accumulation without stress induction. Once we have identified the molecular events that result in lipid accumulation under stress, we should be able to engineer algae that constitutively accumulate large amounts of lipid. Likewise, we should be able to engineer in metabolic enzymes that alter the composition of oils within algae. However, as in any biological system, this is unlikely to be as simple as inserting a single biosynthetic enzyme, as internal control of carbon flux is always operating within any cell.

5.4 Improved Harvestability

Efficient harvesting is a key component of the economics of modern agriculture, and will be a key component of the economics of large-scale algal production. There are a number of strategies for improving harvestability of

algae, including developing strategies for aggregation of algae using molecules produced directly within the algae. This so-called bioflocculation has a number of challenges including developing a strategy that is timed to coincide with the end of the life cycle of the algae, and one that does not detract from the ability of the algae to produce large amounts of oil.

5.5 Improved Nutrient Utilization and Recycling

Algae are already very efficient at taking up nitrogen and phosphate from the surrounding media, but if algae are going to be used to produce significant amounts of fuel, we will need to develop efficient strategies for nutrient recycling. Some of these will certainly require chemical engineering and not necessarily biological alterations, but some may require that the algae utilize forms of nutrients that they do not presently use. Potentially these new catabolic pathways can be engineered into algae from other microorganism that do utilize other forms of nitrogen, phosphate and other macro-elements, and some may simply require the correct transporter system to get these minerals into the cells. In either case, we have the potential to expand the repertoire of nutrients utilized by algae, and therefore improve the overall efficiency of the system.

6 Concluding Remarks

In general the academic efforts to date to genetically engineer eukaryotic microalgae have mainly involved the development of tools to answer a specific biological question. These techniques have then been co-opted by other groups to answer a different question, but few systematic approaches have been undertaken to develop the suite of tools necessary to realize the full potential of algae as a biotechnology platform. As the push for algae biofuels increases, it will not be surprising to see academic groups and companies focus on developing a more comprehensive set of tools that will allow for full genetic manipulation of a variety of algal species. The types of tools that may be developed will most likely take their cues from other genetic systems. This includes, but is not limited to, fluorescent or luminescent reporter systems, tags that promote easy protein purification, improved selection markers, improved expression and inducible systems, and improved homologous gene replacement. In addition vectors that work in a broad range of algae species may be in high demand. The biotechnology revolution has been ongoing for several years, and algae now seem poised to become a part of this new industry and to lead biotechnology into a new green era.

Acknowledgements This work was supported by a grant to SPM from the US Air Force (FA9550-09-1-0336) and a grant to BAR from the San Diego Foundation (C-2008-00296). JAG and MT were supported by Skaggs pre-doctoral fellowships.

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Photobioreactors for Microalgal Biofuel Production

7

Graziella Chini Zittelli, Liliana Rodolfi, Niccoló Bassi, Natascia Biondi, and Mario R. Tredici

1 Introduction

Biofuels have the potential to reduce the world's dependence on fossil fuels, but their production suffers from severe limitations like the requirement of vast areas of land and competition with food production (Brennan and Owende 2010; Mata et al. 2010). To be beneficial, biofuels must be produced without impacting on arable land or tropical rainforests and provide significant greenhouse-gas emissions savings compared to fossil fuels, characteristics which are expected in the so called "second generation biofuels", like cellulosic ethanol. Microalgae present several advantages over higher plants as source of second (or even third) generation biofuels. Microalgae cultivation shows less dependency on seasonal variations and requires less freshwater than conventional agriculture, making cultivation in arid regions possible (Mata et al. 2010; Tredici 2010; Wijffels and Barbosa 2010). Microalgae cultivation does not require herbicide or pesticide applications (Rodolfi et al. 2009; Williams et al. 2009). Microalgae can fix CO_2 efficiently from different sources, including industrial exhaust gases, and can use nutrients contained in wastewaters for their growth (Huntley and Redalje 2007; Wang et al. 2008; Brennan and Owende 2010). The combination of wastewater treatment, CO_2 fixation, and biofuel production through microalgae represents a promising alternative to current CO_2 mitigation strategies. Besides oil, microalgae can accumulate sugars, which can be fermented to produce bioethanol (Huesemann and Benemann 2009) and, above all, these microorganisms can synthesize many valuable co-products such as proteins, vitamins, hormones, polyunsaturated fatty acids that can be commercialized to integrate foods and feed (Brennan and Owende 2010; Tredici et al. 2009). Finally, the whole algal biomass or the residue after extraction of oil, carbohydrate or any other specific product can be anaerobically digested to obtain biogas or be gasified to produce syngas (Huesemann and Benemann 2009; Sialve et al. 2009; Mussgnug et al. 2010). The most important advantage of microalgae as source of biofuels is that they can be cultivated on land unsuitable for agriculture using saline or brackish waters. A limitation is that, differently from plants that obtain carbon from air, algae cultures must be supplied with CO_2 to be productive. The need to dissolve large amounts of CO_2 in the growth medium, wrongly perceived as an advantage, is an energy-intensive and expensive requirement of algae mass cultures, worsened by the fact that very few large-CO_2 emitters are located in the regions more suitable for year-round, large-scale algae production (e.g., dry tropical coastal regions) (Darzins et al. 2010).

In spite of the inherent potential of microalgae as a renewable fuel source and the many promises of recent years, there is no current industrial production of algae biofuel in the world. The higher capital and operating costs of microalgae farming compared to conventional agriculture, the non-sufficiently positive energy balance (after accounting for energy requirements for water pumping, mixing, CO_2 and nutrient supply, biomass harvesting and processing), and the not yet established sustainability (Lardon et al. 2009; Clarens et al. 2010; Borowitzka and Moheimani 2010) still prevent the development of this technology to commercial scale.

Today, microalgae (including cyanobacteria) biomass for commercial exploitation is either harvested from natural habitats or obtained through more or less controlled cultivation processes (Tredici 2004; Tredici et al. 2010). Commercial production of algae amounts to about 20,000 t annually, mainly marketed as high-value human nutritional supplements, specialty animal feeds and pharmaceutical products

G. Chini Zittelli
Istituto per lo Studio degli Ecosistemi, CNR, Via Madonna del Piano 10, 50019 Sesto Fiorentino, Florence, Italy
e-mail: graziella.chinizittelli@ise.cnr.it

L. Rodolfi • N. Biondi • M.R. Tredici (✉)
Dipartimento di Biotecnologie Agrarie, Università degli Studi di Firenze, Piazzale delle Cascine 24, 50144 Florence, Italy
e-mail :mario.tredici@unifi.it; natascia.biondi@libero.it

N. Bassi
Fotosintetica & Microbiologia S.r.l.,
Via dei Della Robbia 54, 50132 Florence, Italy
e-mail: niccolo.bassi@femonline.it

M.A. Borowitzka and N.R. Moheimani (eds.), *Algae for Biofuels and Energy*, Developments in Applied Phycology 5,
DOI 10.1007/978-94-007-5479-9_7,

(Spolaore et al. 2006; Tredici et al. 2009). Commercial plants use one of the following four technologies:

1. extensive ponds (lagoons);
2. raceway and circular ponds;
3. tubular photobioreactors;
4. fermenters (where algae are grown on organic substrates in the dark).

The shallow raceway pond, in which the suspension is mixed with a paddle wheel, is the most common commercial system in use. All these systems have been considered for algae biofuels. Which technology will dominate the field in the future is not yet clear.

Most of the start-ups in the algae biofuel sector focus on photobioreactors (PBR). The reasons of the preference are:

1. PBR are closed to the atmosphere and protect the cultivated alga to some extent (note that by being closed, PBR are less prone, but not immune, to contamination);
2. growth parameters (e.g., temperature) can be better controlled;
3. due to a higher surface-to-volume (S/V) ratio, PBR allow to reach higher volumetric productivities and cell concentrations;
4. closed systems eliminate or strongly reduce evaporation;
5. since PBR have not been engineered to the extent of other bioreactors in commercial use (fermenters) there is room for improvement (Darzins et al. 2010).

Many different PBR designs have been proposed for biofuel production, few of them have been tested at pilot-scale, none developed at the (large) scale necessary for a complete and correct evaluation. Thus the main issues that impact on the reactor's performance (i.e., suitable construction materials, efficient mixing, heating/cooling, CO_2 supply and oxygen removal), although explored at pilot level, still await evaluation at real scale (Darzins et al. 2010).

Although the main limitations of PBR are the high cost and the reduced scalability (Lehr and Posten 2009; Tredici et al. 2010), with few exceptions, R&D on photobioreactor design is aimed at achieving high photosynthetic efficiencies and at pushing productivity beyond that currently attainable. The main strategies explored to this end are intensive mixing (Richmond 2004), light dilution via large external surfaces or internal light conducting structures (Zijffers et al. 2008a, b), and cultivation of improved or genetically modified strains (Radakovits et al. 2010). Most of this development is still in a very early stage and productivity projections are largely based on data from small-scale experiments. In reality, no company in the algae biofuel field seems to possess yet a mature technology able to compete with fossil fuels and be on the market in the near future.

Open ponds are much cheaper to build and operate than PBR, but they are strongly limited by contamination (by other algae, grazers, bacteria), the degree of which depends on climatic conditions (for example it is very difficult to maintain an open algal culture in the tropics during the rainy season), and the specific strain which is cultivated. Growing algae that require extreme conditions (e.g., high salinity or high pH) alleviates the problem. In fact, current commercial production is mainly based on algae such as *Dunaliella* and *Arthrospira* (*Spirulina*) that require extreme media for growth. Selection of a suitable strain and a favorable location for building the plant is fundamental. For example, some areas of the world (e.g., deserts) provide a more uniform environment that reduces the risk of contamination and the necessity of frequent intervention (for draining, cleaning, re-inoculation) (Darzins et al. 2010). Many believe that the solution is in combined systems (Huntley and Redalje 2007; Rodolfi et al. 2009): photobioreactors for inocula production followed by open ponds for bulk cultivation. Thus, even if the final choice for industrial production of algae biofuel will be open ponds, still reactors will be necessary for the first crucial step of producing strong and viable inocula. The main PBR used in commercial plants and tested at pilot level have been described elsewhere (Carvalho et al. 2006; Tredici 2004; Tredici et al. 2010). This chapter focuses on new designs mainly developed with the scope of biofuel production and/or CO_2 biofixation.

2 Recent Advances in Photobioreactor Design for Biofuel Production

In the last years PBR have much evolved and new designs have been proposed, most of them for research or small scale applications (Carvalho et al. 2006; Lehr and Posten 2009; Tredici et al. 2010). The high capital and operating costs of PBR have limited, and still do limit, their commercial application to the production of high-value products (biomass for aquaculture, food supplements, nutraceuticals, pharmaceuticals). Today, even if significant improvements are expected in large-scale PBR design thanks to new materials and automated process control systems, and by integrating skills in PBR engineering and solar technology (Lehr and Posten 2009; Tredici 2010), it is much debated if PBR will ever be used to produce "low-value" products such as biofuels and feed (Tredici et al. 2009; Tredici 2010; Darzins et al. 2010). For example, intensive research has been recently devoted to vertical systems that dilute light minimizing photosaturation and photoinhibition and thus maximize photosynthetic efficiency (PE) and areal productivity (see below). However, the achievement of a significant light dilution effect through vertical reactors requires large illuminated surface areas per unit ground area and consequently impacts heavily on investment and operating costs.

The principles leading to maximum productivities of algae culture systems are well known (Posten 2009; Tredici et al. 2010):

1. adequate mixing to provide a suitable light-dark cycle to the cells and avoid biofouling;
2. high mass transfer capacity to efficiently supply CO_2 and prevent O_2 build-up;
3. high S/V ratio to increase cell concentration and volumetric productivity;
4. control of temperature at or near the optimum for the cultivated organism;
5. accurate control of pH and CO_2 and nutrient concentrations;
6. adequate harvesting regime to maintain the optimal population density.

Most of the new PBR designs efficiently deal with the above requirements, their main drawback remaining a limited possibility of being scaled-up at low cost.

In the last few years, numerous companies targeting the field of microalgae biofuels have been established, with interesting new ideas or innovative applications of old PBR designs. There is also quite a large number of cooperative projects involving large corporations, including ExxonMobil Corp., Chevron Corp., Royal Dutch Shell Plc, Boeing, Raytheon, Honeywell UOP and General Electric.

In the following paragraphs most of the PBR designs tested at pilot level are described and the main current developments in the algae biofuel field are discussed.

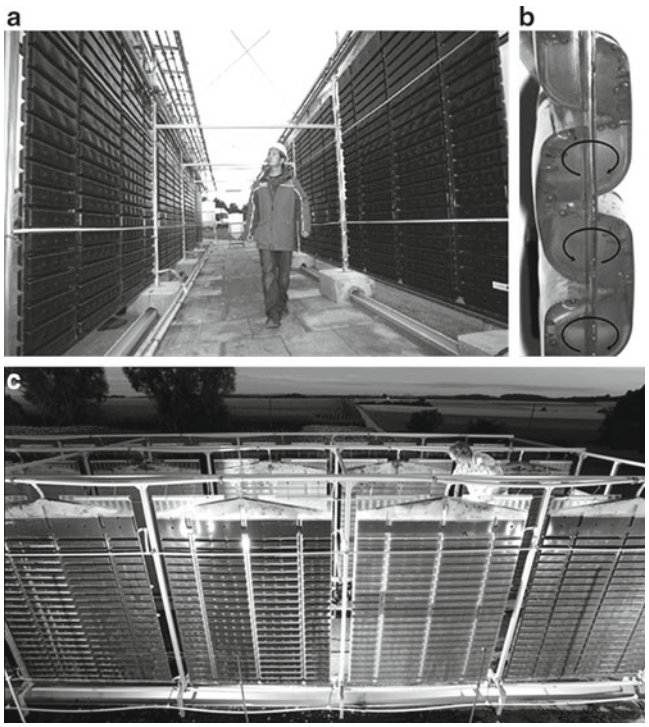

Fig. 7.1 Flat Panel Airlift reactor developed by Subitec GmbH. (**a**) Pilot plant in Hamburg-Reitbrook developed in cooperation with E.ON Hanse AG. (**b**) Side view of the panel with inbuilt baffles for induction of vortices (Courtesy of Subitec GmbH, ©Thomas Ernsting) (**c**) Plant in Eutingen-Weitingen (Stuttgart) developed in cooperation with EnBW AG

2.1 Flat Photobioreactors

Vertical or inclined flat reactors represent very promising culture devices. They can be oriented and tilted at different angles so as to modify the intensity of impinging light and use diffuse and reflected light, which plays an important role in productivity (Qiang et al. 1998; Tredici 2010; Tredici et al. 2010). Flat panels also offer the possibility to be closely packed together and thus attain, by a sort of "lamination" of the culture, high areal productivities (Carlozzi 2003; Wijffels and Barbosa 2010). In vertical or inclined plates, air-bubbling can be adopted for mixing ensuring at the same time adequate turbulence, a good mass transfer capacity, and scouring of the reactor walls. However, for air-bubbling to be efficient, relatively high bubbling rates must be adopted, and the cost of power supply may be high, especially when compared to the cost for mixing in raceway ponds (Bassi et al. 2010). Temperature control may be achieved both by water spraying (evaporative cooling) or by heat exchangers (Rodolfi et al. 2009). Several types of flat photobioreactors have been experimented with at pilot level outdoors (Tredici et al. 1991; Pulz and Scheibenbogen 1998; Degen et al. 2001; Zhang et al. 2001; Aflalo et al. 2007; Rodolfi et al. 2009). Some of the designs showing good scalability are described here.

A Flat Panel Airlift (FPA) photobioreactor, developed and patented in the early 2000s (Degen et al. 2001; Trösch 2002), has been recently implemented and scaled-up by **Subitec GmbH**, a spin-off company of the Fraunhofer-Institute for Interfacial Engineering and Biotechnology (Stuttgart, Germany) (http://en.subitec.com acessed 20 Jan 2011). The reactor is basically a plastic plate divided in large riser zones, in which compressed air is injected, and smaller down-comer zones (Fig. 7.1a, c). The riser zone is subdivided into interconnected horizontal chambers by means of baffles attached alternatively to the two wider sides of the reactor (Fig. 7.1b). The ascending air bubbles induce vortices that move the cells in and out the illuminated layers (Degen et al. 2001). FPA reactors from 3 to 33 L in volume have been successfully employed to grow several microalgae in the laboratory (Degen et al. 2001; Meiser et al. 2004) and outdoors (Schenk et al. 2008). At laboratory scale cell concentrations in the range of 10–16 g L^{-1} and volumetric productivities up to 1.5 g L^{-1} day^{-1} have been achieved with different microalgal species (Ripplinger 2009). Recent developments have been made into the direction of increasing reactor dimensions and reducing power supply from 500 to 200 W m^{-3} (Trösch

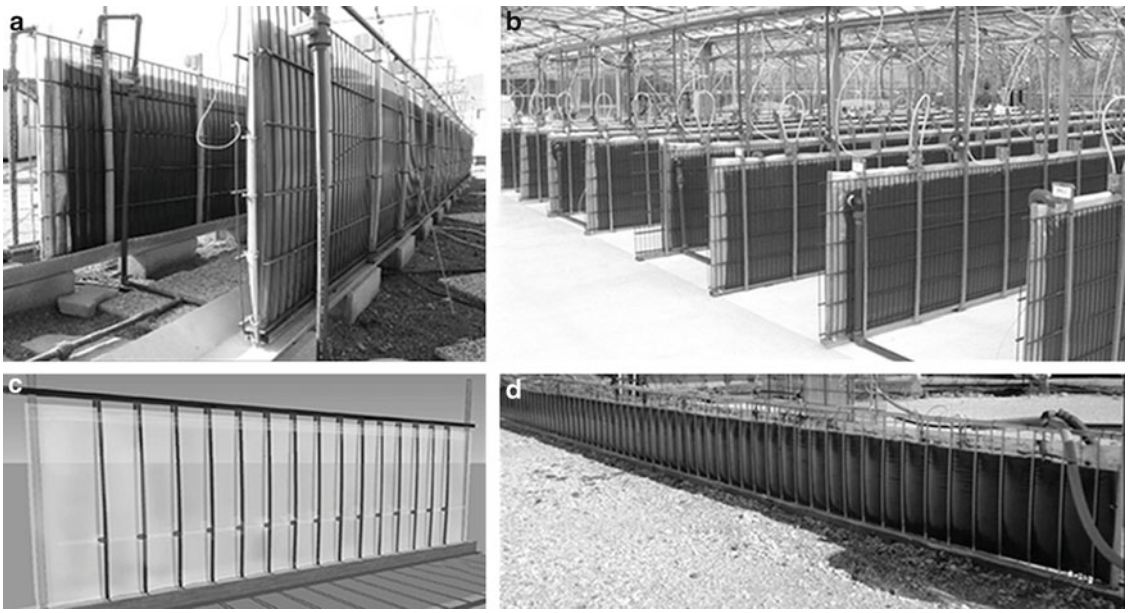

Fig. 7.2 Green Wall Panel (GWP) photobioreactors developed at the University of Florence (2004–2009) and commercialized by F&M S.r.l. (**a**) 20-m-long GWP prototype; (**b**) GWP pilot-scale plant (5,000 L) in operation at Microalghe Camporosso S.r.l. (Italy); (**c**) schematic drawing of a GWP-II; (**d**) 12-m-long GWP-II prototype (Photographs by the authors)

2009). The individual module of FPA reactors of the last generation consists of two deep-drawing polyvinyl chloride (PVC) or glycol-modified polyethylene terephthalate (PETG) shells welded together to form a 2.7-m-high, 5-cm-thick and 1.75-m-long reactor, containing 180 L of culture (Fig. 7.1a, c). The reactor cost is about €1 L^{-1}, equivalent to about €40 m^{-2} of reactor. In 2008 demonstration plants for CO_2 capture from waste gases were built in Hamburg (Germany) (two modules made of four reactors each for a total volume of 1.44 m^3), in cooperation with E.ON Hanse AG (Fig. 7.1a), and in Stuttgart (Germany) (three modules of eight reactors each for a total volume of 4.33 m^3) in cooperation with EnBW AG (Fig. 7.1c). Subitec GmbH is planning the construction of a plant of about 0.5 ha (180 m^3 total volume) in Spain (Ripplinger 2009; Trösch 2009). The main advantages of this system are industrial production of the plastic shells at relatively low-cost, good mixing and short light-path. At 1-ha scale, using 180-L FPA modules and assuming a productivity of 120 t ha^{-1} $year^{-1}$, capital costs of 1.5 M€ (of which 25% for the reactors) and operating costs of €2,200 t^{-1}, a biomass production cost of €4.2 kg^{-1} was calculated.

In 2006 a vertical panel reactor (named Vertigro*)* was developed and patented for production of oil-rich algae (Kertz 2007). The system consists of a series of closely-spaced, vertically-suspended panels made from thin plastic film. Each panel is divided into horizontal channels by welding the film, with the horizontal weldings shortened alternatively at one side or the other so as to allow communication between channels. The culture is pumped at the top of the reactor and circulates by gravity to the bottom in a meandering way. In 2007 a small scale plant was built in El Paso (Texas, USA) by Vertigro Algae Technologies, LLC, a joint venture between **Valcent Products, Inc**. and **Global Green Solutions, Inc**. (http://www.valcent.net/s/NewsReleases.asp?ReportID=344356, accessed 21 Jan 2011) using 3-m-high plastic panels, which were deployed under a greenhouse. The culture was held in an underground tank to maintain a constant temperature. To start the process, a pump pushed the culture up to a holding tank placed 3 m above the top of the plastic panels. Gravity then pulled the culture into the series of panels below. At the bottom, a collection chamber fed back the culture into the underground tank, where oxygen was removed and CO_2 added (Torrey 2008). During a 90-day test, an algal suspension at 1 g L^{-1} was continuously harvested. The company claimed to be able to achieve a productivity of more than 600 tonnes of dry biomass (and approximately 300,000 L of algae oil) per hectare and year (http://findarticles.com/p/articles/mi_pwwi/is_200712/ai_n21152992/?tag=content;col1, accessed 21 Jan 2011) which, if attained solely on solar energy input, is thermodynamically impossible (Tredici et al. 2010). The Vertigro system is interesting for its simplicity, high S/V ratio and verticality. Data on panel durability, oxygen accumulation and energy consumption are not available. Problems in such a high S/V ratio, low-turbulence system may arise at high cell densities that favour biofouling and because of oxygen build-up (Tredici et al. 2010). In 2009 the activity in the algae field of Valcent Products Inc. was closed and Vertigro Algae Technologies LLC dissolved (C. Harding, personal communication).

In the early 2000s the concept of the "disposable panel" was developed by two groups working independently in Italy (Tredici and Rodolfi 2004) and Israel (Boussiba and Zarka

2005). The group in Florence designed and patented the Green Wall Panel (GWP), a flat reactor comprising a culture chamber made of a 0.3-mm-thick flexible low-density polyethylene (LDPE) film enclosed in a rectangular frame of steel grids and vertical uprights. The typical GWP is 1-m-high, 4-cm-thick and 20-m-long and contains about 800 L of culture. Generally, the modules are placed vertically and facing south in parallel rows at a distance of about 1 m that, in Tuscany, prevents shading for most of the year (Fig. 7.2a). For mixing, compressed air is bubbled at the bottom of the reactor through a perforated plastic tube. CO_2 is injected into the culture through gas diffusers placed in unaerated zones. A control unit provides temperature regulation by automatically activating heat exchangers or water spraying on the reactor surface.

The GWP has been used to grow several marine microalgal species outdoors (Rodolfi et al. 2006). With *Tetraselmis suecica* grown in September in panels placed in parallel rows at a distance of 0.8 m wall to wall, it was shown that North-South oriented panels intercepted 22% more solar radiation and achieved a 35% higher areal productivity compared to East-West oriented panels (Rodolfi et al. 2008; Bassi et al. 2010). However, considering that the average annual solar radiation intercepted by vertical full-scale panels at a latitude of 43°N is similar for both orientations (about 7 MJ m^{-2} day^{-1}), annual productivities should not differ significantly between the two arrangements. Sierra et al. (2008) evaluated the fluid-dynamics and mass transfer characteristics of a 1.5-m-high, 2.5-m-long and 0.07-m-thick disposable panel. The study concluded that the low power supply (53 W m^{-3}) and the high mass transfer capacity make bubbled plates preferable to pump mixed tubular PBR. A complete energy analysis for the GWP is reported later in this chapter.

Using GWP reactors, the potential of the marine eustigmatophyte *Nannochloropsis* sp. as a source of renewable oil was thoroughly investigated by Rodolfi et al. (2009). In a two-phase cultivation process (a nutrient sufficient phase to produce the inoculum followed by a nitrogen deprived phase to boost lipid synthesis) the lipid content of the biomass was increased up to 60% and lipid productivity was doubled in comparison with a nutrient sufficient single-phase process (100 and 204 mg lipid L^{-1} day^{-1}, respectively). Experiments carried out in a "full scale" simulation showed that *Nannochloropsis* sp. has the potential for an annual lipid production of 20 t ha^{-1} in the Mediterranean climate (Rodolfi et al. 2009). Nutrient deprivation has been shown to increase neutral lipids up to 48% of dry biomass, with the triacylglycerols (TAGs) representing the most abundant component (Bondioli et al. 2010). Pilot scale GWP reactors for CO_2 bio-sequestration are currently in operation at ENI S.p.A. (Gela, Italy), ENEL Ingegneria e Innovazione S.p.A. (Brindisi, Italy) and Bioscan S.A. (Antofagasta, Chile). The GWP is used for commercial production of algae biomass by Necton S.A. (Olhão, Portugal) and Microalghe Camporosso S.r.l. (Imperia, Italy) (Fig. 7.2b).

The GWP design has been recently modified in order to reduce its cost (Tredici et al. 2011). In the new design (GWP-II) the grids have been removed and the culture chamber is contained within a simple structure made by a base and a number of vertical uprights driven directly into the base or into the ground (Fig. 7.2c, d). A 0.7-m-high, 4-cm-thick and 12-m-long prototype has been tested with *T. suecica*, *Cylindrotheca* sp. and *Scenedesmus* sp. The removal of the grids and the reduction of the culture chamber height from 1 to 0.7 m have allowed the use of a much lighter metal frame and decreased the reactor's cost from €50 to about €25 m^{-2}. A further improvement, that envisages dividing the unique culture chamber into two or more horizontal sections (Tredici et al. 2011) is expected to bring the cost of the reactor to about €5 m^{-2} (Tredici 2010).

The main advantages of the GWP designs are the low construction cost and the capacity to be scaled-up. The main limitation are the high energy expenditure for mixing and cooling (Tredici 2009; Bassi et al. 2010). GWP reactors are commercialized by **Fotosintetica & Microbiologica S.r.l.** (Florence, Italy), a spin-off of the University of Florence (http://www.femonline.it, accessed 23 Jan 2011).

Solix Biofuels® (http://www.solixbiofuels.com, accessed 21 Jan 2011) a spin-off of the Colorado State University (Fort Collins, Colorado, USA), has developed a low-cost reactor, the Algae Growth System (AGS), for biofuel production (Willson et al. 2008, 2009). The G3 design, the last of three generations of AGS (Fig. 7.3), comprises a series of vertical panels made of welded flexible plastic film, which are submerged in a shallow water basin to provide mechanical support and temperature control. Carbon dioxide enriched air is bubbled through sparging tubes to regulate pH, remove dissolved oxygen and provide adequate mixing of the algal suspension (Buehner et al. 2009; Willson 2009a). The system combines large reactor surface areas per land area to reduce light intensity (light lamination) with an external water basin for structural support and thermal regulation (Willson 2009a). In the G4 design under development gas exchange will be achieved by permeable membranes placed inside the culture (Willson 2009b; Willson et al. 2009).

A model-based control system has been developed to estimate microalgae growth, O_2 production and CO_2 consumption as a function of incident light. The model has been validated with *Nannochloropsis oculata* in a 644 L PBR made of four panels, 17-m-long, 0.33-m-high, and 0.03-m-thick (Buehner et al. 2009). Experimental results correlated well with the model and showed that automation is necessary to maximize growth and achieve cost targets (Buehner et al. 2009; Willson et al. 2010). The cost of large-scale oil production with the current AGS technology was estimated to be US$1 L^{-1} (Willson 2009a).

Fig. 7.3 Solix Biofuels® photobioreactor at the Coyote Gulch demonstration facility in Southern Colorado (USA) (Courtesy of Solix Biofuels®)

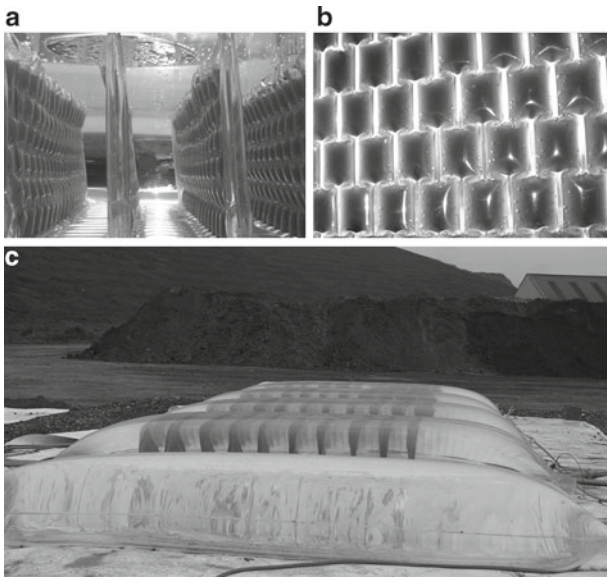

Fig. 7.4 ProviAPT photobioreactor developed by Proviron Holding NV (Belgium). (**a**) submerged panels; (**b**) detail of interconnecting compartments; (**c**) pilot plant in operation at an ex-municipal waste site (Courtesy of Proviron Holding NV)

The practical maximum annual oil production at the latitude of Denver (Colorado, USA) (40°N) and with 50% cell oil content, was calculated to be 44,000 L ha^{-1} (Weyer et al. 2010), a value that exceeds by more than 100% the potential annual lipid production estimated by Rodolfi et al. (2009) for Mediterranean regions. According to company information, microalgae (no details about the species are provided) grown at Solix site in Colorado have yielded 16,800 L of oil ha^{-1} $year^{-1}$ (i.e. 38% of the maximum expected). Currently, a demo plant made of three basins covering a surface area of 0.1 ha each (Fig. 7.3) is in operation at Coyote Gulch in Southern Colorado (USA) (www.solixbiofuels.com; Willson 2009b). The Coyote Gulch demonstration facility is co-located with a coal-bed methane production plant and uses the wastewater generated during coal-bed methane production and the CO_2 produced by the amine plant to feed the microalgae culture.

A water-filled plastic bag PBR that incorporates a series of thin panels (less than 1 cm thick) of low height (about 0.5 m), has been recently developed by **Proviron Holding NV** (Hemiksem, Belgium) (www.proviron.com, accessed 22 Jan 2011). The innovative concept of this design (named ProviAPT) is that the plastic bag, including all the panels and connections, is automatically produced and the setup is limited to rolling out the bag and filling it with water (Michiels 2009). Due to water pressure from outside that balances the interior pressure of the culture suspension, the panels are self-supporting and take on a vertical position without any need of a supporting structure (Fig. 7.4a). Mixing of the culture suspension and pH control are obtained by bubbling CO_2 enriched air. Each panel is internally partitioned to form small interconnecting compartments (Fig. 7.4b). This special pattern reduces the rise velocity of the bubbles ensuring a more efficient utilization of the air stream. Among the advantages of this system there are the short optical path and the large volume (500 L m^{-2}) of water surrounding the panels, which provides temperature buffer without the need of additional thermoregulatory systems. According to company information about 2.5 kg of plastic foil are used per square meter of reactor with an investment cost of less than €10 m^{-2}. The energy input is about 2 W m^{-2}, mainly due to air-bubbling (http://www.proviron.com/algae/pdf/GB/ProviAPT_01.pdf, accessed 22 Jan 2011). A pilot plant that comprises four bags (each bag is 4-m-long and 1.5-m-wide and contains ten panels) and covers about 24 m^2 land area is in operation in an ex-municipal waste site where biogas is produced (Fig. 7.4c) (M. Michiels, personal communication, 2010). A 270-L ProviAPT unit has been installed at AlgaePARC (Bennekom, The Netherlands) (M. Barbosa, personal communication, 2011). The main drawbacks of this design are difficult accessibility to the culture and risks with punctures of the bag that could cause the collapse of the panels. The performance of ProviAPT has been not yet fully tested, and its reliability and scalability remain to be demonstrated.

Fig. 7.5 Accordion photobioreactor developed at the University of Arizona (USA) (Courtesy of Prof. JL Cuello)

At the University of Arizona (USA) the research team of Prof J. Cuello is developing a new design called "Accordion". The system, made of thin polyethylene film, consists of a series of vertical sinuously bent sleeves mounted on a metal framework (Fig. 7.5). The algal suspension is pumped to the top of the system and flows down from section to section (Cuello and Ley 2010). The Accordion has been used to grow *Botryococcus braunii*. The University is negotiating with the Norwegian company Biopharmia AS a licensing option. Among the advantages of the system there are the large illuminated surface area that maximizes the light dilution effect, the high S/V ratio and the possibility to vary the bending angle of the sleeves to modify the velocity of the descending flow and the incidence angle of light and thus irradiance on the reactor surface. Data of productivity, energy consumption, and costs are not available.

A vertical PBR called "Hanging Gardens" has been recently developed and patented by **Ecoduna OG** (Hainburg, Austria) (Mohr and Emminger 2009; http://www.ecoduna.com, accessed 21 Jan 2011). The system consists of a series

Fig. 7.6 Vertical plates developed by Renewed World Energies Corp. (USA) (Reproduced with permission from the company's web site)

of closely spaced, 6-m-high rigid vertical plates hung within a movable structure which allows the plate arrays to track the sun movement. The plates are internally partitioned into interconnected channels by means of inner vertical walls shortened alternatively at the bottom and at the top so as to allow circulation of the culture suspension. CO_2 injected at the bottom of the reactor is used to generate a gas-lift effect, to remove the O_2 produced by photosynthesis and as carbon source. The use of pumps for mixing is minimal. The PBR provides an illuminated surface area of 32 m^2 and a culture volume of about 440 L on each square meter footprint. Among the advantages of the system, the reduction of energy for mixing, the large illuminated surface area and the efficient utilization of land are claimed to be the most important. Besides, it is reported that, since the reactor acts as a solar tracker, mutual shading between the units is avoided. It remains to be proved that CO_2 injection in response to pH increase might be adequate to provide mixing and oxygen removal. Another limitation of this concept is the greatly increased amount of reactor material and supporting structures, which enhances capital costs significantly.

Renewed World Energies (RWE) **Corp.** (Georgetown, South Carolina, USA) developed and patented a photobioreactor, which consists of a series of closely spaced vertical plates held in a rack. Each plate (approximately 1.2-m-wide, 1.8-m-high, and 0.08-m-thick) is made from high-density polyethylene (HDPE) sheets thermoformed to create a flattened tubular serpentine culture chamber (Fig. 7.6). Water, nutrients and gases are circulated through the panels by means of piping header connections (www.rwenergies.com, accessed 22 Jan 2011). An automated process control system regulates algae growth and harvesting with minimal operator inputs. The company presented a prototype of the PBR at the Algae Biomass Summit Conference in San Diego in October 2009 (http://www.youtube.com/watch?v=J2cc2bGBuvo, accessed 21

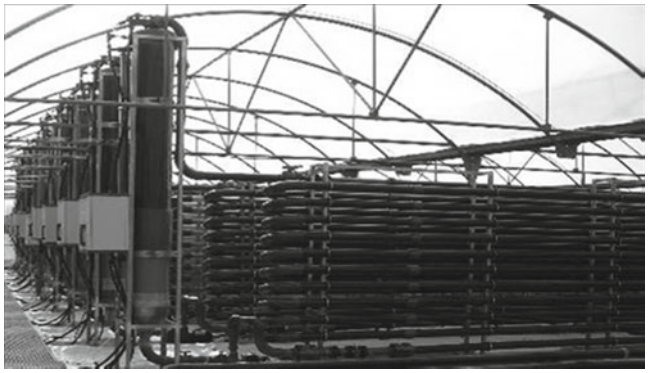

Fig. 7.7 Vertical tubular serpentine PBR developed by the Department of Chemical Engineering of the University of Almeria (Spain) (Courtesy of Prof. E Molina Grima)

Jan 2011). Among the limitations of the system are the heavy structure and the low transparency of the HDPE sheets that form the culture chamber.

2.2 Tubular Photobioreactors

Tubular photobioreactors are the most common design available and the preferred one in commercial algae production (Tredici et al. 2010). These reactors are usually constructed with either glass or plastic tubes in which the culture is circulated with pumps or preferably by means of airlift systems. They can be either serpentines or manifolds and have a horizontal (Chaumont et al. 1988; Molina Grima et al. 2001), vertical (Pirt et al. 1983), inclined (Lee and Low 1991; Torzillo et al. 1993; Tredici and Chini Zittelli 1998; Ugwu et al. 2002) or conical (Watanabe and Saiki 1997) arrangement. Advantages and limitations of tubular photobioreactors have been discussed in numerous reviews (Sánchez Mirón et al. 1999; Janssen et al. 2003; Tredici 2004; Tredici et al. 2010).

Among the pilot-scale plants devised for algae biofuel production, special mention deserves the serpentine reactors developed at the Department of Chemical Engineering of the University of Almeria (Spain) by the research group of Prof Molina Grima and in operation under a greenhouse at the Estación Esperimental de Cajamar "Las Palmerillas" (Almeria) (Fernández-Sevilla et al. 2010). A two-layer, 4,000-L horizontal tubular PBR, made of 10-cm diameter Plexiglas® tubes connected by U-joints to form a single 400-m long loop, has been used for production of lutein-rich biomass of *Scenedesmus almeriensis* (Fernández-Sevilla et al. 2008, 2010). With this freshwater microalga an oil production potential of about 16 t ha^{-1} $year^{-1}$ has been estimated (Molina Grima 2009). The system has been redesigned and is now composed of ten 2.8 m^3 vertical serpentine units. Each unit occupies a surface area of about 50 m^2 and consists of 20-m-long, 9-cm-diameter Plexiglas® tubes running in a fence-like structure (Fig. 7.7). The loop outlet is connected to a 3.2-m-high downcomer connected to the inlet of the loop. The culture suspension is circulated by a centrifugal pump (Molina Grima 2006). The plant is fully automated and the process is controlled by specially-designed software (Molina Grima 2009; Fernández-Sevilla et al. 2010). Adopting a dilution rate of about 35% a mean volumetric productivity of 0.4 g L^{-1} day^{-1} (corresponding to an areal productivity of about 20 g m^{-2} day^{-1}) was attained in winter with *Nannochloropsis* (F.G. Acién Fernández, personal communication, 2010). The biomass production cost in this plant was estimated to be around €25 kg^{-1} (Molina Grima 2009). According to the authors, to reduce the cost of biomass to less than €0.5 kg^{-1} (necessary for energy applications), PBR cost should be less than €1 L^{-1} and personnel should be reduced to less than 0.5 persons per hectare (Acién Fernández 2008).

In Spain, one of the most active companies in the algae biofuel field is **Algaenergy SA** (Madrid) (http://www.algaenergy.es, accessed 22 Jan 2011). Starting from technology acquired from universities (University of Seville, University of Almeria, University of Santiago de Compostela) and research centres (Consejo Superior de Investigaciones Científicas), the company aims at industrial scale microalgae cultivation using different PBR designs and ponds. Algaenergy SA has invested in the construction of the tubular pilot plant in operation at "Las Palmerillas" and is building a 10,000 m^2 pilot plant based on flat panel PBR. (http://rp7.ffg.at/Kontext/WebService/SecureFileAccess.aspx?fileguid=%7B3aa74614-4d8e-4328-883e-6e4c64bd071c%7D, accessed 18 Jan 2011). Two Spanish leaders in renewable energy and biofuels, Iberdrola SA and Repsol S.p.A., are shareholders and technology partners in Algaenergy SA.

Since 2007 **AlgaeLink NV** (Yerseke, The Netherlands) commercializes a horizontal serpentine PBR made of large-diameter transparent plastic tubes (Van de Ven and Van de Ven 2009). The company offers systems from demo (3.8 m^3) to large-scale (140 m^3) size, equipped with feeding and control units, an automatic cleaning device, filters for harvesting and equipment for solar drying (www.algaelink.com, accessed 23 Jan 2011). A 97 m^3 system (1,200 m^2 occupied area) made of 2,000-m-long, 25-cm diameter poly(methyl methacrylate) (PMMA) tubes is sold for €194,000 (http://www.algaeglobal.com/algaelink%20com%20cult.htm, accessed 23 Jan 2011). This is an interesting price (about €160 m^{-2}) for a completely-controlled, self-cleaning, pump-mixed closed PBR, which includes harvesting and solar drying equipment. The company expects to reach, with algae of the genus *Tetraselmis,* productivities of 160 t ha^{-1} $year^{-1}$ in The Netherlands and 300 t^{-1} ha^{-1} $year^{-1}$ in Australia, (Van den Dorpel 2010), which correspond to photosynthetic efficiencies of about 8% on total solar energy, never obtained outdoors at large scale.

GreenFuel Technologies Corp. (GFT) (Cambridge, Massachusetts, USA), founded in 2001 by Massachusetts Institute of Technology (MIT) and Harvard scientists, used a

tubular airlift PBR to cultivate microalgae on gas emissions from a power plant, aiming at simultaneously scrubbing the flue gases and producing biofuels (Tredici et al. 2010). In 2004 the reactor was tested at the MIT cogeneration power plant (Massachusetts, USA). The reactor consisted of a set of riser tubes, gas separators and downcomer tubes arranged in a triangular configuration. The gas was injected at the bottom, and the difference in fluid density between the riser and the downcomer provided the driving force for liquid circulation (Berzin 2005; Vunjak-Novakovic et al. 2005). It was claimed that more than 80% of CO_2 and NOx could be removed from the flue gas and biodiesel productivities of 80 t ha^{-1} $year^{-1}$ were attainable (Vunjak-Novakovic et al. 2005; Tredici et al. 2010). The triangular photobioreactor geometry was later changed to simple inclined tubes and a pilot unit was tested using algae selected for their high oil and starch production potential at the Arizona Public Service Redhawk power plant in 2006. In 2007 a final design, called the 3D Matrix System (3DMS), was tested at the GFT facilities in the Arizona desert (Pulz 2007). According to company press releases, with this technology an average biomass areal productivity of 98 g m^{-2} day^{-1} was achieved, with peak values of over 170 g m^{-2} day^{-1} on good sunny days (Pulz 2007). Even if the 3DMS was reported to have a S/V ratio of 1,500–2,000 m^{-1} and a very high illuminated surface area per areal footprint (that maximized the "light dilution effect"), these productivity values are unrealistic. The peak productivity value would correspond to a photosynthetic efficiency on total solar radiation of about 18%, i.e., 1.5 times the theoretical maximum for algal biomass production (Tredici 2010).

GFT, with its significant efforts to integrate their technology into power plants, in the USA, South Africa and Europe, renewed the interest in algae biofuels, after many years of dormancy probably due to the negative conclusions of the DOE project ended in the 1990s. Cost concerns and the difficulty to fully control algae growth seemingly have hampered the company to continue. GFT officially announced closing down operations in May 2009 (Kanellos 2009). All the details here provided are based on data gathered prior to company's closure.

Recently **Sogepi S.r.l.** (Milan, Italy) and **F&M S.r.l.** (Florence, Italy) have jointly developed a 5 m^3 manifold tubular reactor for CO_2 biofixation and production of algae biomass as feedstock for feed and biofuel (Giudici and Tredici 2010). The tubular section of a module, made of 10 m-long, 9-cm-diameter, PMMA tubes joined by PVC flanges and connected at the end to steel manifolds, occupies about 100 m^2. Circulation and mixing are achieved by means of a rotary lobe pump. Cooling and oxygen removal are obtained by circulating the culture through a cooling tower (Fig. 7.8). In the summer 2010, a 5,000-L prototype was built and tested at F&M S.r.l. experimental field (Florence).

Fig. 7.8 Manifold tubular reactor developed by Sogepi S.r.l. (Milan, Italy) and F&M S.r.l. (Florence, Italy) (Photographs by the authors)

Diversified Energy Corp. (Phoenix, Arizona, USA) is commercializing a closed system called Simgae™ (for simple algae) invented and patented by **XL Renewables Inc**. Aiming at agricultural levels of simplicity, the Simgae™ culture system utilizes a series of transparent, thin-walled polyethylene tubes (named Algae Biotape™) similar to conventional drip irrigation tubes, that are laid across the field in troughs created by means of traditional farming equipment. The tubes are V-shaped at the bottom. CO_2 – enriched air injection, nutrient addition and water circulation is achieved by pumps and piping available in the agriculture industry. Oxygen is removed through vents placed on the top of the tubes. By avoiding complex systems, Diversified Energy Corp. aims to lower capital costs of the technology below US$50,000 ha^{-1}. Simgae™ annual yield is estimated to be about 50 t dry algal biomass per hectare, with an oil content ranging from 20 to 30% (http://www.diversified-energy.com, accessed 23 Jan 2011). The advantages of the system include: (1) a simple design based on common agriculture components and processes; (2) easy installation, operations and maintenance; (3) low capital cost (even below that of raceway ponds). However, the commercial exploitation of the Simgae™ technology for production of biofuel still requires development and optimization of the process (above all improvement in oxygen removal, thermoregulation, biofouling control).

A2BE Carbon Capture, LLC (http://www.algaeatwork.com, accessed 23 Jan 2011) (Boulder, Colorado, USA) is developing a technology that combines algae farming-based CO_2 capture with production of biofuels, animal feed, protein and fertilizer. The core of the technology is the Carbon

Fig. 7.9 Biological Algae Growth Systems (BAGS) developed by MBD Energy Ltd (Australia) (Courtesy of MBD Energy Ltd)

Capture & Recycle Roller-Film photobioreactor (CC&R PBR) for growing and harvesting algae (Sears 2007). Each module is approximately 137 m long and 15 m wide and consists of twin 6-m-wide, 0.25-m-deep, 122-m-long, transparent plastic reactor tubes connected to end gas exchangers. One module covers about 0.2 ha (Sears 2009). Two rollers (0.6 m in diameter, 6-m long each) push and re-suspend the microalgae through the tubes also cleaning the inner tube walls. In addition to moving the culture suspension, the rollers favour degassing by pushing the oxygen bubbles towards the end of the tubes where they are collected and released through vents. Flue gas emissions rich in CO_2 and NOx or pure CO_2 are introduced through perforated membranes placed at the bottom of the water column in which the water flows in opposite direction to bubble movement (Sears 2007). According to company information the annual CO_2 consumption of the CC&R PBR was estimated to be about 250 t ha^{-1} and biomass productivity 140 t ha^{-1} $year^{-1}$, values which appear too optimistic.

The "Biological Algae Growth System" (BAGS), recently developed by **MBD Energy Ltd** (Melbourne, Australia) can be considered a hybrid system, i.e., it combines the characteristics of open ponds and photobioreactors. The BAGS consists of a series of large horizontal bags made of flexible plastic film, partially filled with the culture suspension and including a large gas space above the culture (Fig. 7.9). Injection of flue-gas at the bottom of the culture allows mixing and deoxygenation of the culture suspension, favours distribution of nutrients and maintains the cultivation chamber inflated (www.mbdenergy.com, accessed 24 Jan 2011; Stammbach et al. 2010). The growth of *Nannochloropsis oculata* was tested in a 10-m-long, 3-m-wide bag, filled up to 30 cm so as to have a final culture volume of about 9 m^3. In a 20-day trial the mean productivity obtained was about 1×10^6 cells mL^{-1} day^{-1} (i.e., about 2 g m^{-2} day^{-1}) (Stammbach et al. 2010). In collaboration with the James Cook University (Townsville, Australia), MBD has developed a 5,000-m^2 facility potentially capable of producing 14,000 L of oil and 25,000 kg of algal meal from every 100 t of CO_2 consumed. The company is currently moving from test facility to full scale plants (1 ha) to be built at a number of Australia coal-burning power stations. This reactor couples the advantages of open systems (e.g., inexpensive construction) and closed PBR (e.g., a closed and controlled environment). The inside positive pressure in the bags also helps in limiting contamination. However, further testing on the field scale is necessary for a complete evaluation of the potential of the system at large scale.

2.3 Innovative Concepts

To achieve high biomass areal yields, a PBR should capture as much sunlight as possible and distribute it to the cells in such a way (uniformly and at low irradiances) so as to allow high efficiencies of conversion into biomass. High diurnal irradiances, necessary on the other hand for high areal productivity, make this goal difficult to achieve. A solution seems, at least from the theoretical point of view, to develop systems in which photon capture is physically separated from the cultivation phase and light is then distributed, at adequate intensity, via conducting structures within the culture.

Fibre-optic-based systems in which visible solar light is collected by mirrors, concentrated through lenses and delivered into the bioreactor via an array of flexible, optical fibres or transparent bars or plates, have been developed and tested at laboratory scale (Mori 1985; Ogbonna et al. 1999; Feuermann et al. 2002; Gordon 2002). Wijffels and his team at the Bioprocess Engineering Group of the Wageningen University (The Netherlands) envisaged a rectangular airlift photobioreactor 10-m-high, 250 m^3 in volume, containing 83-cm-thick light-re-distributing plates placed 3 cm apart. Solar light would be collected by parabolic dishes from a field of about two hectares and conveyed through optical fibres to the plates that redistribute visible photons inside the culture at an average irradiance of 1,200 μmol photons m^{-2} s^{-1}. Assuming a 15% photosynthetic efficiency (too high for that irradiance), the authors estimated that the annual productivity of such a system would reach 200 tonnes of dry algal biomass (Janssen et al. 2003), i.e. 100 t ha^{-1} of collecting surface.

More recently, the same group has developed the Green Solar Collector (GSC), a reactor in which sunlight is captured through PMMA Fresnel lenses that are able to rotate over two axes to follow the sun. Light focused on top of PMMA distributing elements (light-guides), refracts into the light-guides and propagates downwards by total internal reflection. Refraction out of the triangular shaped bottom part of the guides distributes light into the culture compartments (Zijffers et al. 2008a, b; Zijffers 2009). Efficient cap-

turing of sunlight and redistribution inside the algal culture can be achieved in the GSC at high elevation angles of the sun, making the GSC suitable for operation at low latitudes with high level of direct irradiance (Zijffers et al. 2008b). Compared to optical fibre systems, the GSC technology appears more efficient because focusing sunlight directly on the light guides reduces losses caused by attenuation in the fibres. Moreover, light reflects internally without loss in intensity over the small distance where it needs to be transported (Zijffers 2009). Due to the very high cost of solar tracking devices and distribution systems, these technologies are prohibitive for low cost algae feedstock production, but expected progress in materials for photon capture and transport makes this approach promising in the long run.

A limiting factor in any system for algae cultivation, whether an open pond or a PBR, is the short penetration of light into the culture. Based on the work of Prof Amos Richmond (Ben-Gurion University of the Negev, Israel) and using the patented "light distribution" technology devised by Zweig in 2010, **Algaenesis Ltd** (Jerusalem, Israel) has developed an innovative system that can be integrated into an open pond and is capable of capturing, without tracking, all incident sunlight and distribute it evenly throughout the culture volume. It consists of an optically transparent light concentrator/redirector device made of a series of rectangular prisms. After refraction on the curved upper surface of each prism, light is propagated into conducting channels and transferred deeply and evenly within the culture (Zweig 2010). According to company information, since impinging light is diluted ten times before being delivered to the culture, the technology would allow to attain efficiencies and productivities five times greater than those attainable with conventional systems (http://www.algaenesis.com, accessed 4 Feb 2011).

Several companies in Europe and USA have developed technologies that exploit the tendency of microalgae to grow attached on solid substrates and form biofilms. A pilot-scale photobioreactor that uses fixed-film membranes has been built at the Ohio University's Coal Research Center (Athens, Ohio, USA) for photosynthetic CO_2 fixation. In this membrane-based photobioreactor, known as Carbon Recycling Facility (CRF), the algae grow on woven-fibre membranes suspended vertically in a reaction chamber where both flue gases and the growth medium are continuously circulated. Parabolic mirrors mounted on the top of the reactor collect sunlight and channel it along fibre-optic cables which in turn deliver light to illuminating panels interspersed between the membranes. By increasing the medium flow a high shearing force is obtained that forces the algae off the membranes (Bayless et al. 2002, 2006; Mears 2008) for harvesting. The bioreactor has been tested with the thermophilic cyanobacterium *Chlorogleopsis* sp. It was reported that the cyanobacterium can be grown on saturated hot flue gas with productivities ranging from 10 to 50 g m^{-2} day^{-1} as a function of irradiance (Bayless et al. 2006). Growing microalgae on membranes minimizes water use and reduces harvesting cost. However, the technology is restricted to microalgae able to grow in attached state. **GreenShift Corp.** (Alpharetta, Georgia, USA) (http://www.greenshift.com, accessed 4 Feb 2011), under a license agreement with the Ohio University, is conducting experiments with the CRF for reducing greenhouse gases emissions from fossil-fuel combustion processes.

On an industrial scale **SBAE Industries NV** (Sleidinge, Belgium) has developed and patented a technology, called DIAFORCE™, for the outdoor production of diatom poly-cultures (www.sbae-industries.com, accessed 4 Feb 2011). The DIAFORCE™, imitating nature, adopts specially designed triangle carriers, on which artificial substrata are mounted, that are placed in the water stream and upon which a community of diatoms can grow. Typically the water stream is wastewater and the flow is controlled (Vanhoutte and Vanhoutte 2009). Harvesting is achieved by an automatic device, which travels the length of the system, lifting the carriers covered with the diatoms out of the water stream, and blows or rinses the biofilm into a collector, then replacing the carriers back into the water channel (Vanhoutte and Vanhoutte 2010). Compared to conventional systems this method reduces the water to be processed by over 95%. According to tests carried out in 200 m^2 plants, a DIAFORCE™ reactor can produce 100 t ha^{-1} $year^{-1}$ of biomass in temperate climates. SBAE has plans to realize facilities (from 5 to 50 ha) in the next 2 years to produce algae feedstock for feed and fuel (Van Aken 2009). Although it can be applied only to poly-cultures, the DIAFORCE seems suitable to grow algae in different environments, with the advantage of low water use and economical harvesting.

A biofilm based approach is also applied by **BioProcess Algae, LLC** (Portsmouth, Rhode Island, USA) for CO_2 capture. The company has developed and patented a system known as Grower Harvester™ bioreactor, for growing attached microalgae under autotrophic, heterotrophic or mixotrophic conditions and harvesting the algal biomass (www.bioprocessalgae.com, accessed 4 Feb 2011; Ahrens et al. 2009; Ahrens 2010). The system includes a plurality of cylindrical containers that can be arranged either vertically or horizontally. Each cylinder contains specially-designed substrata, at least partially submerged in the water, that serve for the attachment of microbial cells. The substrata are supported on a rotary frame to improve utilization of light. The system includes a flushing device that sprays the substrata and removes the attached microalgae (Haley 2010; Ahrens 2010). A demonstration unit installed at the ethanol plant of Green Plains Renewable Energy, Inc. in Shenandoah (Iowa, USA) has been operating continuously since October 2009 using the plant recycled heat, water and CO_2.

OriginOil, Inc. (Los Angeles, California, USA) has recently developed a new technology to produce oil from microalgae. The cultivation system, known as "Helix

BioReactor™" features a rotating vertical shaft with low-energy lights arranged in a helix or spiral pattern, which results in a theoretically unlimited number of growth layers. Each lighting element can produce specific light wavelengths for optimal algae growth (www.originoil.com, accessed 4 Feb 2011; Shigematsu and Eckelberry 2009). This design has been recently applied in pilot systems, which consist of a series of LED light sticks placed inside an 800-L algal culture tank (Sula 2010). The company has also developed a process for algae oil extraction where Quantum Fracturing™ is combined with electromagnetic pulses and pH modification to break the algae cells and release their oil content. The oil rises to the top and can be skimmed, while the remaining biomass settles to the bottom. Recently, the company announced that a process has been developed by which algae oil can be continuously extracted without cell damage in a sort of milking. OriginOil, Inc. has recently entered into a partnership agreement with MBD Energy Ltd, which is regarded as a pioneer in the use of exhaust (flue) gases as feedstock to produce algal biomass.

2.4 Combined Production Processes: Coupling Ponds and Photobioreactors

Raceway ponds are less expensive than PBR. However, being open to the atmosphere, algae cultures in open ponds easily become contaminated with unwanted algal species and grazers. PBR, being closed, minimize air-borne contamination, but have higher installation and operation costs. A combination of both systems seems a promising strategy for cost-effective cultivation of selected strains for biofuel production. Besides, it can be well adapted to two-stage cultivation processes (Rodolfi et al. 2009): the first stage, carried out in the PBR to produce the inocula; the second stage, carried out in the pond, to obtain the main product (e.g., biomass, oil). Since the cultivation in the pond lasts only few days, there will be not time for contaminants to develop and prevail.

Huntley and Redalje (2007) described a coupled process for the production of oil and astaxanthin from *Haematococcus pluvialis*. The plant was made of 25,000-L tubular photobioreactors and 50,000-L open ponds. The module consisted of a 200-m^2 horizontal serpentine reactor made of low-density polyethylene tubing (38 cm in diameter). Temperature was controlled by immersion of the reactor in a water pond. The culture grown in the PBR was used to inoculate the raceway ponds in which the cells, exposed to stresses (high irradiance, low nitrogen), accumulated both astaxanthin and oil. The coupled system achieved an average annual biomass productivity of 38 t ha^{-1} with an oil production rate of about 10 t ha^{-1}.

A dual cultivation process, called ALDUO™ technology, which uses PBR for continuous cultivation and open ponds for batch cultivation has been developed and patented by **HR BioPetroleum, Inc.** (Kailua-Kona, Hawaii, USA) (http://www.hrbp.com, accessed 18 Feb 2011). The technology aims to convert industrial CO_2 emissions into algae biomass to be further processed into biofuels and other useful products (Huntley and Redalje 2010). In 2007 HR BioPetroleum, Inc. and **Royal Dutch Shell Plc** established a joint-venture company, called **Cellana,** to build and operate a 2.5-ha demonstration facility in Hawaii for growing marine algae for biodiesel. In February 2011 Royal Dutch Shell Plc decided to relinquish its stake in Cellana and HR BioPetroleum, Inc. assumed full ownership (Sims 2011).

3 Energy Needs for Algae Biomass Production in a Disposable Panel Reactor

The many different reactor designs here described cannot be fully evaluated because of lack of long experiments able to provide reliable data on productivity, durability, sustainability at large scale. An issue of utmost importance, when biofuel production is the target, is a thorough analysis of the energy balance of the process. In this paragraph the energy balance of algae biomass production in a disposable panel reactor is illustrated.

Recently, based on the experimental data published by Rodolfi et al. (2009) a comparative analysis of the energy life-cycle for production of biomass and oil from *Nannochloropsis* has been published (Jorquera et al. 2010). The net energy ratio (NER), i.e., the ratio of the total energy content of the oil and the residual biomass over the energy content of the system construction plus energy required for all operations, of three different systems (GWP, tubular reactors and ponds) was calculated. The NER in the GWP was largely positive: 4.5 for whole biomass and 1.6 for oil. Harvesting and oil extraction energy costs were not considered. The results of this analysis appear too optimistic as shown by the calculations reported below.

Optimal bubbling rates for *Nannochloropsis* in the GWP vary between 0.15 and 0.45 L (of air) L^{-1} (of culture) min^{-1} (Bassi and Tredici, unpublished). The GWP typically contains about 40 L of culture per meter of panel (equivalent to 40 L of culture per square meter of occupied land area when the panels are deployed at 1 m distance) and has an average cross sectional area of 0.04 m^2. Considering an optimal bubbling rate of 0.3 L L^{-1} min^{-1}, corresponding to a superficial gas velocity of 0.3 m min^{-1}, a power of 1.96 W m^{-2}, equivalent to 85 kJ m^{-2} day^{-1} when the culture is mixed for 12 h a day, is required, according to the formula given by Chisti and Moo-Young (1989) (see footnote). Considering actual air-compression costs (Metcalf & Eddy, Inc, 2003), the cost for mixing rises to 142 kJ m^{-2} day^{-1}. The embodied energy and the energy cost for cooling in the GWP have been calculated to be about 300 and 35 kJ m^{-2} day^{-1}, respectively (Bassi and Tredici, unpublished). A typical produc-

tivity of 20 g m^{-2} day^{-1} and a biomass energy content of 20 kJ g^{-1} will result in an energy output of 400 kJ m^{-2} day^{-1} and in a NER lower than one, even without considering the energy costs for nutrients and harvesting.[1]

A more favourable situation characterizes algae cultivation in raceway ponds. According to Oswald (1988) power consumption for a large, well-designed, paddle-wheel mixed raceway pond is only 15 kWh ha^{-1} day^{-1} (equivalent to 0.06 W m^{-2}). Weissman et al. (1988) reported a much higher power input (0.25 W m^{-2}) to circulate the culture at a velocity of 20 cm s^{-1} necessary to avoid cell deposition with most algae. Even at the higher consumption rate reported by Weissman et al. (1988) mixing a raceway pond is relatively cheap: only 21.6 kJ m^{-2} day^{-1} when mixing is applied for 24 h a day. The embodied energy of a raceway lined with a 12-year-lifespan PVC membrane has been calculated to be about 30 kJ m^{-2} day^{-1} (Bassi and Tredici, unpublished) and since there is no need for cooling, which is provided, within a certain limit, by evaporation, the total energy cost for algae cultivation in ponds amounts to about 50 kJ m^{-2} day^{-1}. It is to note that these calculations have been done without considering harvesting and medium recycling, which are much more expensive in open ponds than in PBR.

Reducing mixing intensity in the GWP seems possible, but it can reduce productivity in sunny days (Bassi 2010), which is not advisable. The only applicable solution to significantly decrease mixing costs in GWP seems to be reducing the light-path of the panels to reduce the amount of air required to mix the culture without decreasing the superficial gas velocity and thus turbulence. New patent-pending panel designs (e.g. GWP-II), with significantly lower embodied energy and reduced culture thickness (1.5–2.5 cm), are being tested in Florence. These improvements allow to reach a NER close to one. GWP-II, as other low light-path PBR, show other advantages, among which the significant reduction of culture medium to be prepared and handled and a much increased cell concentration, which reduces the energy costs for harvesting.

4 Economics of Algae Biofuel Production

If there is one thing certain with respect to the economics of future commercial-scale algal oil production it is its uncertainty. Since large-scale plants for cultivation of oleaginous algae do not exist, any economic estimate of algae oil production must be based on presumed productivities and costs. In a recent analysis, Darzins et al. (2010) examined three different scenarios for algae-to-biofuel using raceway ponds. In the so called "high oil content and low biomass productivity scenario" a biomass productivity of 10 g m^{-2} day^{-1} with a 40% oil content was assumed, which appears plausible. In fact, at pilot level, an algal lipid productivity of 9 g m^{-2} day^{-1}, corresponding to more than 6 g (oil) m^{-2} day^{-1}, has been demonstrated as feasible (Rodolfi et al. 2009). According to this scenario, the oil cost in large-scale systems (about 40 ML $year^{-1}$) using unlined ponds would be over US$2 L^{-1} (~ €1.45 L^{-1}) (i.e., double the cost of soybean oil in the US). Bassi and Tredici (unpublished) analyzed the cost of oil-rich biomass production with *Nannochloropis* adopting the two-step process described by Rodolfi et al. (2009). In a 400-ha plant producing annually about 50 t dry biomass per hectare, the cost of algae biomass varied between €2.7 kg^{-1}, when fertilizers and CO_2 are purchased, and €1.1 kg^{-1}, when fertilizers and CO_2 are obtained from wastewaters and flue gas at the sole cost of delivering them to the culture. Only at the lower biomass cost (€1.1 kg^{-1}) it will be possible to produce oil at nearly the cost calculated in the analysis of Darzins et al. (2010), which is, however, not low enough to compete with fossil fuel. Much higher oil productivities, as for example the 20 g (oil) m^{-2} day^{-1} assumed in the "high productivity scenario" by Darzins et al. (2010), and/or much lower cost of resources are necessary to produce biodiesel at a cost comparable to that of petroleum diesel.

The analysis of Darzins et al. (2010) also showed that when instead of ponds, PBR at a capital cost of US$500,000 ha^{-1} are used to cultivate the algae, the cost of biodiesel increases to more than US$10 L^{-1} and concluded, in agreement with other published estimates, that in order to compete with raceway ponds, the cost of the PBR should decrease to less than US$100,000 ha^{-1} (US$10 m^{-2}).

To produce algal oil at costs not far from the cost of petroleum diesel with current oleaginous algal species, high oil productivities (>10 g m^{-2} day^{-1}) need to be achieved using low-cost "unlined" ponds. However, the suitability of unlined ponds seems dubious since silt re-suspension and interaction of nutrients with the natural substratum will not allow to maintain high growth of the selected strain for as long as necessary. On the other hand, when liners suitable to seal the pond bottom are considered, the cost of production of algal diesel, even in suitable places (e.g. the Australian desert in close proximity to water resources and CO_2 emitters) rises to US$3–4 L^{-1}, depending on the size of the plant and oil content of the biomass (Darzins et al. 2010).

In conclusion, current available technologies for commercial algae production (either PBR or lined ponds) do not allow yet the competitive production of biodiesel from microalgae, and since ponds seem limited in their possibility

[1] The power input in bubbled panels can be calculated from the following formula: $P_G = V_L \rho_L g U_G$ (Chisti and Moo-Young 1989) where:
P_G is the power input due to aeration (W)
V_L is the culture volume (m^3)
ρ_L is the water density (1,000 kg m^{-3})
g is the gravitational acceleration (9.8 m s^{-2})
U_G is the superficial gas velocity (m s^{-1})

of development, research is mainly focusing on PBR. The interest in PBR is increasing, also promoted by the fact that systems like the Simgae™ and the GWP-II may be built at costs not far from, or even lower than, those of lined ponds. However, it is worth noting that operating costs, more than capital costs, make algae biomass (and oil) production in PBR too expensive.

5 Conclusions

World commercial production of microalgae is limited to about 20,000 t year^{-1}. Only few hundred tonnes are produced in closed photobioreactors. There is essentially one reason for preferring open ponds or lagoons of low productivity for algae cultivation, and this is cost. At large scale, PBR are more expensive to build and operate than raceways ponds, which are thus used in the majority of commercial plants.

By providing a closed, more controllable environment and, in many cases, by light dilution, PBR may achieve a higher efficiency of solar energy conversion compared to open systems. However, rarely these advantages translate into a significantly higher areal productivity and compensate the higher cost of PBR. It is true that, because they are closed and have a higher S/V ratio, PBR save water by avoiding evaporation and produce more biomass per liter. However, water savings are more than offset by the need to cool the culture, which in open ponds is obtained at no cost thanks to evaporation, and not always the higher volumetric productivity of a high S/V ratio PBR leads to higher areal productivities. Light dilution in PBR has been shown to reduce photosaturation and photoinhibition and a significant increase of areal productivity has been actually obtained by using vertical reactors on which sunlight is spread, and thus diluted, on a large surface area. But, the drawbacks of a high reactor-area to ground-area ratio (typically between two and ten) are self evident: it requires large reactor transparent surfaces, and this significantly increases capital and operating costs. The real advantage of PBR is that by limiting the risk of contamination they allow more species to be cultivated and that, thanks to their shorter light-path, they achieve higher cell concentrations with significant savings in harvesting and medium preparation and handling.

New PBR designs are emerging, like the Simgae™, the GWP-II and the ProviAPT, that show construction costs similar to or lower than those of lined ponds. However, a low construction cost is not enough to make these systems competitive for large-scale production of algae biomass. The raceway pond is a very efficient culture system that, except in humid climates, does not require cooling and in which sufficient turbulence can be generated at an energy expenditure of less than 5% of the energy stored in the biomass. On the contrary, PBR require energy inputs for mixing and cooling that, together with the reactor embodied energy, may surpass the energy content of the biomass.

Growing microalgae in open ponds is much cheaper. Growing algae in PBR is generally safer and more reliable. It is thus very likely that an industrial plant for cost-effective production of biofuel from microalgae will adopt a strategy that combines both PBR, in which active inocula of the selected species are produced, and ponds for bulk cultivation.

When a biofuel is the target product, the most important issue is the cost of the biomass which will be processed to yield the fuel. The current cost of algal biomass production (US$5 kg^{-1} being the lowest possible with available technologies) exceeds by 20 times that required for economic fuel production (about US$0.25 kg^{-1} prior to conversion to biofuel). Some recent estimates (see for example that of Darzins et al. 2010) confirm that productivities will have to increase and costs decrease significantly to achieve this ambitious goal.

The strategy to decrease the cost of algae biomass is undoubtedly complex. The key issue is strain selection, that should be based on strain productivity, robustness, oil (or carbohydrate) content, harvestability and extractability. The candidate microalga must then be thoroughly studied at a significant large scale outdoors to maximize its productivity in terms of biomass and desired component (e.g., TAGs for biodiesel, sugars for ethanol). Low-cost reactors with automated process control should be adopted for inexpensive production of inocula, and low-cost, lined, large-scale ponds must be designed to carry out the accumulation of the "energetic" compound. It will be fundamental the close proximity of the plant to water resources and that CO_2 is provided "free of charge" at the battery limits of the production facility. When available, and compatible with reliable growth and high productivity of the selected strain, wastewaters should be used as nutrient sources. Also important will be that value is generated from the residual biomass (e.g., used for animal feed).

In conclusion, despite its "appealing" potential, investors should be aware that the algal biofuel technology is not ready yet, a reasonable projection for the establishment of an economically viable process being 10–15 years. The high lipid or carbohydrate productivity per land area and the lack of competition for freshwater and arable land amply justify, however, the renewed interest of researchers and the ongoing large investments in algae biofuel.

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Open Pond Culture Systems

8

Michael A. Borowitzka and Navid Reza Moheimani

1 Introduction

Large-scale microalgae culture systems are broadly classified into 'open' systems where the culture is directly exposed to the environment, and 'closed' systems where the culture is wholly enclosed within the culture vessel (photobioreactor). Open culture systems are the main systems used to produce microalgae commercially as well as in wastewater treatment systems, mainly because they are the most economical culture systems for large-scale microalgae culture.

Open pond culture systems can be classified broadly as:

- Shallow lagoons and ponds
- Inclined (Cascade) systems
- Circular central-pivot ponds
- Mixed ponds
- 'Raceway' ponds

2 Shallow Lagoons and Ponds

Algae lagoons, ponds and ditches have been used as simple wastewater treatment systems for thousands of years. Simple ponds of varying sizes and often fertilized with manure are also a common means of producing algal biomass in the faming of freshwater fish. Some of these 'natural' systems of algae culture have also been used for the production of algal biomass for human consumption. The oldest records are of *Arthrospira* (*Spirulina)* harvesting from natural lakes are from Lake Texcoco, Mexico, where the Aztecs used to harvest this alga (Ciferri 1983). In the 1970s and 1980s Sosa Texcoco Co. grew *Arthrospira maxima* in the outer parts of a spirally-shaped solar evaporator, the 'Caracol' (Spanish for snail) located near Mexico City. The pond area was about 40 ha producing about 300 t of algae per year (Durand-Chastel 1980). Today, in Myanmar (Burma) monocultures of *Arthrospira* (*Spirulina*), which grow naturally in four volcanic craters filled with alkaline waters, are harvested by simple filtration during the growing season (Min-Thein 1993). After further dewatering the algae are dried and sold locally. The current production is about 30 t year^{-1} (Lee 1997).

The largest commercial microalgae production plants in the world are the two *Dunaliella salina* β-carotene plants located at Hutt Lagoon, Western Australia and Whyalla, South Australia and now owned and operated by BASF (Fig. 8.1). Here the algae are grown in very large (up to about 200 ha each) unlined shallow ponds (Borowitzka and Hallegraeff 2007). Mixing is only by wind and convection currents as well as by careful management of water flow through the pond system (Borowitzka and Borowitzka 1989, 1990). The total pond area of each of these plants is in excess of 700 ha. Productivity on an areal basis is low, however the use of a very efficient and low cost harvesting method means that this is the lowest production cost algae production system in the world (Borowitzka 1999b). The maintenance of an almost unialgal culture is possible because of the very high salinity the algae are grown in, and the low cost production is further made possible by an extremely effective system of harvesting the cells from the brines.

3 Inclined Systems

In these ponds the algae culture flows down an inclined surface and then is collected at the bottom and pumped back to the top of the incline (Fig. 8.1). This type of culture system was originally developed by Setlík and coworkers in the 1960s at Trebon in the Czech Republic (Setlík et al. 1970; Doucha and Livansky 1995) and this

M.A. Borowitzka (✉)
Algae R&D Centre, School of Biological Sciences & Biotechnology, Murdoch University, Murdoch, WA 6150, Australia
e-mail: M.borowitzka@murdoch.edu.au

N.R. Moheimani
Algae R&D Centre, School of Biological Sciences & Biotechnology, Murdoch University, Murdoch, WA 6150, Australia
e-mail: N.Moheimani@murdoch.edu.au

M.A. Borowitzka and N.R. Moheimani (eds.), *Algae for Biofuels and Energy*, Developments in Applied Phycology 5,
DOI 10.1007/978-94-007-5479-9_8,

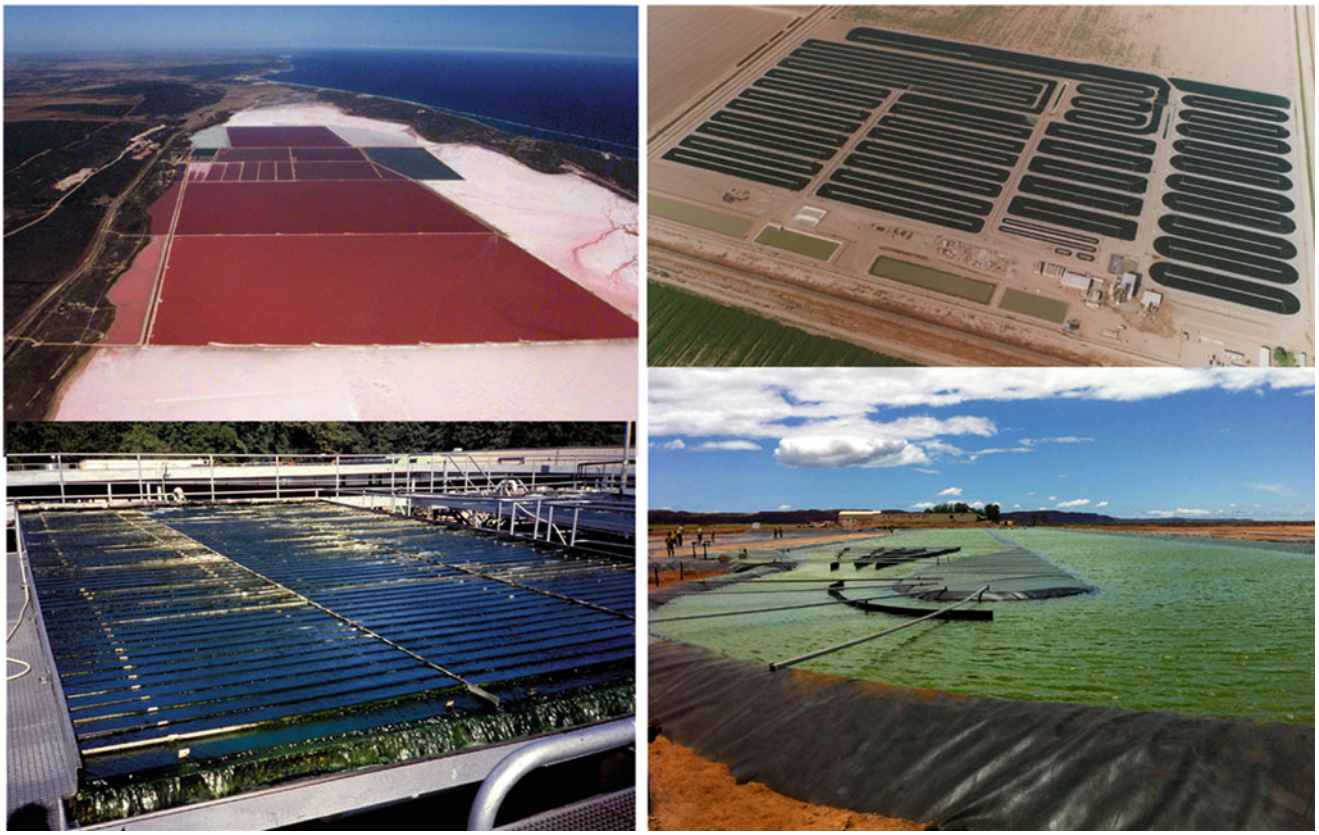

Fig. 8.1 Different types of large-scale and commercial-scale open pond systems. *Top left*; The extensive shallow ponds of the *Dunaliella salina* β-carotene plant at Hutt Lagoon, Western Australia, operated by BASF (total pond area ~ 740 ha); *Top Right*: Paddle-wheel driven raceway ponds at the Earthrise *Spirulina* production plant in Calipatria, California, USA (Courtesy Amha Belay); *Bottom Left*: Sloping shallow cascade system at Trebon, Czech Republic with baffles installed to increase turbulence; *Bottom Right*: Lined raceway-type pond with water-jet circulation designed and operated by Aurora Algae in Karratha, Western Australia (Courtesy Matt Caspari, Aurora Algae)

design has recently been patented (Doucha and Livansky 1999). The Trebon prototypes consisted of a 50 m^2 and a 900 m^2 unit. The culture flowed over a 3% inclined slope made of glass sheets which can be fitted with transverse baffles to create additional turbulent motion. During the day the culture is circulated from the bottoms of the slope to the top, whereas at night the culture is kept in a large tank where it is aerated and mixed. This reduces the overall pumping costs and also reduces the degree of cooling of the culture at night. In Rupite, Bulgaria, a 3,000 m^2 plant with sloping ponds (500, 1,000 and 1,500 m^2) has been constructed. Here, the ponds are made of concrete and covered with iron grids to generate turbulence. Like the Trebon system, the slope of the ponds is 3%. Between April and October (summer) average daily productivities of 18–25 g m^{-2} day^{-1} have been obtained with *Arthrospira* and *Scenedesmus* (Fournadzhieva and Pilarsky 1993; Becker 1994). Near Dongara, Western Australia, a similar system with a greater slope consisting of a 0.5 ha sloping plastic lined pond constructed on a hill side was used to culture a local strain of *Chlorella* for several years and achieved annual average productivities of about 20 g m^{-2} day^{-1} (Borowitzka 1999a).

