Biotechnological exploitation of microalgae

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Short statement

This comprehensive review highlights the use of microalgae for biotechnological exploitation, and discusses production of high value products, genetic engineering of microalgae, downstream processing issues and engineering of synthetic communities

Abstract

Microalgae are a diverse group of single-cell photosynthetic organisms that include cyanobacteria and a wide range of eukaryotic algae. A number of microalgae contain high-value compounds such as oils, colorants and polysaccharides, which are used by the food additive, oil and cosmetic industries, among others. They offer the potential for rapid growth under photoautotrophic conditions, and they can grow in a wide range of habitats. More recently, the development of genetic tools means that a number of species can be transformed and hence used as cell factories for the production of high-value chemicals or recombinant proteins. In this article we review exploitation use of microalgae with a special emphasis on genetic engineering approaches to develop cell factories, and the use of synthetic ecology approaches to maximise productivity. We discuss the success stories in these areas, the hurdles that need to be overcome, and the potential for expanding the industry in general.

1. Introduction

Microalgae are a large and diverse group of photosynthetic organisms ranging from prokaryotic cyanobacteria to eukaryotic algae spread across many phyla (Guiry, 2012). This diversity offers great potential that has yet to be exploited to any great extent. Microalgae are found in freshwater and marine habitats and produce half of the atmospheric oxygen on earth. The ability to grow autotrophically makes their cultivation potentially simple and cost-effective, and microalgae have attracted increasing interest as sources of natural food additives, cosmetics, animal feed additives, pigments, polysaccharides, fatty acids and biomass (Borowitzka, 2013; Hallmann, 2007; Leu and Boussiba, 2014). An important aspect of using microalgae for industrial purposes is the GRAS (generally regarded as safe) status of numerous algae. This is essential for products intended for animal or human consumption and could significantly reduce downstream processing costs. Recently, major advances in developing microalgae as biotech platforms have been made; this is especially the case from a genetic engineering perspective. Here we give an overview of state of the art engineering tools, previous successes with recombinant protein expression and advances made in engineering cyanobacteria for high value compound production. Furthermore, the largely untapped potential of algae grown in synthetic communities and the challenges associated with downstream processing of microalgae are discussed.

2. High-value natural products in microalgae

Microalgae are the source of several forms of high-value compounds such as carotenoids, polyunsaturated fatty acids (PUFAs), proteins, antioxidants and pigments. Characterised by high protein and nutrient contents, some species such as *Arthrospira platensis* (a cyanobacterium, also known as *Spirulina platensis*) and *Chlorella vulgaris* (a green alga) are used as feed, food additives and diet supplements (Yaakob *et al.*, 2014). In other cases specific high-value compounds are isolated from appropriate strains.

Relatively few proteins have been purified from microalgae for commercial use, but *Spirulina* is a rich source of phycocyanin, a protein that constitutes 14% of the dry weight of this cyanobacterium (McCarty, 2007). The US Food and Drug Administration (FDA) has approved phycocyanin from *Spirulina* as a blue food colorant. Moreover phycocyanobilin, the tetrapyrrole chromophore of phycocyanin, manifests fluorescent properties that have been exploited for labelling of antibodies in immunofluorescence and flow cytometry. In mammalian tissues it can be enzymatically reduced to phycocyanorubin (a close homolog of bilirubin) and inhibits the activity of NADPH oxidase, thus reducing the generation of reactive oxygen species. It has been suggested that a regular intake of phycocyanobilin may provide protection against cancer and other diseases (Sørensen *et al.*, 2013). Moreover, recent studies have proven the beneficial health effect of microalgae (*Chlorella, Spirulina)* by increasing natural killer cell levels and stimulating immune and anti-inflammatory system response in humans (Nielsen *et al.*, 2010; Kwak *et al.*, 2012).

Carotenoids are important products that are extracted from microalgae, and indeed the first commercialised product derived from algae was β-carotene. It is produced in very high amounts by *Dunaliella salina*, a halophilic alga growing in saline habitats which makes the cultures less susceptible to contamination. What differentiates *Dunaliella* β-carotene from the synthetic product (present only in the form of all-*trans* isomer) is that it is rich in the 9-*cis* isomer, and a negative effect of the use of all-*trans* isomer, such an effect on plasma cholesterol levels and atherogenesis, has been reported from mice studies (Borowitzka, 2013; Harari *et al.*, 2008). Another example of a carotenoid with a well-established and growing market in the nutraceutical area is astaxanthin from the freshwater green alga *Haematococcus pluvialis*. Astaxanthin is mainly used as a feed supplement and pigmentation source for salmon and shrimp farming, but due to its high antioxidant properties (10-fold greater than other carotenoids) and protective activities it has also many applications in the pharmaceutical and cosmetic industries. Astaxanthin has also been shown to prevent bacterial infection, vascular failure and cancer (Ambati *et al.*, 2014).

Fatty acids are the other natural components produced commercially from microalgae. Several marine algal species are rich in omega-3 long chain polyunsaturated fatty acids (LC-PUFAs),

such as docosahexaenoic acid (DHA; e.g. from *Isochrysis* strain T-iso and *Pavlova lutheri*), eicosapentaenoic acid (EPA; e.g. *Nannochloropsis gaditana*, *Nannochloropsis oculata*) and alpha-linolenic acid (e.g. *Rhodomonas salina*, *Tetraselmis uecica*). Oils from *Nannochloropsis*, *Rhodomonas* and *Tetraselmis* have higher antioxidant properties than fish oils, partly because of a high content of valuable carotenoids (fucoxanthin, lutein, neoxanthin, alloxantin) and polyphenols. Given the fact that intensive fishing endangers many fish species, algal oils may provide an alternative to fish oils in diets in the future (Ryckebosch *et al.*, 2014).

Other algal species such as *Dunaliella salina* and *Botryococcus braunii* can accumulate up to 60% of storage lipids as triacylglycerides (TAGs), potentially making them a valuable source of oil for biodiesel production (Scott *et al.,* 2010). Compared to plants, algae exhibit higher productivities and theoretically could give 10- to 100-fold higher yields of oil per acre, although such capacities have not yet been achieved on a commercial scale (Greenwell *et al.*, 2010). Nevertheless, algae appear to be a potential solution to the controversial food vs. fuel problem that is associated with the use of fertile land to produce plant-derived biofuels. TAGs are not the only way in which microalgae could be exploited for biofuels. Under anaerobic conditions and sulphur depletion, some microalgae produce hydrogen gas, which could be used as an alternative to fossil fuels in the future (Melis *et al.*, 2000; Zhang *et al.*, 2002). Recently, Scoma *et al.* have attempted to produce hydrogen from *Chlamydomonas reinhardtii* using natural sunlight. Although successful, the overall yields did not exceed those obtained in lab-scale settings (Scoma *et al.*, 2012) and so this technology is at an early stage.

While the above compounds are well-established microalgal products with known potential, other microalgae are being studied for new compounds with useful properties. Some cyanobacteria have a poor reputation in the popular press for causing toxic blooms, because they are able to produce hepatotoxins and neurotoxins (e.g. anatoxin, jamaicadine, *L*-beta-N-methylamino-*L*-alanine) (Araoz *et al.*, 2010). However, research on cyanobacteria is undergoing a renaissance, because some identified metabolites and their derivatives have been shown to have potential as next generation antiviral (Huheihel *et al.*, 2002), anticancer (Leao *et al.*, 2013) and antibacterial drugs. Several of these drugs have even successfully reached phase II and III clinical trials (Dixit and Suseela, 2013). A large number of promising natural compounds are derived from filamentous marine genera such as *Lyngbya*, *Symploca* and *Oscillatoria*. Usually they are short peptides built from non-canonical amino acids by the hybrid polyketide synthase (PKS)/non-ribosomal peptide synthetase (NRPS) (Tan, 2013).

Some of the most promising candidate anticancer agents are derivatives of dolastatin 10, a peptide originally isolated from sea hare *Dolabella auricularia*. The first one, TZT-1027 (soblidotin) is a microtubule polymerisation inhibitor that exhibits antitumor activity in

preclinical models, manifesting stronger activity than paclitaxel (Akashi *et al.*, 2007; Watanabe *et al.*, 2006). Another analogue of dolastatin 10 (monomethyl auristatin E) linked to an antibody is already approved by FDA and used in therapies for patients with Hodgkin lymphoma (Deng *et al.*, 2013).

In summary, algae and cyanobacteria are an apparently under-explored source of natural high-value compounds and there is a rising interest in their exploitation. Just as numerous plant secondary metabolites have been used for biotechnological and biomedical purposes, there is huge scope for the identification of correspondingly valuable compounds within the vast microalgal population. New discoveries are made regularly and new compounds and applications for industrial purposes are to be expected in the near future.

3. Genetic tools in microalgae, and the development of microalgal cell factories

As well as containing a range of high-value compounds, microalgae offer real potential as cell factories for the production of other compounds and proteins. With the advancement and availability of algal genome data, transformation protocols have been developed for a number of microalgae and this means that they can now be used to enhance the levels of natural high-value products, or for the expression of genes in order to produce novel products, or recombinant proteins including antibodies, hormones, vaccines and insecticidal protein at economically viable levels (Gong *et al.*, 2011; Hallmann, 2007). To date, there have been reports of successful genetic manipulation of over 40 different microalgae species including the green algae *C. reinhardtii*, *D. salina, C. vulgaris* and *H. pluvialis*, and the diatoms *Phaeodactylum tricornutum* and *Thalassiosira pseudonana* (Walker *et al.*, 2005). However, the genetic toolkits developed for *C. reinhardtii* and *P. tricornutum* are the most advanced, and both nuclear and chloroplast transformation has been achieved, so these species will be reviewed as examples.

