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BIOPROSPECTING MICROALGAE AS POTENTIAL SOURCES OF "GREEN ENERGY"–CHALLENGES AND PERSPECTIVES (REVIEW)

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Microalgae and cyanobacteria are potential foods, feeds, sources of high-value bioactive molecules and biofuels, and find tremendous applications in bioremediation and agriculture. Although few efforts have been undertaken to index the microalgal germplasm available in terms of lipid content, information on suitability of strains for mass multiplication and advances in development of methods for extraction and generating biofuel are scarce. Our review summarizes the potential of microalgae, latest developments in the field and analyzes the "pitfalls" in oversimplification of their promise in the years to come. Microalgae represent "green gold mines" for generating energy; however, the path to success is long and winding and needs tremendous and concerted efforts from science and industry, besides political will and social acceptance for overcoming the limitations. The major advantages of second generation biofuels based on microalgal systems, include their higher photon conversion efficiency, growth all around the year, even in wastewaters, and production of environment friendly biodegradable biofuels.

Microalgae are microscopic photosynthetic organisms that are found in marine and freshwater environments, besides being prevalent in soil and air. They include unicellular, microscopic (2–200 μm), polyphyletic, highly diverse, non-cohesive, oxygen evolving autotrophic organisms which grow by photosynthesis. The term algae has no formal taxonomic standing and is defined as thallophytes (plant body lack of roots, embryos, vascular system, stems and leaves) that have chlorophyll-a as their primary photosynthetic pigment and lack a sterile covering of cells around the reproductive organs [1].

The number of algal species has been estimated to be one to ten million, and most of them are microalgae. It has been estimated that about 200000–800000 species of microalgae exist, of which about 35000 species are described. Over 15000 novel compounds originating from algal biomass have been chemically determined [2]. Most of these microalgal species produce unique products like carotenoids, antioxidants, fatty acids, enzymes, polymers, peptides, toxins and sterols [3]. Their photosynthetic mechanism is similar to land plants, but due to their simple cellular structure and submergence in an aqueous environment, in most cases, where they have an efficient access to water, CO_2 and other nutrients, they are generally more efficient in converting solar energy to biomass than terrestrial plants and are efficient CO_2 fixers. They account for ~50% of global organic carbon fixation.

The evolutionary history and taxonomy of microalgae is complex due to constant revisions as a result of new genetic and ultrastructural evidence. The main criteria for categorizing microalgae are pigmentation, life cycle and basic cellular structure [1]. Algae are classified into 11 divisions comprising 2 prokaryotic divisions – Cyanophyta/Cyanobacteria and prochlorophyta (although the prokaryotic cyanobacteria are frequently included as algae) and 9 eukaryotic divisions (Glaucophyta, Rhodophyta, Heterokontophyta, Haptophyta, Cryptophyta, Dinophyta, Euglenophyta, Chlorarachniophyta and Chlorophyta) [4]. Many algae can switch from phototrophic to heterotrophic growth, and some can also grow mixotrophically [5].

Overview of applications of microalgae. The use of microalgae by human populations goes back to around thousands years ago. The first reported use of ''microalgae'' by humans is that by the Chinese who utilised *Nostoc* and other edible cyanobacteria as an emergency food source some 2000 years ago. But the mass culture of microalgae began shortly after World War II in the USA, Germany and Japan as a potential source of food in a world experiencing a population explosion. Since then, mass culturing of microalgal species have been variously explored in the treatment of wastewater and control of water pollution, for atmosphere regeneration in biospheres (i.e., spacecraft), as renewable fuels for transportation (biodiesel), as a source of high value natural health products (nutraceuticals) and lately in the mitigation of greenhouse gases (GHG) and the production of hydrogen as a fuel source [6].

Microalgae are potentially a great source of natural compounds that could be used as ingredients for preparing foods and enhancing the nutritional food content of humans and animals. Research initially focused on algal biomass as a source of protein and the

Table 1. Selected microalgal species with their products and application areas

Genus/group	Product	Application areas	References
Spirulina (*Arthrospira platensis*)/Cyanobacteria	Phycocyanin, biomass	Health food, cosmetics	[12]
Aphanizomenon flos-aquae/Cyanobacteria	Phycocyanin, biomass	Pharmaceuticals, nutrition	[13]
Lyngbya majuscula/Cyanobacteria	Immunomodulators	Pharmaceuticals, nutrition	[14]
Anabaena/Cyanobacteria	Bioactive metabolites/ hydrolytic enzymes	Agriculture and industry	[10, 11]
Chlorella minutissima/Chlorophyta	Eicosapentaenoic acid, Polyunsaturated fatty acids	Food additive, nutraceuticals	[2]
Chlorella vulgaris/Chlorophyta	Biomass	Health food, food supplement, feed surrogates	[12]
Prototheca moriformis/Chlorophyta	Ascorbic acid	Nutrition	[13]
Dunaliella salina/Chlorophyta	Carotenoids, β-carotene	Health food, food supplement, feed	[14]
Haematococcus pluvialis/Chlorophyta	Carotenoids, astaxanthin, leutein	Health food, pharmaceuticals, feed additives	[8, 14, 15]
Muriellopsis sp./Chlorophyta	Carotenoids, lutein	Health food, food supplement, feed	[14]
Isochrysis galbana /Chlorophyta	Fatty acids	Animal nutrition	[16]
Euglena gracilis/Euglenophyta	Biotin	Nutrition	[17]
Crypthecodinium cohnii/Dinophyta	Lipids, fatty acids	Pharmaceuticals, fuel production	[18]
Nannochloropsis /Eustigmatophyceae	Lipids, fatty acids	Pharmaceuticals	[19]
Odontella aurita/Bacillariophyta	Fatty acids	Pharmaceuticals, cosmetics, baby food	[16]
Phaedactylum tricornutum/Bacillariohyta	Lipids, fatty acids	Nutrition, fuel production	[20, 21]
Porphyridium cruentum /Rhodophyta	Polysaccharides	Pharmaceuticals, cosmetics, nutrition	[22]

systematic examination of algae for biologically active compounds and pharmaceuticals. The high protein content of various microalgal species is one of the main reasons to consider them as unconventional sources of protein. As their cells are capable of synthesizing all amino acids, they can provide the essential ones to humans and animals. They also represent a valuable source of nearly all essential vitamins (e.g., A, B1, B2, B6, B12, C, E, nicotinate, biotin, folic acid and pantothenic acid) [7]. Vitamins improve the nutritional value of algal cells but their quantity fluctuates with environmental factors, the harvesting treatment and the method of drying the cells [9]. They are also rich in pigments like chlorophyll (0.5% to 1% of dry weight), carotenoids (0.1% to 0.2% of dry weight on average and up to 14% of dry weight for β-carotene of *Dunaliella*) and phycobiliproteins. Carbohydrates in microalgae can be found in the form of glucose, starch and polysaccharides. Their overall digestibility is high, which is why there is no limitation to using dried whole microalgae in foods or feeds. The average lipid content of algal cells varies between 1% and 70% but can reach 90% of dry weight under certain conditions [8]. More recently, algae have been used successfully to produce biodiesel, polyunsaturated fatty acids (PUFA), such as docosahexaenoic and eicosapentaenoic acids. Different compounds with anti-bacterial, anti-viral and anti-fungicidal activity can be found in these types of organisms [8–11]. The most frequently used microalgae belong to Cyanophyceae (cyanobacteria/blue-green algae), Chlorophyceae (green algae), Bacillariophyceae (including the diatoms) and Chrysophyceae (including golden algae). A list of selected microalgal species with their products and applications is given in Table 1.