Another variant of this type of culture system was constructed in Sausal, Peru (Heussler 1985). This system was an open concrete raceway-type system consisting of a sloped meandering channel adapted to the terrain gradient. The algae culture is pumped from the lowest point to the top from where it then flows 'down hill' in a shallow layer. This system reportedly achieved maximum productivities of 40 g m^{-2} day^{-1} with and average of about 22 g m^{-2} day^{-1} in semi-continuous operation. In South Africa, A series of ponds of decreasing depths was tested as a means of reducing the nitrogen levels in wastewater from an explosives factory. This system had the advantage of minimising water losses by evaporation and thermal instability and achieving high algal biomass in the final shallowest pond thus making harvesting easier. The final 1 ha demonstration plant produced about 150 kg day^{-1} algal biomass on average (Altona et al. 1983).

4 Circular Central-Pivot Ponds

Some of the oldest pond types used for commercial algae cultivation are the circular ponds used in Taiwan (see Fig. 1.4), Japan and Indonesia for the cultivation of *Chlorella* (Lee 2001). These ponds, which may be of up to 0.5 ha in area and up to 50 m diameter, are constructed of concrete and are mixed by a rotating arm mounted at the center of the pond. This results in much greater mixing at the periphery of the pond because here the arm travels over a much greater distance. In the large ponds a smaller rotating arm is mounted tional mixing in this region.

5 Mixed Ponds

These ponds are mainly used on the production of algae for aquaculture feed (Fulks and Main 1991; Borowitzka 1997). The simplest type of pond/tank is about 50–80 cm deep with aeration from the base of the pond used to provide some mixing of the culture. Because of uneven mixing and the depth of the ponds productivities are low.

An alternative mixing method suitable for rectangular ponds is the drag board. The board, which closes the cross section of the pond except for a small slit of a few centimetres between the bottom of the board and the pond bottom, is dragged from one end of the pond to the other by a motor-driven pulley system and this forces the water to run through the slit creating a turbulent back-whirl. Pond mixed with drag boards have been tested in Chile (Valderrama et al. 1987; Cárdenas and Markovits 1987) for the culture of *Spirulina* and *Dunaliella* and, although this system was found to use less power than paddlewheels, it proved to be unrealiable in the long term because of the complexity of the pulley system which failed regularly.

6 Raceway Ponds

Raceway ponds are the most widely used culture system for the commercial production of microalgae (Fig. 8.1), as well as in the treatments of wastewaters. This is mainly because they generally are the cheapest to construct and operate. The design and construction of raceway ponds has been reviewed by Dodd (1986), Oswald (1988), and Borowitzka (2005).

The simplest type of raceway pond is in the form of ditches dug in the ground with sloping earth berms. Depending on the nature of the soil the ponds may need to have the walls and bottom covered compacted clay the reduce seepage and they may possibly also need to be covered with crushed and compacted rock to reduce erosion, especially at the curving ends of the ponds. However, this type of construction limits the flow rate in the ponds because of potential erosion problems and there is generally a higher amount of suspended materials from the pond surfaces. This suspended material can reduce productivity as it limits light penetration into the pond. Such ponds also are impossible to clean effectively and this puts the long-term stability of the cultures at risk. This simple kind of construction may be suitable for high-rate oxidation ponds for wastewater treatment (e.g., Craggs et al. 2003), but unlined ponds have proven to be not suitable for the production of algal biomass for biofuels or any kind of high productivity algae culture.

It is also possible to cover the ponds, usually with clear plastic. This has several potential advantages and disadvantages. Covered ponds are warmer and this may benefit productivity in cooler locations (Walmsley and Shillinglaw 1984), however in high light locations covered ponds can reach very high temperatures greatly limiting the choice of algae species which can be grown. Covering the ponds also reduces the effects of wind-borne dust and other contaminants compares to un-covered ponds. Covered ponds also enable CO_2 addition by enriching the air above the cultures.

In order to achieve reliable high productivities good water circulation is required and this means that lined or concrete ponds need to be used, despite their greater cost (Borowitzka 2005). Water flow rates of between 20 and 30 cm s^{-1} are most commonly used. The most common raceway pond construction used in commercial algae production is ponds that are lined with plastic, such as HDPE geotextile liners, although PVC liners have also been used. With the correct choice of liner material, and when laid carefully and correctly, these liners have proven to have a long life of at least 20 years. There are several variants in the mode of pond construction; the ponds may be dug into the ground with sloping side walls and lined, or they may be constructed on the surface of the ground with the pond walls constructed of concrete blocks or of formed concrete cast in situ, and then lined. Alternatively, the whole pond may be constructed of concrete, but this is more expensive. Concrete is also generally not suitable for growing algae in saline media such as seawater unless it is epoxy coated to protect the concrete from the salt, further adding to the expense.

The pond liner is the single most expensive component of the construction costs of the ponds and cheaper alternatives have been explored. For example, various spray applied membranes have been tried in the past. These have included both cold applied (two component) and hot applied systems using mixtures of asphalt, rubber and other elastomeric materials both with and without reinforcing fabrics. However, these have as yet not proven to be as durable and effective as the membrane pond liners.

The choice of construction method depends on many factors. For example, relatively homogeneous soils which

can be easily cut with a scraper allow easy construction of excavated ponds which are basically cheaper to construct than ponds with concrete walls. However, at sites where excavation of the ponds is difficult, it may be cheaper to use the concrete wall type of construction. The vertical sides of concrete wall ponds also have some other small, but important, advantages. The vertical walls reduce the amount of dust which can blow into the ponds compared to the 'in ground' ponds. The dust increases the turbidity of the water reducing light availability to the algae thus reducing productivity. Excessive sediment build-up may also lead to instability of the culture. Furthermore, water flow in the ponds is more homogeneous throughout the water column because the pond has the same width at the bottom as at the water surface. The problem of sediment build-up can be reduced by including a depressed section (or ditch) in the ponds (Dodd 1986). This depression acts as a sedimentation basin from which the accumulated sediments can be removed periodically either through a drain or a pump.

The culture in the ponds must be circulated at about 20–30 cm s^{-1} to keep the algae suspended as well as to provide relatively even illumination to the algae and prevent thermal stratification. Many systems have been trialled for circulating and mixing the algae culture in a raceway pond. These include:

- Air lifts
- Archimedes screws
- Propellers
- Pumps (impellers)
- Water jets
- Paddlewheels

6.1 Paddle-Wheels

There are several variants on paddle wheel design (e.g. Fig. 8.2), but experience has shown that an eight blade paddle wheel is generally the best compromise between efficiency, weight and construction costs. The paddle wheel may be over a flat bottom or, in order to reduce backflow and improve efficiency, may sit in an invagination in the pond floor as proposed by Dodd (1986). Generally an 8-blade paddle wheel is considered optimal. In wide ponds (over about 2 m wide) the paddle wheel may be made up of smaller paddle elements with each element offset by several degrees so that some of the blades are always immersed in the culture. This means that there is a constant force on the whole wheel resulting in an even power requirement; If one of the blades are not always immersed then there is an increased power requirement when the blade first enters the water due to the sudden resistance encountered by the blade as it enters the water.

Fig. 8.2 Two types of circulation system for open ponds. *Top*: Paddle wheel; *Bottom*: Empty pond with jets at base of pond (Courtesy of Matt Caspari, Aurora Algae)

6.2 Air-Lifts, Archimedes Screws, Propellers and Water Jets

Air-lifts for pond circulation were first tested by Clement and Van Landeghem (1970) for the culture of *Spirulina*. Their ponds had a ditch-like depression at both ends of the pond. CO_2-rich waste gas was blown into bottom of the ditch at one end of the pond lifting the water from the ditch resulting in flow down the channel; at the opposing end and in the adjacent channel air was similarly introduced. Persoone at al. (1980) also tested airlifts and Märkl and Mather (1985) analysed the design and performance of such air-lift driven systems and concluded that, even at the optimum mixing velocity of 30 cm s^{-1}, the efficiency of the air-lift circulation system will be only about 50%, and the power requirement will be almost twice as high as for paddle wheels.

Propellers have been tested only in small ponds and have been found to have a power demand similar to that of paddle wheels whereas Archimedes screws have a markedly higher power consumption (Laws and Berning 1991).

Water jets have also been tested, mainly in small systems and have generally been found to have a high power requirement (Becker 1994). Recently a jet-type circulation system for algae ponds has been patented (Paresh et al. 2010) and is being used in large raceway-type ponds by Aurora Algae in Australia (Fig. 8.2).

Aside from the generally higher construction and/or operating costs of these systems they all create more shear than

paddle wheels and therefore are unsuitable for many algae species, especially flagellates, which are damaged by the circulation system.

7 Culture Management

The key to reliable production of microalgae in open ponds is to maintain conditions which favour the rapid growth of the species being grown. This is easiest where the species grows in a selective environment such as high nutrients (*Chlorella, Scenedesmus, Phaeodactylum*), high alkalinity (*Spirulina/Arthrospira*) or high salinity (*Dunaliella salina*). However, several species which grow in less 'selective' environments have been cultured successfully for very long periods in open ponds. These include *Nannochloropsis* spp. and *Pleurochrysis carterae*.

Problems with contamination in open ponds are often cited as the reason why reliable commercial microalgae cultivation must be carried out in 'closed' photobioreactors. However, our experience with long-term large-scale cultivation in tubular photobioreactors has shown that contamination can be a significant problem in these systems as well. An important reason for this is that large volumes of air and CO_2 need to be introduced into the culture and partial harvesting at regular intervals means both medium removal and replacement.

A basic requirement for culture stability is good and even mixing throughout the pond so that the algae do not settle out of the culture in regions of low water flow. Homogeneous mixing also means an even distribution of nutrients, pH and O_2 in the pond and all cells regularly move from the deeper parts of the water column where there is less light, to the surface, high light part so that all cells receive the same amount of light thus maximising productivity. Maintaining the culture in the exponential stage of growth is also very important. This can be achieved by regularly harvesting part of the culture and maintaining a constant nutrient supply; i.e. semi-continuous culture. For marine and halophilic species maintaining the salinity in the optimum range for the algae is also critical.

An outdoor open pond algae culture experiences a constantly varying environment, both diurnally and over the annual cycle. It is obvious therefore that for successful reliable outdoor culture the species cultured must have a reasonably broad tolerance in temperature, pH and O_2 concentrations. It must be remembered that the large-scale outdoor culture of microalgae is quite different from the growing of axenic or unialgal cultures in the laboratory. Large-scale microalgae culture is akin to broad acre farming and the algaecultureist has only limited control over many of the environmental factors. Furthermore the cultures are rarely (if ever) unialgal and definitely not axenic. Rather the algae culture in the pond is an ecosystem which needs to be managed carefully to produce the desired outcomes. The correct choice of algae species and strain and its temperature tolerance to match the temperatures encountered in the ponds also is very important to achieving maximum productivities and for easier pond management of competition between species in outdoor ponds (Goldman and Ryther 1976) and the outcome of this competition is modulated by nutrients (D'Elia et al. 1979; Goldman and Mann 1980).

7.1 CO_2 Addition

Most algal cultures are carbon limited and the addition of a carbon source such as carbon dioxide will enhance productivity. In water inorganic carbon is in the form of CO_2 (actually a small amount of $H_2CO_3 + CO_{2(aq)}$), HCO_3^- and CO_3^{2-}. The relative proportion of these three inorganic carbon species is dependent on the pH, salinity and temperature. All algae can take up CO_2 and many (but not all) species can also take up HCO_3^-; none can take up CO_3^{2-} as a carbon source for photosynthesis.

Although bicarbonate salts rather than CO_2 can be used to provide additional inorganic carbon this is not usually done for several reasons. Firstly, the addition of bicarbonate salts (e.g., $NaHCO_3$), although having little effect on the pH of the medium, increases the ionic strength of the medium which may lead to problems over the longer time in freshwater algae culture and, second $NaHCO_3$ is more expensive that CO_2. On the other hand, the addition of CO_2 not only increases the total amount of inorganic carbon in the medium, but also reduces the pH. In cultures without CO_2 addition photosynthetic CO_2 uptake results in the medium becoming more alkaline, and pH values of >pH 9.5 are often reached in dense algal cultures outdoors by mid-day. This high pH means that most of the inorganic carbon is now in the form of CO_3^{2-} and HCO_3^-, and there is little or no CO_2 available for photosynthesis. There is also the possibility of carbonate salts precipitating at the high pH. The addition of CO_2 however, leads to acidification which means that a much larger proportion of the inorganic carbon is in the form of CO_2 and HCO_3^- which can be utilised for photosynthesis by the algae. CO_2 addition can therefore also be used to maintain the pH of the medium at the optimum pH for the algae being grown throughout the day. This is usually done by using a pH-stat system where the CO_2 is added as required to maintain a constant pH.

Efficient addition of CO_2 is important for economic reasons as even 'free' CO_2 from sources such as power station flue gas requires pumping and this is an energy cost to the process.

The transfer of CO_2 through the gas–liquid interface can be described by the equation:

$$Q = kA(C_s - C_d) \tag{8.1}$$

where Q = the mass flux of CO_2 (mM L^{-1}), k = the mass transfer coefficient (M min^{-1}), A = the gas–liquid interface area (m^2), C_s = the saturation concentration of dissolved CO_2 in the liquid in equilibrium with the partial pressure of CO_2 in the gas phase (mM L^{-1}), and C_d = the instantaneous concentration of dissolved CO_2 in the liquid (mM L^{-1}).

From Eq. (8.1) it can be seen that the efficiency of the CO_2 gas transfer to the medium will be affected by the concentration of the CO_2 in the gas phase, the gas–liquid interface area and the time that the gas phase is in contact with the liquid phase. Bubbling the CO_2-containing gas through the medium is the simplest way to supply the CO_2, but in the shallow ponds used for algae culture the residence time of the bubbles in the medium is brief and only a small amount of the CO_2 contained in the gas will be transferred to the liquid phase. The gas transfer efficiency can be improved by using smaller bubbles through the use of sintered porous stones or pipes, resulting in a larger gas–liquid interface area compared to large bubbles. Since in most 'waste' CO_2 streams (i.e. from power station flue gas, cement plants etc.) the CO_2 partial pressure is still relatively low the value of C_s will also be low. The residence time of the bubbles can also be increased by locating the diffusers just upstream of the paddle wheel so that the bubbled are dragged horizontally by the flow of water under the paddle wheel. Pre-concentration of the CO_2 also is an option but this is expensive and generally impractical, especially in an algae-for-biofuels process. Therefore, in order to increase the efficiency of the CO_2 transfer several methods have been developed.

For example, transparent plastic sheets mounted on a frame above the gas diffuser pipes just below the water surface have been used to increase the residence time of the gas phase but were shown to be rather inefficient. Becker (1994) describes a more efficient floating CO_2 injector system based on the system developed by Vasquez and Heussler (1985) and variants of which are used in some commercial algae plants. The device consists of a floating compartment constructed of PVC pipes covered by a transparent plastic sheet and open to the bottom. The CO_2 gas injectors are located at the upstream end of the compartment and spoilers are placed across the injectors to produce turbulence in the water flowing past the injectors so as to enhance gas transfer. The rear (downstream) end of the device is designed to reduce the turbulence to minimise desorption losses to the atmosphere once the water leaves the device. Experiments with dense cultures of *Scenedesmus obliquus* showed that about 4 m^2 of injector area were required per 100 m^2 of pond area to maintain a minimum CO_2 concentration of 0.2mM L^{-1} when pure CO_2 was used. The efficiency of the injector declined over time due to dilution of the CO_2 in the injector by photosynthetically produced O_2 and nitrogen from the atmosphere. Larger areas would also be required if the gas stream contains a lower partial pressure of CO_2.

Several other methods such as hollow fibers for CO_2 supply to open ponds have been tested (Cavalho and Malcata 2001; Su et al. 2008; Gardner 2011). Gas-permeable membranes have also been used in closed photobioreactors but not in open pond systems (Lee and Hing 1989).

7.2 Optimising Productivity

The optimisation of productivity in raceway ponds has been a topic of intense study for many years. The most important limiting factors to growth and productivity are light, carbon supply, oxygen and temperature (Richmond et al. 1990; Borowitzka 1998). In outdoor ponds the process of culture optimisation is complex because all these parameters vary continuously both over the day and also over the year and therefore, unlike most laboratory cultures, outdoor algae cultures are never in a steady state.

Light is clearly the single most important factor affecting productivity. The purpose of photoautotrophic algal culture is to convert the energy in the light to convert CO_2 into chemical energy in the form of organic compounds through photosynthesis. For the production of biofuels and bioenergy using microalgae the organic compounds on interest are lipids, sugars and/or organic biomass. The effective use of the available light (i.e. sunlight in most cases) in order to achieve the maximum photosynthetic efficiency is the fundamental and essential criterion for the sustainable and economic production of biofuels from algae. There is an absolute limit to photosynthetic efficiency and this has been discussed in detail by (Walker 2009; Grobbelaar 2009). Furthermore, light not only provides the energy source for photosynthesis, but too much light can inhibit photosynthesis and potentially damage the photosystems.

Algae, like all plants, use only light in the 400–700 nm wavelengths for photosynthesis. In the light-limited exponential growth phase, the relationship between productivity and the light energy absorbed by the culture (E_oA) can be expressed as follows (Pirt et al. 1980)

$$E_0 A = \mu XV / Y \tag{8.2}$$

where $\boldsymbol{E}_0$ is the light energy, $\boldsymbol{A}$ = the irradiated culture area, $\boldsymbol{\mu}$ = the specific growth rate, $\boldsymbol{X}$ = biomass productivity, $\boldsymbol{V}$ = the total culture volume, and $\boldsymbol{Y}$ = the bioenergetic growth yield.[1]

This equation indicates that the biomass productivity in continuous cultures is determined by the area/volume rela-

[1] The growth yield ($Y_{x/s}$, g cell biomass g^{-1} substrate) is an expression of the conversion efficiency of the substrate to biomass and is defined as the amount of biomass produced (dX) through the consumption of a unit quantity of a substrate (ds), i.e. $Y_{x/s} = dX/ds$. For the bioenergetic growth yield the substate is light.

Table 8.1 Effect of cell concentration at constant irradiance on culture parameters in a steady state culture of *Chlorella ellipsoidea* (Data from Myers and Graham 1958)

Irradiance (kcal day^{-1})	Cell Conc. (mg L^{-1})	Productivity (mg L^{-1} day^{-1})	Photosynthetic efficiency[a] (%)	Specific growth Rate[b] (day^{-1})	Respiration rate (μL O_2 mg^{-1}h^{-1})	Chlorophyll content (%)	E_c[c] (μ Wcm^{-2})
15.2	71	125	4.5	1.76	9.6	1.7	8,800
14.0	104	131	5.1	1.26	7.0	2.2	6,000
14.4	155	139	5.3	0.90	6.2	3.4	4,700
14.1	175	116	4.5	0.66	4.2	3.3	4,000
14.1	202	115	4.5	0.57	4.3	3.6	3,600
14.1	226	100	3.9	0.44	3.3	3.8	3,100
14.5	390	86	3.2	0.22	2.1	4.4	2,000

[a]Productivity × 0.0054; heat of combustion of cells produced is taken as 5.4 cal mg^{-1}
[b]Productivity/cell concentration
[c]Average irradiance per cell

tionship (A/V) so that, in order to obtain high cell densities one must use a reactor with a high A/V ratio. Furthermore, if *Y* for a particular microalga is a constant, the specific growth rate (μ; time^{-1}) can be altered by adjusting *X* without changing any other culture parameters (Richmond 2004).

In dilute cultures (i.e., cultures with low cell densities) at optimal growth conditions of optimum temperature and non-limiting nutrients, the irradiance received by the culture is the only factor which controls growth. Under these conditions the photosynthetic rate of the algae with relation to irradiance follows the classical text-book light response curve (P/E curve). However, dilute cultures have a low productivity as the productivity is a function of the specific growth rate times the cell density; i.e. Productivity = μ × (g L^{-1}). Thus, in order to achieve high productivities, high cell density cultures are required. However, in high cell density cultures not all cells in the culture receive the same amount of light because of *mutual shading* (Tamiya 1957). Light passing through the culture is absorbed by the cells so that it rapidly attenuates and cells deeper in the culture receive less or no light. The higher the cell density the shorter the depth the light penetrates the culture. Oswald (1988) empirically found that the maximum depth that light penetrated in a pond of green algae was 6,000/C_c, where C_c is the cell density in mg dry wt L^{-1}.

In a mixed ponds, such as a paddle wheel driven raceway ponds, the algae are exposed to a more dynamic light environment as mixing of the culture moves individual algal cells between the high-light surface layers of the reactor and the deeper lower-light (dark) layers, exposing the cells to periodic changes in the quantity of light energy received. Figure 8.1 shows a typical light profile in a raceway pond.

Myers and Graham (1958) were the first to show that cell density affected productivity and the efficiency of light utilisation using continuous cultures of *Chlorella* grown in a tubular reactor mixed by gentle aeration (Table 8.1). They found that in the biomass density range from 71 to 390 mg dry wt L^{-1}, maximum productivity was at a biomass density of 155 mg dry wt L^{-1}. At this cell density the highest photosynthetic efficiency was also observed. They calculated the average irradiance (E_c) received by the cells and found that at this cell density E_c was about half of that at the lowest cell density, and about twice as much as available at the highest cell density. This concept of the '*average irradiance*' is very useful when optimising the productivity of algal cultures.

The cell chlorophyll content was also sensitive to the cell concentration (average irradiance) and respiration on a unit biomass basis decreased with increasing cell density. Interestingly, the respiration rate calculated on a culture volume basis remains fairly constant. These results show that at a given irradiance at the culture surface there is an optimum cell density (OCD; in cells mL^{-1}) at which photosynthetic efficiency and productivity are at a maximum. Obviously, the OCD is dependent on the algal species as well as the irradiance and temperature – two parameters which strongly interact – and assumes that the algae are not nutrient limited. The optimum average irradiance is equivalent to the saturating irradiance (E_s) as measured in a photosynthesis/irradiance curve using dilute algal suspensions. The occurrence of an OCD has been demonstrated for many algal cultures such as *Spirulina platensis* (Richmond and Vonshak 1978; Hu et al. 1998b), *Isochrysis galbana* (Hu and Richmond 1994), and *Chlorococcum littorale* (Hu et al. 1998a). Alternatives to the OCD are the optimum chlorophyll concentration (OCC; in g chlorophyll-*a* m^{-3}) (Sukenik et al. 1991) or the optimum areal density (OAD; in g dry weight m^{-2}) (Soeder 1980; Hartig et al. 1988).

Based on the many empirical observations on the variation in the productivity of open ponds with season and cell density/chlorophyll concentration recommendations on changing cell density, pond depth and/or retention time to optimise productivity have been made by various workers (e.g., Azov et al. 1980; Vonshak et al. 1982). Various models of algal biomass production in open ponds and photobioreactors have been developed and such models can be used in culture system management in the changing light environment over the year optimising OCD, OCC and/or OAD in order to achieve the maximum productivity over the whole year. One example if such a model is that developed by

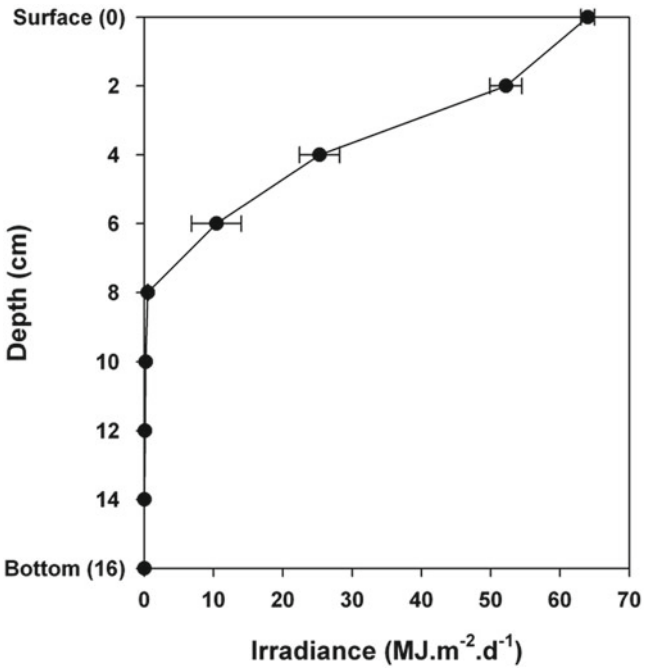

Fig. 8.3 Vertical distribution of light through a *Pleurochrysis carterae* culture in a 16 cm deep raceway pond (cell density = 4.9×10^4 cells. mL^{-1}). (Mean ± SE, $n = 10$) (From Moheimani and Borowitzka 2007)

Sukenik et al. (1991) for the production of *Isochrysis galbana* in outdoor ponds. The model was found to be in good agreement with data from a 100 m^2 pond culture of this alga in Israel (Boussiba et al. 1988). Another, less sophisticated but useful, model for outdoor pond cultures is that of Grobbelaar et al. (1990).

In outdoor cultures the algae are exposed to very high irradiances and this can lead to photoinhibition (Vonshak and Guy 1992) in many species, even in very dense cultures (Hu et al. 1996). Photoinhibition is defined as a light-induced depression of photosynthesis that is manifested as a decrease in the maximum quantum yield of photosynthesis, a decrease in the convexity of the photosynthesis vs. irradiance (P/E) curve and, in the case of prolonged exposure to excessive light, a decrease in the rate of light-saturated photosynthesis (Leverenz et al. 1990; Vonshak and Torzillo 2004). The irradiance at which species are photoinhibited depends on the species. For example, the cyanobacterium *S. platensis* is particularly sensitive to high irradiances whereas the coccolithophorid, *Pleurochrysis carterae* is significantly less sensitive.

Algae can acclimate to higher irradiances[2] and photoacclimation in free-living phytoplankton has been demonstrated in many studies (e.g., Brown and Richardson 1968; Prezelin 1976; Meeson and Sweeney 1982; Olaizola and Yamamoto 1994) as well as in outdoor algal cultures (Moheimani and Borowitzka 2007). Figure 8.2a shows the process of photoacclimation in a *Pleurochrysis carterae* culture which was transferred from a low irradiance indoor environment to a full daylight outdoor environment. The process of full acclimation takes about 4 weeks after which photosynthesis is significantly higher at higher irradiances compared to the indoor low light culture (Fig. 8.2b).

The management of outdoor cultures is further complicated by the rise in $[O_2]$ in the pond due to photosynthetic O_2 production during the day as well as a change in temperature (Fig. 8.3). An increase in $[O_2]$ results in an inhibition of photosynthesis due to photorespiration and is a major factor in reducing productivity (Ogawa et al. 1980). Photorespiration is the light stimulated oxidation of the products of photosynthesis to CO_2 and is due to the oxygenase activity of Rubisco, the essential carboxylating enzyme in autotrophic organisms possessing C3 photobiochemistry. At high O_2 concentrations and low CO_2 Rubisco catalyses ribulose-bis-phosphate to 3-phosphoglyceric acid and 2-phosphoglycolate (Hough and Wetzel 1978). Numerous studies have also suggested that the reactions of dioxygen (O_2) and active oxygen species, such as the superoxide radical (O_2^-), the hydroxyl radical (OH), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$), can cause photoinhibition of photosynthesis (Belay and Fogg 1978; Foy and Gibson 1982; Krause 1994; Singh et al. 1995; Cadenus 2005). Photoinhibition of outdoor algae cultures has been shown in many studies (Lu and Vonshak 1999; Vonshak et al. 2001; Kromkamp et al. 2009; Sukenik et al. 2009; Masojidek et al. 2010).

Photosynthesis and photorespiration are also influenced by temperature, with higher temperatures increasing oxygenase and PSII activities (Belay and Fogg 1978; Verity 1981; Janssen et al. 2002; Morris and Kronkamp 2003). Different algae species have different temperature optima and therefore their response to high irradiance will vary with temperature. For example, Fig. 8.4 shows the effect of temperature at different irradiances on the effect of increasing $[O_2]$ for *Isochrysis galbana*. The gross photosynthetic rate decreased with increasing $[O_2]$ above 50% sat_{air} for all conditions except for the 23°C treatment at 1,200 µmol photons.$m^{-2}.s^{-1}$ which was inhibited at concentrations above 80% sat_{air}. The rate of decrease in gross photosynthetic rate (O_2 inhibition rate) was affected by both irradiance and temperature. The gross photosynthetic rate at 1,200 µmol photons.$m^{-2}.s^{-1}$ was highest at 23°C whereas at 2,500 µmol photons.$m^{-2}.s^{-1}$ the highest temperature specific maximum gross photosynthetic rate was observed at 26°C. Suboptimal temperatures also lead to greater night biomass loss (Torzillo et al. 1991).

For maximum productivity the temperature optimum of the alga cultured should be near the average tempera-

[2] Acclimation, as defined by Raven and Geider (2003), is the change of the macromolecular composition of an organism that occurs in response to variation of environmental conditions.

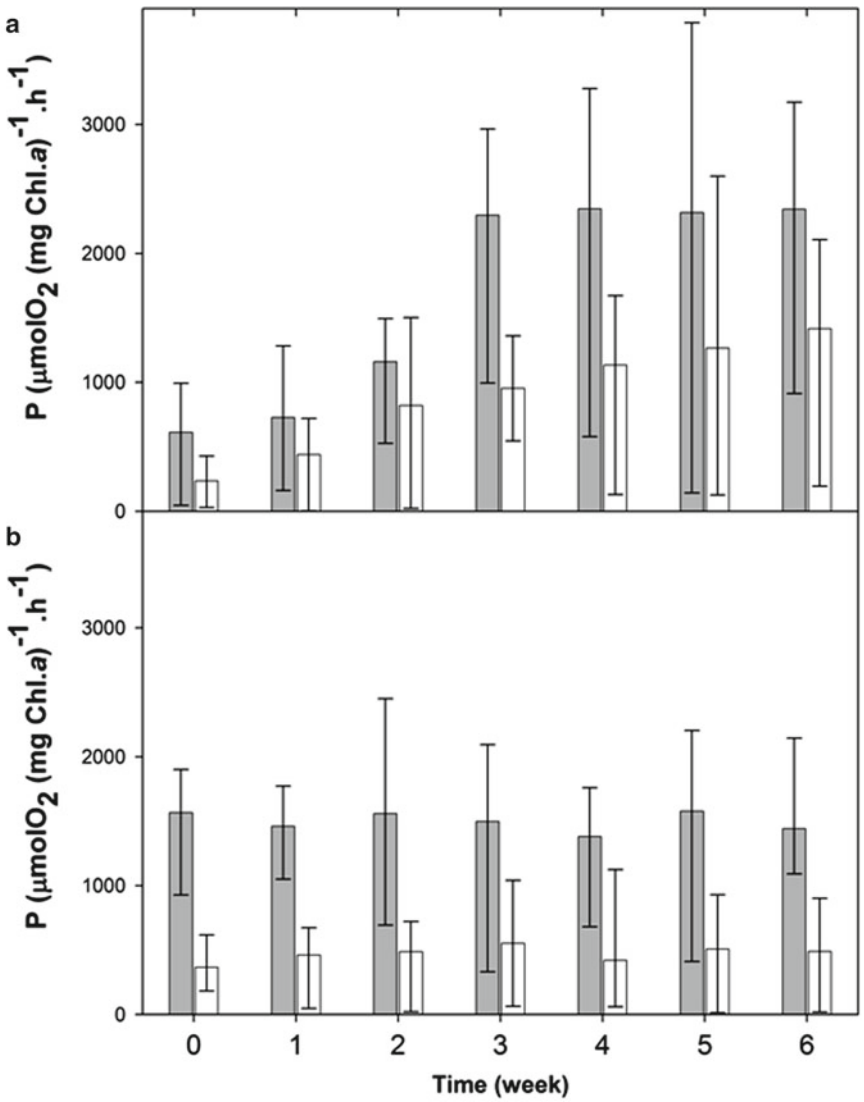

Fig. 8.4 Photoacclimation in *Pleurochrysis carterae*. The algae were: (**a**) grown in an outdoor raceway pond in full daylight starting with an inoculum which had been grown indoors at a low irradiance of 300 µmol photons.m^{-2}.s^{-1}, and (**b**) cultured indoors under a 12:12 light:dark cycle at an irradiance of 300 µmol photons.m^{-2}.s^{-1} and at 25°C. At weekly intervals the photosynthetic rates of the algae were measured at irradiances of 1,800 µmol photons.m^{-2}.s^{-1} (*grey bars*) and 2,300 µmol photons.m^{-2}.s^{-1} (*white bars*). Data are mean and range, n = 3 (From Moheimani and Borowitzka 2007)

ture in the pond during the day and the alga must be able to survive maximum temperature reached in the pond on extremely hot days. When at their optimum temperature algae are better able to utilise the available light and are less likely to be significantly photoinhibited (Torzillo et al. 1996; Borowitzka 1998). The seasonal variation in temperature means that this will, most likely, only be achieved for part of the year. Control of temperature is usually impossible in large outdoor ponds and therefore the selection of the algae strain used must carefully consider the temperature regime in the ponds. For example, in the high solar irradiation environments where very high average annual productivities are possible temperatures in open ponds can reach up to about 35–40°C in summer and they may cool down to less than 5°C at night in winter. Given the seasonal variation in temperature two options to maximise the annual average productivity may be possible: (1) use a strain with a very wide temperature optimum or, (2) change strains between summer and winter. The latter approach has been used successfully at Earthrise Farms in California to extend the growth season of *Spirulina* (Belay 1997). An alternative strategy would be to locate the production plant where the seasonal temperature variation is small such as Hawaii, or to use geothermal energy as is

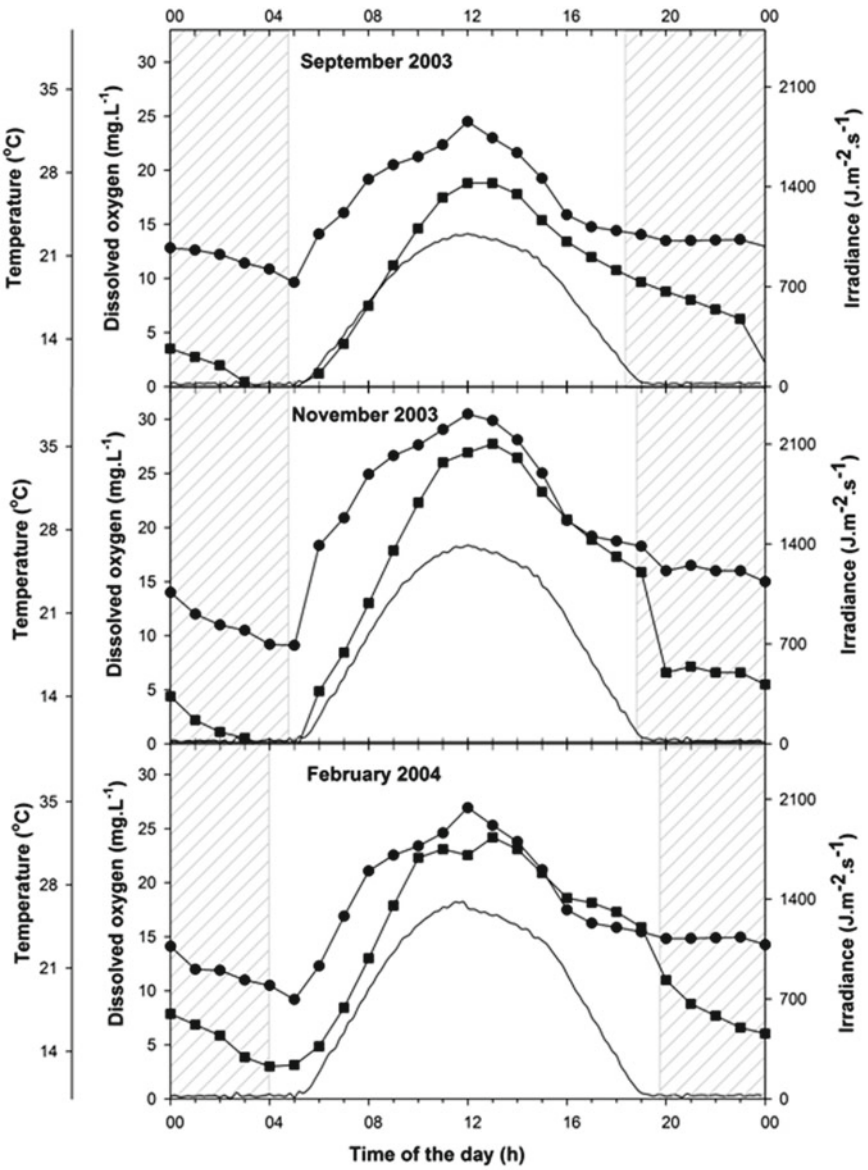

Fig. 8.5 Diurnal pattern in dissolved oxygen concentration (•), irradiance (––), and culture temperature (■) in an outdoor raceway culture of *Pleurochrysis carterae* (From Moheimani and Borowitzka 2007)

being done in Bulgaria (Fournadzieva et al. 1999) and Greece (Fournadzhieva et al. 2002) for the cultivation of *Spirulina* (Fig. 8.5).

The night time temperature is also very important. Higher temperatures usually mean higher respiration and respiration at night results in loss of biomass (Grobbelaar and Soeder 1985), although Torzillo et al. (1991) observed higher night respiration at 25°C (average of 7.6% of total dry weight) than at 35°C (average of 5% of total dry weight) in *Arthrospira* (*Spirulina) platensis* grown in a tubular photobioreactor. This is probably related to the fact that 25°C is suboptimal for this species/strain. They found that night biomass loss depended on the temperature and irradiance during the day as these factors affected the biomass composition. Several studies have found that high daytime irradiance results in increased respiration at night (Banse 1976; Raven 1981). Many studies have found that dark respiration rates increase with increasing irradiance (Falkowski et al. 1985). Respiration in the light is also greater than dark respiration (Weger et al. 1989). However, the observation by Hu et al. (1996) who found that biomass loss due to respiration at night was minimal in *Arthrospira* when the cell density was optimal, suggests that when the algae are not stressed by too high light during the day, respiration at night is reduced thus minimising night biomass loss. It should also be recognised that the

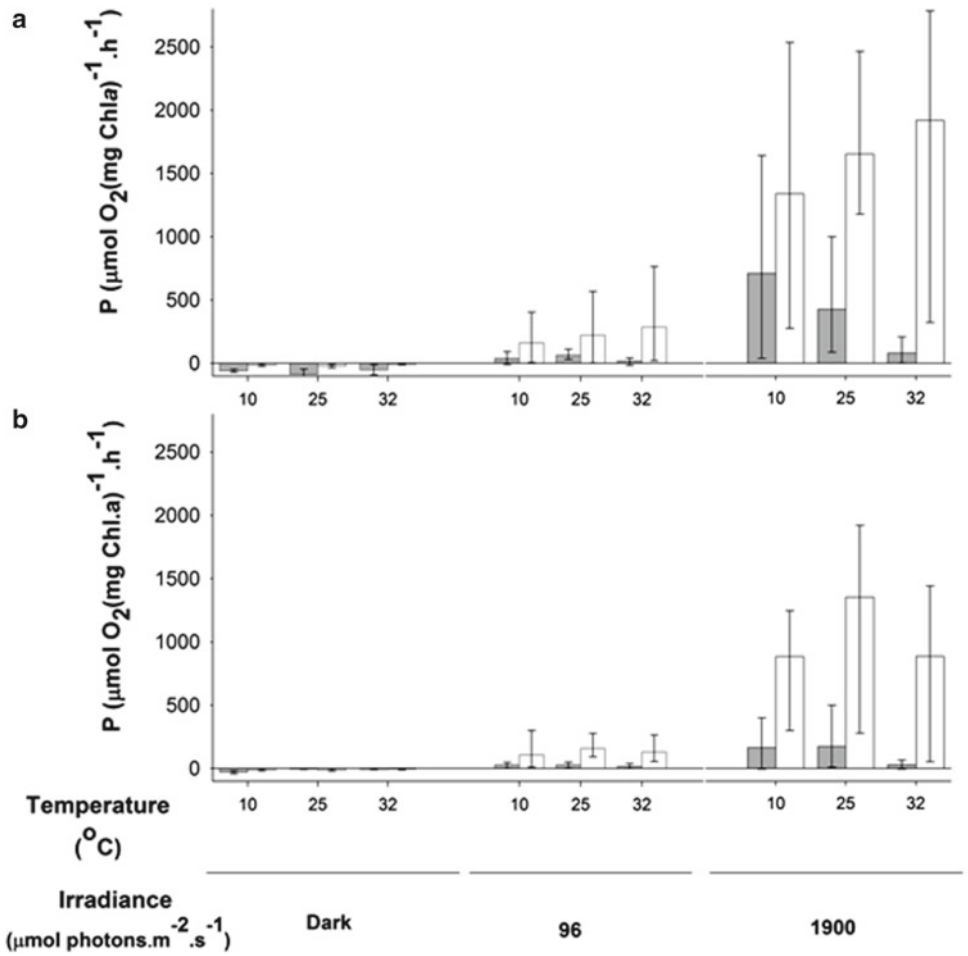

Fig. 8.6 Effect of light and oxygen concentration on photosynthesis of *Pleurochrysis carterae* grown in (**a**) an outdoor raceway pond and (**b**) indoors under a 12:12 light/dark cycle at 300 µmol photons.m^{-2}.s^{-1} and at 25°C. *White bars* = 6–10 mg O_2.L^{-1} and *Grey bars* = 26–32 mg O_2.L^{-1} (mean ± range, n = 3) (From Moheimani and Borowitzka 2007)

algae also respire during the day and higher respiration rates during the day mean a lower net primary production (Geider and Osborne 1989). Maintaining optimum growth conditions reduces respiration rates and will therefore increase productivity.

Cooling of the ponds during the night means that the ponds are below the optimum temperature for algal photosynthesis in the morning (Vonshak et al. 1994). Cultures of *Arthrospira* (*Spirulina), Chlorella* and *Monodus* have been found to show increased photoinhibition at sub-optimal low morning temperatures. An increase in the morning temperature by 4–10°C has been found to significantly increase productivity in many outdoor cultures (Richmond et al. 1980; Vonshak et al. 2001; Moheimani and Borowitzka 2007). Unfortunately it is usually impractical to increase the pond temperature in the morning in commercial-scale outdoor ponds (Fig. 8.6).

7.3 Management and Control of Contaminating Organisms

Open pond cultures of microalgae are constantly exposed to potential contaminating organisms such as bacteria, protozoa, fungi and other algae which can blow in by wind or via the water source. Culture collapse due to phage-like activity has also been reported occasionally in *Arthrospira* (*Spirulina)* pond cultures (Shimamatsu 2004). It is well known that microalgae are easily distributed by wind and rain over large distances (Marshall and Chalmers 1997; Sharma et al. 2007) and therefore other algae species can easily be transferred to the growth ponds. Also, unlike laboratory cultures, it is impossible to sterilise the very large volumes of water needed in commercial-scale production plants. Allelopathic interactions (Inderjit and Dakshini 1994) may also affect the levels

and types of contaminants in the ponds. The management of the cultures to maintain what is essentially a monoculture of the desired species over long periods is therefore a major challenge and is not possible for all species of microalgae.

It is often suggested that unlike open pond systems, 'closed' photobioreactors have the advantage of better control of potential contaminants (e.g., Brennan and Owende 2010). However, the experience so far with large, commercial-scale photobioreactors has shown that these are equally prone to contamination by protozoa and fungi (Borowitzka 1999c; M. Ecke, personal communication), and cannot be maintained in axenic conditions because of the long culture periods (compared to bacteria) required for algae as well as the large quantities of air and water which must be exchanges during the culture process.

For long-term culture stability and minimal contamination problems it is essential that the culture conditions are optimised for the species being grown so that they can outcompete any contaminating species. If the algae grow best in a highly selective environment such as high salinity (*D. salina*), high alkalinity (*Arthrospira*/*Spirulina*) or high nutrients (*Chlorella*) then contamination is less of an issue as long as this environment can be maintained. Some algae also modify the growth environment inhibiting the growth of contaminants. A good example of this is the culture of *P. carterae* which can raise the pond pH during the day to as high as pH 11 (Moheimani and Borowitzka 2006). An increase in the pH to pH 11 can inhibit the growth of unwanted species of algae by restricting the available C_i source and can also be toxic to some protozoa and invertebrates (O'Brien and DeNoyelles 1972; Pedersen and Hansen 2003; Weisse and Stadler 2006). Furthermore, coccolithophorid algae such as *P. carterae* have been shown to produce allelochemicals such as dimethylsulfide (DMS) (Malin et al. 1993; Houdan et al. 2004).

However, for some species such as the freshwater green alga, *Haematococcus pluvialis,* the only option is periodically to empty the pond, clean and disinfect it (if possible) and to restart the pond with a fresh unialgal inoculum. This type of extended batch culture however is more expensive in labour and reduces the overall average productivity as the ponds are non-productive during the cleaning and re-inoculation period. Long-term continuous culture with regular periodic harvesting of part of the biomass is preferred as the algal biomass is cheaper to produce.

If contamination does occur then there are a limited number of possible management options available. Many of these rely on the maintenance specific selective conditions. For example, contamination of *Spirulina* cultures with green algae can be minimised by maintaining the bicarbonate concentrations above 0.2 M and the pH above 10, and operating at high cell densities (Richmond et al. 1982). An effective treatment based on the differential sensitivity of *Spirulina* and *Chlorella* cells to NH_3 is the application of repeated pulses of 1–2 mM NH_3 followed by a 30% dilution of the culture. However, the green alga *Oocystis* sp., which is also an alkaliophile, can still present significant problems (Belay 1997). The application of the herbicide DCMU (3-(3,4-dichlorophenyl)-1,1-dimethyl urea) has also been proposed as a means of inhibiting the growth of other algae in cultures of *Nannochloropsis* (Gonen-Zurgil et al. 1996). The use of formaldehyde (Rothbard 1975; Moreno-Garrido and Cañavate 2001) has been tested to control some ciliates such as *Euplotes* in algae cultures. Rotifers, especially *Brachionus* spp., can be a problem both in freshwater and marine cultures. Becker (1994) recommends lowering the pH to about 3.0 by the addition of acid and allowing the culture to stand at this pH for 1–2 h. Following this, the pH can be readjusted to pH 7.5 with KOH. This treatment does not affect the algal cultures but effectively eliminates the rotifers. This method has also been used successfully in cultures of *Nannochloropsis* (Zmora and Richmond 2004). Rotifers and cladocerans have also been controlled in algae ponds by temporarily raising the free ammonia concentration in the pond to 20 mg L^{-1} (as N) by the addition of ammonium hydroxide (Lincoln et al. 1983). In fact, experience has shown that extremes of pH (either acid or alkaline) are better tolerated by algae than by protozoa and other zooplankton (O'Brien and DeNoyelles 1972; Pedersen and Hansen 2003). Therefore, the high pH which is reached by dense algae cultures during the day due to photosynthetic CO_2 uptake can, by itself, serve to reduce the population of contaminating protozoa. Rotifers have also been shown to be susceptible to extreme pH values (Mitchell 1992). Chlorination has also been applied to eliminate grazers in cultures of *Nannochloropsis* (Zmora and Richmond 2004) and has also been found to be effective for several species by us. Concentrations of active chlorine of about 4–10 ppm are used. Ozonation has also been proposed to eliminate contaminating organisms in *Nannochloropsis* cultures (Weissman et al. 2010). The use of pesticides to control zooplankton has also been tested (Loosanoff et al. 1957), but these have not been used in large-scale cultures as yet and the long-term effects on cultures is not known.

Recently the use of selective herbicides (e.g., glyphosate) and herbicide resistant strains has been proposed as a method to maintain monospecific outdoor algae cultures (Vick 2009). Such an approach may be possible if the algae are grown for biofuels, but would not be acceptable if the product is a nutraceutical or health food.

Larger contaminating organisms such as aquatic insects including the Ephydridae (brine-flies), Corixidae (waterboatmen) and Chironomidae (midges) in freshwater cultures, and brine shrimp (*Artemia*) in saline cultures, can be managed mainly by netting *in situ* and during preharvest screening.

Bacteria and fungi are usually not a problem in intensive 'clean' (i.e. not grown on waste water) algal cultures,

presumably because there is little dissolved organic carbon in the medium which would provide the energy source for these organisms. Many algal viruses have also been isolated and characterized (e.g., Cottrell and Suttle 1991; Van Etten 1995; Chen et al. 2009), however algae seem to have evolved mechanisms of coexistence with these viruses (Thyrhaug et al. 2003). In commercial algae ponds viruses appear not to have presented major problems so far, although they have been isolated from such ponds (Jaquet et al. 2012).

The method of harvesting, and whether or not the medium after harvesting is recycled to the pond, may also affect the level of contamination. Both the method of harvesting and the frequency of harvesting are important. For example, harvesting by filtration using vibrating screens as is done for *Arthrospira* (*Spirulina*) selectively removes the larger filaments (trichomes) of *Arthrospir*a but not smaller, shorter filaments, not smaller unicellular algae such the chlorophytes *Chlorella* or *Oocystis*. This selective harvesting method can lead to the build up of the unharvestable short-filament strain of *Arthrospira* (*Spirulina*) in the pond as well as a build-up of the contaminating green alga and eventually the pond culture has to be restarted from a fresh inoculum.

7.4 Recycling of the Medium

Recycling of the growth medium after harvesting of the cells is generally an important part of the culture process. It is necessary to reduce nutrient costs as the medium after cell harvesting still contains significant amounts of nutrients. If this medium were discarded it would represent not only an economic cost, but the discharge of large volumes of nutrient-rich water would also present an environmental problem. However, recycling of the medium presents some potential hazards.

First, depending on the harvesting process used, the recycled medium may be enriched (compared to the culture in the pond) in contaminating organisms and/or cell types with undesirable characteristics. When returned to the growth pond this may result in an eventual change in the composition of the biomass in the growth pond. For example, one problem with harvesting *Arthrospira* (*Spirulina*) by filtration is that both contaminating algae such as *Chlorella* and short filaments of *Arthrospira* (*Spirulina*) pass through the filters. In time this can result in overgrowth of the pond by the *Chlorella* or by *Arthrospira* (*Spirulina*) with short filaments which cannot be harvested (Belay 1997).

Second, when chemical flocculation is used in harvesting, the recycled medium may be contaminated with the flocculant. This may result in unwanted flocculation of the algae in the growth pond reducing productivity. It may also lead to product contamination as, for example, with Al when alum is used as a flocculant.

Third, cell breakage during harvesting and the pumping of the culture can lead to a build up of organics in the water which can lead to increased bacterial growth and potential culture loss (Belay 1997).

Recycling of medium may also lead to the build-up of growth inhibitory substances either released by the algae themselves (autoinhibitors – (Harris 1971) or as a result of cell breakage and therefore reduce the growth of the algae (Rodolfi et al. 2003). Free fatty acids have been shown to be compounds commonly leading to autoinhibition in microalgae cultures (Yamada et al. 1993; Ikawa et al. 1997; Bosma et al. 2008; Yingying et al. 2008). Therefore, if medium recycling is undertaken, then the medium may need to be preconditioned or otherwise treated (Belay 1997).

7.5 Using Waste Water

The use of waste waters presents an environmentally beneficial and potentially economic source of nutrients for algae culture. The use of microalgae in wastewater treatment is well established (see Chapter 9) and the culture of microalgae on various wastewater streams has been extensively studied – for example: *Chlorella* (Cheeke et al. 1977; Rohani et al. 1994; Li et al. 2012), *Arthrospira* (*Spirulina)* (Kosaric et al. 1974; Tanticharoen et al. 1993; Laliberté et al. 1997; Phang et al. 2000; Olguin et al. 2003), *Botryococcus* (Sawayama et al. 1992, 1994; An et al. 2003), *Dunaliella* (Rose and Cowan 1992), *Phormidium* (Cañizares-Villanueva et al. 1994) and *Oocystis* (de la Noüe et al. 1984). These studies show that wastewater or the effluent arising after anaerobic digestion of wastes can be used potentially for the cultivation of microalgae for biofuels.

One alternative 'open pond wastewater-treatment system' is the algae turf scrubber (ATS) developed by Adey (1982) which uses attached, mainly filamentous, algae also known as periphyton (Sladekova et al. 1983). The possible application of this system for producing biofuels is discussed in the recent review by Adey et al. (2011).

7.6 Productivity of Outdoor Open Pond Systems

For economic and sustainable production of biofuels from algae high and reliable annual average biomass and lipid productivity is essential (Borowitzka and Moheimani 2010). An obvious, and important, question is: "What is the maximum achievable productivity?" There has been much debate and speculation about this value. In 1991 Sukenik and colleagues

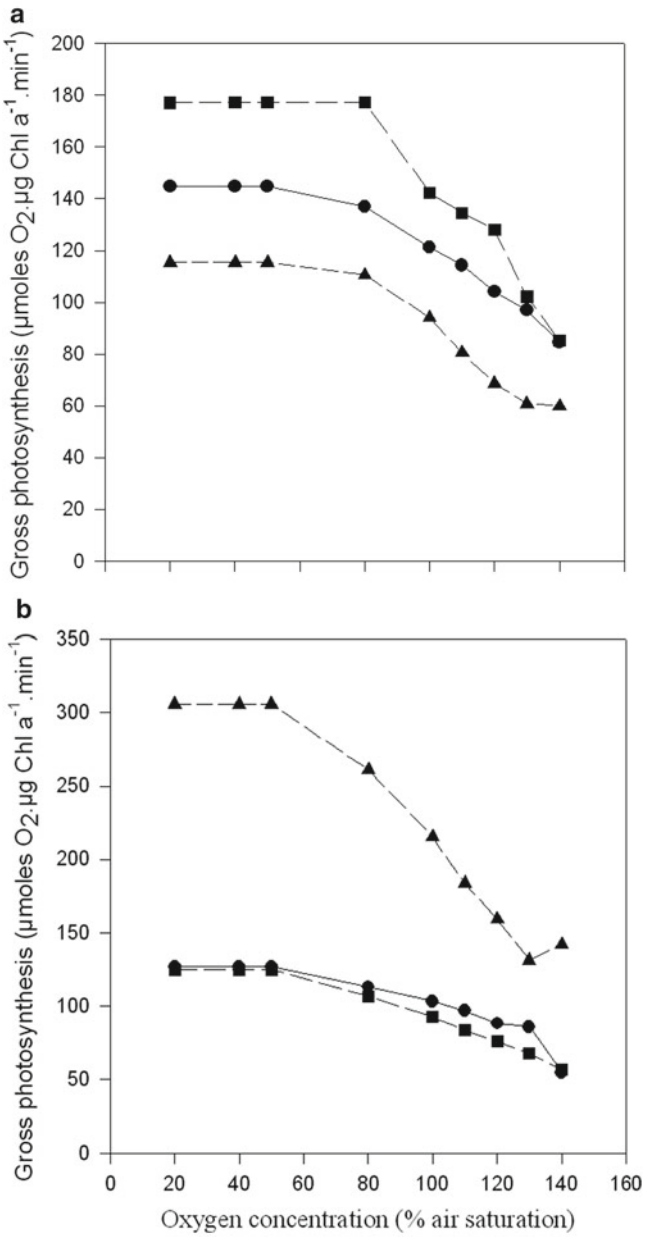

Fig. 8.7 The effect of oxygen concentration on gross photosynthesis in *Isochrysis galbana* at different irradiances and temperatures. (**a**) 1,200 µmol photons m^{-2} s^{-1} (**b**) 2,500 µmol photons m^{-2} s^{-1}. ●=20°C, ■=23°C, ▲=26°C

predicted an annual average productivity of 9.7 g(C) m^{-2} day^{-1} for *Isochrysis galbana* in open ponds in Eilat, Israel (29°33′N, 34°57′E) (Sukenik et al. 1991). Using a somewhat different approach based on available solar energy and a realistic microalgae photosynthetic efficiency Ritchie (2010) calculated that the maximum achievable biomass productivity is no more than 10 g(C) m^{-2} day^{-1}. This equates to a theoretical maximum biomass productivity of 20 g (ash-free dry weight) m^{-2} day^{-1} based on an average microalgal biomass C content of 50% (Sánchez Mirón et al. 2003) (Fig. 8.7).

The maximum quantum yield (Φ_{max}) is generally accepted to be 8 photons or 0.125 mol C (mol quanta)$^{-1}$ (Wozniak et al. 2002) and this equates to a maximum areal productivity of no more than 12 g(C) m^{-2} day^{-1}, although in cultures with a short light path and high degrees of mixing (i.e. intermittent light/dark frequencies of >10 ms) at photosynthetic efficiencies of 2 and 8% the theoretical productivity may be increased to 50 and 200 g dry weight m^{-2} day^{-1}, respectively (Grobbelaar 2009). These calculations are based on locations in regions with high irradiance and therefore productivities would be lower in regions with lower solar irradiance.

Data on actual long term productivities in open ponds are scarce, and there is even less data for closed photobioreactors. Table 8.2 summarises published data on long-term (≥3months) productivities in open systems. The maximum sustainable biomass productivities in open ponds is in the range of 20–25 g (dry weight) m^{-2} day^{-1} with the highest productivities in summer ranging from 26.5 to 37 g m^{-2} day^{-1} (Table 8.2). On exceptional days productivities of up to 50 gm^{-2} day^{-1} have been achieved in outdoor ponds (Heussler et al. 1978; Borowitzka and Moheimani, unpublished results). In winter (lower light, shorted days and lower temperatures) productivities can be up to five times or more lower than summer productivities. The lower summer productivities of 10–15 g m^{-2} day^{-1} can probably be attributed mainly to operational factors such as CO_2-limitation, O_2 build-up, poor mixing and/or suboptimal cell densities.

Almost all of the systems summarised in Table 8.2 are small-scale systems and unfortunately there is little information available on commercial-scale algae production (e.g. from companies such as Earthrise, Cyanotech and BASF), but there is no reason to assume that they are achieving annual average productivities in excess of 20 g (ash-free dry weight) m^{-2} day^{-1}, and productivities are likely to be less. Earthrise report annual average productivities of about 8.2 g m^{-2} day^{-1} for their cultivation of *Arthrospira* (*Spirulina)* in California, USA (Belay 1997).

Mixotrophic cultivation outdoors with *Chlorella* is also undertaken in Taiwan using acetate as the organic carbon source (Iwamoto 2004) and productivities in summer of 30–35 g m^{-2} day^{-1} on sunny days, 20–25 g m^{-2} day^{-1} on cloudy days, and 10–15 g m^{-2} day^{-1} on rainy days have been reported. These productivities are about 1.5–2.0 times higher than those of photoautotrophic cultivation (Iwamoto 2004).

8 Conclusion

The cultivation of microalgae in open outdoor culture systems such as raceway ponds was first attempted in the early 1950s (Gummert et al. 1953; Mituya et al. 1953; Krauss 1962) and has been a reality since the 1960s and is the method used in most commercial microalgae production plants around the

Table 8.2 Reported biomass productivities of algae in outdoor grown in open pond culture for periods of 3months of greater

Cultivation system	Culture volume (L)	Culture period (months)	Productivity range		Species	Location	References
			Areal (g dry weight.m^{-2}.day^{-1})	Volumetric (g dry weight.L^{-1}.d^{-1})			
Raceway[a]	300	+3	9.4–23.5	≈ 0.031–0.078	*Anabaena* sp.	Spain	Moreno et al. (2003)
Raceway (in greenhouse)	260	2	13.2	0.05	*Chlorella* sp.	Japan	Hase et al. (2000)
Raceway	–	–3	9	–	*Cyclotella* sp. CM1-1 (reduced antenna size)	USA	Huesemann et al. (2009)
Raceway	–	–3	12	–	*Cyclotella* sp. wild type	USA	Huesemann et al. (2009)
Raceway	600	12	5–40	0.008–0.060	*Tetraselmis* sp.	Japan	Matsumoto et al. (1995)
Raceway[a]	?	+3	1.6–3.5	?	*Dunaliella salina*	Spain	Garcia et al. (2003)
Raceway[a]*	110	12	20–37	0.22–0.34	*Dunaliella salina*	Perth, Australia	Moheimani and Borowitzka (2006)
Raceway	200	–3	14.7–18.1	0.183–0.226	*Gloeotrichia natans*	Israel	Querijero-Palacpac et al. (1990)
Raceway	100	0.67	12.9 ± 0.16	0.129	*Muriellopsis* sp.	Italy	Blanco et al. (2007)
Raceway[a]*	160–200	12	16–33.5	0.11–0.21	*Pleurochrysis carterae*	Perth. Australia	Moheimani and Borowitzka (2006)
Raceway	–	12	15	–	*Scenedesmus obliquus*	Bangkok, Thailand	Payer et al. (1978)
Raceway[a]	750	+3	15–27	0.06–0.18	*Spirulina platensis*	Israel	Richmond et al. (1990)
Raceway*	–	12[a]	8.2	–	*Spirulina platensis*	USA (California)	Belay (1997)
Raceway	13,200–19,800	12	14.5 (5.8–24.2)[b]	0.03–0.12	*Spirulina platensis*	Antofagasta, Chile	Ayala et al. (1988)
Raceway[a]*	282	+3	14.47 ± 0.16	0.183 ± 0.02	*Spirulina platensis*	Italy	Pushparaj et al. (1997)
Raceway[a]	135,000	+3	2–17	0.006–0.07	*Spirulina* sp.	Spain	Jimenez et al. (2003)
Raceway[a,d]	?	+3	9–13	?	*Spirulina* sp.	Mexico	Olguin et al. (2003)
Raceway[e]	500	12	11.2	0.024	*Tetraselmis* sp.	Japan	Matsumoto et al. (1995)
Raceway	300–600	6	5–26	0.01–0.05	*Tetraselmis suecica*	Italy	Pedroni et al. (2004)
Raceway	120	12	5.0–24.0[c]	0.04–0.20	*Porphyridium cruentum*	Israel	Cohen et al. (1988)
Inclined thin l ayer pond	1,000	+3	10–30	1–3	*Chlorella* sp.	Czech Republic and Spain	Doucha and Livansky (2006)
Inclined thin l ayer pond	~2,500	+2	23	–	*Scenedesmus* sp.	Rupite, Bulgaria	Vendlova (1969)
Inclined thin layer pond	~2,500	7	19	–	*Scenedesmus obliquus*	Rupite, Bulgaria	Dilov et al. (1985)
Inclined thin layer pond	~2,500	+2	12	–	*Scenedesmus* sp.	Tylitz, Poland	Vendlova (1969)
Continuous flume (raceway)	4,150	+3	2.4–11.3	0.0028–0.13	*Phaeodactylum tricornutum*	Hawaii	Laws et al. (1983)
Circular central pivot pond	1,960	+3	1.61–16.47	0.02–0.16	*Chlorella* sp.	Japan	Kanazawa et al. (1958)
Circular central pivot pond	1,960	+3	2.43–13.52	0.03–0.13	*Scenedesmus* sp.	Japan	Kanazawa et al. (1958)
Circular central pivot pond	Up to 11,500	12	21.5[f]		*Chlorella* sp. (mixotrophic culture)	Japan	Tsukuda et al. (1977)
Open culture system[g]	2,400–16,200	12	19–22[f]		*Chlorella* sp.	China	Tsukuda et al. (1977)

Note that productivities other than those marked with a * are on a dry weight rather than an ash-free dry weight basis and since the ash content (i.e. non-organic content) of microalgae ranges from about 2–10% of dry weight they are therefore are an overestimate

[a]This figure is the annual average productivity at Earthrise farms, but the growth season is only 8months.

[b]Data are the annual mean and the summer and winter mean in brackets. Culture depth was 12 cm in winter and 18 cm in summer

[c]Data shown are the highest productivity achieved in winter and summer

[d]The algae were grown on the effluent after anaerobic digestion of piggery waste water

[e]Recalculated from Fig. 8.4 in paper

[f]Recalculated from annual production figures (the range shown is the variation between different production plants)

[g]The exact culture system is not stated

world. Although it is easiest to grow species such as *Dunaliella, Arthrospira* (*Spirulina)* and *Chlorella* which live in highly selective environments many other species have been grown successfully for extended periods. Under optimum conditions annual average productivities of over 20 g dry weight m^{-2} day^{-1} are achieved. However, the large-scale 'farming' of microalgae is still less than 60 years old compared to the thousands of years of land-based farming of crops such as wheat and rice and much remains to be learned in microalgae farming and the management of large-scale outdoor cultures of microalgae.

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Wastewater Treatment and Algal Biofuel Production

9

Rupert J. Craggs, Tryg J. Lundquist, and John R. Benemann

1 Introduction

Oswald and Golueke (1960) first proposed the large-scale production of algae as a biofuel feedstock using high rate algal ponds (HRAP), with wastewater providing the make-up water and nutrients. HRAP have since been developed as a low-cost efficient wastewater treatment technology (Oswald 1988a; Craggs 2005). The concept of producing biofuel from algae harvested from wastewater treatment HRAP effluent is a niche opportunity that could be economical today, since the harvested algal biomass would essentially be a "free" by-product of wastewater treatment.

2 Wastewater Treatment Ponds

Wastewater treatment ponds rely on algal photosynthesis to harness sunlight energy and provide oxygen to drive aerobic bacterial degradation of organic compounds. The wastewater nutrients that are released are, in turn, assimilated into algal biomass (Oswald 1988a). However facultative ponds, the unmixed, ~1 m deep ponds that are widely used throughout the world for wastewater treatment do not consistently provide a high level of nutrient removal and have very low algal biomass productivity. For example, in New Zealand, such ponds have an average annual production of little more than 10 tonne ha^{-1} $year^{-1}$ (Davies-Colley et al. 1995; Craggs et al. 2003), well below that required for economical biofuel production. High Rate Algal Ponds (HRAP) have much higher treatment performance and algal productivity, and could provide sufficient algal biomass to be economically used as a biofuel feedstock.

2.1 High Rate Algal Ponds

HRAPs are paddlewheel mixed, shallow, open channel raceways (Fig. 9.1) that were developed in the late 1950s for wastewater treatment and resource recovery by Oswald and co-workers at the University of California at Berkeley (Oswald et al. 1957). Oswald applied HRAP technology to treat the wastewater of several northern California cities over the next 40 years. For example, St Helena (in 1967) (Fig. 9.2) and Delhi (in 1997), and both systems still operate today. HRAPs are used at wastewater treatment plants around the world and have been shown to be capable of treating a variety of agricultural and industrial wastes (Oswald 1988a; Craggs 2005).

HRAPs are also used by the majority of algal farms that produce algae for high value health food supplements, pigments and chemicals (Borowitzka and Borowitzka 1988; Weissman et al. 1988). Earth-lined HRAPs have much lower capital costs than closed photobioreactors but at current fossil fuel prices it is unlikely that even the relatively simple HRAP could be economically used for algal biofuel production alone, without producing high value co-products or providing beneficial functions (such as wastewater treatment) (Oswald and Golueke 1960; Benemann and Oswald 1996; Benemann 2003).

2.2 Algal Production in HRAPs

New Zealand HRAPs treating domestic wastewater have been shown to have algal yields of about 0.2 tonne ML^{-1} of

R.J. Craggs (✉)
National Institute for Water and Atmospheric Research,
PO Box 11-115, Hamilton, New Zealand
e-mail: r.craggs@niwa.co.nz

T.J. Lundquist
Civil and Environmental Engineering Department,
California Polytechnic State University,
San Luis Obispo, CA 93407, USA
e-mail: tlundqui@calpoly.edu

J.R. Benemann
Benemann and Associates, Walnut Creek, CA, USA
e-mail: jbenemann@aol.com

M.A. Borowitzka and N.R. Moheimani (eds.), *Algae for Biofuels and Energy*, Developments in Applied Phycology 5,
DOI 10.1007/978-94-007-5479-9_9,

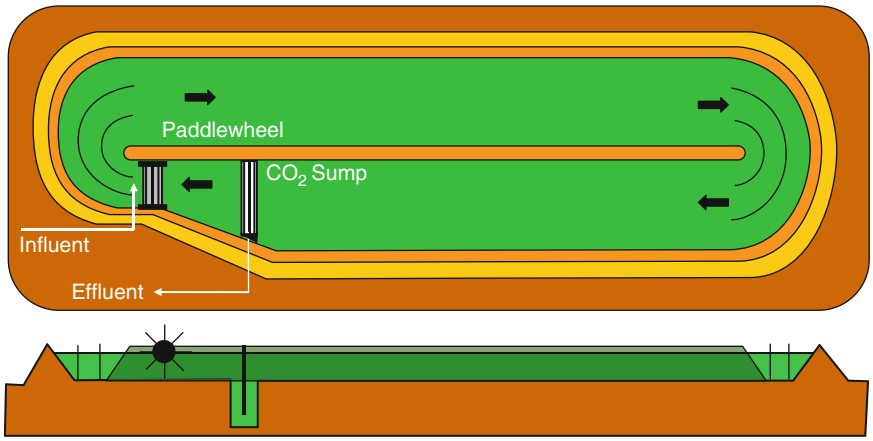

Fig. 9.1 Plan view and side elevation of a high rate algal pond with CO_2 addition

wastewater and productivities of almost 30 tonne ha^{-1}·year, which is between two to three-fold that of facultative wastewater treatment ponds (Craggs et al. 2003). However, algal production in such ponds is severely carbon-limited due to the low C:N ratio of wastewaters (typically 4:1 for domestic wastewater) compared to algal biomass, which can range from about 10:1 to 5:1, depending, respectively, on whether N is limiting or not (Benemann 2003; van Harmelen and Oonk 2006; Lundquist 2008). Thus domestic wastewaters contain insufficient carbon to remove all of the nitrogen by assimilation into algal biomass. Carbon-limitation in wastewater treatment HRAPs is indicated by elevated daytime pond water pH, resulting from the use of bicarbonate ions as a CO_2 source for algal photosynthesis, releasing hydroxide ions which can increase pond water pH to >10 (Oswald 1988a; Garcia et al. 2000; Craggs 2005; Kong et al. 2010; Park and Craggs 2010a).

The growth of many algal species is increasingly inhibited at a pond water pH greater than 8 (Kong et al. 2010). For example, the productivity of the alga *Chlorella* sp. declined by 22% when pH was raised from 8 to 9 (Weissman et al. 1988). Although, some algae are still capable of growing under carbon limited conditions, including many of the desmids, e.g. *Ankistrodesmus* sp. which grows well at pH 10 (Weissman et al. 1988).

The high pH inhibition of algal growth could in part be due to high free ammonia concentrations at high pH (Azov and Goldman 1982; Azov et al. 1982; Konig et al. 1987). For example, growth of the alga *Scenedesmus obliquus* at free ammonia concentrations of 34 and 51 g m^{-3} (pH 9.5 and 20–25 °C) was reduced by 50 and 90% respectively (Azov and Goldman 1982). Intense photosynthesis in HRAP also increases daytime pond water dissolved oxygen levels to >200% saturation. High oxygen levels have also been shown to reduce algal productivity (Weissman et al. 1988).

High pond water pH also inhibits the growth of aerobic heterotrophic bacteria that oxidize organic matter to CO_2 in wastewater treatment HRAP (Weissman and Goebel 1987; Oswald 1988a; Craggs 2005). These bacteria typically have an optimum pH of ~8.3, above which bacterial activity is increasingly inhibited (Craggs 2005).

Addition of CO_2 to wastewater treatment HRAPs would therefore augment carbon availability and avoid pH inhibition to enhance algal production and nutrient removal by assimilation into algal biomass. This can be simply achieved through control of the HRAP water maximum pH to below 7.5–8.0 by CO_2 addition. There is little published information on CO_2 addition to wastewater treatment HRAPs, however, CO_2 addition has been shown to more than double the productivity of algal cultures (Benemann et al. 1980; Azov et al. 1982; Benemann 2003; Lundquist 2008; Park and Craggs 2010a) and is practiced at all commercial algal farms (van Harmelen and Oonk 2006). The first small-scale experiments on CO_2 addition to wastewater HRAP were conducted at Richmond, California (Benemann et al. 1980). Initial trials of CO_2 addition to HRAP treating agricultural drainage waters were conducted by Gerhardt et al. (1991) and a large-scale trial was later successfully operated over several years.

For wastewater treatment HRAP the source of CO_2 could be the flue gas from the power generated using the biogas produced by anaerobic digestion of solids removed from the wastewater, both as settled raw sewage sludge (during "primary treatment") and the algal biomass harvested from the HRAPs (Benemann 2003). Further sources of CO_2 could be from the digestion of the biomass residues resulting from the conversion of algal biomass to other biofuels, such as ethanol or biodiesel; the digestion of other organic waste sources such as household or garden waste; or directly using waste gaseous emissions, such as power plant flue gas (van Harmelen and Oonk 2006; Chisti 2008; Lardon et al. 2009). The use of HRAP to purify biogas (scrub CO_2 and H_2S) using cost-effective apparatus for mixing the gas into pond water has been demonstrated (Conde et al. 1993; Mandeno et al. 2005). Recent pilot-scale research during New Zealand summer conditions has shown that CO_2 addition to wastewater HRAP

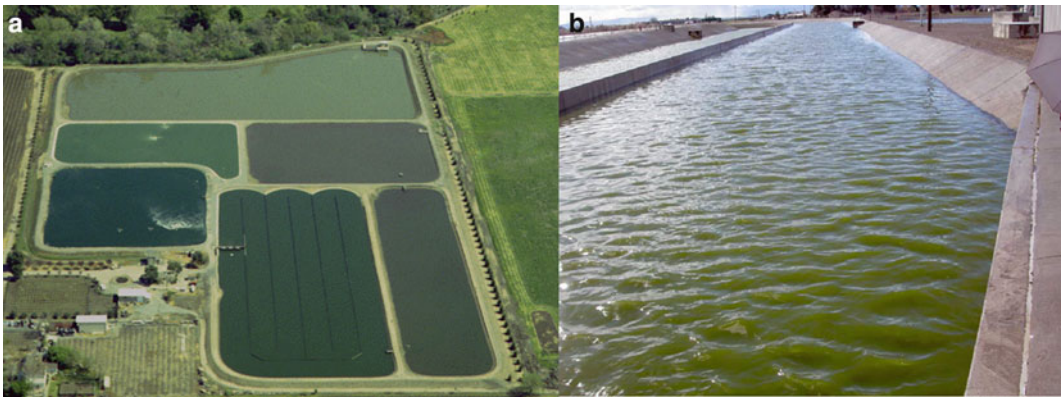

Fig. 9.2 Wastewater treatment HRAPs operating at St Helena (**a**) and Hilmer (**b**) in California

Fig. 9.3 One of the four wastewater treatment HRAP (1.25 ha) with CO_2 addition at Christchurch, New Zealand

can increase algal production by 30–100% (Yield: up to 0.3 tonne ML^{-1}; projected productivity: as high as 60 tonne ha^{-1}·year) (Heubeck et al. 2007; Park and Craggs 2010a) (Fig. 9.3).

A major disadvantage of wastewater treatment HRAPs is the relatively large land requirement compared with electro-mechanical treatment systems (e.g. activated sludge), however HRAPs have a smaller footprint than conventional facultative wastewater pond systems. The algal biomass production potential from wastewater treatment HRAP is limited by daily insolation and temperature, and hence the area necessary for effective year-round wastewater treatment increases with increasing latitude (Bouterfas et al. 2002; Jeon et al. 2005; Voltolina et al. 2005). For example a HRAP area of ~1.7 ha is required per ML·day of wastewater flow under suitable Southern Californian climatic conditions, increasing to ~2.7 ha ML^{-1}·day under the limiting climatic conditions of New Zealand. Algal productivity increases are needed to improve the economics of algal biofuels, and could be achievable through research on HRAP design and operation, and selection of algal strains that thrive in the HRAP environment (high sunlight, high daytime pH, temperature and super-saturated dissolved oxygen).

2.3 Algal Grazers and Pathogens

Herbivorous zooplankton such as rotifers and cladocerans graze on HRAP algae, and when present at high density, can reduce pond water algal concentrations to low levels within a few days (Benemann et al. 1980; Picot et al. 1991; Cauchie et al. 1995; Nurdogan and Oswald 1995; van Harmelen and Oonk 2006; Smith et al. 2009). For example, rotifers and cladocerans at densities greater than 100 per litre were found to reduce wastewater treatment HRAP algal concentration by 90% within 2 days (Oswald 1980), and several days of

grazing by a population of the cladoceran, *Daphnia* sp., reduced the chlorophyll *a* concentration of a pond by 99% (Cauchie et al. 1995). Algae are also susceptible to fungal parasitism and bacterial or viral infection which can also reduce the pond algal population within a few days and can result in changes in algal cell structure, diversity and succession (Wommack and Colwell 2000; Short and Suttle 2002; Kagami et al. 2007).

Therefore, to maximize HRAP algal productivity, populations of zooplankton grazers, parasitic fungi, and infective bacteria and viruses must be controlled. Zooplankton grazer populations may be limited by application of chemicals or invertebrate hormone mimics, or by increasing pond water pH to 11, particularly if the pond water has a high ammoniacal-N concentration (O'Brien and De Noyelles 1972; Schluter and Groeneweg 1981; Oswald 1988b). There are no practical control methods for fungal paracitism or bacterial and viral infections, and further research is required to fully understand their influence on algal productivity in wastewater treatment HRAP.