In recent years there have been rapid developments of genetic tools for microalgae in an attempt to generate more effective microalgal cell factories. Microalgae have several distinct advantages compared with plant-based production and some other bioreactor systems. Firstly, many microalgae can double their biomass in less than 24 hours, with some species such as *Chlorella sorokiniana* having a doubling time under optimum conditions of less than three hours (Sorokin, 1967). Therefore only a short period of time is required for large-scale production compared with plants. Furthermore, the time from creation of a new transgenic line to industrial scale up can be as short as two months (Mayfield *et al.*, 2007; Schmidt, 2004). Secondly, genes for recombinant proteins can be expressed from the nuclear or chloroplast genomes (León-Bañares *et al.*, 2004), and eukaryotic microalgae possess post-translational modification pathways which allow glycosylated proteins to be produced and secreted out of the cell (Hempel and Maier,

2012; Lauersen *et al.*, 2013a, 2013b). Thirdly, many microalgae can be grown either phototrophically or heterotrophically in enclosed photobioreactors, preventing transgenes from escaping into the environment. This is a particular concern for transgenic plants where transgenic DNA might spread to soil bacteria or to related plant species by means of pollen transfer (Gong *et al.*, 2011). Prior to introducing the desired gene into an algal genome, a few fundamental factors such as DNA delivery method, selection method and control of gene expression have to be taken into account.

3.1. DNA Delivery Method.

A variety of different methods have been employed for delivering transgenes into algal cells (**Table 1**), and the method of choice is very much determined by the cell size and nature of the cell wall of the chosen algal species (Stevens and Purton, 1997). The first demonstrations of stable transformation of an alga involved the bombardment of *C. reinhardtii* cells with DNA-coated microprojectiles (so-called biolistics) and proved successful for the delivery of DNA into both the chloroplast (Boynton *et al.*, 1988) and nucleus (Debuchy *et al.*, 1989). Biolistics was also used to transform the mitochondrial genome of *C. reinhardtii* (Randolph-Anderson *et al.*, 1993).

Subsequently, the nuclear genome of *C. reinhardtii* has been genetically transformed using other delivery methods including agitation in the presence of glass beads or silicon carbide whiskers, electroporation, *Agrobacterium*-mediated transformation, and, more recently, positively-charged aminoclay nanoparticles (Brown *et al.*, 1991; Dunahay, 1993; Kim *et al.*, 2014; Kindle, 1990; Kumar *et al.*, 2004). Unlike biolistics or electroporation, methods using glass beads, silicon carbide whiskers or *Agrobacterium* do not involve any specialised equipment and can be done in any laboratory at low cost, although silicon carbide whiskers can be difficult to obtain and may potentially be a health hazard (León-Bañares *et al.*, 2004). The glass bead method remains popular, and has also been employed for chloroplast transformation as a simple alternative to biolistics (Economou *et al.*, 2014; Kindle *et al.*, 1991). For nuclear transformation by electroporation, cell wall-less strains (either mutants or cells treated with autolysin) are normally used (Brown *et al.*, 1991; Shimogawara *et al.*, 1998). However, *C. reinhardtii* with an intact cell wall was recently transformed using a series of multi-electroporation pulses (Yamano *et al.*, 2013). The cheaper *Agrobacterium*-mediated gene transfer technique can also be used with walled strains and has been reported to give high transformation frequency compared to glass bead transformation (Kumar *et al.*, 2004), and this can be increased further by inducing the *Agrobacterium* prior to infection using acetosyringone and glycine betaine (Pratheesh *et al.*, 2014).

Nuclear transformation using aminoclay nanoparticles (positively charged nanoparticles based on 3-aminopropyl-functionalized magnesium phyllosilicate) is still new, but it has also been reported to give high transformation rates, and again this simple method can be used with wild-type strains possessing a cell wall (Kim *et al.*, 2014). This method might have general applicability to other algal species that have traditionally been transformed using particle bombardment (Walker *et al.*, 2005) since the aminoclay nanoparticles (45 nm) are several orders of magnitude smaller than the gold particles (1 μm to 3 μm) used in the bombardment The size of particles is an important factor, because smaller particles increase the chance of cell wall penetration and genome integration (Kim *et al.*, 2014).

The nuclear genome of the diatom *P. tricornutum* is most commonly transformed via particle bombardment (Apt *et al.*, 1996; Falciatore *et al.*, 1999). Diatoms are generally quite difficult to transform due to challenges such as controlling and manipulating their life cycles, as well as penetrating their rigid cells walls (Falciatore *et al.*, 1999). Nonetheless, recently the *P. tricornutum* nuclear genome was successfully transformed by electroporation (Miyahara *et al.*, 2013; Zhang and Hu, 2014). More remarkable, given the four membranes that surround a diatom chloroplast, is a recent demonstration that electroporation can be used to deliver DNA into the chloroplast genome (Xie *et al.*, 2014).

Chloroplast genome engineering is particularly attractive given the ability to target transgenes into specific, predetermined loci via homologous recombination, and the high levels of expression that can be achieved. To date, chloroplast transformation by biolistics has been reported for five other algal species: *Dunaliella tertiolecta*, *Euglena gracilis*, *Haematococcus pluvialis*, *Porphyridium* sp. and *Tetraselmis cordiformis* (Cui *et al.*, 2014; Purton *et al*., 2013).

3.2. Selectable Markers, Reporter Genes and Promoters

Different types of selectable marker and reporter genes can be used in identifying putative transformants from the population of untransformed cells (**Table 2**). Endogenous selectable markers for nuclear transformation of the haploid *C. reinhardtii* allow the rescue of auxotrophic or non-photosynthetic mutants with examples for nuclear transformation being *ARG7*, *NIT1* and *OEE1* that allow rescue of recessive mutants defective in arginine biosynthesis, nitrate metabolism and photosynthesis, respectively (Stevens and Purton, 1997). A wide range of dominant markers are commonly used in *C. reinhardtii* and confer resistance to various antibiotics or herbicides, as detailed in **Table 2**. For chloroplast transformation, several dominant markers (e.g. *aadA* and *aphA6*) have been developed (**Table 2)**. In addition, endogenous chloroplast genes have been used as selectable markers for phototrophic rescue of the corresponding chloroplast mutant: for example the *atpB* gene rescues an *atpB* mutant defective in the ATP synthase. The use of phototrophic markers is not possible for nuclear or

chloroplast transformation of wild-type *P. tricornutum* since this diatom is an obligate phototroph. However, an engineered strain expressing a transgene encoding a glucose transporter has been described that is capable of heterotrophic growth on the sugar (Zaslavskaia *et al.* 2001), and this potentially could be used to develop non-phototrophic recipient strains. Reporter genes based on fluorescent proteins, luciferases or colorimetric enzymes have been developed for both the nucleus and chloroplast (**Table 2**), with the key to good expression of such transgenes being the use of synthetic genes that are codon-optimised for expression in the algal host (Fuhrmann *et al.*, 1999; Zaslavskaia *et al.*, 2000; Purton *et al.* 2013).

For transgene expression in the nucleus or the chloroplast, promoters and untranslated regions (UTRs) from highly expressed endogenous genes are typically used. For nuclear expression these include genes for subunits of the photosynthetic complexes, ribulose bisphosphate carboxylase or components of the light-harvesting apparatus (Walker et al. 2005), and for chloroplast expression, genes for core photosynthetic subunits such as *atpA*, *psbA*, *psaA* and *psbD* (Purton, 2007). However, such chloroplast genes are typically under feedback control via *trans*-acting factors that bind to the 5' untranslated region (UTR). Recently, (Specht and Mayfield, 2013) have shown that re-engineering these 5'UTRs can overcome such control and result in elevated levels of transgene expression.

With the advancement of microalgal biotechnology, many tools and techniques for the genetic manipulation of microalgae have been developed. However, nuclear transformation still faces problems such as poor transgene integration, codon bias, positional effects and gene silencing which can lead to poor or unstable transgene expression. Moreover, there are still limited molecular genetic tools for many of the microalgal species that have high commercial value. Chloroplast transformation is a promising strategy for precise engineering and high-level expression of transgenes, but its application is still largely limited to *C. reinhardtii*, and again there is a pressing need to develop reliable methods for commercial species. Hence, more work has to be carried out so that microalgae can be exploited as light-driven bioreactors in the future.

4. Production of Recombinant Proteins in Microalgae

Several previous studies have shown that microalgae are promising production platforms for recombinant proteins. Major successes and recent advances in recombinant protein production are summarised in the following.