Significance of biofuels. Energy is an indispensable factor to sustain our economic growth and quality of living standards. A rapidly growing world population and rising consumption of fossil fuels is increasing the demand for both food and biofuels [23], which can lead to energy shortage. Producing biofuels requires huge amounts of both fossil energy and food resources, which will intensify conflicts among these resources. Global warming is caused by indiscriminate use of resources, in particular of fossil fuel for a variety of human needs and is largely responsible for climate change. The current definition of progress is largely confined to economic well being of humankind dictated by access to modern technologies, which are driven by modern energy carriers. Along with the increased demands of the burgeoning populations, the production and use of fossil fuel based energy sources has led to the degradation of the environment.

Among the GHG, which are responsible for global warming, CO_2 is the most prominent one. According to information given in World Energy Outlook-2009 of the International Energy Agency (IEA), the energy sector contributes 84% of global CO_2 emissions and 64% of the world's GHG emissions. If no action is initiated, the contributions will increase to about 91% of the global CO_2 emissions by 2030 and the share in GHG emissions is likely to reach 71%. In an absolute sense, energy-related emissions are expected to increase from 28.8 Gt in 2007 to 40.2 Gt in 2030. To limit the global average temperature increase of 2°C, the concentration of GHG in the atmosphere has to be stabilized at a level of around 450 ppm CO_2. The energy sector contribution is expected to be very significant to achieve this target. According to the IEA, in this scenario, the global energy-related CO_2 emissions are expected to peak at 30.9 Gt by 2020 and decline thereafter to 26.4 Gt in 2030. Enhancing the energy efficiency is expected to be the largest contributor to abatement of CO_2 emissions till 2030 [24].

Biofuel can be broadly defined as solid, liquid, or gas fuel consisting of/or derived from biomass. In 1900, Rudolph Diesel first demonstrated the use of biodiesel from a variety of crops. However, the widespread availability of inexpensive petroleum during the 20th century determined otherwise. Now, biofuels are a key focus of developmental efforts globally. Biofuels are ecofriendly, fossil energy independent, carbon neutral, non-toxic, biodegradable and renewable resources [23, 25, 26]. Their use leads to a decrease in the harmful emissions of carbon monoxide, hydrocarbons and SO_x emissions, with a consequent decrease in the greenhouse effect. Biofuels can play an essential part in reaching targets to replace petroleum based transportation fuels with a viable alternative, and in reducing long-term CO_2 emissions, if environmental and economic sustainability are considered carefully. They can be direct and immediate replacements for the liquid fuels used in transport and can be easily integrated to the logistic systems that are operating today. In recent years, the use of liquid biofuels in the transport sector has shown rapid global growth, driven mostly by policies focused on achievement of energy security, and mitigation of GHG emissions [27].

Types of biofuels. Oil seeds/cells of many plants/algae have been extensively evaluated as sources of biofuels. Biofuels are derived from food crops such as sugarcane, sugar beet, maize (corn), sorghum, rapeseed, sunflower, soybean and palm, although other forms of biomass can be used, and may be preferable. The most significant concern is the efficiency and sustainability of these first generation biofuels. In contrast, the second generation biofuels are derived from non-food feedstock [28, 29]. They are extracted from microalgae and other microbial sources, lignocellulosic biomass, rice straw and bioethers, and are a better option for addressing the food and energy security and environmental concerns. However, the lack of enough land space to grow crops for food and feed as well as for biofuels on one hand, and the need to retain the forests and other land uses that sequester carbon in huge quantities, on the other is a complex issue. According to one estimate, to replace worldwide petroleum use with biofuel, 10.8 million square miles of farmland with the highest yielding biofuel crops are needed, but unfortunately, we have only 5.8 million square miles of farmland on earth. A major criticism often leveled against biomass, particularly against large-scale fuel production, is that it will consume vast swaths of farmland and native habitats, drive up food prices, and result in little reduction in GHG emissions. However, this so-called ''food vs. fuel'' controversy appears to have been exaggerated in many cases [30]. Credible studies show that with plausible technology developments, biofuels could supply some 30% of global demand in an environmentally responsible manner without affecting food production. As a matter of fact, average biodiesel production yield from microalgae can be 10–20 times higher than the yield obtained from oleaginous seeds and/or vegetable. The search for renewable carbon neutral energy sources has spurred research and development (R&D) in this area globally, into various forms of solar energy transformations like solar thermal, photovoltaic, photocatalysis, and photosynthetic processes. Out of this, biofuel derived from cellulose and lipid materials of higher plants, has drawn considerable commercial entrepreneurship in recent times. Corn, sugar cane, jatropha etc. (Fig. 1) have been used as feedstock for production of fuels like ethanol and biodiesel. Brazil, USA and Europe already produce significant quantities of biofuel based on these feedstock. Algae as a feedstock is emerging at the forefront of biofuel research with the increasing awareness of global energy uses and production limitations of agriculture based oilseed crops. Khan with coworkers [31] undertook a methodical analysis of a maximum algal oil production rate from a theoretical perspective. They found that a theoretical maximum of 354000 l ha^{-1} $year^{-1}$ of unrefined oil, as against reported estimates of 40700–53200 l ha^{-1} $year^{-1}$ of unrefined oil. However, the full potential of microalgae is yet untapped.

Present scenario of biofuels. The twenty-first century has brought forward two major obstacles in the path of advancement of human civilization, namely clean environment to live in and eco-friendly, sustainable source of energy to fuel the modernization. According to a World Bank report (2008), 6.5 billion liters of biodiesel was produced worldwide in 2006, 75% of which by the European Union and 13% by the USA. The current contribution of biodiesel to global transportation fuel consumption is, however, only 0.14% and the favorable policies of major countries in the world are expected to increase this contribution by 5 times by 2020. The use of renewable energy source is becoming increasingly necessary to mitigate the de-

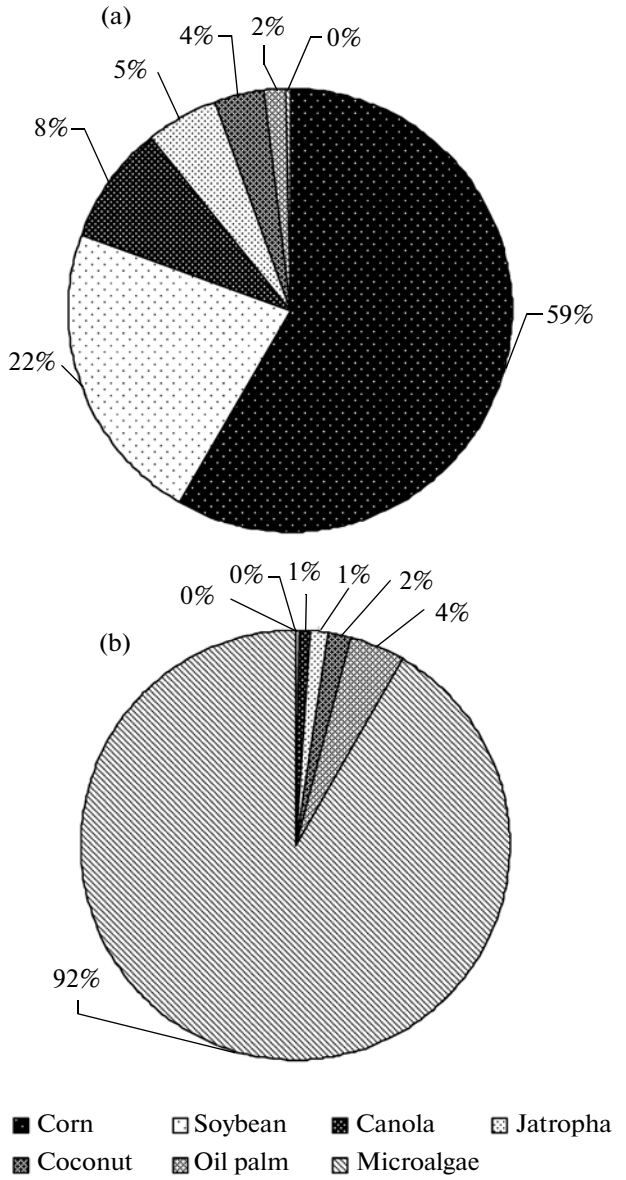

Fig. 1. Comparison of different crops and microalgae in terms of area (a, in ha) required for oil crop production and oil yield (b, in l/ha).

pletion of fossil fuels and increasing global warming. It is estimated that there will be a 60% increase in global energy requirement by 2030 over its present consumption level. Out of this 45% will be accounted by India and China alone [28].