3 Wastewater Treatment in HRAPs

HRAPs can be used to provide effective aerobic treatment (oxidation of organic matter) and assimilate soluble nutrients from many types of wastewater (e.g., anaerobic pond effluent, domestic wastewater pre-treated to the primary or secondary level, agricultural wastewaters, etc.).

3.1 Aerobic Treatment

HRAP aeration efficiency in terms of the power required for gentle paddlewheel mixing and photosynthetic oxygen production by the algae, varies between 0.04 and 0.15 kWh_e kg^{-1} O_2 produced depending on season, insolation and other factors (Benemann et al. 1980; Oswald 1988b; Green et al. 1995). This equates to 50–110 kWh_e ML^{-1} of wastewater. In comparison, activated sludge requires from 230 to 960 kWh_e ML^{-1} (based on 0.4–1.7 kWh_e kg^{-1} O_2) (Owen 1982; Metcalf and Eddy, Inc. 1991; Green et al. 1995). HRAP integrated wastewater treatment amortized capital and operation costs (~US450,000 ML^{-1}) are only 25–33% of those of secondary-level activated sludge treatment (Green et al. 1995; Downing et al. 2002).

3.2 Nutrient Removal

Nitrogen removal by nitrification-denitrification is a common electromechanical nutrient removal process, but it is costly and for a typical wastewater primary effluent nitrogen concentration of 30 g N m^{-3} requires additional aeration energy of ~400–1,000 kWh_e ML^{-1} of wastewater (Owen 1982). HRAPs with CO_2 addition could provide energy efficient tertiary-level nutrient removal for little additional energy cost (Benemann et al. 1980; Nurdogan and Oswald 1995; Woertz 2007; Park and Craggs 2010a). Algal biomass can exhibit N:P ratios ranging from nearly 4:1 (under nitrogen limiting conditions) to almost 40:1. These N:P ratios correspond to algal biomass N and P content percentages typically of 10% N and 1% P and 4% N and 0.4% P (under nitrogen limiting conditions). Due to this large, almost tenfold, range in microalgal N:P ratios, N removal is the key issue in HRAP tertiary-level treatment (nutrient removal), since efficient P removal does generally not require additional algal biomass production above that needed for N assimilation. Near-complete assimilation of both N and P into algal biomass from wastewater is therefore theoretically possible (Benemann 2003).

Nutrient assimilation rates can reach 24 kg N ha^{-1}·day and 3 kg P ha^{-1}·day, based on the typical algal nutrient composition and a maximum productivity of 30 g m^{-2}·day algal biomass (dry weight). These removals are achieved at much lower capital and operation costs compared to conventional mechanical treatment technologies (Owen 1982; Craggs et al. 1999). A critical issue for tertiary-level nutrient removal is the maintenance of high algal productivity even when dissolved N has been reduced to low levels (e.g. <1 g m^{-3}). This has been demonstrated in preliminary trials by supplying nutrients as required (Benemann 2003), but more research is needed.

Wastewater treatment HRAP nutrient removal processes that occur at high pond water pH such as ammonia volatilisation and phosphate precipitation with cations (Nurdogan and Oswald 1995; Garcia et al. 2000; Craggs et al. 2003; Heubeck et al. 2007) are greatly reduced by CO_2 addition to the pond. However, this can be offset by the increased assimilation of nutrients into algal biomass with the CO_2 enhanced algal production. For example recent laboratory-scale (Woertz et al. 2009) and pilot-scale (Park and Craggs 2010a, b) studies have shown near complete nutrient removal with CO_2 addition to wastewater HRAP. Park and Craggs (2010b) demonstrated that daytime control of maximum pH to below 8 with CO_2 addition reduced nitrogen loss by ammonia volatilization from 24% (in a control HRAP without CO_2 addition) to ~9%.

3.3 Disinfection

Disinfection by chlorination requires little energy (3 kWh ML^{-1} of wastewater treated) for mixing, however, 20–540 kWh ML^{-1} is required to generate the chlorine depending on the quality of the effluent being disinfected (Owen 1982). Dechlorination of the treated effluent to remove potentially carcinogenic chlorinated hydrocarbons further adds to disinfection costs. Ultraviolet (UV) disinfection uses less electricity (20–100 kWh ML^{-1}) than chlorination, but requires a high level of pre-treatment so that the influent

has low turbidity. Ozonation is also preferable to chlorination but uses slightly more electricity (100–200 kWh ML^{-1}) than UV disinfection (Owen 1982). Gently mixed High Rate Algal Ponds promote sunlight-driven disinfection mechanisms without the need for chemicals or additional power requirement. Algal photosynthesis augments the sunlight-driven mechanisms by elevating daytime pond water pH (>10) and dissolved oxygen concentrations (>300 %) (Davies-Colley 2005).

Despite their high efficiency, HRAP still remain to be widely applied for wastewater treatment. This is due to a combination of: a lack of wide-spread HRAP knowledge and design skills in the engineering profession, the relatively large land area required compared with electromechanical treatment systems, and availability of low cost fossil fuel derived electricity to operate electromechanical treatment systems. Moreover, wastewater treatment HRAP were not initially optimised for efficient nutrient removal and cost-effective algal harvest and were only recognised as a secondary-level rather than tertiary-level treatment system.

4 Wastewater Treatment HRAP Design

Depending on climate, HRAPs are typically designed with an organic loading rate of between 100 and 150 kg BOD_5 ha^{-1} day. HRAPs depths vary between 0.2 and 0.8 m depending upon climate and clarity of influent wastewater. A paddlewheel is used to circulate the wastewater around the HRAP raceway at a horizontal velocity of 0.15–0.30 m s^{-1} which is sufficient to keep the algal colonies in suspension. Algal concentrations of 100–400 g TSS m^{-3} are common depending upon season. At the higher concentrations almost all of the available sunlight (PAR) is absorbed within the top 0.20 m of the HRAP, leaving the rest of the pond depth in the dark. However, the paddlewheel, along with turbulent eddies formed when the water flows around the raceway corners promote vertical mixing through the pond depth ensuring that the algal biomass is intermittently exposed to sunlight.

5 Harvest of Wastewater Treatment HRAP Algae

Effective and low-cost removal of algal biomass from HRAP effluent is imperative to achieve both a high effluent quality and economical production of harvested algal biomass (Benemann and Oswald 1996; Benemann 2003; Molina Grima et al. 2003; van Harmelen and Oonk 2006). Wastewater treatment HRAP algal harvest is challenging due to (1) low and varying cell concentration (typically <400 g m^{-3}); (2) cell densities similar to water (1.08–1.13 g mL^{-1}); (3) small cell size (5–20 μm); and (4) strong negative surface charge particularly during exponential growth phase (Moraine et al. 1979; Lavoie and de la Noue 1987). Various harvesting methods, including centrifugation (3 kWh_e kg^{-1} algae), filtration or microstraining, sedimentation, and dissolved air flotation (0.6 kWh_e kg^{-1} algae, in addition to the chemical flocculants required) can be used to remove algae from HRAP effluent (Shen et al. 2009; Tampier 2009; Brennan and Owende 2010; Mata et al. 2010). However, these processes are either not applicable (e.g. filtration, microstraining), are not economical for algal harvesting from wastewater treatment HRAPs, or increase parasitic energy losses, as indicated (Benemann 2003). Chemical flocculation forming large (1–5 mm) sized floccs (e.g. with metal salts or polyelectrolytes) is the process currently used to enable algal recovery from facultative oxidation pond effluents (Benemann and Oswald 1996). However, the chemical reactions are highly sensitive to pH and the high doses of flocculants required produce large amounts of sludge and may leave a residue in the treated effluent. The high energy requirement of centrifugation makes it only economically viable for secondary thickening of harvested algae (1–2 % solids) up to 30% solids (Tampier 2009).

The gentle paddlewheel mixing in wastewater treatment HRAPs selects for genera (including: *Scenedesmus* sp., *Micractinium* sp., *Actinastrum* sp., *Pediastrum* sp., *Dictyosphaerium* sp., and *Coelastrum* sp.) that form large (50–200 μm) colonies (Oswald 1988a; Banat et al. 1990; Benemann 2003; Wells 2005; Heubeck et al. 2007; Park and Craggs 2010a). Algal colonies settle reasonably well under quiescent conditions (50–90 % removal) (Benemann et al. 1980; Green et al. 1996; Craggs et al. 2003). This settling phenomenon may be augmented by self-flocculation of the algal cells (bioflocculation) which seems to be promoted by stress conditions, such as nutrient (e.g. N) limitation and CO_2 addition, and can produce a concentrated algal slurry (1–3 % DM) (Eisenberg et al. 1981; Benemann and Oswald 1996; Sheehan et al. 1998). Bioflocculation/aggregation of the algal and bacterial biomass and selection for easily settleable algal genera can be enhanced by recycling of a portion of the most easily settled algal/bacterial biomass back to the HRAP in a similar way to sludge recycle in the activated sludge process (Benemann et al. 1980; Tillett 1988; Benemann and Oswald 1996; Benemann 2003; Park and Craggs 2010a). However, further research is required to refine this low-cost harvest and algal selection process.

6 Economic and Environmental Benefits of Wastewater Treatment HRAP Algal Production

Wastewater treatment HRAP and commercial farm HRAP algal production are compared in Table 9.1. The main advantage of wastewater treatment HRAP is that the costs of algal production and harvest are essentially covered by the wastewater treatment plant capital and operation costs. Algal production

Table 9.1 Comparison between algal production HRAP and Wastewater treatment HRAP

Parameter	Algal production	Wastewater treatment
Capex cost	US $100,000 (unlined) to US $250,000 ha^{-1} (lined)	Algal production system part of wastewater treatment
Opex costs (annual)	US $20,000 $ha^{-1} \times year^{-1}$	Free algal production and harvest
Land requirement	Depends on productivity	Part of wastewater treatment
Application	High value food supplements	Wastewater treatment
Most costly parameters	Water, nutrients, CO_2 Mixing, harvesting, processing	Algal biomass by-product of wastewater treatment
Algal productivity	30 g m^{-2}·day (but not yet achieved in practice)	15–20 g m^{-2}·day annual average possible at higher and lower latitudes, respectively
Water footprint	High (if potable water use, non-potable water need make-up)	Not applicable unless effluent is reused
Risk of grazing and infection	High	High
Algal harvest	Costly, low concentration, small size (<20 μm), neutral buoyancy	Gravity settling promoted by aggregation of colonial algae with bacteria
Algal species control	Difficult but possible by use of selective media (e.g. high alkalinity or salinity) or inoculation	Difficult but possible with recycle of harvested biomass or inoculation
Blowdown treatment/disposal	May be required	Not applicable

as a by-product of wastewater treatment HRAP has fewer environmental impacts in terms of water footprint, energy use, fertiliser use, and blow-down water disposal (Borowitzka 1999, 2005; Benemann 2003; Tampier 2009; Clarens et al. 2010).

7 Wastewater Algal Biofuel Production

Biofuel conversion of harvested wastewater treatment HRAP algal biomass could involve one or a combination of four main pathways: (1) Anaerobic digestion to produce biogas (methane); (2) Extraction and transesterification of algal lipid triglycerides to produce biodiesel; (3) Fermentation of algal carbohydrates to ethanol or butanol and (4) Super critical water, gasification, pyrolysis or other thermochemical conversions of algal biomass to produce hydrocarbon gases and bio-crude oils (Craggs et al. 1999; USDoE 2005; Heubeck et al. 2007). Below we describe these four biofuel conversion pathways and potential GHG emission abatement from fossil fuel substitution; however the parasitic energy consumption and associated GHG emissions for conversion are not included.

7.1 Biogas Methane

Harvested algal biomass and algal residues remaining following lipid (oil) extraction or ethanol fermentation (see below) could be anaerobically digested to produce biogas. Oswald and Golueke (1960) found that algae could be digested to biogas (~60 % methane) with an average yield of about 0.30 m^3 (0.20 kg) CH_4 kg^{-1} algal biomass with 50–60% volatile solids conversion. Lower yields have been attributed to both the relatively refractory nature of some algal cell walls and ammonium inhibition. Pretreatment (e.g. heating) of algal biomass has been shown to improve digestibility of algal biomass under mesophylic conditions (Chen and Oswald 1998). Inhibition of anaerobic digestion can occur at free ammonia concentrations of 4,000–6,000 g NH_3-N m^{-3} (Siegrist et al. 2005). Algal biomass can contain over 50% protein (Becker 1988) of which up to 70 % may be released as ammonia during digestion (Golueke and Oswald 1959). Therefore, to avoid free ammonia toxicity of algal digestion, the algal biomass could be (1) concentrated to no more than 6–8% solids or (2) mixed with other organic waste substrates with a lower nitrogen content (e.g. sewage sludge) (Yen and Brune 2007). The largest and longest running (decades) demonstration of co-digestion of facultative wastewater pond algae with primary settled sewage sludge in a heated mesophylic mixed digester is at the Sunnyvale wastewater treatment plant, California, USA. Co-digestion of HRAP algae biomass with the wastewater solids removed by primary treatment could potentially double the overall methane production from HRAP integrated wastewater treatment (Benemann and Oswald 1996; Heubeck and Craggs 2007).

Cost-effective anaerobic digestion could be achieved using simple ambient temperature covered digester ponds, which could be fed with algal biomass harvested by bioflocculation (typically at ~3% solids concentration), compared to the 5–10% solids required for conventional, and more expensive, mesophylic heated mixed digesters (Oswald 1988a). Moreover, biogas production rates from laboratory-scale ambient temperature covered digester ponds have been shown to be similar to those of heated mixed digesters; 0.21–0.28 m^3 CH_4 kg^{-1} algal volatile solids (VS) added (Sukias and Craggs 2011).

Biogas methane can be used directly for heating (33.8 MJ or 9.39 kWh_{heat} m^{-3} CH_4 at STP) or for electricity generation at 30% conversion efficiency (2.82 $kWh_{electricity}$ m^{-3} CH_4) and concomitant heat generation (4.70 kWh_{heat} m^{-3} CH_4). Essentially ~1 kWh_e can be generated from the biogas

produced from 1 kg algae (Oswald 1988a, b). Electricity generation can either be used to displace electricity requirements of the wastewater treatment plant; be exported to the national grid (requires a larger capital investment for transformers, line upgrade, etc.); or be used for peak load generation earning highest prices. Biogas may be cleaned (desulphurised, stripped of CO_2, and dried) and compressed (>20 MPa) for export into a natural gas pipeline or use as transport fuel. Each cubic meter (0.67 kg) of biogas methane has an energy value (34 MJ) equivalent to ~1 L of petrol.

7.2 Biodiesel

Biodiesel production from oils extracted from algae grown in HRAPs was the main research focus of the 30 year U.S. Dept. of Energy Aquatic Species Program (Weissman and Goebel.1987; Benemann and Oswald 1996; Sheehan et al. 1998). The program concluded that in suitable climates, algae have higher oil yields than most terrestrial crop plants due to their high productivity, with between 50 and 100 tonne algae dry matter ha^{-1}·year and a 25–50 % oil (as triglycerides) content thought to be attainable, with the lower range being what is currently thought to be feasible and the higher values projecting what is thought to be possible in the future by applying the modern tools of molecular biology to algal mass cultivation (Benemann and Oswald 1996; Benemann 2003). Algal oil content and quality varies between species and even strains within a species, and with culture conditions, e.g. nitrogen limitation often greatly increasing oil content (Feinberg 1984; Benemann and Oswald 1996; Barclay et al. 1987; Coleman et al. 1987; Cooksey et al. 1987; Tillett 1988; Chelf 1990; Schenk et al. 2008; Guckert and Cooksey 1990; Brennan and Owende 2010). However, nitrogen limitation to stimulate lipid accumulation in algal cells may in turn reduce algal growth (Barclay et al. 1987; Coleman et al. 1987; Tillett 1988; Chelf 1990; Weyer et al. 2009), suggesting that the two conditions of high lipid and high algal productivity are likely to be mutually exclusive. A major issue is the economical extraction of the oil from the algae, if drying of the biomass is required this will add significantly to the overall costs, even for sun drying (the only plausible method). Algal oil contains a relatively low proportion (50%) of the mono-, di-, and triglycerides that are suitable for transesterification (Feinberg 1984), and the remaining long chain polyunsaturated fatty acids produce a viscous biodiesel that may polymerize over time into waxy solids, reducing engine efficiency and clogging filters and injectors (Feinberg 1984). In the case of algal biomass grown on wastewaters, maximizing oil content and yield would not be a priority, and, for the present, a yield of 0.12 L biodiesel kg^{-1} algae biomass would be a reasonable near-term goal for wastewater grown algae biomass.

7.3 Bioethanol

Bioethanol is produced from the carbohydrate portion of algal biomass by yeast fermentation followed by distillation to separate it from the other fermentation products. However, bioethanol production from algae is limited by the carbohydrate content of algae biomass (typically 20% of dry matter) and the portion of the carbohydrate that can be converted to fermentable sugars and then to ethanol (typically half to two thirds of the carbohydrate fraction). Thus the fermentable carbohydrate content of algae biomass (~13% DM) is low compared with other bioethanol crops (e.g. ~65% DM for maize) (Sheehan et al. 1998) and an average yield of 0.13 L bioethanol kg^{-1} algal biomass appears reasonable. As in the case of algal oil production, a higher content of fermentable starch or other carbohydrates can be induced by means of nitrogen (and other nutrient) limitation. However, this option has received relatively little attention, compared to oil production and is unlikely to be a high priority for algal biofuels production in conjunction with wastewater treatment.

7.4 Bio-Crude Oil

A novel technology for the conversion of algal biomass to biofuel is the super critical water reactor (SCWR) that mimics processes that may have produced fossil oil by using intense heat (~374 °C) and pressure (~22.1 MPa) to disassociate water and degrade organic compounds (Chandler et al. 1998; Yesodharan 2002; Matsumura et al. 2005). SCWR conversion has similar advantages to anaerobic digestion in that the algal biomass does not have to be dried (5–30% solids) and conversion is of the whole algal biomass rather than just the lipid or carbohydrate fraction. The SCWR produces a 'bio-crude' oil (with a conversion potential efficiency of ~30%) from which a range of fuel products could be derived. An average yield of 0.4–0.5 L bio-crude oil kg^{-1} algal biomass might be achievable, but much more research is required to demonstrate the viability of this technology.

7.5 Other Algal Uses

7.5.1 Feeds

Algal biomass also has potential for use as high-protein feed supplements for aquaculture and livestock (chickens, pigs and ruminants) (Becker 1988). Microalgae can contain over 50% crude protein with a yield that is 25-fold higher than soy beans, the most widely cultivated protein crop (de la Noüe and de Pauw 1988). Therefore an average yield of 0.5 kg protein kg^{-1} algal biomass is reasonable.

7.5.2 High Value Products

High value products such as β-carotene, the polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), alloxanthin and pigments (chlorophylls and carotenoids) which all have much higher value (~US$5,000 tonne^{-1} algae) than biofuel could potentially be extracted to increase the value to wastewater grown algal biomass (Apt and Behrens 1999; Wickfors and Ohno 2001).

8 Greenhouse Gas (GHG) Emission Abatement

Algal biofuel production from wastewater treatment HRAP abates greenhouse emissions by several mechanisms (Green et al. 1995; Benemann 2003):

8.1 Offset Equivalent Fossil Fuel Use GHG Emissions

Production of 1 tonne of algal biomass in wastewater treatment HRAP assimilates approximately 1.8 tonnes of CO_2 (assuming an algal carbon content of 46 % dry weight) (Benemann 2003). Once converted to biofuel, this offsets the CO_2 GHG emissions from equivalent fossil fuel use, which is dependent upon the type of fuel it replaces. For example, generation of electricity from biogas methane abates 0.4 kg CO_{2EQV} kWh_e^{-1} from natural gas electricity generation compared to 1.0 kg CO_{2EQV} kWh_e^{-1} from coal electricity generation. Actual substitution depends on the marginal source of power. GHG emission abatement from the substitution of diesel fuel and heavy bunker fuel with algal biodiesel and algal bio-crude oil are 2.68 kg CO_{2EQV} L^{-1} and 2.99 kg CO_{2EQV} L^{-1}, respectively (NZMED 2007).

8.2 Reduced CO_2 Emissions from Wastewater Treatment Through Lower Electricity Use

Low energy use HRAP wastewater treatment using sunlight energy through photosynthesis abates between 100 and 200 tonne of CO_2 per ML treated compared to fossil energy that would have powered electromechanical treatment (e.g., activated sludge; Green et al. 1995; Benemann 2003). Nitrogen removal in HRAP would abate a further 100–200 tonne of CO_2 per ML treated. HRAP, in addition to BOD_5 reduction and nutrient removal, also promote solar disinfection decreasing the need for chemical or electromechanical disinfection (Davies-Colley 2005) and associated GHG emissions.

9 Fertiliser Recovery

As discussed, algal biomass is high in nitrogen (7% of dry matter for a non-limited culture), phosphorus (about 0.8% of dry matter) and micronutrients, thus 1 kg of algae biomass contains on average 70 g of N and 8 g of P. Algae harvested from wastewater HRAP effluent would allow the recycling of these nutrients, and reduce fossil-fuel consumption required for ammonia fertiliser synthesis and phosphate rock mining (Metting and Pyne 1986; Oswald 1988a). Typically, the manufacture of 1 kg of ammonia fertiliser requires the equivalent of 16 kWh and the processing of 1 kg of phosphate fertiliser requires the equivalent of 4.5 kWh of energy (Wood and Cowie 2004). Moreover, the manufacture of 1 tonne of nitrogen (N) fertiliser will release 3.15 tonne CO_{2EQV} from natural gas and the mining of 1 tonne of phosphate fertiliser will release 1.39 tonne CO_{2EQV} (West and Marland 2001; Wood and Cowie 2004). Therefore the use of 1 kg of algae (7%N, 0.8% P) as fertiliser would reduce CO_2 emissions from inorganic fertiliser manufacture by 0.23 kg CO_{2EQV} kg^{-1} Algae. Thus even at 7% N content, the energy savings, and greenhouse gas abatement from the use of algae biomass biofuel residues as fertiliser would be equivalent to those from the use of the algae biofuel.

10 Conclusions

Algal biofuel production in combination with wastewater treatment using HRAP provides a niche opportunity for community-level algal biofuel production that has several advantages over other approaches. Wastewater treatment HRAP provide energy efficient and effective tertiary-level wastewater treatment with significant cost savings over electromechanical wastewater treatment technologies. Wastewater is an excellent growth medium (water, nutrients and buffering) for the growth of naturally occurring algae, especially when augmented with CO_2 addition from biogas produced and used in the treatment plant. Harvested algal biomass is a by-product of the HRAP wastewater treatment plant and the wastewater treatment function essentially funds the capital and operation costs of algal production. As wastewater treatment HRAPs naturally select for productive colonial algae, low-cost algal harvest can be achieved through gravity settling. Algal harvest can be improved by promoting biofloculation/aggregation of the algal-bacterial biomass. Several pathways are available to convert the harvested algal biomass to biofuel, however, those that use the whole algal biomass and require little or no dewatering of the harvested algae appear to be most appropriate for use in combination with wastewater treatment. In particular, anaerobic digestion of algal biomass along with

the settled wastewater solids makes economic sense as the capital and operation costs of anaerobic digestion and biogas use infrastructure may also be funded by the wastewater treatment plant. Additional financial and environmental incentives for wastewater treatment HRAP are from fertilizer recovery and GHG abatement. Harvesting algae from wastewater treatment HRAP effluent enables recovery of wastewater nutrients that can be recycled as fertilizer after biofuel conversion. Wastewater treatment HRAP also provide GHG abatement from a combination of low energy wastewater treatment, renewable fuel production and fertiliser recovery.

Since wastewater treatment HRAP systems are already a viable technology for efficient tertiary-level wastewater treatment, they provide a 'testing ground' to develop and refine full-scale algal production, harvest and biofuel conversion technologies that may be implemented in the future when higher fossil fuel costs make stand alone HRAP systems for biofuel production economical.

Acknowledgements The authors wish to thank Jason Park, Stephan Heubeck and Ian Woertz who provided valuable contributions to this chapter. NIWA funding was provided by the New Zealand Foundation for Research Science and Technology.

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Harvesting, Thickening and Dewatering Microalgae Biomass

10

Stephen L. Pahl, Andrew K. Lee, Theo Kalaitzidis,
Peter J. Ashman, Suraj Sathe, and David M. Lewis

1 Introduction

The recovery and processing of microalgae biomass from a culture media is an essential component for the production of almost all microalgae products. However, despite extensive research carried out to date, costs related to harvesting, thickening and dewatering of microalgae biomass are high (Borowitzka 1999; Fon Sing et al. 2011; Molina Grima et al. 2003; Uduman et al. 2010). The economic recovery of microalgae biomass is a challenging area and costs must be reduced to an acceptable level before microalgae based biofuels can be commercially viable. The difficulties with harvesting, and consequently the high biomass recovery costs, are associated with several factors, namely: (a) the nature of the microalgae cells (i.e.) size, specific gravity, charge and morphology; (b) the low concentration of biomass typical in large-scale culture systems; and, (c) high capital equipment costs, especially when the microalgae are cultivated in saline systems.

A wide range of solid-liquid separation techniques is available and the aim of this chapter is to compare the technologies and assess the technical and economic considerations for each option. The major challenge in selecting an appropriate technology for biofuels production from microalgae is that traditional microalgae concentration processes have generally used energy-intensive unit operations that are expensive. Since the 1960s microalgae harvesting and dewatering methods have been extensively reviewed on many occasions by Golueke and Oswald (1965), Henderson et al. (2008), Mohn (1980, 1988), Mohn and Contreras (1990), Molina Grima et al. (2004), Shelef et al. (1984) and Uduman et al. (2010), and whilst no revolutionary advances have been made, process optimization can significantly reduce operating costs.

Irrespective of the cultivation system(s) employed (i.e. open ponds or enclosed photo-bioreactors), the concentration of a microalgae cultures is low and generally lies between 0.1 and 4.0 g L^{-1} ash free dry weight (AFDW). Due to the requirements of downstream processing operations and/or the economic packaging and transportation of microalgal products, the concentration of microalgae biomass must generally be increased substantially to produce concentrated slurry, a paste or a dried solid.

The effectiveness of a solid-liquid separation process can be described in terms of the recovery efficiency (RE) and the concentration factor (CF) (Lee et al. 2009). The recovery efficiency (see Eq. 10.1) is defined as the ratio of the mass of cells recovered in the final product to the total mass of cells in the initial culture. Thus, separation processes with high recovery efficiencies capture most of the microalgae biomass present in the feed so that the concentration of microalgae in the dilute stream is low. The concentration factor (see Eq. 10.2) is the ratio of the concentration of microalgae biomass in the final product to the initial concentration in the culture.

$$\text{Recovery efficiency (RE)} = \frac{\text{mass of cells recovered}}{\text{mass of cells initial culture}} \tag{10.1}$$

$$\text{Concentration factor (CF)} = \frac{\text{concentration of algae in final product}}{\text{initial concentration of algae in culture}} \tag{10.2}$$

All solid-liquid separation techniques are based by constraining either the liquid or solids. The most common industrial solid-liquid processes are shown in Fig. 10.1 and include filtration, sedimentation and flotation, which are described in more detail below. Any microalgal biomass recovery system that is to be used to harvest and concentrate the microalgae

S.L. Pahl • A.K. Lee • T. Kalaitzidis • P.J. Ashman • D.M. Lewis (✉)
School of Chemical Engineering, The University of Adelaide,
Adelaide, SA 5005, Australia
e-mail: david.lewis@adelaide.edu.au

S. Sathe
School of Chemical Engineering, The University of Adelaide,
Adelaide, SA 5005, Australia

M.A. Borowitzka and N.R. Moheimani (eds.), *Algae for Biofuels and Energy*, Developments in Applied Phycology 5,
DOI 10.1007/978-94-007-5479-9_10,

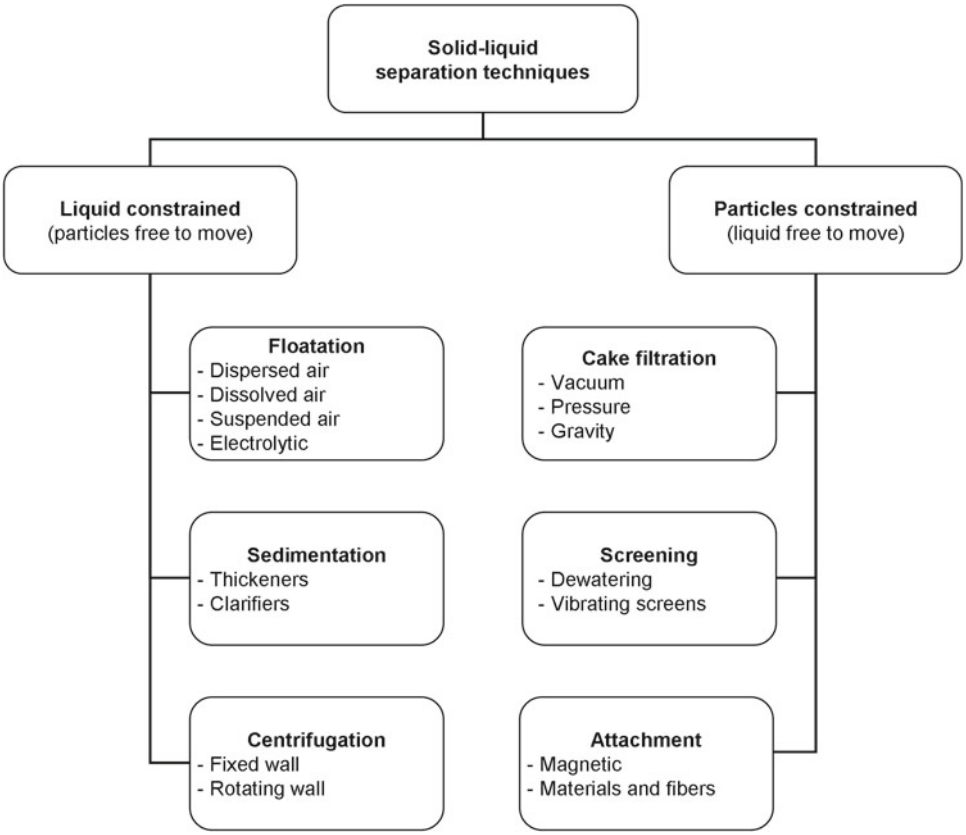

Fig. 10.1 Common industrial solid-liquid separation techniques

(i.e. solids) from the culture fluid (i.e. liquid) for biofuel applications must (1) be able to handle extremely large volumetric throughputs, (2) be highly reliable, (3) have low capital and operating costs, (4) be easily managed and (5) be constructed with materials that are compatible with the culture media. In addition, microalgae biomass recovery systems must allow recycling of the growth medium and also be sufficiently flexible to handle any upstream physical, biological and environmental changes. This will likely require an integrated system which is adaptable to any output changes from the culture system. Potential output changes include volumetric throughput, biomass/solids concentration, particle properties (size, specific gravity, zeta-potential) and the fluid properties (i.e. composition, nutrients, ionic strength, salinity, pH, dissolved gases, specific gravity and temperature). In addition, consideration must be given if the chosen microalgae recovery system leads to deterioration in the quality of the final product.

Microalgae recovery techniques can be used individually (single-stage) or in combination (multi-stage) and the choice is often dependent on the species of microalgae, desired product concentration and product quality. Multi-stage or sequential processing steps can be designed to increase the microalgae concentration by employing the most suitable, economical and sustainable method for each stage and so that each previous stage reduces the load on each subsequent stage. While there is often a considerable overlap in the actual concentration of microalgae between stages, the general stages in the recovery of microalgae biomass are shown in Fig. 10.2.

- **Stage 1** (harvesting/primary concentration): increases the biomass concentration by a concentration factor of 10–20. The material retains its fluid like consistency.
- **Stage II** (thickening): thickens the primary concentrate by an additional concentration factor of 10 and generates a material with slurry-like consistency.
- **Stage III** (dewatering): dewaters the thickened biomass to approximately 15–25% solids and generates a wet paste.
- **Stage IV** (drying): removes unbound and possibly bound water, generating a dry solid and may increase stability by minimise spoilage.

The fundamental properties of microalgae which influence their recovery include (a) particle shape (filamentous, rods, spheres, or chains), (b) particle size (generally between 2 and 20 μm), (c) specific weight (generally between 1.05 and 1.1) and (d) charge (usually negative). These properties depend on the microalgae, the growth conditions and the age of the culture. Due to the colloidal nature, microscopic size of microalgae and the small density difference between live

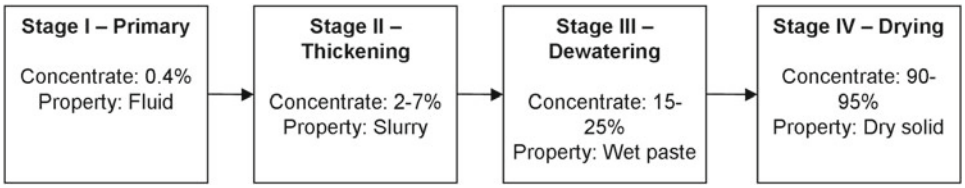

Fig. 10.2 Block flow diagram for the recovery of microalgae biomass, showing typical biomass concentration achieved and the physical state of the output stream

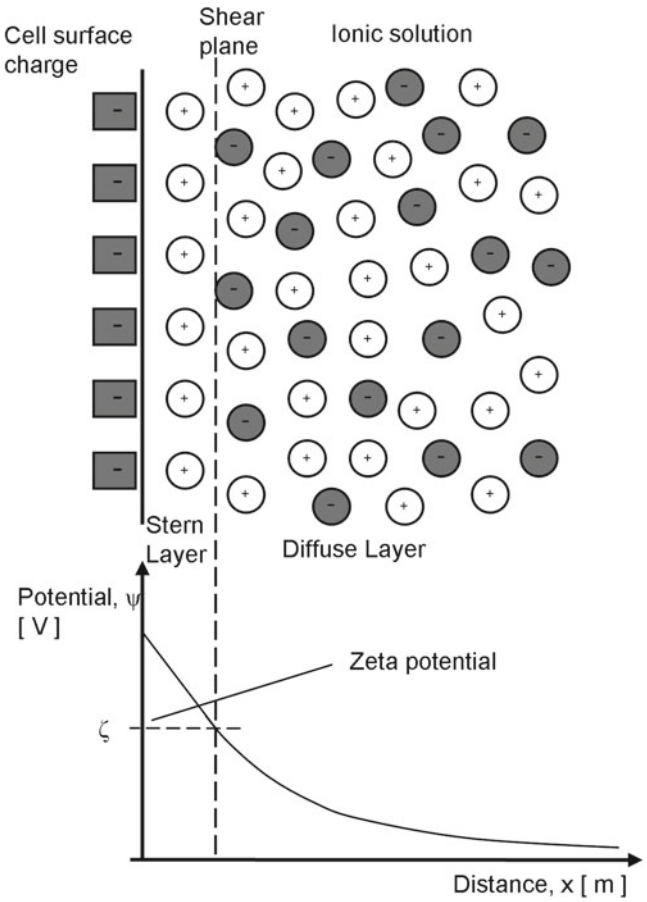

Fig. 10.3 Visulalisation of the Electric Double Layer (EDL)

microalgae and the culture fluid, most solid-liquid separation techniques do not function effectively without some form of microalgae pre-treatment or conditioning. Pre-treatment can improve the recovery efficiency (RE) and concentration factor (CF) and is often based on destabilisation and aggregation of the microalgae cells through coagulation and flocculation. Coagulation is a method of destabilising individual microalgal cells so that the destabilised microalgae cells can be flocculated into larger particles. Flocculant particles or "flocs" facilitate their recovery as they are larger, often heavier and have a smaller surface area per unit volume than the individual cells.

The low cost recovery of microalgae, whilst conceivable simple, has proven to be difficult to achieve due to the fact that microalgae suspensions are colloidal and consist of highly disperse particles (discontinuous phase) distributed uniformly throughout a dispersion medium (continuous phase). Interfacial effects dominate as the surface of the microalgae cells interact with the dissolved ions in the continuous phase and results in an electrical surface charge at the cellular level. The origins of this surface charge are a combination of (a) preferential adsorption of the dissolved ions in solution, (b) adsorption-desorption of lattice ions, (c) direct dissociation or ionisation of active surface groups and (d) charge-defective lattice (isomorphous substitution).

Microalgae exhibit a slight negative surface charge (Molina Grima et al. 2003; Shelef et al. 1984) which repels negative ions (co-ions) in the surrounding solution whilst attracting positive ions (counter-ions) from the solution, thereby forming a tightly-bound layer of ions called the *Stern layer*. Whilst the remaining positively charged counter-ions are still attracted to the negative surface charge of the cell wall, they are also repelled by the positive Stern layer, whereas the negatively charged co-ions are repelled from the negative surface charge of the cell wall and are attracted to the positive Stern layer. A dynamic equilibrium of charges (counter-ions and co-ions) is established and is free to move around the Stern layer to form a *diffuse layer*. The diffuse layer extends from the edge of the Stern layer to some distance into the surrounding media until the concentration of counter-ions and co-ions are identical and there is a zero net charge. The net result is the formation of an electrical double layer (EDL) around the microalgae cells. Furthermore an electrical potential will exist wherever there is a difference in ion concentration and the magnitude of the electrical potential can be visualised as a function of distance as shown in Fig. 10.3.

An important parameter of any colloid is the potential at the interface (shear plane) of the Stern layer and diffuse layer, known as the *zeta potential*. The zeta potential of microalgae is typically negative and is usually within the range −10 to −35 mV (Henderson et al. 2008). The magnitude of the zeta potential is dependent on a number of factors including the pH and ionic strength of the culture media. The zeta potential reduces with salinity, as shown in Fig. 10.4.

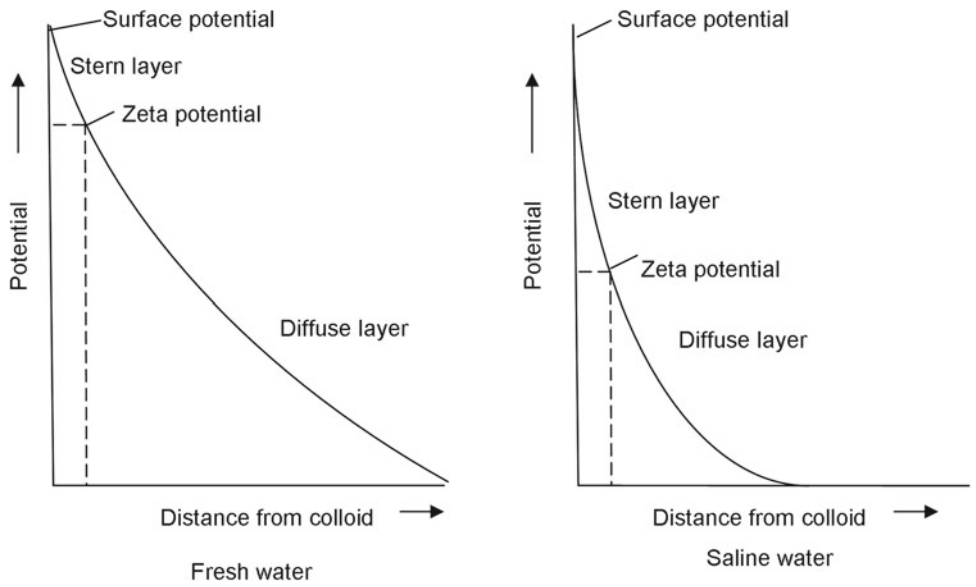

Fig. 10.4 Effect of salinity on zeta potential

2 Coagulation and Flocculation

Coagulation and flocculation are two distinct unit processes. Coagulation involves the addition of a chemical coagulant(s) in order to condition suspended, colloidal, and dissolved matter for subsequent processing. Flocculation involves the aggregation of destabilised particles and the precipitation products formed by one or more coagulants into larger particles known as flocculant particles or, more commonly, "flocs". The resultant flocs are generally easier to recover by the traditional solid-liquid separation techniques shown in Fig. 10.1. In addition, coagulant aids and flocculant aids can be used to provide additional nucleation sites for floc formation and to enhance the floc aggregation process, respectively. Coagulation and flocculation can also be differentiated on the basis of time required for each process. Coagulation occurs quickly typically in less than 10 s whereas flocculation occurs over a longer, typically 20–45 min (Crittenden et al. 2005). Coagulation and flocculation processes are frequently employed in low saline systems such as water purification and wastewater treatment.

Coagulation and flocculation may be initiated through the use of inorganic coagulants, organic coagulants (often polymers) or by using autoflocculation, bioflocculation, ultrasound and electrocoagulation procedures. Coagulants destabilise the microalgal cells in suspension by reducing or neutralising the cell surface charge, after which the destabilised microalgae can flocculate. Coagulation mechanisms can take four forms including (1) electrical double-layer compression, (2) adsorption and charge neutralisation, (3) adsorption and interparticle bridging, and (4) entrapment in a precipitate, or "sweep floc". Coagulation relies on the interaction between the negatively charged microalgae, the coagulant and any coagulant aids (if used). Consequently, the extent of coagulation, microalgae recovery and cost are ultimately dependent on the microalgae species and initial microalgae concentration, initial surface charge of the microalgae cells, the coagulant(s) and coagulant aid(s) used, the coagulant dose, degree of mixing and cultivation media parameters such as alkalinity, ionic strength, pH and temperature. Flocculation relies on the collision, interaction and aggregation between destabilised microalgae cells and any flocculant aid(s) (if used).

2.1 Inorganic (Chemical) Coagulation and Flocculation

Many inorganic chemicals can be used to coagulate microalgae prior to recovery. A number of common inorganic coagulants and their optimal pH ranges which are used in water and wastewater treatment systems for removing colloids are shown in Table 10.1. Aluminium and iron coagulants are frequently chosen due to their high efficiency and effectiveness in removing colloidal particles. The addition of the inorganic coagulants trigger the formation and precipitation of inorganic metal hydroxides (including aluminium hydroxide ($Al(OH)_3$), ferric hydroxide ($Fe(OH)_3$) and magnesium hydroxide ($Mg(OH)_2$)) and calcium carbonate ($CaCO_3$). In addition to neutralising the negative charge on the microalgae, these precipitates may also act as nucleation sites for microalgae attachment and microalgae removal may also be accomplished via entrapment (Shammas 2005). The anion present in the coagulant has also been shown to influence the extent of biomass recovery, with the chloride salts of

Table 10.1 Inorganic chemicals and optimum pH range commonly used in wastewater processing

Chemical	Formula	Optimal pH range
Alum	$Al_2(SO_4)_3{\cdot}18H_2O$	4.0–7.0
Ferric chloride	$FeCl_3$	3.5–6.5 and >8.5
Ferric sulphate	$Fe_2(SO_4)_3{\cdot}3H_2O$	3.5–7.0 and >9.0
Ferrous sulphate	$FeSO_4{\cdot}7H_2O$	>8.5

Table 10.2 Effect of ionic strength on optimum chemical coagulation dosage for removal of *Isochrysis galbana*

Medium ionic strength (M)	Optimal dosage for coagulation (mg L^{-1})	
	Alum (pH 5.5)	Ferric chloride (pH 5.0)
0.1	0	15
0.2	75	30
0.4	96	59
0.6	150	–
0.7	225	120

Adapted from Sukenik et al. (1988)
Note: Natural seawater has an ionic strength of ~0.7 M

aluminium, iron and zinc being more effective than the sulphate salts in coagulating cultures of the freshwater green alga *Chlorella minutissima* (Papazi et al. 2010).

The optimal coagulant dose depends on the system and must be determined on a case-by-case basis. Optimal coagulant doses are frequently determined by the use of standard jar test apparatus and the effectiveness of a dose can be based on the recovery efficiency or the concentration factor. The ionic strength of the cultivation media has a significant effect on the optimal coagulant dose with higher ionic strength systems requiring larger coagulant doses (see Table 10.2) and subsequently higher operational cost. In many wastewater treatment processes the Alum ($Al_2(SO_4)_3{\cdot}18H_2O$) dose varies between 50 and 600 mg L^{-1} (Crites et al. 2005).

Commercially, the use of Alum, or any chemical coagulant, is often combined with a dissolved air flotation module as they are more compact and can have a higher loading rate (Wang et al. 2005). The major concerns with the use of inorganic coagulants for the recovery of microalgae for biofuels are high operating costs, residual metal salts dissolved in the growth media (which must be reused) and the metal salts incorporated in the recovered biomass. For example, the high aluminium concentrations typical in Alum flocculated biomass renders any residual biomass unsuitable for use as animal feed. In 1998, the Aquatic Species Program (ASP) concluded that inorganic flocculation was too expensive for the production of biofuels from microalgae (Sheehan et al. 1998), and while standard jar testers are readily available and can be used to facilitate determining the optimum flocculant dose, the above concerns remain.

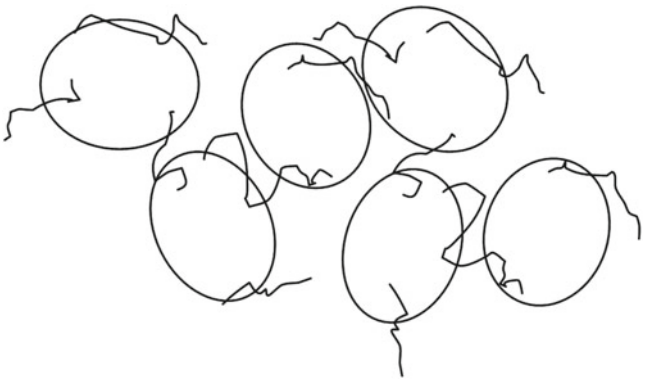

Fig. 10.5 Bridging between particles

2.2 Organic (Chemical) Coagulation and Flocculation

Organic coagulants, which are derived from naturally occurring substances or synthetic, are generally polymer based and may contain ionisable functional groups including carboxyl, amino or sulphonic structures or are non-ionisable. Polymers without ionisable groups are non-ionic, whereas, ionisable polymers (known as polyelectrolytes) may be anionic, cationic or amopholytic. Organic coagulants are used to recover microalgae by themselves or more frequently are used in conjunction with inorganic coagulants to aid in interparticle bridging. Bridging, shown in Fig. 10.5, links small particles and flocs together and thereby produces larger flocs to aid in floc recovery.

A number of organic coagulants are commercially available and they vary in molecular weight and charge density. Organic coagulants are generally considered to be non-toxic. Cationic polyelectrolytes have generally received the most attention in the recovery of microalgae as they can facilitate bridging and assist in neutralising the negative surface charge on microalgae. Whilst anionic polyelectrolytes can destabilise negative colloids (Shammas 2005; Tilton and Murphy 1972), their efficiency when tested with microalgae was poor (Tilton and Murphy 1972). However, similar to inorganic coagulants, the effectiveness of many polyelectrolytes (see Fig. 10.6) decrease as the ionic strength of the water is increased. This decrease in flocculation effectiveness is a result of the polyelectrolyte structure collapsing or folding (Bilanovic et al. 1988), and this change in shape reduces the extent of bridging.

While organic coagulants are more expensive than inorganic coagulants, this cost can be generally be offset by lower dose (Shammas 2005). The optimal dose of inorganic coagulations, similar to inorganic coagulants, needs to be determined on a case-by-case basis. The maximum performance of organic coagulants can also be hampered by their narrow operability window, with concentrations lower than required resulting poor flocculation and concentrations higher than required resulting in charge-reversal and re-stabilisation (Shammas

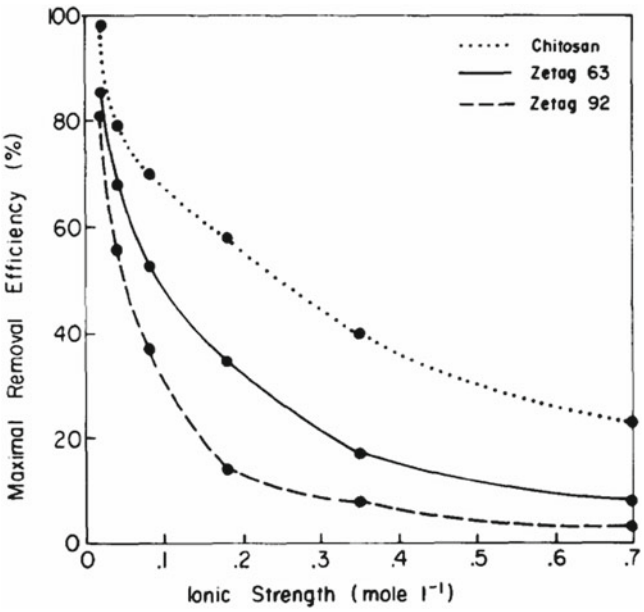

Fig. 10.6 Efficiency of three polyelectrolytes as the ionic strength is increased (Reproduced from Bilanovic et al. 1988). Natural seawater has an ionic strength of ~0.7 M

2005). In saline systems, microalgae recoveries of between 70 and 95% have been achieved when Chitosan was dosed at 40 and 150 mg L^{-1} (Heasman et al. 2000) while recoveries around 70% were reported with 1 mg L^{-1} Praestol® (Pushparaj et al. 1993).

2.3 Autoflocculation

Microalgae may sometimes self-flocculate naturally (autoflocculation) without the addition of chemical coagulants. Incidences of autoflocculation have been induced under non-ideal cultivation conditions, generally high pH and with the presence of phosphate and divalent cations (Sheehan et al. 1998). Autoflocculation occurs through a co-precipitation of magnesium, and calcium carbonate salts (Becker 1994) which have a positive surface charge and help reduce or neutralise the negative surface charge on the microalgae. The performance of autoflocculation is difficult to predict and is species dependent (Becker 1994). Furthermore, as the precipitation of magnesium and calcium carbonate salts generally only occur when the pH >10 (Irving 1926), this method is unsuitable for continuous cultures which are frequently maintained near neutral pH for maximum productivity.

2.4 Bioflocculation

Bioflocculation can be achieved through the use of biologically excreted organic compounds, often termed extracellular polymeric substances (EPS). Some microalgae and bacteria can be induced to excrete EPS, which are usually polysaccharides of uronic or pyruvic acids, during extreme temperature, pH or nutrient stress conditions (Lee et al. 2009; Mishra and Jha 2009; Shipin et al. 1988). While microalgae are known to excrete EPS, the EPS generally only occur under non-ideal growth conditions and this method is unsuitable for continuous cultures which are managed for maximum productivity.

As previously mentioned EPS can come from a range of other sources and while the addition of a crude or purified EPS is unlikely to be economically viable, due to the complexity and high costs associated with EPS separation and purification, several trials have been successful to co-culture bacteria for EPS production with microalgae (Eisenberg et al. 1981; Lee et al. 2009). Bacteria, unlike autotrophic microalgae, require a suitable organic carbon source as a substrate for growth, and this requirement can be exploited without affecting the microalgae biomass productivity. While the use of EPS prevents the risk of contamination by inorganic coagulants and can achieve similar flocculating efficiencies (Lee et al. 2009), the performances are often unpredictable (Li and Yang 2007). Lee et al. (2009) estimated that the cost of bioflocculation could be AUS$0.13 m^{-3} of culture.

2.5 Ultrasound

Standing acoustic waves can cause material to flocculate by forcing the material into the pressure nodes. Ultrasound induced flocculation can be advantageous in some situations as it can be operated continuously, has a small footprint, lack of freely moving parts and the flocculation mechanism does not rely on the addition of chemicals or envoke any detrimental shear stresses. Bosma et al. (2003) investigated the use of ultrasound to flocculate the microalga, *Monodus subterraneus*, and concluded that while ultrasound was successful in flocculation the microalga, the energy requirements were too high for the recovery of microalgae. Technical challenges also lie in the capacity of resonators, with the largest size resonator at the time handling 1,000 L day^{-1} and the difficulty in preventing the entrainment of air bubbles which interfere with the stability of the standing acoustic waves (Bosma et al. 2003).

2.6 Electrocoagulation

Electrocoagulation of microalgae can be achieved by passing an electrical current between two electrodes; a sacrificial metal anode (most commonly aluminum or iron) and a cathode. Half-reactions occur at each electrode and the dominate anode and cathode half reactions are shown in Eqs. 10.3, 10.4 and 10.5.

$$\text{Anode half reaction I}: \mathrm{M}_{(s)} \rightarrow \mathrm{M}^{n+}_{(aq)} + n\mathrm{e}^{-} \qquad (10.3)$$

$$\text{Anode half reaction II}: 2H_2O_{(l)} \rightarrow O_{2(g)} + 4H^+_{(aq)} + 4e^- \quad (10.4)$$

$$\text{Cathode half reaction I}: 2H_2O_{(l)} + 2e^- \rightarrow H_{2(g)} + 2OH^- \quad (10.5)$$

where M is the metal anode, n is the charge of the metal ion

Hydrogen gas is generated at the cathode and the sacrificial anode releases cations which can destabilise microalgae by reducing or neutralising the negative surface charge. The destabilised microalgae can then flocculate. Depending on the design of the system the flocs once formed may sink to the bottom of the vessel, or attach to the hydrogen bubbles produced at the cathode and float to the surface. Electroflocculation has an advantage over inorganic (chemical) flocculation as whilst metal cations are released from the anode, no anions are introduced to the growth media and less 'coagulant' may also be required (Vandamme et al. 2011).

The major factors which affect the electroflocculation of microalgae include voltage, current, residence time, electrode material and system design. High biomass recoveries are possible, with biomass recovery up to 99 and 98% being observed under laboratory conditions for *Tetraselmis* sp. and *Chlorococcum* sp., respectively (Uduman et al. 2011). The greatest issues surrounding the use of electroflocculation is the contamination of the recovered biomass and growth media with metal salts from the sacrificial anode, the high cost of anode replacement and the formation of an oxide layer on the cathode. While the energy consumed during electroflocculation can be calculated based on the applied current, voltage, residence time and volume of growth medium, the reported energy consumptions vary significantly and depend on the system design and operational parameters. The initial biomass concentration, mode of biomass recovery (i.e. flotation or sedimentation), percent biomass recovery and the salinity of the media have a significant bearing on the operational cost. Freshwater systems generally have a higher energy demand over saline systems as the electrical resistance in freshwater systems is higher and a higher voltage is required in order to achieve the same current.

2.7 Flocculation Summary

Coagulation and flocculation are suitable methods to increase particle size and may improve the ease of solid-liquid separation; however not all microalgae require flocculation to be effectively removed, as some species can be readily filtered (i.e. *Arthrospira* sp.) or separated from the culture media through attachment to suitable materials or by utilising phototactic, natural buoyancy, or any other cellular response. If flocculation is beneficial for the removal of microalgae, then the method of flocculation will influence the structure of the flocs, and this in turn will alter the performance and load on any subsequent dewatering process and can also impact on the end uses of the product (such as high Aluminium concentrations rendering any residual biomass unsuitable for use as animal feed). The optimal coagulant dose may not necessarily scale linearly with biomass concentration, as higher biomass concentrations can facilitate a shift towards bridging and entrapment mechanisms. While tight flocs have stronger attractive forces, are less fragile to breakage and retain less water than loose flocs, they may not release the retained water as readily during dewatering process. A range of tools including jar testers, zeta meters, particle size analysers and small scale dewatering systems (i.e. filtration systems) exist and can be used to assist in determining the optimal flocculation process conditions.

The practice of chemical coagulation and subsequent flocculation is a widely used and remains a key process to remove microalgae in water and wastewater industries. The application of electrocoagulation and other electrokinetic techniques is a promising development; however several issues remain especially regarding the design of electrocoagulation systems for continuous operation, high cost of anode replacement and accumulation of metal ions in the biomass and growth media.

Coagulation and flocculation processes are susceptible to changes in the feed characteristics (microalgal species, loading rate and water chemistry) and consequently the coagulation performance, floc formation and floc structure is highly variable and has been difficult to predict. The inherent variability increases the risk that successful flocculation and thus harvesting process may fail. With the use of any coagulant a greater understanding is required to determine how the coagulant incorporated with the biomass will impact on its end use as well as how any residual coagulant present in the growth media will impact on the cultivation process when the growth media is re-used.

3 Liquid Constrained Systems

Liquid constrained systems include centrifugation, sedimentation and flotation and require a density different between the particle and the surrounding media. The forces exerted on a single particle in a gravitational field are shown in Fig. 10.7 and include the external (in this case gravitational) force (F_E), buoyancy force (F_B) and drag force (F_D). The buoyancy force acts parallel to the external force, but in the opposite direction, whereas the drag force appears whenever there is relative motion between the particle and fluid. The net force on the particle determines the direction of movement.

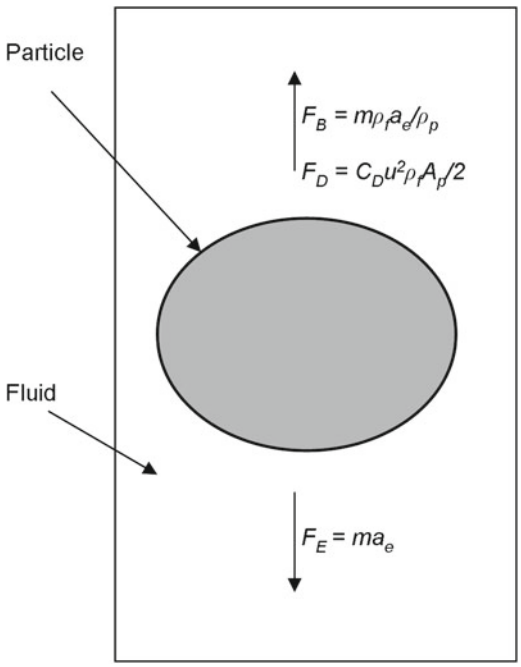

Fig. 10.7 Forces exerted on a particle

3.1 Sedimentation

Sedimentation is one of the simplest forms of solid-liquid separation. The major advantages of sedimentation processes are low power consumption, low design cost and low requirement for skilled operators. The major disadvantages of sedimentation processes for the recovery of microalgae include the slow sedimentation rates and consequently large footprint required, and the low solids concentration achieved. The sedimentation rate is controlled by the net force acting on the microalgae and for a small inert spherical isolated particle in a Newtonian fluid the sedimentation rate is governed by Stokes Law, and the terminal velocity for such a particle is given by Eq. 10.6.

$$V = \frac{g(\rho_s - \rho_f)d^2}{18\mu} \tag{10.6}$$

where V is the terminal velocity (m s^{-1}), g is the acceleration due to gravity (m s^{-2}), ρ_s is the density of the particle (kg m^{-3}), ρ_f is the density of the fluid (kg m^{-3}), d is the diameter of the spherical particle (m) and μ is the fluid viscosity (kg m^{-1} s^{-1}).

Aside from size, shape and density, the sedimentation rate of microalgae can also depend on cell motility, water turbulence and water upwelling (due to wind, temperature stratification and interference with other settling bodies). As most microalgae are not spherical a suitable equivalent spherical diameter must be used. The sedimentation rates of most microalgae are too low for practical operations and microalgae are usually flocculated to increase the particle size and density.

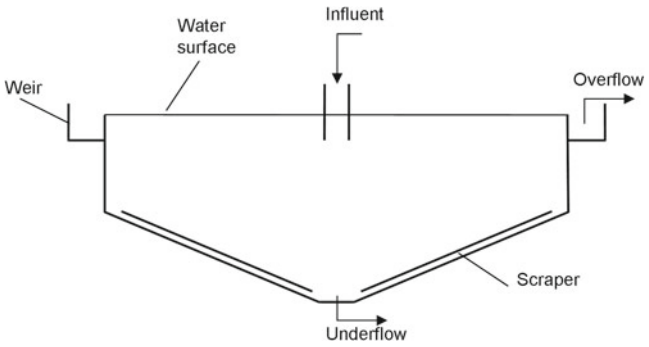

Fig. 10.8 Cross-sectional view of a circular gravity thickener

3.1.1 Gravity Thickeners

Gravity thickeners rely on the natural tendency for higher density particles to settle out of the liquid, with a final solids concentration of 2–3% generally being maintained. Gravity thickeners may be rectangular or circular in shape, although circular gravity thickeners is more commonly used as they are generally cheaper due to lower construction, equipment installation, operation and maintenance costs. The influent is generally pumped up through the middle section of the thickener and is released at low velocity near the surface. As the particles settle, several zones are created. The top zone consists of relatively clear media (clarified supernatant), and the lower zones consists of particles exposed to free settling, hindered settling particles and compaction settling. Gravity thickeners frequently have a sloped base and scrapers to collect the settled solids for removal and a peripheral weir to handle the overflow. Gravity thickeners are normally sized on the basis of suitable hydraulic and solids loading capacities. A typical solids loading capacity for a gravity thickener is 25–35 kg m^{-2} day^{-1}. The process is continuous with a retention time governed by the dimensions of the thickener and the settling rate of the particles. Long retention times in gravity settlers can lead to product degradation. A schematic a circular gravity thickener is shown in Fig. 10.8.

3.1.2 Enhanced Gravity Sedimentation

The settling rate in a sedimentation tank can be increased through the use of inclined channels, plates or tubes. The term Lamella® is a registered trademark (held by Parkson Corp, Fort Lauderdale, Florida, USA) for a settling tank with inclined plates to promote enhanced-gravity sedimentation. Unlike traditional sedimentation where the distance particles need to travel is large, these gravity-enhanced settlers use a series of angled plates or tubes and shorten the distance a particle needs to travel before hitting a surface. Once particles hit the surface, they may slide down. The easy at which particles slide depends on the nature of the particles and the angle of the plates or tubes. A recent report suggested that Lamella® settlers could be used to recover some microalgae (Nakamura et al. 2005).

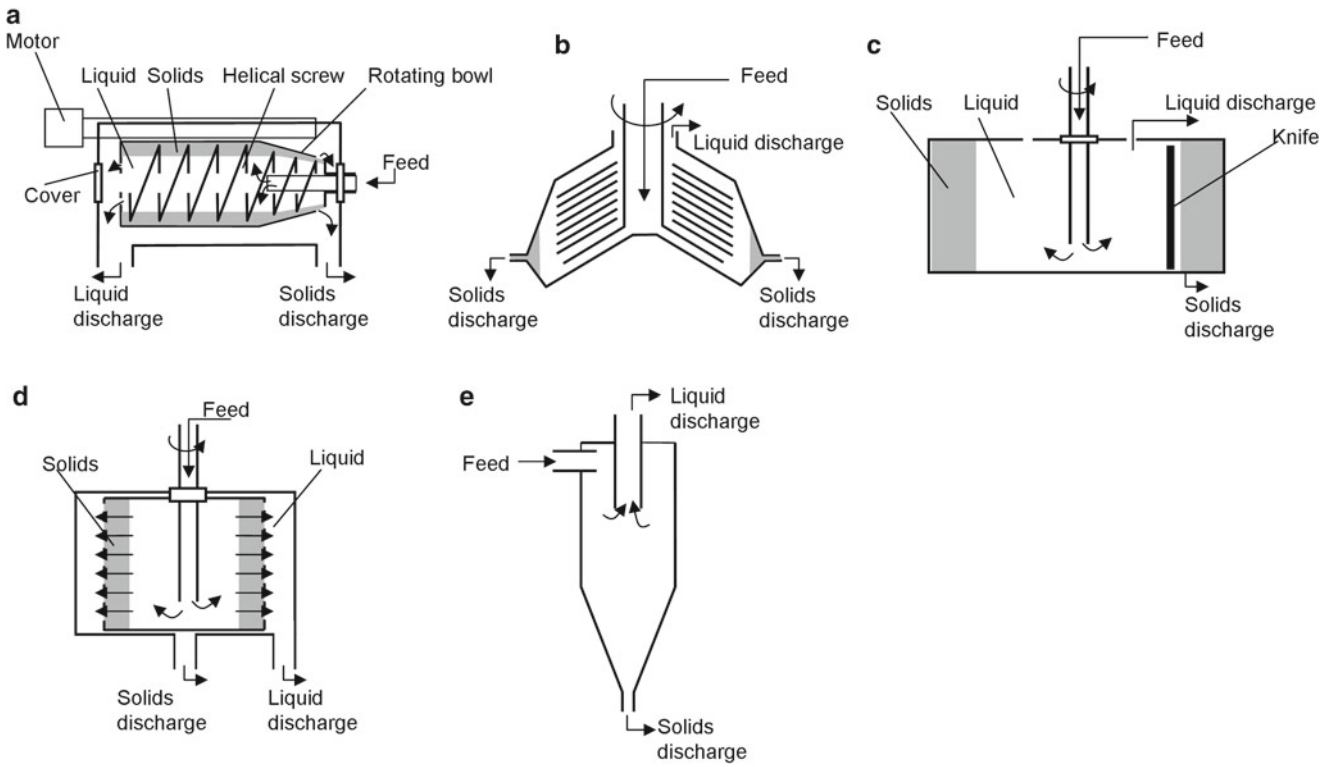

Fig. 10.9 Cross sectional view of a (**a**) decanter type centrifuge, (**b**) disc stacked centrifuge, (**c**) imperforated basket centrifuge, (**d**) perforated basket centrifuge and (**e**) hydrocyclone

Whilst enhanced settling is commonly used in waste water treatment processes, it does rely on there being significant density differences between the liquid and suspended solids. The density difference between individual cells and the growth media is not sufficient in many biological systems, but may be enhanced through the use of flocculants.

3.2 Centrifugation

Centrifugal processes rely on the generation of a centrifugal force which acts radially and accelerates the movement and separation of particles based on a density difference between the particle and the surrounding medium. If the particle is denser then the media the particle will migrate outwards, otherwise it will migrate inwards. Where sedimentation under gravity is a slow process, the use of centrifugal force will quicken the settling process, thereby increasing throughput and reducing the footprint. Five basic types of centrifuges, namely disc stack centrifuges, perforated basket centrifuges, imperforated basket centrifuges, decanters (or scroll centrifuges) and hydrocyclones, as shown in Fig. 10.9, are used on industrial scales.

The decanter or scroll centrifuge is one of the most promising centrifugal devices for the recovery of microalgae as they can operate continuously, have high capacity and lower maintenance requirements; however the high capital cost and energy demand often limits their application to higher valued products. Decanters essentially consist of two concentric rotating elements surrounded by a stationary casing or cover. The tapered outer rotating element, or 'bowl', and the inner element, or 'screw', rotate at slightly different speeds. Solids entering the decanter settle on the bowl wall and are conveyed along the bowl wall to the discharge, while the clarified liquid is discharged at the opposite end. The solids concentration on discharge is dependent on the feed properties (particle size, particle-fluid density difference) and can be modified by altering the feed flow rate, the rotational speeds of the bowl and screw and the bowl length. The maximum discharge concentration typically achieved in continuous centrifugation processes are 10–20% solids, however higher concentrations are expected with some of the latest 'sedicanters' (Pers. Comm. Flottweg Australia).

Hydrocyclones consist of an upper cylindrical section joined to a conical base. Feed is injected tangentially through an inlet opening near the top of cylindrical section and the particles experience the radial centrifugal force. If the radial centrifugal force is greater than the drag force then the particles will be separated from the fluid and leave through the conical base, otherwise the particles will be retained and leave through the upper outlet. Unlike centrifuges, hydrocyclones are relatively cheap and have no moving parts. Hydrocyclones require precision engineering and are somewhat inflexible once installed as the separation performance is

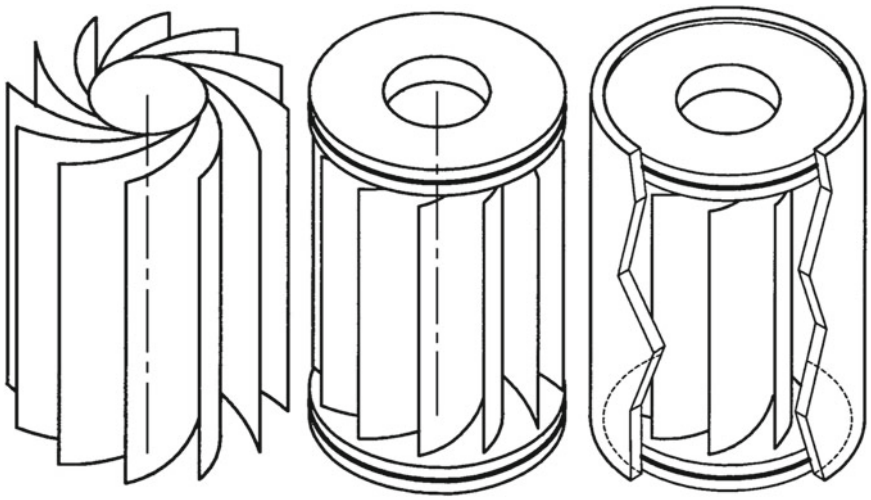

Fig. 10.10 Spiral plate rotor (Courtesy Evodos™)

very sensitive to the fluid and particle dynamics within the hydrocyclone. While hydrocyclones have shown some promise for the primary concentration of microalgae, their reliability is poor (Mohn 1980).

The cost, biomass recovery and ease of solids discharge ultimately depends on centrifuge design and operational parameters. The sticky nature of microalgae can make solids discharge difficult. Historically, many disc stacked centrifuges and decanters use a liquid to support the discharge of collected material, which results in some of the liquid being discharged with and diluting the solids. As one of the primary objective of centrifugation in the harvesting and dewatering of microalgae is to reduce the water content this presents several challenges with conventional designs. However, more recently, Evodos™ have developed 'Spiral Plate Technology' by using rotating curved plates inside a sliding cylindrical drum (see in Fig. 10.10) to reduce particle settling distances and a control system to remove any remaining process liquid from the drum prior to solids discharge. The net result is an improvement in the efficiency of dewatering microalgae and a reduction in the typical maintenance and operational costs (Boele 2008).

Although centrifugation is a proven technology for the fast and effective means of harvesting most microalgae, its use and the high capital and operational costs, must be considered in conjunction with the scale and value of the product. Design features can differ substantially amongst centrifuge manufacturers and consequently process design and centrifuge performance should be evaluated using test units and after close consultation with manufacturers. While centrifuge performance, capital cost and operating cost are ultimately determined by the process; the typical energy requirements and performance of the various centrifugation processes are reported in Table 10.3.

Table 10.3 Summary of centrifugal equipment

Device	Energy requirements (kWh m^{-3})	Biomass concentration in solid discharge (%)	Reliability
Disc stacked and nozzle	0.7–1.3	2–15	Very good
Decanter	8	22	Very good
Hydrocyclones	0.3	0.4	Poor
Evodos Spiral Plate Technology	data not available		

Based on Mohn (1980), Molina Grima et al. (2003), Oswald (1988) and Evodos

3.3 Flotation

Flotation is a separation process based on air or gas bubbles adhering to the particles, which are then carried to the liquid surface where they can be separated, usually via skimming. Flotation is frequently used in wastewater treatment and mineral processing industry and the gas bubbles can be introduced into, or generated within, the flotation cell and is often used in conjunction with flocculation. Air is the most common gas which is introduced into the flotation cell and the most popular flotation mechanisms include dispersed air flotation, dissolved air flotation (DAF), bioflotation and electrolytic flotation. Smaller gas bubbles are more efficient than larger gas bubbles, as they have a larger surface area per unit volume and lower buoyancy (de Rijk et al. 1994) thus increasing the likelihood of collisions between an air bubble and a particle. The major design factors and operational parameters for all flotation systems include the air to solids ratio, hydraulic loading, solids loading, weir overflow rate and handling of floated solids and any sediment.

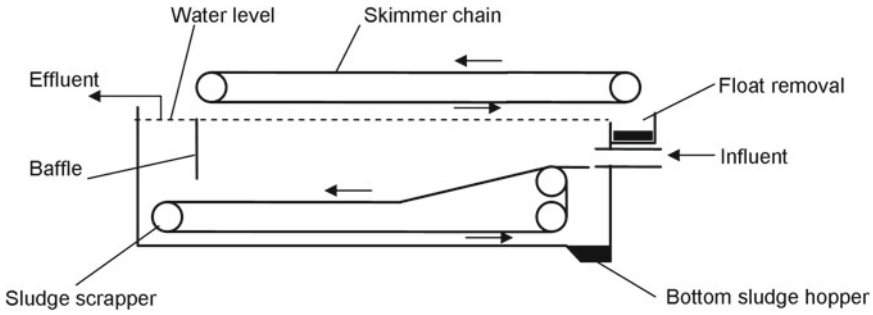

Fig. 10.11 Cross sectional view of a typical DAF unit with surface skimmer

3.3.1 Dispersed Air Flotation

Air can be continuous pumped into a flotation cell through a process of froth or foam flotation. The bubble size in dispersed air flotation systems is typically large (often >1 mm diameter) and results in low flotation efficiencies. The pH of the culture can also govern the stability of the air-microalgae mixture and while concentration factors of between 50 and 200 have been reported (Levin et al. 1962), dispersed air flotation is not widely used for the recovery of microalgae.

3.3.2 Dissolved Air Flotation (DAF)

Higher flotation efficiencies are obtained if air-supersaturated water is injected under pressure into a flotation cell, upon which small air bubbles (often 60–200 μm diameter) are formed by the reduction in pressure. Known as dissolved-air flotation (DAF), this process is often chosen in preference to sedimentation as higher volumetric capacities per unit area are achievable. A typical cross-sectional view of a DAF unit is shown in Fig. 10.11 and approximately 5–20% of the effluent leaving the flotation tank is recycled, supersaturated with air and used as the pressurised flow. Particles attach to the small air bubbles and float to the surface. In addition to the increase in volumetric capacity, the solid concentration typically achieved with DAF systems is higher, cf. 7%, compared to 2–3% for sedimentation. Operational costs of DAF systems are generally higher than sedimentation due to the high energy cost of supersaturating the water with air under pressure (Féris and Rubio 1999).

3.3.3 Suspended Air Flotation (SAF)

Suspended air flotation (SAF) is similar to dissolved air flotation in that small bubbles are used to float particles to the surface of the water. However, SAF units utilise chemicals (often cationic surfactants) to create the small bubbles, eliminating the need for a compressor and saturator thus reducing energy costs (Wiley et al. 2009). In addition, the flotation bubbles can be electrically-charged to increase the stability of the float. The increased stability of the float can significantly increase the acceptable hydraulic loading rate and solid loading rate. Suspended air flotation is a relatively new technology and at the present time it is not known whether the chemicals used to create the flotation bubbles, although often non-toxic, will effect subsequent medium recycling.

3.3.4 Autoflotation

Mechanical methods used to provide the flotation gas are often energy intensive and this disadvantage can be overcome by using photosynthetic oxygen (autoflotation) (Arbelaez et al. 1983; Koopman and Lincoln 1983). Dissolved oxygen (DO) concentrations exceeding 14–16 mg L^{-1} are essential for successful autoflotation (Arbelaez et al. 1983) and can be obtained by careful withdrawal of the oxygen saturated surface layer in unmixed and stagnant ponds. As the autoflotation process requires very high dissolved oxygen concentrations it cannot be used as a method to continuously harvest algae and is therefore not a feasible option in recovering microalgae biomass for bioenergy.

4 Particle Constrained Systems

Particle constrains systems include cake filtration, screening and attachment. Filtration and screening systems rely on the microalgae being retained based on size, whereas attachment is governed by the physical, chemical or electrical interactions between the microalgae and a media.

4.1 Filtration

Filtration processes require a filter media and operate under gravity, pressure or vacuum and the operation can be continuous or discontinuous. Most industrial filters are either pressure filters or vacuum filters with gravity filters reserved for simple separations including the screening of large particles through a course mesh or the filtration through a bed of course particles such as sand. However, slimy or very fine particles can form a dense, impermeable cake that will quickly plug any filter media which is fine enough to retain them. Filtration of such particles can often be improved through the use of filter aids which allow satisfactory cake

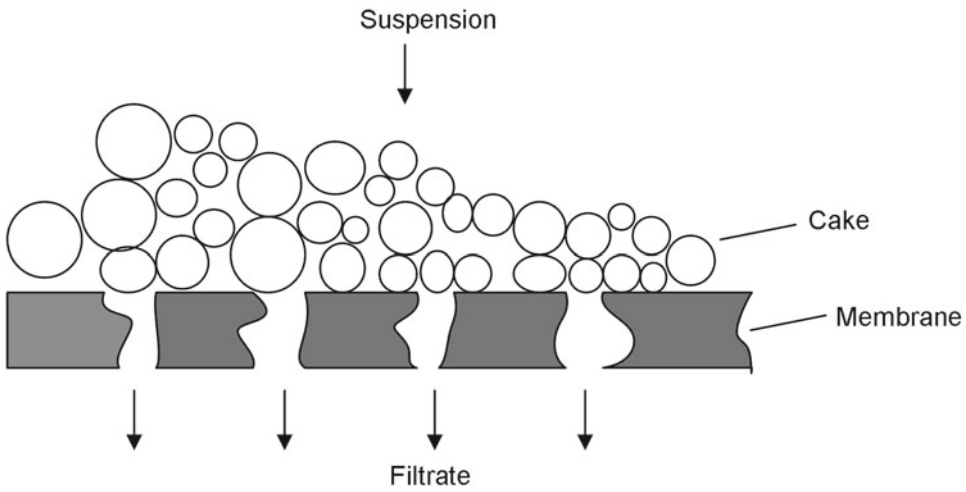

Fig. 10.12 Mechanism of cake filtration

porosity to be maintained. Filter aids can be incorporated into the particle suspension prior to filtration or added as a precoat to the filter media. A generalised schematic of a filtration process is shown in Fig. 10.12 and some published microalgae filtration performances are reported in Table 10.4. While a magnitude of filtration equipment has been developed to meet different applications, the most common industrial filtration equipment include belt filters, drum filters, filter presses, tangential (cross) flow membrane filters and rotary filters. It is worth noting that filamentous microalgae including *Arthrospira* (*Spirulina*) are often recovered through filtration under gravity or low pressure.