Monoclonal antibodies are complex molecules used to treat several human diseases. To date most antibodies are produced in mammalian cells due to their ability to carry out post-translational modifications, particularly glycosylation and disulphide bond formation. Recently,

efforts have also been directed towards producing them in bacteria and yeast (Spadiut *et al.*, 2014). The first expression of a functional antibody in microalgae was achieved in the chloroplast of *C. reinhardtii*. A large single chain antibody directed against the herpes simplex virus (HSV) glycoprotein D was successfully expressed in this green alga. The genetic construct was integrated into the chloroplast genome via homologous recombination and expression was driven by *atpA* or *rbcL* promoters and 5' UTRs. The antibodies were shown to accumulate as soluble proteins in the chloroplast and could bind to HSV proteins in ELISA experiments. Heavy and light chains assembled by forming disulphide bonds, however, no other post-translational modifications were detected (Mayfield *et al.*, 2003). In another study, a fully functional monoclonal antibody against anthrax protective antigen 83 (PA83) was expressed in the *C. reinhardtii* chloroplast. Separate constructs for the heavy and light chain were introduced and the antibody was shown to assemble spontaneously by forming the necessary 16 disulphide bonds (Tran et al., 2009).

The production of antibodies in microalgae is, however, not restricted to *Chlamydomonas*. A monoclonal antibody was also successfully expressed in the endoplasmic reticulum (ER) of the diatom *P. tricornutum*. It was directed against the Hepatitis B virus surface protein and accumulated to 8.7 % of total soluble protein. Heavy and light chains, retained in the ER by DDEL retention peptides, were shown to assemble into complete antibodies and were glycosylated (Hempel *et al.*, 2011). When the same group omitted the ER retention signal from their constructs, functional antibodies were efficiently secreted and accumulated in the culture medium in an active form (Hempel and Maier, 2012).

Another group of valuable therapeutics are immunotoxins, which are most commonly used in the treatment of cancer. Immunotoxins are antibodies coupled to eukaryotic toxins and are difficult to produce in both prokaryotic and eukaryotic hosts. Tran *et al.* (2012) were able to show that fully functional immunotoxins can be expressed in algal chloroplasts. An antibody directed against the B-cell surface epitope CD22 was genetically linked to Exotoxin A from the human pathogenic bacterium *Pseudomonas aeruginosa* and expressed in the chloroplast of *C. reinhardtii*. It was shown that the immunotoxins were enzymatically active, bound specifically to CD22 on B-cells and inhibited cell proliferation *in vitro*. Tests in mouse xenograft models showed reduced tumour progression (Tran *et al.*, 2012). In another study the successful expression of an immunotoxin comprising of an antibody recognizing B-cell CD22 coupled to gelonine, a ribosome inhibiting protein, in the *C. reinhardtii* chloroplast was demonstrated. The immunotoxin was shown to inhibit the survival of two B-cell lymphoma lines (Ramos and CA-46) while leaving Jurkat T-cells intact, thereby proving their specificity (Tran *et al.*, 2013).

Several vaccine antigens have been produced in the chloroplast of *C. reinhardtii* (Demurtas et al., 2013; He et al., 2007; Sun et al., 2003). The GRAS status of the alga makes the production of antigens without costly purification from residual toxins or impurities attractive and could even be exploited for oral delivery of vaccines. An algal produced *Staphylococcus aureus* vaccine was shown to protect orally vaccinated mice against sub-lethal and lethal doses of *S. aureus* infection (Dreesen *et al.*, 2010). Malaria transmission blocking vaccines were also successfully expressed in the *C. reinhardtii* chloroplast (Gregory *et al.*, 2012; Gregory *et al.*, 2013; Jones *et al.*, 2013). In addition, Bayne *et al.* showed that it is possible to produce influenza haemagglutinin (HA) in the heterotrophic alga *Schizochytrium sp.* thereby presenting an alternative to current egg-based vaccine production platforms (Bayne *et al.*, 2013).

Other proteins expressed in microalgae include mammary-associated serum amyloid (M-SAA) which could be used as viral and bacterial prophylaxis in new born mammals (Manuell *et al.*, 2007); domains 10 and 14 of human fibronectin which have potential as antibody mimics; proinsulin and vascular endothelial growth factor, as well as the high mobility group protein B1 involved in wound healing (Rasala *et al.*, 2010). Furthermore, a human selenoprotein, Sep15 (Hou *et al.*, 2013) and antitoxins against botulinum neurotoxin have been successfully produced (Barrera *et al.*, 2014).

Further efforts to develop microalgae as a production platform have been made. Other than the therapeutic proteins mentioned, several enzymes have been successfully produced in *C. reinhardtii*. The industrially relevant Xylanase 1 was expressed in the nucleus of *C. reinhardtii* both in a soluble and secreted form (Rasala *et al.*, 2012). The first membrane-bound enzyme expressed in the *C. reinhardtii* chloroplast was CYP79A1 from *Sorghum bicolor*. It was shown to be active and produced p-hydroxyphenylacetaldoxime, the precursor for a plant natural product, from endogenous tyrosine (Gangl *et al*., 2014). In another study, a bifunctional diterpene synthase was expressed in the chloroplast of *C. reinhardtii*, and is the largest recombinant protein expressed to date. This synthase is of interest for exploring the potential of terpenoid production in microalgae (Zedler *et al.*, 2014; see section 5 for details).

Despite the huge variety of recombinants successfully produced in algae to date, only one report exists of transferring recombinant protein production to a larger scale. A milk Amyloid A-producing strain of *C. reinhardtii* was grown in three 100 L bags in a greenhouse setting and the maximum rate of MAA production achieved was 0.051 ± 0.016 g/L/day (Gimpel *et al.*, 2014). The lack of other examples shows that the knowledge transfer from the lab scale to industrially relevant growth conditions for recombinant protein production in microalgae is still not established.

5. Metabolic engineering of microalgae to produce other high value compounds

Microalgae offer additional potential as light-driven cell factories for the production of novel metabolites. Plant secondary products are one of the most important forms of target compound. Plants are a source of a broad spectrum of diverse bioactive compounds, also known as secondary or specialised metabolites. Often their biosynthesis is restricted to specific developmental stages, tissues or even cells and they can be involved in many processes important for plant growth and survival, such as protection from pathogens or herbivores, attraction of pollinators and adaptation to environmental stress. A large number of specialised metabolites are now used by the pharmaceutical, chemical and food industries, and approximately 50% of all approved medicines including anticancer drugs are of natural origin (Dai *et al.*, 2014; Lassen *et al.*, 2014a).

Many of these plant-derived compounds can be produced by chemical synthesis, but in some cases due to complexity of their structure they require difficult multistep regio- and stereo-specific reactions, therefore overall yields can be very low. This applies particularly to the synthesis of terpenoids – a particularly large, complex and important family of plant secondary metabolites. One solution to this problem can be extraction from the plant source, but if a product is accumulated in scarce amounts, the result may not be economically viable. An example is paclitaxel, a cytostatic drug known under the commercial name Taxol. Its accumulation in *Taxus baccata* depends on the maturity of the tree and ranges from 0.064 g (27 year old tree) to 8.038 g (136 year old tree) per tree (Nadeem *et al.*, 2002), whereas chemical synthesis is complicated and requires 40 different steps (Nicolaou *et al.*, 1994).

5.1 Towards engineering metabolic pathways

An alternative method for production of high-value chemicals is to use synthetic biology or metabolic engineering tools. Both terms are often used interchangeably, however there are significant differences between the two fields. Synthetic biology is more about designing and using defined synthetic DNA parts (biobricks), constructing genetic circuits and molecular switches to control the expression and metabolism, whereas metabolic engineering is a broader term involving protein engineering and pathway optimisation in order to improve production yields of desired product (Stephanopoulos, 2012). Nonetheless, both are understood as transferring biosynthetic pathways from one organism to another (or combining pathways from different organisms).

In order to produce some specialized high-value compounds such as terpenoids, it is often necessary to perform highly specific and complex enzymatic reactions, which can be catalysed by cytochromes P450 (P450s). For example, biosynthesis of both artemisinin and paclitaxel

requires the involvement of P450s. Unfortunately the expression of plant P450s in *E. coli* and *S. cerevisiae* is not always simple due to special requirements for post-translational modification and protein localisation, and in consequence plant P450s are often inactive upon expression in these hosts (Chemler and Koffas, 2008).

P450s are monooxygenases performing stereospecific hydroxylations and they are anchored in the plant endoplasmic reticulum (ER). In order to function they need to be powered by single electron transfers from NADPH-dependent cytochrome P450 reductase (CPR). The expression level of these proteins is relatively low and the activity is often limited by NADPH and substrate pool (Jensen *et al.*, 2011). It has been demonstrated that these energy-consuming reactions can be bypassed by relocating P450-dependent metabolic pathways to the thylakoid membranes of tobacco chloroplasts, where the reducing power generated by photosystem I (PSI) in the form of reduced ferredoxin (Fd) is a cheap and essentially unlimited source of electrons for the P450s (Nielsen *et al.*, 2013) (Fig. 1). More recently, it has been shown that a P450 can be expressed in the chloroplasts of transgenic *C. reinhardtii* and it was furthermore shown that the enzyme was targeted into the chloroplast membranes and highly active (Gangl *et al.*, 2014). This strongly suggests that it will be possible to produce pharmacologically important terpenoids not only in plant chloroplasts (Bock and Warzecha, 2010), but also in microalgal chloroplasts.