However, diversion of agricultural or forest land for the cultivation of biofuel crops, has drawn strong criticism of late, due to its impact on food supply and net carbon footprint. Under these circumstances, photosynthetic organisms of microalgae species, which have productivity many orders higher than the conventional biofuel crops, do not require agricultural land and can sequester CO_2, have seen intense R&D inputs in the last few years for their commercialization. In recent years, with the boom in oil prices, some firms have already invested money in US, Israel, Australia and New Zealand for setting up pilot scale operations in algae cultivation and extraction of value added products including biodiesel. India started its biofuel initiative in 2003. This approach differs from other nations' in its choice of raw material for biofuel production—molasses for bioethanol and non-edible oil for biodiesel. Cyclicality resulted in a fuel ethanol program from sugar and molasses which suffered from inconsistent production and supply. However, except for sporadic R&D efforts on culturing and characterization, no major initiatives have been undertaken in scaling up and studying the economics of deriving biofuel from appropriate algae species in the Third world countries.

Microalgae as biofuels. The last few decades have seen a growing interest in using microalgae, cyanobacteria and other photosynthetic bacteria as potential producers of biorenewable fuels, such as biodiesel, biohydrogen and biogas. Biodiesel production from microalgae is a relatively novel concept. Microalgae (as opposed to other plants) are a natural choice for maximum-yield biofuels because they (1) intrinsically offer the greatest flux tolerance and photosynthetic efficiency as a consequence of a minimum of internally competitive plant functions (2) have fast reproductive cycles, (3) have limited nutrient requirements, and (4) can readily be exposed to temporal and spectral irradiation distributions and intensities that are not encountered in nature but are optimal for bioproductivity via cleverly crafted photonic systems. Alternative approaches for biofuel generation have identified aquatic microalgae as fast-growing species. Some microalgae exhibit carbon fixation rates and solar conversion efficiencies an order of magnitude greater than those of typical land-based plants [32]. Exploitation of microalgae for bioenergy generation (biodiesel, biomethane, biohydrogen), or combined applications for biofuels production and CO_2 mitigation, by which CO_2 is captured and sequestered, are under research [33–42]. An integrated strategy was proposed to enhance the economics, cost effectiveness and environmental sustainability by combining the benefits of biofuel production, CO_2 mitigation, waste heat utilization, waste water treatment and novel bioproduct production using the microalgal cultivation processes [43–46].

Several reviews on the commercial applications of microalgae are available [3, 47] especially those focusing particularly on biofuel [38, 48–51]. However, a critical evaluation of the prospects of microalgae as sources of biofuels is scarce.

Technologies for use of microalgae as sources of biofuels. Microalgae are found in diverse environmental conditions and habitats where light and water are available—lacustrine, brackish, freshwater, hypersaline, wastewater maturation ponds, dams, rivers, ma-

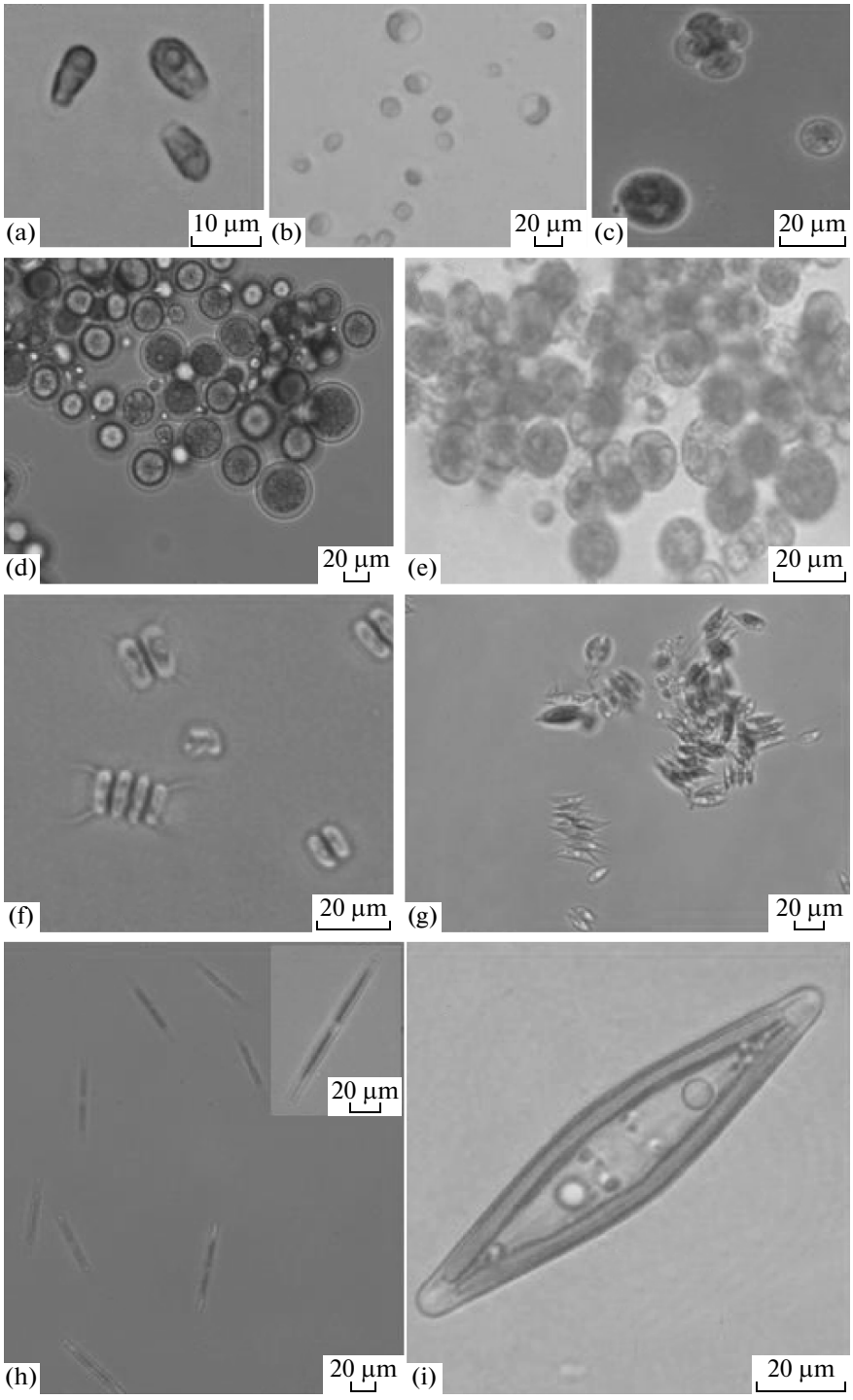

Fig. 2. Light micrographs of potential microalgal species for biofuel production: a – *Chlamydomonas* sp.; b, c – *Chlorella* sp.; d, e – *Chlorococcum* sp.; f, g – *Scenedesmus* sp.; h – *Pinnularia* sp.; i – *Navicula* sp.

rine and coastal areas. Fig. 2 provides an insight into their morphological diversity. Due to selection pressure and changing environmental conditions, there is a wide range of microalgal species worldwide found in extreme environments and these natural ecosystems have immeasurable value as sources of hyper-lipid producing microalgae [52]. In bioprospecting, it is important to collect microalgal samples temporally and spatially so as to determine if there are any successional tendencies in the habitat. Microalgal biomass has shown exhibit clear temporal and spatial patterns during the heterogeneous conditions of the open and

closed phases in estuaries. The microalgae are found as a mixed consortium and their population dynamics is complex in any habitat [53]. Different types of microalgal strains require different habitats.