4.1.1 Filter Presses

A filter press consists of sets of plates interspaced with filter media. It utilises a dead end filtration process where the suspension is pumped into each press compartment and flows perpendicular to the filter media. Particles retained by the filter media build up to form a *cake*. When the cake consumes the void between the plates, the filter press is full and filtration ceases. The filtration process must be temporarily stopped to allow the plates to be separated and the cake discharged. While filter presses can be automated to minimise operator requirements they are infrequently used to recover microalgae.

4.1.2 Tangential (Cross) Flow Filtration

Tangential flow filtration (TFF) using ultrafiltration (UF; typically 1–100 nm pore size) or microfiltration (MF; typically 100–10,000 nm pore size) membrane technology can be used to retain particles and several studies, including Danquah et al. (2009) and Petrusevski et al. (1995), have successfully harvested and concentrated freshwater and marine microalgae using this technology. A schematic of a tangential flow filtration system is shown in Fig. 10.13. The

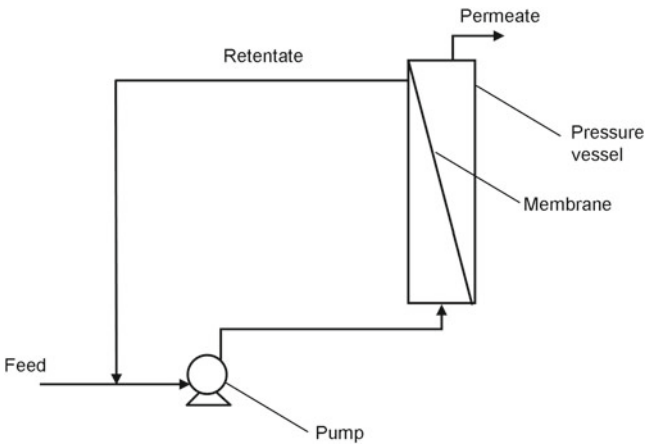

Fig. 10.13 Schematic of batch tangential flow filtration

suspension is pumped into pressure vessels holding the membrane filters and the bulk of the flow is parallel to the membranes.

The flow parallel to the membrane surface creates a shear force which assists in keeping the membrane clean by minimising a cake formation. Permeate fluxes are influenced by the membrane technology, pore size, feed flow rates, feed concentration, transmembrane pressure and any membrane fouling. There is a concern that the high shear force in some membrane technology may result in cell damage. Permeate fluxes of between 15 and 60 L m^{-2} h^{-1} are typical with UF membranes operating at low pressure and low velocity, while permeate fluxes up to 120 L m^{-2} h^{-1} for UF and MF are possible when higher operating pressures and velocities are used (Rossignol et al. 1999).

Energy requirements, while dependent on the system design, operating pressures and feed characteristics, have been estimated to be 3–10 kWh m^{-3} (Rossignol et al. 1999). While it is likely that tangential flow filtration has often been

Table 10.4 Performance of some pressure and vacuum filters used in harvesting of microalgae

Type	Machine and make	Operation mode	Concentration procedure	Algae	Suspended solids in concentrate (%); (concentration factor in parentheses)	Energy consumed (kWh m^{-3})	Relative harvesting cost[a]	Reliability
Pressure filter	Netzch chamber filter; Netzch	Discontinuous	One step	*C. proboscideum*	22–27 (245)	0.88	0.4	Very high
	Belt press; Bellmer	Continuous	Need pre-concentration	*C. proboscideum*	18 (180)	0.5	1.1	
	Suction filter; Seitz	Discontinuous	One step or pre-concentration	*C. proboscideum*	16 (160)	–	–	Good
	Cylindrical sieve rotators; Engelmann	Continuous	One step and for pre-concentration	*C. proboscideum*	7.5 (75)	0.3	1.9	Sufficient
	Filter basket; Seitz Dinlinger	Discontinuous	For pre-concentration	*C. proboscideum*	5 (50)	0.2	0.48	Good
Vacuum filter	Non pre-coat vacuum drum filter: Dorr Oliver	Continuous	One step	*C. proboscideum*	8 (180)	5.9	3.9	Low
	Potato starch pre-coat vacuum drum filter; Nivoba	Continuous after pre-coating	Need 2–15 fold pre-concentration	*C. proboscideum Scenedesmus*	37 (2–18.5)			Good
	Walther suction filter	Discontinuous	One step	*C. proboscideum*	8 (80)	0.1	4.5	Satisfactory
	Belt filter; Dinglinger	Continuous	For pre-concentration	*C. proboscideum*	9.5 (95)	0.45	0.88	Good
	Filter thickener; Schenck	Discontinuous	For pre-concentration	*C. proboscideum Scenedesmus*	5–7 (50–70)	1.6	3.2	Satisfactory

Adapted from Molina Grima et al. (2003)

[a]Does not include labor. Relative harvesting cost are calculated on the basis of operational cost of a self cleaning plate separator being 1.0

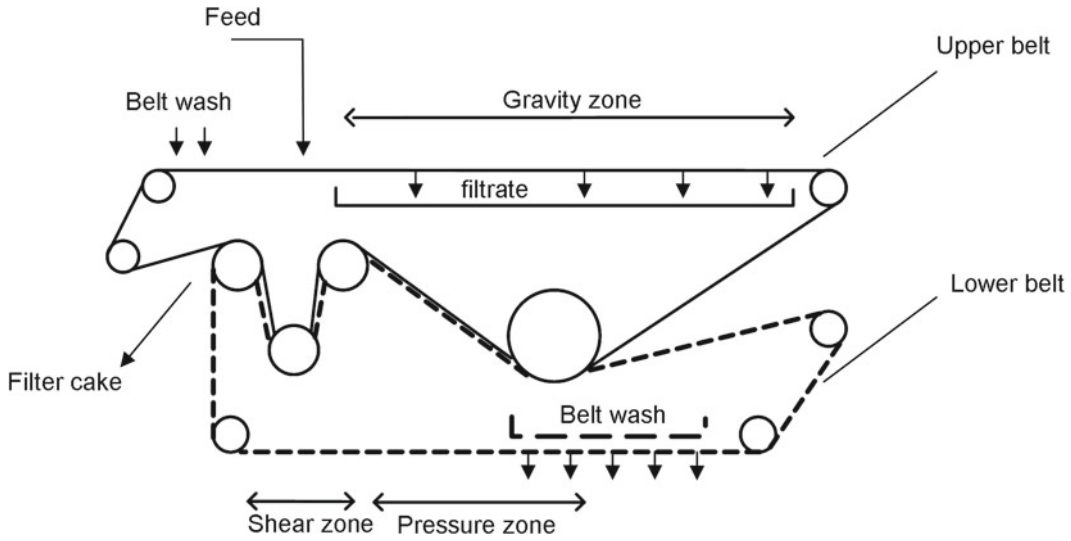

Fig. 10.14 Schematic of a combined gravity belt thickener and dewatering unit (Ashbrook Simon-Hartley Ltd., UK)

overlooked since MayKay and Salusbury (1988) reported that centrifugation may be more economical than cross-flow microfiltration, membrane technology has developed and more recently, energy costs of 0.38–0.51 kWh m^{-3} with permeate fluxes of ~20 L m^{-2} h^{-1} and concentration factors of 20–46 have been reported (Danquah et al. 2009). This, when compared to the energy required for seawater desalination (cf. 4.0–5.0 kWh m^{-3}), provides some promise. However, membrane fouling especially with long-term use, which reduces permeate fluxes, membrane life and possibly product quality, is a significant problem in many other industries and remain a major concern. There is little information on the long-term use of MF membranes for removal of microalgae.

4.1.3 Gravity Belt Filters

Gravity belt filters are generally continuously operated and consist of a fabric mesh (filter media) that moves over rollers driven by a variable speed drive. Microalgae feed streams may have already been primary harvested and have often been flocculated to increase the particle size and dewaterability. Particle thickening occurs by gravity drainage of any free water through the fabric mesh. Blades, or ploughs, can be installed along the gravity drainage zone to turnover the slurry thereby increasing the amount of drainage. Gravity belt thickeners generally have a relatively low power consumption and low capital cost. While the final moisture content can be controlled to some extent by varying the drainage time on the belt, the extent of thickening is strongly dependent on the floc characteristics and the way that the water is bound to the slurry. Belt thickeners are often designed for a maximum 5–7% solids concentration (Tchobanoglous et al. 2003).

4.1.4 Combined Gravity Belt Thickener and Dewatering

The solids concentration from gravity belt thickeners can be dewatered by incorporating a second 'belt'. The second belt may directly contact the slurry or act through capillary action. A combined gravity belt filter thickener and dewatering unit typically receives a feed stream containing 1–4% solids and generates a concentrated stream ('cake') with 12–35% solids (Shammas and Wang 2007).

When the second belt is in direct contact to the slurry, pressure is exerted through additional rollers located above and below the belts and forces some of the liquid that was initially retained in the slurry to pass through the belts, whilst containing the solids between the belts. A schematic of a gravity belt thickener and dewatering unit with the second belt in direct contact to the slurry is shown in Fig. 10.14. An alternative is for the second belt to operate on capillary action. In this case the second belt is in direct contact with the lower side of the main belt as shown in Fig. 10.15. Algae Ventures™ have recently designed a gravity belt thickener and dewatering system specifically to thicken and dewater microalgae without the need of flocculants. The Algae Ventures™ system incorporates a super-absorbent capillary belt which draws water through the main belt and away from the slurry without added mechanical pressure, leaving a cake which can then be heated with a waste heat stream to produce dry flakes. Whilst this enhanced technique can achieved high recovery efficiency and concentration factor at relatively low operating costs, they have only been used in small scale operations and have not been tested on all microalgae species.

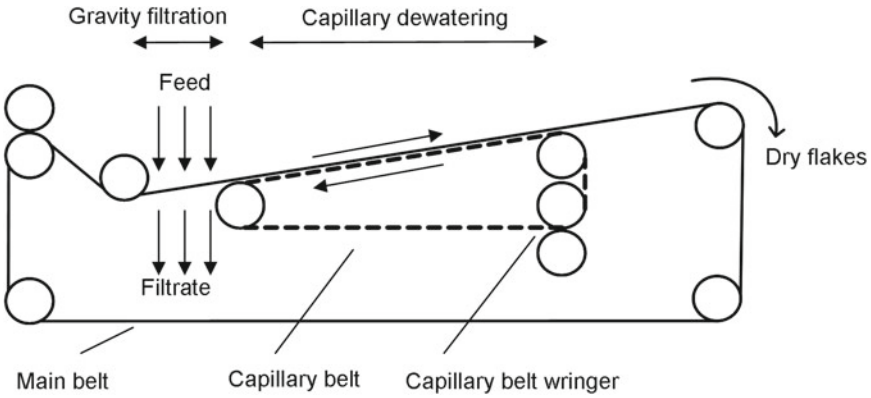

Fig. 10.15 Cross-sectional view of a capillary belt filter

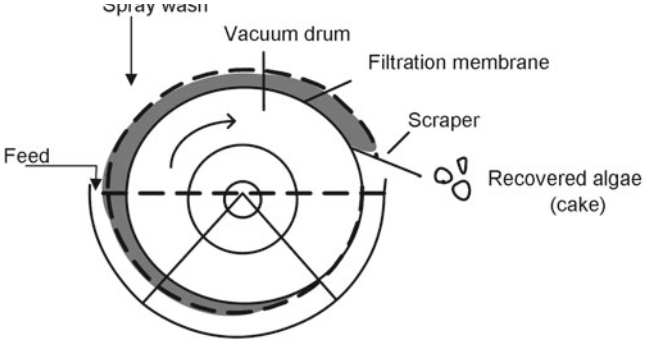

Fig. 10.16 Cross-sectional view of a vacuum drum filter

4.1.5 Vacuum Filters (Rotary Drum)

Vacuum filters almost exclusively employ a rotating drum filters. Important design variables include the size and type of filter, cake discharge mechanism, vacuum level, cycle time and feed conditioning. Majority of feed streams are flocculated with a solids concentration of 0.5–5%. Water removal is typically 40–60% with a biomass recovery rate over 90% (Pers. Comm. Parkson Corporation). A cross-sectional view of a typical vacuum drum drier is shown in Fig. 10.16.

4.1.5.1 Mechanical Presses

Mechanical presses or thickeners are filtration devices which make use of slow rotating screens or screws which generate high pressure and shear zones through volume reduction to dewater slurries. Common mechanical presses include rotary presses and screw presses. Mechanical presses, as opposed to using filter presses or belts, generally have higher throughputs, lower footprints and a minimal requirement for wash-water. In a rotary press the solid suspension is fed through a channel between two parallel revolving screens. The retained solids are pushed forward inside the channel by rotating blades and eventually form a cake, while the filtrate passes through the revolving screens. Screw presses can be either horizontal or inclined and consists of a rotating screw inside a cylindrical perforated screen. Through a progressive reducing pitch and as the screw rotates, the solid suspension fed from a hopper is subjected to a gradually increasing pressure. This forces the liquid out through the perforated screen whilst the retained solids moves toward the exit end of the press. New materials, including elastic capillary membranes on porous water absorbing materials, are being developed to aid in the dewatering process. The moisture content in the dewatered cake can be controlled by the discharge pressure and rotational speeds. Cross-sectional view of a rotary press and a horizontal screw press are shown in Figs. 10.17 and 10.18.

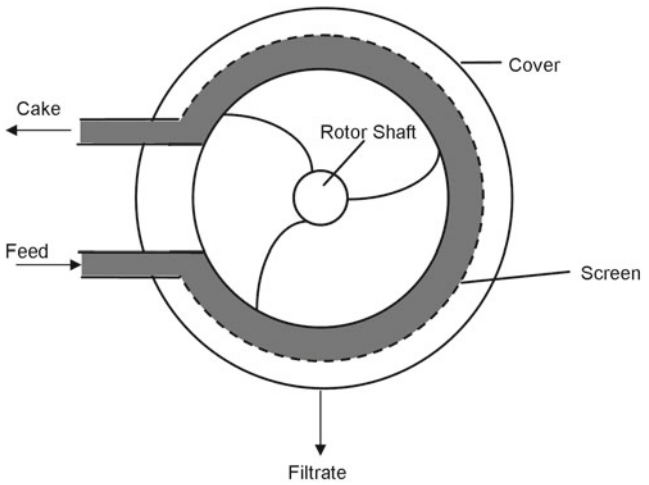

Fig. 10.17 Cross-sectional view of rotary filter press

4.1.6 Linear Electro-dewatering (EDW)

Mechanical removal of water from microalgae biomass is generally limited to 10–25% solids. If higher levels of dewatering are required then this has generally been accomplished through the use of energy intensive thermal drying (evaporation) systems. Electrokinetic dewatering units, whilst not fully understood, exploit the behaviour of charged particles in electric fields and when combined with mechanical dewatering systems can improve the dewatering process and

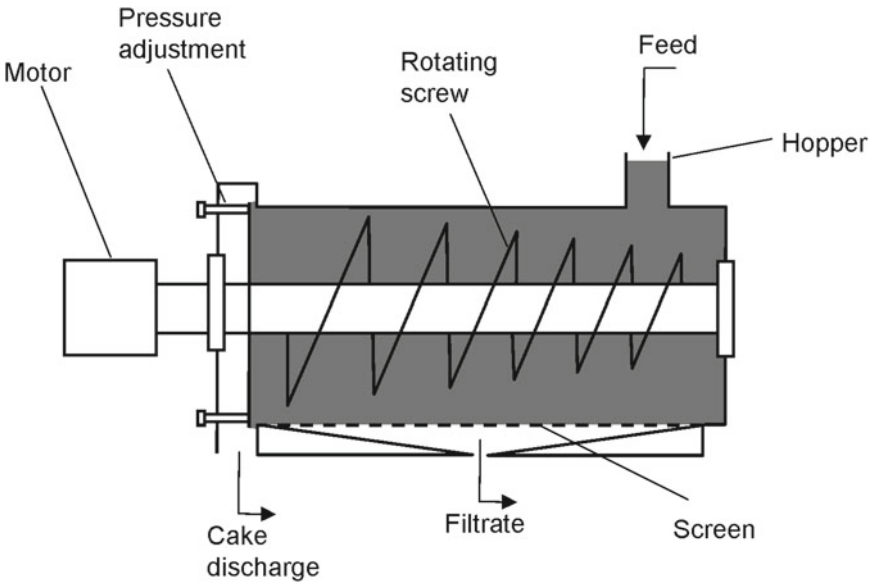

Fig. 10.18 Cross sectional view of a horizontal screw press (From Vincent Corporation, FL, USA)

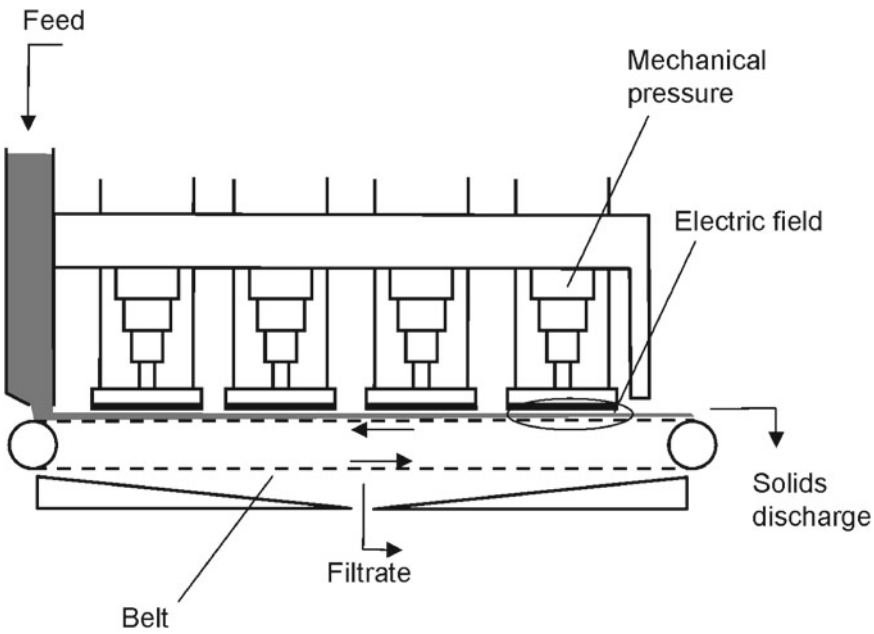

Fig. 10.19 Linear electro-dewatering system (Courtesy Elcotech)

increase the final solids content. Enhanced electrokinetic dewatering techniques include systems based on electro-osmosis, electromigration and electrophoresis principles, and whilst many systems were designed for dewatering sludges in wastewater treatment plants, there may be scope for its use in dewatering microalgae slurries. Elcotech's Linear Electro-Dewatering units use mechanical pressure and electro-osmosis to dewater sludges up to and above 50% dry solids (Fig. 10.19).

4.1.7 Filtration Summary

Whilst there are countless filtration techniques most have significant shortcomings when applied to harvesting microalgae on a large scale. The filtration method, filtration media and the physical properties of the feed significantly affect filtration efficiency, filtration capacity requirements and cost. The typical energy requirements for several filtration systems and achievable microalgae concentration factors are reported in Table 10.5. Low biomass recovery and severe

Table 10.5 Energy costs for continuous filtration based operations

Filtration method	Concentration procedure	Feed conc.	Final solids conc. (%)	Concentration factor	Reliability	Energy requirements (kWh m^{-3})
Micro-strainer	Primary		1.5	15	Good	0.2
Vibrating screen	Primary		6	60	Good	0.4
Filter press (discont.)	Primary/secondary		22–27	245	Very high	0.88
Belt filter press	Secondary	4%	18	180	Poor	0.5
Cylindrical sieve	Primary		7.5	75	Moderate	0.3
Vacuum drum	Primary/secondary		18	180	Low	5.9
Belt filter	Primary		9.5	95	Good	0.45

Adapted from Mohn (1980)

fouling of the filtration media are two of the greatest problems when harvesting small unicellular microalgae. The performance of most stand-alone filtration systems for the recovery of unicellular microalgae is poor and requires the feed to be flocculated and conditioned before being processed.

Filtration is generally only acceptable when large (filamentous, colonial or flocculated) microalgae are to be recovered. In addition, consideration must be even to minimise any selectivity in the harvested microalgae. For example, the long term filtration of *Spirulina* can lead to a progressive enrichment of species with short and narrow filaments (Vonshak and Richmond 1988). Large amounts of energy have historically been used for wash water and to provide sufficient mechanical pressure to overcome the clogging of filter media. However, with appropriate feed conditioning, improved filter media characteristics and with enhanced dewatering methods, filtration systems may offer new promises for the efficient and low cost harvesting of microalgae.

4.2 Attachment

Materials or fibers may also be deployed directly into the culture system and the microalgae cells removed through attachment via physical, chemical or electrical attraction. High gradient magnetic separation can be used to recover microalgae through the addition of magnetic particles (usually Fe_3O_4, magnetite) (Shelef et al. 1984). In 2001 the use of superconducting magnetic separators were investigated in a wastewater treatment facility and the energy requirement was estimated at 6.5 kWh m^{-3} (Isogami et al. 2001). It has also been reported that *Dunaliella* can be recovered by absorption into hydrophobic materials (Darzins et al. 2010).

4.3 Drying

Mechanical dewatering processes are typically capable of increasing the solids content up to 10–25%, leaving 75–90% water. If additional drying is required prior to biomass processing then this can be undertaken by thermal processes and solid concentrations greater than 85% can often be achieved. While there is a large range of drying systems, they must all provide sufficient energy to overcome the high enthalpy of evaporation. Waste heat from industrial processes, such as power stations, requires expensive infrastructure to move large quantities of hot air (typically 400 °C) and to collect any particle fines. Solar drying is another possibility and is frequently used in many industries including wastewater for sludge processing. Large drying surface areas and regular agitation of the drying biomass is required. Drying processes, if required, must consider product quality degradation and odor generation. Thermal drying is an energy intensive process and is generally considered too expensive for microalgae based biofuels.

4.4 Process Equipment Selection

In order to select the most appropriate equipment for harvesting and dewatering microalgae, consideration must be given to the scale of the operation, the initial and desired solids content, the preferred operation cycle (batch vs. continuous) and the capital and operating cost. While operational costs can vary significantly, a summary of typical operational costs in reported in It is also useful to have some knowledge of the physical characteristics of the microalgae and its growth media, including the salinity (conductivity), relative density, pH, viscosity and zeta potential, as well as the size and shape of the microalgae cells. It is therefore necessary to conduct a number of tests on the microalgae in the laboratory to determine these and other characteristics (Table 10.6).

4.4.1 Laboratory Testing

A number of laboratory tests, including flocculation jar testers, settling tubes (Imhoff cones) and leaf filters can be used to develop an understanding of the flocculation, flotation, filtration, or sedimentation ability of the microalgae under various process conditions. Sedimentation rates and filtration rates (see Figs. 10.20 and 10.21, respectively) can be used to design sedimentation and filtration systems.

Table 10.6 Summary of harvesting operational costs

Process	Estimated operating cost (US$/Mgal) (1976)	Quality of recovered biomass	Reliability	Relative energy requirements	Removal efficiency	Concentration factor
High-gradient magnetic separation	US$600/Mgal (1976)	Poor	Very good	Very high	95	Unknown
Coagulation – clarification – filtration	600	Poor	Very good	Moderate	95	–
Centrifugation	500	Good	Good	Very high	80	40
Coagulation – floatation	450	Poor	Good	High	90	85
Coagulation – Flocculation – sedimentation	400	Poor	Good	High	85	50
Ultrafiltration	350	Good	Very good	High	95	50
Intermittent sand filtration	250	Poor	Fair	Low	80	–
Direct filtration without coagulants	200	Fair	Poor	Moderate	40	–
Intermittent discharge ponds with chemicals	150	None	Unknown	Very low	90	–
Micro-strainer	50	Good	Poor	Low	50	35
Sedimentation	50	Fair	Poor	Low	80	Unknown

Adapted from Benemann et al. (1980)

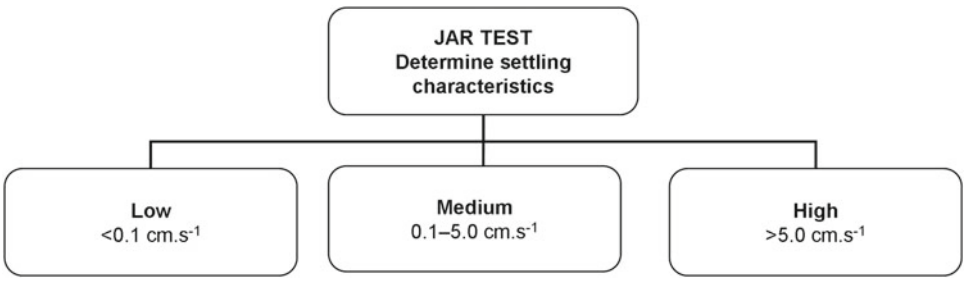

Fig. 10.20 The settling characteristics can be determined by a jar test

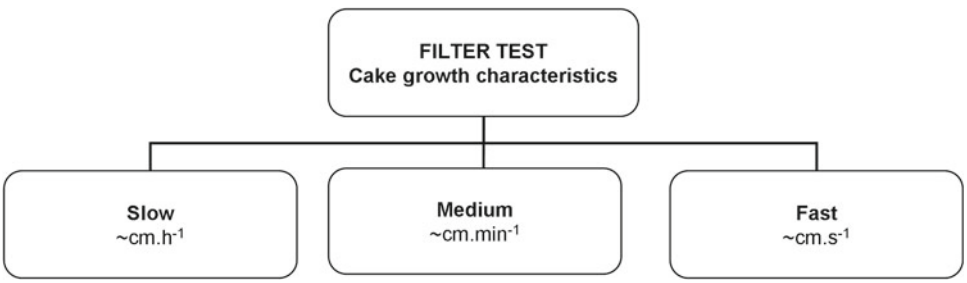

Fig. 10.21 The cake growth and filtration rate can be determined from a leaf vacuum test

4.4.2 Specification and Shortlist

A specification for the harvesting equipment can be developed based upon the requirements in terms of scale (throughput), operation and performance objectives, as outlined in Fig. 10.22.

Based on the duty specification and information gained from laboratory tests, an initial shortlist of suitable equipment can be selected. Consideration should also be given to predicted energy consumption, using Fig. 10.23 as a guide.

Consideration must also be given to the capital cost, expected downtime and maintenance requirements and hazards introduced (i.e. noise, chemicals, moving and rotating items) with any equipment and process. Specification

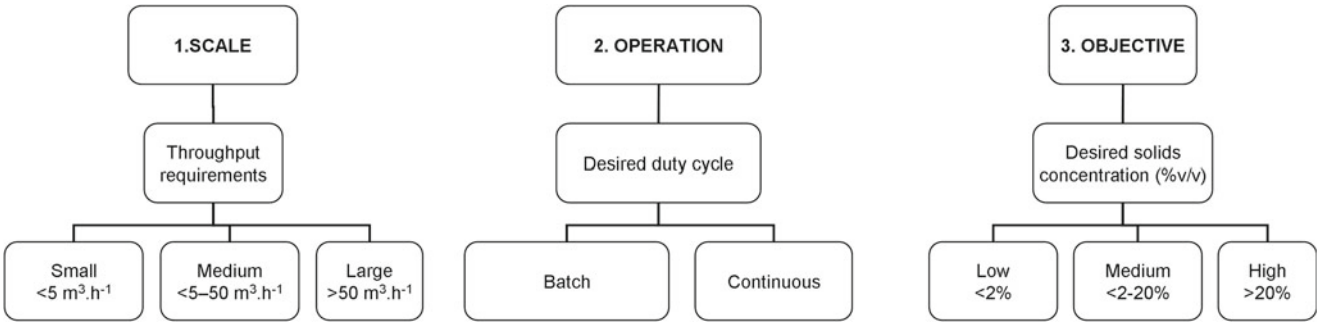

Fig. 10.22 Duty specification for harvesting system

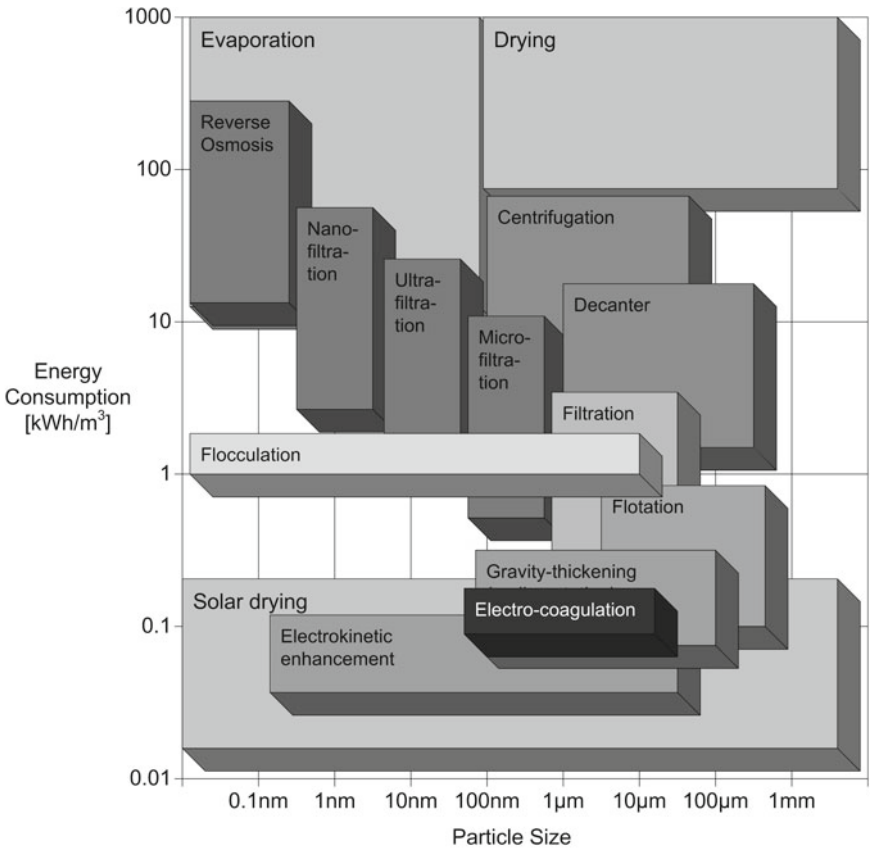

Fig. 10.23 Energy consumption and particle size for various separation techniques

must reference any applicable standards relating to the design of the specific equipment.

4.4.3 Pilot-Plant and Computer Simulation

Before investing in expensive large-scale harvesting equipment, it is often useful to develop a computer simulation of the system along with building a pilot-scale plant which can be used to predict and fine-tune performance of short-listed equipment under various conditions. Computer simulation involves developing a mathematical model of the system using various software packages available. The parameters of the model are determined through measurement from the pilot plant and subsequent calculations. The computer model can then be used to predict the behaviour of the system. By comparing the results of the simulation against actual data obtained from the pilot plant, it is possible to economically refine the design of both the pilot plant and the model to develop a process which meets the specified requirements under various operational scenarios.

4.4.4 Scale-Up, Construction and Operational Considerations

Selection of the most suitable equipment for scale-up must take into account a life-cycle assessment of costs, including design, construction, installation, operation and eventual demolition and disposal. Information should be sourced from discussions with potential suppliers, equipment manufacturers as well as other users to ensure a clear understanding the risks and expectations. Negotiations with suitable contractors for the construction and installation should also begin early to ensure the best qualified resources are allocated to the project.

4.5 Conclusion

The ease and costs associated with the recovery of microalgae biomass will ultimately depend on the microalgae strain(s) and the culture system. For the commercial-scale recovery of microalgae biomass as a source of renewable biofuels, the biomass recovery process must be effective at large scale, highly reliable, easily managed and have low capital and operating costs. Many small-scale harvesting and dewatering processes have proven to be very species specific, expensive and not readily scalable. A single best method, universal process, or a series of processes, is yet to be identified that will be suitable in all situations and for all microalgae species. Consequently, harvesting and dewatering processes must be tailored based on the species of microalgae and its growth conditions, whilst being adaptable to handle the natural variations in the output from the culture system. However, given that most species that are currently of interest as potential biodiesel feedstocks are cultivated as individual cells which are too small for a simple filtration and have a low density difference to the growth media, the first step in a biomass recovery system is likely to consist of a two stage harvesting module (e.g. flocculation, followed by flotation or gravity sedimentation). While flotation is often preferred over gravity sedimentation, as higher solid concentrations can be achieved with a smaller footprint, operational costs are generally higher. Significant scope exists for more research and development in the area of harvesting and dewatering technologies which combine traditional equipment which are proven and economic, with enhanced techniques such as electrokinetics (electro-osmosis, electro-flocculation and electro-dewatering) and enhanced filtration.

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Solvent Extraction for Microalgae Lipids

11

Emilio Molina Grima, María José Ibáñez González,
and Antonio Giménez Giménez

1 Introduction

Algae produce a great variety of fatty acids and lipids which must be extracted before their conversion into fuels. The composition of microalgae lipids is qualitatively different from that of common vegetable oils and the conventional technologies for processing them may be unsuitable as they incurs in huge losses. In addition, the unusually high content of free fatty acids in the oil, unsaponifiable constituents, phospholipids, glycolipids and its dark colour, all cause difficulties in the necessary crude lipid refining process. To date, recovery of algal oils has usually been carried out at laboratory scale, following typical analytical procedures used for terrestrial plants, animal tissues and microbial cellular lipids, which in many cases are ineffective and of little use due to the large differences with microalgae biomass. Microalgae biomass differs from traditional biomass feedstocks in several respects, such as the chemistry of cell wall and plasma membrane, the presence of a large amount of water and the small size of the cells.

Extraction of lipids from microalgae at laboratory scale has been attempted by physical methods (with a screw press) or chemically with organic solvents, water (by three-phase partitioning extraction methods) and supercritical methods; and solvent extraction has proved to be the most workable approach so far. The extraction of lipids from algae requires attention to their polarity. Polarity is related to the distribution of lipids within the algal cell and the association of lipids to non-lipid molecules. Lipids present in algae can be classified as (Pohl and Zurheide 1982): (i) neutral lipids (NLs) (triacylglicerols, TAGs, wax esters, hydrocarbons, fatty acids, FAs, and sterols); (ii) phospholipids (PLs) (phosphatidylcholine, PC, phosphatidylethanolamine, PEA, phosphatidylserine, PS, phosphatidylglycerol, PG, and phosphatidylinositol, PI); and (iii) glycolipids (GLs) (sulfoquinovosyldiacylglycerol, SQDG, monogalactosyldiacylglycerol, MGDG, and digalactosyldiacylglycerol, DGDG). TAGs are usually regarded as energy storage products, whereas PLs and GLs are structural lipids, contained mainly in the cell membranes. On the other hand, the major fatty acids are saturated and the cis isomers of unsaturated, with 12–22 carbon atoms and 0–6 double bonds, Table 11.1.

Typically, chain lengths of over 18 carbons are found in marine microalgae and the presence of polyunsaturated fatty acids (PUFAs) is also usual, the main ones being eicosapentaenoic acid (EPA, C20:5n3), and docosahexaenoic acid (DHA, C22:6n3). These fatty acids are susceptible to oxidation during storage, which makes them less suitable for use in biodiesel. Autoxidation, the spontaneous free radical reaction of organic compound with oxygen, is the responsible for the deterioration of lipids during processing and storage. Reasonable rates of autoxidation of fatty acid methyl esters (FAMEs) can only be achieved at high temperature in the case of saturated fatty acids (C16:0, C18:0, C20:0), while the autoxidation of monounsaturated fatty acids (C16:1, C18:1 and C20:1), and above all PUFAs (C16:3, C18:2, C18:3 C20:5, C22:6, etc.) occurs readily at room temperature. The final result of autoxidation is the transformation of lipids into some other deteriorated lipid macromolecules that do not meet the legal specifications for using as biodiesel (Porter et al. 1995).

The microalgae species and the time of harvesting will determine the lipid composition of algae. Algae harvested in the exponential growth phase (which is usual when production is in continuous operational mode) will contain more polar lipids (GLs and PLs) than those harvested in the late stationary phase of growth (usual for batch cultures), which contain more neutral lipids (TAGs). The lipid fractionation procedure and the selection of solvent, or solvent system, will depend on the particular classes of lipids present. Overall, neutral lipids are recovered by non-polar solvents, whereas polar lipids are recovered by polar ones. Therefore, to select a solvent system we need to match the polarity index and the

E.M. Grima (✉) • M.J.I. González • A.G. Giménez
Department of Chemical Engineering,
University of Almería, 04120 Almería, Spain
e-mail: emolina@ual.es; mjibanez@ual.es; agimenez@ual.es

M.A. Borowitzka and N.R. Moheimani (eds.), *Algae for Biofuels and Energy*, Developments in Applied Phycology 5,
DOI 10.1007/978-94-007-5479-9_11,

Table 11.1 Fatty acids in major microalgae groups (Adapted from Ackman and Tocher 1968; Kayama et al. 1989)

Division/classes	Saturated and monounsaturated	Polyunsaturated
Cyanophyta/ Cyanophyceae	C16:0; C16:1n7; C18:1n9	C18:2n6; C18:3n3
Rhodophyta/ Rhodophyceae	C16:0	C18:2n6; C20:4n6; C20:5n3
Chlorophyta/ Chlorophyceae	C16:0; C16:1n7	C18:2n6; C18:3n3
Dinophyta/ Dinophyceae	C16:0	C20:5n3; C22:6n3
Chrysophyta/ Chrysophyceae	C16:0; C16:1n7; C18:1n9	C20:5n3; C22:6n3
Haptophyta/ Haptophyceae	C16:0; C16:1n7; C18:1n9	C18:2n6; C18:3n3; C22:6n3
Bacillariophyta/ Bacillariophyceae	C16:0; C18:1n9	C20:5n3; C22:6n3
Xanthophyta/ Xanthophyceae	C14:0; C16:0; C16:1n7	C20:5n3
Eustigmatophyta/ Eustigmatophyceae	C16:0; C16:1n7; C18:1n9	C18:2n6; C18:3n3; C20:4n6;C20:5n3

solubility parameters of the solvents and the lipids classes to be extracted.

This chapter analyzes the solvent extraction and fractionation of algal oil for biofuel. Initially the basic thermodynamic principles are outlines for the dissolution of materials into solvents and for the solvent selection. The possible pretreatments of biomass are then studied. The core of the chapter is devoted to analyzing the extraction of lipids and how to solve some problems that occur due to the nature of lipids present and the possibility of their prior fractionation. The following section discusses an alternative to the extraction of lipids for biofuel, namely the direct extraction of fatty acids from biomass by means of a direct saponification of biomass and their eventual fractionation. The final section studies the direct production of FAMEs through a direct transesterification of wet biomass.

2 Thermodynamics of Solvent Extraction

Solvent extraction has been defined as a process for transporting materials from one phase to another for the purpose of separating one or more mixtures. In the case of microalgae biomass, crude lipids are separated by a solvent, or a co-solvent mixture, from the rest of the cell biomass. The solvent extraction theory, based on the laws of thermodynamics, has been explained by Sekine and Hasegawa (1977). During dissolution, two separate substances (the oil and the solvent) form a molecular mixture. Dissolution is always accompanied by a negative Gibbs energy change. Gibbs energy change (ΔG) is related by Gibbs equation to enthalpy variation (ΔH), absolute temperature, T, and entropy variation (ΔS) as:

$$\Delta G = \Delta H - T\Delta S \tag{11.1}$$

Methods of estimation Gibbs energy change from thermodynamics tables are not accurate. Van Kreevelen and Chermin (1951) suggested to estimate this by:

$$\Delta G = A + BT \tag{11.2}$$

where A and B are determined by considering the different group contributions of lipid molecule. Values of A and B whether for hydrocarbon and non-hydrocarbon groups can be found also in Perry et al. (2003). In addition, constant B should be corrected by taking into account the symmetry of the group: $R \cdot T \cdot ln\sigma$, where σ represent the number of indistinguishable positions in space the molecule may be made to take up by a simple rigid rotation (for example, σ = 4 for carbon tetrachloride (CCl_4) and 2 for acetone) and the number of optical isomers of the group: R T *ln*η, where η represent the number of such as isomers. $R \cdot T \cdot ln\sigma$ and $R \cdot T \cdot ln\eta$ should be summed to constant B. In Eq. (11.2) T is in Kelvin and ΔG is in Kcal mol^{-1}. By using Eq. (11.2) ΔG can be estimated with an accuracy of ± 5 kcal mol^{-1}.

Because dissolution involves mixing of two substances, an increase in their disorder (i.e. a positive entropy change) occurs. Dissolution involves two endothermic processes and one exothermic one. First, lipid molecules separate into isolated molecules. This is an endothermic process and its energy is small for nonpolar TAGs and slightly higher for polar lipids (i.e. PLs and GLs). The separated lipid molecules are next dispersed into solvent and energy is required to dissociate the solvent molecules. The energy required increases with increasing intermolecular interaction in the solvent in the following order: non-polar solvent < polar solvent < hydrogen-bonded solvent. In the third process, which is exothermic, the dispersed lipid molecules interact with neighbouring solvent molecules. The energy released increases in the following order of lipid-solvent interactions: both solvent and lipid molecules are non-polar < one is polar and the other is non-polar < both molecules are polar < lipid molecules are solvated by solvent molecules.

The overall enthalpy change is more negative (exothermic) if energy losses of lipid-lipid and solvent-solvent interactions are greater than the energy gain in the lipid-solvent interaction. When lipids molecules are strongly bound to each other, they are highly soluble only in solvent whose lipid-solvent interactions are also large. When solvent molecules are highly interassociated, as with water, the lipids dissolve well only if dissolution results in a stronger lipid-solvent interaction. Therefore, the solubility of TAGs in water is very small, because TAG molecules interact with

water only very weakly and the energy gained from the TAG-water interaction cannot compensate for the large amount of energy required to break the intermolecular hydrogen bonds of water. However, solubility of oil in hexane is high because of the stronger TAG-solvent interaction which compensates for energy losses in the first and second stages. A general principle for the dissolution of materials, is that "like dissolves like"; i.e. a non-polar lipid is more soluble in a non-polar solvent, and vice-versa. However, some polar solvents can dissolve certain non-polar lipids, such as methanol dissolving TAGs. The energy required to disrupt a solvent-solvent interaction may be great, but the energy gained in the TAG-solvent interaction is even greater.

In seeking the best solvent or solvent system for lipid extraction, a quantitative approach, less complex than determining the different terms of Eqs. (11.1) and (11.2), is to calculate the solubility parameters (polarity index, solubility parameters and dipole moments) for the solvent and lipid class to be extracted. However, the problem lies in the fact that lipids such as TAGs are usually located in cytoplasmatic packed bodies while PLs and GLs are mainly located in the plasma membrane and chloroplast membranes, respectively. In addition, polar lipids (PLs and GLs) are usually associated among themselves and to membranes and proteins; which is an added problem because the solvent (or solvent system) has to disrupt the cellular membrane and the linkages between lipids and membrane proteins, followed by the dissolution of the polar and neutral lipids. A large body of literature exists on data for polarity index, solubility parameters and dipole moments for organic solvent; however, similar data must be estimated for the different microalgae lipid classes being extracted. When solubility parameters for solvent and lipid are similar, the solvent will be a good candidate for a given application. Polarity index and solvent parameters for 82 solvents can be found in Snyder (1974); only polarity index can be found in Gupta et al. (1997) and Lalman and Bagley (2004); solubility parameters in van Dyck et al. (1989); Tijssen et al. (1976), and Stefanis and Panyiotu (2008), and dipole moments in Lide (2001).

The solubility parameter (δ) is an important concept that can be used for a rational design of solvent system. Both the total solubility parameter and its constituent partial solubility parameters (Hansen solubility parameters, Hansen 2008) are widely used for the selection of appropriate solvent systems for given solutes. The total solubility parameter (δ) is defined by Eq. (11.3):

$$\delta = \sqrt{\frac{\Delta H_v - R \cdot T}{V_m}} \tag{11.3}$$

where, ΔH_v is the enthalpy of vaporization of the solvent, T is the absolute temperature, R is the universal gas constant and V_m is the molar volume of the solvent at temperature T.

For polar compound, as well as for compounds that contain hydrogen bonds, the total solubility parameter is not sufficient to describe their solubility behavior. This occurs because, in these compounds, three kinds of intermolecular forces can be present: dispersion, polar, and hydrogen-bonding forces. A significant enhancement of the predictive capacity and usefulness of the solubility parameter has been developed by the introduction of Hansen parameters (or partial solubility parameters) (Hansen 1967, 2008). Specifically, each molecule is given three Hansen parameters, each generally measured in $MPa^{0.5}$. Equation (11.4) describes the relation between the total solubility parameter, δ, and the Hansen solubility parameters, δ_D, δ_P, and $\delta_{H:}$

$$\delta^2 = \delta_D^2 + \delta_P^2 + \delta_H^2 \tag{11.4}$$

where δ_D, δ_p and δ_H are the dispersion, polar and hydrogen-bonding Hansen parameters, respectively.

These three parameters can be treated as co-ordinates for a point in three dimensions also known as the Hansen space. The nearer two molecules are in this three dimensional space, the more likely they are to dissolve into each other (Hansen 1967). To determine if the parameters of two molecules (usually a solvent and a solute, in our case a microalgal lipid class) are within range a value called interaction radius (R_0) is given to the lipid being dissolved. This value determines the radius of the sphere in Hansen space and its center is the three Hansen parameters. To calculate the distance (R_a) between Hansen parameters in Hansen space the following formula is used:

$$\left(R_a\right)^2 = 4 \cdot \left(\delta_{D2} - \delta_{D1}\right)^2 + \left(\delta_{P2} - \delta_{P1}\right)^2 + \left(\delta_{H2} - \delta_{H1}\right)^2 \tag{11.5}$$

In this equation the subscript "1" might refer to the microalgal lipid class to be extracted and the subscript "2" might refers to the potential solvent or solvent system. Combining this with the interaction radius gives the relative energy difference (RED) of the system:

$$\mathrm{RED} = \frac{R_a}{R_o} \tag{11.6}$$

In this equation, R_o is the radius of Hansen solubility parameter sphere and represents the largest value for R_a allowed where solubility is allowed. Good solvent systems have a RED number less than 1. If RED = 1 the system will partially dissolve, and if RED > 1 the system will not dissolve.

There is enough literature for prediction of Hansen solubility parameters (for example Tables 1a and 1b in the Handbook of Lo et al. (1983)). Also we can use specific thermodynamic software based on the method of contribution of groups: Molecular Modeling Pro (http:www.chemsw.com/13052a.htm). But one of the most comprehensive guideline in this respect is the one of Stefanis and Panayioutu

Table 11.2 Neutral and polar lipid composition of *Isochrysis galbana* (Molina-Grima et al. 1994), *Phaeodactylum tricornutum* (Cartens et al. 1996) and *Porphyridium cruentum* (Giménez-Giménez et al. 1998) cultured in external tubular photobioreactors in continuous operational mode

	Isochrysis galbana	*Phaeodactylum tricornutum*	*Porphyridium cruetum*
Lipids	% of total lipids[a]		
Neutral	26.5 (43.0)	23.2 (51.0)	39.5 (47.0)
Polar	73.5 (57.0)	76.8 (49.0)	60.5 (53.0)
Glycolipid	59.3 (37.0)	49.1 (35.0)	45.0 (43.0)
Phospholipid	14.2 (20.0)	27.7 (14.0)	15.5 (10.0)

[a]For measurement of the percentage of lipids we converted all the extracted lipids to FAMEs via acid-catalyzed transesterification following a modification of the Lepage a Roy method (Rodríguez-Ruíz et al. 1998). In this way the percentage of lipids is referred to the saponifiable lipid fraction. Using FAMEs precluded the unsaponifiable lipids (chlorophylls and carotenoids) which are included in gravimetric methods, but that are not generally of interest for biofuel

Data in parenthesis correspond to batch cultures in the late stationary phase of growth (López Alonso et al. 1998)

(2008). These authors have introduced a group contribution method which allows the direct prediction of Hansen parameters of organic compounds. The solubility parameters are calculated using simple linear equations. Only the molecular structure of the lipids classes to be extracted and the molecular structure of the potential solvent (or solvent system) are required for the prediction, and no experimental data are needed. Using the predicted solubility parameters values, the selection of appropriate solvents for each lipid class is possible. By calculating the distance R_a the best existing solvent for each lipid class can be found (Stefanis and Panayioutu 2008). Nonetheless, it should be noted that the above guideline for solvent extraction is incomplete because it does not consider the additional association among the lipid classes and membrane proteins (hydrogen bonds) that are more difficult to estimate and are one of the reasons for which 100% extraction efficiency cannot be guaranteed.

3 Ideal Solvent Characteristics

The solvents should be selected bearing in mind also several factors: they should be volatile (for easy removal later), free from toxic or reactive impurities (to avoid reaction with the lipids), able to form a two-phase system with water (to remove non-lipids), and be ineffective extractors of undesirable components (e.g., proteolipid protein, small molecules). The range of their effectiveness on the different classes of lipids and their cost must also be considered. A solvent with high solubility at elevated temperature and low solubility at ambient temperature may be desirable, because phase separation of oil from solvent would occur without necessity of evaporation. The lower alcohols exhibit this characteristic. The second most important characteristic is that the solvent be nontoxic to workers. Selectivity of a solvent is also a very important characteristic. When the concern is only about oil for biofuels, it is desirable to use solvents which extract only TAGs and leave polar lipids (PLs and GLs), fatty acids, waxes, and pigments in the spent biomass. However, as will be discussed later, we do believe that this approach would reduce the interest of microalgae for biofuels, since in some cases the polar lipid content is over 50% of algae oil (Table 11.2). In this last case, alternative co-solvent mixtures need to be explored, and even an economic balance must be performed considering pros and cons. If the use of the polar lipid fraction were still discouraged, other alternatives should be considered such as the extraction of fatty acids by a previous saponification of the biomass, or even a direct transesterification of total lipids from the biomass to obtain fatty acids methyl esters (FAMEs) directly, as we will discuss later. To date the cost is a favourable factor for hexane use; however, concerns about its availability, tighter emission restrictions, safety and its testing as a harzadous air pollutant have stimulated interest in alternative solvents. At present aqueous extraction processing wherein water is used as a separation medium, is attracting more attention. Obviously, when using water as a solvent it is not necessary to dry the biomass, the major constraints being the difficulty to eliminate the emulsions formed, the need for several extraction stages and the high time consumed. In addition to being an organic-solvent-free process and enabling simultaneous extraction of oil and protein, AEP has the potential of producing extracted oil that requires less refining due to low phospholipids content (Jung et al. 2009). In the future, the high increase in bioethanol production, obtained by fermentation processes, as well as favourable regulations regarding its production, may change the current factors that encourage the use of hexane.

4 Cell Wall and Plasma Membrane of Microalgae

The properties of the plasma membrane (PM) and cell wall (CW) play an important role in the solvent extraction process. For example, the presence of CW may prevent direct contact between the solvent and cell membrane and impede

extraction. Knowledge of the cell wall chemistry of microalgae may therefore be useful because it can determine the implementation of pre-treatment procedures. The CW gives cells rigidity and strength, offering protection against mechanical stress. The CW is an extracellular polymeric structure composed of polysaccharides, proteoglycans, peptides, proteins, and associated inorganic elements. The CW of marine algae is relatively rich in negatively charged non-crystalline polysaccharides, which are often sulphated (Tomaselli 2004). Electron-microscopy studies have shown that the CW is made up of two major components: (1) an organized fibrillar structure embedded in, (2) a continuous matrix. The microfibrills are irregularly interwoven in a continuous network over the wall and consist mainly of cellulose, forming the skeleton of the CW. This fraction contains polysaccharides composed of monosaccharides other than glucose, but in some cases this cellulose component is replaced by mannans or xylans (Lee 1989). The matrix, which chemically corresponds to substances soluble in dilute sodium hydroxide, is mainly made up of hemicellulose, protein, amino sugars, amorphous mucilaginous components and possible lipids. The amorphous mucilaginous components occur in the greatest amounts in Phaeophyta and Rodophyta. The most usual ones are: alginic acid; fucoidin and galatans (Lee 1989). The CW may be silicious (in the case of Bacillariophyceae) or calcified (as in the coccolithphoris algae), and may be strengthened with plates and scales. However, some species are naked, lacking of cell wall, for example: *Dunaliella salina*, *Isochrysis galbana* or *Ochromonas danica*, etc. The presence of insoluble non-hydrolizable biopolymers in the outer cells walls of *Nannochloropsis* are unusual resistant to drastic non-oxidative chemical treatment (Allard and Templier 2000). These biopolymers, termed algaenans (Gelin et al. 1997); have a highly aliphatic structure that serves as a protective coat surrounding the cells (Mendes Pinto et al. (2001). Algaenan-containing microalgae are known to be resistant and difficult to break. Additionally, the spherical shape and size (1–2 μm) of *Nannochloropsis* make them especially difficult to lyse. Similar results have been reported for *Haematococcus*, which is also especially resistant to cell wall disruption because of the thick sporopollenin wall (Mendes Pinto et al. 2001).

The plasma membrane (PM) marks the boundary between the CW on the outside and cytoplasm on the inside. PM is a thin unit membrane which surrounds the protoplasm directly beneath the CW and a layer of mucilage. It is in contact with the thylacoid membrane with forms an interconnecting network of concentric shells, merging only at the inner surface of the PM. GLs are the dominant lipid of the eukaryotic algal chloroplast membranes and PLs in the PM. Given the large variety of CW structures that can be found in microalgae, it is necessary to carry out a complete characterization of them before setting up an eventual pre-treatment unit for biomass.

5 Biomass Pretreatment

Lipids from microalgae may often be extracted in the wet state directly after harvesting. The cells do not need to be homogenized since they are readily broken on suspension in the extracting solvent (the organic solvent seems to create channels trough the cell wall). In some cases, breakage of the CW and PM may be necessary to:

1. reduce the extraction time,
2. avoid the use of high temperatures and pressures to push the solvent into contact with the lipids within the cell,
3. reduce solvent consumption,
4. allow the solvent to easily penetrate into the cell and release cell contents into the bulk medium for increasing the lipid yield.

This may be accomplished by using one of the well established cell-disruption techniques such as sonication, homogenization in a tissue grinder, blender or in high-pressure flow device, freezing and grinding with a pestle and mortar, autoclaving microwaves and osmotic shock.

One of the earliest major reviews of the bead mill technique was reported by Chisti and Moo-Young (1986) and a comprehensive study of disruption in bead mills was also reported by the same group (Garrido et al. 1994). For large-scale cell disruption for the disintegration of algae, the mechanical method of shearing the cells with a bead mill appears more suitable. Bead mills have been used to disintegrate the algae *Scenedesmus obliquus* and *Spirulina platensis* (Hedenskog and Ebbinghaus 1972). Among the various pretreatment procedures studied for lipid recovery in *Botryococcus braunii* UTEX 572, the most effective method was cell disruption with a bead mill (Lee et al. 1998). In microscopic observation, most of the cells were destroyed by bead mills but not by other methods such as sonication, homogenization and French press. Recently, Cerón-García et al. (2008) demonstrated that disruption was necessary for the algae *Scenedesmus almeriensis*. This work compares three different methods: mortar, bead mill, and ultrasound. The best option among, bearing in mind their potential industrial application, proved to be the bed mill with alumina in a 1:1 w/w proportion as disintegrating agent for 5 min (Cerón-García et al. 2008). In general bead mill may be the pretreatment choice for microalgae cells with strong CW.

Ultrasound is another liquid-shear method that has also been used at laboratory scale for microalgal disruption. At high acoustic power, ultrasound is known to cause disruption of microbial cells in suspension (Chisti and Moo-Young 1986). The cell disruption mechanism derives from the inten-

sive shear induced by sonicating the suspension at sound frequencies above 20 kHz. For the study of lipid classes and fatty acids of the green algal classes Chlorophyceae and Prasinophyceae, Dunstan et al. (1992) extracted the lipids with chloroform-methanol–water (1:2:0.8 v/v/v) and between each extraction the samples were exposed to ultrasound from a sonic probe in a water bath at 20 °C. In general sonication is a very high cost pre-treatment which should be disregarded at industrial level and, in any case, should only be used for microalgae with less resistant CW.

Within the mechanical disruption methods, homogenizers (pumping of microalgae slurry through a restricted orifice valve) such as French press, are less time consuming than other methods, easy to use and can ensure almost complete breakage of small cells. It is recommended for microalgae with the strongest cell wall which require up to 1,000 bar. Homogenization of freshly harvested paste biomass of *Nannochloropsis gaditana* (86% moisture) has been carried out in our laboratory, noticing a marked increase in the recovery of saponifiable lipids when using non-polar solvents such as hexane, because homogenization breaks the cell wall allowing access of hexane to intracellular lipids. Due to the polar nature of some of the structural components present in the cell walls of *N. gaditana*, the yield of lipid extraction (quantified as saponifiable lipids) with hexane at room temperature is very low (around 7%), while if the biomass is homogenised at 1,000 bar or 1,850 bar, the yield reached 53.5 and 70% respectively.

Freeze-drying breaks up the cells and turns the algal material into a loose, fine powder, making homogenization unnecessary (Ahlgren and Merino 1991). We have also compared the lipid recovery yield of freeze-drying *Phaeodactylum tricornutum* with that obtained using fresh paste biomass (after harvesting by centrifugation). The direct extraction of fatty acid from wet *P. tricornutum* biomass with ethanol (96%) produced only slightly lower yields (90.4%) than those obtained from lyophilized biomass (96.2%), see lines 7 and 9 of Table 11.5, therefore lyophilization may be omitted, saving the high energy consumption in that step and thereby diminishing the cost of extraction (Molina Grima et al. 1996).

Cooney et al. (2009) have explored many chemical (dissolution in 1M $NaOH_{(aq)}$, hydrogen peroxide treatment, or adding detergents) and physical techniques (sonication, glass bead beating, liquid nitrogen + griding the frozen mixture) applied to wet and dried *Nannochloropsis* and *Tetraselmis* biomass. Except for the grinding of cells in liquid nitrogen, none of the physical and chemical methods tested were effective in cell disruption. The same authors have tested the suitability of grinding cell after oven drying and compared it with wet cell that had been ground and not ground. The solvent system used extracting lipid was chlorofom:methanol:water (1:2:0.8 v/v/v). The best lipid recovery yield was achieved from solvent applied to oven dried and ground cells (21.5%), but the extraction of wet cells that had been ground was 20%, suggesting that the presence of water has the effect of blocking solvent access. As control, the extraction was also applied to wet cells that were not ground and the lipid recovery yield was 16.5% (Cooney et al. 2009).

In a very recent study, Lee et al. (2010) compare several methods for effective lipid extraction from three different microalgae: *Botryococcus* sp., *Chorella vulgaris*, and *Scenedesmus* sp. Among the five methods used (autoclaving, bead mills, microwave, sonication, and osmotic shock with a 10% NaCl solution), the microwave oven method (at about 100°C and 2,450 MHz for 5 min) appears to be the most simple, easiest and most efficient method for lipid extraction from microalgae.

6 Extraction of Lipid from Microalgae

For complete extraction, all the linkages between the lipids and other non-polar lipids cell components must be disrupted and, at the same time, the disruption agent used must not cause any degradation of the lipid extracted. There are three main types of associations in which lipids participate:

(i) hydrophobic or van der Waals interactions, in which neutral or non -polar lipids, such as triacylglycerols are bound by relatively weak forces through their hydrocarbon chains to other lipids and to hydrophobics sites of proteins,
(ii) hydrogen bonding and electrostatic association by which polar lipids are bound to proteins, and
(iii) covalent association, although this type of interaction is less frequent.

The energy of the weak hydrophobic interactions which link the stored lipids is never over of 2 kJ mol^{-1}, so they may be disrupted by non-polar organic solvents, such as chloroform, hexane or ether (Chuecas and Riley 1969). Hydrogen bonds of membrane-associated polar lipids have an energy of 0.84–50.16 kJ mol^{-1} and may be disrupted only by polar organic solvents, such as methanol, ethanol and other alcohols, and also by water (a solvent with a high dielectric constant). To extract lipids linked by stronger electrostatic forces such as ionic bonds, it is necessary to shift the pH value somewhat toward the acidic or alkaline region (Kates 1986; Zhukov and Vereshchagin 1981).

Lipids may also be retained in living matter by mechanical confinement. For example, in the case of cell with poor permeability of the CW to solvents, the lipid recovery yield may be increased by adding a small amount of water to the extraction phase. Water causes swelling of the cellular structures rich in polysaccharides, thereby increasing the extent of dispersion of the living systems and facilitating access of extraction

solvents to the lipids. Thus the presence of water in the extractant is absolutely necessary for the extraction of polar lipids to be quantitative (Zhukov and Vereshchagin 1981).

Some biological materials contain enzymes which cause degradation of the lipids during the extraction process. In general, the use of alcohol-containing solvent mixtures is sufficient to inactivate many of the phosphatidases and lipases (Zhukov and Vereshchagin 1981; Kates 1986). Therefore, alcohol is an essential component of the extracting solvent, being required for disruption of lipid-protein complexes, dissolution of the lipids and inactivation of degradative enzymes. However, alcoholic solvents also extract some cellular contaminants such as sugars, amino acids, salts, hydrophobic proteins and pigment, and therefore it is essential that the crude extract be treated to remove these water-soluble contaminants (Kates 1986). The method of Bligh and Dyer (1959), which uses a quantitative extraction technique that uses a monophasic ternary system: chloroform: methanol:water in a ratio 1:2:0.8 v/v/v, is the most commonly used methods for the extraction of lipids from microalgae at analytical level in the last 50 years (over 23,500 journal citation currently, Burja et al. 2007). Usually the solvent mixture is added to dry biomass, although Lewis et al. (2000) have demonstrated that the lipid extraction was significantly more efficient when solvent were added in the order of increasing polarity (Chloroform then methanol then water). In a recent study, Cooney et al. (2009) have extensively investigated the Bligh and Dyer system on the algae *Chlorella protothecoides*. The lipid recovery was 15% when using one extraction stage. When using a second extraction stage, by re-suspending the spent biomass in the additional solvent mixture, the recovery yield increased an additional 7%, up to a total of 22%, but far from the 51% reported by another external laboratory which used an acid hydrolysis method certified by the Association of Analytical Community (AOAC 922.06, http://www.aoac.org/). Cooney and co-workers suggest that when designing co-solvent systems to extract lipids, the use of more polar solvent to improve the range of lipids extracted may also decrease the carrying capacity of the solvent because, in general, solvent that extract polar lipids (GLs and PLs) are less miscible with relatively high ratios of non-polar lipids (as in the case of *C. protothecoides* biomass) and lower miscibility leads to lower carrying capacity, Cooney et al. 2009). This group has also extracted lipids from microalgae at lab scale level by using mixtures of inonic liquids (ILs) and methanol (Young et al. (2010). Mixtures of ILs and methanol successfully dissolved biomass leaving lipid insoluble. This process is the subject of a U.S patent application (Cooney and Young 2009). Interest in ILs is rapidly increasing due their potential application as "green solvents". The same IL-methanol mixture has also been used by Kim et al. (2011) to extract lipids from *Chlorella vulgaris* biomass. IL-methanol showed higher extraction efficiencies than the conventional Bligh and Dyer method: 12.5 and 11.2%, respectively (Kim et al. 2011).

To carry out the extraction in large quantities, an extraction tank with a reflux apparatus, equipped with cell homogenizing apparatus such as polytron homogenizer, may be employed. Usually, the non-polar and the polar solvents are mixed in a ratio of the former to the latter ranging from approximately 2:1 to 1:1 by volume. The extraction may be carried out at 60 °C by using from 5 to 20 parts by volume of the mixed solvent per part by volume of dry biomass. Although there is no limit, the extraction period usually ranges from 30 min to 3 h. After extraction, the solvent may be removed by distillation under reduced pressure at temperatures ranging from 40 to 60 °C to provide a lipid composition that contains TAGs, PLs, GLs, glycol-phospholipids, chlorophylls, beta-carotene, sterols, etc.

Once the lipids have been extracted, the crude extract obtained must be purified before commencing the conversion of lipids to methyl esters of fatty acids. The usual laboratory scale methods for purifying lipids are based on the difference in affinity of the polar lipids and their contaminants for a certain solvent. The crude extract is treated with non-polar solvents such as chloroform (Folch et al. 1957; Bligh and Dyer 1959; Kates 1986), hexane (Molina Grima et al. 1994) or diethyl ether, in which the non-lipid contaminants are less soluble. These procedures do not attain the complete extraction of most polar lipids (e.g., proteolipids) because of their low solubility in these solvents. An improved method for washing the crude lipid extract was proposed by Hara and Radin (1978). In searching for useful washing systems for removing the non-lipids from lipid extracts, they first tried water. This produced a considerable loss of lipid into the aqueous layer. The addition of KCl to water reduced lipid loss but an appreciable amount of KCl entered the hexane rich phase. However, when using a solution of sodium sulphate (1 g of anhydrous salt per 15 mL water) the lipids were purified in the upper of the hexane layer. No precipitate was visible at the interface layer when using this salt concentration, which otherwise was found to be critical (Hara and Radin 1978). Other less used purification methods are molecular adsorption on silicic acid, on alumina and the use of ion-exchange and gel filtration (Zhukov and Vereshchagin 1981). None of these methods guarantee the obtention of completely pure lipids without any degradation.

Processes for refining of crude algal lipid extracts (mainly the removal of the phospholipids fraction), similar to those existing for terrestrial plants (Nasirullah 2005; Rajam et al. 2005), have not been developed to date. Unlike terrestrial feedstocks, the unusual content of microalgal oils in fatty acids, non-saponifiable constituents, PLs, GLs and its dark colour, may all cause difficulties in the refining processes. An alternative to the refining may be the lipid fractionation and to properly process each lipid classes separately.

7 Lipid Fractionation

Lipid extraction depends largely on the particular classes of lipids present. Usually, most animal and microbial cellular lipids consist of about 60–85% of polar lipids, whereas terrestrial seed lipids have considerably higher proportions of neutral lipids (Kates 1986). Lipid extracts rich in polar lipids are commonly fractionated, at laboratory scale, by countercurrent distribution, ion exchange chromatography and adsorption chromatography on silicic acid and aluminium oxide. This technique ensures the separation of non-polar and polar lipids and separation of the latter into fractions. The crude extract is applied to a silica gel column (250–400 mesh) and solvent, with increasing polarity (e.g. chloroform, acetone and methanol) are used to elute increasing polar lipids: neutral lipids, low-polarity lipids (e.g. galactolipids) and polar lipids (such as phospholipids) (Kates 1986). For microalgae, the expected composition of each fraction is: (i) Chloroform: hydrocarbons, carotenoids, chlorophylls, sterols, TAGs, wax esters, long-chain alcohols, aldehydes and fatty acids; (ii) Acetone: mono- and digalactosyldiacylglycerols (MGDG and DGDG), cerebrosides and sulfolipids, and (iii) Methanol: PLs and traces of GLs.

For lipid class separation Sukenik and Carmeli (1989) eluted the algal oil from 6 g of *Nannochlorpsis* sp with 30 mL of chloroform for neutral lipids, 120 mL of chloroform:methanol (5:1 v/v) for glycolipids and 45 mL methanol for phospholipids. As an example, Table 11.2 details the fractionation of the lipid extracts from an indoor batch, in its late stationary-phase of growth, for *I. galbana, P. tricornutum* and *P. cruentum* into neutral lipids and polar lipids. Additionally, the lipid fractionation of the three biomasses from external cultures grown in tubular photobioreactor, running in continuous operational mode, is also presented in Table 11.2. TAGs often accumulate in times of nutritional excess or under stress. Biomass taken from batch cultures, in late stationary phase of growth, when some nutritional resource was depleted, accumulated storage lipids (mainly TAGs). A dramatic change was observed in lipid composition between indoor (batch cultures) and outdoor cultures (continuous cultures) of the three microalgae. The NLs fraction in *P. tricornutum* shifted from 51 to 23.2%, from 43 to 26.5% in *I. galbana,* and from 47 to 39.5% in the case of *P. cruentum* in the indoor and outdoor cultures, respectively. The polar lipids (Gls and PLs) fraction increased significantly in outdoor cultures (from 49 to 76.8% for *P. tricornutum*, from 57 to 73.5% for *I. galbana,* and from 53 to 60.5% for *P. cruentum,* in the indoor and outdoor cultures respectively. Such profound changes in type of lipids are to be expected as a consequence of specific growth conditions. The outdoor cultures were from continuous tubular photobioreactor cultures, under natural sunlight, with high irradiances in spring and summer in southern Spain. The outdoor cultures were therefore in nearly optimal growing conditions, so that there was probably a high rate of metabolism producing a large amount of the lipid classes characteristic of young cells (i.e., GLs which are the main chloroplast lipids). In these three microalgae, a greater proportion of PUFAs is found in the polar lipids. This result may be useful for PUFA purification strategies.

Although little has been published about PUFA purification from microalgae biomass, currently there are basically two strategies. First, direct saponification of microalgae biomass followed by PUFA purification, as will be detailed later; and, second, recovering of highly pure PUFAs from the PLs fraction of lipid, previously isolated (Cohen and Cohen 1991).

Seto and Yamashita patented a procedure for preparing high grade PUFAs, in which a relatively easy lipid fractionation (at pilot plant) is described (Seto and Yamashita 1986). In this patent, lipids may be fractionated by adequate contact of dry powder of marine *Chlorella* with a co-solvent mixture. The non-polar organic solvents were hexane, chloroform, ether or a mixture thereof, and the polar solvents may include a lower alcohol, such a methanol, ethanol or a mixture thereof. Neutral lipids, sterols and chlorophyll are transferred to the non-polar solvent. This extraction may generally be carried out with a solvent at a rate ranging from 5 to 20 parts by weight per part by weight of the crude lipid extract, at temperatures from approximately 20–40 °C, for approximately 1 h. After extraction the solvent fraction is removed by filtration and the residue is then brought into contact with acetone, usually at a temperature of approximately 4 °C, and is left overnight. The extraction with acetone prevents the entire PLs contained in the crude lipid extract from dissolving in the acetone. The acetone phase is then collected and the solvent (acetone) is then removed by distillation at low pressure and temperature, providing a fraction of polar lipids containing nearly no PLs and consisting mainly of GLs and glyco-phospholipids. Fractionation using acetone as solvent, combined with temperature control, has a long history of use to produce acetone-insoluble PLs that are precipitated and recovery essentially free of TAGs, fatty acids and other neutral lipids.

8 Lipid Extraction from *Dry* Microalgae Biomass

One of the first and most efficient extraction systems used for microorganisms was the mixture of chloroform:methanol 2:1 v/v (Folch et al. 1957; Kochert 1978). This procedure was simplified and improved by Bligh and Dyer (1959), the reference method used for extracting lipids over the last

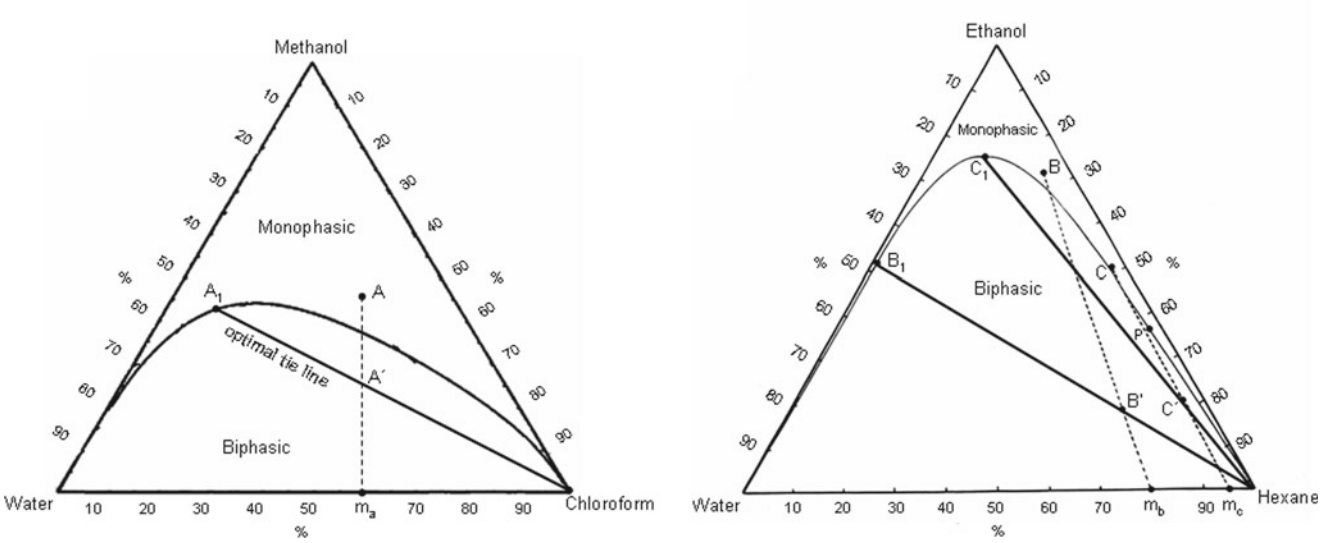

Fig. 11.1 Ternary diagrams for systems: (**a**) chloroform/methanol/water and (**b**) hexane/ethanol/water. Binodal curves at 25 °C and atmospheric pressure

50 years. This method uses a single-phase extraction, chloroform:methanol:water 1:2:0.8 v/v/v (point *A* of Fig. 11.1a), and then diluted it with a chloroform and water mixture (point *m*, Fig. 11.1a) to form a biphasic system in a proportion 1:1:0.9 v/v/v (point *A′*, Fig. 11.1a) chosen in such a manner that the lower layer is practically 100% chloroform and contains the purified lipids and the upper layer nearly all methanol–water and contains the non-lipids (Fig. 11.1a). It is a simple, fast and gentle method (the temperature is kept at around room temperature). For all practical purposes, total lipid extraction was complete and the separation of lipids and non-lipids was nearly quantitative. The method is applicable for many materials (of animal, vegetal or microorganic origin), but with microorganisms (including microalgae), homogenization is not necessary and the extraction takes only 1 or 2 h at room temperature (Kates 1986). This is the most used procedure for microalgae as a routine method of lipid extraction at analytical scale. The ternary mixture chloroform, methanol and water has been widely used and the optimal tie line, which provides the optimal partitioning of the lipids in the chloroformic phase, is the only one represented in Fig. 11.1a). It has the disadvantage that chloroform and methanol are toxic solvents (chloroform can produce tumours in animals and it is well known that methanol damages the eyesight (Hara and Radin 1978). Therefore, large-scale and workable lipid extraction using this method is prohibited due to environmental and health risks. In another recent study, Burja et al. (2007), tested various analytical lipid extraction methods for dry powder of the heterotrophic alga *Thraustochytrium* sp. The Bligh and Dyer method and three modifications of it (Bligh-Dyer ultrasonic bath, Bligh-Dyer ultrasonic probe, miniaturized Bligh-Dyer) were compared with direct saponification of biomass (this method of fatty acid extraction is detailed in the next section), by using KOH in ethanol or in hexane:ethanol (Burja et al. 2007). The highest recovery yield of fatty acids (71.4%) was achieved using the Bligh-Dyer ultrasonic probe technique, similar the yield obtained by direct saponification by using KOH (69.7%).

Guckert et al. (1988), using *Chlorella*, compare the lipid recovery yield of three lipid extraction procedures with the purpose of determining the most suitable procedure for biochemical analysis: (1) Soxhlet method (methylene chloride/methanol, 3 h reflux); (2) hexane:isopropanol 3:2 v/v, and (3) the Bligh and Dyer method. This study demonstrated that the Bligh and Dyer method provided the most quantitative and reproducible recovery yield of all *Chlorella* lipid classes. The hexane:isopropanol method was selective for algal neutral lipids with poor recovery of membrane lipids (mainly GLs and PLs). Finally the Soxhlet method had significantly lower recovery yield of many PUFAs. Hexane:isopropanol 3:2 v/v, butanol and ethanol are also low-toxicity solvents, easy and safe to handle, relatively cheap and sufficiently volatile that have also been used for the extraction of microalgal lipids. The lipid extract contains a significant amount of PLs that should be further purified, for example, by a chloroform:methanol:water phase separation.