5.2. Cyanobacteria as a tool for synthetic biology

During the last decade, cyanobacteria have been gaining renewed interest as a chassis for metabolic engineering and synthetic biology approaches. Currently many efforts are being undertaken to use genetically modified cyanobacteria as a production platform for biofuels such as isoprene (Zhou and Li, 2010), bioplastics like polyhydroxybutyrate (Wang *et al.*, 2013) and polylactic acid (Angermayr *et al.*, 2014), fatty acids (Ruffing, 2014), ethylene (Guerrero *et al.*, 2012; Ungerer *et al*, 2012) and sugars (Jacobsen and Frigaard, 2014). In many cases reported yields are not yet satisfactory, however, the ability to perform photosynthesis and the idea of converting sunlight and carbon dioxide into high value chemicals are undoubtedly attractive.

As indicated above, expression of foreign pathways involving P450s in *E. coli* often causes problems and is ineffective, due to difficulties with proper folding, posttranslational modifications and targeting to the membranes. Unlike most native bacterial P450 enzymes that are soluble, cyanobacterial P450s are membrane bound, exactly like plant P450s (Robert *et al.*, 2010). All the above considerations indicate that cyanobacteria and eukaryotic microalgae can be suitable candidates for expression of foreign P450s.

Expression of p-coumarate-3-hydroxylase from *Arabidopsis thaliana* in the cyanobacterium *Synechococcus* sp. PCC 6803 for the production of caffeic acid was the first reported successful expression of a plant cytochrome P450 in cyanobacteria (Xue *et al.*, 2014). Further genetic strain modifications resulted in a 25-fold increase of production rate of *p*-coumaric acid (a precursor for caffeic acid) giving yields of 82.6 mg/L secreted to the media (Xue *et al.*, 2014). Another example of stable expression of a P450 enzyme (CYP79A1 from the dhurrin pathway) in *Synechococcus* sp. PCC 7002, and direction of the protein to the thylakoid membranes by fusing the protein with the PsaM subunit of PSI has recently been reported (Lassen *et al.*, 2014). Successful heterologous expression of the mevalonic acid (MVA) pathway in *Synechocystis* sp. PCC 6803 (Bentley *et al.*, 2014) and production of *β*-phellandrene (Bentley *et al.*, 2013) also opens up the opportunity to express terpenoid biosynthetic pathways in cyanobacteria. Moreover, excretion of produced metabolites into the growth media (Lassen *et al.*, 2014; Xue *et al.*, 2014) suggests that cyanobacteria have an unexplored potential as good platforms for metabolic engineering. Overall, it is clear that these organisms have potential as production hosts but it is fair to say that improvements are required before they can attain mainstream status as production chassis.

6. Techniques to engineer algal communities

In their natural environment, algae live intimately with many other organisms. These communities exhibit complex interactions including metabolite exchange, cell aggregation and biofilm formation (Jagmann and Philipp, 2014). For example, at least half of all algal species require an exogenous supply of vitamin B_{12}, and bacteria can be a source for this co-factor in return for a carbon source, thus forming a tightly regulated symbiosis (Croft *et al.*, 2005; Kazamia *et al.*, 2012). Further examples include the symbiosis between a unicellular prymnesiophyte alga and a cyanobacterium (UCYN-A) with the former providing a carbon source in return for fixed nitrogen (Thompson *et al.*, 2012), and the dynamic symbiosis between the haptophyte alga *Emiliania huxleyi* and the bacterium *Phaeobacter gallaeciensis* BS107, which can be mutualistic or parasitic according to environmental conditions (Seyedsayamdost *et al.*, 2011).

In contrast, algal biotechnology has traditionally involved large-scale cultivation of axenic cultures (Day *et al.*, 2012; Scott *et al.*, 2010). This difference has been widely recognised and consequently there are many reviews outlining the potential benefits of using consortia in algal biotechnology. The main advantages proposed include an increase in algal productivity (accrued biomass) of mixed cultures compared to axenic cultures, enhanced crop protection from pathogens and pests due to the competitive exclusion principle, reduction of energy inputs, increased stability of the crop to include population control and resilience to environment

perturbations, and finally the division of labour between consortium members leading to increased ability to engineer complex and broader metabolic pathways (Table 3) (Jagmann and Philipp, 2014; Kazamia *et al.*, 2014; Nalley *et al.*, 2014; Ortiz-Marquez *et al.*, 2013; Pandhal and Noirel, 2014; Smith and Crews, 2014).

Although the proposed benefits of creating algal communities are great, engineering the communities in practice is likely to be complex. We therefore focus on examples where engineering of efficient algal communities has been shown to be effective.

6.1. Genetic engineering for symbiosis

Genetic manipulation can be used to engineer organisms to interact in a clearly defined way by the trading of metabolites. Examples of this include engineering the nitrogen-fixing bacterium *Azotobacter vinelandii* to secrete fixed nitrogen into the growth media, replacing the need for addition of synthetic nitrogen inputs for algal growth (Ortiz-Marquez *et al.*, 2014; Ortiz-Marquez *et al.*, 2012), and the engineering of the cyanobacterium *Synechocystis sp.* PCC 6803 to secrete a carbon source for *Escherichia coli* which it can utilise to produce low- to high-value compounds (Niederholtmeyer *et al.*, 2010). The benefits of using one organism to provide nutrients for another include the potential to lower production costs (nutrient inputs and energy consumption) and reduce the carbon footprint of the process. However, creating these communities may require considerable investment of time and resources, limiting the size to a small number of interactions therefore limiting the benefit of the competitive exclusion principle (Table 3). Given the limited ability to exclude contaminants, this strategy, if applied on its own, would be best suited for contained cultures (with low risk of contamination) for production of high-value products in contrast to cultures exposed to the environment (for low-value product) where contaminants will easily occupy niches and even take advantage of the engineered community.

6.2. Screening symbioses

Screening of naturally developing populations can offer a simpler method of identifying mutually beneficial symbioses from a large number of organisms. For example, Do Nascimento *et al.* showed an increase of up to 30% in chlorophyll, biomass and lipid accumulation in the oleaginous microalga *Ankistrodesmus* sp. strain SP2-15 when co-cultured with the bacterium *Rhizobium* strain 10II (Do Nascimento *et al.*, 2013). The positive interaction was identified by subculturing non-axenic algal cultures (absent of organic carbon) for three years, which was followed by the isolation of bacterial strains able to utilise carbon exudates from the algal strains. The bacteria were then screened for induction of positive growth effects on various algal strains via growth curves. Le Chevanton *et al.* used a similar approach where bacteria were isolated from algal cultures (Le Chevanton *et al.*, 2013). However, the cultures were initially

screened with a high-throughput optical technique to identify interactions that gave the highest chlorophyll *a* fluorescence. Le Chevanton *et al.* thus identified two bacterial strains enhancing biomass accumulation and nitrogen provision for *Dunaliella* sp. The final example given here highlights the importance of screening a diverse range of organisms for symbiosis. Lorincz *et al.* serendipitously created an artificial tripartite consortium based on the known bipartite symbiosis involving *Chlamydomonas reinhardtii* (alga) trading carbon to *Azotobacter vinelandii* (bacterium) in exchange for nitrogen. *Alternaria infectoria* (fungus) had spontaneously contaminated the co-culture, which led to a positive growth effect by providing amino acids (in particular cystathionine) to the consortium (Lorincz *et al.*, 2010). Analysis of naturally developing populations is therefore advantageous in its potential for screening of a large range of interactions. However, significant investment in time, materials and manpower may be required for success.

6.3. Environmental selection for symbiosis

A novel approach to form communities is through manipulation of the environmental conditions to force organisms to form symbioses. For example Hom and Murray (2014) created an obligate mutualism between the yeast *Saccharomyces cerevisiae* and *C. reinhardtii* by testing a range of different conditions to identify those that allowed both organisms to grow. Under the successful conditions, *S. cerevisiae* provided carbon dioxide by metabolising glucose and in return *C. reinhardtii* provided ammonia by metabolising nitrite. The authors reproduced this mutualism with many additional algal and yeast species, which gives a sense of optimism about applying this technique to a range of other organisms (Hom and Murray, 2014). Similarly Ortiz-Marquez and colleagues created an artificial symbiosis between a strain of *Azotobacter vinelandii* engineered to secrete fixed nitrogen (ammonium) and the alga *Chlorella sorokiniana* by co-culturing the species in nitrogen- and carbon-deficient media (Ortiz-Marquez *et al.*, 2012). An important similarity between these two examples was the requirement of a solid support for the symbiosis to occur (i.e. a lack of agitation or shaking), outlining the importance of cell-cell proximity and spatial structure. It should be noted that this approach is new and only a few positive examples have been reported, so it remains to be seen how widely the approach can be applied. It also remains to be seen how robust symbioses generated in this way will be.