The crucial step is to search, collect and identify hyper-lipid producing strains. Selection of productive strains, fast-growing, optimized for the local climatic conditions is very important for the success of any algal mass culture and particularly for high-value products such as biodiesel. It is also important to evaluate harvesting costs at the time of choosing the species. Low-cost harvesting requires large cell size, high specific gravity compared to the medium and reliable autoflocculation for successful biofuel production [54].

The idea of using microalgae as a source of transportation fuel is not new. It was first proposed in the 1950s [55] and, since the 1970s, several publicly funded research programs in different countries (USA, Australia, Japan) have investigated microalgae cultivation for producing renewable liquid fuels [36, 56–58]. Although the net energetics of the process appeared in some cases favorable, the projected costs for algal oil were several-fold higher than fossil oil prices, even with the most optimistic assumptions [36]. From 1978 to 1996 the U.S. Department of Energy invested more than US$ 25 million in the Aquatic Species Program (ASP) to develop renewable transportation fuels from microalgae [36]. The major focus of the program was to isolate high lipid content microalgae that could be cultivated in open ponds using CO_2 from coal fired power plants for wide-scale renewable fuel (biodiesel) production. The major conclusions were (1) oil accumulation in the algal cell attained through nitrogen deficiency does not increase oil productivity, since the higher oil content is more than offset by the lower productivities attained under nutrient shortage; (2) given the low cost requirements associated with fuel production, there is little prospect for any alternative (i.e., closed reactors) to the open pond design for large-scale production of microalgae; (3) maintaining mono-specific cultures of laboratory selected organisms in open ponds for more than a few weeks or months is very difficult because these are not robust enough to withstand contamination under field conditions. To overcome the latter limitation, it was suggested to allow native species to take over the culture. This solution, however, would conflict with the approach of genetically modifying microalgae attempted in ASP to reach higher, hopefully near-theoretical, conversion efficiencies of sunlight into biomass and to accumulate high levels of neutral lipids.

One of the biggest challenges in algae culture for biodiesel is to find a suitable strain with high lipid content and growth rate. Microalgae, by contrast, have received scant attention. The productivity of microalgae in nature, on an aerial basis, exceeds that of terrestrial plants by approximately one order of magnitude. The biodiversity of microalgae is large but the most of it remains biochemically and metabolically unexplored. To date, only very few number of species have been cultivated at industrial scale.

It is worthwhile to review in some detail the history of research and development on bioproducts from microalgae. Agriculture began more than 5.000 years ago. Industrial microbiology was a global business by the mid-twentieth century. By contrast, the first attempts at large-scale cultivation of microalgae began only 50 years ago. Their potential for bioenergy production was not recognized until the 1970s, and the resources devoted to this potential have been trivial by comparison to those lavished upon alternatives. Major advances in the biochemistry of microalgae were made in the 1980s and 1990s. Models of bioenergy production based on laboratory results showed great promise, and significant funding flowed into further studies, especially in Japan and the USA. However, with the increasing importance of microalgae in biodiesel production, several countries are vying with each other in the race for developing a suitable cost effective technology, by identifying the right alga, its cultivation and biodiesel production.

Sampling and isolation techniques. The microalgal sampling and selection process is well established although it requires specialized equipment and may be time-consuming [53]. Collection is mainly depends up on both biotic and abiotic factors, parameters measured onsite, type of aquatic system and sampling equipment. The equipment required for microalgal sampling includes a knife, mesh net (2 μm mesh), scooping jar, vials for collecting samples, scalpels, water analyzer kit measuring dissolved CO_2 and O_2 analyzer with a data logger, light meter, GPS, salinity meter, multiprobe system (measuring pH, temperature, turbidity, conductivity and light intensity simultaneously). Heavy duty equipment includes a suitable vehicle for rough terrain with enough space for the collected samples. There is no definite sampling procedure documented in literature though researchers can follow simple and cheap methods of collecting microalgal samples. Ideally samples can be collected from the natural substrata by chipping, scrapping, and by brushing from rock surfaces and bottom sediments. The brushing method was reported to be effective and reproducible method of collecting microalgal cells and also that it does not damage them. Sampling in deep freshwater lakes and dams requires systematic sampling, whereby water samples are scooped from at least three depth levels to the bottom of the lake or dam. This will allow selection of microalgae which prefer different light intensities. Bottom sediments are also major habitats of benthic microalgae and therefore should be collected together with pieces of detritus and mud. Stringent regimes and protocols need to be exercised when sampling. Therefore an all encompassing sampling regime is essential to undertake collection and isolation of microalgae from aquatic environments.

The isolation of microalgae from nature has a long history. The first microalga to be isolated and grown in pure culture was the freshwater microalga, *Chlorella vulgaris*. Over the next several decades, hundreds of species were gathered and maintained in very small quantities to form permanent culture collections, but very few species were cultivated in volumes of 50 ml or more. The chemical composition of microalgae could not be studied until the 1930s, when a new technique for "large-scale cultivation" made it possible to collect sufficiently large samples for analysis [59]. A key consideration is the choice of algal strain. There are many screening programs around the globe surveying algal species in different locations for suitable strains, very often building on the pioneering studies in the aquatic species program (ASP) during the 1980s and 1990s and a culture collection of more than 3000 strains were maintained. On the basis of oil content and high growth rate 300 species were screened [60]. Japan committed about US$ 117 million [61] to conduct research on microalgal CO_2 utilization in the 1990s in a program entitled Research Institute of Innovative Technology for the Earth, funded by the Ministry of International Trade and Industry through the New Energy Development Organization. Like the ASP, the program focused on both species collection and characterization [62–64] and development of cultivation technology and it has maintained marine microalgal culture collection comprising 1393 strains.

Isolation of microalgae into culture can be done by means of either the traditional methods or advanced methods or a judicious mix of both. The traditional methods are well established. Some species, often called weeds, are easy to isolate and cultivate, whereas others are difficult or seemingly impossible to grow. The first step towards successful isolation is the naturally occurring environmental conditions, which depends up on the nature of environment, quality of water, temperature, salinity etc. The second step is aimed towards the elimination of contaminants. The collection method is sometimes crucial for success, because damaged or dead cells lead to failure.

The most common method for single-cell isolation is by micropipette, although automation is more advantageous. Micropipette isolation is usually performed with a Pasteur pipette or a glass capillary having a straight or bent or curved tip. The goal of micropipette isolation is to pick up a cell from the sample, deposit it without damage into a series of sterile droplets, until a single algal cell, free of all other protists, can be confidently placed into the culture medium. Subsequently, the sample can be examined microscopically in a glass or plastic dish, in a multiwell plate, or on a microscope slide. However, the microalgal droplets can be placed on agar to reduce evaporation but this step depends on the size of the cells. Furthermore, the single cell can be pipetted and discharged into the sterile rinsing droplet and before the cell can settle, it should be picked up and transferred. Skill of the technique is important not to shear or damage the cell. For flagellates, cessation of swimming sometimes indicates damage. For diatoms, broken frustules can refract light differently than for intact cells. Leakage of protoplasm is an obvious sign of severe damage. Rogerson and co-workers, [65] employed repeated introduction and ejection of cells, suspended in a 1% crude papain solution, into and from a micropipette to generate ca. 10% naked cells of *Coscinodiscus asteromphalus*. These naked cells were re-isolated via micropipette into fresh medium. The traditional method of micropipette isolation can be successfully employed with certain precautions. Ultraclean droplets for rinsing are necessary, because the tiny cells cannot be easily distinguished from particulate material present, especially when working with seawater.