All these solvent systems have been used, at gram scale, for lipid extraction from *I. galbana* lyophilized biomass (Table 11.3) in which the Bligh and Dyer (control method) method was compared to the biocompatible ternary system

Table 11.3 Yields (%) of extracts and raffinates (in parentheses) of lipids extraction from dry *Isochrysis galbana*, and polarity index for the solvent system used

Solvent system	Yields (%)[a]	Polarity index[b]
$Cl_3CH/MeOH/H_2O$ (1:2:0.8 v/v/v)	92.9 (0.9)	5.66
Hexane/EtOH (96%) (1:2.5 v/v)	52.2 (27.4)	3.81
Hexane/EtOH (96%) (1:0.9 v/v)	49.5 (8.3)	2.52
Butanol	70.4	3.90
EtOH (96%)	84.4	5.35
EtOH (96%)/H_2O (1:1 v/v)	63.3	4.93
Hexane/Isopropanol (1:1.5 v/v)	66.0	2.58

[a]Calculated as in Table 11.2

[b]The Polarity Index was calculated from the pure components (Hexane, 0; Chloroform, 4,1; Methanol, 5,1; Ethanol, 5,2; Isopropanol, 4,3; Butanol, 3,9; Water, 9 (Snyder 1974). For solvent mixtures the Polarity Index was calculated as: $PI_{mix} = \sum_{i=1...n} X_i \cdot PI_i$, where, PI_{mix} and PI_i are Polarity Index of the mixture and the component i, respectively, and X_i is the volumetric fraction of component i in the solvent mixture

Table 11.4 Yields (%)[a] of fatty acid extracts by direct saponification from dry *Isochrysis galbana*

Solvent mixture + KOH (47.5 mL solvent/g KOH (85%))	60 °C-1 h	Room-8 h
$Cl_3CH/MeOH/H_2O$ (1:2:0.8 v/v/v)	–	–
Hexane/EtOH (96%) (1:2.5 v/v)	81.0	75.0
Hexane/EtOH (96%) (1:0.9 v/v)	48.0	47.1
Butanol	9.0	–
EtOH (96%)	79.8	79.2
EtOH (96%)/H_2O (1:1 v/v)	46.5	45.5
Hexane/Isopropanol (1:1.5 v/v)	62.0	61.1

[a]Calculated as in Table 11.2

hexane/ethanol (96%) 1:2.5 v/v, and hexane-ethanol (96%) 1:0.9 v/v, Molina-Grima et al. (1994). The phase equilibrium of the hexane-ethanol water system is represented in Fig. 11.1b. The major difference with respect to the Bligh and Dyer diagram (Fig. 11.1a) is that the extraction area (monophasic area) is much lower with this system. The monophasic mixtures (point B or C, Fig. 11.1b) were used, first, to extract lipids from *I. galbana* lyophilized biomass and then to purify them by adding hexane and water (point *mb* or *mc*, Fig. 11.1b) to form a biphasic system (point *B′* or *C′*, Fig. 11.1b) in a proportion chosen in such a manner that the lower layer is practically 100% hexane and contains the purified lipids and the upper layer, nearly all ethanol-water, contains the non-lipids (point *B1*, or *C1* Fig. 11.1b). Although a high lipid yield was obtained with system B (79.6%), high proportion of them (27.4%) remained in the ethanol-water phase together with the non-lipid contaminants (Table 11.3), implying that a greater consumption of hexane is required to extract them. On the other hand, the yield of lipid extract with system C is low (57.8%), although partitioning (point *C′*) is more favourable (49.5:8.3; Table 11.3). This would seem to point out that lipid extraction may be carried out with a system such as B, or with an even greater ethanol content (bearing in mind the yield of 84.4% reached with ethanol (96%)) followed by formation of a biphasic system similar to C′, i.e. with a high proportion of hexane to enhance lipid partitioning in the extract (note that the optimal tie line with the hexane:ethanol:water system has not been determined yet, although the possibilities for optimizing it are more reduced with this ternary system than that of the Bligh Dyer, due the much lower extraction area). The order of efficiency of the yields obtained (Table 11.3) shows that lipid yield increases when the alcohol content and the polarity index of the solvent mixture also do so, Table 11.3. This may account for the high yield of the Bligh and Dyer method (system A in Fig. 11.1a), as methanol has a slightly higher polarity than ethanol, as does chloroform over hexane. These results are in agreement with the fractionation of the lipid extract from *I. galbana* into neutral lipids (26.5%) and polar lipids (73.5%) (see Table 11.2). This argument is in accordance with the low yield reported by Ahlgren and Merino (1991) in the extraction of lipids from *Spirulina* with hexane:isopropanol 3:2 (v/v), due to the low polarity of the solvent mixture that was therefore not recommended for lipid extraction. The isotherm saturation curve (binodal curve) is of paramount importance in all cases and must be determined experimentally. Alternatively, information of equilibrium data for ternary systems for more than 50 solvent extraction systems can be found in Bonner (1910).

9 Fatty Acid Extraction by Direct Saponification of *Dry* Microalgae Biomass

Direct saponification of microalgae biomass enables fatty acids to be obtained as potassium or sodium salts instead of as crude lipids in a first step. The fatty acid profiles and overall yields obtained with each of the solvent systems of Table 11.3 are presented in Table 11.4. Direct saponification was performed by adding 1 g of KOH (85%) per 47.5 mL of solvent mixture used for lipid extraction. The extraction/saponification was performed a room temperature for 8 h or at 60 °C for 1 h. After saponification, the unsaponifiable fraction was extracted with hexane and then, the hydroalcoholic phase, containing the fatty acid salts, was acidified by HCl addition to pH 1, and the fatty acids obtained were recovered with hexane. Such direct saponification during extraction from the biomass is faster, and reduces cost and operating time compared to lipid extraction followed by saponification, although more intensive operating conditions are necessary (1 h at 60 °C or 8 h at room temperature). The resulting fatty acid extraction yields (Table 11.4) are somewhat lower than for lipid extraction with the same solvent

system (Table 11.3) and the order of efficiency is similar to that obtained for lipid extraction. It may therefore be assumed that direct saponification of biomass could ideally be induced in two stages, first lipid extraction with a solvent, and second alkaline hydrolysis of the extracted lipids to render fatty acid salts. The yields obtained from fatty acid extraction carried out in this way would depend mainly on the suitability of the extraction solvent.

Biocompatible systems such as ethanol (96%) and hexane:ethanol (96%), 1:2.5 v/v, have also been used to extract fatty acids by direct saponification from *Phaeodactylum tricornutum* lyophilized biomass (Cartens et al. 1996). Ethanol (96%) gave a fatty acid recovery yield of 96.2% and was more efficient than hexane:ethanol (96%) 1:2.5 v/v (fatty acid recovery yield of 86%) due to the higher polarity of the former, as shown by the fractionation of the lipid extract from *P. tricornutum* into neutral lipids (23.2%) and polar lipids (76.8%) (see Table 11.2).

Three major conclusions arise for either lipid or fatty acid extraction when using dry microalgae biomass, Tables 11.3 and 11.4: (1) it seems that alcohol content is the main factor which determines the lipid extraction yield. The higher the polarity of the solvent mixture, the higher the extraction efficiency (Table 11.3); (2) for each solvent system the yield of lipid extraction (Table 11.3) is slightly higher than the yield of fatty acid extraction (Table 11.4), and (3) it may thus be assumed that the direct saponification of biomass could ideally be induced in two stages the lipid extraction being the bottleneck. In other words: it seems that attention should be centred on selecting the optimal solvent mixture because the saponification is an instantaneous reaction. Another important problem observed when using dry biomass is the large amount of solvent used: 144 mL g^{-1} of dry biomass. Research aimed at lowering solvent volumes is summarized in the next section.

10 Optimization of Lipid and Fatty Acid Extraction from *Paste* Microalgae Biomass

One of the pioneering studies which use freshly harvested paste biomass (15–20% of dry biomass) for extracting lipids was performed by Nagle and Lemke (1990). The lipid extraction was carried out by using 1-butanol, ethanol and a mixture of hexane and isopropanol. They used two microalgae species: *Monoraphidium minutum* and the diatom *Chaetoceros muelleri* with no previous biomass pre-treatment. Paste biomass was mixed with the different solvents, or solvent systems, in the proportion 1:3 w/w and heated to near boiling and maintained at that temperature for 90 min. The solvent was then removed by distillation. The lipid fraction was further polished by adding the ternary mixture chloroform:methanol:water phase separation 1:1:0.9 v/v/v and then a second distillation was performed to remove the solvent from the purified lipids. The yields for the extraction of crude lipids were 94, 82 and 87% (compared to the Bligh-Dyer method that was used as control) for 1-butanol, ethanol and hexane:isopropanol 2:3 v/v, respectively. On the other hand, the extraction of lipids from *M. minutum* was carried out with 1-butanol and the extraction recovery yield was 81%. The subsequent transesterification of these crude lipids to produce fatty acids methyl esters (FAMEs), was carried out by using 0.6 N hydrochloric acid-methanol catalyst using the above extracted lipids from *C. muelleri*. The transesterification yield reaction was 68% (170 mg of FAMEs from 250 mg of lipids). The total amount of solvent used in the crude lipid extraction and its purification was about 50 mL g^{-1} of dry biomass (Nagle and Lemke 1990).

We have carried out a comprehensive study for optimizing the fatty acid extraction from wet biomass and its purification, Ibáñez-González et al. (1998). Fatty acid extraction was performed by a three-step method shown in Fig. 11.2: (1) direct saponification of wet biomass, followed by (2) extraction of unsaponifiable constituents and, finally, (3) extraction of purified fatty acids. It should be noted that this study was conducted using ethanol (96%) as the extraction system as it gave rise to fewer problems than hexane:ethanol (96%) 1:2.5 v/v, with respect to the formation of emulsions, which is the major bottleneck of lipid extraction. In a typical experiment 500 g of wet biomass (100 g of dry biomass) were treated with 1,000 mL (790 g) of ethanol (96%), containing 40 g of KOH (85%), in a 2.5 L reactor that was jacketed for temperature control. Saponification was carried out at 60 °C for 1 h, with constant agitation in an N_2 atmosphere. The mixture obtained was then filtered through a 100–160 μm microporous glass filter and the biomass residue was washed with 500 mL (345 g) of ethanol (96%). The unsaponifiable extraction was performed by adding water to the soap solution, which contained 11% w/w of water and a concentration of fatty acid salts of 47 ± 2 mg L^{-1}, to obtain a solution with 40% w/w water and unsaponifiables were then extracted in absence of direct light at 20 °C by adding hexane and shaking (Ibañez-González et al. 1998). Unsaponifiables were separated from the soap solution in several extraction steps with different volumes of hexane. Finally, the hydroalcoholic phase (40% w/w water) containing fatty acids salts was made acidic by adjusting pH to 6 by adding HCl. Extraction of fatty acid was quantitative (roughly 100% of fatty acids were extracted) using a relatively low hexane/alcoholic phase ratio of 0.2 v/v in four cocurrent contact L-L steps (Ibañez-González et al. 1998). The fatty acid concentration of the overflow solution leaving the L-L extraction stage was 903 mg L^{-1}. This solution was transparent with a weak yellow colour almost free of pigments. This water concentration in the hydroalcoholic phase

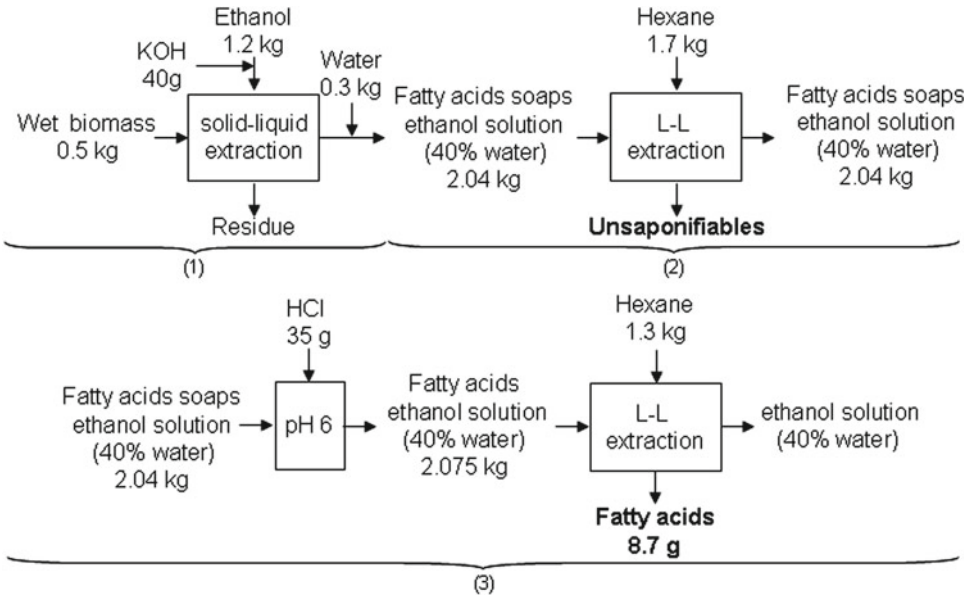

Fig. 11.2 Block diagram for the fatty acid extraction by direct saponification from wet biomass: (*1*) saponification + solid–liquid extraction of fatty acids soaps followed by extraction of (*2*) unsaponifiables and (*3*) fatty acids

Table 11.5 Influence of ethanol (96%) to wet biomass ratio on the fatty acid yield obtained by direct saponification of wet *Phaeodactylum tricornutum* (Ibáñez-González et al. 1998)

Ethanol (96%)/ wet biomass (mL mg^{-1})	Ethanol (96%)/ dry biomass (mL mg^{-1})	% Ethanol (v/v)	Yield[a] (%)
1.05	5	55	86.0
2.09	10	70	87.0
2.93	14	76	86.3
3.97	19	80	86.9
7.94	38	87	88.5
11.91	57	90	90.6
18.88	76	92	90.4
19.86	95	92	90.0
Lyophilized	76	96	96.2

[a]These fatty acid yield corresponds to the saponification step, which coincides with the fatty acid yield of the entire process (saponification, extraction of unsaponifiable and fatty acid extraction) since the yield of the L-L fatty acid extraction was quantitative (100%)

(40%) was also later found to produce the optimal extraction recovery yields for lipids (Ramirez-Fajardo et al. 2007).

Table 11.5 shows that the fatty acid yield decreased by about 4% when the ethanol (96%) to wet biomass ratio decreases from 19.86 to 1.05 mL g^{-1}, and subsequently, the ethanol concentration decreased from 92 to 55% v/v as a consequence of the water present in the paste biomass. Therefore an ethanol to wet biomass ratio of 1.05 mL g^{-1}, which is much lower than those used previously reported (76 mL g^{-1} of lyophilized biomass, Cartens et al. 1996), seems to be enough. However 2.09 mL of ethanol (96%) per gram of wet biomass (equivalent to 10 mL g^{-1} of dry biomass) was finally used because the subsequent filtration, after the saponification reaction stage, took a long time when using an ethanol (96%) to paste biomass ratio of 1.05 mL g^{-1}. When the extraction was made from lyophilized *P. tricornutum* biomass, a fatty acid yield of 96.2% was obtained (Table 11.5). The decrease in the fatty acid extraction yield obtained with wet biomass, 87% when using 2.09 mL of ethanol per gram of wet biomass, can be compensated by the decrease in cost of the drying/lyophilization process.

Figure 11.2 also shows the amount of paste biomass used, solvent and fatty acid amount of the overflow leaving the liquid-liquid (L-L) fatty acid extraction stage. These amounts of solvents correspond to those employed in a cocurrent contact. If the L-L extraction stage were carried out in a counter current contact, as is usual at industrial level, the amount of solvent used would have been substantially reduced (by roughly 40% compared to those shown in Fig. 11.2) to achieve the same fatty acid extraction yield (i.e. 87%). Alternatively, if the same amount of solvent were used in a countercurrent L-L extraction mode, the fatty acid extraction yield would significantly increase up to about 100%.

For the unavoidable calculations to be made in the scale-up of a process for direct saponification of wet biomass, the subsequent extraction of unsaponifiable material and fatty acids (Fig. 11.2), it is recommended to determine first the quantity of fatty acid soap solution in the underflow (solid slurry) leaving the saponification reactor which is a function of the concentration of the fatty acid soap solution in the overflow leaving the reactor. For the unsaponifiable and the fatty acid L-L extraction stages, we also need the equilibrium

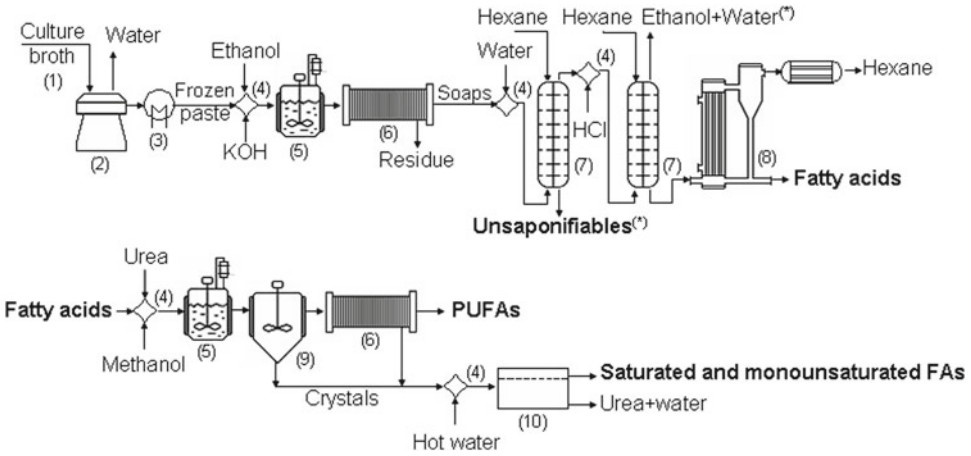

Fig. 11.3 Indicative flowchart for direct saponification of wet biomass and fatty acid fractionation.(*1*) Culture from photobioreactor; (*2*) centrifugation; (*3*) freezer (this step can be omitted if the paste biomass is used immediately after centrifugation); (*4*) mixer; (*5*) stirred tank reactor under nitrogen; (*6*) cake filtration; (*7*) solvent extraction; (*8*) evaporator; (*9*) crystallizer; (*10*) decanter. (*) The hexane from the unsaponifiable solution is recovered by evaporation and the ethanol is recovered by vacuum distillation

distribution of both unsaponifiables and fatty acids between the alcoholic and the hexanic phase (for *P. tricornutum* biomass, and the example detailed in Fig. 11.2, these equilibrium data can be seen in Ibañez-González et al. 1998). Calculations for scaling up this process and estimating, step by step, all the unknown flows and concentrations can be carried out using basic chemical engineering principles of S-L and L-L extraction (Coulson and Richardson 1968). Calculations may be performed either analytically or graphically. However we would recommend the latter, which are easier, more practical and more intuitive and allow us to visualize problems of the type "what will happen" if we change a solvent extraction flow rate, the fatty acid concentration of the feed, the flow rate of paste biomass to be processed, etc.

With respect to the previous sections, in which we used *dry* lyophilized biomass, the use of *wet* biomass reduces the fatty acids recovery to 87%, as compared to 96.2% for dry biomass (last line in Table 11.5); but we have reduced the amount of solvent employed by 90% for hexane, and 84% for the alcohol. The solvent to dry biomass ratio was 24 as opposed to 144 of the previous work. Although this method has been optimized for the diatom *P. tricornutum* it could be extended to other microalgae with hardly any modification

11 Fractionation of Fatty Acids

In the case of an unacceptable amount of polyunsaturated fatty acids (PUFAs) for biofuels, as is usual for marine microalgae, Fig. 11.3 shows a flowchart of a relatively cheap process for isolation of PUFAs from monounsaturated and saturated fatty acids from various algae biomasses (Molina-Grima et al. 1996). The biomass produced in outdoor photobioreactors (1) is concentrated by centrifugation (2) and the wet paste (with about 20% (w/w) of biomass) is conserved until needed at low temperature (3). Fatty acids are extracted by direct saponification of paste biomass with KOH (85%)-ethanol (96%) (in the proportion of 5 kg of paste—or 1 kg of dry biomass—1.6 kg of KOH (85%) and 76 L of ethanol (96%)) (4), the mixture is filtered (5) to remove the biomass residue to prevent the formation of emulsions (Cartens et al. 1996). Before extracting the unsaponifiable lipids with hexane (6), water is added to shift the equilibrium distribution of unsaponifiables to the hexane phase (Ibañez-González et al. 1998). Hexane is recovered by evaporation (7) and reused. The ethanolic solution of soaps is acidified with HCl, decreasing the pH down to pH 6, and the free fatty acids are extracted with hexane (6). The ethanolic solution of soaps is acidified with HCl and fatty acids are recovered with hexane. Ethanol is also recovered by distillation and the solution of fatty acid in hexane is concentrated by vaporization of hexane under vacuum (8). Fatty acids are added to a hot urea-methanol saturated solution (4) and the urea-fatty acids adducts are crystallized (9) (Robles-Medina et al. 1995a, b). The crystals are separated by filtration (5) and the filtrate, which is a high grade PUFA solution, is concentrated by vaporization of methanol under vacuum (7). Methanol is then recycled and PUFAs are extracted from the hydromethanolic phase with hexane (7). On the other hand, the crystals, mainly containing mono- and saturated fatty acids, are re-dissolved with an aqueous hydrochloric acid solution to decompose urea, washed with warm water, monounsaturated and saturated fatty acids remaining in the upper layer of the decanter (10). The PUFAs mixture may be later upgraded by using proper chromatographic procedures, according to the degree of purification needed (Robles-Medina et al. 1998). The urea complexation conditions, concentration factors (i.e. PUFA

Table 11.6 Fatty acid composition of extract, urea concentrate and urea crystals from *Phaeodactylum tricornutum* (Molina-Grima et al. 1996)

Fatty acid	Extract[a]	Urea concentrate[b]	Urea crystals[b]
14:0	4.9	0.1	13.8
16:0	13.4	0.1	37.8
16:1n7	18.2	0.7	25.9
16:2n4	7.1	6.9	3.7
16:3n4	7.1	20.7	0.0
16:4n1	2.2	6.0	0.0
18:2n6	2.6	0.5	3.2
18:3n3	0.4	0.0	0.5
20:4n6	1.3	3.0	0.0
20:5n3	36.3	50.5	10.0
24:0	1.9	0.0	5.3
22:6n3	2.3	4.7	0.0
Others	0.3	0.5	0.0

[a]Obtained by direct saponification of wet biomass with KOH (85%)-ethanol (96%) (1 h, 60 °C)
[b]Urea/fatty acid ratio 4:1, crystallization temperature 28 °C, solvent methanol

Table 11.7 Fatty acid composition of extract, urea concentrate and urea crystals from *Isochrysis galbana* (Robles-Medina et al. 1995a)

Fatty acid	Extract[a]	Urea concentrate[b]	Urea crystals[b]
14:0	10.7	0.3	15.7
16:0	18.9	0.2	27.9
16:1n-7	23.3	4.3	32.2
18:1n-9	1.7	0.2	2.4
18:1n-7	3.2	0.8	4.3
18:2n-6	0.9	0.2	1.3
18:3n-3	1.3	0.8	1.5
18:4n-3	7.3	22.6	0.0
20:4n-6	0.7	1.1	0.5
20:5n-3	22.4	39.4	13.0
22:4n6	1.3	3.6	0.0
22:6n-3	6.8	23.4	0.0
Others	1.5	2.8	0.8

[a]Obtained by direct saponification of biomass (hexane/ethanol (96%), 1/2.5 v/v, 8 h, room temperature
[b]Urea/fatty acid ratio 4:1, crystallization temperature 4 °C, solvent methanol

concentration in the urea concentrate to PUFA concentration in the free fatty acids extract ratio) and recovery yields obtained are collected in Molina Grima et al. (1999). Tables 11.6 and 11.7 show the fatty acid profile of the fatty acid extract as well as the monounsaturated and saturated fatty acid and the PUFAs fractions for two marine microalgae whose fatty acid profiles are unacceptable for direct conversion into biodiesel. It can be seen that the fatty acid profiles of the mono- and saturated fraction (urea crystals fraction) are closer to the requirements for biodiesel than the crude extract of fatty acid before the urea fractionation. Note that for biodiesel purposes, shorter chain, saturated and monounsaturated fatty acids are preferred, as they have lower melting points and higher cetane number and are less prone to oxidation.

12 Direct Transesterification of Wet Biomass for Producing Fatty Acid Methyl Esters (FAMEs)

Figure 11.4 shows a relatively low expense process for recovering and fractionation of fatty acids methyl esters from microalgae. The process was developed at the University of Almería, Spain, by Belarbi et al. (2000). All aspects of the process have been demonstrated at lab scale. The process was initially developed for the production of high grade polyunsaturated fatty acid methyl esters and Fig. 11.4 is a simplification of that process, which operates in batches. The direct production of FAMEs uses either wet or dry biomass. The flowsheet also shows a cell disruption step that is optional for *Phaeodactylum tricornutum*, but necessary for processing *Monodus subterraneus* or *Scenedesmus almeriensis* with stronger cell walls.

In a typical run, biomass paste (500 g, about 80% moisture by wt) was added to a mixture of methanol (1 L) and acetyl chloride (50 mL). In the case of *S. almeriensis* we have also tested as acid catalysts H_2SO_4 10% w/w, and no difference was observed in FAMEs recovery yield (E Molina

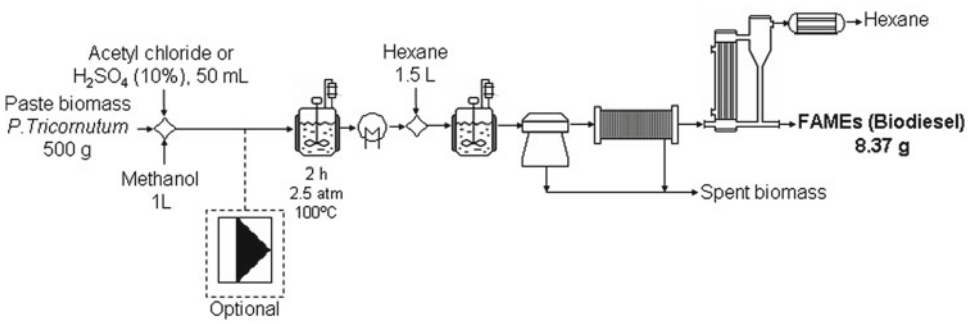

Fig. 11.4 Flowchart for FAMEs transesterification and extraction from wet biomass

Table 11.8 Crude FAMEs produced by direct transesterification of wet biomass of *P. tricornutum*, and fractions obtained by chromatography in an argentated silica gel column (Belarbi et al. 2000)

Fatty acid	Crude FAMEs	FAMEs with low content PUFA	FAMES with a high content PUFA
14:00	6.3	7.4	0.0
16:00	1.3	16.7	0.0
16:1n7	20.5	26.8	0.0
16:2n4	9.1	11.1	0.0
16:3n4	10.3	12.3	0.0
16:4n1	3.8	2.6	0.0
18:2n6	1.0	1.1	0.0
20:5n3	27.2	8.2	96.4
22:5n3	2.0	2.9	0.0
22:6n-3	2.4	0.0	0.6
Others	3.9	10.8	2.4
Amount (g)	8.37	6.11	1.66
% Fatty acids	100	73.0	19.8

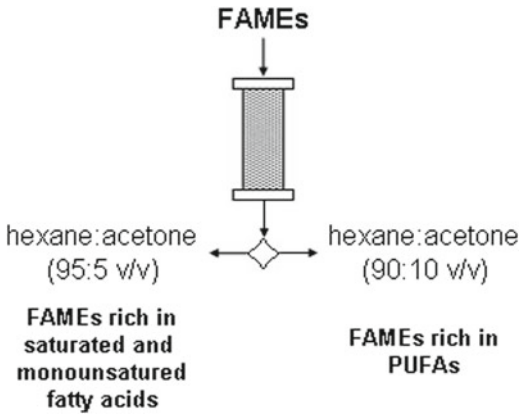

Fig. 11.5 Fractionation of FAMEs by argentated silica gel chromatography

Grima, personal communication). The resulting slurry was placed in a stainless steel pressure vessel and kept in an ultrasonic bath for 10 min. The pressure vessel was then transferred to a boiling water bath and kept there for 120 min from the time the pressure reached its maximum value of 2.5 bar. After cooling to ambient temperature, hexane (1 L) was added to the slurry reaction mixture and the slurry was allowed to settle by leaving overnight at 4 °C. The next day, the hexane layer was removed using a peristaltic pump and without disturbing the settled solids. Alternatively, the extraction time may be reduced by using a gentle level of agitation in the reactor vessel and forcing the separation of biomass slurry from the hexanic FAMEs solution by centrifugation. The crude FAMEs extract is further polished by filtration and the hexane is finally recovered by evaporation. However, we have not yet been able to remove all the pigment in the crude extract, and a final polishing distillation of FAMEs is still needed to improve the colour of the final product (E Molina Grima, personal communication). The FMAEs recovery yield ranged from 90 to 92% for *S. almeriensis* and *P. tricornutum*, respectively. Table 11.8 shows the fatty acid profile of the crude FAMEs extract produced from the diatom *P. tricornutum*. Note that the relatively high percentage of PUFAs in this extract is unacceptable for biodiesel purposes. This need not be a significant limitation. The unsaturation of FAMEs can be reduced by catalytic hydrogenation by using the same technology that is commonly used in the food industry (Shahidi 2005). Alternatively, for oils with an unacceptable content of PUFAs, such as those produced by the diatom *P. tricornutum*, and in general for almost all marine algae, an option that could also be taken into account could be the one represented in Fig. 11.5.

In this figure the crude FAMEs solution could be fractionated by using an argentated silica gel column, which in the case of *P. tricornutum*, would yield 73% of low grade PUFA methyl esters and 19.8% of high grade PUFA methyl esters, Table 11.8. These results that can also be explained on the basic of the polarity index of the two solvent systems used for eluting low grade PUFA methyl esters and high grade PUFA methyl esters. The polarity index of the hexane:acetone 95:5 v/v and 90:10 v/v are 0.27 and 0.54, respectively (the PI has been calculated as shown in Table 11.3, and considering that the PI for hexane is 0 and for acetone is 5.3; Synder 1974). The lower the polarity index of the solvent system the higher is the suitability for extracting the less polar fatty acid methyl ester mixture (saturated and monounsaturated fatty acid methyl esters are less polar than PUFAs methyl esters).

Lewis et al. (2000) have studied the extraction of lipids from freeze-dried biomass of two lipid-producing microheterotrophs (tentatively identified as thraustochystrids) by two procedures: (i) the extraction of lipids from biomass by the Bligh and Dyer method followed by the transesterification of fatty acids (extraction-transesterification); and (ii) the direct transesterification of biomass to produce fatty acid methyl ester (i.e. without the initial extraction step). They demonstrated that direct transesterification of biomass was most efficient than that of the most efficient method for extraction of fatty acids prior transesterification. Recently this direct acid-catalyzed biomass transesterification has also been proven to be a promising technology for the production of biodiesel from feedstock that contains high amounts of free fatty acids such as *Jatropha curcas* seed oil, Shuit et al. (2010). The yield of FAME extraction obtained with this seed oil was even greater (99.8%) than the one obtained for wet microalgae biomass (92%) and even greater that the traditional two step process followed for oil from oleaginous plants: extraction of the oils followed by transesterification.

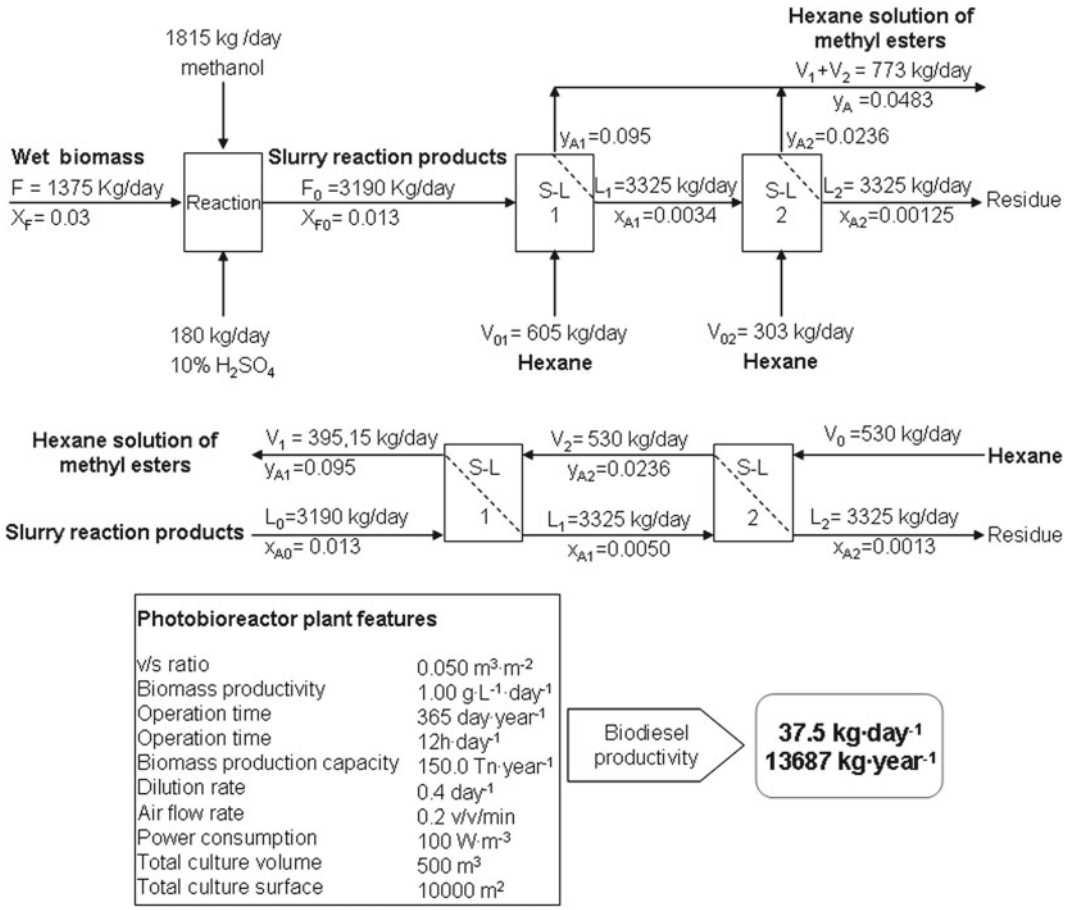

Fig. 11.6 Case study: block diagram for processing by direct transesterification of the wet biomass produced in 1 ha plant tubular photobioreactors whose characteristics are detailed in the box above. *X* refers to mass fraction of FAMEs in the underflow (raffinates) and *Y* refers to mass fraction of FAMEs in the overflow (extracts), except for X_F, which refers to mass fraction of fatty acids in the wet biomass entering to the process. *V* refers to overflow (FAMEs extract) and *L* refers to underflow (raffinates) flow rates. V_0 refers to pure hexane flow rate. Subscripts *1* and *2* refer to first and second extraction step respectively

Therefore, acid-catalyzed direct transesterification of biomass can be an important technology for biodiesel production not only for microalgal oils, as reported in this work, but also with other feedstocks.

13 Case Study

Figure 11.6 shows a block diagram and calculations made for processing the wet biomass produced in a 1 ha plant of tubular photobioreactors, with a volume of photobioreactor to surface ratio of 50 L m^{-2}, major operational conditions are shown in the box of this figure. If we were able to keep the same fluid dynamic conditions in the extractor vessel, and the same contact time between the co-solvent mixture and the biomass, a cocurrent contact extraction strategy, similar to the one developed in Fig. 11.4, would produce roughly 13,700 kg of biodiesel per ha and year. The amount of hexane required per day being over 900 kg. If the S-L extraction were carried out in a countercurrent system, as is usual at industrial level, the annual biodiesel produced would be the same but the amount of hexane needed would be 41% less. Alternatively, if we used the same amount of hexane the recovery yield would be higher and as result so would the annual productivity of FAMEs.

14 Concluding Remarks

While for many oleaginous terrestrial crops there are defined procedures for extraction and recovery of oils, there is no well-defined demonstration or industrial plants for processing algal oils. Solvent extraction uses relatively inexpensive solvents and is a workable process as a unit operation that exits in the food engineering and can be used for an industrial scale microalgal cultivation process covering many of the

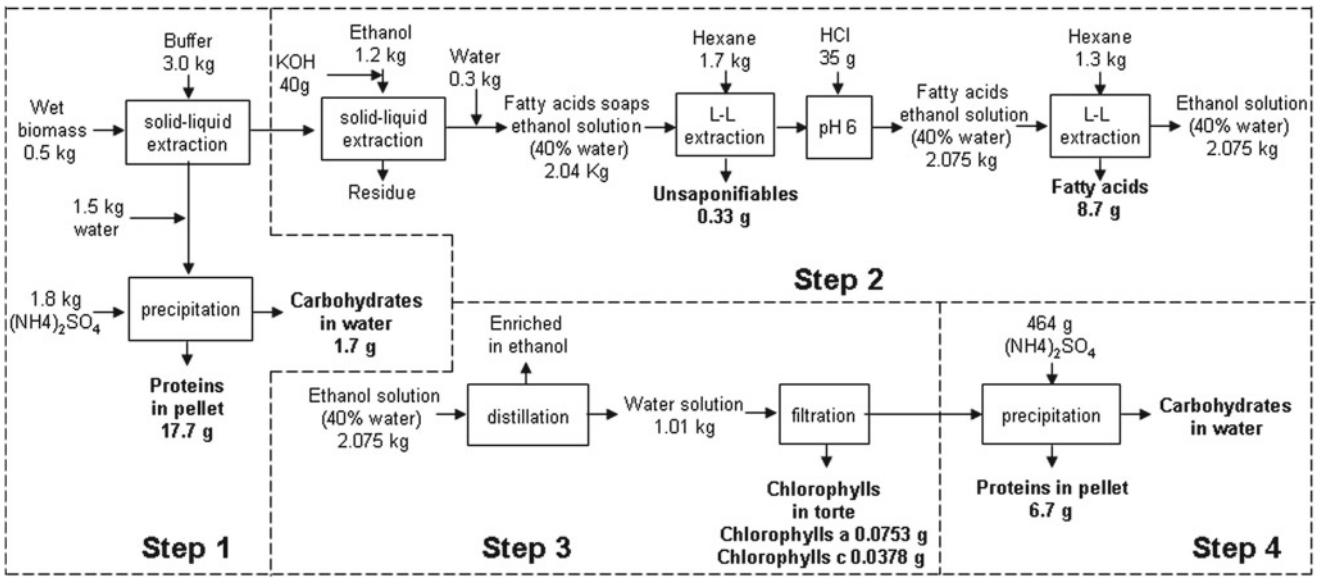

Fig. 11.7 Block diagram for a proposed multi-step process for an integral use of biomass. After initial S-L extraction of the soluble proteins and carbohydrates in a buffer solution, the proteins are later precipitated from the water solution, recovering the carbohydrates in the liquid phase. The biomass slurry is then subjected to a direct saponification of the algal oil in another S-L extraction unit. Once water is added, the carotenoids are recovered in a co-current contact extraction process. The hydroalcoholic phase is subjected to another L-L extraction of fatty acids. The underflow phase leaving the L-L extraction unit is then distilled to produce a high grade alcohol solution and a bottom residue that after filtration, allows the separation of the chlorophylls from the rest of the proteins and carbohydrates

multitasks needed for an integral exploitation of biomass. However, most organic solvents are highly flammable and/or toxic, and solvent recovery is expensive and energy intensive. We have tried to extract lipid from microalgae by other more environment friendly methods. However, in spite of the efforts made to extract lipids by mechanical methods from microalgae that do not involve any solvents (oil press), or with supercritical CO_2, which are workable processes for oil seed extraction, their use for microalgae has proved to be unworkable. This work highlights the technical feasibility of solvents for recovering lipids, fatty acids, fatty acid methyl esters and their fractionation using either dry or wet microalgae biomasses. The processes are more or less developed at lab scale, but now is time to pay attention to setting up a pilot, or demonstration plant. It is unlikely that any pilot plant will be efficient or cost effective, but it will surely evolve to greater efficiency and lower operating costs. The direct transesterification of wet microalgae (Fig. 11.4) seems to be the most suitable method for processing lipids from biomass for biofuels purposes. In fact the most efficient method for extraction lipid (Table 11.3) or fatty acid (Table 11.4) prior to transesterification yielded significantly less fatty acids than the direct transesterification of either dry or paste biomass (90–92%). Nonetheless, direct transesterification of biomass still has several constraints that need additional research, principally, the formation of emulsions and the difficulty of removing pigments from the crude FAMEs extract, which at present requires use an additional distillation step. Fatty acid extraction and their subsequent fractionation using the urea complexation method (Fig. 11.3) is apparently a longer method which uses more stages and solvent, but it yields a final fatty acid solution that is very clean and easy to convert into FAMEs. In addition, this approach provides the unsaponifiable fraction (carotenoids, pigments, sterols) as a by-product. Finally, lipid extraction from biomass and its subsequent conversion into FAMEs by transesterification would be a very attractive method if there is no need for previous lipid refining, which is unlike, due to the unusual high content of fatty acids in the microalgal oil, unsaponifiable constituents, phospholipids, glycolipids and its dark colour. But lipid extraction followed by conversion into FAMEs may be very useful when considering a prior fractionation of lipid classes in such a way that the enriched saturated and monounsaturated fatty acids fractions would be exploited for biofuels and the high grade PUFA fractions, which can be sold at much higher prices than biofuel (depending of the PUFA grade), would be used in dairy or nutraceutical industries. The three methods have their pros and cons and all usually require biomass pre-treatment.

Finally other components such as carbohydrates and proteins may need multi-step processes. It is obvious that the concurrent production of co-products with biofuel production has significant potential. The large scale production of microalgae for biofuel will increase the availability of these

by-products. One of the many possibilities that could be proposed for an integral use of the biomass is shown in Fig. 11.7. The process has been developed at laboratory level and involved four major working areas. The first one focused on the extraction of the soluble fraction of proteins and carbohydrates, the second unit on recovering fatty acids and carotenoids from the residual biomass slurry, the third one on recovering pigments and, finally, the last one was focused on recovering the remaining proteins and carbohydrates.

Acknowledgements Our most sincere recognition to our colleagues of the Marine Microalgae Biotechnology group, of the University of Almería, which over the past 20 years have been co-working with us on the topics treated in this chapter. Especially, to Prof Robles-Medina and Dr Belarbi EH and Dr González-Moreno by their help, suggestions and criticisms on the contents of this chapter.

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Production and Properties of Biodiesel from Algal Oils

12

Gerhard Knothe

1 Introduction

Biodiesel, an alternative to conventional petroleum-derived diesel fuel (petrodiesel), is most commonly derived from classical commodity vegetable oils such as palm, rapeseed/canola, soybean and sunflower. Since the supply of these feedstocks can only replace a few percent of petrodiesel, additional triacylglycerol-containing feedstocks have found increasing interest in recent years. These feedstocks include animal fats, used cooking oils, and less common vegetable oils such as jatropha. Algae are among the feedstocks having generated the most interest in recent years.

This chapter will deal with the production of biodiesel once the oil has been obtained as well as with the fuel properties of biodiesel derived from algal oils.

2 General Aspects of Biodiesel

Biodiesel (Knothe et al. 2010; Mittelbach and Remschmidt 2004) is defined as the mono-alkyl esters of vegetable oils or animal fats per the American biodiesel standard ASTM D6751. The American standard and the European standard EN 14214 have served as guidelines for the development of standards elsewhere around the world. Besides serving as a transportation fuel, biodiesel is also of interest as a heating fuel. A separate standard, EN 14213 in Europe, when using biodiesel as heating oil has been developed. The American standards ASTM D975 (diesel fuel oils) ASTM D396 (fuel oils, i.e., for heating purposes) now cover blends of up to 5% biodiesel meeting the specifications of ASTM D6751. Triacylglycerol feedstocks have also been used untransesterified as fuel. Another fuel that has recently gained interest is "renewable diesel", whose composition resembles petrodiesel, which is obtained from a lipid feedstock by a hydrodeoxygenation reaction (for a comparison of biodiesel and renewable diesel see Knothe 2010a).

Biodiesel is technically competitive with petrodiesel. Advantages of biodiesel include that it is derived from a renewable, domestic resource, miscibility with petrodiesel at all blend levels, positive energy balance, reduction of most regulated exhaust emissions with the exception of nitrogen oxides (NO_x), biodegradability, little or no sulfur and aromatics content, high flash point, and inherent lubricity, the last property being of special interest in connection with modern ultra-low sulfur diesel fuels which possess poor lubricity. Major technical problems with biodiesel include poor cold flow properties and oxidative stability, which are discussed below briefly.

The reaction, commonly termed transesterification, by which biodiesel is produced, is discussed below. An important aspect of biodiesel production is that the fatty acid profile of biodiesel corresponds to that of its parent oil. Many fuel properties of biodiesel, including some limited in standards, are determined by the fatty acid profile. The aforementioned technical problems associated with biodiesel can often be traced to the fatty acid profile. With knowledge of the properties of the individual components of biodiesel, it is therefore possible to relatively accurately predict the properties of a biodiesel fuel.

The market expansion of biodiesel has been impaired by unfavorable economics due to the high price of the vegetable oil feedstocks compared to petroleum. This issue has often been addressed by legislative and regulatory subsidies and incentives designed to increase production of and demand for biodiesel. These subsidies and incentives are subject to frequent change by legislative and regulatory bodies, thus influencing the production of and demand for biodiesel.

Biodiesel production has increased significantly worldwide in the last decade. Estimated biodiesel production in the United States in 2008 was 700×10^6 gal (= 2.646×10^9 L;

G. Knothe (✉)
Agricultural Research Service, U.S. Department of Agriculture,
National Center for Agricultural Utilization Research,
1815 N. University St, Peoria, IL 61604, USA
e-mail: gerhard.knothe@ars.usda.gov

M.A. Borowitzka and N.R. Moheimani (eds.), *Algae for Biofuels and Energy*, Developments in Applied Phycology 5,
DOI 10.1007/978-94-007-5479-9_12,

approximately 2.33×10^6 t), with the greatest production increase occurring in the last few years (National Biodiesel Board). In June 2009, production capacity in the United States was given as 2.69 10^9 gal (approximately 10.17×10^9 L; approximately 8.97 a 10^6 t) (National Biodiesel Board). In Europe (EU countries), estimated biodiesel production in 2008 was 7.715×10^6 t (European Biodiesel Board), with Germany being the largest biodiesel producer in the EU and the world and France being the second largest producer in the EU Biodiesel production has increased significantly in other countries around the world, for example Brazil and Malaysia.

Besides the possibility of being used as neat fuel, biodiesel is very commonly used in blends with conventional, petroleum-derived diesel fuel (petrodiesel). These blends are usually designated "BX", with X defining the amount of biodiesel in the blend. Thus, a 5% blend of biodiesel with petrodiesel is termed B5.

Several driving forces are causing the search for additional feedstocks for biofuels including biodiesel. Briefly, these issues are to increase the potential supply of energy feedstocks, to provide domestic sources of renewable energy, and to enhance economic development by providing a source of jobs and income. The latter aspect is of particular interest in more indigent sections of developing countries. The so-called food vs. fuel discussion, i.e., that sources of food should not be used as sources of fuel (although it may be noted that this discussion does not seem to affect other non-food uses such as lubricants, polymers, etc.) and the carbon footprint of some feedstocks have also become major issues. Especially the food vs. fuel discussion has shifted attention towards feedstocks that yield inedible oils.

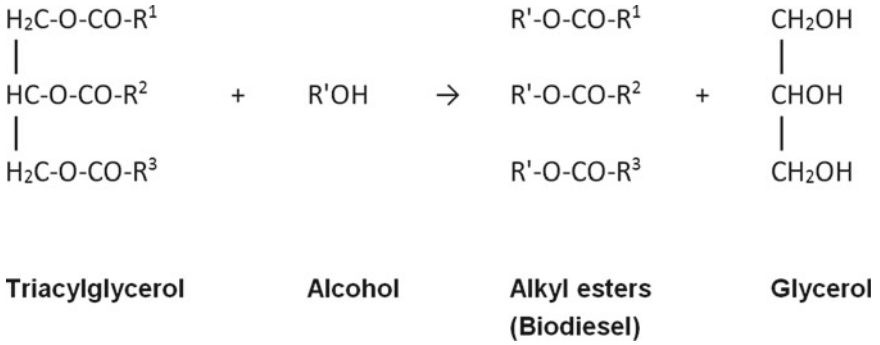
Among the sources of oil that yield inedible oils, algae have been finding increasing attention due to their production potential per unit of land. Biodiesel from algae has been the subject of some recent reviews (e.g., Chisti 2007; Hu et al. 2008; Mata et al. 2010). Historically, an initial program on algae as source of fuel was conducted by the U.S. Department of Energy from the late 1970s into the 1990s (Sheehan et al. 1998).

2.1 Biodiesel Production. General Aspects

Transesterification. Base catalysis is the most common process for transesterifying an oil or fat with an alcohol to give biodiesel (Scheme 12.1 depicts the principle of the transesterification reaction) because base catalysis generally is considerably faster than acid catalysis. Bases such as NaOH or KOH have been commonly used as catalysts. However, alkoxides such as $NaOCH_3$ have the advantage that water formation according to the reaction $XOH + ROH \rightarrow H_2O + ROX$ (X = Na or K, R = alkyl) is not possible. Both water and free fatty acids should be kept to a minimum in base-catalyzed transesterification (Freedman et al. 1984). Lower quality feedstocks such as used cooking oils have high free fatty acid content, necessitating an acid-catalyzed pre-treatment to reduce the free fatty acid content (thereby converting the free fatty acids to alkyl esters) to a level suitable for carrying out base-catalyzed transesterification on the remaining material (Canakci and Van Gerpen 1999). Besides the desired mono-alkyl esters, glycerol is formed as co-product during transesterification.

A major advantage of base-catalyzed transesterification is the mild reaction conditions, which for the production of methyl esters typically are 1h at 60–65°C and ambient pressure, 1% catalyst and a molar ratio of alcohol to oil of 6:1. The reaction mixture has two phases at the outset (methanol if using this alcohol and oil) and two when the reaction is complete (methyl esters, glycerol). The heavier glycerol phase at the end of the reaction can be easily separated from the methyl ester phase. The methyl ester product is typically washed with water. The yield from this transesterification procedure is typically very high, usually more than 96%, and generally does not depend on the fatty acid profile of the feedstock. When using alcohols other than methanol, some changes to the reaction conditions are necessary such as higher reaction temperature (around the boiling point of the alcohol) and other effects may occur such as the possible formation of emulsions when producing ethyl esters. Small amounts of triacylglycerol starting material and mono- and diacylglycerols formed as intermediates can remain in the finished product as do traces of glycerol co-product, alcohol and catalyst. These minor components (impurities) are limited in biodiesel standards by corresponding specifications. These minor components can influence fuel properties.

Besides the typical base-catalyzed transesterification, numerous other catalysts and catalytic procedures have been developed for the synthesis of biodiesel. These procedures include heterogeneous catalysis and enzymatic processes. A goal of these procedures often is to permit lower quality feedstocks to be used directly or to enable the use of other alcohols without significant change of reaction conditions or to facilitate catalyst separation. Numerous reviews in the literature provide an overview of various aspects of the transesterification reaction and the various catalysts and procedures (Adamczak et al. 2009; Akoh et al. 2007; Al-Zuhair 2007; Behzadi and Farid 2007; Bondioli 2004; Di Serio et al. 2008; Fjerbaek et al. 2009; Fukuda et al. 2001; Gutsche 1997; Haas et al. 2002; Helwani et al. 2009a, b; Hoydonckx et al. 2004; Lee et al. 2009a; Leung et al. 2010; Lotero et al. 2005, 2006; Ma and Hanna 1999; Marchetti et al. 2007; Mbaraka and Shanks 2006; Meher et al. 2006; Nakazono 2003; Nielsen et al. 2008; Nikiema and Heitz 2008; Ranganathan et al. 2008; Robles-Medina et al. 2009; Schuchardt et al. 1998; Shah et al. 2003; Vyas et al. 2010; Zabeti et al. 2009).



Scheme 12.1 The transesterification reaction. The feedstock, be it a vegetable oil, animal fat or algal oil, is represented by the triacylglycerol (triglyceride; R^1, R^2 and R^3 may or may not be identical). The most commonly used alcohol is methanol ($R' = CH_3$)

2.2 Biodiesel Production from Algae

One of the first reports of a methyl ester fuel (now termed biodiesel) from microalgae states that the best yield of fatty acid methyl ester from algal lipids was obtained with 0.6N hydrochloric acid-methanol at 0.1 h for 70°C using extracted lipid from *Chaetoceros muelleri* (Nagle and Lemke 1990). That acid catalysis gave better results in this case was attributed to the high free fatty acid content of the algal lipid.

More recent reports on the preparation of biodiesel have also utilized acid transesterification. For example an acid-catalyzed transesterification was carried out on the material extracted from dried heterotrophic cell powder of *Chlorella protothecoides* due to the high acid value (Miao and Wu 2006). The optimum process was 100% catalyst (based on oil weight) with 56:1 M ratio of methanol to oil at 30°C, about 4 h reaction time (Miao and Wu 2006; Xu et al. 2006; Cheng et al. 2009).

In another report (Johnson and Wen 2009) two methods were described. One method (extraction-transesterification) consisted of oil extraction with chloroform-methanol followed by transesterification with a mixture of methanol, sulfuric acid, and chloroform added to the algal oil, heated at 90°C for 40 min. The other method (direct transesterification) consisted of freeze-dried algal biomass or wet algal biomass mixed with methanol and sulfuric acid. Depending on experimental design, solvent (chloroform, hexane, or petroleum ether) was added. The mixture then was heated at 90°C for 40 min, followed by work-up and gravimetric determination of biodiesel yield. An in situ acid-catalyzed (H_2SO_4) transesterification method for dried algal biomass has recently been described (Haas and Wagner 2011).

2.3 Fatty Acid Profile and Fuel Properties of Biodiesel. General Aspects

Ultimately, the properties of the biodiesel fuel derived from any lipid feedstock will determine the viability of this feedstock for biodiesel production. These properties are mainly influenced by the fatty esters largely comprising biodiesel, although minor components can also play a significant role. Therefore, the essential fuel properties of biodiesel as influenced by the fatty acid profile as well as minor components are discussed here briefly.

Two biodiesel standards, ASTM D6751 in the United States and EN 14214 in Europe, generally serve as guidelines for development of other standards around the world and for assessing biodiesel fuel quality. The specifications contained in these standards can be traced to several factors influencing biodiesel fuel properties, namely (a) specifications mainly influenced by the fatty acid (ester) profile of the fuel, (b) specifications influenced mainly by the transesterification reaction and (c) specifications influenced mainly by extraneous factors, although there is some overlap between these categories. This chapter will deal mainly with those properties that are influenced by the fatty acid (ester) profile.

Table 12.1 contains specifications (fuel properties) from the standards ASTM D6751 and EN 14214 that are directly affected by the fatty acid profile of the biodiesel. It is important to note that the European standard EN 14214 contains some further restrictions on the fatty acid profile. These include direct restrictions on fatty acids with more than three double bonds (restricted a maximum of 12%) and fatty acids with more than four double bonds (restricted to a maximum of 1%). The major reason for this is the easy oxidation of fatty acid chains with methylene-interrupted double bonds (–CH=CH-CH_2-CH=CH-; *bis*-allylic CH_2 positions), the oxidation occurring more readily. A more indirect restriction on the fatty acid profile in EN 14214 is the iodine value (IV), which is a crude measure of the total unsaturation and all double bonds, in monounsaturated and polyunsaturated fatty acid chains, contribute to its value. The use of the IV is problematic as an infinite number of fatty acid profiles can give the same IV, it is molecular weight-dependent (for example, ethyl esters will have a lower IV than methyl esters although this does not necessarily

Table 12.1 Standard specifications for fuel properties largely determined by fatty esters

Property	ASTM D6751	EN 14214
Cetane number	47 min	51 min
Cold flow	Cloud point: report	Cold filter plugging point: varies by location and time of year
Kinematic viscosity; 40°C; mm^2s^{-1}	1.9–6.0	3.5–5.0
Oxidative stability; h	3 min	6 min

C16:0), stearic (octadecanoic; C18:0), oleic (9(*Z*)-octadecenoic; C18:1), linoleic (9(*Z*),12(*Z*)-octadecadienoic; C18:2), and linolenic (9(*Z*),12(*Z*),15(*Z*)-octadecadienoic; C18:3). Fuel-related properties of the methyl esters of these acids, together with esters of some other acids occurring in fats and oils, are compiled in Table 12.2. These fuel properties are the cetane number, kinematic viscosity, oxidative stability and cold flow specifications in biodiesel standards, complementing the specifications in standards given in Table 12.1. Minor components of biodiesel can also influence these properties, especially oxidative stability and cold flow, and are briefly discussed in the following relevant sections.

Table 12.2 Fuel properties of common fatty acid methyl esters[a]

Fatty acid methyl ester (trivial name; acronym)	Cetane number	Kinematic viscosity (40°C; $mm^2\ s^{-1}$)[b]	Melting point (°C)	Oxidative stability (h)[a]
Methyl decanoate (caprate; C10:0)	51.6	1.72	−13.5	>24
Methyl dodecanoate (laurate; C12:0)	66.7	2.43	4.3	>24
Methyl tetradecanoate (myristate; C14:0)	nd	3.30	18.1	nd
Methyl hexadecanoate (palmitate; C16:0)	85.9	4.38	28.5	>24
Methyl 9(*Z*)-hexadecenoate (palmitoleate; C16:1)	56.6; 51.0	3.67	−34.1	2.11
Methyl octadecanoate (stearate; C18:0)	101	5.85	37.7	>24
Methyl 9(*Z*)-octadecenoate (oleate; C18:1)	56.6; 59.3	4.51	−20.2	2.79
Methyl 9(*Z*),12(*Z*)-octadecadienoate (linoleate: C18:2)	38.2	3.65	−43.1	0.94
Methyl 9(*Z*),12(*Z*),15(*Z*)-octadecatrienoate (linolenate; C18:3)	22.7	3.14	−45.5	0.00
Methyl eicosanoate (arachidate; C20:0)	–	–	46.4	nd
Methyl 11(*Z*)-eicosenoate (gondoate; C20:1)	73.2	5.77	−7.8	nd
Methyl docosanoate (behenate; C22:0)	–	–	53.2	nd
Methyl 13(*Z*)-docosenoate (erucate: C22:1)	74.2	7.33	−3.0	nd

[a]Data from Knothe et al. (2003), Knothe and Steidley (2005a, b), Knothe (2008), Knothe and Dunn (2009), Moser et al. (2009)
Nd = not determined. Cetane number usually determined as derived cetane number. Cetane numbers and kinematic viscosity of methyl eicosanoate and methyl docosanoate not determined due to high melting points. Oxidative stability per Rancimat test (EN !4112), experiments terminated after 24 h; oxidative stabilities of methyl tetradecanoate, eicosanoate and docosanoate, although not determined, likely > 24 h

affect the reactivity of the fatty acid chain) and treats all double bonds equally (Knothe 2002).

Although cold flow specifications in standards are "soft" as no hard or variable limits are given ("by report" in ASTM D6751; variation by location and time of year in EN 14214), this is still one of the most problematic issues with biodiesel as briefly discussed below.

The most common fatty acids in the fatty acid profiles of vegetable oils and animal fats are palmitic (hexadecanoic;

2.3.1 Cetane Number and Combustion

The cetane number is a dimensionless descriptor of the ignition quality of a diesel fuel conceptually similar to the octane number used for gasoline (petrol). It is related to the ignition delay the fuel experiences upon injection into the combustion chamber of a diesel engine. The shorter the ignition delay time, the higher the cetane number and vice versa. Higher cetane numbers are generally associated with better ignition and combustion characteristics. The high-quality

reference compound on the cetane scale is hexadecane (trivial name cetane, giving the cetane scale its name) with an assigned cetane number of 100. The low-quality reference compound on the cetane scale is 2,2,4,4,6,8,8-heptamethylnonane with an assigned cetane number of 15. This also serves to show that branching in the hydrocarbon chain reduces the cetane number. Cyclic compounds such as aromatics also tend to possess low cetane numbers, although the cetane number will increase with increasing length of an unbranched alkyl side chain. Overall, straight-chain alkanes possess the highest cetane numbers and under this aspect are the "ideal" components of a petrodiesel fuel.

The cetane numbers of esters of fatty acids depend strongly on compound structure. Generally, cetane number increases with increasing chain length and decreasing unsaturation (Table 12.2). Saturated long-chain esters such as methyl palmitate and methyl stearate have high cetane numbers close to that of hexadecane or other long-chain alkanes (CN of methyl stearate approximately 100, that of methyl palmitate approximately 85, that of methyl laurate around 65). One double bond reduces the CN considerably, thus the CN of methyl oleate is in the range of 55–59. The cetane numbers of the more unsaturated esters methyl linoleate and methyl linolenate are even lower, in the ranges of 38–40 and 22–25, respectively. The cetane number of a mixture such as biodiesel is related to the cetane numbers of the individual components taking their amounts into consideration proportionally. Thus, most biodiesel fuels possess cetane numbers in the range of high 40s to lower 60s. The specifications for minimum cetane number of biodiesel are 47 in ASTM D6751 and 51 in EN 14214. Generally, higher cetane numbers are more desirable.

Another important issue that is related to combustion is that of exhaust emissions. While levels of exhaust emissions are not specified when operating an engine on biodiesel, or on any other fuel, they are regulated by the agencies empowered by legislation to carry out this task. While exhaust emissions generated by fuel combustion are composed of a great variety of compounds, generally only four types are addressed by regulations. These are nitrogen oxides (NO_x), particulate matter (PM), carbon monoxide (CO), and hydrocarbons (HC). Similar to the effect of compound structure on the cetane number, levels of exhaust emissions are also affected by compound structure (McCormick et al. 2001; Knothe et al. 2006). A correlation if cetane number with some exhaust emissions has been discussed (Ladommatos et al. 1996). Most notably, NOx exhaust emissions in the past have been stated to apparently increase with increasing level of unsaturation, which is of significance for biodiesel due to the presence of unsaturated fatty acid chains, but this effect may not hold upon closer inspection (McCormick et al. 2006) and PM exhaust emissions are significantly reduced when operating a diesel engine on biodiesel. For blends of biodiesel with petrodiesel, the influence of biodiesel on exhaust emissions is approximately linear to the blend level (U.S. Environmental Protection Agency 2002). It appears possible that the issue of exhaust emissions differences between biodiesel and petrodiesel will diminish in the future due to increasing market penetration of new exhaust emissions reduction technologies as a result of increasingly stringent regulations which all fuels will need to meet.

2.3.2 Kinematic Viscosity

The high viscosity of vegetable oils, approximately an order of magnitude greater than that of petroleum-based diesel fuel (petrodiesel), is the major reason why these feedstocks are transesterified to biodiesel. The high viscosity of vegetable oils, influencing penetration and atomization of the fuel in the combustion chamber, leads to operational problems such as engine deposits. Biodiesel fuels possess viscosity values closer to those of petrodiesel, which often exhibit kinematic viscosity values in the range of 2.0–3.0 $mm^2\ s^{-1}$. However, the range of kinematic viscosity in biodiesel standards extends beyond this typical range observed for biodiesel fuels (Table 12.1) as most biodiesel fuels exhibit kinematic viscosity in the range of 4.0–5.0 $mm^2\ s^{-1}$ at 40°C. Note, however, that the kinematic viscosity range prescribed in the European biodiesel standard EN 14214 is tight and there appears to be no technical justification for the high lower limit (3.5 $mm^2\ s^{-1}$) as this limit is above the kinematic viscosity of most petrodiesel fuels. There appears to be no technical reason why the kinematic viscosity could not be the same for biodiesel and petrodiesel.

Compound structure also significantly influences viscosity. Viscosity increases with chain length and decreasing *cis*-unsaturation (Table 12.2). However, compounds with *trans* double bonds exhibit higher kinematic viscosity the *cis* isomers. For example, the kinematic viscosity of methyl elaidate, the *trans* isomer of methyl oleate, is 5.86 $mm^2\ s^{-1}$, almost identical to that of the corresponding saturated C_{18} compound, methyl stearate (Knothe and Steidley 2005a). Thus the kinematic viscosity of a biodiesel fuel depends on its fatty acid profile with the individually components affecting viscosity approximately proportional to their individual amounts. Kinematic viscosity is also strongly temperature-dependent, increasing significantly at lower temperatures.

2.3.3 Oxidative Stability

Unsaturated fatty acid chains, especially the polyunsaturated species (containing so-called *bis*-allylic CH_2 positions located between two double bonds), i.e., esters of linoleic and linolenic acids, are susceptible to oxidation, i.e. reaction with the oxygen in ambient air. Relative rates of oxidation given in the literature (Frankel 2005 and references therein) are 1 for oleates (C18:1), 41 for linoleates (C18:2), 98 for linolenates (C18:3) and 195 for C20:4. When setting a relative

rate of oxidation for linoleate (C18:2) as 1, the relative rates reported in the literature are 2.1 for C18:3, 2.9 for eicosatetraenoate (C20:4; arachidonate) and 5.1 for docosahexaenoate (C22:6; DHA) (Frankel 2005). Therefore, other polyunsaturated fatty acid chains, such as octadecatetraenoate (C18:4; also known as stearidonate), eicosapentenoate (C20:5, EPA), and docosapentaenoate (C22:5) will also be very susceptible to oxidation. Small amounts of unsaturated fatty esters probably affect oxidative stability more than their small amounts indicate. Oxidation of fatty acid chains is a complex reaction, consisting initially of the formation of hydroperoxides followed by secondary reactions, during which products such as acids, aldehydes, ketones, hydrocarbons, etc., can be formed.

Oxidative stability is addressed in biodiesel standards primarily by the corresponding specification which prescribes the use of a Rancimat instrument. This instrument permits an accelerated test to be conducted with the goal of judging the oxidative stability of a sample. The lower the so-called induction time by this method, the less oxidatively stable the sample. Minimum induction times prescribed in biodiesel standards by this test are 3 h (ASTM D6751) and 6 h (EN 14214). However, an antioxidant additive will almost always be needed to achieve these specifications as the induction time of methyl oleate is 2.79 h, that of methyl linoleate 0.94 h and that of methyl linolenate 0 h, while methyl esters of saturated fatty acids possess induction times >24 h (see Table 12.2). No typical oxidative stability times for specific biodiesel fuels can be given can be given as oxidative stability is strongly influenced by factors such as the presence of extraneous materials such as traces of metals or minor components such as free fatty acids. Besides presence of air and minor components, other factors significantly influencing oxidative stability are size of contact area with air, temperature and presence of light.

Besides the Rancimat method, an oxidative stability test that is frequently mentioned in the literature is the OSI (oil stability index) method, which is also a standard method of the American Oil Chemists' Society. The principle of these two methods is nearly identical, including the suggested test temperature of 110°C.

As mentioned above, to achieve the mentioned minimum induction times in prescribed in standards, the use of antioxidants is almost always necessary as mentioned above. Synthetic antioxidants such as butylated hydroxytoluene (BHT; 2,6-di-*tert*-butyl-4-methylphenol), *tert*-butylhydroquinone (TBHQ; 2-*tert*-butylhydroquinone) and others are more effective than naturally occurring antioxidants such as tocopherols (Dunn 2008) As mentioned above, the European biodiesel standard EN 14214 contains some specifications that can also be related to the phenomenon of oxidative stability. These specifications are the iodine value, a crude measure of total unsaturation of a sample, a maximum of 12% for linolenic acid methyl esters and a maximum of 1% for esters of fatty acids with more than three double bonds.

2.3.4 Cold Flow

One of the major problems associated with the use of biodiesel is poor low-temperature flow properties, documented by relatively high cloud points (CP) and pour points (PP). The CP, which usually occurs at a higher temperature than the PP, is the temperature at which a fatty material becomes cloudy due to formation of crystals and solidification of saturates. Solids and crystals rapidly grow and agglomerate, clogging fuel lines and filters and causing major operability problems. With decreasing temperature, more solids form and the material approaches the pour point, the lowest temperature at which it will still flow. Besides CP and PP, other test methods for the low-temperature flow properties of conventional diesel fuels, namely the low-temperature flow test (LTFT; used in North America) and cold filter plugging point (CFPP).

Saturated fatty compounds have significantly higher melting points than unsaturated fatty compounds and within a homologous series the melting points generally increase with chain length (Table 12.2). Thus, in a mixture the saturated esters crystallize at higher temperature than the unsaturated ones. The nature and amount of saturated fatty compounds is a determining factor of the cold flow properties of biodiesel (Imahara et al. 2006). Thus biodiesel fuels derived from fats or oils with significant amounts of saturated fatty compounds will display higher CPs and PPs. For example, the cloud point of the methyl esters of soybean oil is around 0°C (with approximately 15% saturated fatty acids, about two-thirds thereof C16:0, the remainder largely C18:0) and that of the methyl esters of palm oil (with approximately 40% C16:0, but also some other saturated fatty acids) is around 15°C. Minor components of biodiesel can significantly affect low temperature properties. Examples are monoacylglycerols (monoglycerides, especially of those of saturated fatty acids) (Yu et al. 1998) and sterol glucosides (Bondioli et al. 2008).

Several approaches to improving the low-temperature properties of biodiesel have been investigated including blending with petrodiesel, winterization, additives, branched-chain esters, and bulky substituents in the chain. The latter approach may be considered a variation of the additive approach as the corresponding compounds have been investigated in biodiesel at additive levels. Winterization is the physical removal of higher melting components by repeated cooling and filtration cycles. Regarding the use of branched-chain esters, alcohols other than methanol can be used to prepare biodiesel, thus giving esters such as ethyl or *iso*-propyl. Branched esters such as *iso*-propyl, *iso*-butyl and 2-butyl possess lower melting points than methyl esters. Consequently, biodiesel prepared with the corresponding alcohols showed a lower TCO (crystallization onset temperature) for the isopropyl

esters of soybean oil by 7–11°C and for the 2-butyl esters of SBO by 12–14°C compared to methyl esters (Lee et al. 1995). This effect is also reflected in the cloud point. However, economically only iso-propyl esters appear attractive although even they are more expensive than methyl esters. Branching in the ester chain does not have any negative effect on the CNs of these compounds.

Lubricity. Historically, diesel engines had relied on the lubricating properties of the petrodiesel fuel itself to lubricate important engine parts such as injectors and fuel pumps. New ultra low-sulfur petrodiesel fuels have poor lubricity properties compared to their high sulfur precursors. Adding biodiesel at levels around 1–2% to low-lubricity petrodiesel fuels restores the lubricity, although some minor components of biodiesel such as monoacylglycerols and free fatty acids also play a role at these low levels (Knothe and Steidley 2005a, b). This ability of biodiesel to restore lubricity has provided a technical incentive to the use of low-level blends of biodiesel in petrodiesel such as B2. Lubricity is a property not prescribed in biodiesel standards but is contained in petrodiesel standards such as ASTM D975 and EN 590.

2.3.5 Summary

In summary, oxidative stability and combustion properties of biodiesel improve with increasing saturation of the fatty acid chains, while cold flow properties are negatively affected. Conversely, cold flow properties improve with increasing unsaturation while oxidative stability and combustion are negatively affected. These observations also hold for mixtures such as biodiesel. Higher content of saturated fatty esters improves oxidative stability and combustion while worsening cold flow, while higher content of unsaturated fatty esters, especially polyunsaturated fatty esters, improves cold flow but leads to poorer oxidative stability and combustion. Chain length of the fatty acid chain is another structural factor significantly influencing fuel properties. While chain length has comparatively little effect on oxidative stability (except for the number of possible sites for double bonds), the effect on cold flow is pronounced as the melting point of fatty acid chains generally increases significantly with increasing chain length. The cetane number of a fatty ester also increases with chain length although saturated neat C_{10} and C_{12} esters already exhibit acceptable cetane numbers. It appears that decanoic (C10:0) and palmitoleic (C16:1) acids are the most desirable to highly enrich in a fatty acid profile in order to overall improve biodiesel fuel properties (Knothe 2008).

2.4 Fatty Acid Profiles of Algae-Derived Biodiesel and Effect on Fuel Properties

As discussed above, fuel properties will ultimately determine if biodiesel from a specific feedstock is commercially feasible / viable. The influence of compound structure on individual fatty esters and mixtures thereof, such as biodiesel, was also discussed above. It is therefore of interest to evaluate the fatty ester profiles of biodiesel derived from algal lipids under this aspect.

Some literature reports on the potential of algae for production of lipids suggest specific algal strains with regard to biodiesel. For example, in a critical review on the potential of microbial and algal oils for biodiesel production, several oleaginous yeasts and oleaginous phototrophic algae cultivatable in marine or brackish waters were identified as having the greatest potential due to their high lipid content (Ratledge and Cohen 2008). The algal species identified in that work are *Botrycococcus braunii*, *Isochrysis galbana*, *Haematococcus pluvialis*, *Nannochloropsis* sp., *Nitzschia* sp., *Parietochloris incisa* and *Pleurochrysis carterae*. In another review, the oil content (by % dry cell weight) of several microalgal species was compiled (Li et al. 2008) with the high oil-content species listed there including *Chlorella emersonii*, *Chlorella minutissima*, *Chlorella vulgaris*, *Neochloris oleoabundans*, *Parietochloris incisa* and *Nitzschia laevis*.

As indicated above, such suggestions are usually based on the potential yield of lipids from these algae. The fatty acid profiles of these lipids and their potential impact on fuel properties have found less attention. Furthermore, not all algae may yield lipid fractions useful for biodiesel production. For example, the fatty acids of autotrophic *Botryococcus braunii* ranged from C_{14} to C_{30} with C_{16}, C_{18} and C_{28} (palmitic, oleic, octacosenoic; also C_{26} monoenoic acid and a C_{28} dienoic acid) predominating and small amounts of branched fatty acids also being present (Douglas et al. 1969), however, only 0.014% fatty acids were reported in a natural bloom of this. The majority of reports on *B. braunii* appear to deal with hydrocarbons obtained from this alga. Thus *B. braunii* is more likely a source of hydrocarbons (Banerjee et al. 2002), which would yield petrodiesel-like fuels, rather than a feedstock for biodiesel. The hydrocarbons and ether lipids from *B. braunii* have been reviewed (Metzger and Largeau 2005).

For comparison purposes, the fatty acid profiles of biodiesel (methyl esters) derived from two common commodity oils may be noted here. The fatty acid profile of soybean oil consists of palmitic (usually 10–11%), stearic (4–6%), oleic (21–25%), linoleic (50–55%) and linolenic (around 8%) acids, that of palm oil of palmitic (around 40–45%), stearic (4–5%), oleic (around 40%) and linoleic (around 10%) with lesser amounts of other fatty acids. As mentioned above, the differences in the fatty acid profiles are manifested in different fuel properties.

While the number of reports in the scientific literature on using algal oils to produce biodiesel fuels, there appears to be little or no comprehensive determination of the properties

Table 12.3 Effect of temperature on the fatty acid profile of Australian algae

		Saturates			Monounsaturates				Polyunsaturates						
Alga		14:0	16:0	Total	16:1 Δ9	18:1 Δ9	18:1 Δ7	Total	16:3-ω4	18:2	18:3	18:4	20:5	22:6	Total
Rhodomonas sp. (NT 15)	25°C	7.9	12.4	25.2	3.9	1.0	3.8	8.7	0.7	1.3	19.8	28.1	7.7	3.8	64.7
	33°C	12.4	19.0	40.3	2.1	5.8	4.0	11.9	2.6	5.7	14.6	9.5	4.7	2.6	45.0
Cryptomonas sp. (CRFI01)	25°C	4.6	5.9	25.7	3.1	1.4	2.2	6.7	1.3	0.9	21.0	26.6	7.2	4.1	63.9
	30°C	16.6	18.8	29.5	3.1	2.0	2.4	7.5	1.3	1.2	20.2	20.9	7.7	4.3	57.8
Isochrysis sp. (T. ISO)	25°C	25.9	12.6	39.0	11.3	7.4	1.8	20.5	–	4.1	7.7	14.5	1.5	5.4	37.4
	33°C	27.2	13.1	41.5	8.4	7.6	2.0	18.0	–	3.6	9.6	11.9	0.8	5.8	37.3
Chaetoceros sp. (CS 256)	25°C	23.6	9.2	33.5	36.5	1.7	1.2	39.4	2.6	0.4	0.5	0.6	8.0	1.0	19.5
	35°C	28.3	8.4	38.2	33.5	1.8	1.2	36.5	4.8	1.2	1.0	0.3	6.6	0.3	20.4

Data from Renaud et al. (2002); more data, including at additional temperatures, given in this reference

of these biodiesel fuels. Therefore, in most cases the properties of biodiesel fuels from algal lipids must be estimated by comparison with biodiesel derived from other feedstocks and/or from general correlations of structure–property relationships.

The fatty acid profiles of algal oils are very sensitive to the parameters of algal growth such as temperature, light intensity, growth medium and time of growth. The composition of the final fatty acid profile can also depend on the nature of the transesterification method and if wet or dry algal biomass was used as feedstock in the transesterification as shown for *Schizochytrium limacinum* (Johnson and Wen 2009). To illustrate the influence of these parameters, some literature data related to the effect of temperature on the fatty acid profile of Australian algae (Renaud et al. 2002) are extracted in Table 12.3. A more comprehensive collection of fatty acid profile data from the literature is contained in Table 12.4. In a few cases, influences of growth parameters on the fatty acid profile are also noted in Table 12.4. It may be noted that one review article has also compiled some data on the fatty acid profiles of various algal species (Hu et al. 2008).

Some algal oils contain odd-numbered fatty acids or other less common fatty acids which are not discussed here but are mentioned in some footnotes in Table 12.4. In any case, inspection of the fatty acid profiles in Tables 12.3 and 12.4 reveals several consistent features for many algal oils. Palmitic acid appears to be the most common fatty acid. In some cases, myristic acid is also present in enhanced amounts. As a result, the vast majority of biodiesel fuels derived from algal sources would likely exhibit moderate to very poor cold flow properties. A more extreme example appears to be *Schizochytrium* strains with palmitic acid content >35%, complemented by smaller amounts of myristic acid. *Nannochloropsis* sp. is an algae species with high oil yield potential (Rebolloso-Fuentes et al. 2001; Rodolfi et al. 2009). A study on the growth and lipid content of *Nannochloropsis occulata* and *Chlorella vulgaris* in dependence on temperature and nitrogen concentration showed that *N. oculata* accumulated very high amounts of palmitic acid (52–62%) with the "classical" C18 acids comprising most of the other part of the fatty acid profile (Converti et al. 2009). Thus the potential of *Nannochloropsis* is affected by the issue of cold flow properties.

Besides these saturated fatty acids, many fatty acid profiles show significant amounts of polyunsaturated fatty acids. In contrast to the saturated fatty acids, which are mainly palmitic and myristic acids as mentioned above, the polyunsaturated fatty acids display more variety. The major polyunsaturated acid in some algae such as *Isochrysis galbana* and *Nannochloropsis* is C20:5, in other algae such as *Schizochytrium* it is C22:6, in *Parietochloris incisa* it is C20:4 in *Dunaliella* it is C18:3 and C16:4 (for C16:4 see footnotes in Table 12.4). In many cases lesser amounts of polyunsaturated fatty acids such as C16:3, the aforementioned C16:4, C18:4 and others are observed (see footnotes in Table 12.4). It may be noted that it is frequently stated in the literature that some of these species are the richest source of specific polyunsaturated fatty acids, for example, *Parietochloris incisa* for C20:4 (Bigogno et al. 2002), for which C20:4 can reach 59–62% under nitrogen-deficient and low-light-conditions (Solovchenko et al. 2008 and references therein). Biodiesel derived from these oils with high amounts of polyunsaturated fatty acids would likely display poor oxidative stability per the Rancimat test in EN 14112 and not meet the minimum values prescribed in standards besides not meeting the restrictions on such kinds of acids in the European biodiesel standard EN 14214.