6.4. Trait-based engineering

Trait-based engineering is the creation of communities by organisms with unique but complementary growth requirements. This is based on the principle of resource-use complementarity, which allows, in principle, individuals to cohabit and lead to an increase in productivity. Examples of trait-based complementarity include the co-culturing of algal species with varying accessory light harvesting pigments maximising the utility of the visible light spectrum (Kazamia *et al.*, 2014; Nalley *et al.*, 2014). Trait-based engineering could be applied

559 to make use of the varying temperatures that occur during a 24-hour cycle as well as seasonal
560 fluctuations, by co-culturing organisms that optimally grow at a range of temperatures, such as
561 *Detonula confervacea* with a temperature optimum of 11^0C to *Chlorella pyrenoidosa*, with a
562 temperature optimum of 40^0C (Eppley, 1985; Myers, 1984). Another feature that could be
563 considered in this context is the introduction of species that control organisms such as
564 zooplankton, which are known for their predation on algal species. Kazamia *et al.* and Nalley *et*
565 *al.* outlined the use of fish as a means to control zooplankton (Kazamia *et al.*, 2014; Nalley *et*
566 *al.*, 2014). Trait-based engineering has particular benefits for open-raceway production, where
567 algal species are exposed to fluctuating environmental conditions. However, this approach will
568 require a thorough understanding of the surrounding environment of the raceway pond to be
569 able to handpick organisms for optimal community growth.
570
571 ***6.5. Directed evolution***
572 Another approach is to take advantage of artificial selection as a means of creating efficient co-
573 cultures. Selecting for the ability of organisms to work together over many generations may be
574 an efficient and productive means of achieving certain traits from mixed communities. This
575 approach has been applied to fermentation, anaerobic digestion, bioremediation, and the
576 production of polyhydroxyalkanoate and polyphosphates (Johnson *et al.*, 2009; Sabra *et al.*,
577 2010; Serafim *et al.*, 2008; Zeng *et al.*, 2003). Mooij *et al*. (2013) used this method to select for
578 carbon storage molecules in the form of starch and lipids in algae. Algal inocula were taken
579 from several different surface waters and cultured in a carbon dioxide-rich light period (nitrogen
580 absent) and then a nitrogen-rich dark period. Cycling between these two conditions over many
581 generations selected for organisms able to produce energy storage compounds (starch/lipids) to
582 power nitrogen assimilation in the dark periods (Mooij *et al.*, 2013). This particular example,
583 due to the controlled environmental conditions (nitrogen cycling), may be best utilised with
584 photobioreactors. However, directed evolution could be applied for production of algae in open
585 raceway ponds for the selection of resistance to extreme conditions such as high pH or salt
586 concentrations as applied to the production of *Arthrospira* sp. and *Dunaliella salina*
587 respectively.
588
589 In summary, metabolic engineering has the advantages of precisely engineering metabolite
590 trading as well as the ability to fine tune population dynamics (Kerner *et al.*, 2012; You *et al.*,
591 2004). However, it is labour intensive as a way of creating symbiotic interactions and obtaining
592 regulatory approval for genetically modified organisms may prove a barrier. The metabolic
593 engineering approach is further limited to organisms whose genomes are readily manipulated.
594 Screening and environmental selection for symbiosis has the advantage of using a potentially
595 large range of organisms to form symbioses, and trait-based engineering and directed evolution
596 approaches offer the potential to create highly complex communities with improved

productivity. However, these techniques are in their infancy and further evidence of their efficacy is required.

Algal community research has made great progress in recent years and a number of additional technologies will be utilised alongside the engineering approaches discussed above. Some of these emerging technologies include the monitoring of algal populations through the use of qPCR; crop protection using traditional chemicals such as pesticides and herbicides (McBride *et al.*, 2014); the highly parallel screening of beneficial and novel microbial symbiotic interactions through the use of microdroplet technology (Park *et al.*, 2011); development of online databanks or libraries detailing functional traits of bacteria and algae so communities can be designed for optimum trait functionality (Guiry *et al.*, 2014); and accurate modelling of communities to help predict environments that induce and stabilise microbial interactions (Grant *et al.*, 2014; Kim *et al.*, 2008; Klitgord and Segre, 2010).

7. Downstream processing of microalgal products

As described above, microalgae are light-driven cell factories that can produce a wide spectrum of natural products, or which can be engineered to produce diverse high-value compounds. Extraction and purification of these products are critical processes that can contribute to up to 60% of the total production cost (Molina Grima *et al.*, 2003). Research to date has focused on developing methods for downstream processing of algal biomass and for oil extraction and biofuel production. Many have reviewed downstream processing for biofuel production from different algae including *Chlorella*, *Dunaliella*, *Nannochloris*, *Nannochloropsis*, *Porphyridium*, *Schizochytrium* and *Tetraselmis* (Ahmad *et al.*, 2014; Chen *et al.*, 2011; Halim *et al.*, 2012; Kim *et al.*, 2013; Lee *et al.*, 2012; Mata *et al.*, 2010; Pragya *et al.*, 2013; Rawat *et al.*, 2013; Rios *et al.*, 2013). In this section, we review the common steps for processing microalgae in order to prepare biofuels, recombinant proteins and other high-value products.

7.1 Downstream processing methods for biofuels production

Typically, downstream processing of microalgae used for the production of biodiesel consists of harvesting in a two-step operation in order to separate the biomass from the culture media using solid-liquid separation technologies. The first step is bulk harvesting where the biomass is concentrated by flocculation, flotation or gravity sedimentation reaching up to 7% total solids; the second step is thickening where the biomass slurry is concentrated into a paste by more energy-intensive processes like centrifugation or filtration (Brennan and Owende, 2010). Such technologies are chosen according to the characteristics of the microalga species and the nature and quality of the final product (Amaro *et al.*, 2011), however, they are limited in their abilities

to separate biomass from the media and the operating cost that can fluctuate between 20 and 30% of the total production cost (Gudin and Therpenier, 1986).

From this point, the harvested biomass paste can undergo two different pre-treatments depending on the final product requirements, the so-called dry and wet route. The dry route, which involves technologies such as spray drying, drum drying, freeze-drying or sun drying, is the preferred method to obtain dry biomass as it offers high extraction yields, albeit with high costs and energy use (e.g. harvesting and drying combined with extraction cost is 50% of the total production cost (Pragya *et al.*, 2013)).

An alternative to the dry route is the wet route, in which wet biomass needs to be disrupted first to release the intracellular products. Cell wall composition plays an important role in this route; cell walls from microalgae are typically a thick and rigid matrix of polysaccharides and glycoproteins that require costly downstream processing steps during the production of bio-products (Kim *et al.*, 2013). For example, *Haematococcus pluvialis* possesses a thick cell wall that makes this alga highly resistant to chemical and physical disruption, thus significantly increasing astaxanthin production costs (Hagen *et al.*, 2002). The unit operations for the wet route can be: mechanical (ultra-sonication, high pressure homogenisation, microwave, bead beating, and electroporation); chemical (acids, alkalis and organic solvents); biological (enzymes), or osmotic shock. Selection of methods depends again on the biomass characteristics. Assessment of the dry and wet routes have demonstrated that both have a positive energy balance for production of biofuels. This evidence also shows that the wet route has more potential for high valuable biofuels whereas the dry route seems more attractive for short term biofuel productions (Xu *et al.*, 2011). Whether the biomass goes into the dry or wet route, the physical state of the output is a dried biomass or disrupted concentrate; this stream is processed for lipid extraction. Different methods are available including: (i). Organic solvents: algal oil can be recovered by using solvent such as chloroform, methanol, benzene, diethyl ether and n-hexane. Other metabolites such as β-carotene and astaxanthin are also extracted by solvents (Molina Grima *et al.*, 2003). However, solvent extraction can result in high toxicity if the product is used for animal or human consumption. (ii). Supercritical fluid extraction: this is a green technology based on CO_2 at supercritical conditions used as a non-toxic extracting solvent in order to separate the lipids from matrix (dried biomass or disrupted concentrate). It has been applied to extract lipids and other high valuable compounds from algae, however, the operational cost is high (Mendes *et al.*, 1995; Thana *et al.*, 2008).

7.2. Strategies to facilitate downstream processing of recombinant protein produced in microalgae

The potential of *C. reinhardtii* to produce recombinant proteins for industrial, nutritional, and medical uses has been discussed above, but it is notable that hardly any reports have described large scale production or recovery of target proteins. Moreover, downstream processing of engineered microalgae is largely based on laboratory scale techniques. More than 30 proteins with biotechnological applications have been expressed in *Chlamydomonas* (Rasala and Mayfield, 2014) including a number that have been expressed in the chloroplast. Although the chloroplast has been shown to be a robust platform for commercial production of proteins, it has some limitations as chloroplast-expressed proteins cannot be secreted, thus, cells must be processed after cultivation to recover and purify the product. Regardless of the nature of the bio-product produced in the microalgae, downstream processing follows the same route as in biofuels; therefore the operations to recover high value products that require high purity (recombinant proteins, pigments, carotenoids, PUFAs, terpenoids, etc.) include harvesting, cell disruption (if the compound is intracellular) and purification. Figure 2 illustrates the recovery and purification process of recombinant proteins produced in microalgae.

Since recombinant protein production in *Chlamydomonas* is largely limited to small-scale cultivation, the current harvesting is mainly done by conventional centrifugation. Harvested biomass can be processed using the approach of dry and wet routes. For the dry route, the drying biomass has potential in the production of edible vaccines and enzymes; research suggests that dry algal extract is an ideal vehicle for oral delivery of vaccines and enzymes as the cell wall protects the antigens and enzymes (Yoon *et al.*, 2011). Production of vaccines that can be administrated orally is benefited by the effectiveness of freeze-drying to preserve antigen-expressing *Chlamydomonas*; some vaccine-producing strains have been reported to be stable for up to 20 months; therefore the cost is reduced by eliminating extraction and purification steps (Specht and Mayfield, 2014). Algal-based recombinant vaccines administered orally as dry biomass have demonstrated effectiveness in two studies, namely protection against *Staphylococcus aureus* infection (Dreesen *et al.*, 2010) and a potential malaria vaccine (Gregory *et al.*, 2013).