Screening of microalgae. The screening stage of bioprospecting focuses on isolation and identification of algal species capable of substantial lipid production, targeting organisms with rapid growth rate and tolerance to environmental parameters. The conventional method used for lipid determination involves solvent extraction and gravimetric determination. A major disadvantage of the conventional method is that it is time-consuming, labor-intensive and has a low throughput screening rate. Moreover, approximately 10–15 mg of wet weight of cells must be cultured for the extraction and derivatization [66]. Consequently, there is greater interest on a rapid in situ measurement of the lipid content [67]. Nile red (9-diethylamino-5H-benzo[a]phenoxazine-5-one), a lipid- soluble fluorescent dye, has been commonly used to evaluate the lipid content of animal cells and microorganisms [65] and especially microalgae [67, 68]. Nile red possesses several characteristics advantageous to in situ screening. It is relatively photostable, intensely fluorescent in organic solvents and hydrophobic environments. The emission maximum of Nile red is blue-shifted as the polarity of the medium decreases, [67, 69, 70] which allows one to differentiate between neutral and polar lipids at the excitation and emission wavelengths. Elsey et al. [71] showed the technical emission spectra for Nile red in various solvents. The peak emission intensity of Nile red in hexane is located near 576 nm when excited at 486 nm. The chloroform and ethanol peaks excitation were recorded at 600 and 632 nm, respectively [68]. In acetone Nile red is excited at 488–525 nm and the fluorescent emission is measured at 570–600 nm using various instruments [67]. Measurement of neutral lipids using the Nile red application requires the instrument to be calibrated using the stain dissolved in an organic solvent and account for the nonlinear intensity emission with respect to time. Measurements of lipid per unit cell require a calibration curve that correlates fluorescence to lipid content, whether determined gravimetrically or by use of lipid standards [68]. Thick cell walls of microalgae inhibit the permeation of Nile red and may indicate the

absence of oil, even though gravimetric analysis shows high yields of neutral lipids. It has been noted that the permeation of Nile red dye is also variable among algal species, requiring the use of high levels of DMSO (20–30% vol./vol.) and elevated temperatures of 40°C [72]. Stockenreiter et al., [73] observed that analysis of microalgae lipid content with Nile red fluorescence along with imaging flowcytometer (Flow CAM^R) offers the unique advantages of estimating the lipid content of each cell without the physical separation of algal cells.

Alternatively, the lipophilic fluorescent dye BODIPY 505/515 (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4adiaza-s-indacene) has recently been used as a vital stain to monitor algal oil storage within viable cells. Lipid bodies are stained green and chloroplasts appear red and are visualized in live oleaginous (oil-containing) algal cells [73]. The advantage of BODIPY 505/515 is that high lipid yielding cells may be identified and isolated microscopically using a micromanipulator system, flow cytometry or a fluorescence-activated cell sorter [72]. Subsequently, pure cultures may be propagated from the isolated viable cells. BODIPY 505/515 has been shown to have a narrower emission spectrum than Nile red, making it potentially more useful for confocal imaging, where fluorescence contrast enhancement of lipid bodies is important for image resolution [73]. Unlike Nile red, BODIPY 505/515 has the advantage that it does not bind to cytoplasmic compartments other than lipid bodies and chloroplasts. A recent study [74] demonstrated the use of Fourier transform infrared microspectroscopy (FTIR) to determine lipid and carbohydrate content of freshwater microalgae. FTIR was shown to be an efficient and rapid tool for monitoring lipid accumulation of microalgae. This study has reported highly significant correlations between the FTIR- and Nile red-based lipid measurements. For the purposes of bioprospecting for high lipid yielding microalgae, a rapid throughput of sample processing is required. The semi-quantification of neutral lipids using Nile red or BODIPY 505/515 and fluorescence microscopy allows for an initial rapid screening and visualization of lipid globules. FTIR spectroscopy may be used thereafter to quantify the yield of lipids. Once the high lipid producing microalgae have been identified, isolated and purified, a further step in the screening would be to determine the photosynthetic efficiency of the culture.

Subsequent to screening, understanding the physiology of the algal isolate is imperative. Pulse Amplitude Modulated (PAM) chlorophyll- a fluorescence measurements are widely used as a simple, rapid, and non-invasive method to assess the physiological state of microalgae. It is also a valuable tool to assess the optimum growth conditions required to maximize the biomass yield and to quantify the effect of nutrient or other extreme environmental stresses (salinity, temperature, photosynthetically active radiation, PAR and pH) on the algal culture. Neutral lipid synthesis is stimulated under nutrient depleted or limited conditions. Many microalgae have the ability to produce triacylglycerols (TAG) which comprise almost 80% of dry cell weight as a storage lipid [3, 38] under nutrient or other environmental stress. The PAM fluorometer parameters (electron transport rate, maximum quantum efficiency of Photosystem II [FV/Fm], and non-photochemical quenching) may be used as indicators of nutrient stress and consequently the possibility of neutral lipid synthesis and can be a valuable instrument in the screening process. Neutral lipid synthesis is likely to occur during the stationary phase of growth due to nutrient limitation [39]. The screening process of microalgae bioprospecting has to be comprehensive in assessing the lipid producing potential as well as the kinetics of growth and tolerance. The success of downstream processing is dependent on reliable biochemical and physiological screening tools such as the BODIPY 505/515 lipid stain, FTIR spectroscopy and PAM fluorometry.

Realizing the importance of microalgae in biodiesel production, several countries like China, Taiwan, Germany, France, Brazil, Australia, Canada, New Zealand, Italy and Israel are vying with each other in the race for developing a suitable cost effective technology by identifying the right alga, its cultivation and biodiesel production. A list of microalgae strains with potential to be used for the production of oils for biofuel is presented in Table 2.

Effect of different parameters on microalgal oil production. The yield of biodiesel from microalgae depends on both the biomass concentration of the cultures and the oil content of individual cells [8, 38, 98, 99]. One option for enhancing the metabolic flux into lipid biosynthesis is by applying artificial physiological stresses and producing biodiesel from microalgae that accumulate high amounts of oil.

Oleaginous algae produce only small quantities of TAG under optimal growth or favorable environmental conditions [100]. The interest in microalgae for oil production is due to the high lipid content of some species, and to the fact that lipid synthesis, especially of the non-polar TAG, which are the best substrate to produce biodiesel, can be modulated by varying growth conditions. The total content of lipids in microalgae may vary from about 1–85% of the dry weight, with values higher than 40% being typically achieved under stress conditions [8, 38, 101]. The lipid content in some microalgae could be modified by various growth conditions such as nitrogen deprivation [39, 52, 95, 102, 103], silicon deficiency [104, 105], phosphate limitation [23], high salinity [106] and some heavy metal stress such as cadmium [107].