In connection with the strains mentioned above, it can be noted that *Dunaliella* contains virtually no fatty acids $>C_{18}$, although among the "classical" five fatty acids, the profile is enriched in C16:0 and C18:3 (Table 12.4).

Examples of algae from Table 12.4 whose fatty acid profiles are largely dominated by saturated and polyunsaturated fatty acids are *Dunaliella*, *Nannochloropsis* and *Schizochytrium* (for a mass spectrometric study using acetonitrile chemical ionization of this alga see Michaud et al. 2002) with the latter appearing to be the most extreme example. Unfortunately, biodiesel derived from oils with such fatty acid profiles would likely display poor cold flow

Table 12.4 Fatty acid profiles of algae reported in the literature[a]

Alga	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:4	20:5	22:5	22:6	Reference
Chlorella protothecoides[b]	1.31	12.94		2.76	60.84	17.28		0.35					Xu et al. (2006)
Chlorella protothecoides (glucose feedstock)[c]			11.34		53.75	19.48		0.51					Cheng et al. (2009)
Chlorella protothecoides (sugar cane juice hydrolysate feedstock)			12.49		52.56	10.33							Cheng et al. (2009)
Chlorella protothecoides (sweet sorghum juice feedstock)[d]			12.66		66.80	15.12							Gao et al. (2010)
Chlorella vulgaris[e]	0.1	15–18	0.5–1	3–5	20–25	13–18	13–18	0.1–0.2					Matucha et al. (1972)
Dunaliella parva[f]	0.6–0.9	19.3–24.6	1.3–1.9	0.2–2.2	5.8	10.1–10.7	38.3–39.3						Evans et al. (1982)
Dunaliella tertiolecta[g]	0.5–0.8	14.8–15.2	1.7–2.0	1.0–2.3	2.4	3.6–9.2	34.7–44.9						Evans et al. (1982)
Dunaliella tertiolecta[h]	0.5	19.3	0.2	0.5	6.4	9.5	35.1						Delaunay et al. (1993)
Isochrysis galbana[i]	4.63–16.39	7.30–27.39	10.15–24.28	0–2.31	0–1.56	0–1.70		0–2.74	0–2.12	13.19–31.93	0	4.25–13.36	López Alonso et al. (1992)
Isochrysis galbana[j]	6.59–14.51	18.45–25.75	9.54–27.65	3.29–0.64	9.03–2.84	0–2.44		1.96–0.67	0–0.72	23.08–10.46	0–0.62	7.51–6.65	Molina Grima et al. (1994)
Isochrysis galbana[k]	11.1–16.4	11.5–15.5	1.1–2.0	0.7–2.0	18.4–28.6	3.6–7.4	3.6–6.4					9.9–14.6	Poisson and Ergan (2001)
Neochloris oleo abundans[l]	0.4	19.4	1.9	1.0	20.3	13.0	17.5						da Silva et al. (2009)
Neochloris oleoabundans[m]	1.6	15.0	3.5	11.0	36.0	7.4		2.1					Tornabene et al. (1983)
Nannochloropsis sp[n]	4.1–5.7	24.4–31.8	25.1–27.9	0.5–1.2	7.9–10.0	2.9–4.8		0.2–0.3	2.1–3.5	18–25.3		1.3–1.4	Hu and Gao (2003)
Na nnochloropsis sp[o]	4.2–5.9	23.9–27.9	27.1–32.4	1.1–2.1	7.1–10.4	1.1–1.9			1.8–5.4	20.1–30.9		0.1–0.5	Xu et al. (2004)
Nitzschia laevis[p]	15.1–20.0	28.5–34.6	23.2–28.1	0.1–0.2	5.0–8.5	3.1–5.5	0.8–1.3		2.4–3.3	6.3–8.2			Chen et al. (2008)

(continued)

Table 12.4 (continued)

Alga	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:4	20:5	22:5	22:6	Reference
Nitzschia ovalis[q]	2.6	17.2	71.5		2.2					4.0			Orcutt and Patterson (1975)
Parietochloris incisa[r]		8.4–13.3	Tr	3.1–3.7	15.3–18.0	10.4–14.1	0.4–1.0		43.0–47.1	0.7–1.0			Bigogno et al. (2002)
Parietochloris incisa[s]	0.1–0.4	11.2–14.8	1.7–3.2	1.4–1.8	5.1–9.1	15.0–21.2	3.1–6.5		33.1-41.8 (ω6)				Liu et al. (2002)
Scenedesmus obliquus[t]	1.5	21.8	6.0	0.4	17.9	21.7	3.7						da Silva et al. (2009)
Schizochytrium limacinum (soap-free medium)	2.06–4.77	35.50–52.64		0.81–1.04							5.66–8.58	35.89–53.05	Pyle et al. (2008)
Schizochytrium limacinum (soap-containing medium)	2.59–3.14	38.35–42.35		1.10–2.79	3.93–9.17	17.68–21.50	2.72–4.85				2.93–4.07	18.26–24.49	Pyle et al. (2008)
Schizochytrium limacinum[u]	4.00–5.33	38.06–56.51									4.78–5.61	23.51–35.15	Johnson and Wen (2009)
Schizochytrium mangrovei[v]	3.38 ± 0.02	50.57 ± 0.80		0.82 ± 0.01	0.04 ± 0.01		0.08 ± 0.01		0.37 ± 0.01	0.12 ± 0.01	8.24 ± 0.69	29.74 ± 1.70	Fan et al. (2007)
Spirulina (Arthrospira) platensis[w]	12.0	19.4	10.2	1.6	3.4	1.0	7.5			39.0		0.4	Bravi et al. (2006)
Tetraselmis sp.[x]	0–0.38	17.04–25.47	2.76–1.57	0.72–0.46	23.02–24.30	5.03–6.57	18.52–13.99		0.81–2.02	4.75–10.27			Molina Grima et al. (1994)
Tetraselmis suecica[y]	6.3	22.8	10.2		6.9	14.9			2.0	6.2			Servel et al. (1994)

[a]Fatty acid profiles in this table do not necessarily add to 100%. Reasons for this can be rounding, unidentified species or incomplete fatty acid profiles given in the literature. Abbreviation: tr = traces. Acronyms refer to fatty acid structures as given in Table 12.2. Isomers of these structures are listed in the footnotes below

[b]0.89% C17:0; 0.36% C19:1 Δ10; 0.42% C20:1 Δ11

[c]1.48% C17:0; 0.75% C19:1

[d]0.54% C17:0; 4.30% 16-methylheptadecanoic acid

[e]0.1% C12:0; 0.5% C14:1; 0.1% C14:2; 0.1% C14:3; 0.1% anteiso-15; 0.5% C15:0; 3–5% C16:2; 10–12 C16:3; 1–1.5% anteiso-17; 0.2% anteiso-C19; 0.2% C20:1; 0.2% C20:2; traces of fatty acids higher than C20

[f]0.3–0.4% C14:1; 0.6–1.7% C15:0; 0–1.7% C15:1; 0.3% C16:2; 11.2% C16:4; 0.4–0.9% C17:1; 2.0–4.2% C18:3ω6; 0.5–0.8 C18:4; single values given for C18:1 and C16:4 because of chromatographic co-elution in one case, a combined value for C18:1 and C16:4 for co-elution is 24.1%

[g]0.2–0.4% C14:1; 1.0–2.0% C15:0; 1.0–2.0% C15:1; 0.6–3.3% C16:2; 17.1% C16:4; 0.9–1.9% C17:1; 3.8–5.5% C18:3ω6; 0.9–2.7 C18:4; single values given for C18:1 and C16:4 because of chromatographic co-elution in one case, a combined value for C18:1 and C16:4 for co-elution is 24.1%

[h]0.1% Ca5:0; 0.1 C17:0; 0.8% C16:1 Δ7; 2.6% C18:1 Δ11; 2.0% C16:2ω6; 16.3% C16:4ω-3; 0.1% C18:2 ω4; 3.5% C18:3 ω6; 0.5% C18:4 ω3; 0.1% C20:2 ω6

[i]Values as percentages of total lipid. 0–0.43% C16:1Δ7; 0–2.20% C16;3; 0.49–7.03% C18:1Δ11; 4.54-13.88% C18:4ω3; 0–3.18% C20:1Δ11; 0–2.06% C22:1Δ11; 0–2.15% C22:1Δ13; 0–3.31% C22:3

[j]Values for 16:1 combined from C16:1Δ7 and C16:1Δ9; values for C18:1 combined form C18:1Δ9 and C18:1Δ11; 1.45–0.29% C16.2; 1.80–0.47% C16:3; 0.26–0.25% C18:4; 1.49–2.67% C20:1; 0–0.88–0% C22:0; 0.–1.72% C 22:3; 0–1.17–0% C24:0; values given for light intensity of 42.4–218.4 Wm^{-2}; there may be maximum or minimum values between these light intensity values, which is indicated by three values for those components not detected at the 42.4 or 218.4 Wm^{-2}

[k]Grown in different culture media (Jones, Provasoli 1/3) and different times (Provasoli 1/3 medium). Values for C18:3 are for ω-3 and ω-6. 5.7–7.2 $ C18:4
[l]1.7% C16:2; 1.0% C16:3; 7.2% C16:4; 2.1% C18:4
[m]0.4% C14:1; 1.0% iso-C15:0; 2.5% C16:2; 8.4% iso-C17:0; 3.3% C17:0; 1.0% C17:1; 0.3% C19:0; 0.5% iso C19;); 0.1 C19:1; 2.5% C20:1
[n]0.6–1.2% C 20:1; all values combined for photoautotrophic and mixotrophic growth
[o]Values combined from batch culture, batch culture with addition of glucose, nitrate and glucose + nitrate
[p]Values combined from cultures at 15, 19 and 23°C. 0.9–1.7% C14:1; 1.3–1.7 C18:3 ω6. Data for TAG species. MAG and other lipid classes contain higher amounts of EPA
[q]Value for C18:1 is a combination C16:3 and C18:1, likely due to GC peak overlap. 2.6% C16:2
[r]Values combined from logarithmic and stationary phases. 0.4–0.5% C16:1 Δ5; tr C16:2; tr-0.4% C16:3; 4.0–6.8% C18:1 Δ11; 0.7–1.1% C18:3 ω6; 1.1–1.5% C20:3 ω6; 1.7–2.4% C20:5ω3
[s]1.1–2.7% C16:1 Δ5; 0.3–0.3% C16:1 Δ7; 1.3–1.7% C16:2; 0.0–5.2% C18:1 Δ11; 0.4–1.1% C18:3 ω6; 0.3–1.1% C20:2 ω6; 0.5–0.7% C20:3 ω6; 1.7–2.4% C20:5ω3
[t]4.0% C16:2; 0.7% C16:3; 0.4% C16:4; 0.2% C18:4
[u]Fatty acid profiles in this work depend on transesterification method and the use of wet vs. dry algal biomass
[v]5.20 ± 0.02% C15:0; 1.01 ± 0.05% C17:0; 0.26 ± 0.002% C22:0; 0.08 ± 0.01% C20:3ω
[w]0.9% C18:4
[x]Values for 16:1 combined from C16:1Δ7 and C16:1Δ9; values for C18:1 combined form C18:1Δ9 and C18:1Δ11; 1.89–1.67% C16.2; 0–0.72% C16:3; 11.12–3.64% C18:4; 0.46–1.18% C20:1Δ11; 0.68–1.06% C18:1. Values given for light intensity of 42.4–218.4 Wm^{-2}; there may be maximum or minimum values between these light intensity values
[y]0.7% C12:0; 1.6% C18:3 ω6; 21.6 C18:4 ω3

and poor oxidative stability simultaneously. As mentioned above, the cold flow properties would be determined by the amount and nature of the saturated fatty acid chains, the oxidative stability largely determined by the high amounts of polyunsaturated fatty acid chains without a compensating effect by the other types of fatty acids.

Regarding cetane number, the situation is different. The cetane number of a mixture of compounds is largely an aggregate of the cetane numbers of the individual components taking the relative amounts into consideration. Thus even in the case of "extreme" fatty acid profiles consisting of high amounts of saturated and polyunsaturated fatty acid chains, the high cetane number of methyl palmitate may largely compensate for the low cetane number polyunsaturated esters It must be noted in this connection that the cetane numbers of C18:4, C20:4, C20:5, C22:5 and C22:6 esters are likely lower than that of C18:3 (methyl linolenate), although no such data on neat esters appears to be available in the literature. If biodiesel with such fatty acid profiles meets the minimum specifications in standards would depend on the exact amount of each component and would need to be, of course, be ascertained by cetane testing. This effect would likely also be observed for exhaust emissions, with benefits of one type of fatty acid at least partially compensating for the other type.

Viscosity is also determined, similar to cetane number, by the aggregate of the individual components accounting for their individual amounts. Thus a high viscosity component can be offset by a lower viscosity component to meet the specifications in biodiesel standards. However, it appears likely that kinematic viscosity is not problematic with biodiesel derived from algal oils. The esters of the saturated components exhibit viscosity values within the range of biodiesel standards. While viscosity increases with chain length as discussed above (also Table 12.2), polyunsaturation in the long-chain fatty acids in algal oils will likely cause reduced kinematic viscosity of these compounds (see kinematic viscosity of C18:3 vs. C18:0 in Table 12.2). In this connection, it may be noted that biodiesel produced from *Schizochytrium limacinum* with high saturation and polyunsaturation levels was reported to possess a kinematic viscosity of 3.87 (Johnson and Wen 2009).

Some algal oils are enriched in palmitoleic acid (Table 12.4). High enrichment of palmitoleic acid could be desirable for "designer" biodiesel fuels (Knothe 2008). However, at the levels found in algal oils and considering the other components of algal oils, the impact of palmitoleic acid may be limited, an effect shown for biodiesel from a nut oil moderately enriched in palmitoleic acid (Knothe 2010b).

In comparison to algal oils it is interesting to note properties of biodiesel prepared from fish oil, as fish oils generally display fatty acid profiles similar to those of algal oils. In this connection, some fuel properties of biodiesel prepared from waste salmon displayed have been reported (Chiou et al. 2008). The fatty acid profile reported in that study showed about 6.8% C14:0, 14.9% C16:0, 6.1% C16:1 3.1% stearic acid, 18% C18:1 (combining oleic with Δ10 and Δ11), 11.5% C18:3, 3.3% C20:4, 11.1% C20:5, 3% C22:5 and 13.7 C22:6 besides smaller amounts of numerous other components. Interestingly, the biodiesel prepared from this salmon oil showed higher OSI values (see text above for OSI vs. Rancimat) than biodiesel from corn oil despite higher content of polyunsaturates in the salmon biodiesel, however, the test was performed at 60°C instead of 110°C (Chiou et al. 2008), which can influence results. The cloud point was in the range of −2 to −3°C.

For sake of completeness, some fatty acid profiles of algal oils not reported in Tables 12.3 and 12.4 are discussed briefly below.

In a study on 35 *Arthrospira* (*Spirulina*) strains, it was reported that linoleic, γ-linolenic (ω-6) and palmitic acid comprised 88–92% of the fatty acid profile (Mühling et al. 2005). The range for palmitic acid was 42.4–47.6%, which shows that any biodiesel from this strain would likely have poor cold properties. For comparison, in Table 12.4 *Spirulina (Arthrospira) platensis* showed a fatty acid profile with significant amounts of saturated fatty acid chains and polyunsaturated fatty acids was reported.

The observation that palmitic acid is probably the most common fatty acid in algal oils is confirmed by the fatty acid profiles of the oils of eleven blue-green algae identified as *Trichodesmium erythaeum*, *Coccochloris elabens*, *Microcoleus chtonoplastes*, *Nostoc muscurom*, *Anabena variabilis*, *Agmenellum quadruplicatum*, *Plectonema terebrans*, *Oscillatoria williamsii*, *Lyngbya lagerhamii*, *Anacystis marina*, and *Anacystis nidulans* (Parker et al. 1967). Other common fatty acids were palmitoleic and oleic, being present in all oils investigated. Linoleic and linolenic acids were present in most oils, sometimes in moderate (11–19%), sometimes in lower amounts. Myristic and stearic acids were present in all samples, though in most cases in lower amounts. Similarly, palmitic and palmitoleic acids were the major fatty acids observed in another study on the blue-green algae strains of the genera *Synechococcus*, *Aphanocapsa*, *Gloeocapsa*, *Microcystis*, and *Chlorogloea* (Kenyon 1972). The major fatty acids in five marine dinoflagellates (*Scripsiella* sp., *Symbiodinium microadriaticum*, *Gymnodinium* sp., *Gymnodimium sanguineum*, *Fragilidium* sp.) were given as myristic (2.0–6.4%), palmitic (9.0–24.8%), stearidonic (C18:4 ω3; 2.5–11.5%) 18:5 ω3 (7.0–43.1%), 20:5 ω3 (1.8–20.9%) and 22:6 ω3 (9.9–26.3%), with total polyunsaturates ranging from 51.3 to 83.0% (Mansour et al. 1999). *Phaeodactylum tricornutum* and *Porphyridium cruentum* displayed palmitic acid (13.3 and 14.1%, respectively), palmitoleic acid (17.4 and 16.7%, respectively), and C20:5ω3 (35.5 and 32.3%, respectively)

as major fatty acids with more moderate amounts of myristic acid (4.3 and 2.7%, respectively), C16:2ω4 (4.8 and 5.8%, respectively) and C16:3ω4 (2.1 and 6.7%, respectively) present (López Alonso et al. 1998). Diatoms of the genera *Nitzschia*, *Phaeodactylum*, *Amphora*, *Navicula*, *Thallassiosira*, *Biddulphia*, *Fragilaria* contained myristic, palmitic, palmitoleic and C20:5 as the most common fatty acids (Orcutt and Patterson 1975). In a study conducted with six marine microalgae, the strains *Chaeoteceros calcitrans*, *Skeletonema costatum*, *Porphyridium cruentum, Nannochloropsis* sp. (see also Table 12.4) and *Isochrysis galbana* (see also Table 12.4) all contained myristic (6.9–16.8%) and/or palmitic acids (11.1–30.9%) as well as polyunsaturated fatty acids (EPA in four cases, *Isochrysis galbana* and *Tetraselmis suecica* being the exceptions; C18:3 and C18:4 in *I. galbana* and *T. suecica*) as major fatty acids (Servel et al. 1994).

Utilizing the data discussed here, it is of interest to evaluate the algae which likely will provide biodiesel fuels with better properties. It appears that *Chlorella protothecoides* and *Nitzschia ovalis* (under the conditions as given in the corresponding literature references; the issue of restrictions in standards, especially EN 14214, also requires attention) possess more preferable fatty acid profiles than the other strains. Other algae not discussed here may also provide some preferable profiles. However, even the more favorable profiles could likely benefit from further enhancement according to "designer" biodiesel (Knothe 2008).

It may be noted that other issues besides the fatty acid profile of algal oils will likely need further investigation. One such issue is the content of heteroelements of algal oils and how they may transfer to biodiesel. Elements that are limited to low levels in biodiesel standards are Na, K, Ca, Mg, S and P. For example, for biodiesel from *Schizochytrium limacinum*, a level of 69 ppm sulfur was reported (Johnson and Wen 2009), which exceeds the maximum sulfur levels permitted in the biodiesel standards ASTM D6751 and EN 14214.

3 Outlook

Judging by the fatty acid profile information available for algal oils, it appears that biodiesel from this source would not possess advantageous fuel properties compared to biodiesel from other sources. On the contrary, some properties, especially cold flow and oxidative stability may be even more problematic for biodiesel from many alga oils. Hardly any publications address the issue of enhancing the fatty acid profile of algal oils, except for one abstract which mentions optimizing fatty acid composition (Lee et al. 2009b), but no details are reported. Genetic manipulation to enhance lipid production has also been suggested (Roessler et al. 1994; Dunahay et al. 1996). Such an approach may be needed to improve the fuel properties of biodiesel from algal oils. Other issues such as content of heteroelements will also require further study.

Disclaimer: Product names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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Energy Considerations of Photobioreactors

13

Anna Jacobi and Clemens Posten

1 Introduction

Production of microalgae for different purposes has gained rapidly growing interest in the last years represented both as scientific results and as huge amount of money dedicated to new pilot and production plants. In the field of biofuel production the technical breakthrough has not been reached yet (Clarens et al. 2010; Hankamer et al. 2007; Wijffels and Barbosa 2010; Norsker et al. 2011). In a recent feasibility study (Stephens et al. 2010) the authors come to the conclusion that closed photobioreactors are already potentially economically successful in the production of high value products, but their energy consumption has historically made them unsuitable for providing low-value energy products solely, with electrical energy being able to represent over 50% of the harvested chemical energy. While scaling up can contribute to overcoming cost problems, the energy issue has its origin in the single reactor modules itself (Jorquera et al. 2010). Reliable values or precise energy balances are quite rare especially from pilot plants. In cases where such data are provided at least in public talks or personal communications the balance borders or access points of free energy or material flows are often not clearly defined, making upscale calculations impossible. The challenge for reactor optimization goes into two directions, one is to provide optimum physiological conditions for the algae cells to come close to their theoretical photo-conversion efficiency (PCE) of about 9–10% (Tredici 2010), the other is to achieve this with a minimum of auxiliary energy. Even for the next stepping stone in microalgae technology – the production of middle value products for bulk chemicals, food, and feed – the energy issue is critical. A new generation of photobioreactors optimized with respect to productivity and energy usage is required to overcome this problem. In this chapter the different reasons for this high demand of auxiliary energy will be outlined and points for further improvement are considered. However, a vital step forward feasibility could be reached by an integrated approach of biological and technical means.

A. Jacobi • C. Posten (✉)
Division of Bioprocess Engineering, Karlsruhe Institute of Technology, Institute of Engineering in Life Science,
Fritz-Haber-Weg 2, Geb. 30.44, D-76131, Karlsruhe, Germany
e-mail: anna.jacobi@kit.edu; clemens.posten@kit.edu

2 Basic Energy Flows in Photobioreactors

Cultivation of microalgae for the production of bulk materials or fuels implies harvesting of solar energy over large areas, bringing light and CO_2 to the algae cells and maintenance of moderate conditions inside the medium (Borowitzka 1999; Molina Grima et al. 1999; Eriksen 2008; Lehr and Posten 2009). Finally the cells are harvested and have to be further processed. All of these steps require the employment of auxiliary energy and work only with a limited efficiency. The basic task of assessment of the concept "closed photobioreactor" should be, to list these energy streams and try to obtain quantitative knowledge about how far they are inevitable, how far they contribute to productivity, or how far they can be minimized. Energy exchange inside the reactor and with the environment depends strongly on reactor design parameters. Some energy flows are volume related, some scale with reactor surface, while others depend directly on biomass concentration. It is also obvious that the interaction between cell physiology and conditions inside the reactor are the key parameters for reactor optimization and are strongly influenced by directed mass and energy flows.

The energetic efficiency of microalgae production is basically determined by the efficiency of the cells themselves with respect to photosynthesis and cell anabolism. Theoretical values for maximum photo-conversion efficiency have recently been published (Walker 2009; Tredici 2010; Weyer et al. 2010; Zhu et al. 2010). The photobioreactor has to support the cells in coming close to these values by maintaining optimum physiological conditions (Fig. 13.1). The overall energetic efficiency of the process will depend on the

M.A. Borowitzka and N.R. Moheimani (eds.), *Algae for Biofuels and Energy*, Developments in Applied Phycology 5,
DOI 10.1007/978-94-007-5479-9_13,

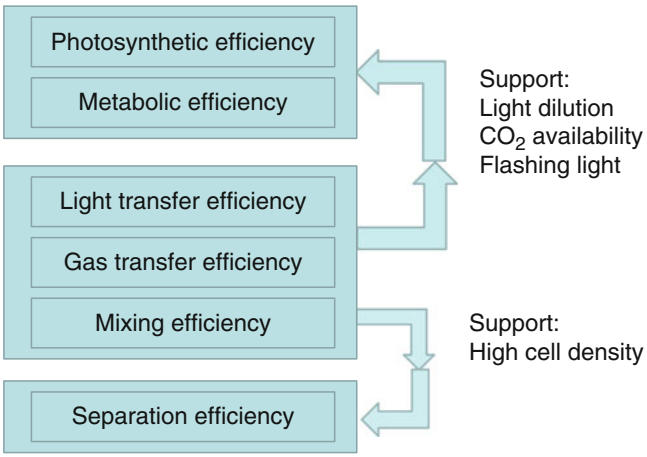

Fig. 13.1 Interactions between reactor performance, cell physiology and downstream processing

energy needed for maintaining these conditions and on engineering means to minimize energy losses by the reactor to achieve this goal. Here some points are mentioned, not only to understand influencing parameters of the upper limit, but also to be aware of accessible engineering means for proper treatment and for energetic considerations.

The gross energy of the whole plant must be provided entirely by incident solar irradiation, which is dependent on the region on the earth because of the latitude and regional atmospheric conditions. In central Europe this is 1,000 kWh m^{-2} $year^{-1}$ = 114 W m^{-2}, and world peak values reach 2,500 kWh m^{-2} $year^{-1}$ = 285 W m^{-2}. Even with the maximum PCE of 9% the maximum areal continuous power output of an algae plant would be 10.3 W m^{-2} for central Europe and 25.7 W m^{-2} best site value (for a world map see Tredici (2010)). For reactor design especially with respect to energetic considerations this span means double areal productivity in sunny regions justifying a bit higher amount of auxiliary energy, but possibly other problems may occur like overheating during day time, what makes water cooling necessary, or lacking infrastructure e.g. for carbon dioxide supply.

Because of the fact that only a small band of the full solar energy spectrum is photosynthetically active (PAR, 400–740 nm), because of relaxation of higher excitation states and energy demand during carbohydrate synthesis, the photoconversion efficiency (PCE) is reduced to 12.6% (Zhu et al. 2008; Zhu et al. 2010). In this context it has also to be mentioned, that the energetic efficiency of the cells depends on the product. This is due to thermodynamics, as many algae have a calorific value of about 20 MJ kg^{-1}, while oil rich algae can reach 30 MJ kg^{-1}. With constant PCE of 5% for example this leads to lower dry biomass values for the oil-rich algae. Furthermore, the formation of oil or other molecules requires ATP-dependent steps, leading possibly to lower PCE values. Reliable measurements are not available, but the present strategies to come to high oil contents with long limited growth phases are probably not energetically profitable.

The portion of dark respiration within the whole algal metabolism differs from species to species, depends on culturing conditions, light intensity and temperature and can therefore only be estimated with difficulty. If we assume a minimum average respiration portion of 20% (Tredici 2010), the PCE would further decrease to 8.8%. Furthermore, the key enzyme of photosynthesis (Ribulose-1,5-bisphosphate carboxylase) shows an oxygenation side-activity at low CO_2 partial pressure, leading to metabolic pathways known as photorespiration (Spalding 1989; Spalding 2008). But as this side-activity is competitively inhibited by CO_2, these losses could eventually be avoided for microalgae cultures by appropriate gassing, keeping CO_2-partial pressure high and keeping O_2 partial pressure low. Also lipid accumulation can be forced by high CO_2 partial pressures (Chiu et al. 2009). Exact physiological values, which would allow for a precise adjustment of dissolved gases according to the physiological demands of different species and growth conditions, are not available yet. A general value of 0.1 kPa CO_2-partial pressure to avoid carbon limitation by *Chlorella* is given by Doucha et al. (2005). This value could be different for different algae strains. In any case, a better physiological performance of the cells means a trend towards higher energy expenditure of the reactor.

Another reason, why the theoretical upper PCE-value of ~9% is not reached in praxis, is that the photosynthesis rate increases linearly with light intensity only at low photon fluxes and stays constant or even diminishes at higher levels (see Beardall and Raven Chapter 5 this book). Typical measurement data for continuous cultivation are presented in Fig. 13.2 (Posten 2009). An optimum reactor operation is only possible when irradiance in normal direction to the reactor fences does not exceed the value of the higher edge of the linear part. This is usually reached by light dilution over the height of the reactor and by arrangement in north/south-direction (Carlozzi 2003). Again, this is a starting-point for elaborate reactor and process design with respect to high light dilution properties by employing large surface to footprint ratios (Posten 2009). A large outer reactor surface means of course higher expenditure for reactor construction and maintenance as well as higher fluid coverage being the medium volume per footprint area. This leads again to a higher specific need for mixing energy.

The effect of photosaturation and photoinhibition can be partially diminished by turbulences inside the medium. Local turbulence caries the cells more or less randomly through well-illuminated volume elements near the glass wall and poorly illuminated reactor zones remote from light incidence, so each individual cell is exposed to statistical dark/light cycles. As soon as the frequency is high enough, e.g. >10 Hz, these cycles support growth. This phenomenon is called the flashing light effect. For the physiological reasons see (Grobbelaar et al. 1996; Wagner et al. 2006), for a recent model-based investigation see (Yoshimoto et al. 2005;

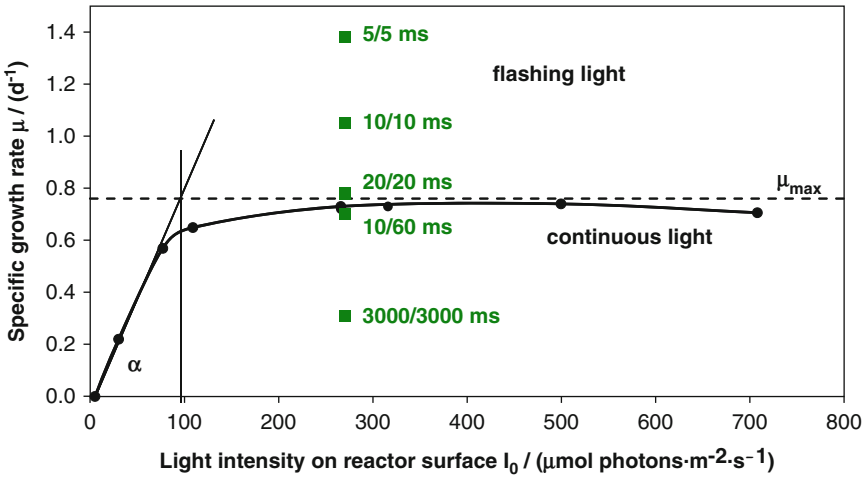

Fig. 13.2 Growth kinetics of *Porphyridium purpureum* in continuous culture; *circles* are measurements under constant light conditions, *squares* are measurements under light/dark-cycles

Barbosa et al. 2003; Janssen et al. 2000). For slow cycles (compare Fig. 13.2) lower growth rates have to be expected. For the energy balance of the reactor this means that a certain mixing energy cannot be avoided.

Similarly, the different energy flows in the photobioreactor, which can be directly influenced by technical means, have to be revisited to define minimum requirements high enough to fulfill the physiological demands of the cells. In order to reach the ceiling of 9% PCE, photobioreactors have to be almost ideal with respect to mass and light transfer. On the other hand, this has to be fulfilled with minimum auxiliary energy and minimum areal-based reactor costs to meet economic efficiency constraints. These conflicting priorities raise different requirements for closed photobioreactors as tools for biofuel production.

Growth of microalgae in closed photo-bioreactors depends on appropriate mass transfer of CO_2 and O_2. The volumetric gas transfer rates in microalgae cultures amount to only a few percent compared to heterotrophic microbial cultivation. Nevertheless, due to the specific geometry of photobioreactors and, due to the rigorous limitations in energy expenditure, it turns out to be a problem. Sufficient mass transfer can be assured by bubbling. In the case of plate reactors or bubble columns, the ascending bubbles assure sufficient mixing at the same time. Here it has to be mentioned that, energetically speaking, bubbling is the most expensive way to mix fluids, as the energy stored in surface tension of the bubbles is lost. In most engineering applications like microbial cell cultivation in bubble columns the energy aspect does not play a role, but in microalgae cultivation for biofuel production it has to be accounted for. To achieve an appropriate degree of aeration and mixing, energy inputs of about 15–50 W m^{-3} are reported (Sierra et al. 2008). Values in the same order of magnitude (estimated) or higher can also be found (Camacho Rubio et al. 2004; Chini Zittelli et al. 2006; Merchuk et al. 2007a, b). In addition, good mixing substantially helps to prevent the cells from staying too long in dark or bright zones of the reactor. Thus, a thorough understanding of fluid dynamics is needed for reactor optimization and assessment (Luo and Al-Dahhan 2004). A rigorous mathematical analysis of the relation between productivity and hydrodynamics is given by Pruvost et al. (2008). The results indicate a direct relationship between performance and energy expenditure. To break up such undesired constraints technology changes will be necessary, here a decoupling of gas supply and mixing.

In any case, the photon transmission efficiency has to be close to 100%, which means reflection on the surface and adsorption on support frames or other reactor equipment has to be avoided. Values of up to 30% depending on design and local conditions are under discussion. Future reactor designs should minimize these losses by an appropriate choice of the material or by arrangement of the panels to capture reflected light of one panel by the next one. From energetic and engineering viewpoint, here the big energy flux of 40% of infrared (IR) light has to be somehow used in the future and not only converted to heat.

All algae strains show a temperature profile of growth with a distinct optimum (Baumert and Petzoldt 2008). Heat exchange with the environment is therefore a factor to be seriously considered. The impact of temperature control on the energy balance of the process is highly dependent on the reactor system used, the algae strain, and most of all the geographical location of the plant. While 95% of the areal specific light energy is converted to heat, the reactor surface exchanges heat energy with the surrounding air. A high surface to footprint ratio helps to keep the temperature at the ambient temperature which is useful especially in cool locations of high altitude. Cooling in regions of high irradiation is a major issue. Spraying with cooling water to the outer wall of the reactor is usually necessary in summer even at locations in central Europe. Therefore cooling water which

Table 13.1 Hydrodynamic parameters of tubular photobioreactors

Reactor type	V_R [m^3]	l_R/ d_R [m^3]	u_F [m s^{-1}]	P_W [W m^{-3}]	k_La [s^{-1}]	$c_{O2,max}$ [%]	Reference
Horizontal tubular, air lift	0.200	80/0.06	0.5	–	0.007	300	Acién Fernández et al. (2001)
Helical, air lift	0.075	106/0.03	0.28	2,000	0.003	280	Hall et al. (2003)

has to supplied may be used from remote places and has to be pumped through the nozzles. But also heating in cool temperate climate zone regions could improve overall productivity especially in spring, when irradiation is already high but temperature is still too low for high growth rates. Here again a lack in reliable data has to be deplored both for the technical expenditure of temperature control and the temperature dependent energetic efficiency of the cell growth itself.

Furthermore, energetic costs in the peripheral processes have to be accounted for. Supply of CO_2, nutrients, and water from possibly remote resources are critical points in the upstream part of the process, while solid/liquid separation or waste water disposal are main contributors to energy losses in the downstream part. These points cannot be calculated independently from the phototrophic process itself, as process intensification e.g. by reaching high biomass concentrations in the reactor, can lower the peripheral energy fluxes remarkably. Current economically operated microalgae production plants use often energy and material flows from the specific environment of the production site. That could be a waste water stream to deliver nutrients or heat.

3 Situation in Recent Reactors Designs

Tubular reactors have proven their viability for many years. Actually the world's biggest closed photo-bioreactors are tubular reactors. The *Haematococcus* plant in Kibbutz Ketura, Israel, with about 1 ha overbuilt with pipes (Algatechnologies Ltd 2010) is only a bit outnumbered by the plant in Klötze, Germany, with 500 km glass pipes on 1.2 ha holding about 600 m^3 of algae suspension (Algomed 2011). A new plant was built in 2011 by the IGV in Jerez, Spain. With 85 m^3 of culturing volume in 35 km glass tubes this is the world largest closed algae production plant with a footprint area of 1,000 m^2 (IGV GmbH Potsdam 2011). The excellent light distribution is owed to the fact that two dimensions, namely the two radial directions of the tube, are used for light transfer, while mass transfer has to be guaranteed by convective flow in axial direction, which is the scaling axis as well. The flow can be regarded in first approximation as pure plug flow. That means that volume elements are treated as single batch reactors underlying their own reaction dynamics while moving through the tube. Backward and forward mixing is present, but small compared to the tube length (Camacho Rubio et al. 1999). However, that is the weak point with respect to energy efficiency. While the single volume elements flow through the reactor, CO_2 is used up and oxygen accumulates. Due to the dualism between the time scale and the spatial scale, axial stationary gradients of c_{CO2}, c_{O2}, and pH build up. While efficient supply of a carbon source is important, oxygen build-up is the greatest problem due to toxicity and limits scalability. To avoid such strong gradients axial fluid velocities between 0.1 and 0.4 m s^{-1} are usually chosen. These high velocities are additionally necessary to guarantee for high mixing quality in radial direction and support flashing light effect (Acien Fernandez et al. 2003; Babcock et al. 2002). This is occasionally supported by employing static mixers (Ugwu et al. 2005). High axial velocities and high radial dispersion coefficients have their costs in form of high pressure losses in axial direction, which have to be compensated by mechanical energy (Table 13.1) and allow only for production of algae which are not very sensitive to shear stress.

In practical installations (compare Table 13.1) power consumption between 400 and 2,000 W m^{-3} are reported (Acién Fernández et al. 2001; Hall et al. 2003; Norsker, 2011). While tubular reactors are often installed in high fences, the water coverage is with typically 200 L m^{-2} quite high, leading to an area specific power consumption of >100 W m^{-2}. This value comes close to the power density of the incident light and exceeds several times the highest possible gains of chemical energy bound in the biomass even in sunny areas. Lower flow rates would actually reduce pressure losses, but would also reduce the maximum tube length making additional technical measures necessary. Molina Grima et al. (2000) give an elaborated scale up criterion based on the light/dark cycles which are evoked by velocity dependent turbulences. In this example it becomes evident that increasing productivity has to be gained by increasing energy expenditure.

In contrast to the negative energy balance the good performance criteria are undisputed. The good results for tubular reactors are also underlined by Pulz (2001) and Carvalho et al. (2006). They found a better performance compared to bubble columns. However, the high energy consumption is surely one of the major drawbacks. While power consumption and high cost exclude tubular reactors for production e.g. of pure energy products, the possibility for monoseptic operation and the well-defined illumination conditions inside the reactor, make them a good choice for production of high value compounds.

The flat plate reactors are surely the most robust designs (Richmond et al. 2003; Zou and Richmond 2000). Roughly speaking, two transparent plastic sheets have to be glued

Fig. 13.3 CFD simulation of a plate photobioreactor of 1 m height; even moderate aeration leads to sufficient axial mixing ($T_{mix} \approx 100$ s) as indicated by the *streamlines*

together to make a flat plate reactor with any desired light path length in the range from a few mm up to 70 mm. Thereby surface to volume ratios of up to 200 m^{-1} for one single plate and about 50 m^{-1} for practical installations are reached. Reactors with considerable costs are available like the "Green Wall Panel" (Tredici and Rodolfi 2004; Rodolfi et al. 2009). Flat panel reactors work with respect to mass transfer similarly to bubble columns (Sánchez Mirón et al. 2000; Oncel and Sukan 2008), with the vertical axis being the main transport and scaling axis. While this reactor design has already been employed for decades, hydrodynamic parameters have been studied intensively only in the last years. A recent comprehensive process engineering characterisation is available from Sierra et al. (2008). Mixing and CO_2-supply is accomplished by sparging with CO_2-enriched air. For the pilot scale example reactor (0.07 m wide, 1.5 m height, 2.5 m length) the authors report air flow rates of 0.25 v v^{-1} min^{-1} leading to an axial mixing time of the medium of 150 s – in the range of residence times of tubular reactors – what is enough to prevent formation of strong axial gradients, compare Fig. 13.3. Even under low aeration rates ($v_g = 0.004$ m s^{-1}) the flat plate behaves as a mixed-tank system, which means that it can be characterized by dispersion coefficients and tank cascade considerations are not necessary. Considering the CO_2-demand of the cells, such low aeration rates should be high enough for sufficient mass transfer.

Power supply for bubbling is typically in the range of 50 W m^{-3} and even in quite compact arrangements of several modules close to each other (Chini Zittelli et al. 2006) an areal specific power supply of 2 W m^{-2} is required. This means that the auxiliary energy demand could eventually count up to one third of the possible chemical energy finally harvested (see Table 13.2). But also in flat plate bubble reactors there are some drawbacks with respect to energetic efficiency. Some authors (Brindley Alías et al. 2004; Wang et al. 2005) have reported much higher aeration rates up to 2.0 v v^{-1} min^{-1} with positive effects on biomass productivity. The reason is not yet clear, but probably the bubbles contribute to a better light penetration inside the medium and to a better fluid dispersion in the normal direction to the plates, what means along the light gradient, thus supporting the flashing light effect. Even a low gas hold-up of 1% leads to changes of 10% in the internal irradiance in comparison to a gas-free operating system (Sánchez Mirón et al. 1999). The authors observe that for higher aeration rates the bubble size has a significant influence on light penetration and shear stress. So here is a point of further research to find out, under which conditions optimum productivities can be achieved at low aeration rates. Finally, a decoupling of mixing and gas supply could turn out to be necessary for low energy reactors.

The height of pneumatically agitated reactors is usually chosen as variable parameter for increasing the volume of a single installation with high light dilution properties. Nevertheless, this increases the specific power consumption of the system due to a higher hydrodynamic pressure against which the bubbles for aeration have to be generated. Furthermore, a higher superficial gas velocity is necessary. That can be understood from the fact that carbon dioxide has to be transported through the lower part of the reactor to supply the upper part, and removed oxygen from the lower part has to be transported through the upper part. Strictly speaking, the axial dispersion coefficient has to be higher. Besides employing the airlift effect to overcome this problem, flat plate reactors could be developed towards a "low-ceilinged" design with small vertical dimension. For harvesting the sun light of large areas of land, such reactors have to be aligned very close to each other. An early example of such kind of horizontal plate reactor has been invented by Sato et al. (2006). The light distribution is achieved in this case by deformation of the plate to cylinders or spheres. This is one of the rare examples, where a design has been conducted from scratch by CFD simulations. The performance has been claimed to be more than $P_G > 20.5$ g m^{-2} day^{-1} for one device and it is mentioned, that the overall productivity for an area with several domes is only half of that value. Such specifications are missing in many other publications.

A reactor type, which has already been patented and characterized (Degen et al. 2001), attempts to the increase of the longitudinal mass transfer and mixing in normal direction to

Table 13.2 Hydrodynamic parameters of bubbled photobioreactors

V_R [m^3]	$w_R/h_R/l_R$ [m]	v_G [v v^{-1} min^{-1}]	u_G [m s^{-1}]	E [–]	t_M [s]	k_La [s^{-1}]	P_E [W m^{-3}]
Flat Plate (low values), (Sierra et al. 2008)							
0.25	0.07/1.5/2.5	0.15	0.00357	0.008	105	0.0025	15
Flat Plate (high values), (Sierra et al. 2008)							
0.25	0.007/1.5/2.5	0.32	0.0076	0.018	150	0.0063	53
Bubble column, (Camacho Rubio et al. 2004)							
0.06	0.193/2.3	–	0.03	–	60	–	–
Inclined airlift, (Merchuk et al. 2007a, b)							
–	0.14/2.12	–	0.01	0.005	–	0.004	–
Annular reactor, (Chini Zittelli et al. 2006)							
0.12	0.045/0.5/1.9	0.23	0.007	0.020	–	0.010	–

Abbreviations
$c_{O2,max}$ [%] maximum concentration of O_2
d_R [m] diameter, reactor geometry
h_R [m] height, reactor geometry
E [–] gas holdup
k_La [s^{-1}] mass transfer coefficient
l_R [m] length, reactor geometry
P_E [W m^{-3}] specific power input
P_W [W m^{-3}] specific power input
t_M [s] mixing time
u_G [m s^{-1}] superficial gas velocity
u_F [m s^{-1}] liquid velocity
V_R [m^3] reactor volume
v_G [v v^{-1} min^{-1}] gassing rate
w_R [m] width, reactor geometry

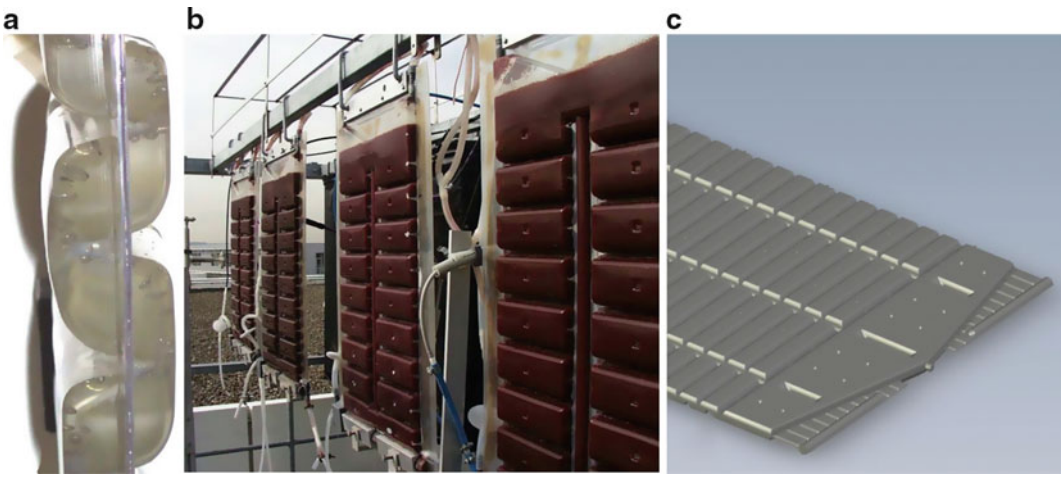

Fig. 13.4 The flat panel airlift reactor as built by the company Subitec GmbH, with kind permission of P. Ripplinger (Subitec GmbH 2010)

the plate using a purely pneumatic energy regime (Fig. 13.4). In this so-called flat panel airlift reactor, a directed flow is generated using the airlift principle. Cylindrical eddies are evoked using horizontal baffles as static mixers located in the interior of the panels at right angles to the flow, thus transporting the algae in a highly defined frequency from the dark to the light area. The baffles act also as light conducting structure. The individual reactor modules consist of deep-drawn PVC panels with module sizes of 35–180 L. The authors claim that in this reactor type not only a random mixing but a regular mixing pattern has been applied for the first time. That should improve productivity on the one hand and reduce required mixing energy on the other. Further activities are under development by the company Subitec, Stuttgart (Subitec GmbH 2010) into the direction of reducing specific power input to 200 W m^{-3}. According to company information, working with *Haematococcus pluvialis* and *Phaeodactylum tricornutum*, concentrations of over 10 g L^{-1} and average volumetric productivity of over 0.5 g L^{-1} day^{-1} were achieved where PCE was specified as 4.75% under Central-European

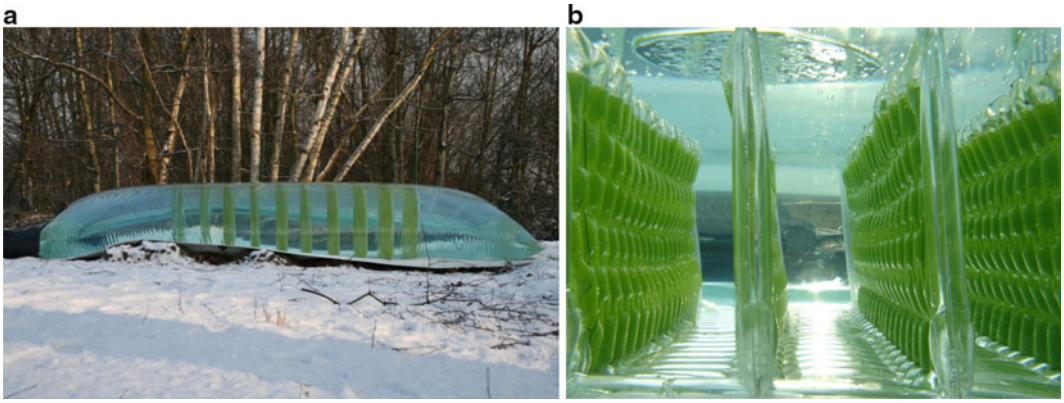

Fig. 13.5 Reactor module of the ProviAPT technology from the Proviron company (Proviron 2010)

outdoor conditions for full sunlight spectrum. A 100 m^2 demonstration plant is in operation, with an estimated fluid volume to footprint area of 30 L m^{-2}. So the good performance is achieved with an energy expenditure of about 6 W m^{-2}, which is still too high for pure energy production, while an economical operation for the production of high and middle price products is proven, leaving biomass residues for energy purposes.

4 Process Integration and Further Developments

In a recent study (Wijffles 2010) the authors conclude that even in large (hypothetical) algae production plants the need for auxiliary energy will make up 42% of the costs. A positive energy balance still needs to be reached. Other authors calculate the amount of auxiliary energy needed to at least 30% only for mixing and aeration. This is especially unacceptable, since this energy is in contemporary plants gained from electricity, thus an energy form of much higher value per MJ with respect to entropy and costs. However, this is not the last word, as many options for remarkable improvements are not exploited yet. The energy balance of the reactor is closely linked to the energy balance of the downstream part of the process and to energy flows in the environment. These factors can be positively influenced by an adept employment of additional technical means, by applying rigorous engineering tools for reactor design, and by carefully integrating the plant into the specific situation of the operation site. Some examples will be given in the next paragraphs.

The first step for reactor improvement with respect to energy saving will be to consider the findings of hydrodynamics (Perner-Nochta and Posten 2007) and light transfer, namely to go for designs with small vertical dimension and short light-path lengths (compare also Fig. 13.3). Further cost reduction should be approached by saving costly support frames of the single modules (Willson 2009). This idea has been followed by the company Proviron (2010) in the ProviAPT design. Each reactor module is one big translucent plastic bag, which contains multiple vertical panes of 1 cm thickness (see Fig. 13.5) mounted in an artificial pond, with the surrounding water acting as a scaffold, temperature regulation and light diffuser at the same time. The reactor can be unrolled from a big coil without any additional supports. The company claims to reach typically 5 g L^{-1} up to 10 g L^{-1} biomass concentrations due to this small light path length. The investment is stated to be 200 k€ ha^{-1} (= 20 € m^{-2}) and the need for auxiliary energy to be 20 kW ha^{-1} (= 2 W m^{-2}). This would be approximately 30% of the expected chemical energy for a site in Middle Europe. Even lower values are prospected. A pilot plant started operation, a larger facility is planned.

Productivity data are not available yet. The weight of the plastic bags is given as 2.5 kg m^{-2}. Although this value is probably smaller than in other designs the CO_2-footprint for production has a value of about 1.5 kg m^{-2}. Assuming a high CO_2-fixation rate of 50 g m^{-2} d^{-1} by the microalgae cultures, it takes 30 days to fix the same amount of CO_2 by the algae. Assuming the same calorific value for plastic and microalgae calculation with this same example leads to 100 days to fix enough sun energy to pay it back. In any case the energy stored in the reactor material contributes to the energy balance in the life cycle analysis.

The photobioreactor has to provide ideal conditions for the microalgal cells with respect to a desired physiological state under the constraints of incoming light or other given external parameters. This can be done by measurement of physical conditions inside the medium and controlling technical variables like gas supply. Two pO_2, pH and pCO_2 sensors along the main reactor axis – this means along the strongest gradient – should be mandatory. However, the topic of measurement and automation is a bit neglected but could be implemented even in current installations. As the cells are the one and only reason for maintaining the process, online measurement of optical density (OD) and fluorescence

(PAM) (Gardemann 2005) can help to assess the physiological state and react with online optimization of mixing, gassing, or diluting thus reducing aeration rates and improving productivity. Other options from process operation strategy which have been proposed and applied already are reduction of aeration in the night to supply only the necessary oxygen or harvesting in the afternoon to minimize losses by respiration in the night. The effect of these means could be roughly estimated to save 20% of the spent auxiliary energy.

In the "water bed reactors" of Solix Biofuels a series of water embedded synthetic bags is provided, where the surrounding water similarly as in the Proviron reactor can attenuate day/night temperature changes. This concept could be even improved by employing so called phase changing materials (e.g. PCM from BASF (2010)). These materials are commercially produced in the paper hangings for flats to control the room temperature at the given value of phase transition. For photo-bioreactors that means temperature control not only at the day/night average but at an adjustable value. Another technical approach to reduce the heating problem is the avoidance of IR radiation. IR reflecting glass or plastic is already available and is used to reduce heat in parked cars or to reduce heat radiation from light bulbs. The most efficient ways of course would be to make directly use of this energy.

One step in this direction is the plant of Algenol (2011) (Radzinski 2010) where the heat is directly used to evaporate the produced ethanol from the algae cultivation, thus avoiding any costs for solid/liquid separation. The company claims to reach already over 5% PCE and will come close to 10% in a projected pilot and production plant. Hydrogen or extracellularly produced hydrocarbons could be other options to avoid costs and energy expenditure during cultivation and downstream operation.

Membrane gassing is one concept to reduce auxiliary energy (Fan et al. 2007; Cheng et al. 2006) as is done in animal cell cultures, in that case to reduce shear effects of the bubbles to the cells. Dissolving of CO_2 is no longer effected at the boundary layer between gas bubbles and the culture medium and gassing and mixing are decoupled, what gives more degrees of freedom in process operation. Instead, a gas permeable membrane creates the surface for CO_2 dissolving and O_2 removal directly at the gas intake. This saves the energy for the formation of gas bubbles which is lost when the bubble collapses at the top of the reactor. It also lowers the loss of CO_2 via the off gas. This results from the homogenous gas supply over large parts of the reactor surface. However, by using membrane gassing, gas dispersion from the membrane to the opposite side of the reactor will still require soft agitation. The already mentioned reactor from Solix will be equipped with membrane gassing.

For the time being microalgae plants for energy production can use energy flows from the environment to support a positive energy balance. Examples are the cultivation in waste water streams, where the kinetic energy is already there and an additional value – the cleaned water – is obtained. Cooling water from power stations could be used for temperature control. Actually, low temperature heat is available in industrial regions in excess. But there are more specific examples. Van Aken (2010) described a plant for production of diatoms as fish food. Water from a natural stream is let over the culture, thus saving energy and costs for mixing and mineral supply. The cells themselves are attached to carries, which are simply drawn out of the water for harvesting. So, only 3–5% of water has to be processed. Using this periphyton goes beyond the normal process engineering paradigm that cells should be suspended as good as possible. The integration aspect includes using environmental circumstances and solving technical problems on the biological level.

Reactor design and operation mode have also a direct influence on energy cost of the downstream operations, especially the solid/liquid separation as the first step. Three general principles exist: floatation, filtration, and sedimentation based methods. Flotation is actually the cheapest method and is applied in some algae companies. It relies on a hydrophobic interaction between cells and gas bubbles. Concentrations up to 200 g L^{-1} dry mass can be reached with 0.2–0.3 kWh electrical energy per kg algae dry mass. Sedimentation separates particles according to a difference in specific density, while with filtration particle size and surface play an important role. Solid–liquid-separation of organic matter is already standard in large scale. Baker's yeast is concentrated with separators and filter units to dry mass contents up to 35%. Also blue-green algae for food are harvested and dewatered by screens and spray-drying (Earthrise Nutritionals LLC 2009). But other than for food or high value products, for biofuel production constraints are much tighter in respect to energy demand for the separation process, while standard techniques have been highly developed in a way that it is unlikely to achieve a major drop in the energy demand of the separation principle as such. Employing centrifugation, the rotational speeds sufficient for algae separation in a small scale disk stack centrifuge consumes approximately 5 kWh m^{-3} at 1 m^3 h^{-1} (manufacturer communication), scale up may lower the energy demand to 1–3 kWh m^{-3} (Molina Grima et al. 2003). Assuming a dry biomass concentration of 2 kg m^{-3} this means that for a realistic average value of 2 kWh m^{-3} an energy expenditure of about 3.6 MJ kg^{-1}. Even lower values are claimed by Evodos B.V. (2011). Filtration requires less power than centrifugation, but the success of filtration of microalgae depends strongly on the properties of the strain and the type of membrane (Rossignol et al. 1999). Membrane fouling, pressure drop and the required filter surface must be considered when applying filtration for microalgal separation. Recent developments are belt filtration or dynamic shear field filtration (ANDRITZ KMPT

GmbH 2011). Energy costs of separation depend beside on residual moisture mainly on the amount of water running through the devices. From these facts it is clear that the solution for the energy problem cannot be found in developing new separation techniques, but in increasing the biomass concentration in the photobioreactor. In closed bioreactor systems 20 g L^{-1} dry biomass concentrations are already reached or at least projected. Beside the energetic advantages during cultivation, this would reduce the amount of water going to the separation unit remarkably with the corresponding energy savings.

5 Conclusions

Although economically viable, photobioreactors are in the present form not able to perform with an energy balance good enough for biofuel production at large scale. Accounting for all energy fluxes from the reactor over downstream to logistics an only slightly positive balance can be obtained. Nevertheless, current activities show that commercial breakthrough has already been done in specific cases or can be expected in the next few years. This is achieved by making clever use of environmental conditions and by changing the biological and technical paradigms. However, also for 'classical' biomass or biodiesel production with suspended cells, the potential for considerable steps forward has been shown. The final goal of biofuel production with a highly positive area-specific energy balance and comparable cost to fossil fuels can be reached by exploiting this potential. Among the technical means to do so are the decoupling of mixing and gassing using membranes, restriction of mixing to some desired frequencies, achievement of high biomass concentrations, making use of the IR part of the spectrum, and of course by employing all kind of synergisms with the natural and technical environs of the plant. This will empty into specifically parameterized designs for optimum combination of specific cells, products, and sites.

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Greenhouse Gas Balance and Algae-Based Biodiesel

14

Anne Flesch, Tom Beer, Peter K. Campbell, David Batten, and Tim Grant

Abbreviations

A$	Australian dollars, average value across the 2008–2009 financial year.
CED	Cumulative Energy Demand – method used in life cycle analysis to calculate the total (primary, HHV) energy used.
CH_4	Methane – a greenhouse gas released naturally during the anaerobic breakdown of organic material, especially in wet environments.
CO_2	Carbon Dioxide – the primary greenhouse gas.
CO_2-e	Carbon Dioxide equivalent units. Indicates the amount of global warming that would result from the introduction of a certain mass of carbon dioxide over a 100 year timescale. Also see GWP.
CSIRO	The Australian Commonwealth Scientific and Industrial Research Organisation.
GHG	Greenhouse Gas – a gas which when released to the atmosphere is believed to contribute to global warming and climate change.
GWP	Global Warming Potential. A measure of how much a given mass of greenhouse gas contributes to global warming, relative to CO_2 (which is by definition 1). Kyoto Protocol values are used in this chapter, i.e. 21 for methane and 310 for nitrous oxide.
ha.a	hectare.annum – an area of land used for a given purpose over a certain number of years. For example, 10 ha.a could indicate the occupation of 10 ha of land over 1 year, 5 ha over 2 years, etc.
HHV	Higher Heating Value – the amount of energy released during fuel combustion once the products have returned to a temperature of 25 °C. This takes into account the latent heat of vaporisation of water.
IPCC	Intergovernmental Panel on Climate Change.
JCU	James Cook University – a public university based in Townsville, Queensland, in Australia.
LCA	Life Cycle Assessment – a study of the total impacts from 'cradle to grave' resulting from the supply of a given product or service.
LHV	Lower Heating Value – as with HHV, but the cooling process is stopped at a temperature of 150 °C, meaning the heat embodied in water vapour is lost. This is typically the case in vehicles, where the water vapour is lost to the atmosphere via the tailpipe.
NO_2	Nitrous Oxide – a greenhouse gas emitted from the breakdown of animal dung and urine in soil, as well as nitrogen fertilisers in wet environments.
PBR	Photobioreactor – a device for growing plants or organisms (especially algae) that admits light, but otherwise operates with a system closed to the environment (no direct exchange of gases or water, generally).
SETS	Solar Environmental Tube System – sausage-shaped plastic PBRs used by Victorian company in one of the scenarios examined.
t.km	Tonne-Kilometre – a unit to measure the quantity of freight transportation. For example, 20 tkm

A. Flesch
Veolia Environnement, Paris, France
e-mail: Anne.FLESCH@veolia.com

T. Beer (✉)
Transport Biofuels Stream, CSIRO Energy Transformed Flagship, PB1, Aspendale, VIC 3195, Australia
e-mail: tom.beer@csiro.au

P.K. Campbell
Information Technology Resources, University of Tasmania, Private Bag 120, Hobart, TAS 7001, Australia
e-mail: P.K.Campbell@utas.edu.au

D. Batten
Low Cost Algal Fuels, CSIRO Energy Transformed Flagship, PB1, Aspendale, Vic 3195, Australia
e-mail: david.batten@csiro.au

T. Grant
Life Cycle Strategies,
125 Swanston Street, Melbourne, Vic 3000, Australia
e-mail: tim@lifecycles.com.au

M.A. Borowitzka and N.R. Moheimani (eds.), *Algae for Biofuels and Energy*, Developments in Applied Phycology 5,
DOI 10.1007/978-94-007-5479-9_14, © Springer Science+Business Media Dordrecht 2013

could indicate 20 t of freight transported 1 km, 10 t transported 2 km, etc.

ULS Ultra Low Sulfur – fuel (generally diesel, ULSD) containing under 50 ppm sulfur.

wos Wildly optimistic scenario – a scenario where every variable results in the best result possible (in this case, resulting in maximal algal growth).

XLS Extra Low Sulfur – fuel (generally diesel, XLSD) containing under 10 ppm sulphur. In Australia legislation requires diesel fuel to be XLS from 1 January 2009.

1 Introduction

Concerns about energy security, climate change, and soaring oil prices have driven people to develop alternative energy sources that should allow them to break their dependence on foreign oil. Algae have attracted attention due to their potential in the production of feedstock for biofuels and chemicals (Chisti 2007, 2008; Ratledge and Cohen 2008; Stephens et al. 2010; Brennan and Owende 2010).

Microalgal biofuels are a potential energy source that can reduce GHG (greenhouse gas) emissions. But whether this potential is, or is not, realised depends on the exact nature of the production system that is used. To undertake a proper carbon balance of a biofuel production system it is necessary to use life cycle analysis and to then examine the results and determine their acceptability, which transforms an analysis into an assessment. This chapter discusses the use of life cycle assessment (LCA) in relation to algal biofuels by first of all describing life cycle assessment (LCA) as a process that considers the whole process chain from biomass production to the biodiesel combustion.

The chapter continues with an example in which the methods of life cycle assessment are used to analyse the potential greenhouse gas emissions and energy balance of biodiesel production from microalgae. The design chosen in this study focuses on commercial scale, plastic-bag reactors and the biofixation of CO_2 from a nearby power station to assist in the growth of the algae. Eight different scenarios involving photobioreactors are examined that involve two methods for harvesting the algae (mechanical harvesting and chemical harvesting by flocculant); two methods for oil extraction (solvent extraction and high pressure extraction), and also two end-uses for the algal cake that remains after oil extraction (animal feed and production of energy through methane combustion after anaerobic digestion). The scenario results are compared with previous scenarios of biodiesel from microalgae grown in ponds, and also with diesel and canola biodiesel production.

Enclosed photobioreactors and open ponds are the two predominant methods for growing algae (Ratledge and Cohen 2008; Schenk et al. 2008). Each has advantages and disadvantages; the major difference between the two is the trade-off between cost and control. Closed systems allow better control of the cultivation conditions than open systems; better biomass productivities are obtained and contamination can be prevented more easily (Chisti 2007). The higher concentration of the biomass also decreases the energy required for the harvesting.

However, most studies refute the economic feasibility of photobioreactor microalgae culturing for biofuels production (Borowitzka 1992), and, as yet, no viable commercial-scale algal biofuel project exists. Photobioreactor designs vary widely, but there is still room for improvement so as to make systems simple and cheap enough to be scaled up while maintaining higher productivity and control over the culture than ponds allow. Even though some of the key costs are the energy requirements involved in algal harvesting, water circulation, heating and cooling (if needed), the use of cheap materials such as plastic offers the potential to improve the economics and the environmental impacts of the process.

In addition, the development of technologies to control the emission of anthropogenic greenhouse gases has become critical to ensure the integrity of the biosphere and, ultimately, the future of our climate. Recently, there has been renewed interest in microalgae biofixation of carbon dioxide. Many reports deal with the potential and bio-economics of algal biomass to generate fuels and most of these are based on the premise that one would utilise the CO_2 emitted from fossil-fuelled power stations or other industrial sources of CO_2 (Benemann 1993, 1997; Hughes and Benemann 1997; Vunjak-Novakovic et al. 2005; Greque de Morais and Costa 2007; Ratledge and Cohen 2008), because biomass generation can be dramatically stimulated by the presence of additional levels of carbon dioxide.

Irrespective of the choice of reactor design, all studies agree that an integrative approach where energy requirements are kept low and co-products are generated is necessary for either ponds or photobioreactors to be economic (Chisti 2007).

This chapter examines the GHG (greenhouse gas) balance, namely the emissions and environmental impacts, of the production of biodiesel from microalgae. Such an assessment needs to be based on a Life Cycle Assessment (LCA). The design chosen in this study focuses on plastic-bag reactors and the biofixation of CO_2 (carbon dioxide) from a nearby power station. It also deals with an integrative approach with use of the co-products. Table 14.1 lists all of the scenarios tested in this analysis. Scenarios 1–6 deal with the production of microalgae in photobioreactors (Tredici 1999). Scenarios 7 and 8 use the results of Campbell et al. (2011) in relation to production in ponds. Scenarios 9 and 10 provide the baselines for comparison by examining diesel and canola biodiesel production.

Table 14.1 Scenarios for the production of diesel/biodiesel tested in this study with details of harvesting and extraction methods and use of the algal cake

Scenario number	Scenario name	Algae harvesting method	Oil extraction method	Use of the algal cake (co-product)
1	**PBR**, mechanical harvesting and extraction, 175 t.ha^{-1} year^{-1}	Mechanical, Centrifuge	Mechanical extraction (high pressure)	Waste
2	**Worst case scenario PBR**, mechanical harvesting and extraction, 109 t.ha^{-1}.year^{-1}	Mechanical, Centrifuge	Mechanical extraction (high pressure)	Waste
3	**PBR**, mechanical harvesting and extraction, +cake for animal feed, 175 t.ha^{-1}.year^{-1}	Mechanical, Centrifuge	Mechanical extraction (high pressure)	Animal feed (avoided product lupin meal)
4	**PBR**, Victorian commercial project, mechanical harvesting and extraction, +cake for animal feed, 375 t.ha^{-1}.year^{-1}	Dewatering technology including centrifuge	High pressure device	Animal feed (avoided product lupin meal)
5	**PBR**, flocculation/solvent harvesting and extraction, 175 t.ha^{-1}.year^{-1}	Flocculation	Solvent	Waste
6	**PBR**, flocculation/solvent harvesting and extraction, +anaerobic digestion, 175 t.ha^{-1}.year^{-1}	Flocculation	Solvent	Energy Production (anaerobic digestion)
7	**Ponds**, flocculation/solvent harvesting and extraction, 1 09 t.ha^{-1}.year^{-1}	Flocculation	Solvent	Waste
8	**Ponds**, flocculation/solvent harvesting and extraction, +anaerobic digestion, 109 t.ha^{-1}.year^{-1}	Flocculation	Solvent	Energy Production (anaerobic digestion)
9	**Diesel (ULS)**	N/A	N/A	N/A
10	**Canola biodiesel**	N/A	Solvent	Animal feed (avoided product lupin meal)

PBR Photobioreactors

The greenhouse gas balance on its own does not guarantee a sustainable operation. The economic and social aspects of the photobioreactors scenarios are also taken into consideration. A comparison of the different photobioreactors scenarios with pond systems and diesel is also undertaken. This transforms the greenhouse gas balance study to a triple bottom line study by linking the potential environmental benefits of the process to the reality of economics.

2 Life Cycle Assessment Overview

A general introduction to life cycle analysis may be found in Horne et al. (2009), while the international standards on LCA, contained in the 14040 series (International Standards Organisation 2006) provide a basic framework in which to undertake LCA. When LCA is applied to the emissions from the use of different transport fuels, both combustion and evaporative emissions need to be included, as well as the full life cycle of the fuel (Beer et al. 2002). A full life cycle analysis of emissions takes into account not only the direct emissions from vehicles (which are referred to as downstream emissions) but also those associated with the fuel's: extraction, production, transport, processing, conversion, and distribution. These are referred to as upstream emissions. In the context of automobile fuels they are also referred to as pre-combustion emissions.

3 System Boundary for LCA

In this study not all elements of the production system are included in the study. Some of the reasons that some elements are excluded from the study are:

- the process is considered small enough to ignore given the aims of the study, and

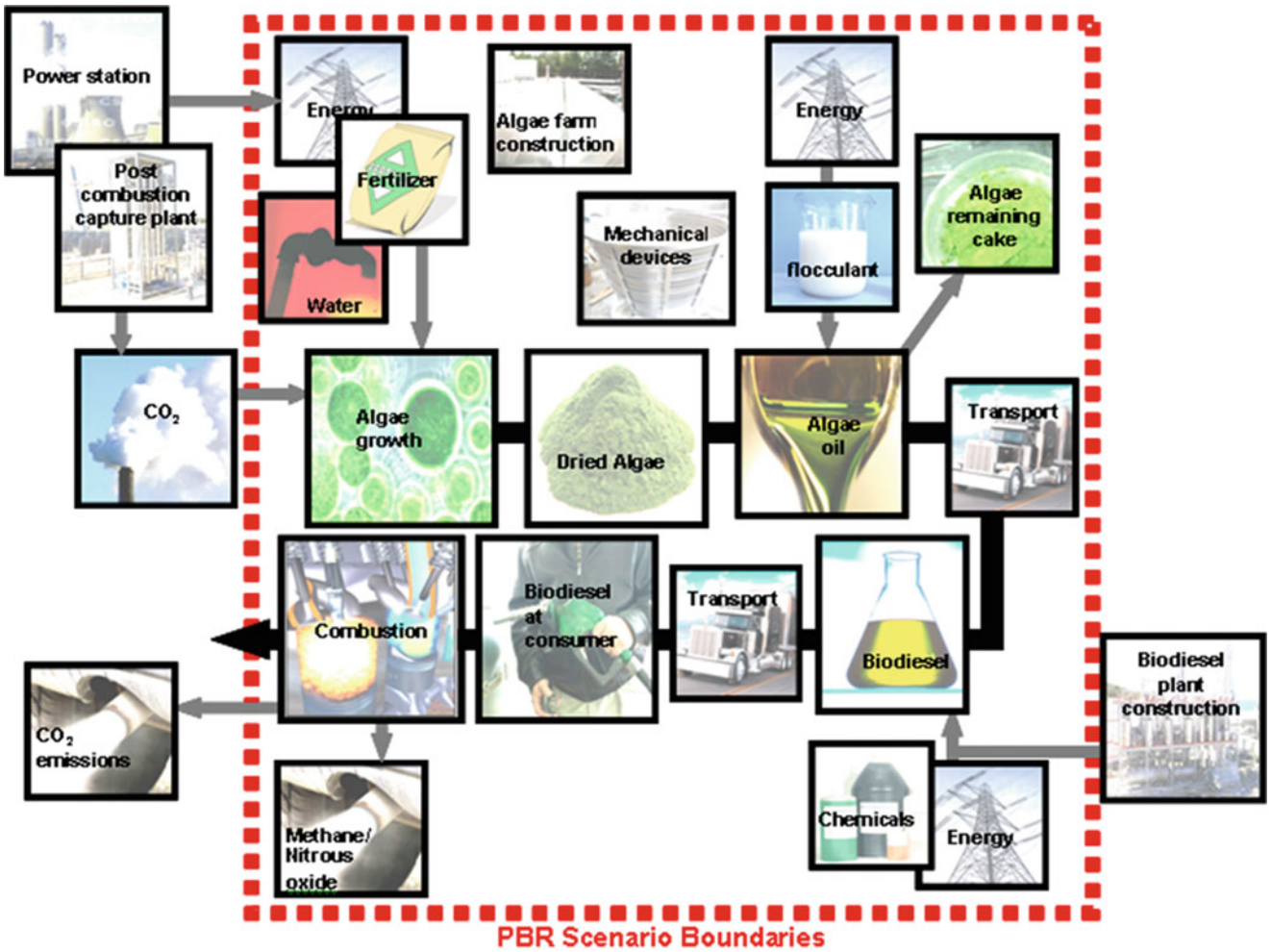


Fig. 14.1 System boundaries for microalgal biodiesel production. Processes situated inside the rectangle are considered in this study

- the impacts of the process belong to a different product system entirely, which is the case with waste products which attract little or no revenue in their disposal;
- the results will be used in a comparative assessment so that the analyst does not need to consider those items of production or infrastructure that are the same in all of the systems being compared.

Figure 14.1 shows a simplified outline of the system elements for the fuels studied, and places a boundary around those included in the study. Capital equipment and infrastructure is universally excluded from the study, based on its expected low contribution to the overall environmental impact of the fuel used. The impacts derived from capital goods are expected to be similar for each of the fuels studied. Though the capital goods in fuels delivery and filling could have substantial impacts if radically different filling infrastructure is required that is not the case in this work where the method used is that of a comparative LCA, in which algal biodiesel is compared to diesel or Canola diesel.

The comparative LCA took into account all the processes from biomass production in plastic photobioreactors through to the biodiesel combustion (Gnansounou et al. 2009). The scenarios differ by the production process and the variable uses of the co-products. The purpose of this analysis is to identify the best option(s) and to quantitatively compare it with the diesel production process. The study also provides a comparison with biodiesel production from microalgae grown in ponds and with Canola biodiesel produced in Australia with the objectives being to quantify the implication of each step of the process in the GHG emissions, to identify the key processes that need to be improved, and to determine their priority.

The scenarios tested in this study include all the processes from the biomass production to the biodiesel combustion. The construction of the algal farm is also included in the study. Figure 14.1 presents the system boundaries of scenarios 1–7. In Scenario 8 (diesel) and 9 (Canola biodiesel) the construction of the diesel/biodiesel plant is not included.

Table 14.2 global warming potentials factors used in the IPCC 1990 method

	GWP weighting factors
Carbon dioxide	1
Methane	21
Nitrous oxide	310

4 LCA Method and Functional Unit

To undertake the Life Cycle Assessment the SimaPro software[1] (Version 7.0) was used. The software offers a module to examine greenhouse gas emissions according to the guidelines of the Intergovernmental Panel on Climate Change (IPCC). This module disaggregates greenhouse results, based on a 100 year timeframe. The main greenhouse gases considered are carbon dioxide, methane and nitrous oxide. The global warming potential (GWP) is used to express the mass of each gas as a CO_2 equivalent (CO_2-e). The GWP factors are given in Table 14.2. They represent the radiative forcing role of each gas based on the relative importance of absorption of infrared radiation and the residential time of each gas in the atmosphere. The values are those agreed upon by the United Nations Framework Convention on Climate Change,[2] are used in the Kyoto Protocol, and are the values used by the Australian Government at the time of writing.

To compare the biodiesel produced from microalgae grown to any other fuel, or to biodiesel from other processes, the chosen functional unit is 1 t-km (t.km). It represents the combustion of fuel required for the transport of 1 t of freight over 1 km in an articulated truck (the most common form of road-freight transport in Australia). The equivalence in energy content and volume for both fuels is given later in this Chapter.

4.1 Origins of the Data Used for the Environmental Study

Because there is no commercial production of algal biofuels, no industrial data of the processes are available at the moment. For this reason, the set of data used in this analysis is based on different sources. Table 14.3 gives an overview of the sources of data.

Publicly available general industrial documentation of an Australian company planning to produce microalgae in photobioreactors[3] has been used for the simulation of one scenario (Table 14.1, Scenario 4).

[1] http://www.pre.nl/simapro/

[2] http://unfccc.int/ghg_data/items/3825.php

[3] http://www.mbdenergy.com/

5 Algal LCA Review

There is a view that if it were possible to capture carbon dioxide flue gases from a power station and use the gases to grow algae for biofuels production then this process would qualify as a GHG capture mechanism. This view is only partially correct, since the permissible carbon credits do not arise from the flue gas that is captured because the algae-derived fuel will eventually be burnt and the captured carbon returned to the atmosphere. The carbon credits arise as a result of the displacement of the fossil fuel that would have been used if the biofuel had not become available. In addition, if electricity is produced from the algal by-products such as spent algal biomass, then additional carbon credits may become available through the displacement of any coal, gas, etc. that would have been used for electrical production.

Detailed life-cycle calculations of the processing energy needed to make the biofuel, quantify the GHG emissions at each stage of the process. This enables us to determine whether the process does indeed emit less CO_2 than the use of fossil fuels and if this is the case to quantify the associated greenhouse gas savings.

Kadam (2001) applied life cycle methods to microalgal production from power plant flue gases but examined only the energy balance – not the greenhouse gas emissions. Posten (2013) (Chap. 13 in this volume) provides the detailed energetics of modern photobioreactor designs. Jorquera et al. (2010) used life cycle analysis to examine the energy return of algal grown using photo-bioreactors (PBRs) and algae grown using ponds. They found that the nett energy return of PBRs was less than unity and thus, according to them, uneconomical whereas that of ponds was greater than unity. The result that the nett energy return on algal ponds is positive agrees with the life cycle energy analysis of Campbell et al. (2011) – who also examined the greenhouse gas emissions associated with biodiesel manufacture from algal biomass.