The wet route for extraction offers the possibility of recovery of recombinant proteins with high purity. However, cell disruption techniques can be limited since the proteins of interest can be compromised during harsh conditions. Cell lysis can be accomplished by combining mechanical, physical and chemical methods. The most common methods are freezing, sonication and lysis with a buffer, and any combination of them will result in a disrupted concentrate that must go through more purification steps. Cell disruption methods adopted by the community vary considerably, some common approaches are: freezing followed by lysis buffer (Gregory *et al.*, 2013), and lysis buffer coupled with sonication (Demurtas *et al.*, 2013; Rasala *et al.*, 2012).

There is an alternative third route in the process, termed the "supernatant route". This route is only possible when nuclear-expressed proteins are coupled with a signal peptide in order to be secreted into the culture media and thereby facilitate product recovery (Table 4). After cultivation, biomass is discarded as the recombinant proteins are present in the media. The proteins are then concentrated by filtration, lyophilisation or precipitation and further purified. Lauersen et al. (2015) optimized culture conditions and parameters of an engineered *Chlamydomonas* strain able to secrete an ice-binding protein with potential use in the food industry; they found that photomixotrophic cultivations led to accumulation of ~10 mg L^{-1} of this protein in a small scale. Larger scale experiments (10 L) conducted in plastic bags under the same conditions resulted in ~12 mg L^{-1} of recombinant protein accumulated in the medium. Compared with other microbial systems where secretion of recombinants proteins into the culture media is well established, microalgae still needs further develop and optimization in order to have comparable secretion yields.

Thus, although microalgae appear to be an attractive platform for biomanufacturing of high value proteins for industrial, nutritional and medical uses, downstream operations have technical challenges and such processes are still limited to small-scale proof of concept studies. On the other hand, the high cost of downstream processing of high value products from transgenic algae can be avoided in applications where no product purification is required, thus resulting in less expensive processing. The next step is to explore higher scale production levels in order to make the platforms competitive with other expression platforms. The first (and so far only) example of large-scale production of an algal-expressed therapeutic protein was MAA in a volume of 100 L in a greenhouse (Gimpel *et al.*, 2014).

8. Conclusions

In this review we have highlighted the biotechnological potential of microalgae from several different angles. Microalgae are a valuable source of natural products, including carotenoids, antioxidants and pigments, in addition to being used as feed stock or for the production of biodiesel. Advances in genetic engineering also mean that some species can be transformed and used as cell factories for other high-value products, including recombinant proteins. Several vaccine antigens, antibodies and some enzymes have now been produced in the model alga *C. reinhardtii*. In addition, efforts have been directed towards metabolic engineering in algae and cyanobacteria. On a different note, we have also summarised the current research into engineering algal communities and the potential benefit of co-culturing bacteria and algae to increase productivity, reduce energy inputs and protect cultures from pathogens. Considering these aspects, the great potential of microalgae in biotechnology becomes evident. However,

large-scale production remains a challenge and microalgae still struggle to compete with existing platforms. It remains to be seen whether microalgae will be used for a wide range of industrial applications or only for more specialised applications, where there are severe shortcomings in competing platforms. The niche for microalgae still needs to be developed. The GRAS status of many microalgae, their inexpensive culturing and potential for large-scale growth in bioreactors are definitely distinctive advantages of these photosynthetic microorganisms and upcoming years will reveal where the industry is headed.

9. References

Ahmad AL, Yasin NHM, Derek CJC Lim JK. 2014. Comparison of harvesting methods for microalgae *Chlorella* sp. and its potential use as a biodiesel feedstock. Environmental Technology **35**, 2244-2253.

Akashi Y, Okamoto I, Suzuki M, Tamura K, Iwasa T, Hisada S, Satoh T, Nakagawa K, Ono K, Fukuoka M. 2007. The novel microtubule-interfering agent TZT-1027 enhances the anticancer effect of radiation in vitro and in vivo. British Journal of Cancer **96**, 1532-1539.

Almaraz-Delgado AL, Flores-Uribe J, Pérez-España VH, Salgado-Manjarrez E, Badillo-Corona JA. 2014. Production of therapeutic proteins in the chloroplast of *Chlamydomonas reinhardtii*. AMB Express **4**, 57.

Amaro HM, Guedes AC, Malcata FX. 2011. Advances and perspectives in using microalgae to produce biodiesel. Applied Energy **88**, 3402-3410.

Ambati RR, Phang SM, Ravi S, Aswathanarayana RG. 2014. Astaxanthin: sources, extraction, stability, biological activities and its commercial applications – a review. Marine drugs **12**, 128-152.

Angermayr SA, Van der Woude AD, Correddu D, Vreugdenhil A, Verrone V, Hellingwerf KJ. 2014. Exploring metabolic engineering design principles for the photosynthetic production of lactic acid by *Synechocystis* sp. PCC6803. Biotechnology for Biofuels **7**, 99.

Apt KE, Kroth-Pancic PG, Grossman AR. 1996. Stable nuclear transformation of the diatom *Phaeodactylum tricornutum*. Molecular and General Genetics **252**, 572-579.

Aráoz R, Molgó J, Tandeau de Marsac N. 2010. Neurotoxic cyanobacterial toxins. Toxicon **56**, 813-828.

Barrera DJ, Rosenberg JN, Chiu JG, Chang Y-N, Debatis M, Ngoi S-M, Chang JT, Shoemaker CB, Oyler GA, Mayfield SP. 2014. Algal chloroplast produced camelid V_HH antitoxins are capable of neutralizing botulinum neurotoxin. Plant Biotechnology Journal **13**, 117-124.

Bayne A-CV, Boltz D, Owen C, Betz Y, Maia G, Azadi P, Archer-Hartmann S, Zirkle R, Lippmeier JC. 2013. Vaccination against influenza with recombinant hemagglutinin expressed by *Schizochytrium* sp. confers protective immunity. PLoS ONE **8**, e61790.

Bentley FK, García-Cerdán JG, Chen H-C, Melis A. 2013. Paradigm of Monoterpene (*β*-phellandrene) Hydrocarbons Production via Photosynthesis in Cyanobacteria. BioEnergy Research **6**, 917-929.

Bentley FK, Zurbriggen A, Melis A. 2014. Heterologous expression of the mevalonic acid pathway in cyanobacteria enhances endogenous carbon partitioning to isoprene. Molecular Plant **7**, 71-86.

Berthold P, Schmitt R, Mages W. 2002. An engineered *Streptomyces hygroscopicus aph 7*" gene mediates dominant resistance against Hygromycin B in *Chlamydomonas reinhardtii*. Protist **153**, 401-412.

Bock R, Warzecha H. 2010. Solar-powered factories for new vaccines and antibiotics. Trends in Biotechnology **28**, 246-252.

Borowitzka MA. 2013. High-value products from microalgae – their development and commercialisation. Journal of Applied Phycology **25**, 743-756.

Boynton JE, Gillham NW, Harris EH, *et al.* 1988. Chloroplast transformation in *Chlamydomonas* with high velocity microprojectiles. Science **240**, 1534-1538.

Brennan L, Owende P. 2010. Biofuels from microalgae - A review of technologies for production, processing, and extractions of biofuels and co-products. Renewable and Sustainable Energy Reviews **14**, 557-577.

Brown LE, Sprecher SL, Keller LR. 1991. Introduction of Exogenous DNA into *Chlamydomonas reinhardtii* by Electroporation. Molecular and Cellular Biology **11**, 2328-2332.

Chang M, Li F, Odom OW, Lee J, Herrin DL. 2003. A cosmid vector containing a dominant selectable marker for cloning *Chlamydomonas* genes by complementation. Plasmid **49**, 75–78.

Chemler JA, Koffas MA. 2008. Metabolic engineering for plant natural product biosynthesis in microbes. Current Opinion in Biotechnology **19**, 597-605.

Chen C-Y, Yeh K-L, Aisyah R, Lee D-J, Chang J-S. 2011. Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: A critical review. Bioresource Technology **102**, 71-81.

Chiaiese P, Palomba F, Tatino F, Lanzillo C, Pinto G, Pollio A, Filippone E. 2011. Engineered tobacco and microalgae secreting the fungal laccase POXA1b reduce phenol content in olive oil mill wastewater. Enzyme and Microbial Technology **49**, 540-546.

Croft MT, Lawrence AD, Raux-Deery E, Warren MJ, Smith AG. 2005. Algae acquire vitamin B-12 through a symbiotic relationship with bacteria. Nature **438**, 90-93.

Cui YL, Qin, Jiang, P. 2014. Chloroplast transformation of *Platymonas* (*Tetraselmis*) *subcordiformis* with the *bar* gene as selectable marker. PLoS ONE **9**, e98607.

Cullen M, Ray N, Husain S, Nugent J, Nield J, Purton S. 2007. A highly active histidine-tagged *Chlamydomonas reinhardtii* Photosystem II preparation for structural and biophysical analysis. Photochemical and Photobiological Sciences **6**, 1177–1183.