Factors such as temperature, irradiance and, most markedly, nutrient availability have been shown to affect both lipid composition and lipid content in many algae [48, 107, 108]. Research is going on to identify

Table 2. List of microalgal strains (with their oil content) having potential for biofuel production

Microalgae	Oil content, % dry wt	Reference
Ankistrodesmus sp.	28–40	[75]
Botryococcus braunni	25–86	[33, 36, 69, 76]
Chaetoceros calcitrans	40	[52]
Chaetoceros muelleri	34	[52]
Chlamydomonas reihardtii	25	[77]
Chlorella emersonii	63	[78]
Chlorella minutissima	56–57	[79, 78]
Chlorella protothecoides (autotrophic/ heterotrophic)	15–55	[80]
Chlorella pyrenoidosa	55	[70, 80, 81, 82]
Chlorella vulgaris	19–56	[41, 52, 70, 78, 83, 84]
Chlorella zofingiensis	79	[83]
Chlorella sp.	28–48	[36, 38, 52]
Chlorococcum littorale	34	[85]
Chlorococcum sp.	19	[52]
Crypthecodinium cohnii	20	[38]
Cyclotella sp.	42	[36]
Cylindrotheca sp.	16–37	[38]
Dunaliella primolecta	23	[38]
Dunaliella salina	28	[81]
Dunaliella tertiolecta	15–42	[37, 41, 78, 86]
Haematococcus pluvialis	25	[87]
Hantzschia sp.	66	[36]
Isochrysis sp.	25–33	[36, 38, 88]
Monallanthus salina	20	[38]
Nannochloris sp.	63	[36]
Nannochloropsis sp.	31–80	[36, 38, 52]
Nanochloropsis oculata	36–60	[52, 89]
Nanochloropsis salina	72	[90, 91]
Neochloris oleoabundans	35–65	[38, 78, 92]
Nitzschia sp.	28–50	[38, 93]
Phaeodactylum tricornutum	20–30	[36, 38]
Pseudochlorococcum sp.	52	[94]
Scenedesmus dimophus	16–40	[78]
Scenedesmus obliquus	31–55	[78]
Scenedesmus sp.	21–45	[36, 52]
Schizochytrium sp.	50–77	[38]
Stichococcus sp.	9–59	[36, 95]
Tetraselmis suecica	15–32	[36, 82, 89, 96]
Thalassiosira pseudonana	21–31	[97]

the environmental/abiotic factors which cause stress. Among chemical environmental stimuli, nutrient starvation (nitrogen and phosphate), salinity and pH of growth medium are the most investigated. It is important to take into consideration physical/environmental stimuli—temperature and light intensity, growth phase and/or aging of the culture. The point of concern is to identify stimuli which can enhance oil/lipid accumulation in microalgae without affecting their growth rate. A number of algal strains, with good potential for making biodiesel have been identified, which include *Botryococcus* sp., *Chlorella* spp., *Chlamydomonas* sp., *Scenedesmus* sp., *Crypthecodinium* sp., *Nannochloropsis* sp., *Nannochloris* sp. etc.

Nutrients. The strategy of enhancing lipid production of microalgae by controlling the nutritional or cultivation conditions (e.g., temperature, pH, and salinity) is aimed at channeling metabolic flux generated in photobiosynthesis into lipid biosynthesis. Nutrient starvation has so far been the most commonly employed approach for directing metabolic fluxes to lipid biosynthesis of microalgae. In this scenario, microalgae accumulate lipids as a means of storage under nutrient limitation when energy source (i.e., light) and carbon source (i.e., CO_2) are abundantly available and when the cellular mechanisms for the photobiosynthesis are active. While a number of nutrients such as phosphorus and iron deficiency have been reported as being able to cause cell growth cessation and channel metabolic flux to lipid/fatty acid biosynthesis, nitrogen is the most commonly reported nutritional limiting factor triggering lipid accumulation in microalgae. Nitrogen starvation has been observed to lead to lipid accumulation in a number of microalgal species. For instance, *Chlorella* usually accumulates starch as storage material. However, it was observed by Illman et al. [103] that *C. emersonii*, *C. minutissima*, *C. vulgaris*, and *C. pyrenoidosa* could accumulate lipids of up to 63%, 57%, 40%, and 23% of their cells on a dry weight basis, respectively, in low-N medium. Under nitrogen-deficient conditions, *Neochloris oleoabundans* was reported to be able to accumulate 35–54% lipids of its cell dry weight and its TAG comprised 80% of the total lipids [109]. Yamaberi et al., [110] also observed that TAG accumulated in *Nannochloris* sp. cells could be 2.2-fold more than in the cells in nitrogen sufficient cultures. Li et al. [39] showed that sodium nitrate was the most favourable nitrogen source for cell growth and lipid production of *N. oleoabundans* among the three tested nitrogen-containing compounds, i.e., sodium nitrate, urea, and ammonium bicarbonate. It was observed that lipid cell content decreased with the increase of sodium nitrate in the medium in the range of 3–20 mM concenteration. The trend that lower nitrogen source concentration in medium led to higher lipid cell content was hypothetically explained by the fact that nitrogen would have exhausted earlier at low cell density when the initial concentration of nitrogen source in medium was low. As a result, cells started to accumulate lipid when light had good penetration (at low cell density), when individual cells were exposed to a large quantity of light energy, resulting in more metabolic flux generated from photosynthesis to be channeled to lipid accumulation on an unit biomass basis.

Nutrient deficiency (particularly nitrogen and silicon) has been regarded as the most efficient approach to increase lipid content in algae. Enhanced lipid accumulation (TAG) in various algal taxa and numerous species has been observed under nitrogen limitation. As green algae require more nitrogen source for growth, nitrogen limitation is considered more beneficial for increasing lipid content in them. Spoehr and Milner [102] demonstrated that a nitrogen starved *C. pyrenoidosa* culture was able to accumulate up to 85% lipid in its biomass, while the typical content of exponential cultures was only about 5%. An increase of lipid content up to 70% of the dry biomass has been reported with several species in response to limiting nitrogen supply in batch cultures, with TAG mainly containing saturated and monounsaturated fatty acids forming the bulk (up to 80%) of the lipid fraction in the starved cells [108, 110]. However, a large variability exists in the response to nitrogen deficiency. Generally, diatoms, which have relatively high log-phase lipid content, do not respond to nitrogen starvation by increasing their lipid content [1, 90]. Green microalgae show a variety of responses, from several-fold increase from log-phase values (e.g., in *C. pyrenoidosa*), to no change or even a slight reduction (e.g., in some *Dunaliella* spp. and in *Tetraselmis suecica*) [110]. Within the same genus (e.g., *Chlorella*) some strains were found to accumulate starch under nitrogen starvation, whereas others accumulated neutral lipids.

A stronger stimulation of lipid production occurs in response to conditions of nitrogen limitation, which potentially can occur in all known microalgae. Nitrogen-starved cells can contain four times lipid content as compared with N-sufficient cells [91, 110, 111], and optimization of the lipid production of pond bioreactors therefore depends on their operators' ability to induce N-limitation in the resident algal cells reliably and consistently. Resource-ratio theory and the principles of ecological stoichiometry provide additional new insights into the control of algal biomass and lipid production in pond bioreactors [112, 113]. As demonstrated by Rhee [114], the nutrient limitation status of microalgae can be directly controlled by regulating the ratio of nitrogen and phosphorus (N : P). A transition between N- and P-limitation of phytoplankton growth typically occurs in the range of N : P supply ratios between ca. 20 : 1 to ca. 50 : 1 by moles [114]. Such shifts between N- and P-limitation have extremely important implications for algal biofuel production because diverse species of microalgae grown under nitrogen-limited conditions (i.e. low N : P supply ratios) can exhibit 3 times more the lipid content than cells grown under conditions of phosphorus limitation

(high N : P supply ratios) [113]. Total phosphorus and nitrogen concentration in the nutrient feed to pond bioreactors should therefore impact algal biodiesel production, because the N : P ratio of incoming nutrients will strongly influence algal biomass production [99] as well as the cellular lipid content. An inverse relationship was observed between N : P and cellular lipids [115], and a positive, hyperbolic relationship observed between N : P and microalgal biomass [99]. Thus, it can be concluded that optimal lipid yield (in terms of mass of lipid produced per unit of bioreactor volume per day) occurs at intermediate values of the N : P supply ratio. From the strong apparent interactions between the effects of nitrogen and carbon dioxide availability on microalgal lipids, and the effects of N : P supply ratios on volumetric lipid production, it can be surmised that this might be even greater if the bioreactors are simultaneously provided with supplemental CO_2 [99].