Lardon et al. (2009) examined the life-cycle emissions of greenhouse gases resulting from the use of algae as a biofuel in Europe. Clarens et al. (2010) undertook a similar analysis for the United States and compared algae to ethanol by using the heating value of the respective fuels. This fails to account for the subsequent transesterification of the algal oil to produce a speciality fuel (biodiesel) rather than combustible biomass. Stephenson et al. (2010) used life cycle assessment to examine the operation of the freshwater alga *Chlorella* and concluded that the greenhouse gas emissions and energy balances were particularly sensitive to:

(i) the yield of oil achieved during cultivation, (ii) the velocity of circulation of the algae in the cultivation facility, (iii) whether the culture media could be recycled or not, and (iv) the concentration of carbon dioxide in the flue gas. These

Table 14.3 Sources of the data used in the photobioreactors scenarios: production processes and co-product use (anaerobic digestion and animal feed)

	Sources			
	Estimates extrapolated from laboratory scale studies	Campbell et al. (2011)	A Victorian commercial project	CSIRO industrial LCA database
Construction of the farm			X	x
Algae growth	x	x	X	x
Algae harvesting	x	x	X	x
Oil extraction	x	x	X	
Transesterification				x
Transport				x
Combustion				x
Algal cake; anaerobic digestion	x	x		
Algal cake; animal feed	x		x	x

results highlight the crucial importance of using life-cycle assessment to guide the future development of biodiesel from microalgae

Batan et al. (2010) used the GREET biofuel LCA model to examine the energetics and greenhouse gas emissions of *Nannochloropsis* grown in photobioreactors and obtained a positive energy return, with 1.08 MJ of energy produced per MJ of energy consumed. Campbell et al. (2011) show that if the algal cake is used to produce electricity through anaerobic digestion then the nett energy return is much higher. Batan et al. (2010) also note that their system avoids 75 g of carbon dioxide equivalents per MJ of energy produced. In this chapter we assume that the transport of a load of 1 t over 1 km requires 1.12 MJ so that their results correspond to 85 g CO_2-e per tonne-km, which is considerably higher than the results given in this Chapter.

6 Production System Details

At present Earthrise operates 44 ha of ponds in California for *Spirulina* production, and Cyanotech operates 36 ha of ponds in Hawaii for similar products. We assume that there are no economies of scale for ponds or photobioreactor systems of size greater than 100 ha (Stephens et al. 2010), so that our scenarios assume that a pure culture of microalgae is achieved in photobioreactors in a facility covering 100 ha. Larger algal growth systems can be treated as multiples of 100 ha units. Of the land area, 80 % is covered with photobioreactors. The remaining 20 % is devoted to work space, harvesting and pumping facilities. Borowitzka and Moheimani (2011) give an overview of the methods used to produce sustainable biofuels from algae. Based on their review, Fig. 14.2 provides a diagrammatic representation of the process that is envisaged in the analysis that follows in this chapter.

6.1 Locations and Distances

The land chosen for this study is located in the Australian State of Victoria. It is assumed that the algae farm is situated 1 km from a brown-coal fired power station on the outskirts of the city of Traralgon, in south-eastern Victoria. The algae farm is situated 60 km from the sea. The processed waste water is assumed to end up in the sea via existing wastewater treatment operations.

The biodiesel plant is assumed to be located in Melbourne, Australia (the capital of Victoria), 170 km from the farm. The average distance from the biodiesel plant to the consumer is considered to be 50 km. The LCA assumes that the extracted oil will be trucked from the farm to the plant and the biodiesel from the plant to the consumer.

6.2 Microalgae Strains and Production Yields

Based on laboratory-scale data, pilot plant research and the published literature, several microalgae species could potentially be used for industrial production of oil. For biodiesel generation criteria to be met include (i) fast algal growth, (ii) a significant production rate when kept under standard conditions of nutrients, CO_2 and light, and (iii) a high oil content. For certain strains, the hydrocarbon content can exceed 75% by weight of dried biomass (Maxwell et al. 1968), but those algae usually have a very slow growth rate. Oil levels of 20% of dry weight are common (Chisti 2007). The best candidates for biodiesel production are strains combining high oil content (preferably greater than 30%) and growth rate. Other criteria relate to the qualities of the cell walls: the oil extraction must be easy and consume the lowest amount of chemicals and energy possible, but the cell must be resistant enough to withstand mixing during cultivation.

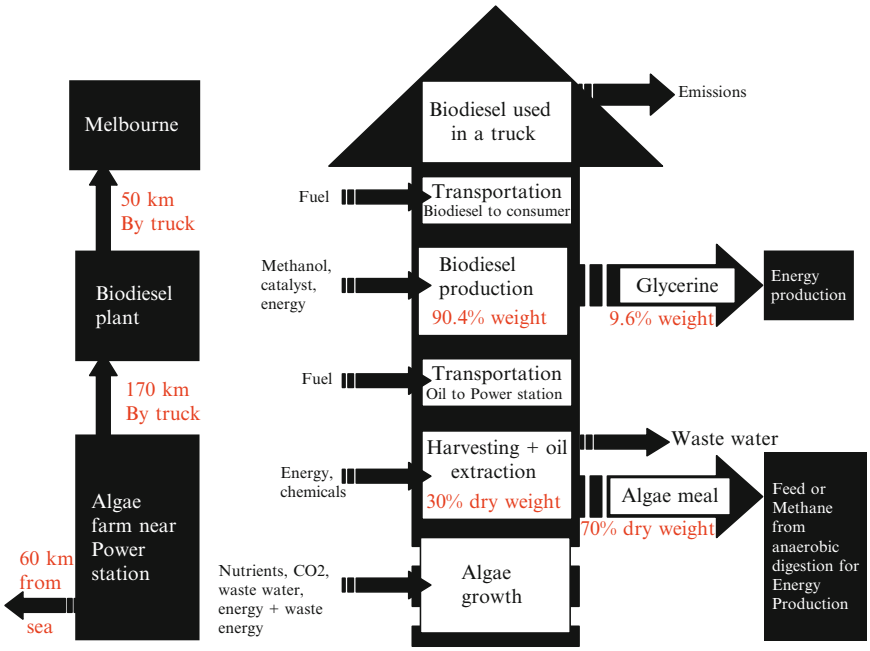


Fig. 14.2 Production scenario overview for the photobioreactor scenarios tested in this study from biomass production to combustion, including possible co-products uses and locations

Table 14.4 Actual and estimated yield for *Nannochloropsis oculata* and *Phaeodactylum tricornutum* grown in photobioreactors in literature, extrapolation from laboratory-scale data, commercial project estimates

Environment of growth	Strain	Yield (t ha^{-1} year^{-1})	Quality of the data	Reference
Ponds	*Dunaliella salina*	109	Estimate	Campbell et al. (2011)
Photobioreactors	*Phaeodactylum tricornutum*	115	Extrapolation of laboratory-scale data	Chisti (2007), Sanchez Miron et al. (1999), Contreras Gomez et al. (1998), Garcia Camacho et al. (1999), Lewin et al. (1958), Mann and Myers (1968), Molina et al. (1999, 2001), Acien Fernandez et al. (2001)
Photobioreactors	*Phaeodactylum tricornutum*	175	Estimate based on laboratory-scale data on optimisation of the space	Chisti (2007) from Sanchez Miron et al. (1999), Miron et al. (1999), (2001), Acien Fernandez et al. (2001)
Photobioreactors	*Nannochloropsis oculata*	109	Laboratory-scale data	Victorian commercial project with research based at James Cook University
Photobioreactors	*Nannochloropsis oculata*	375	Estimate of improvement possible	Victorian commercial project

Taking all these criteria into account, the microalgae chosen for this study are *Nannochloropsis salina*, *N. oculata* and *Phaeodactylum tricornutum*. The first two are some of the best species proposed by Cheng-Wu et al. (2000), Zittelli et al. (1999, 2000, 2003), Rodolfi et al. (2003); Sukenik et al. (1997), Gitelson et al. (2000), Hanhua and Kunshan (2006) and Xu et al. (2004). *Phaeodactylum tricornutum* has shown good and consistent results (Chisti 2007; Miron et al. 1999; Gomez et al. 1998; Camacho et al. 1999; Lewin et al. 1958; Mann and Myers 1968; Molina et al. 1999, 2001; Fernandez et al. 2001), and is able to tolerate high levels of salinity (Fernandez et al. 2000).

Table 14.4 gives an overview of actual or estimated production yields for these strains. An optimistic algae yield of 175 t ha^{-1} year^{-1} is assumed for this analysis. The worst case scenario tested in this study (Scenario 2) has a growth rate of 109 t ha^{-1} year^{-1}. This yield was reached by Gomez et al. (1998); Molina et al. (2001) and Fernandez et al. (2001). Though their experiments were over short time scales we assume that with biological and technological advances it will be possible to achieve such a yield on an annual basis. We assume in the other scenarios (1, 3, 4, 5, 6 and 7) that this rate can be improved by new technologies and scaling-up. This worst case yield also happens to be similar to the optimistic

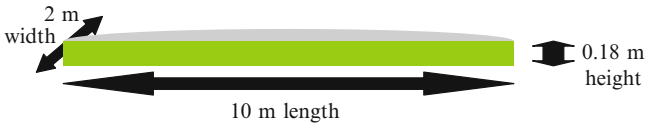


Fig. 14.3 Design and dimensions of one plastic photobioreactor

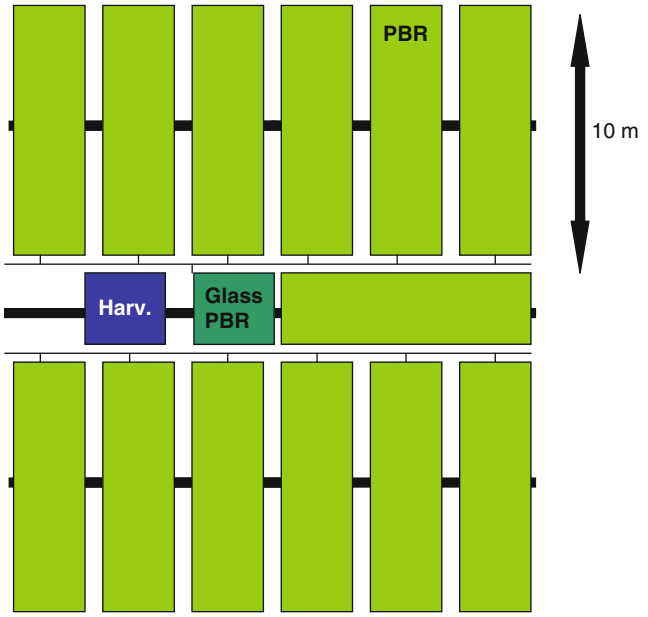


Fig. 14.4 Potential spatial organisation of photobioreactors. *Harv.* area reserved for the harvesting system, *PBR* plastic photobioreactors, *Glass PBR* 4,000 L glass photobioreactors used to inoculate the PBR

growth yield for ponds (Campbell et al. 2009). The oil content of *Nannochloropsis* ranges from 30 to 68 % by mass (Ratledge and Cohen 2008). *Phaeodactylum* reaches a yield of 30 % oil content (Chisti 2007). For the purpose of this analysis we will assume that 30 % by dry weight can be extracted from the algae.

6.3 Algae Culture

6.3.1 Photobioreactor Design Details

The photobioreactors are made of transparent, flexible plastic in the form of a horizontal bag akin to a water-bed. The depth of the water is 0.18 m. There are 362 units per hectare, which represents 8,000 m^2 land area (80 % coverage). The 362 units are fed by four 4,000 L glass photobioreactors. This design is partly inspired by the Victorian commercial project. Figure 14.3 illustrates that one photobioreactor unit has dimensions of 2 m width, 0.18 m height, and 10 m length. Figure 14.4 describes the complex and gives an example of a possible organisation of a small area (360 m^2) of the farm. The carbon dioxide, water and fertilizers are distributed or harvested by a basic piping system.

6.3.2 Fertilization

A substantial amount of nitrogen and phosphorous are added to the system as fertilizer. The calculations for the fertilizer requirements are based on a global composition formula of an alga (Lardon et al. 2009; Sheehan et al. 1998; Chisti 2008; Geider and La Roche 2002): C (48 %), N (4.6 %), P (0.99 %), K (0.8 %). Based on these values it is possible to estimate the nitrogen and phosphorus requirements. It is assumed that there is a 5 % loss of nitrogen by volatilisation. Phosphorus is supplied by superphosphate fertilizers and nitrogen by superphosphates and urea. Thus NPKS 32 10 fertiliser at 17.3 $t.ha^{-1}.year^{-1}$ and 5.57 $t.ha^{-1}.year^{-1}$ of urea needs to be added. The distance from the production sites to the regional store is considered to be 200 km. It is assumed that the total amount of nutrients is used with a perfect efficiency (apart from the aforementioned nitrogen) and that micronutrients such as magnesium or sodium are provided in sufficient quantities in the water (Geider and La Roche 2002; Geldenhuys et al. 1985).

Illman et al. (2000) and Lardon et al. (2009) report that certain species, including *Nannochloropsis* spp. can maintain a high productivity and have a higher proportion of lipids under nitrogen-limiting conditions (Lardon et al. 2009; Illman et al. 2000). However this technique has not been used in this analysis.

6.3.3 Water

It is assumed that the algae farm, situated 1 km from the power station, uses the waste cooling water coming out of the station. The waste heat carried out by the water reduces the energy required to stabilize the temperature during winter. In summer the water can be allowed to cool, using the existing power plant cooling ponds, for a few days before utilisation. Because of the proximity to the station the pumping costs for the water are much reduced. The release of waste water to the sea is done by gravity and does not require any energy.

The quantity of water required per hectare per annum varies between the scenarios. The quantity of media harvested (algae, water and remaining chemicals) per hectare per annum is 92,100 m^3. The photobioreactors have a capacity of 1,300 m^3 ha^{-1}. Every day 20 % of the reactor volume is harvested. Because photobioreactors are enclosed, evaporation is low.

In the scenario, when the harvesting is done by centrifugation it is assumed that the water is clean enough to be reintroduced into the system. A 30 % loss is considered on the whole cycle of the water (in the centrifugation, evaporation, water remaining in the algae harvested). Twice a year the water of the whole system is changed to avoid contamination.

When the harvesting is done by flocculation the water is not recycled and thus must be renewed continuously.

6.3.4 Carbon Dioxide

Flue gas from the power station can be used to introduce levels of CO_2 to the site appropriate for algal production (Benemann 1993, 1997; Hughes and Benemann 1997; Vunjak-Novakovic et al. 2005; Greque de Morais and Costa 2007; Ratledge and Cohen 2008). It is important to note that the post combustion capture (PCC) plant is assumed to be already in existence and is not included in these calculations (Feron 2010). It is hoped to be able to capture 85 % of the CO_2 in the incoming flue gas and remove SO_x and NO_x (though NOx could serve as a possible nutrient source for the algae). The capacity could reach 100–500 kg.h^{-1} CO_2 capture.

According to the algal composition, for each 1 g of algae produced, 1.8 g of CO_2 is required. This means that 2 g CO_2. g^{-1} of algae needs to be introduced into the system as 10% is assumed to be lost or unable to be captured by the algae. A total of 350 t of CO_2 per ha per annum is provided to the system from the flue gas. It represents 35,000 t of carbon dioxide for the 100 ha farm.

The CO_2 is pumped 1 km. It is then introduced in each photobioreactor from the bottom of the photobioreactor along the whole 10 m length of the reactor. This design is supposed to permit a better capture of the gas by the algae and promote mixing, thus greatly reducing the energy required for this purpose.

6.3.5 Energy

The energy consumed by the system is mainly due to the mixing and pumping of nutrients, water and CO_2 on the site. Numbers used in this analysis are based on previous pond reports (Campbell et al. 2009; Benemann and Oswald 1996) and industrial public information[4] (including a commercial project in Victoria, Australia). Pumping energy costs are dependent on the design (gas introduction system into the algae mixture, etc.) and location (1 km of pumping). The station's waste heat may eventually be recycled for purposes in addition to heating the water during winter. Thus the amount of energy for mixing and pumping is assumed to be 9,200 kWh.ha^{-1}.a^{-1}. This represents 0.10 Wh.L^{-1} treated.

6.4 Harvesting and Oil Extraction

For harvesting purposes several options are taken into consideration. Harvesting is one of the bottlenecks (both physical and economic) in algal production (Mohn 1988; Danquah et al. 2009). More effective dewatering techniques are required to enhance maximum lipid extraction. High-density algal cultures such as those in reactors can be concentrated by either chemical flocculation or centrifugation. The oil is most likely extracted by solvent, often hexane. In this study the flocculation harvesting is combined with the regular solvent extraction (scenarios 5, 6, 7 and 8). The second option assumes the use of centrifuge harvesting and a pressure system for the extraction (scenarios 1, 2, 3 and 4).

6.4.1 Flocculation Harvesting and Solvent Extraction

Flocculation is a method to separate algae from the medium by using chemicals to force the algae to clump together and come out of suspension in the water, either then floating to the top or sinking to the bottom of the liquid for easier collection (Yahi et al. 1994; Oh et al. 2001; Gutzeit et al. 2005; Danquah et al. 2009; Campbell et al. 2009). The main disadvantage of this separation method is the additional chemicals that can be difficult to remove from the separated algae. Flocculating agents are chemicals promoting conglomeration from the solution by causing colloids and other suspended particles to aggregate. Aluminium chloride is the flocculant assumed to be used in this study. An amount of 425 kg of flocculant is used per ha per annum. It represents 2.43 kg of aluminium chloride per tonne of algae. It is then fed into a DAF (dissolved air flotation) system to further concentrate algae before it is heated and fed into a centrifuge. According to Benemann and Oswald (1996) the electricity requirement for this step is around 8,000 kWh.ha^{-1}.

The oil is then extracted with hexane. In this case the oil extraction is similar to any other oil seed extraction such as Canola. An amount of 1.5 g of hexane is consumed for each kg of dried algae treated. This process consumes 12,300 kWh.ha^{-1}.

6.4.2 Centrifugation Harvesting and Pressure Extraction

Centrifugation is a method of separating algae from the medium by using a centrifuge to cause the algae to settle on the side of the device. Pressure extraction is realised by applying high pressure on the wall cells. The Victorian commercial project (scenario 4) proposes this new extraction method. They have stated that the electricity requirement for the centrifuge is 16,000 kWh.ha^{-1} (Mohn 1988). We will assume for the mechanical harvesting and extraction scenarios (scenarios 1, 2 and 3) 0.25 Wh.L^{-1} of media harvested. Thus the electricity requirement is 23,000 kWh.ha^{-1}.

6.5 Biodiesel Conversion

Oil extraction leads to two products: crude oil and algal cake. They differ in their carbon and energy content. The algae oil will be transesterified (Mittelbach and Remschmidt 2005) like any other plant oil. The biodiesel plant is located 170 km from the algae farm. In this study, the transesterification of

[4] http://www.mbdenergy.com/

Table 14.5 Functional unit, volume and energetic equivalence between biodiesel and diesel

	Functional unit (t.km in an articulated truck)	Quantity of fuel (mL)	Energy (MJ)
Biodiesel	1	27.1	0.89
Diesel		23.1	

algal oil is done with methanol and sodium methoxide serving as the catalyst. The quantities of reactants and energy required for this process are given in Table 14.7.

6.6 Combustion

Transport impacts from the biodiesel plant to the consumer are considered in this study. It is assumed that the fuels are trucked an average of 50 km to the consumers. Emissions are generated by the combustion of the fuel in an articulated truck. Because there is currently no data about emissions generated by the combustion of microalgae biodiesel, it is assumed that the tailpipe emissions are similar to those of Canola biodiesel.

In this study only the displacement of fossil carbon dioxide is considered. The consumption of CO_2 from the power station during the growing phase results in no long-term displacement of fossil CO_2 (because the bio-product is eventually combusted) and thus is outside the system boundaries of the LCA as shown in Fig. 14.1. For the combustion step the same logic applies: only the biogenic emissions are recorded. It means that the tailpipe emissions do not effectively comprise CO_2 (for GHG purposes only methane and nitrous oxide contribution from the tailpipe are added to the upstream processing emissions).

To compare the biodiesel produced from microalgae grown in photobioreactors to any other fuel the chosen functional unit is 1 t.km (tonne-kilometre). Table 14.5 gives the equivalence in energy content and volume for both fuels.

6.7 Co-products and Utilisation

Two main co-products are generated during the microalgae biodiesel production. Glycerol is a co-product of the transesterification process. The algal cake is the product that remains after the oil extraction.

6.7.1 Glycerol

Optimistic studies consider that glycerol produced during the transesterification of vegetable oil can be considered as pharmaceutical grade, which means 99.5 % + pure. In reality, to reach this level of purity, substantial washing is required (Mittelbach and Remschmidt 2005). These processes can be expensive and energy demanding. Therefore for the purpose of this LCA the glycerine produced will be combusted to produce energy. During the reaction 96 kg of glycerol are produced per 1.037 t of oil treated. Glycerol energy content is 17.9 $MJ.kg^{-1}$ Hence, for each tonne of oil treated the energy produced by glycerine combustion corresponds to 1,657 MJ avoided from natural gas.

6.7.2 Algal Cake

Algal cake remains the main co-product by its volume and composition. It represents in this analysis 70 % of the dried algae weight. At the moment, two uses have been proposed for the algal cake: anaerobic digestion and animal feed (Sialve et al. 2009). One purpose of this study is to test the environmental impact of these two main potential uses.

Anaerobic digestion is a series of processes in which micro-organisms break down biodegradable material in the absence of oxygen. It is widely used to treat wastewater sludges and organic waste because it reduces the volume and mass of the input material. In this case, biogas is the ultimate waste product of the bacteria feeding off the input biodegradable feedstock, and is mostly methane (65 %) and carbon dioxide. Methane can be burned to produce both heat and electricity, usually with a reciprocating engine or microturbine, often in a cogeneration arrangement. The conversion of algal cake into methane is one of the best CO_2 reduction measures. We assume in this study that the infrastructure required for turning biogas into energy is provided by the power station.

According to Borowitzka (1992), 60 % of the carbon is recovered in the lipids, leaving 40 % in the cake (dry matter). Sewage sludge submitted to anaerobic digestion in treatment plants has a carbon content ranging from 6 to 35 %; 20 % on average (Smernik et al. 2003; Macias-Corral et al. 2008), as such algal cake has a higher carbon content than the average sludge treated by anaerobic digestion. According to Gray (2008), in Californian electricity production of 730–1,300 $kWh.t^{-1}$ of food waste and 560–940 $kWh.t^{-1}$ of municipal waste has been achieved. However, Benemann and Oswald (1996) and Campbell et al. (2011) use a value of 1,980 $kWh.t^{-1}$ for electricity produced by anaerobic digestion of algal cake, assuming 32 % conversion efficiency. For the purpose of this LCA we have considered an average of 1,330 $kWh.t^{-1}$ of algal cake treated (equivalent to 930 $kWh.t^{-1}$ of algae produced) which can be considered to be pessimistic. It includes the energy required for the process. We also made

Table 14.6 Victorian commercial project data (scenario 4)

Flow	Unit	Value	Comment
Product and co-products			
Dried algae	t	30,000	Equivalent to 375 $t.ha^{-1}.year^{-1}$
Algal cake	t	20,000	
Algae oil	t	10,000	33 % oil for *Nannochloropsis oculata*
Resources production of algae			
Occupation, industrial area	ha.a	80	
Water from power station	t	70,000	875 $t.ha^{-1}.year^{-1}$
Fertilizer NPKS 32 % 10 % at regional store	t	3,000	37. $t.ha^{-1}.year^{-1}$
Carbon dioxide	t	70,000	796 $t.ha^{-1}.year^{-1}$
Electricity, High voltage Victoria	MWh	660	8,250 $kWh.ha^{-1}$
Resources for harvesting and extraction of algae			
Electricity, High voltage Victoria for dewatering system	MWh	640	8,000 $kWh.ha^{-1}$
Electricity, High voltage Victoria for high pressure extraction	MWh	2,000	25,000 $kWh.ha^{-1}$
Co-production			
Equivalence energy from Natural gas from glycerol combustion	GJ	16,586	188 $GJ.ha^{-1}$
Avoided production of Lupins	t	20,000	250 $t.ha^{-1}$

the assumption that the whole process would be realized with a generator with 32 % efficiency, which is conservative given advances in modern generators (Campbell et al. 2009). The electricity produced is fed back into the grid at the power station. An alternative means of generating electricity from the algal cake is for it to be co-fired along with coal in the nearby power station after drying; however this option is not considered due to the lack of hard data available on how much energy this would actually produce.

The second potential use for the algal cake is animal feed. Analyses and nutritional studies (Becker 2007) have demonstrated that algal proteins are comparable to conventional vegetable proteins. A large number of nutritional and toxicological evaluations demonstrated the suitability of algae biomass as a valuable feed supplement or substitute for conventional protein sources (soybean meal, lupin, etc.). The target domestic animal is poultry, mainly because the incorporation of algal cake into poultry rations offers the most promising prospect for their commercial use in animal feeding. In the context of this LCA the product the closest to this meal is lupin. The energy and protein (36.59 %) contents in lupin meal are very close to algae meal (Woods and Fearon 2009). Hence with algae meal as a co-product, its production and distribution would allow one to avoid the production of the same mass of lupin meal.

6.8 Australian Commercial Project Scenario Located in Victoria (Scenario 4)

Publicly available data from a Victorian company project have been compiled to create scenario number 4. The numbers are given in Table 14.6. The pilot plant project is based in Victoria adjacent to Loy-Yang power station [Latitude: 38°14′S; Longitude: 146°34′E]. The company expects to generate 375 $t.ha^{-1}.a^{-1}$ of dry algae. The planned algae farm uses plastic bag photobioreactors grouped into SETS (Solar Environmental Tube System, see Figs. 14.3 and 14.4). The SETS are 3.4 m in diameter, 50 m long and the height of the media is 0.6 m. The harvesting system is currently proprietary information, but consists of a new dewatering system technology combined with centrifugation. It is completed with treatment of waste water and a high pressure extraction device that can be compared to a homogenizer. Communicated data give total electricity consumption for the 80 ha complex of 3,300 MWh of which 20% is dedicated to the algae culture, mixing and pumping, and 80% to the harvesting/extraction. Of this 80%, 60% is expected to be consumed by the high pressure extraction and the 20% by dewatering and centrifuge. If not specified in Table 14.6, other assumptions concerning the Victorian commercial project are similar to scenario 3. The design of the farm is also very close to the one given in Figs. 14.3 and 14.4.

Table 14.7 Data for the scenarios 1, 2, 3, 5, 6

Flow	Unit	Value for scenarios 1, 3, 4, and 5 if concerned	Worst case scenario (scenario 2)	Scenarios concerned
Product and co-products				
Dried algae	t	175	109	1, 2, 3, 5, 6
Algal cake	t	122	122	1, 2, 3, 5, 6
Algae oil	t	53	53	1, 2, 3, 5, 6
Resources for the production of algae				
Occupation, industrial area	ha. year^{-1}	1	1	1, 2, 3, 5, 6
Water from power station	t	3,370	92,100	1, 2, 3, 5, 6
Fertilizer NPKS 32 % 10 % at regional store	t	17.3	17.3	1, 2, 3, 5, 6
Urea at regional store	t	5.57	5.57	1, 2, 3, 5, 6
Carbon dioxide	t	350	350	1, 2, 3, 5, 6
Electricity, High voltage Victoria	kWh	9,210	12,000 (+30 %)	1, 2, 3, 5, 6
Resources for harvesting and extraction of algae				
Electricity, High voltage Victoria for Mechanical harvesting and extraction	kWh	23,000	27,000 (+22 %)	1, 2 and 3 (Mechanical harvesting + extraction)
Electricity, High voltage Victoria for flocculation harvesting	kWh	8,000	N/A	5 and 6 (flocculation harvesting + solvent extraction)
Flocculant	kg	425	N/A	5 and 6 (flocculation harvesting + solvent extraction)
Steam from natural gas for solvent extraction	kg	326	N/A	5 and 6 (flocculation harvesting + solvent extraction)
Electricity, High voltage Victoria for solvent extraction	kWh	12,300	N/A	5 and 6 (flocculation harvesting + solvent extraction)
Hexane for solvent extraction	kg	263	N/A	5 and 6 (flocculation harvesting + solvent extraction)
Co-production				
Electricity produced by anaerobic digestion, High voltage, Victoria	kWh	163,000	N/A	6 (Anaerobic digestion of the algal cake)
Equivalence energy from Natural gas from glycerol combustion	MJ	87,078	87,078	1, 2, 3, 5, 6
Lupins	t	122	N/A	3 and 4 (use of the cake for animal feed)
Biodiesel conversion				
Electricity, High voltage, Australian average	kWh	1,509	1,509	1, 2, 3, 5, 6
Energy from natural gas	MJ	69,865	69,865	1, 2, 3, 5, 6
Methanol	t	4.35	4.35	1, 2, 3, 5, 6
Caustic soda	kg	109.35	109.35	1, 2, 3, 5, 6
Sodium methoxide (catalyst)	t	1.22	1.22	1, 2, 3, 5, 6
Hydrogen chloride	kg	382.9	382.9	1, 2, 3, 5, 6
Water delivered	t	18	18	1, 2, 3, 5, 6

7 Worst Case Scenario (Scenario 2)

The worst case scenario describes a pessimistic case. It has lower assumptions in terms of algae production yield: 109 t. ha^{-1}.a^{-1}. The more energy demanding system (centrifuge and mechanical extraction) has been chosen. Furthermore the energy requirement for pumping/mixing and harvesting has also been increased by 20–30 %. No use of the algal cake has been selected. Table 14.7 gives the data used for this scenario.

8 Construction of the PBR Algal Farm

A simplified model of the construction of the PBR algal farm has been created in order to understand better the whole environmental impact of the process. It is assumed in this study that the plastic bag photobioreactors have a 5 year life-time. The plastic chosen is a high density polyethylene (HDPE) and is recyclable. One photobioreactor is made from 12.4 kg of extruded plastic. Other equipment such as pipes, metallic equipment or glassware is expected to have a 20-year lifespan. The centrifuge, high pressure device, and other metallic objects are mostly made of steel. Four devices are required on each hectare for the algae farm with mechanical harvesting or extraction (scenarios 1, 2 and 3). Only one centrifuge is assumed for the flocculation harvesting because the volume to process by centrifugation has already been much reduced by the flocculation process (scenarios 5 and 6). On site, 750 m of HDPE pipes (0.1 m width) per hectare are considered necessary to deliver the CO_2, the water and for harvesting. On each hectare four small 4,000 L glass photobioreactors are built to provide inoculum. Finally a 10,000 L tank per hectare is used to mix water and fertilizer or/and to temporarily stock the media before centrifugation or treatment in case of technical problems with the photobioreactors.

Data from the Victorian project (scenario 4) describes a design very close to the one described above. It includes 2.40 m^3 of metal (steel and iron) devices per hectare and devices are based on concrete pads. On site, 188 m^3 of soil are excavated and only 300 m of pipes are assumed per ha. Other design details not specified are assumed to be the same as in scenarios 1, 2 and 3.

The pond algae farm (scenarios 7 and 8) has a much lower capital investment. The construction is reduced to excavation of the ponds (8,000 $m^2.ha^{-1}$ to the depth of 1 m) and one centrifuge is required along with flocculation harvesting. Only 90 $m.ha^{-1}$ of pipes are assumed necessary in this case.

The construction of the biodiesel plant (scenarios 1–8 and 10) and the diesel plant (scenario 9) are not included in this analysis because they are already in operation. The construction and operation of the post combustion capture plant at the power station is also assumed to exist and thus not included in these calculations.

9 Scenarios Compared by LCA

Tables 14.1, 14.7, and 14.6 contain all the data and the scenarios compared in this Life Cycle Assessment. The combination of different processes for the harvesting, the oil extraction and the various uses of algal cake have different emission impacts. We seek to determine, for example, the energy requirements and the GHG emissions difference between using algal cake for anaerobic digestion or for animal feed. Between flocculation combined with solvent extraction or centrifugation with pressure extraction.

It is important to note that not all of the production scenarios are compatible with some uses of the main co-product, algal cake. The solvent extraction, for instance, involves the uses of chemicals that remain in the cake after extraction. Flocculant can be hard to remove. Hexane is toxic and the total removal of this chemical can be highly costly and energy demanding. These issues informed the choice of the given scenarios. Thus it is assumed that only the centrifuge harvesting is compatible with the "animal feed" use of the cake. On the other hand the anaerobic digestion does not require this purification step; it can be combined with any process.

Biodiesel created from the algal oil produced in the photobioreactor scenarios has been compared with Ultra Low Sulphur (ULS) diesel[5] (Beer et al. 2003) and Canola biodiesel (produced in Australia). A comparison with algal biodiesel resulting from a pond model utilising power station flue gas (Campbell et al. 2009, 2011) was undertaken. The production yield in that model was assumed to be 109.6 t. $ha^{-1}.year^{-1}$, and the algal cake treated by anaerobic digestion. The model, based on sites in other States of Australia, has been adapted to Victorian electricity (which is primarily generated by brown coal) for a more accurate comparison with the photobioreactor scenarios.

10 LCA Results and GHG Potential Emissions

The results, based on the entire life cycle of a fuel for the greenhouse gas emissions for 1 t.km in an articulated truck, are given in Fig. 14.5. Table 14.8 gives the breakdown of the emissions for the greenhouse gases (carbon dioxide, methane, nitrous oxide, other) for each scenario (in g CO_2 equivalent). Negative values indicate that carbon credits are being awarded for the displacement of fossil fuels (mainly coal and natural gas) used in electricity production in Victoria. Table 14.9 illustrates the energy requirement for each fuel in $MJ.tkm^{-1}$ and breakdowns to energy from fossil oil, fossil gas and fossil coal. These data were based on the LCA Cumulative Energy Demand method (CED), which calculates the total (primary) energy use through a life cycle.

10.1 Comparison with Culture in Ponds, Diesel and Canola Biodiesel

The first observation is that all the algal biodiesel scenarios have better GHG emissions results than the diesel reference scenario. In terms of energy requirement diesel

[5] Diesel with a sulfur content of less than 50 ppm.

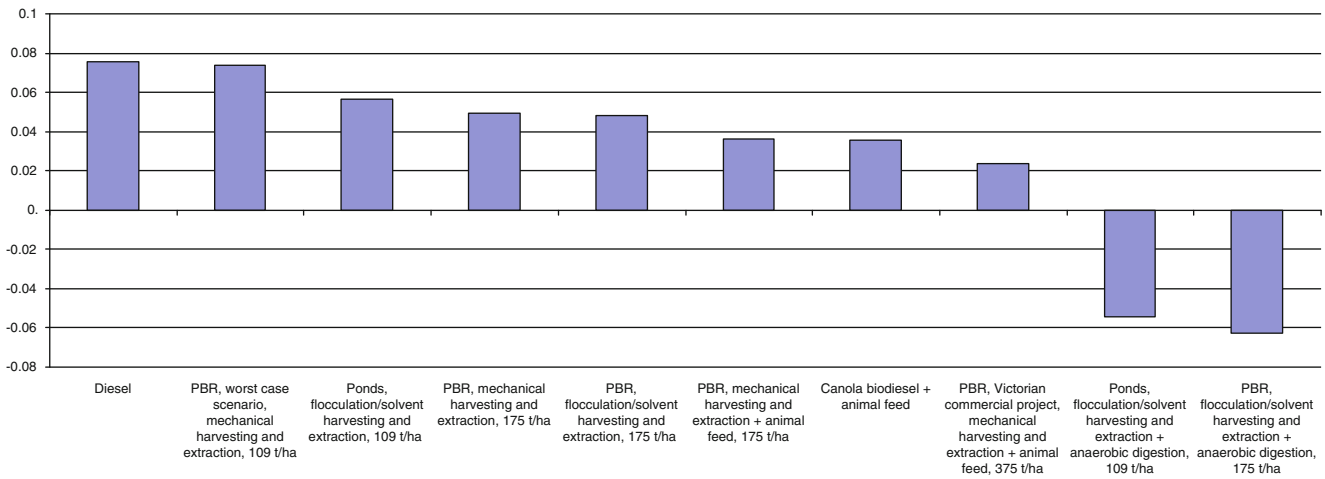


Fig. 14.5 Comparison of the scenarios for GHG emissions (in kg CO_2–e) for a journey of 1 t-km. From *left* to *right* scenario 9, 2, 7, 1, 5, 3, 10, 4, 8 and 6

Table 14.8 GHG emissions per scenario with breakdown into carbon dioxide, methane, nitrous oxide and other GHG (all g CO_2-e tkm^{-1})

Scenario name	Scenario number	Carbon dioxide	Methane	Nitrous oxide	Other	Total
Diesel, ULS	9	74.0768	0.8082	0.4752	0.0003	**75.3605**
PBR, worst case scenario, mechanical harvesting and extraction	2	72.2843	0.0010	0.0007	0.0029	73.9995
Ponds, flocculation/solvent harvesting and extraction	7	55.3497	0.0007	0.0006	0.0017	56.6834
PBR, mechanical harvesting and extraction	1	47.9262	0.0009	0.0006	0.0025	49.3933
PBR, flocculation/solvent harvesting and extraction	5	46.4131	0.0009	0.0006	0.0025	47.8855
PBR, mechanical harvesting and extraction, + animal feed	3	41.2973	0.0009	–0.0057	0.0024	36.5081
Canola Biodiesel	10	33.6065	0.0008	0.0014	0.0034	**35.8270**
PBR, Victorian commercial project, mechanical harvesting and extraction, +animal feed	4	27.6317	0.0007	–0.0061	0.0020	23.4898
Ponds, flocculation/solvent harvesting and extraction, +anaerobic digestion	6	–54.8738	0.0006	0.0001	0.0017	–54.1798
PBR, flocculation/solvent harvesting and extraction, + anaerobic digestion	8	–63.8103	0.0007	0.0001	0.0025	–62.9777

(1.1242 $MJ.tkm^{-1}$) is also higher than other models. Even the worst case scenario (scenario 2) has slightly lower results than the diesel scenario.

If the use of algal cake is not taken into consideration, the ponds scenario is worse than the photobioreactor ones. The GHG emissions difference between ponds (56.7 g CO_2-e) and the equivalent PBR scenario with flocculation and solvent extraction (scenario 5, 47.9 g CO_2-e) can be partly explained by the smaller energy requirement for the mixing in PBR thanks to the CO_2 injection system. It is also due to more efficient use of fertilizer (no loss). Moreover less water has to be mixed and treated for the same quantity of algae produced, which result in a better use of the resources than in ponds. But this result is also partly due to the different assumptions (e.g. locations, fertilizer requirements) between the pond scenarios which were built into previous studies (Campbell et al. 2009) and the PBR. This chapter assumes an idealised location at the power station for the installation of the PBR and allows for a large reduction in the electricity requirement of the farm (e.g. from reduced energy for piping, use of waste heat energy from the station).

Canola biodiesel (35.8 g CO_2-e) has almost half the GHG emissions of the diesel scenario. Because the cultivation of

Table 14.9 Energy requirement in MJ tkm^{-1}

Scenario number	Scenario name	Energy fossil oil	Energy fossil gas	Energy fossil coal	Total fossil energy required in MJ tkm^{-1}
9	**Diesel**, ULS	1.1	0.0197	0.0045	1.1242
2	**PBR, worst case scenario**, mechanical harvesting and extraction	0.0872	0.436	0.468	0.9912
7	**Ponds**, flocculation/solvent harvesting and extraction	0.0863	0.336	0.33	0.7523
1	**PBR**, mechanical harvesting and extraction	0.0774	0.395	0.268	0.7404
5	**PBR**, flocculation/solvent harvesting and extraction	0.0838	0.397	0.254	0.7348
3	**PBR**, mechanical harvesting and extraction, + animal feed	−0.035	0.458	0.275	0.698
10	**Canola Biodiesel**	0.133	0.388	0.0707	0.5917
4	**PBR, Victorian commercial project**, mechanical harvesting and extraction, +animal feed	−0.0305	0.364	0.1688	0.5022
8	**Ponds**, flocculation/solvent harvesting and extraction, +anaerobic digestion	0.0843	0.321	−0.629	−0.2237
6	**PBR**, flocculation + solvent, anaerobic digestion	0.0818	0.382	−0.705	−0.2412

PBR photobioreactors

Canola does not require much energy or any infrastructure emissions, microalgae biodiesel scenarios have difficulties competing with it in terms of GHG emissions. Most of the algae scenarios that do not include the use of algal cake do not have a better GHG balance than Canola biodiesel. The centrifuge scenario with the use of algal cake for animal feed has slightly higher emissions than Canola (36.5 g CO_2-e). The Victorian commercial project (scenario 4) and anaerobic digestion scenarios (6 and 8) are the only ones to present a better GHG balance than the Canola biodiesel scenario. Note that Canola seed cake use has been taken into consideration in this study and dedicated to animal feed.

11 Influence of Design Considerations on Potential GHG Emissions

11.1 Flocculation/Solvent or Centrifuge/High Pressure?

According to the GHG balance the cumulative energy demand (CED) of flocculation and solvent extraction show better results than centrifugation and pressure extraction (comparing scenarios 1 and 5). The difference is only 1.51 g CO_2-e/t.km in favour of the flocculation, however. This small difference can be explained by the fact that the flocculation step does not require energy while the last few steps of the harvesting, such as heat and centrifugation, are energy demanding. In the flocculation and solvent scenarios 18 % of the emissions of the dried algae production are due to electricity for harvesting. The solvent extraction step also consumes chemicals and energy.

For the Victorian commercial project (scenario 4), the energy required for harvesting/extraction appears to be very low and would be the best option if the numbers advanced by the company can be reached. The feasibility of this new technology seems to be dependent on the energy consumption assumptions.

11.2 Anaerobic Digestion of the Cake or Use for Animal Feed?

The use of algal cake as animal feed is a good option. It decreases the emissions per t.km by 26 %, i.e. −12.9 g CO_2 tkm^{-1} (comparing scenarios 1 and 3). However anaerobic digestion is a much better option based on a GHG point of view. Emissions are reduced by 232 % which means −111 g CO_2 t.km (comparing scenarios 5 and 6). The balance becomes negative and indicates that carbon credits are being awarded for the displacement of fossil fuels. This is shown in Table 14.9.

In the light of these results, the option with the lowest GHG emissions appears to be one with anaerobic digestion of the remaining cake. The harvesting would preferably be done by flocculation but mechanical harvesting and extraction seems to be a better than expected and remains a possible alternative.

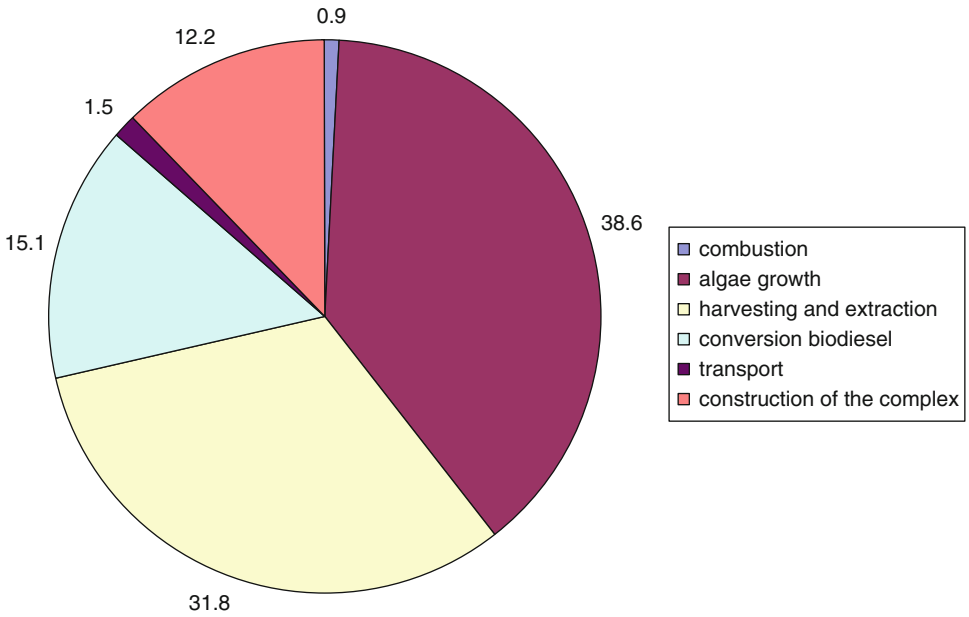


Fig. 14.6 Contribution (in %) of each process responsible for the GHG emissions for the PBR with mechanical harvesting and extraction scenario

11.3 Variation in Potential Yield and Electricity Consumption: Worst Case Scenario (Scenario 2)

In this case the GHG emissions are lower, but very close to the diesel model; only 1.37 g CO_2-e tkm^{-1} separates the two. Note that the worst case scenario energy requirement has been increased by 25 % for algae cultivation and harvesting. Thus, a higher electricity requirement and/or a lower growth rate than 109 $t.ha^{-1}.a^{-1}$ would result in higher GHG emissions than ULS diesel.

12 Identification of the Implications of Each Step of the Process on Potential GHG Emissions

12.1 Photobioreactor Scenarios

Figure 14.6 illustrates the proportions of the different processes responsible for the GHG emissions for the mechanical harvesting and extraction scenario (number 1) of 49.4 g CO_2-e tkm^{-1}. Algal cultivation is responsible for more than a third of the GHG emissions. The harvesting and extraction participates for a third. The transport of the oil/fuel and the combustion (reduced to biogenic carbon) are only responsible for 1.5 % and 0.9 % respectively. Finally the construction of the algae farm, considering a 5 year lifespan plastic, is 12 % even though the plastic is recycled. In the algae growth process 67 % of the emissions are created by the fertilizers and 33 % by the electricity consumption. In terms of harvesting 100 % of the emissions are due to electricity consumption.

Note that the proportions for the flocculation harvesting and solvent extraction in scenario 5 (47.8 g CO_2-e tkm^{-1}) are very similar to scenario 1. The harvesting and extraction contribute less due to lower energy requirements. Of the 29.6 %, 11.9 % is due to the flocculation harvesting (11.35 % for the electricity and 0.55 % for the chemical flocculant) and 17.7 % to the solvent extraction (17.5 % for electricity and steam from natural gas/0.2 % from the hexane).

12.2 Ponds Scenarios

Figure 14.7 lists the contribution of the different processes in the GHG emissions for the pond scenarios (56.7 g CO_2-e tkm^{-1}). It is clear that ponds have a higher percentage of the emissions coming from the algae growth: more energy to pump and mix more water, more fertilizer (to balance the volatilisation). The construction of the farm has a very small impact, only 0.3 %, but this is mainly due to the lack of data at the time the report was written on the GHG contributions from the farm construction; more complete data could result in this value increasing.

12.3 Construction of the PBR Algal Farm and Life Time of the Plastic Photobioreactors

It has been assumed that the algae farm lifespan is 20 years and the plastic photobioreactors 5 years. However, with

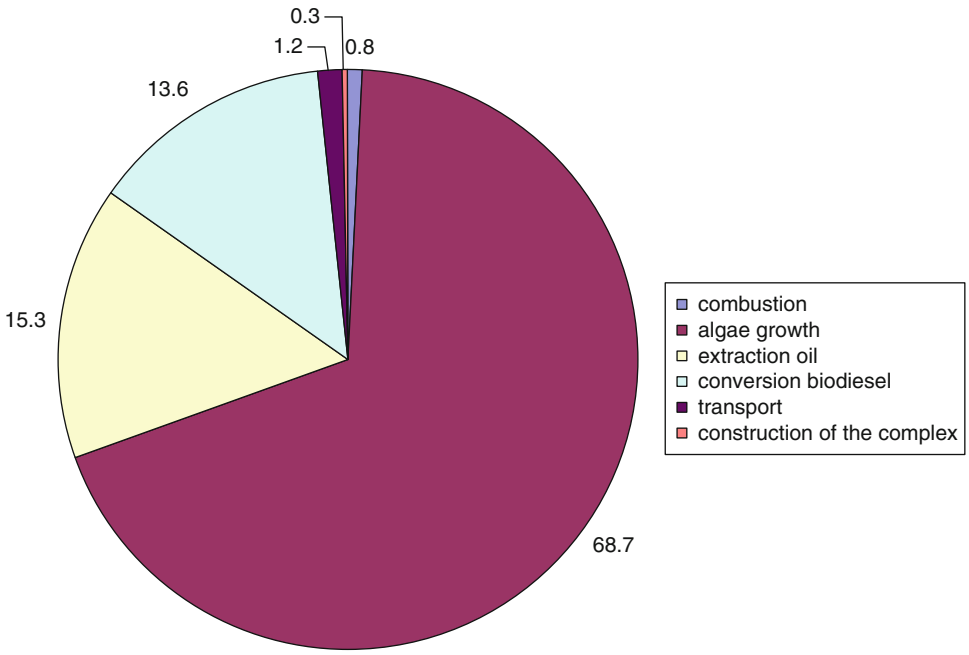


Fig. 14.7 Contribution (in %) of each process responsible for the GHG emissions for the ponds scenario

3 mm thick high density polyethylene one could expect that the PBR would last at least 10 or even 15 years. Table 14.10 shows that for 5 or 10 years, it is the plastic that is responsible for more than 94 % of the potential emissions in all the cases. Hence, an increase in the lifetime of the plastic from 5 to 10 years could almost halve the impacts of the capital investments. It means that for the flocculation cases (scenario 5 and 6) the emissions are reduced by 3.7 g CO_2-e tkm^{-1}. The choice of the plastic and its thickness are important criteria in the GHG emissions as the emissions during construction cannot be disregarded.

The recyclability of the plastic is also essential. Absence of recycling multiplies by 2.16 the emissions of the construction of the farm per t.km; the flocculation case would increase its emissions by a sizeable 16.1 g CO_2-e tkm^{-1}. In this context, the worst case scenario produces more GHG emissions than the ULS diesel reference.

13 Economics Analysis

13.1 Algae Biomass Production Economic Drivers

The production of biodiesel through microalgae has attractive commercial potential due to the high growth rates of microalgae. There is substantial competition presently to develop the first viable commercial scale algal biofuel production system that can generate substantial investor returns. However most people agree that this emerging technology is extremely challenging, highly variable and subject to dynamic and speculative markets. Hence, while improving growth and harvesting technologies it is also important to understand the economic drivers of such a system. A commercially successful production system depends upon cost minimisation of the following economic variables:

- Availability and cost of the land
- Value of the product (biodiesel) and co-products
- Total capital costs
- Operation and maintenance costs

13.2 Total Cost for the Algal Oil Production and Comparison with Other Studies

A cost estimate comparison was made in a recent report from the US Department of Energy (2009) that was based on scientific publications, company data and government numbers. In most cases the cost of algal oil production ranged from US$10 gal^{-1} to US$40 gal^{-1} which corresponds to US$2.64 L^{-1} to US$10.57 L^{-1} (or A$3.30 L^{-1} to A$12.85 L^{-1}). This range agrees with Stephens et al. (2010) who also note that an internal rate of return (IRR) of 15 % is achievable. In general, the estimate of costs of biofuels made from algae grown in PBR are higher than for algae grown in ponds. Oils produced in enclosed systems usually have an estimated cost higher than US$30 gal^{-1} (A$9.60 L^{-1}). Based on these cases, it is assumed that the price per gallon would almost double from US$17 gal^{-1} to US$32 gal^{-1} when going from ponds to PBR. Molina et al. (2003) even estimated that

Table 14.10 Contribution of the different components of the construction of the mechanical harvesting and extraction farm to the GHG emissions (in %)

Material	PBR from scenarios 1,2,3,5 and 6		Victorian commercial project PBR
Lifespan of the plastic PBR	5 years	10 years	5 years
metal device	0.1	0.2	0.2
plastic photobioreactors	97.2	94.6	98.1
tank/small hall	0.2	0.4	0.2
pipes	1.7	3.4	0.7
glass photobioreactors	0.7	1.3	0.7
Other	<0.1	<0.1	<0.1

Table 14.11 Total costs for algal oil for the Victorian commercial project (scenario 4)

Total capital cost (A$ ha^{-1}.year^{-1})	29,700
Total operating cost (A$.ha^{-1}.year^{-1})	300,000
Total cost (A$ ha^{-1}.year^{-1})	329,700
Oil production (L.ha^{-1}.year^{-1})	137,500
Total oil cost (A$ L^{-1})	2.40

the cost of their PBR design would result in an oil price of US$1,127 gal^{-1}.

Other studies have lower estimates. Chisti (2007) stated that oil recovered from the lowest-cost biomass produced in PBR is estimated to cost A$3.35 L^{-1}. This study also assumed that the production price of algae oil recovered from ponds was 30 % higher than for photobioreactors. Campbell et al. (2011) stated in 2009 that, in order to be profitable, a "realistic" cost for biodiesel produced in ponds (scenarios 7 and 8) was around A$1.45 L^{-1} (A$0.72 L^{-1} algal oil), cf. A$1.56 L^{-1} for Canola-derived biodiesel.

Capital costs for an algal biodiesel production system are a major commercial concern. Estimates for algae system capital costs vary widely, with ranges from A$30,000 to A$300,000 (Diversified Energy Corporation 2009). According to Benemann (2008), the simplest open pond systems including harvesting and biomass processing would cost at least A$120,000. Ponds farms (scenario 7 and 8) have a capital cost that ranges from A$93,600 to A$199,000 ha^{-1}.a^{-1}.

The Victorian PBR project (scenario 4) is at the top of these estimates and ranges from $A290,000 to 310,000 ha^{-1}. The price of the land is assumed to be zero in this scenario (being offered for free or at minimal rent by the power station). It also assumes that 30 % of the capital costs are due to the plastic PBR. In the same way Diversified Energy Corporation (2009) estimates that the algae growth system or PBR accounts for 34 % of the capital costs. The CO_2 infrastructure accounts for 13 % and the harvesting 35 %. It has been assumed that the algae farm lifespan is 20 years and the plastic photobioreactors 5 years. Hence 30 % of the capital costs have to be recovered every 5 years.

Operational costs have been estimated at A$300,000 ha^{-1}.a^{-1} by the Victorian project engineers. For ponds, 'realistic' operating costs have been estimated at between A$17,400 and A$47,900 ha^{-1} a^{-1}, but the production yield is expected to be lower. Algal production can be labour intensive, being required for photobioreactor maintenance, monitoring of the culture, harvesting and extraction. However, if the design of the process is improved and the operations are automated, the number of workers required could decrease without unreasonably increasing the capital costs (Tapie and Bernardt 1988). The Victorian commercial project expects to have 20 people working on its 80 ha farm: 1 person per 4 ha.

Table 14.11 gives the total estimated cost for the algae oil according to the Victorian project numbers. The costs of co-products are not included in this table. These results and those of other studies are given in Fig. 14.8, which lists the prices for a litre of algal oil produced in photobioreactors.

13.3 Algae Meal Economic Value: Improvement of the Oil Price

Co-products from microalgae and their commercial value are an important part of the commercial biorefinery approach. In scenario 4 it is assumed that 250 t.ha^{-1}.year^{-1} of cake is produced. Depending upon the end-life chosen for the algal cake (energy production or animal feed), the energy accumulated in the cake can be monetarily estimated.

Anaerobic digestion of the cake produced in scenario 4 would result in the production of electricity. In Victoria, industries and companies pay and sell electricity for 7.5–10c. kWh^{-1}. Note that assigning this value for electricity produced may be conservative if the electricity produced in this fashion could be considered "green" and thus capable of attracting RET (Renewable Energy Target) credits.

Selling the remaining algal cake to farmers for animal feed is highly dependent on the price of the meal on the market. Lupin meal, which is the closest substitute on the

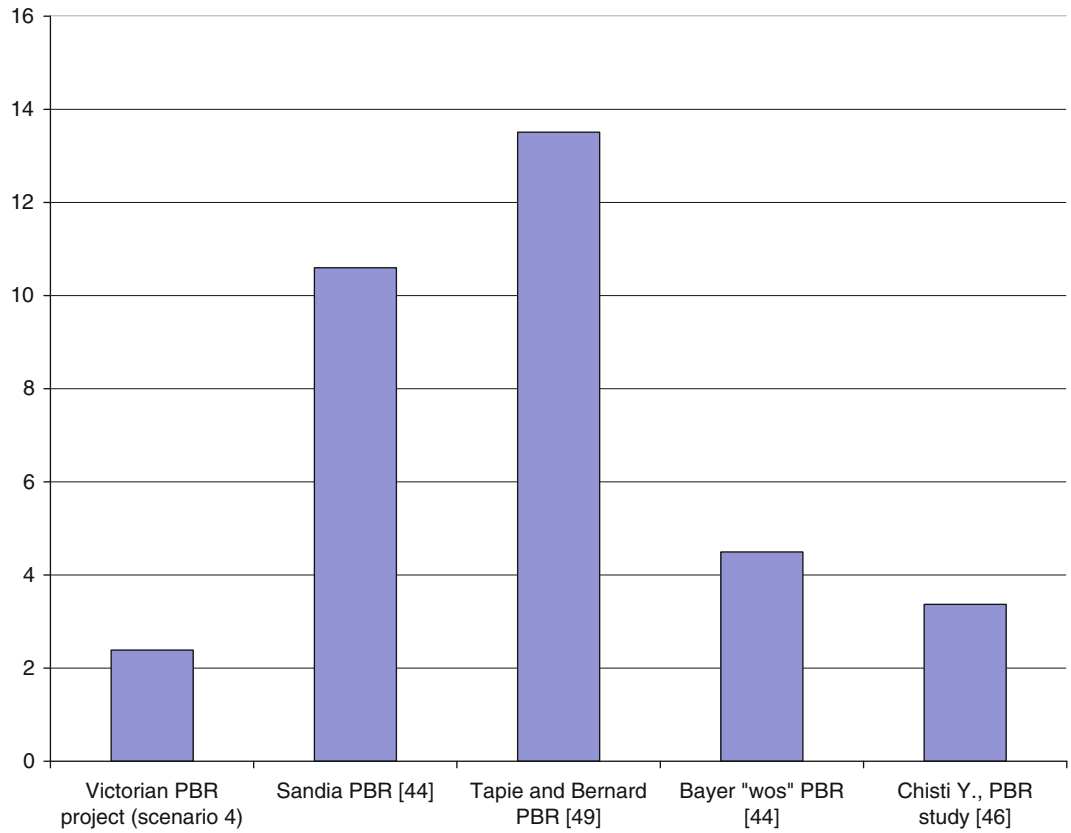


Fig. 14.8 Price of a litre of algal oil produced in PBR for different studies and scenario 4 (A$). The acronym "wos" stands for "wildly optimistic scenario"

Table 14.12 Minimal, maximal and average values for the algal cake in AU$ t^{-1}, AU$ ha^{-1} and AU$ L^{-1} oil produced for the two end-life options of the algal cake (animal feed and anaerobic digestion)

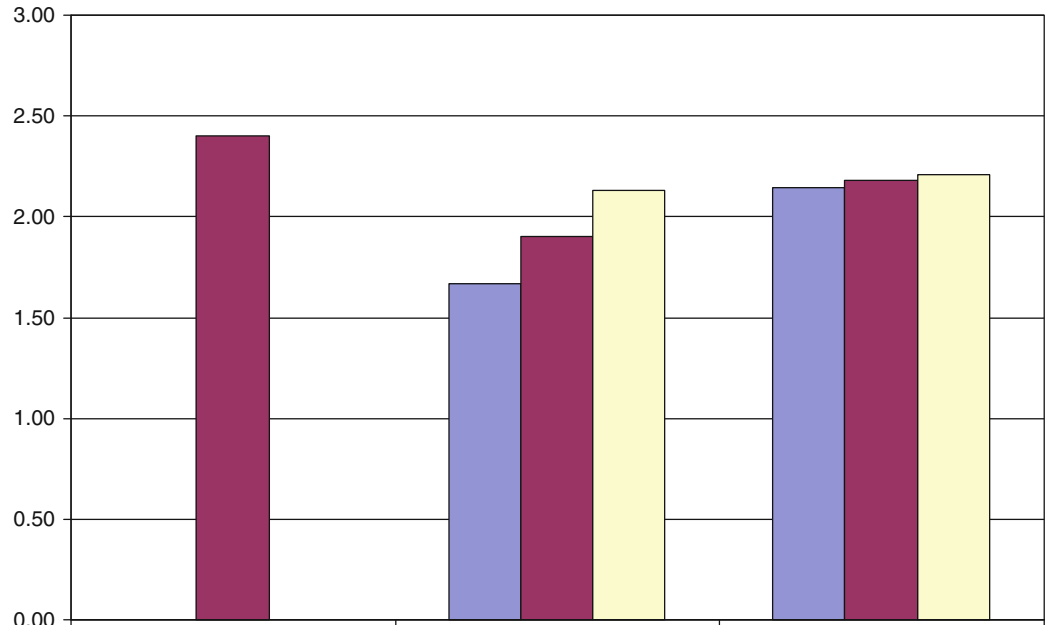
		Best value	Average value	Worst value
Algal cake use: **meal/ animal feed**	Value of the algal meal (AU$ t^{-1})	300	225	150
	Amount received from the algal meal (AU$ ha^{-1})	100,000	68,750	37,500
	Amount received from the algal meal (AU$ L^{-1} oil)	0.73	0.50	0.27
Algal cake use: **energy production-anaerobic digestion**	Value of the electricity (c kWh^{-1})	10.00	8.75	7.50
	Amount received from the algal meal (AU$ ha^{-1})	34,928	30,562	26,196
	Amount received from the algal meal (AU$ L^{-1} oil)	0.25	0.22	0.19

market currently, ranges from A$150 to 400 t^{-1} (Glencross 2001). Adopting these ranges, the minimal, maximal and average values of the product are given in Table 14.12 in A$ t^{-1} of algal cake, A$ ha^{-1} and A$ L^{-1} of oil produced.

Figure 14.9 gives an overview of the prices of a litre of algal oil according to the end-life chosen for the algal cake. Both Table 14.12 and Fig. 14.9 show that the use of the main co-product of the biodiesel process significantly decreases the price of the algal oil production. Although algal cake can be sold for animal feed or processed to produce electricity, the lowest costs arise from algal cake used for animal feed, which decreases the price of the oil by 30 % in the best case. However, this option can be considered only if no chemicals have been involved in the oil extraction (pure mechanical extraction). Nevertheless, anaerobic digestion is also a viable economic alternative, decreasing the price of the oil by 8 % in the worst case assumption.

14 Discussion and Conclusion

As demonstrated here, microalgae biodiesel has the potential to displace liquid transport fuels derived from petroleum. For the designs suggested in this study, environmental figures are



	No use of algae cake	Algae cake use : meal/animal feed	Algae cake use : energy production via anaerobic digestion
Best value algal oil (A$/L)		1.67	2.15
Average value algal oil (A$/L)	2.40	1.90	2.18
Worst value algal oil (A$/L)		2.13	2.21

Fig. 14.9 Algal oil price according to the end-life chosen for the algal cake and range of values

very favourable for algal fuel. Both ponds and photobioreactors show lower greenhouse gas emissions and better energetic balance than petrodiesel. Chosen designs for photobioreactors are very competitive and point to a better use of the resources than pond designs from previous studies. However, it is important to note that this analysis assesses the life cycle of a process that does not yet exist at an industrial scale.

For photobioreactors, algae production scenarios that range from 109 $t.ha^{-1}.a^{-1}$ (worst case scenario) to 375 $t.ha^{-1}.year^{-1}$ (Victorian commercial project, best case scenario) have been tested. The environmental analysis shows the importance of improving the growth rate in order to reduce the GHG emissions. These emissions become lower than Canola biodiesel when a production rate of 375 $t.ha^{-1}.year^{-1}$ is reached. Note that if the production rate is actually lower than 109 $t.ha^{-1}.year^{-1}$ and the inputs higher than the ones described for the worst cast scenario (scenario 2: high energy demanding mechanical extraction and no use of the algae meal) then the production of microalgae biodiesel would become more energy demanding and GHG emitting than petrodiesel. Hopefully this would not be likely to happen.

Several algae harvesting systems and oil extraction methods have been analysed. Mechanical harvesting and extraction was compared to chemical harvesting and extraction. Flocculation followed by solvent extraction is the common method preferred in terms of GHG emissions. If an inorganic flocculant is used then this option is incompatible with the use of the cake as animal feed. Organic flocculants such as chitosan or starch do not have this problem. The new (proprietary information) harvesting system from the Victorian commercial project analysed in scenario 4 exhibits even better results. The method is based on a new dewatering system technology combined with centrifugation.

One of the significant results of this life cycle assessment is the importance of the main co-product of the process: the algal cake remaining after extraction. It represents in mass the main output of the system (around 70 %). The energy stored in the cake is a potential material for anaerobic digestion, producing biogas that can be directly used to provide heat and electricity. The other option tested in this LCA was to sell the cake as animal feed. Both options show an important reduction of the GHG emissions arising from the overall process. The use of algal cake as animal feed is a good option as it decreases the emissions by 26 %. In terms of environmental impact anaerobic digestion is a much better alternative, with emissions being reduced by 232 %; the balance becomes negative which indicates that carbon credits are being awarded for the displacement of fossil fuels.

However the economics study based on the data from the Victorian commercial project shows another point of view.

Being rich in good quality proteins, the algae meal can be sold to farmers at a price that could reach A$400 t^{-1}. At this price the price of algal oil decreases by 30 %. On the other hand, at its best, anaerobic digestion only decreases the price by 10 %. This difference can vary according to the assumptions made for the "anaerobic digestion" and "animal feed" given in this study. The choice of end-use for the algae meal has more impact on the economics and the GHG emissions than switching from one system of harvesting/extraction to another. With regards to these results this appears to be the key issue of the life cycle.

Another important point shown in this study is the part played by the construction of the farm on the economics and environment. The cost of this capital is higher than the average pond or photobioreactor algal farm costs found in literature. The capital investment of the photobioreactor algal farm contributes around 12% of the GHG emissions. Of those 12%, 11.3% are due to the plastic that has to be renewed every 5 years. However, the main advantage of the plastic is its recyclability. Hence, this analysis indicates that the absence of recycling multiplies by 2.16 the emissions of the construction of the farm.

Biodiesel production of microalgae is an emerging technology that is considered promising. However the economics of the process still need to be improved to be competitive with petrodiesel. Improvements in the harvesting system, algae farm construction, algae yield, and integrative end-life of the co-products are shown to be the key challenges.

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Techno-Economic Modeling for Biofuels from Microalgae

15

Michael A. Borowitzka

1 Introduction

Techno-economic modeling is a valuable and widely used process for guiding research and development efforts in order to achieve an economically viable outcome. In the case of algal biofuels, techno-economic modeling can be used and to provide important information on the best path to commercialization and to provide an estimate of the cost of biofuel production. These models integrate complex technical and economic information for a given process or processes. Techno-economic modeling can be used the evaluate and compare alternate processes, to help in defining the project scale and scope for economic value, measure uncertainty of project technical and financial risks, for the assessment of the sensitivity to changes in prices/efficiencies on project worth,[1] for the economic evaluation of project worth, and for the calculation of expected returns and risks to capital investment.

Given the present state of the technology of the production of biofuels from microalgae, and the many alternative options available at every step of the production process (i.e. choice of species and strain, culture system, harvesting and dewatering system, extraction, co-products etc.), techno-economic models have great value as strategic planning and decision making tools to help direct the technical development of the production of biofuels from algae towards establishing an economically viable process.

Many economic models estimating the production costs of microalgae have been published, especially in the last few years (e.g., Tapie and Bernard 1988; Borowitzka 1992; 1999b; Matsumoto et al. 1995; Benemann and Oswald 1996; Molina Grima et al. 2003, 2004; Stephens et al. 2010; Richardson et al. 2010; Lundquist et al. 2010; Norsker et al. 2011). Greenhouse gas life-cycle modeling is a particular case of techno-economic modeling where, in effect, the currency is CO_2 (see Chapter 14 in this volume). The modeling process and structure of these type of models are similar, and as with the economic models, the various recent carbon dioxide life-cycle models are based on the same or very similar assumptions (e.g., Jorquera et al. 2010; Stephenson et al. 2010; Campbell et al. 2011). Recently, other models and technical assessments also have focused on water and nutrient requirements as well as energy balances (e.g., Mulder et al. 2010; Borowitzka and Moheimani 2010; Yang et al. 2011; Fon Sing et al. 2011; Rösch et al. 2012; Beal et al. 2012). All of the models above vary in the level of detail and in the assumptions made; however, as almost all are based on small scale (laboratory) and short term studies, rather than large scale operations, these models must be considered preliminary. However, they do provide a guide for effective research and development to achieve economical and environmentally friendly biofuels from algae. Probably the most realistic and up-to-date techno-economic analysis recently published is that by Lundquist et al. (2010).

This chapter describes the process of techno-economic modeling and highlights some of the findings of existing models, but it does not provide another new model as available data still are insufficient and inadequate.

2 The Process of Techno-Economic Modelling

The process of techno-economic modeling when used as a strategic planning tool, should have four, iterative, steps (Fig. 15.1):

1. scoping the project,
2. modeling the project,
3. evaluating the results of the model,
4. refining the model –and then repeating the process to continue to improve the model.

[1] Project worth as measured by the return on investment (ROI), the internal rate of return (IRR) and/or the net present value (NPV) – see definitions at end of this chapter.

M.A. Borowitzka (✉)
Algae R&D Centre, School of Biological Sciences,
Murdoch University, Murdoch, WA 6150, Australia
e-mail: M.borowitzka@murdoch.edu.au

M.A. Borowitzka and N.R. Moheimani (eds.), *Algae for Biofuels and Energy*, Developments in Applied Phycology 5,
DOI 10.1007/978-94-007-5479-9_15, © Springer Science+Business Media Dordrecht 2013

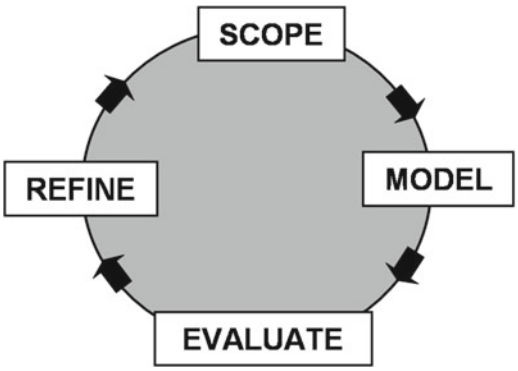

Fig. 15.1 The techno-economic modelling cycle

When the model is used primarily as a tool for determining research needs and to help set research priorities the results of the model and of the associated sensitivity analysis can be examined to prioritise research activities. The model should be periodically refined and updated based on the results of the research to help in the re-prioritisation of the next research steps.

2.1 Scoping the Project

This is a very critical step. The level of detail required depends on the purpose of the model. However, for commercial-scale algae biofuels production, there is the need to define all the steps (and their possible alternatives) of the whole process – from the start of production to the final sale of the product. Consideration must also be given to the likely scale of the production – e.g. how many barrels of algal biodiesel or tonnes of algal biomass per year will need to be produced? What species and strain of alga will be used and what are the physical, physiological and biochemical properties of this species and strain? What culture process will be used, how will the biomass be harvested and dewatered, and what processing of the harvested biomass will be required to produce the final product(s)? What is/are the final product(s) (e.g. algal lipids for biodiesel production, the biodiesel produced, biomass for conversion of fuel by other methods, algal lipids and other co-products etc.)? Where will the production plant be located (climate, land availability, water source(s), local available infrastructure, distance to market etc.)? What environmental and other regulatory issues are there which must be resolved?

A key part of the scoping of the project will be to determine what data are required for the modeling and what data are available. At this stage it is likely that the project will also be divided into sub-projects to make the task more manageable.

2.2 The Model

Models can be as simple or as complex as required. The available data, as determined during the scoping step, are also an important consideration in determining the complexity of the model. The model is generally best constructed as a group of sub-models, one for each of the major steps or processes. This allows for alternative possible process steps (e.g., different harvesting methods) to be more easily evaluated and compared. The creation of process flow diagrams with expected mass balances and flows is important to visualize the various elements of the process and their relationships, and will aid in the creation of the numerical economic model. Although a range of commercial process modeling software packages are available these are generally not essential as spreadsheets such as Excel (Microsoft) are sufficiently powerful tools for most modeling.

Project costs can be separated into the capital expenses (CapEx) and the operating expenses (OpEx). CapEx is defined as '*Funds used by a company to acquire or upgrade physical assets such as property, industrial buildings and equipment*'. CapEx is depreciated over time and the depreciation for the various capital items needs to be considered within the confines of allowed accounting practices. OpEx is defined as '*Expenditure that a business incurs as a result of performing its normal operations*'. Operating expenses are not depreciated over time.

A decision needs to be made whether CapEx is included in the model or not, and this depends, to some degree, on the aims of the modeling. In investment analysis, for example, depreciation and taxes are often not considered initially, although they do need to be considered once a decision to invest has been made.

2.3 Sensitivity Analysis

Sensitivity analysis is used to seek more information on the possible variations of outcomes for different variations of input. This method can be used to determine those input variables which have the most effect on the outcome. This sensitivity analysis can be simple; i.e. where only one variable is varied at a time while the other parameters remain fixed. This is the simplest and most commonly carried out method in economic modeling of algal processes (see for example: Borowitzka 1992; Stephens et al. 2010; Davis et al. 2011; Norsker et al. 2011). As this level of sensitivity analysis is very basic and, in order to obtain a greater understanding, it is better to calculate the variance of the outcome for a given variation of the input parameter. If this is calculated for all input variables, then it is possible to calculate a normalized

variance for each parameter by dividing that parameters variance by the total variance (i.e., the sum of the variances of all parameters). The normalized contribution of input parameter j, p_j, to the total variance of the outcome can be calculated from the following equation:

$$p_j = \frac{\sigma_j^2}{\sum_{j=1}^{m}\sigma_j^2} = \frac{\frac{1}{n}\sum_{i=1}^{n}(x_{ij}-\mu^2)}{\sum_{j=1}^{m}\left(\frac{1}{n}\sum_{i=1}^{n}\left(x_{ij}-\mu^2\right)\right)} = \frac{\sum_{i=1}^{n}\left(x_{ij}-\mu^2\right)}{\sum_{j=1}^{m}\sum_{i=1}^{n}\left(x_{ij}-\mu^2\right)} \quad (15.1)$$

where σ_j^2 = the variance originating from varying input of parameter j; m = the number of input parameters varied, n = the number of tests (i.e., the number of different input values tested for the given parameter), x_{ij} = outcome for test i and with varying input parameter j, μ = mean out come of all tests.

This method is usually used for the first evaluation of the modeled process as is it quite easy to carry out. Importantly, it can be used to determine the key input parameters which are required for the global sensitivity analysis (see below).

A more comprehensive 'global' sensitivity analysis can also be done once the key input parameters have been identified. In a global sensitivity analysis the different key input parameters are varied according to a predefined probability density function (PDF) such as a Gaussian, triangular or uniform distribution. The choice of PDF and the range over which each parameter will be varied (e.g. the standard deviation in the case of a Gaussian distribution) will be very important. A Monte Carlo method is used to sample the very large number of possible outcomes for the model – in each step of this simulation a random probable value for each of the key input parameters is generated according to their PDF. This determines the distribution of all possible outcomes and using this distribution the probability of an outcome can be determined within predefined boundaries. For example, if the business case is being analysed, one could search for the probability of a positive net present value. Furthermore, this global sensitivity analysis can provide detailed information on the impact of the key input parameters on the trend or reliability of the results.

An example of this methodology can be found in the paper by Richardson et al. (2010) which illustrates the type of output data which can be obtained. Unfortunately the conclusions reached by this model are of limited value as the model used concentrates on the production of the biomass in two alternate systems in detail (raceways and photobioreactors), but does not also model the harvesting, dewatering, lipid extraction and processing processes which are critical components of the whole process and which interact with the production system and can have a significant effects on the final product cost.

3 Some Findings of Published Techno-Economic Models

It has been known for a long time that algae are relatively expensive to produce (Tamiya 1957; Borowitzka 1999a) and all recently published reasonably realistic techno-economic models for microalgae biofuels production have reached the same conclusions, irrespective of the model details and the assumptions used.

The various published techno-economic models of commercial-scale microalgae production come to a range of conclusions. This is not surprising as the details of the models vary as do many of the assumptions used. Almost all input data for these models are based on small-scale studies and not on data from the existing commercial operations as the data from the commercial operations is commercially sensitive and therefore not publicly available. However, there are several important common findings which point to the key factors affecting the cost of microalgae and microalgal lipid production.

In all cases the algae growth rate (μ; specific growth rate) and the content of the product (Q) in the algal cells are critical factors as they define the productivity, i.e.

$$Productivity = \mu Q / V \quad (15.2)$$

where **V** is the volume of the culture or the pond area. For biodiesel production **Q** is the product content in the cells (in the case of biofuels this is usually the lipid or triglyceride content).

A common conclusion of all models to date is that the production of biofuels from algae using existing methodologies is uneconomic. This is not really surprising given that the estimated production costs of algal biomass by existing commercial producers ranges from about \$5 kg^{-1} to over \$1,000 kg^{-1} (Borowitzka 1999b). The lowest cost is for *Dunaliella salina* β-carotene production in the very large plants in Australia, whereas the highest cost is for the production of algae in aquaculture hatcheries and grow-out facilities all of which are relatively small scale operations. Some models use unrealistic assumptions on biomass productivities and lipid content, and use the maximum values reported for these, assuming that these values can be maintained over the whole year in a production-scale operation. The best long-term productivities which have been attained in outdoor raceway ponds are productivities of about 20 g ash free dry weight m^{-2} day^{-1} with a total lipid content of about 35–40 % of ash free dry weight (see table 8.2 in

Chapter 8). This is equivalent to 73 t dry algal biomass ha^{-1} $year^{-1}$ and 25.55–29.20 t total lipid ha^{-1} $year^{-1}$ (= 28,389–32,444 L of total lipids, assuming a density of 0.9). Of course, not all of this lipid is convertible to biodiesel or a similar fuel. It must also be noted that these productivities have mainly been achieved in small raceway ponds of up to 200 m^2 in area and it is not known whether they can be achieved on a larger scale. With the right algal strain and at the right location it may be possible to increase the average biomass productivity somewhat, with a sensible target being an annual average productivity of 30 g m^{-2} day^{-1}. Higher productivities have been achieved for short periods of one to several days but, due to the seasonal change in daylength and irradiance, are not achievable as an annual average.