Dai Z, Liu Y, Guo J, Huang L, Zhang X. 2014. Yeast synthetic biology for high-value metabolites. FEMS Yeast Research **15**, 1-11.

Davies JP, Weeks DP, Grossman AR. 1992. Expression of the arylsulfatase gene from the β_2-tubulin promoter in *Chlamydomonas reinhardtii*. Nucleic Acids Research 20, 2959–2965.

Day JG, Slocombe SP, Stanley MS. 2012. Overcoming biological constraints to enable the exploitation of microalgae for biofuels. Bioresource Technology **109**, 245-251.

Debuchy R, Purton S, Rochaix JD. 1989. The argininosuccinate lyase gene of *Chlamydomonas reinhardtii*: an important tool for nuclear transformation and for correlating the genetic and molecular maps of the ARG7 locus. The EMBO Journal **8**, 2803-2809.

Demurtas OC, Massa S, Ferrante P, Venuti A, Franconi R, Giuliano G. 2013. A Chlamydomonas-derived Human Papillomavirus 16 E7 vaccine induces specific tumor protection. PLoS ONE **8**, e61473.

Deng C, Pan B, O'Connor OA. 2013. Brentuximab Vedotin. Clinical Cancer Research **19**, 22-27.

Dixit RB, Suseela MR. 2013. Cyanobacteria: potential candidates for drug discovery. Antonie van Leeuwenhoek **103**, 947-961.

Do Nascimento M, De los Angeles Dublan M, Ortiz-Marquez JCF, Curatti L. 2013. High lipid productivity of an *Ankistrodesmus–Rhizobium* artificial consortium. Bioresource Technology **146**, 400-407.

Dreesen IAJ, Charpin-El Hamri G, Fussenegger M. 2010. Heat-stable oral alga-based vaccine protects mice from *Staphylococcus aureus* infection. Journal of Biotechnology **145**, 273-280.

Dunahay TG. 1993. Transformation of *Chlamydomonas reinhardtii* with silicon carbide whiskers. BioTechniques **15**, 452-460.

Economou C, Wannathong T, Szaub J Purton S. 2014. A simple, low-cost method for chloroplast transformation of the green alga *Chlamydomonas reinhardtii*. Methods in Molecular Biology **1132**, 401-411.

Eichler-Stahlberg A, Weisheit W, Ruecker O, Heitzer M. 2009. Strategies to facilitate transgene expression in *Chlamydomonas reinhardtii*. Planta **229**, 873-883.

Eppley RW. 1985. Citation Classic - Temperature and Phytoplankton Growth in the Sea. Current Contents/Agricluture, Biology & Environmental Sciences **37**, 20-20.

Falciatore A, Casotti R, Leblanc C, Abrescia C, Bowler C. 1999. Transformation of Nonselectable Reporter Genes in Marine Diatoms. Marine Biotechnology **1**, 239-251.

Fuhrmann M, Hausherr A, Ferbitz L, Schödl T, Heitzer M, Hegemann P. 2004. Monitoring dynamic expression of nuclear genes in *Chlamydomonas reinhardtii* by using a synthetic luciferase reporter gene. Plant Molecular Biology **55**, 869-881.

Fuhrmann M, Oertel W, Hegemann P. 1999. A synthetic gene coding for the green fluorescent protein (GFP) is a versatile reporter in *Chlamydomonas reinhardtii*. Plant Journal **19**, 353-361.

Gangl D, Zedler JAZ, Włodarczyk A, Jensen PE, Purton S, Robinson C. 2015. Expression and membrane-targeting of an active plant cytochrome P450 in the chloroplast of the green alga *Chlamydomonas reinhardtii*. Phytochemistry **110**, 22-28.

Gimpel JA, Hyun JS, Schoepp NG, Mayfield SP. 2014. Production of recombinant proteins in microalgae at pilot greenhouse scale. Biotechnology and Bioengineering **112**, 339-345.

Goldschmidt-Clermont M. 1991. Transgenic expression of aminoglycoside adenine transferase in the chloroplast: a selectable marker of site-directed transformation of *Chlamydomonas*. Nucleic Acids Research **19**, 4083–4089.

Gong YM, Hu HH, Gao Y, Xu XD, Gao H. 2011. Microalgae as platforms for production of recombinant proteins and valuable compounds: progress and prospects. Journal of Industrial Microbiology & Biotechnology **38**, 1879-1890.

Grant MAA, Kazamia E, Cicuta P, Smith AG. 2014. Direct exchange of vitamin B-12 is demonstrated by modelling the growth dynamics of algal-bacterial cocultures. The ISME Journal **8**, 1418-1427.

Greenwell HC, Laurens LM, Shields RJ, Lovitt RW Flynn KJ. 2010. Placing microalgae on the biofuels priority list: a review of the technological challenges. Journal of the Royal Society Interface **7**, 703-726.

Gregory JA, Li FW, Tomosada LM, Cox CJ, Topol AB, Vinetz JM, Mayfield S. 2012. Algae-Produced Pfs25 Elicits Antibodies That Inhibit Malaria Transmission. PLoS ONE **7**, e37179.

Gregory JA, Topol AB, Doerner DZ, Mayfield S. 2013. Alga-Produced Cholera Toxin-Pfs25 Fusion Proteins as Oral Vaccines. Applied and Environmental Microbiology **79**, 3917-3925.

Gudin C, Therpenier C. 1986. Bioconversion of solar energy into organic chemicals by microalgae. Advances in Biotechnology Processes **6**, 73-110.

Guerrero F, Carbonell V, Cossu M, Correddu D, Jones PR. 2012. Ethylene synthesis and regulated expression of recombinant protein in *Synechocystis* sp. PCC 6803. PLoS ONE **7**, e50470.

Guiry MD. 2012. How Many Species of Algae Are There? Journal of Phycology **48**, 1057-1063.

Guiry MD, Guiry GM, Morrison L, *et al.* 2014. AlgaeBase: an on-line resource for Algae. Cryptogamie, Algologie **35**, 105-115.

Hagen C, Siegmund S, Braune W. 2002. Ultrastructural and chemical changes in the cell wall of *Haematococcus pluvialis* (Volvocales, Chlorophyta) during aplanospore formation. European Journal of Phycology **37**, 217-226.

Halim R, Danquah MK, Webley PA. 2012. Extraction of oil from microalgae for biodiesel production: A review. Biotechnology advances **30**, 709-732.

Hall LM, Taylor KB, Jones DD. 1993. Expression of a foreign gene in *Chlamydomonas reinhardtii*. Gene **124**, 75–81.

Hallmann A. 2007. Algal Transgenics and Biotechnology. Transgenic Plant Journal **1**, 81-98.

Harari A, Harats D, Marko D, Cohen H, Barshack I, Kamari Y, Gonen A, Gerber Y, Ben-Amotz A, Shaish A. 2008. A 9-cis beta-carotene-enriched diet inhibits atherogenesis and fatty liver formation in LDL receptor knockout mice. The Journal of Nutrition **138**, 1923-1930.

He DM, Qian KX, Shen GF, Zhang ZF, Li YN, Su ZL, Shao HB. 2007. Recombination and expression of classical swine fever virus (CSFV) structural protein E2 gene in *Chlamydomonas reinhardtii* chroloplasts. Colloids and surfaces B: Biointerfaces **55**, 26-30.

Hempel F, Lau J, Klingl A, Maier UG. 2011. Algae as protein factories: expression of a human antibody and the respective antigen in the diatom *Phaeodactylum tricornutum*. PLoS ONE **6**, e28424.

Hempel F, Maier UG. 2012. An engineered diatom acting like a plasma cell secreting human IgG antibodies with high efficiency. Microbial cell factories **11**, 126.

Hom EFY, Murray AW. 2014. Niche engineering demonstrates a latent capacity for fungal-algal mutualism. Science **345**, 94-98.

Hou Q, Qiu S, Liu Q, Tian J, Hu Z, Ni J. 2013. Selenoprotein-transgenic *Chlamydomonas reinhardtii*. Nutrients **5**, 624-636.

Huheihel M, Ishanu V, Tal J, Arad SM. 2002. Activity of *Porphyridium* sp. polysaccharide against herpes simplex viruses in vitro and in vivo. Journal of Biochemical and Biophysical Methods **50**, 189-200.

Jacobsen JH, Frigaard NU. 2014. Engineering of photosynthetic mannitol biosynthesis from CO_2 in a cyanobacterium. Metabolic engineering **21**, 60-70.

Jagmann N, Philipp B. 2014. Design of synthetic microbial communities for biotechnological production processes. Journal of Biotechnology **184**, 209-218.

Jensen K, Jensen PE, Møller BL. 2011. Light-driven cytochrome p450 hydroxylations. ACS Chemical Biology **6**, 533-539.

Johnson K, Jiang Y, Kleerebezem R, Muyzer G, Van Loosdrecht MCM. 2009. Enrichment of a mixed bacterial culture with a high polyhydroxyalkanoate Storage Capacity. Biomacromolecules **10**, 670-676.

Jones CS, Luong T, Hannon M, Tran M, Gregory JA, Shen Z, Briggs SP, Mayfield SP. 2013. Heterologous expression of the C-terminal antigenic domain of the malaria vaccine candidate Pfs48/45 in the green algae *Chlamydomonas reinhardtii*. Applied Microbiology and Biotechnology **97**, 1987-1995.