Other types of nutrient deficiency that promote lipid accumulation include phosphate and sulfate limitation. Phosphate limitation was observed to cause enhancement of lipid accumulation of *Monodus subterraneus* [105]. With a decrease in phosphate availability, the cellular total lipid content of starved cells increased, mainly due to the drastic increase in TAG levels. In the absence of phosphate, the proportion of phospholipids reduced from 8.3% to 1.4% of total lipids, and the proportion of TAG increased from 6.5% to 39.3% of total lipids. Studies have shown that sulfur deprivation enhanced the total lipid content in the green algae *Chlorella* sp. and *C. reinhardtii* [116].

In diatoms, silicon is an important nutrient that affects cellular lipid metabolism. For example, silicon-deficient *Cyclotella cryptica* cells had higher levels of neutral lipids (primarily TAG) and higher proportions of saturated and mono-unsaturated fatty acids than silicon-replete cells [117].

Micronutrients. In recent years, the function of micronutrients in microalgal growth and lipid accumulation has been investigated by many researchers. Micronutrients, including metals (iron, manganese, zinc, cobalt, copper, molybdenum, nickel, and cadmium) and the metalloid selenium, influence microalgal growth and lipid accumulation, because of their role as limiting micronutrients. Iron has a key function in regulating phytoplankton biomass in oligotrophic waters near the Equator and further south [118]. Furthermore, iron deficiency has also been reported to stimulate lipid accumulation in microalgae *C. vulgaris*, which accumulated up to 56.6% lipid of biomass by dry weight under the optimal condition of 1.2×10^{-5} M $FeCl_3$ [98].

Temperature. Temperature has a significant effect on the fatty acid composition of algae. A general trend towards increasing fatty acid unsaturation with decreasing temperature and increasing saturated fatty acids with increasing temperature has been observed in many algae and cyanobacteria [48, 119].

Light intensity. Algae exhibit remarkable changes in their gross chemical composition, pigment content and photosynthetic activity during growth at various light intensities. Typically, low light intensity induces the formation of polar lipids, particularly the membrane polar lipids associated with the chloroplasts, whereas high light intensity decreases total polar lipid content with an increase in the amount of neutral storage lipids, mainly TAG [97, 120].

Growth phase and physiological status. Lipid content and fatty acid composition are also subjected of variability during the growth cycle. In many algal species examined, an increase in TAG is often observed during stationary phase. For example, in the chlorophyte *Parietochloris incise*, TAG increased from 43% (total fatty acids) in the logarithmic phase to 77% in the stationary phase [121] and in the marine dinoflagellate *Gymnodinium* sp., the proportion of TAG increased from 8% in the logarithmic phase to 30% in the stationary phase of growth [122]. Coincident increases in the relative proportions of both saturated and mono-unsaturated 16 : 0 and 18 : 1 fatty acids and decrease in the proportion of PUFA in total lipids were also associated with growth-phase transition from the logarithmic to the stationary phase.

Salinity. Takagi et al. [123] observed that TAG content increased in a marine alga, *Dunaliella*, under high salinity conditions. An initial NaCl concentration higher than 1.5 M was found to markedly inhibit cell growth. However, when the initial NaCl concentration increased from 0.5 M (equal to seawater) to 1.0 M, it resulted in higher intracellular lipid content (67%) in comparison with 60% for initial salt concentration. Addition of 0.5 or 1.0 M NaCl at mid-log phase or at the end of log phase during cultivation further increased the lipid content to 70%.

A commonly suggested procedure is to use a two-stage cultivation strategy, dedicating the first stage for cell growth/division in nutrient-sufficient medium and the second stage for lipid accumulation under nutrient starvation or other physiological stress. A well formulated medium such as proposed by Li et al. [42] would achieve the two-stage lipid production "naturally" as the cells will be able to grow quickly before the exhaustion of the limiting substrate (N, in this particular case) and then switch to lipid accumulation under N starvation conditions. Furthermore, a hybrid closed photobioreactor/open pond microalgal cultivation system [89] was suggested to be potentially the appropriate engineering solution accommodating the two-stage strategy with the photobioreactors dedicated to nutrient-rich inoculum build-up and the open ponds to low-nutrient lipid accumulation. It was also pointed out that employment of low-nutrient media in open ponds is not only beneficial for lipid accumula-

tion and contamination control, but also environmentally friendly.

Nevertheless, deficiency of these nutrients may slowdown photosynthesis of microalgal cells one way or the other, resulting in lowered overall lipid productivity. Many of the commonly used limiting nutrients are essential for photosynthesis of microalgae and the depletion of which may severely impede the photosynthesis responsible for generating the metabolic flux for lipid production. For instance, it was observed in studies that chlorophyll, the essential pigment for light capturing in the biosynthesis of green alga *N. oleoabundans*, was consumed for cell growth when nitrogen was exhausted from the medium, resulting in a sharp drop of chlorophyll cell content [39]. Phosphorus is essential to the cellular processes related to bioconversion of energy (e.g., photophosphorylation). Of particular relevance, photosynthesis requires large amounts of proteins (notably Rubisco) and proteins are synthesized by phosphorus-rich ribosomes [124]. As a result, channeling metabolic flux to lipid biosynthesis by the means of phosphate starvation may have a severe impact on photosynthesis. There is apparently a dilemma in the biochemical engineering strategy i.e., the very reason that stimulates lipid accumulation in cells may result in severely impeded cell growth and photosynthesis and hence lowered overall lipid productivity. This dilemma could likely be solved by employing metabolic engineering approaches.

Recent studies have also indicated that the diversity of primary producer systems is often positively linked to biomass production and lipid accumulation. Stockenreiter et al. [73] showed that lipid production increased with increasing diversity, in both natural and laboratory microalgal communities and the underlying reason seemed to be resource use complementarity. Such ecology related dynamics can provide a cost-effective and resource conserving technique to improve biofuel production.

Genetic engineering approaches. Although biotechnological processes based on transgenic microalgae are still in their infancy, researchers and companies are considering the potential of microalgae as green cell-factories to produce value-added metabolites and heterologous proteins for pharmaceutical applications. The commercial application of algal transgenics is beginning to be realized and algal biotechnology companies are being established. It was predicted that microalgae, due to the numerous advantages they present, could offer a powerful tool for the production of commercial molecules in the near future. The fast growing interests in the use of transgenic microalgae for industrial applications is powered by the rapid developments in microalgal biotechnology. The genome sequencing projects of the red alga *Cyanidioschyzon merolae* [124], the diatoms *Thalassiosira pseudonana* [125] and *Phaeodactylum tricornutum* [126] and the unicellular green alga *Ostreococcus tauri* [127] have been completed. Nuclear transformation of various microalgal species is now a routine; chloroplast transformation has been achieved for green, red, and euglenoid algae, and further success in organelle transformation is likely as the number of sequenced plastid, mitochondrial, and nucleomorph genomes continues to grow [128]. Various genetic transformation systems have been developed in green algae such as *Chlamydomonas reinhardtti* and *Volvox carteri* [129].