A key limitation on the production of algal biofuels is the efficiency of photosynthesis, i.e. the amount of the available light energy which is converted to algal biomass (Walker 2009). Algae use light in the range of 400–700 nm, the photosynthetically active radiation (PAR) which is about 48 % of the total solar radiation at ground level (Goldman 1979; González and Calbó 2002). The theoretical photosynthetic efficiency of algae is about 11 % of solar irradiation (Huntley and Redalje 2007; Weyer et al. 2010), but actual efficiencies observed lie between about 2–4 %, with cultures grown in tubular photobioreactors generally lying at the upper end of this range (data from: Laws et al. 1983; Pushparaj et al. 1997; Molina-Grima et al. 1997; Tredici and Zittelli 1998; Del Campo et al. 2001; Moreno et al. 2003; Moheimani and Borowitzka 2006). Long-term photosynthetic efficiencies of 6.56 % PAR (or 2.8 % solar) have been observed for *Chlorella* cultures grown in small raceway ponds in a glasshouse in Sendai, Japan (Hase et al. 2000). There are further energetic losses due to metabolic requirements of the algae cells etc. (Zijffers et al. 2010). The model developed by Goldman (1979) predicted that a productivity of 60 g m^{-2} day^{-1} was theoretically possible, but that only 30–40 g m^{-2} day^{-1} was practically possible. The simulation model of Sukenik et al. (1991) predicting the production rates of *Isochrysis galbana* in outdoor raceway ponds and based on experimental data of Falkowski et al. (1985) and Dubinsky et al. (1986) gave production rates of between 10.2 and 27.6 g dry weight m^{-2} day^{-1}, values which were is agreement with actual outdoor culture data (Boussiba et al. 1988). Experimental data for a range of algae species have shown productivities of up to ~50 g m^{-2} day^{-1} in raceway ponds over very short periods (days), but with annual average productivities not exceeding 20–22 g m^{-2} day^{-1} (Sheehan et al. 1998; Moheimani and Borowitzka 2006). Theoretical estimations of the maximum productivity of range from 230,000 to 53,200 L algal oil ha^{-1} $year^{-1}$ (Weyer et al. 2010; Zemke et al. 2010), with the higher value based on an assumed 50% oil content.

The harvesting, thickening and dewatering of microalgae is also an expensive but essential step and has been extensively reviewed by Shelef (1974), Moraine et al. (1980), Mohn (1988), Mohn and Cordero-Contreras (1990), and Molina Grima et al. (2004) and also in chapter 10 of this volume. The selection of the harvesting and dewatering method will depend on the species to be harvested, the culture process and the biomass concentration at harvest, the degree of dewatering required for the subsequent extraction step and whether or not the medium will be recycled.

The lipid extraction step (if there is one) is probably the least understood for modeling as none of the proposed methods have been tested at scale meaning that major assumptions have to be made in any model exercise. The first question is whether the biomass has to be dried before extraction or a 'wet' extraction process will be used. Aside from conventional hexane extraction a range of solvent systems have been proposed such as hexane/ethanol (Cartens et al. 1996), and hexane/isopropanol (Nagle and Lemke 1990). Switchable polarity solvents which are lipophilic in the non-ionic form and hydrodrophilic in the ionic form (Handy 2003; Phan et al. 2009) have also been suggested and Samori et al. (2010) have used these to extract hydrocarbons from *Botryococcus braunii*. Other 'green' solvent systems include subcritical water extraction and supercritical fluid extraction with CO_2 (Herrero et al. 2006). The efficiency of these extraction methods will be influenced very much by the algae species, especially by the nature of the cell covering. For many species, as for example *Nannochloropsis, Chlorella* and *Tetraselmis*, the cell covering needs to be ruptured for effective extraction of the lipids. Again, there are a range of potential options for rupturing the cells which include mechanical processes such as bead mills, sonication, cavitation and autoclaving and non-mechanical methods such as freezing, osmotic shock, enzymatic digestion, use of organic solvents, and acid or base reactions (Kanel and Guelcher 1999; Pernet and Tremblay 2003; Cravotto et al. 2008; Cooney et al. 2009; Lee et al. 2010; Ranjan et al. 2010).

Alternative methods to lipid extraction for biofuel production from microalgae are direct thermochemical liquefaction (Demirbas 2010), direct transesterification (Ehimen et al. 2010; Levine et al. 2010; Xu and Mi 2010) and pyrolysis (Peng and Wu 2000; Miao and Wu 2004; Grierson et al. 2009). The cost of several of these methods has been modeled recently by de Boer et al. (2012).

Many of the published models to date do not consider important factors such as the exact location of the production plant which has a very important impact on both CapEx and OpEx. Location is not only important because if affects the productivity of the algal cultures because of climate, but also because of land cost and the availability of land suitable for the construction of the production plant. The distance for the supply of key inputs such as water and CO_2 and the distance to market are also location dependent. There are few loca-

tions where suitable land, an adequate water supply and a source of CO_2 are co-located (Borowitzka et al. 2012). Furthermore, as a production plant capable of producing a relevant quantity of algal biofuels using raceway ponds or a similar production system will be at least 600 ha in area (Borowitzka and Moheimani 2011) the cost of the water and CO_2-supply system of the plant has a major impact on production costs and needs to be considered. The question of how and where the large quantities of waste water produced both in the algae culture process (water cannot be recycled indefinitely because of the build-up of salts) and the extraction process must be considered as this has the potential to add significant costs.

Most of the published models are also 'generic', i.e. they do not take account of the algae species to be used and this is a major limitation of these models. The species of alga used clearly will affect harvesting and extraction processes and will also have an impact on the composition of the lipids which affects the fuel quality and the conversion efficiency of the lipids to fuel such as biodiesel (See Knothe 2005, 2006 and Chapter 12 this book). Whether there are also other valuable by- and co-products which can be exploited will also depend on the species used.

4 Reducing Algal Biofuels Production Costs

A number of cost estimates for microalgae production based on pilot-scale studies are summarised in Table 15.1. Similarly Table 15.2 summarises the findings of detailed modelling by Molina Grima et al. (2004) for the production of biomass of *Phaeodactylum tricornutum* in a tubular photobioreactor. This detailed model shows the relative costs of various components of the biomass production process. The conclusion from all of these cost estimates and models which are based on real productivities achieved at a range of scales of operation further highlight the current main problem with the production of algae for biofuels and bioenergy – it is too expensive to produce the algae biomass.

Irrespective of the model assumptions it is clear that algae biofuels production costs must be significantly reduced. As the algal biomass is the 'feed-stock' for the process reducing the cost of producing this biomass is the critical requirement, irrespective of the costs of the later processing steps. In order to achieve the lowest production cost an annual average high lipid productivity by the cultured algae must be attained. It has long been recognised that seasonal changes in irradiance and temperature greatly affect algae productivity (Sasa et al. 1955) and clearly the site for algae biomass production must be located where there is high insolation and an acceptable temperature range in both summer and winter so as to maximise the annual average productivity. In order to achieve a high annual average lipid productivity and to cost-effectively employ the high capital investment (fixed costs) in the production plant the algae strain used must be grown all year round. Selecting the correct strain suited to the climatic conditions at the production site is therefore a critical step (see Chapter 4 this book) and, in turn, the species selected will influence the choice of harvesting and downstream processes.

A significant reduction in the cost of algal biofuels will only be achieved is all steps in the production process from algal biomass production to the conversion of the biomass to fuel are fully integrated and optimised. Modeling can be used as a preliminary assessment of the cost and benefits of alternate technologies and processes (see for example: Brentner et al. 2011) and to help guide research and development priorities. Table 15.2 summarises the findings of one modelling effort by Molina Grima et al. (2004).

Recent models have clearly confirmed that nutrients and water present both economic and sustainability challenges (Borowitzka and Moheimani 2011; Yang et al. 2011; Rösch et al. 2012). These suggest that using algae able to grow over a wide range of salinity is preferred to minimize competition for fresh water, and that aside from nutrient recycling in the production process, the ability to use nutrient-rich waste waters, if available, should be and important target for sustainable algal biofuels production (Table 15.2).

Nutrient costs can also be reduced if a wastewater stream is used for the algae culture, or if the algae produced are actually used for wastewater treatment directly thus having an added economic benefit (i.e. the value of the waste water treatment). This strategy is being widely examined (Lundquist et al. 2010; Adey et al. 2011; Park et al. 2011). However, the economics of this are still uncertain and will greatly depend on location; i.e. climate, waste stream, land availability etc.

The capital costs of algae production systems also needs to be markedly reduced, possibly through the use of cheaper materials (i.e. pond liners) and lower cost construction methods. Improved automation of the whole process is also needed to reduce labour costs and should also improve reliability.

4.1 Co- and By-products

In order to make algae biofuels (biodiesel) production commercially viable many people have proposed the need to also produce a range of co- and by-products, with many of those proposed having a higher market value that the algal oils themselves.

The major by-product considered by many is methane which is produced by fermenting the algal biomass remaining after the oil extraction, or using the whole biomass for methane production rather than extracting the lipids

Table 15.1 Estimates of the cost of algal biomass grown in raceway ponds from published studies (All costs are converted to 2011 US$ and have been adjusted for inflation by the annual USA inflation rates)

Alga	Culture System	Culture Area/ volume	Productivity (g m^{-2} day^{-1})	Estmated Cost ($US kg^{-1})	References
Scenedesmus	Raceway	4 ha	20	6.74	Becker and Venkataraman (1980)[1]
Chlorella (Photoautotrophic)	Raceway	10 ha	25–30	11.07	Kawaguchi (1980)[2]
Chlorella (Mixotrophic)	Raceway	10 ha	25–30	11.26	Kawaguchi (1980)[3]
Spirulina	Raceway	2 ha	12	11.20	Rebeller (1982)
Porphyridium	Tubular PBR	10 ha	16	9.10	Tapie and Bernard (1988)
Spirulina	Raceway	5 ha	3.2	18.00	Jassby (1988)
Dunaliella salina	Raceway	2 ha	4	11.36	Mohn and Cordero-Contreras (1990)
Chlorella	Thin-layer Cascade	1 ha	18	19.05	Data from Pilot-scale facility at Dongara, Western Australia[4]
Microalgae	Tank Culture	20,000 L		70.90	Fulks and Main, (1991)
Microalgae	Biocoil	2400 L	0.06 g L^{-1} day^{-1}	24.50	Unpublished Data[5]
Spirulina	Raceway	1.5 ha	15	11.90	Tanticharoen et al. (1993)[6]
Nannochloropsis	Raceway	0.2 ha	16 (summer), 8 (winter)	49.00	Zmora and Richmond (2004)[7]

[1]Based on experience of Indo-German project in Mysore, India
[2]freeze-dried
[3]Spray-dried
[4]Includes harvesting and spray-drying costs – no depreciation of capital costs
[5]Does not include harvesting and drying costs – no depreciation of capital costs
[6]grown on sago starch factory wastewater
[7]Only biomass production cost. Harvesting costs etc. not included

first. The latter process has recently been modeled by Zamalloa et al. (2011). Their model showed that the latter process could produce energy (levelised cost of energy; International Energy Agency 2010) in the order of €0.170–0.097 kWh^{-1} (~US$0.119–0.061), taking into account a carbon credit of about €30 t^{-1} CO_2 (~US$21).

An alternative to methane production from the residual biomass is to use this biomass as animal feed. There are extensive literature reports on the suitability of microalgal biomass as a feed for a wide range of animals (e.g., Brune and Walz 1978; Lipstein and Hurwitz 1980; Yap et al. 1982; Ross and Dominy 1990; Herrero et al. 1993; Chowdhury et al. 1995). However, the likely value of this biomass as an animal feed (protein) source is likely to be only about US$1000 t^{-1} at best. The extraction of the oils before using the remaining biomass as animal feed (i.e. a protein-rich food source) reduces the value of this feed supplement by reducing the fat content, whereas the whole algae probably have a higher value than the biofuels. However, for use in feed there is the additional cost of drying the residual algal biomass unless extruded animal feeds using this biomass are produced on site. Lundquist et al. (2010) have estimated that if the biomass residue is 65 % of the biomass with a 20 mJ kg^{-1} energy content half if this energy could be recovered as methane. With a methane electricity generation equivalent of 10,000 kJ kWh^{-1} and a value of US$0.1 kWh^{-1} this would generate electricity with a value of about US$100 t^{-1} of biomass residue. Furthermore, the nutrients in the residue (10 % N), with a value of US$500 t^{-1}, are worth another US$50 t^{-1} of residue. This is about equivalent to the likely value of the residue as animal feed.

Other co-products which have been suggested are carotenoids (i.e. astaxanthin, β-carotene, lutein etc.), long chain polyunsaturated fatty acids (e.g., eicosapentaenoic acid and docosahexaenoic acid), polysaccharides and steroids - all of which have a much higher value, but also a very much smaller market, than the algal oils for biodiesel (see Borowitzka 1988a, b, 1995, 1999c, 2010). For some of these potential co-products (for example: steroids and polysaccharides) applications and markets would have to be developed. Other potential problem in the co-production of some of these products with algal oils for biodiesel also exists. For example, in the case of the carotenoids, these are mainly found within the lipid droplets in the algal cells and they are usually sold in the form in a lipid solution or

Table 15.2 Summary of projected costs of producing algal biomass (*Phaeodactylum tricornutum*) using horizontal tubular photobioreactors in Spain

	Total Cost (in Euro)	Percentage (including depreciation etc.)	Percentage (excluding depreciation etc.)
Total capital investment	**2,490,264**		
Depreciation + tax + insurance	271,660	34.2	
Production cost			
Raw materials	109,940	13.8	21.0
(medium)	(38,530)		
(CO_2)	(45,209)		
Utilities (Power only)	4,997	0.6	1.0
Wastewater Treatment	6,164	0.8	1.2
Total labour	237,182	29.8	45.3
Other operating	165,210	20.8	31.5
Total operating[a]	**795,153**		

Data recalculated from Molina Grima et al. 2004
Plant size 26.2 t $year^{-1}$
[a]Cost of biomass is € 30.25 kg^{-1} when depreciation of the capital expenditure is included, and € 19.98 kg^{-1} when capital expenditure is excluded

as suspensions in lipids. Therefore their extraction also means the extraction of the lipids which are now not available for biofuel production. The long-chain polyunsaturated fatty acids and sterols also are part of the cells lipids and would have to be concentrated which could result in some 'waste' lipids which could be used for biodiesel production. There is also the problem of a mismatch in the relative market sizes of these high value products and of the algal oils for biofuels. For example, it would require only several tens of thousands of tonnes of algal biomass to supply all of the world's β-carotene and astaxanthin markets, whereas the demand for biofuels requires at least hundreds of thousands of tonnes of algal biomass. The highest value products are used in the pharmaceutical, nutraceutical and health food areas and thus require a high level of purity which means a high processing cost. If they are sold as animal feed supplements their value is significantly lower.

If the co-products are to be sold for use as nutraceuticals or cosmaceuticals there are also many quality assurance and regulatory issues which must be considered (Gellenbeck 2012).

Of course there is the option of part of the algae biomass produced in a commercial facility being used to manufacture high value products, and the other portion of the biomass being used to produce biofuels with the possibility that this allows economics of scale to be achieved which reduce the overall biomass production cost. This would mean that the high value products subsidise the cost of production of the low value biofuels – this would seem to be an unrealistic business model. However, there is a limit to the economics of scale which can be achieved in any algae production facility (see for example Stephens et al. 2010), and the production of the co-products requires additional infrastructure and has implications for the range of expertise needed for marketing these products. Interestingly, most modeling studies indicate that there is no further reduction in production costs due to economics of scale once a production plant reaches between 200 and 300 ha in area.

5 Conclusion

Techno-economic modeling is a powerful tool for guiding research priorities and assessing the economics, environmental impact and sustainability of algae biofuels production. However, it is also important to be cognisant of the assumptions made during a particular modeling process and to fully appreciate the degree of uncertainty these assumptions confer upon the outcomes of the model. Good practice in modeling is to test the impact of these assumptions on the outcomes. Methods such as assessing the variance of the outcome during the sensitivity analysis as outline in Sect. 2 above provide powerful tools for this. Ultimately the model must be tested and verified using real data and modified if required.

The recent surge in techno-economic modeling of algae production processes is an important and essential step to the ultimate aim of developing economic and environmentally sustainable processes for algal biofuels. However, all of the models published rely on very similar assumptions many of which are extrapolated from lab-scale studies only and what is required are reasonable pilot-scale studies so that the models can be refined using much better values for the cost estimates of the various process steps, especially those relating to downstream processing.

Definitions

Return on Investment (ROI):

This is a metric which describes the rate of return on money invested in an economic activity over a period of time i.e.:

$$ROI(\%) = \frac{Net\ Profit}{Investment} \times 100\% \qquad (15.3)$$

Net Present Value (NPV):

The net present value is the present value of all cash incomes, less the present value of all cash outflows (including the initial investment).

$$NPV = \sum_{t-1}^{T} \frac{C_t}{(1+d)^t} - C_0 \qquad (15.4)$$

where T=time (usually in years), C_t is the net cash flow (i.e. cash in – cash out) during period t, C_0 is the initial investment and d=the discount rate (i.e. the rate used to discount future cash flows to the present value). Clearly the choice of the value for d is important. One useful value is the rate which the capital needed for the project would return if invested in an alternate venture or if the money were borrowed. Note that the value of d does not need to be constant, but can vary from year-to-year. In this case the equation for NPV would be:

$$NPV = C_0 + \frac{C_1}{1+d_1} + \frac{C_2}{(1+d_1)(1+d_2)} + \frac{C_3}{(1+d_1)(1+d_2)(1+d_3)} + \cdots \qquad (15.5)$$

where d_1, d_2, d_3 … are the discount rates in year 1, 2, 3 etc.

Internal Rate of Return (IRR):

The IRR is the rate of return that would make the present value of future cash flows plus the final market value of the investment equal the current market price (the Net Present Value – NPV) of the investment. The IRR is a measure of the efficiency or yield of an investment and is often used to assess possible investments. If the IRR is greater than the established minimum acceptable rate of return or cost of capital then the investment may be considered acceptable.

The IRR can be calculated from the following equation derived from Equation 15.5 above

$$NPV = \sum_{t-1}^{T} \frac{C_t}{(1+r)^t} - C_0 = 0 \qquad (15.6)$$

where r=the internal rate of return, T=time (usually in years), C=the cash flow. The value of r usually needs to be calculated using graphical methods or numerical methods. Microsoft Excel contains a function for estimating the IRR.

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Standard Methods for Measuring Growth of Algae and Their Composition

16

Navid Reza Moheimani, Michael A. Borowitzka, Andreas Isdepsky, and Sophie Fon Sing

1 Introduction

The application of standardized and validated methods for measuring the basic characteristics of microalgae in culture such as their growth, biomass and proximate composition is essential for any study of the application of microalgae. Although methods to do this have been established for a long time and are well described in the literature (see for example Stein 1973) it is clear from published papers that, at times, some of the methods, their limitations, and their alternatives are not well understood. This aim of this chapter is to provide an easily accessible reference and guide to the fundamental laboratory methods in applied phycology in a format which is easy to follow together with some notes on specific issues which may need to be considered. The methods described here are based on the standard methods we have validated and used for many years in our laboratory.

The Chapter is divided into three main sections covering the measurement of the physical environment (i.e. irradiance), the measurement of algae growth and measurement of the proximate composition of the algae cell.

Of course the decision on a culture medium to use and its preparation is a fundamental first step in culturing algae. The key media and preparation details can be found in Borowitzka (1988) and on the web sites of the main culture collections:

- CCAP: http://www.ccap.ac.uk/media/media.htm
- NCMA: https://ncma.bigelow.org/node/58
- UTEX: http://web.biosci.utexas.edu/utex/media.aspx
- SAG: http://epsag.uni-goettingen.de/cgi-bin/epsag/website/cgi/show_page.cgi?id=4
- CSIRO: http://www.marine.csiro.au/microalgae/methods/

2 The Physical Environment – Irradiance

Light as the driver of photosynthesis is the most important factor affecting the growth of microalgae. For plant photosynthesis the irradiance (photon flux density; PFD) must be measured in quantum units in the photosynthetically active radiation (PAR) range (i.e. 400–700 nm). This requires a quantum light meter and the units used are μmol photons m^{-2} s^{-1} (which is equivalent to μE m^{-2} s^{-1} – but the use of Einsteins is discouraged). Lux (lumens m^{-2}) is a photometric unit which measures illuminance and not irradiance, and is not acceptable for studies of photosynthesis. Lux meters may be cheap, but they are

N.R. Moheimani (✉) • M.A. Borowitzka • A. Isdepsky
Algae R&D Centre, School of Biological Sciences and Biotechnology, Murdoch University, Murdoch 6150, WA, Australia
e-mail: N.moheimani@murdoch.edu.au; M.borowitzka@murdoch.edu.au; A.isdepsky@murdoch.edu.au

S. Fon Sing
Algae R&D Centre, School of Biological Sciences and Biotechnology, Murdoch University, Murdoch 6150, WA, Australia

School of Chemical Engineering, University of Adelaide, Adelaide, SA 5005, Australia

M.A. Borowitzka and N.R. Moheimani (eds.), *Algae for Biofuels and Energy*, Developments in Applied Phycology 5,
DOI 10.1007/978-94-007-5479-9_16,

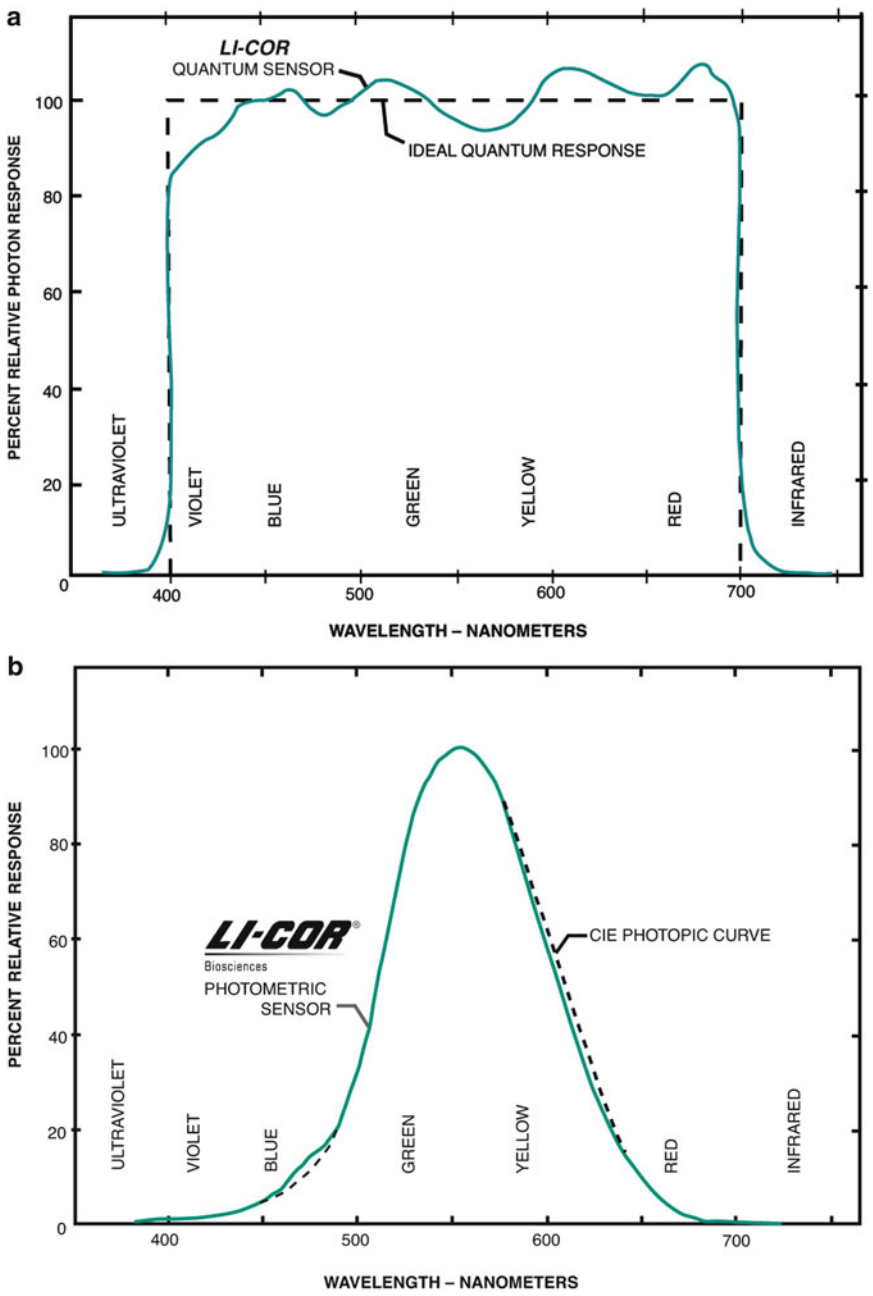


Fig. 16.1 (**a**) Typical spectral response of a Li-Cor quantum sensor vs. wavelength and the ideal quantum response (equal to all photons in the 400–700 nm waveband) and (**b**) spectral response of a photometric sensor (Images courtesy of LI-COR Biosciences)

not equally sensitive over the whole PAR spectrum which is used by plants for photosynthesis, as they are sensitive predominantly around 555 nm – see Fig. 16.1 which compares the sensitivity of a quantum PAR sensor and a photometric (Lux) sensor. Unfortunately Lux cannot be directly converted to μmol photons m^{-2} s^{-1} as the relationship varies with different light sources (i.e. light sources with different spectra). The irradiance for algae photosynthesis therefore must be measured with a PAR quantum sensor for accuracy. However, if absolutely necessary, an approximate conversion of Lux to μmol photons m^{-2} s^{-1} for different light sources can be achieved using the data in Table 16.1 (Thimijan and Heins 1983).

The choice of PAR sensor is also of some importance. Two main types of sensors are available for commercially available quantum light meters: (a) a flat surface sensor, known as a 2π sensor, which measures only the light directly impinging upon the flat surface of the sensor and is quite sensitive to the angle at which the sensor faces the light source, and (b) a spherical sensor, known as a 4π sensor, which measures light from almost all directions. The latter sensor type is preferred for measurement of irradiance in algae ponds as it more accurately measures the light environment experienced by the algae cells in the pond.

Table 16.1 Conversion factors for PAR and Lux (From Thimijan and Heins 1983)

PAR to Lux		Lux to PAR	
Sunlight	54	Sunlight	0.0185
Cool white fluorescent lamps	74	Cool white fluorescent lamps	0.0135
High pressure sodium lamps	82	High pressure sodium lamps	0.0122
High pressure metal halide lamps	71	High pressure metal halide lamps	0.0141
Multiply the PAR by the conversion factor to get Lux		*Multiply the Lux by the conversion factor to get PFD*	

3 Measuring Growth

The growth of algae cultures can be determined by measuring the abundance of the algal cells or the increase in algal biomass in the form of dry weight, ash-free dry weight or chlorophyll *a* content. Turbidity or absorbance is also used as a measure of algae abundance, but this must be used with caution and carefully calibrated with an alternative method such as direct cell counts (see Sect. 3.1 below). Protocols for these measures of algae abundance are presented below as are the equations for calculating algae growth. In some cases the increase in total packed cell volume determined using a haematocrit centrifuge (Tsuzuki et al. 1990; Becker 1994) may also be a useful measure of growth.

Care must also be taken in sampling the culture so the sample is truly representative of the culture. This is easiest in small, well mixed cultures, but can be very difficult in cultures where the algae tend to stick to surfaces or to each other and in very large-scale cultures. If necessary, the initial sampling error needs to be estimated and it needs to be determined if multiple samples need to be taken to achieve a particular level of statistical accuracy. This can be extremely important when sampling large-scale culture systems such as raceway ponds.

3.1 Cell Counting

Depending on the size of the alga, and as long as the algae are single celled and not colonial or chain-forming, cell numbers can be determined using a Neubauer haemocytometer (Guillard and Sieracki 2005) or Sedgwick-Rafter chamber (McAlice 1971). The haemocytometer is suitable for cells in the size range of blood cells (i.e. less than about 100 μm in diameter) and for cell densities less than 10^5 cells mL^{-1}. Larger cells or cell densities greater than 10^5 cells mL^{-1} are better counted using the Sedgwick-Rafter chamber.

If using the haemocytometer a minimum of 300 cells must be counted for each sample to achieve a less than 10% error and it is recommended that triplicate samples are counted to reduce sampling errors. Motile cells can be immobilised with Luogol's Iodine solution[1] (1 drop mL^{-1}). If the microalgae are clumping together then the sample must be homogenised using a Potter homogenizer or similar prior to counting. If there is still clumping of the cells then the cell counts with the haemocytometer will be highly variable and inaccurate and direct cell counting may not be the best method for determining the cell density in the culture.

Direct cell counting has the advantage over other methods (e.g. optical density or using a particle counter) in that one is also closely observing the cells and any anomalies such as changes in cell morphology and/or the presence of contaminants will be detected easily; however it is also time-consuming.

Neubauer haemocytometer: The haemocytometer (Fig. 16.2) is a special, rather thick, microscope slide originally developed for counting blood cells, but which has proven excellent for counting single celled algae. There are various types of haemocytometers with different depths but the Improved Neubauer haemocytometer is the version used mainly in phycology (see Guillard and Sieracki 2005). The chamber of the haemocytometer has a graph paper-like grid etched onto it, which can be seen under the microscope using a 10 or 40x objective (because of the thickness of the slide higher power objectived generally cannot be used). In the Neubauer haemocytometer the chamber depth is 0.1 mm and the main grid is made up of nine large 1 mm × 1 mm squares. Each of these squares is subdivided into smaller squares. i.e. each tiny square is 0.05 mm × 0.05 mm and the "medium" squares (4 × 4 tiny squares) are 0.2 mm × 0.2 mm. Because the dimension of each type of square is known and the distance between the slide and the coverslip is fixed, the volume of each type of square under the

[1] Lugol's Iodine solution is prepared by dissolving 10 g I_2 (toxic !) and 20 g KI in 200 mL distilled water. Add 20 mL concentrated glacial acetic acid a few days before use. Store in the dark, preferably in a glass bottle with a glass stopper. This solution, if properly stored, should remain effective for about 12 months.

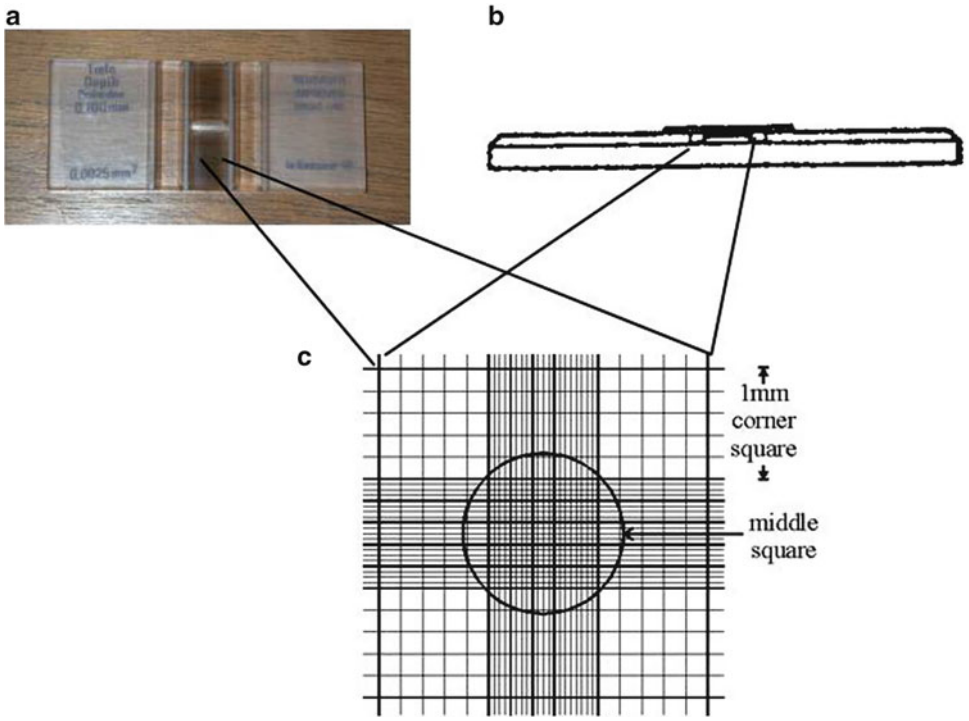


Fig. 16.2 Improved Neubauer haemocytometer slide: *a* Surface of slide, *b* Section of slide showing coverslip in position and *c* central part of grid

cover slip is also known. Because the volume of each "square" is known, the number of cells per unit volume can be calculated. For example, each large square is 1 mm^2 and the chamber depth is 0.1 mm; therefore the volume overlying each square is 0.1 mm^3 (or 0.0001 mL = 0.1 μL). The type of square chosen for counting depends on the density of cells although for most microalgae the 1 mm squares are usually used, and it may be necessary to dilute the original cell suspension to be able to count the cells easily, however one must remember to include this dilution when doing calculations later. The procedure for using a haemocytometer is shown in Method 16.1.

3.2 Absorbance (Optical Density)

Absorbance is used widely as a rapid method for measuring growth of algae cultures. To avoid interference with absorbance by chlorophyll or other photosynthetic pigments, the usual wave lengths used are 550 or 750 nm (i.e. wavelengths where the absorption by chlorophyll and most other pigments is at a minimum). While this is an extremely fast method for estimating culture cell density, it is not recommended for cultures if algae tend to clump (e.g., cultures of *Porphyridium* and some diatoms). If absorbance is used, it is essential first to establish the correlation between cell count and absorbance and how reliable this relationship is under different culture conditions. This relationship will change with changing cell size which can change with growth rate (e.g. at log phase cells can be smaller than stationary phase), as well as other factors which affect the absorbance and is therefore only an approximation of the cell number. In our laboratory we have found that absorbance is not a very reliable method for measuring the cell density of several types of algae (i.e. the coccolithophorids where the abundance of coccoliths on the cell surface affects the absorbance, and colonial algae such as *Botryococcus* spp.). Griffiths et al. (2011) also have found that changes in the cell pigment content during growth can also increase in the error of using absorbance as a growth measurement method.

3.3 Flow Cytometry and Particle Counters

Particle counters such as the Coulter® counter have been available for a long time and can be very convenient. However they can only be used for single cells and may also be inaccurate for cells which easily deform, such as *Dunaliella* cells. In our experience direct cell counting using a haemocytometer has proven easier overall and more reliable. A new and recently

available instrument, the FlowCam, which is a type of digital image analyser, may be a better alternative (Steinberg et al. 2011) but as yet has not been widely used.

Flow cytometry can also be used for measuring cell density (Collier 2000; Marie et al. 2005). Flow cytometers measure light scattering and fluorescence characteristics of the fluid stream while they cross the light beams at a very high rates (up to 1,000 cells.s^{-1}). An advantage of flow cytometry is that not only can the number of particles (algae) be determined but a range of other parameters can be measured simultaneously including variable fluorescence yields (Sosik et al. 2010), lipid content (Cooksey et al. 1987) and lipid composition (Guzmán et al. 2010).

3.4 Dry Weight and Ash-Free Dry Weight

Before measurements of dry weight and ash-free dry weight and also before the extraction step for analysis of the proximate composition of the cells, the cells must be concentrated and this can be by centrifugation or filtration. We have found that filtration (usually on GF/C or GF/F glass fibre filters) is the quickest method.

Algal biomass is determined by measuring either the dry weight (DW) or the ash-free dry weight (AFDW). Gravimetric measurement of ash-free (organic) dry weight (AFDW) is by far the most reliable method for measuring growth and also calculating the productivity of algae cultures and is the best for comparing different algae. The ash content of dry microalgae is usually at least 8–10 % of the dry weight. Correct determination of AFDW minimises the confounding effects of inorganic components of cells such as the silica of diatom frustules or the $CaCO_3$ coccoliths of the coccolithophorid algae, as well as salts from the medium trapped in the interstitial space between cells and in the filter, especially when using marine and hypersaline media. Additionally, autoclaving of the medium (especially if the phosphate has already been added), and particularly seawater-based media, can lead to the precipitation of insoluble salts, as well as the precipitation of carbonates and phosphates during growth of the culture at high pH, and these also add to the 'ash' component of the dry weight.

For freshwater algae the cells on the filters can be gently washed with dilute HCl (~0.01 M) to remove adhering salts and carbonates without breaking the cells. For marine algae Zhu and Lee (1997) have shown that washing of the filtered cells with isotonic ammonium formate greatly reduces the overestimation of both the DW and the AFDW. If the algae culture has experienced a pH > pH 9 it is recommended to use an acidic ammonium formate solution of about pH 5.5 for washing. If the salinity of the medium (measured as NaCl content) is in the range of 0.05–0.2 M NaCl, the ammonium formate concentration required can be calculated based for the equation: $M_{\text{Ammonium Formate}} = 2.8659 \times 10^{-16} + 13 \times M_{\text{NaCl}}$. where M is the molarity.

Dry weight and ash-free dry weight are determined using the method summarised in Method 16.2.

3.5 Calculating Growth Rates

Growth rates of algae cultures are usually given as either the doubling time (i.e. d_2 – the time for the cell number or biomass to double) or the specific growth rate i.e. (μ; $time^{-1}$ – the proportion increase in cell number or biomass per unit time); Note: that biomass can be measured as dry weight, ash-free dry weight, chlorophyll *a* concentration, absorbance etc.

The specific growth rates of batch cultures (μ; $time^{-1}$) can be determined by measuring the doubling time in the exponential growth phase from semi-log plots of cell density or biomass. The specific growth rate can be calculated using Eq. 16.1:

$$\mu = \frac{Ln\ 2}{d_2} = \frac{0.693}{d_2} \tag{16.1}$$

where d_2 is the time taken for the cell number to double (i.e. the doubling time). Note that the time is usually expressed in days.

Alternatively, the growth rate (K'; divisions $time^{-1}$) can be calculated using the following Eq. 16.2:

$$K' = \frac{Ln(N_{t_2} / N_{t_1})}{t_2 - t_1} \tag{16.2}$$

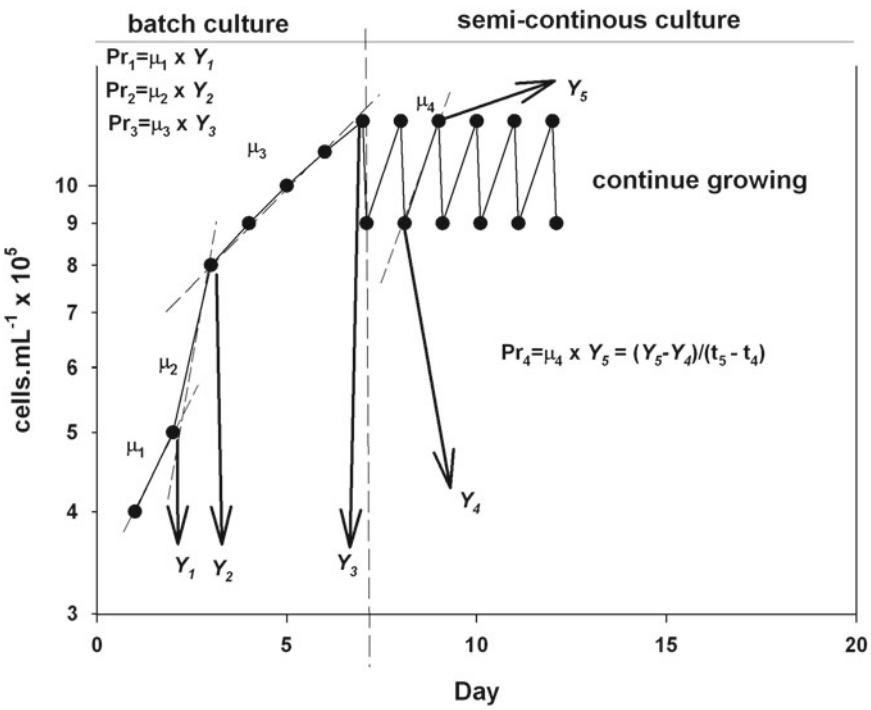


Fig. 16.3 Hypothetical growth curve and productivity equations of microalgae culture under batch and semi-continuous mode

where N_1 and N_2 are the cell densities (or biomass) at time 1 (t_1) and 2 (t_2), respectively. K′ can be converted to the specific growth rate μ by division by Ln 2:

$$\mu = K' / \text{Ln } 2 \tag{16.3}$$

The productivity of a batch culture in the logarithmic growth phase (Fig. 16.3) can be calculated by using Eq. 16.4:

$$\text{Pr}_{batch} = \mu \tag{16.4}$$

where Pr_{batch} (Fig. 16.3) is the productivity in g L^{-1} day^{-1} for volumetric productivity, or g m^{-2} day^{-1} for areal productivity, and Y is the yield of the culture (g L^{-1} or g m^{-2} for volumetric or areal productivity, respectively).

The volume of a culture is easily and unambiguously determined irrespective of the culture system used, however the culture area – which is easy to determine for open ponds – can be more difficult to determine for closed photobioreactors. For example, in the literature people have used either the illuminated area of the reactor, or the projected land area ('footprint') of the reactor; therefore the type of area used in the calculation must be clearly defined. In general. investigators studying closed photobioreactors use volumetric productivity (e.g., Moheimani 2012), whereas open pond productivities are usually compared on the basis of areal productivity (e.g., Moheimani and Borowitzka 2006), although this measure does not take account of pond depth.

If the purpose is to grow algae for biofuel, the operation will most likely be using either continuous or semi-continuous cultures rather than batch cultures so as to maintain the culture in the exponential phase of growth.

For semi-continuous cultures (Fig. 16.3) productivity can be calculated using Eq. 16.5:

$$\text{Pr}_{semi-continuous} = \frac{Y_A - Y_B}{t_A - t_B} \tag{16.5}$$

where $Pr_{semi\text{-}continous}$ is the productivity in g L^{-1} day^{-1} or g m^{-2} day^{-1}, Y is yield (g L^{-1} or g m^{-2}) and t is time (d) and the subscripts A and B refer to t after (A) and before (B) harvest (Fig. 16.3, P_r4).

Algae cultures also can be operated in continuous culture mode. In continuous cultures, some of the culture volume is continuously removed and the removed volume is replaced with fresh medium. The dilution rate is defined as:

$$D = \frac{Medium\ Flow\ Rate}{Culture\ Volume} = \frac{F}{V} \tag{16.6}$$

At steady state the dilution rate (D) equals μ, the specific growth rate. The dilution rate is determined on the basis of either the concentration of a single limiting nutrient (= chemostat), or so as to maintain a particular cell density (= turbidostat). Since D = μ, productivity can be calculated according to Eq. 16.4 above.

4 Measuring the Composition of Microalgae

The proximate composition is often presented as a percentage of dry weight, however because of the variable amount of 'ash' content between samples and between species (see Sect. 3.4 above) it is best to express the proximate composition as a percentage of ash-free dry weight (see Method 16.2).

Measuring the composition of algae (i.e. pigment, protein, lipid or carbohydrate content etc.) requires an extraction step. The extractability of microalgae depends very much on the nature of the cell covering and may also be affected by the growth stage of the algae. For example, 'naked' cells such as *Dunaliella* and the motile cells of *Haematococcus* are easy to extract, whereas cells with a substantial cell wall such as *Chlorella*, *Scenedesmus*, *Nannochloropsis* and *Tetraselmis* and the aplanospore stage of *Haematococcus,* can be difficult to extract, and the extractability of diatoms also varies greatly. For effective extraction the cell covering of these cells must be ruptured and this can be achieved by sonication, grinding with sand, or similar methods. Although repeated freeze-thawing can be useful we have not found it a useful method for many microalgae species. The best and most effective method we have found is to grind the cells with a glass pestle when frozen with liquid N_2. Although somewhat labour intensive, this method has given the most reliable and repeatable results. Irrespective of whether the cells are ruptured or not before extraction, it is highly advisable to test the effectiveness of the extraction method before routine use for each new species of alga – it is not recommended just to rely on previously published methods. This is best done by repeating the extraction step three or more times and determining how much additional material is extracted at each step. A total extraction of over 90% is generally acceptable.

4.1 Storing Samples Before Extraction

If required, the filter papers with the filtered algae can be stored after filtration and washing by folding in two, blotting gently to remove any excess water and placing them in small plastic bags in a closed container and storing at −20 °C in the dark until extraction and analysis (cf. Method 16.3, step 2).

4.2 Sample Treatment Before Extraction

As pointed out in Sect. 4 above, rupture of the cells wall is essential for most microalgae to achieve efficient and complete extraction. For extraction, the frozen filtered samples are placed in individual glass or plastic test tubes. If required, for instance for cells with a substantial cell wall, a small amount of liquid nitrogen can be poured on samples to aid in cell wall rupture (Method 16.3).

4.3 Chlorophyll Determination

Chlorophyll *a* concentration has been used widely as a method for measuring the growth and abundance of algae and it can be determined using the 90% acetone extraction method of Jeffrey and Humphrey (1975) as shown in Method 16.4, which also includes equations for the other forms of chlorophyll. Recently Ritchie (2006, 2008) derived an universal set of equations for measuring chlorophyll *a, b, c* and *d* for 90% (v/v) acetone and for 100% ethanol solvents (Method 16.4). Although 100% methanol can also be used to extract the pigments, he found that the algorithms can be affected severely by the presence of phaeophytin and therefore 100% methanol is not recommended.

4.4 Lipids

4.4.1 Total Lipid Analysis

The hydrophobic nature of lipids provides a convenient means of separating them from other water soluble compounds in an aqueous sample matrix. Extraction of lipids in nonpolar solvents is universally employed. Microalgae total lipid is usually determined gravimetrically using either the Bligh and Dyer (1959) method as modified by Kates and Volcani (1966) and Mercz (1994), or the method of Folch et al. (1957). Since these methods use different solvent systems they give somewhat different results and the modified Bligh and Dyer method is preferred. These methods are summarised in Method 16.5. Following lipid extraction the quantity of the lipids is measured gravimetrically. It is important to extract a sufficiently large sample of algae so that there is sufficient lipid extracted for accurate weighing. The lipid sample also needs to be dry.

The heterogeneous nature of microalgae lipids means that much information can be gained by quantifying individual classes (Debelke et al. 1995) and in particular quantifying the amount of neutral lipids, especially the triglycerides, is important when studying microalgal lipids as a feedstock for biofuels. A variety of chromatographic methods is available. Of these, thin layer chromatography (TLC) has the advantage of requiring a minimum amount of equipment and can be done in most laboratories. TLC gives a rapid and reproducible separation of the oil into several fractions (i.e. triglycerides, waxes, free fatty acids) and the simple TLC method (Method 16.7) described here is based mainly on Fried and Sherma (1999) and Ackman (1991), and is useful for semi-quantitative separation and determination of the major lipid classes.

For quantitative analyses other methods such as high performance liquid chromatography, HPLC (Christie 1985) or the Iatroscan thin-layer chromatography flame–ionisation detection method (Iatroscan TLC-FID) (Volkman et al. 1989; Ackman et al. 1990) are available. These methods can be combined with mass spectrometry to analyse the lipid molecular species (Hudson et al. 2001; MacDougall et al. 2011). More sophisticated methods such as 2D-UPLC-QToF-MS method are also becoming available (Netto et al. 2012).

4.4.2 Nile Red Neutral Lipid Visualisation

Nile Red (9-diethylamino-5 H-benzo[α]phenoxazine-5-one) is an intensely fluorescent dye which has unique properties which makes it an ideal stain for detecting lipid droplets (neutral lipids) in microalgae (Greenspan and Fowler 1985). However, Nile Red does not penetrate all types of algal cells to the same degree and this can lead to variable results when comparing species. Microscopic visualisation of lipid droplets in the algal cells can be carried out by using the Nile Red fluorescent dye as outlined in Method 16.6. Lipid droplets are seen as bright golden spots which, under prolonged exposure to the UV light (>5s), fade rapidly due to degradation of the dye. Nile Red staining for microalgae was originally established as an qualitative method for identifying oil droplets (Cooksey et al. 1987; Lee et al. 1998), but can also be used for qualitative measurement of microalgae neutral lipids (Chen et al. 2009). Nile Red staining is most useful during rapid screening of microalgae for their neutral lipid content as the method is quick.

4.4.3 Extracellular Hydrocarbon Measurement

For the green alga *Botryococcus braunii* which accumulates hydrocarbons in the extracellular matrix, the hydrocarbons can be selectively extracted and measured by the modified Eroglu and Melis (2010) method shown in Method 16.8.

4.5 Total Carbohydrates

Total carbohydrates are usually measured by the phenol-sulphuric acid method developed by Kochert (1978) and Ben-Amotz et al. (1985). Mercz (1994) further optimised this method as described in Method 16.9.

4.6 Total Protein

The protein content of microalgae can be measured by either colorimetric dye-binding methods or by methods that measure the concentration of elemental nitrogen. The most widely used method for total protein determination in microalgae the Lowry method (Lowrey et al. 1951) as adapted by Dorsey et al. (1978). An alternative colorimetric method for measuring total protein content of microalgae is that of Bradford (1976). A comparison of these two methods by Barbarino and Lourenço

(2005) found that the Lowry method was more reliable for measurement of the protein content of algae. Protocols for both these Lowrey and the Bradford methods are summarised in Method 16.10.

An alternative method is to measure the nitrogen content of cell using a method such as the Kjeldahl method (Owusu-Apenten 2002) which is less susceptible to interference than the dye-binding methods. However, it is extremely important to determine the nitrogen to protein ratio of the selected microalgae species (González López et al. 2010) for accurate conversion of the nitrogen concentration to protein.

5 Conclusion

The available methods are constantly evolving and new procedures and equipment are always being developed. However, it is good laboratory practice to regularly test and revalidate the methods being used and to be aware of the possible problems and the limitations of the methods being used. The methods given above represent what we have found to be the most suitable methods in our laboratory, but they are not the only methods. They are also the most basic methods and often more detailed analyses (i.e. fatty acid composition, amino acid composition, pigments other than chlorophylls) will be necessary.

Method 16.1 Cell counting using a Haemocytometer

1. Carefully clean haemocytometer and the coverslip with 70% (v/v) ethanol and wipe dry with lens tissue

↓

2. Place the coverslip (note haemocytometers use a special type of coverslip) over the grid on your haemocytometer

↓

3. Shake or stir the suspension in a standard way to ensure homogeneity of the sample

↓

4. Place a small drop of the suspension containing the algae at the edge of the coverslip (it will be drawn under the coverslip by capillary action)

↓

5. Wait a few minutes for the cells to settle down (if necessary)

↓

6. Choose the appropriate size square on the haemocytometer grid and count the number of cells in at least 3 separate squares. You should count at least a total of 100 cells for accuracy. To achieve this number you may need to count more than 3 separate squares. Record the numbers of cells counted and the number of squares counted.

Note: *Cells will invariably be found on the lines dividing the cells. In order to standardize results, any cells found on the left and top lines should be included, and any cells found on the right and bottom lines of each square (No matter how far in or out the cells may appear) should be ignored.*

For the Neubauer Haemocytometer the volume over one of the 1 mm squares is 0.0001 mL and therefore the cell number per mL can be calculated as follows:

$$Cell\ Number\ (cells\ mL^{-1}) = \frac{Total\ number\ of\ cells\ counted}{Number\ of\ squares\ counted} \times 1000$$

Method 16.2 Dry weight and ash-free (total organic) dry weight determination

GF/C filter pretreatment

For dry weight and ash-free dry weight determination the glass fibre filters used to concentrate the algae must be pre-combusted.

1. Pre-combust Whatman GF/C (2.5cm diameter) filters at 100°C for 1 h.
2. Store filters in vacuum dessicator over $KMnO_4$ crystals until use.

Precautions

The dried algae can be very hygroscopic and care must be taken during weighing. If the weight seems to be increasing slightly during weighing then re-dry the sample in the dessicator for 24 h over fresh KOH or another dessicant.

DRY WEIGHT DETERMINATION

1. Carefully weigh pre-combusted filters to 5 decimal places.
↓
2. Place filters in filter unit and filter culture (generally about 10 mL of culture) until filter appears completely dry
↓
3. Wash filter with 10 mL of distilled water (freshwater spp) or isotonic ammonium formate solution for saline species (0.65 M for marine spp)
↓
4. Remove filter from filter unit and dry at 100°C for 1 h and then place in vacuum desiccator over dessicant overnight
↓
5. Weigh dried filter containing algae to 4 decimal places using a 5 digit balance
↓

Dry weight (DW) = (weight of filter plus algae) - (weight of filter)

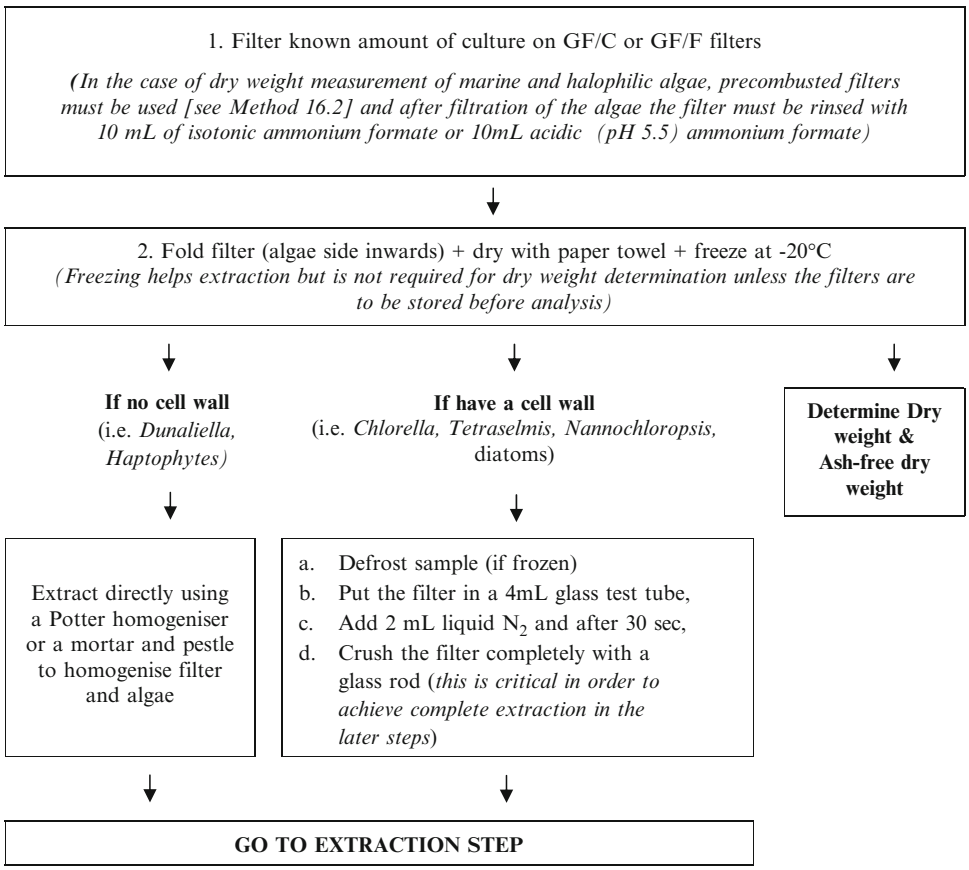
ORGANIC DRY WEIGHT (ASH-FREE DRY WEIGHT) DETERMINATION

1. Take filters from step 5 of dry weight determination and ash at 450°C in a muffle furnace for 5 h
↓
2. Cool filters in a vacuum dessicator over dessicant
↓
3. Rapidly and carefully weigh filter
↓

Ash-free dry weight (AFDW) = Dry Weight - Weight after ashing

Method 16.3 Sample preparation steps for analysis of proximate composition

Precautions :
Some care must be taken when filtering delicate cells such as those of *Dunaliella* so that they are not ruptured during filtration leading to a loss of cell components.



Method 16.4 Chlorophyll assay

General Precautions:

- **Work in dim light** - pigments are easily bleached by lights, especially daylight and fluorescent light
- If using acetone add a pinch of $MgCO_3$ to the acetone before you grind the tissue. This will remove any traces of acids that may come from the algae or from the glassware. Acids remove the Mg^{2+} from chlorophylls, forming phaeophytin
- Keep extracts cool at all times to prevent pigment breakdown. Use chilled solvents, and grind and store algae and extracts in an ice-bath in the dark

1. Add 5-10 mL of 90% (v/v) acetone or 100% ethanol to sample from Extraction Step of Method 16.3

↓

2. Pour off supernatant (if using filtered algae) into a graduated glass centrifuge tube and, if necessary, re-extract with fresh solvent. Pour off this supernatant and combine the supernatants.

↓

3. Make sure the solvent extracts are well mixed and then centrifuge them in a bench centrifuge to get rid of any particulate matter.

↓

4. Measure the volume of the supernatant carefully.

↓

5. Measure absorbance at the required wavelengths (see equations below)

↓

6. Calculate the concentrations of chlorophyll using the equations below
Note: Two different alternate sets of equations are provided

The concentrations of chlorophylls *a*, *b* and *c* in mixtures are calculated using the equations below, where for example, E_{664} = the absorbance of the extract measured through 1 cm of solution at 664 nm. (Results are in $\mu g.mL^{-1}$). *Remember to adjust for dilution in your calculations if necessary*

(A) Equations of Jeffrey and Humphrey (1975) :

a. For green algae which contain chlorophylls *a* and *b*
chlorophyll *a* = 11.93 E_{664} - 1.93 E_{647}
chlorophyll *b* = 20.36 E_{647} - 5.50 E_{664}

b. For diatoms, chrysomonads and brown algae (containing chlorophyll *a* and chlorophylls c_1, and c_2 in equal proportions) USE 100% acetone as the solvent
chlorophyll *a* = 11.47 E_{664} - 0.40 E_{630}
chlorophyll $c_1 + c_2$ = 24.36 E_{630} - 3.73 E_{664}

c. For dinoflagellates and cryptomonads (containing chlorophylls *a* and c_2)
chlorophyll *a* = 11.43 E_{663} - 0.64 E_{630}
chlorophyll c_2 = 27.09 E_{630} - 3.63 E_{663}

d. For mixed phytoplankton populations (containing chlorophylls *a* and *b*, and equal amounts of chlorophylls c_1 and c_2)
chlorophyll *a* = 11.85 E_{664} - 1.54 E_{647} - 0.08 E_{630}
chlorophyll *b* = -5.43 E_{664} - 21.03 E_{647} - 2.66 E_{630}
chlorophyll $c_1 + c_2$ = -1.67 E_{664} - 7.60 E_{647} - 24.52 E_{630}

e. For red algae, containing chlorophyll *a* only
chlorophyll *a* = 11.41 E_{664}

(B) Ritchie (2006 and 2008) equations for determining chlorophyll *a*, *b*, *c* and *d*:

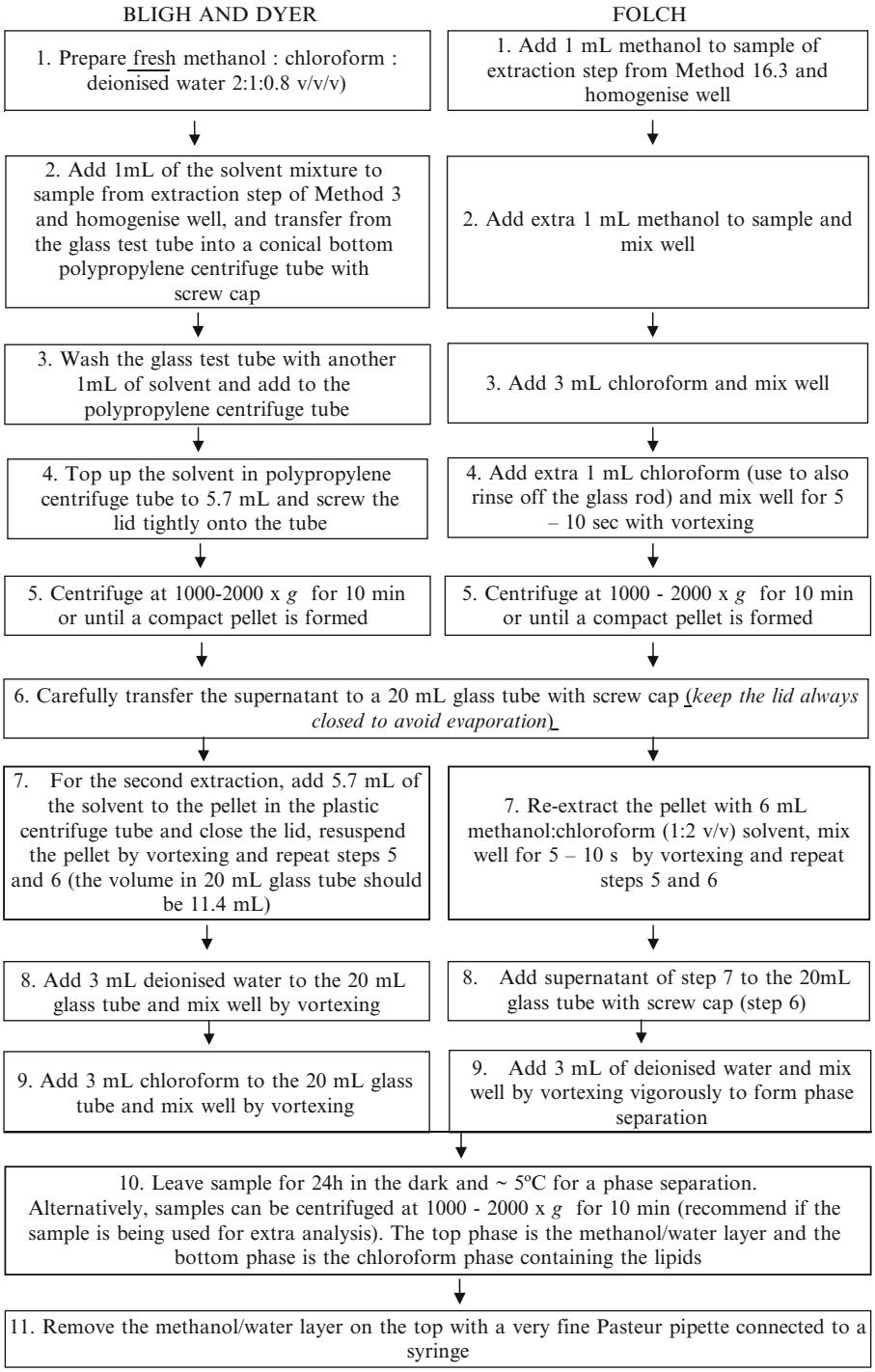
If 90% acetone is used as a solvent:
chlorophyll *a* = -0.3319 E_{630} - 1.7485 E_{647} + 11.9442 E_{664} - 1.4306 E_{691}
chlorophyll *b* = -1.2825 E_{630} + 19.8839 E_{647} - 4.8860 E_{664} -2.3416 E_{691}
chlorophyll c_{total} = 23.5902 E_{630} - 7.8516 E_{647} - 1.5214 E_{664} -1.7443 E_{691}
chlorophyll *d* = 21.3877 E_{630} + 10.3739 E_{647} + 5.3805 E_{664} + 5.5309 E_{691}

If 100% ethanol is used as a solvent:
chlorophyll *a* = 0.0604 E_{630}-4.5224 E_{647}+13.2969 E_{664}-1.7453 E_{691}
chlorophyll *b* = -4.1982 E_{630} + 25.7205 E_{647} - 7.4096 E_{664} -2.7418 E_{691}
chlorophyll c_{total} = 28.4593 E_{630} - 9.9944 E_{647} - 1.9344 E_{664} -1.8093 E_{691}
chlorophyll *d* = 24.1209 E_{630} + 11.288 E_{647} + 3.7620 E_{664} + 5.8338)E_{691}

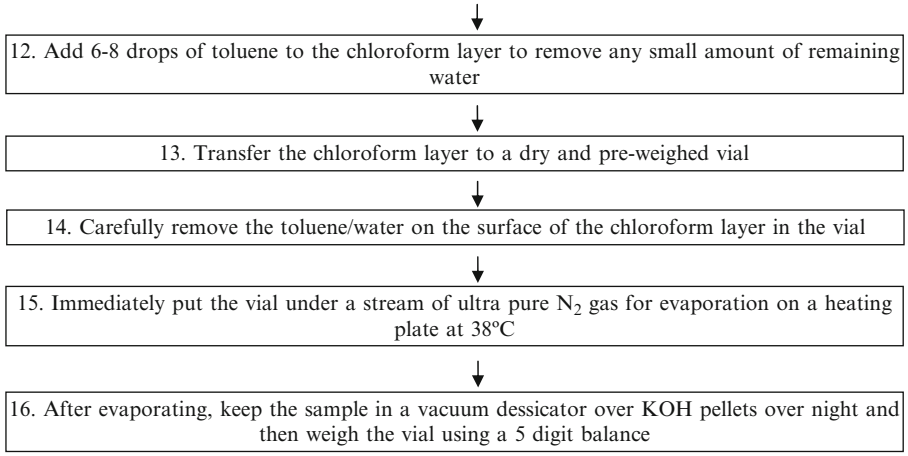
Method 16.5 Modified Bligh and Dyer and Folch methods for total lipid extraction and quantification

Precautions:

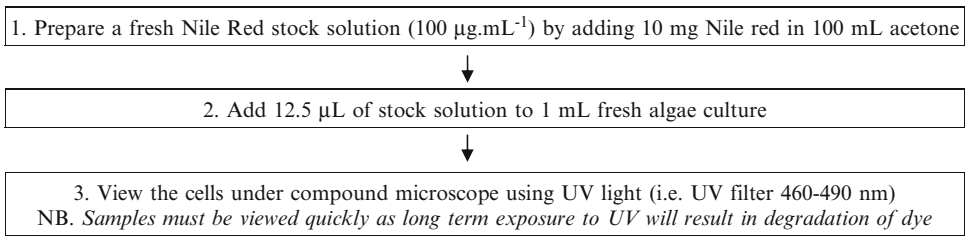
- When applying this method to a new species of algae the effectiveness of extraction should be checked in preliminary experiments (see section 4).
- The extracts can be somewhat hygroscopic and samples must be stored over dessicant and weighing should take place in a dry atmosphere. In our experience changes in atmospheric humidity can affect the results.



Method 16.5 (continued)



Method 16.6 Nile Red lipid visualization method (Dempster and Sommerfeld 1998)



Method 16.7 Thin layer chromatography of total lipids from microalgae (TLC)

Precautions:

All work should be carried out in a flame-proof fume hood.
Note that Iodine vapour is toxic

A. TLC Plate activation and chamber preparation

1. Pre-heat oven at 120° C

2. Verify running direction of the plate by looking at the production marks on the aluminium back of the TLC plate (e.g. aluminium-backed TLC plates (Merck TLC Silica Gel 60F254))

3. Orient the plate such that production marks are vertical and draw a faint baseline and reference line 10 mm each from the plate edge with a soft (2B) pencil and clean ruler

4. Place the plate upright in between 2 clean and grease-free glass plates in the carrier to prevent warping.

5. Activate (dry) the plate for 1h at 120°C.

6. After activation, cool the plate down in a moisture-free environment while keeping the plate sandwiched between the glass plates

7. Prepare developing chamber in a fumehood ensure that it is free of any dust, chemicals and solvents other than those used for the chromatographic run

8. Add in the freshly prepared solvent mixture. Place the filter paper in the chamber and allow the solvent vapor to equilibrate in the chamber for ~1 h

B. Sample loading and TLC run

9. After total lipid extraction (Method 16.5) fully dissolve the dried lipid sample in vials in 20 µL of chloroform for loading the TLC plate

10. While waiting for TLC tank to be saturated, load the activated TLC plate with the lipid sample

11. Pipette 20 µL of lipids dissolved in chloroform and apply as a discrete spot on the baseline and allow the chloroform to evaporate completely

12. Quickly transfer the loaded TLC plate in the saturated developing chamber

13. Allow the chromatographic process to run undisturbed for approx. 50 min or until the solvent front has reached the reference line

c. Derivatisation and lipid class identification

14. Remove the plate from the developing chamber and allow to dry for approx. 30 min in a fume hood

15. Carefully transfer the plate to the iodine chamber and allow for the derivatisation process to complete until all spots on the TLC plate are clearly revealed

16. Carefully outline the visible spots with a pencil and calculate the Rf value of each spot by using the equation:

$$Rf = \frac{\textit{distance of the spot from baseline}}{\textit{distance between baseline and reference line}}$$

17. Identify the groups of lipids by comparing calculated Rf values with those published in the literature where a similar solvent system has been used or comparison with a standard lipid (oil) run on the same system

18. Derivatised plate can be scanned and saved electronically using a scanner

Method 16.8 External hydrocarbon measurement method (Adapted from Eroglu and Melis 2010)

1. Prepare a sample of ~1 g wet cell weight (approximately 0.2 g AFDW) of *Botryococcus braunii* by filtration or centrifugation

↓

2. Mix the sample with 1 g of glass beads and 10-20 mL of n-heptane (alternatively hexane) for 1-3 min

↓

3. Add 10-15 mL of growth medium to the mixture resulting in two phase partition - the aqueous phase will contain cells and solvent phase will contain external *B. braunii* hydrocarbons

↓

4. Remove the solvent the layer and measure absorbance at 190 nm (UV-spectrophotometer) against a squalene standard curve)

Method 16.9 Total carbohydrate determination

Precautions :

- When applying this method to a new species of algae the effectiveness of extraction should be checked in preliminary experiments (see section 4).
- This method uses concentrated acid and phenol. Appropriate safety equipment must be worn

Reagents

Glucose standard solution: 0.1 g L^{-1}

Phenol stock solution: 50 g L^{-1}

1M H_2SO_4

Concentrated H_2SO_4

Carbohydrate Standard curve

1. Prepare carbohydrate standard curve (see bottom of this flowchart) in acid resistant test tubes.

A fresh standard curve must be prepared for each set of samples

Assay method

1. Homogenise sample- from extraction step of Method 16.3 in 0.5 mL 1M H_2SO_4 in a 10 mL acid resistant plastic test tube with screw lid.

↓

2. Top up to 5 mL with 1M H_2SO_4

↓

3. Tighten the lid and incubate in a 100°C water bath for 60 min.

↓

4. Cool to room temperature (~30min) and centrifuge at 1000 – 2000 x *g* for 5-10 min

↓

5. Pipette 2 mL of the supernatant into another acid resistant test tube.

↓

6. In fume hood, add 1 mL of phenol solution and rapidly mix well using a Vortex stirrer

↓

7. In fume hood, rapidly add 5 mL concentrated H_4SO_2
(*Notes* : (*a*) *To avoid splashing acid, keep the pipette tip in a light angle against the test tube wall,* (*b*) *acid resistant protective equipment must be worn*).

↓

8. Close the test tube lid (tighten very well) and mix well by vortexing

↓

9. Cool the test tube for 30 min at room temp.

↓

10. Mix the samples well manually.

↓

11. Read the absorbance at 485 nm and calculate the carbohydrate content from the standard curve using the equation:

$$\textit{Carbohydrate yield } (mg.L^{-1}) = \frac{\textit{Carbohydrate value from standard curve}}{\textit{volume of digested material} \times \textit{culture volume}}$$

Suggested Glucose Standard Curve

Glucose (µg)*	0	40	80	120	160	200
Standard glucose solution (mL)	0	0.4	0.8	1.2	1.6	2
dH_2O (mL)	2	0.16	1.2	0.8	0.4	0

**If the standard curve is NOT linear use a lower range of concentrations*

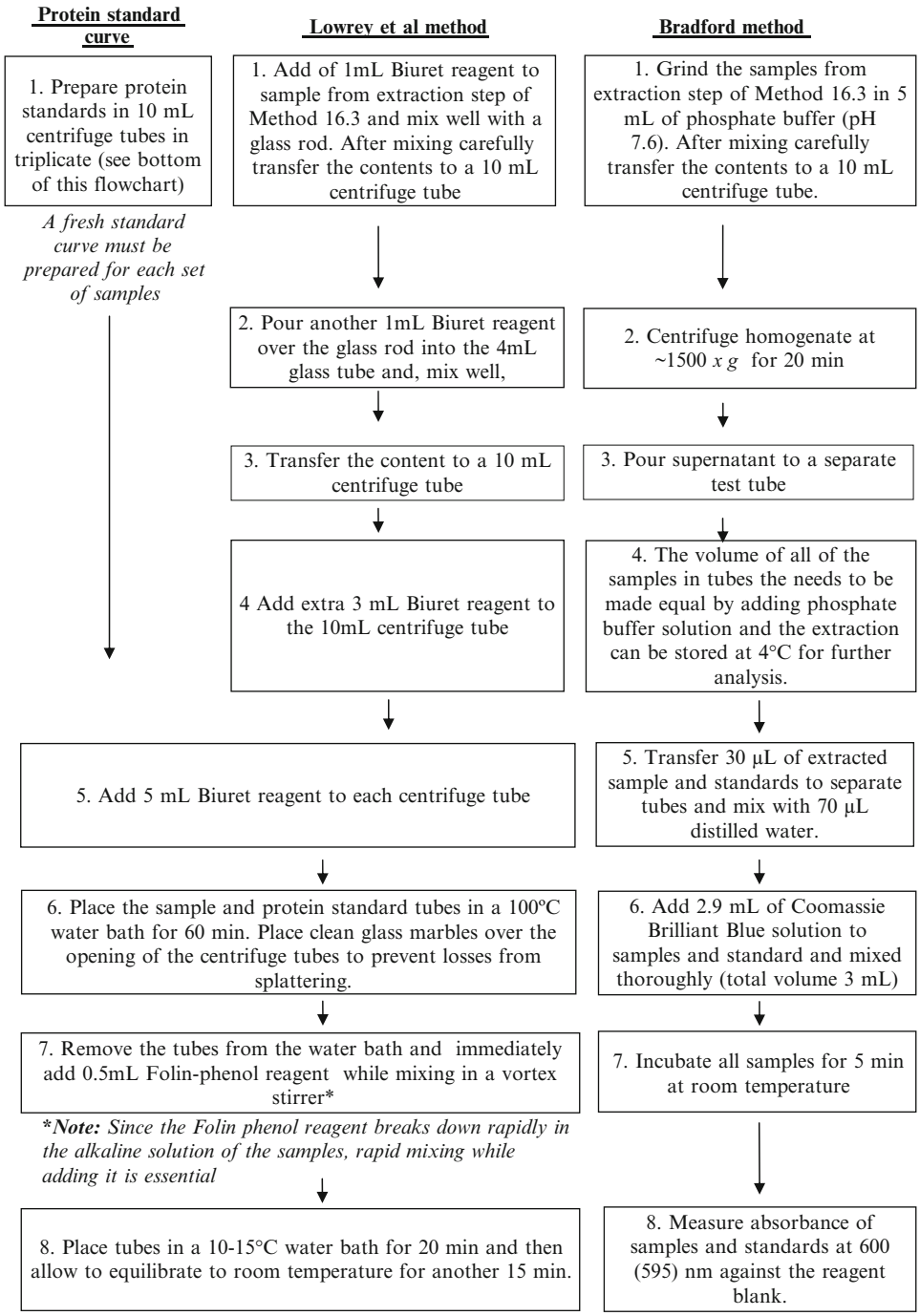
Method 16.10 Total protein determination methods

Precaution:

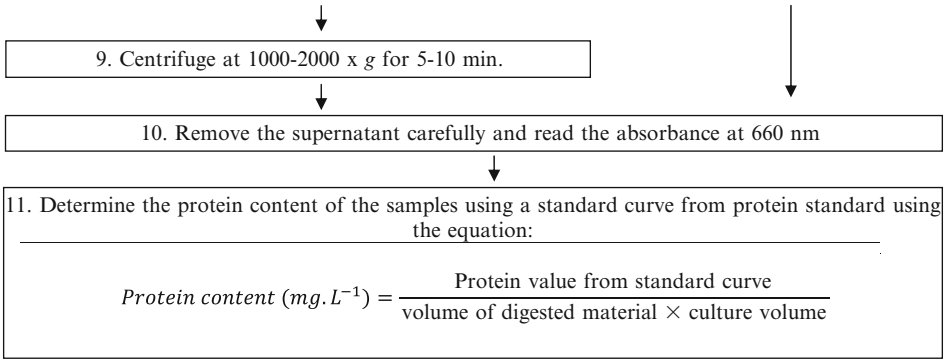
When applying this method to a new species of algae the effectiveness of extraction should be checked in preliminary experiments (see section 4).

Reagents

1. Bovine serum albumin (BSA) Fraction V -stock solution: 2.5 BSA L^{-1}
2. Stock solutions for Biuret reagent
(a) Na_2CO_3 = 200 $g.L^{-1}$
(b) NaOH = 40 $g.L^{-1}$
(c) NaK tartrate = 200 $g.L^{-1}$
(d) $CuSO_4.4H2O$ = 50 $g.L^{-1}$
3. Biuret reagent preparation:
Using the stock solutions above, add 20 mL of (a) + 20 mL of (b) + 160 mL of deionised water, mix well and then add 2mL of (c) and 2 mL of (d)
4. Folin-phenol reagent preparation: Dilute Folin reagent 1:1 with deionised water



Method 16.10 (continued)



Suggested Protein Standard Curve

Protein (μg)	0	50	100	150	200	250	300	350
BSA V (mL)	0.00	0.02	0.04	0.06	0.08	0.10	0.12	0.14
dH2O (mL)	0.14	0.12	0.10	0.08	0.06	0.04	0.02	0.00

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Index

M.A. Borowitzka and N.R. Moheimani (eds.), *Algae for Biofuels and Energy*, Developments in Applied Phycology 5,
DOI 10.1007/978-94-007-5479-9, © Springer Science+Business Media Dordrecht 2013