The fast pace of developments in microalgal biotechnology permit the isolation and use of key genes for genetic transformation. The key enzyme in regulating fatty acid synthesis, acetyl-CoA carboxylase (ACC), was first isolated from the microalga *Cyclotella cryptica* in 1990 by Roessler [118] and then successfully transformed by Dunahay et al. [130] and Sheehan et al. [39] into the diatoms *C. cryptica* and *Navicula saprophila*. The ACC gene, *acc*1, was overexpressed with the enzyme activity enhanced to 2–3-fold. These experiments demonstrated that ACC could be transformed efficiently into microalgae, although no significant increase of lipid accumulation was observed in the transgenic diatoms [129]. It also suggests that overexpression of ACC enzyme alone might not be sufficient to enhance the whole lipid biosynthesis pathway [36]. Even though there is no success story with respect to lipid overproduction of microalgae using the genetic engineering (GE) approach up to now, a solid understanding towards the global TAG biosynthesis pathway, which is generally accepted to be identical throughout all species except the differences in the location of reactions and the structure of some key enzymes, has been established.

Large scale cultivation. Photobioreactors are different types of tanks or closed systems in which algae are cultivated. Algal cultures consist of a single or several specific strains optimised for producing the desired product. Water, necessary nutrients and CO_2 are provided in a controlled way, while oxygen has to be removed. Algae receive sunlight either directly through the transparent container walls or via light fibres or tubes that channel it from sunlight collectors. A great amount of developmental work to optimise different photobioreactor systems for algae cultivation has been carried out and is reviewed in Carvalho et al. [131], and Hankamer et al. [132]. It has also been suggested to grow heterotrophic algae in conventional fermentors instead of photobioreactors for production of high-value products [18]. Instead of light and photosynthesis, heterotrophic algae rely on utilizable carbon sources in the medium for their carbon and energy generation.

Open pond systems. Open pond systems are shallow ponds in which algae are cultivated. Nutrients can be provided through runoff water from nearby land areas or by channeling the water from sewage/water treatment plants. The water is typically kept in motion by paddle wheels or rotating structures, and some mixing

Table 3. Comparison between open pond and photobioreactor system for mass cultivation of algae Modified from [133, 134]

Parameter or issue	Open ponds and raceways	Photobioreactors
Space requirement	High	Low
Water loss	Very high, may also cause salt precipitation	Low
CO_2 loss	High, depending on pond depth	Low
Oxygen concentration	Usually low enough because of continuous spontaneous outgassing	Build-up in closed system requires gas exchange devices (O_2 must be removed to prevent inhibition of photosynthesis and photooxidative damage)
Temperature	Highly variable, some control possible by pond depth	Cooling often required (by spraying water on Photo bioreactor (PBR) or immersing tubes in cooling baths)
Shear	Low (gentle mixing)	High (fast and turbulent flows required for good mixing, pumping through gas exchange devices)
Cleaning	No issue	Required (wall-growth and dirt reduce light intensity), but causes abrasion, limiting PBR life-time
Contamination risk	High (limiting the number of species that can be grown)	Low
Biomass quality	Variable	Reproducible
Biomass concentration	Low, between 0.1 and 0.5 g l^{-1}	High, between 2 and 8 g l^{-1}
Production flexibility	Only few species possible, difficult to switch	High, switching possible
Process control and reproducibility	Limited (flow speed, mixing, temperature only by pond depth)	Possible within certain tolerances
Weather dependence	High (light intensity, temperature, rainfall)	Medium (light intensity, cooling required)
Startup	6–8 weeks	2–4 weeks
Capital investment	Low	Very high
Operating costs	Low (paddle wheel, CO_2 addition)	Very high (CO_2 addition, pH-control, oxygen removal, cooling, cleaning, maintenance)
Harvesting cost	High, species dependent	Lower due to high biomass concentration and better control over species and conditions
Current commercial applications	5000 t of algal biomass per year	Limited to processes for high added value compounds or algae used in food and cosmetics

can be accomplished by appropriately designed guides. Algal cultures can be defined (one or more selected strains), or are made up of an undefined mixture of strains. For an overview of systems used, see Borowitzka [9].

Comparison of the different production systems. The high capital cost associated with producing microalgae in closed culture systems is the main challenge for commercialization of such systems [8]. Open systems do not require expenses associated with sterilization of axenic algal cultures. However, this leads to high risk of contamination of the culture by bacteria or other unwanted microorganisms. A common strategy therefore to achieve monocultures in an open pond system is to keep them at extreme culture conditions such as high salinity, nutrition or alkalinity [12]. Consequently, this strictly limits the species of algae that can be grown in such systems. To our knowledge, based on available literature, currently only *Dunaliella* (high salinity), *Spirulina* (high alkalinity) and *Chlorella* (high nutrition) have been successfully grown in commercial open pond systems [12]. The necessity for a large cultivation area has been pointed out as a limitation in using open ponds to grow microalgae for mitigating the CO_2 released from power generating plants. It has been estimated that a raceway pond requires 1.5 km^2 to fix the CO_2 emitted from a 150 MW thermal power plant [132]. The large area requirements are partly due to the comparable lower productivity of open pond systems. It was pointed out that improving the control of limiting parameters in open ponds such as culture medium, temperature and contamination and thereby increasing productivity could be accomplished by using a transparent cover over the ponds, such as a greenhouse. Selection of a suitable production system clearly depends on the purpose of the production facility.

For example, closed bioreactors will not be suitable for wastewater treatment, because the costs for treating wastewater in this system will be too high in relation to the low value added during the production process. On the other hand, high quality/value products that are produced only in small amounts might require production in bioreactors. A comparison of the different production systems is presented in Table 3.

Carbon dioxide mitigation and sequestration. To use microalgae to fix CO_2 released from power plants via the exhaust gas and thereby mitigate the amount of carbon released into the atmosphere is an attractive idea. However, there are several major challenges before this idea becomes practical. It is known that growth of algae is negatively influenced by increasing CO_2 [135]. Strains that grow well at CO_2 concentrations of 5–10% show a drastic decrease in their growth rate above 20% [136]. An important task therefore has been to identify strains that can cope with very high CO_2 concentrations and also have high growth rates. Screening has yielded strains that grow well in CO_2 concentrations between 30% and 70% saturation [137]. Also, results by Olaizola [18] indicate that by controlling the pH changes in the culture and releasing CO_2 to the algae on demand, growth could be sustained even at 100% CO_2. Another important property that would need to be optimized is the ability of algal strains to have high thermal stability. It has been suggested that the hot flue gases introduced in the algal cell cultures may influence the temperature [138].

There is a worldwide awareness about global warming as a result from the rising levels of different greenhouse gases such as CO_2 released from the burning of fossil fuels. Different methods have been suggested as to how CO_2 could be sequestered or immobilised through filtering or other mechanical/chemical processes and subjected for long-term storage to avoid release into the atmosphere. In this respect, the idea of biological sequestering by growing algae and take advantage of their photosynthetic machinery of capturing carbon dioxide has been suggested by many researchers as an alternative method of reducing the amount of CO_2 released in the atmosphere [36, 38, 41, 89, 139–141].

The Aquatic Species Program (ASP) funded from 1978 through 1996 by the Office of Fuels Development started out as a project investigating the possibilities of using algae to sequestering CO_2 emissions from coal power plants [36]. The main direction of the program over time became focused on the specific application of developing a production of high-quality diesel from algae utilizing the CO_2 in the exhaust gas from these plants. The project screened for algae that could produce high amount of oils as well as could grow at severe conditions regarding temperature, pH and salinity. In Europe, the Emissions Trading Scheme is one of the policies introduced across Europe to tackle emissions of carbon dioxide and other greenhouse gases and combat the serious threat of climate change. Aquatic species could benefit from trading given their potential to mitigate and/or sequester carbon and this would contribute to the economics of production.

Globally, a lot more needs to be done before the utilization of microalgae can become a reality. An urgent need exists to set up facilities comprising "banks" for maintenance of algal germplasm useful as sources of biofuels, and trained manpower (biologists, engineers and technocrats) needs to be developed for effective utilization of these valuable sources of "green energy".

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