Kazamia E, Czesnick H, Thi TVN, Croft MT, Sherwood E, Sasso S, Hodson SJ, Warren MJ, Smith AG. 2012. Mutualistic interactions between vitamin B12-dependent algae and heterotrophic bacteria exhibit regulation. Environmental Microbiology **14**, 1466-1476.

Kazamia E, Riseley AS, Howe CJ, Smith AG. 2014. An engineered community approach for industrial cultivation of microalgae. Industrial Biotechnology **10**, 184-190.

Kerner A, Park J, Williams A, Lin XN. 2012. A programmable *Escherichia coli* consortium via tunable symbiosis. PLoS ONE **7**, e34032.

Kim HJ, Boedicker JQ, Choi JW, Ismagilov, RF. 2008. Defined spatial structure stabilizes a synthetic multispecies bacterial community. Proceedings of the National Academy of Sciences **105**, 18188-18193.

Kim J, Yoo G, Lee H, Lim J, Kim K, Kim CW, Park MS, Yang JW. 2013. Methods of downstream processing for the production of biodiesel from microalgae. Biotechnology advances **31**, 862-876.

Kim S, Lee YC, Cho DH, Lee HU, Huh YS, Kim GJ, Kim HS. 2014. A simple and non-invasive method for nuclear transformation of intact-walled *Chlamydomonas reinhardtii*. PLoS ONE **9**, e101018.

Kindle KL, Schnell RA, Fernández E, Lefebvre PA. 1989. Stable nuclear transformation of *Chlamydomonas* using the Chlamydomonas gene for nitrate reductase. The Journal of Cell Biology **109**, 2589-601.

Kindle KL. 1990. High-frequency nuclear transformation of *Chlamydomonas reinhardtii*. Proceedings of the National Academy of Sciences **87**, 1228-1232.

Kindle KL, Richards KL, Stern DB. 1991. Engineering the chloroplast genome: techniques and capabilities for chloroplast transformation in *Chlamydomonas reinhardtii*. Proceedings of the National Academy of Sciences **88**, 1721-1725.

Klitgord N, Segre D. 2010. Environments that induce synthetic microbial ecosystems. PLoS Computational Biology **6**, e1001002.

Kovar JL, Zhang J, Funke RP, Weeks DP. 2002. Molecular analysis of the acetolactate synthase gene of *Chlamydomonas reinhardtii* and development of a genetically engineered gene as a dominant selectable marker for genetic transformation. The Plant Journal **29**, 109-117.

Kumar SV, Misquitta RW, Reddy VS, Rao BJ, Rajam MV. 2004. Genetic transformation of the green alga – *Chlamydomonas reinhardtii* by *Agrobacterium tumefaciens*. Plant Science **166**, 731-738.

Kwak JH, Baek SH, Woo Y, Han JK, Kim BG, Kim OY, Lee JH. 2012. Beneficial immunostimulatory effect of short-term *Chlorella* supplementation: enhancement of *Natural Killer* cell activity and early inflammatory response (randomized, double-blinded, placebo-controlled trial). Nutrition Journal **11**, 53.

Lassen LM, Nielsen AZ, Olsen CE, Bialek W, Jensen K, Møller BL, Jensen PE. 2014a. Anchoring a Plant Cytochrome P450 via PsaM to the Thylakoids in *Synechococcus* sp PCC 7002: Evidence for Light-Driven Biosynthesis. PLoS ONE **9**, e102184.

Lassen, LM, Zygadlo Nielsen A, Friis Ziersen BE; Gnanasekaran T, Møller BL, Jensen PE. 2014b. Redirecting photosynthetic electron flow into light-driven synthesis of alternative products including high-value bioactive natural compounds. ACS Synthetic Biology **3**, 1-12.

Lauersen KJ, Berger H, Mussgnug JH, Kruse O. 2013a. Efficient recombinant protein production and secretion from nuclear transgenes in *Chlamydomonas reinhardtii*. Journal of Biotechnology **167**, 101-110.

Lauersen, KJ, Vanderveer TL, Berger H, Kaluza I, Mussgnug JH, Walker VK, Kruse O. 2013b. Ice recrystallization inhibition mediated by a nuclear-expressed and -secreted recombinant ice-binding protein in the microalga *Chlamydomonas reinhardtii*. Applied Microbiology and Biotechnology. **97**, 9763-9772.

Lauersen KJ, Huber I, Wichmann J, Baier T, Leiter A, Gaukel V, Kartushin V, Rattenholl A, Steinweg C, von Riesen L, Posten C, Gudermann F, Lütkemeyer D, Mussgnug JH, Kruse O.

2015. Investigating the dynamics of recombinant protein secretion from a microalgal host. Journal of Biotechnology (in press).

Le Chevanton M, Garnier M, Bougaran G, Schreiber N, Lukomska E, Bérard JB, Fouilland E, Bernard O, Cadoret, JP. 2013. Screening and selection of growth-promoting bacteria for *Dunaliella* cultures. Algal Research **2**, 212-222.

Leão PN, Costa M, Ramos V, Pereira AR, Fernandes VC, Domingues VF, Gerwick WH, Vasconcelos VM, Martins R. 2013. Antitumor activity of Hierridin B, a cyanobacterial secondary metabolite found in both filamentous and unicellular marine strains. PLoS ONE **8**, e69562.

Lee AK, Lewis DM, Ashman PJ. 2012. Disruption of microalgal cells for the extraction of lipids for biofuels: Processes and specific energy requirements. Biomass and Bioenergergy **46**, 89-101.

León-Bañares R, González-Ballester D, Galván A, Fernández E. 2004. Transgenic microalgae as green cell-factories. Trends in Biotechnology **22**, 45-52.

Leu S, Boussiba S. 2014. Advances in the production of high-value products by microalgae. Industrial Biotechnology **10**, 169-183.

Lőrincz Z, Preininger É, Kósa A, Pónyi T, Nyitrai P, Sarkadi L, Kovács GM, Böddi B, Gyurján I. 2010. Artificial tripartite symbiosis involving a green alga (*Chlamydomonas*), a bacterium (*Azotobacter*) and a fungus (*Alternaria*): Morphological and Physiological Characterization. Folia Microbiologica **55**, 393-400.

Manuell AL, Beligni MV, Elder JH, Siefker DT, Tran M, Weber A, McDonald TL, Mayfield SP. 2007. Robust expression of a bioactive mammalian protein in *Chlamydomonas* chloroplast. Plant Biotechnology Journal **5**, 402-412.

Mata TM, Martins AA, Caetano NS. 2010. Microalgae for biodiesel production and other applications: A review. Renewable and Sustainable Energy Reviews **14**, 217-232.

Mayfield SP, Franklin SE, Lerner RA. 2003. Expression and assembly of a fully active antibody in algae. Proceedings of the National Academy of Sciences **100**, 438-442.

Mayfield SP, Manuell AL, Chen S, Wu J, Tran M, Siefker D, Muto M, Marin-Navarro J. 2007. *Chlamydomonas reinhardtii* chloroplasts as protein factories. Current Opinion in Biotechnology **18**, 126-133.

McBride RC, Lopez S, Meenach C, Burnett M, Lee PA, Nohilly F, Behnke C. 2014. Contamination management in low cost open algae ponds for biofuels production. Industrial Biotechnology **10**, 221-227.

McCarty MF. 2007. Clinical potential of *Spirulina* as a source of phycocyanobilin. Journal of Medicinal Food **10**, 566-570.

Melis A, Zhang L, Forestier M, Ghirardi ML, Seibert M. 2000. Sustained photobiological hydrogen gas production upon reversible inactivation of oxygen evolution in the green alga *Chlamydomonas reinhardtii*. Plant Physiology **122**, 127-136.

Mendes RL, Fernandes HL, Coelho JP, Reis EC, Cabral JMS, Novais JM, Palavra AF. 1995. Supercritical CO_2 extraction of carotenoids and other lipids from *Chlorella vulgaris*. Food Chemistry **53**, 99-103.

Miyahara M, Aoi M, Inoue-Kashino N, Kashino Y, Ifuku K. 2013. Highly efficient transformation of the diatom *Phaeodactylum tricornutum* by multi-pulse electroporation. Bioscience Biotechnology and Biochemistry **77**, 874-876.

Molina Grima E, Belarbi E-H, Acién Fernández FG, Robles Medina A, Chisti Y. 2003. Recovery of microalgal biomass and metabolites: process options and economics. Biotechnology Advances **20**, 491-515.

Mooij PR, Stouten GR, Tamis J, Van Loosdrecht MCM, Kleerebezem R. 2013. Survival of the fattest. Energy and Environmental Science **6**, 3404-3406.

Myers J. 1984. Citation classic - Nutrition and growth of several blue-green algae. Current Contents/Life Sciences **45**, 22-22.

Nadeem M, Rikhari HC, Kumar A, Palni LM, Nandi SK. 2002. Taxol content in the bark of Himalayan Yew in relation to tree age and sex. Phytochemistry **60**, 627-631.

Nalley JO, Stockenreiter M, Litchman E. 2014. Community ecology of algal biofuels: complementarity and trait-Based approaches. Industrial Biotechnology **10**, 191-201.

Nelson JAE, Lefebvre PA. 1995. Targeted disruption of the NIT8 gene in *Chlamydomonas reinhardtii*. Molecular and Cellular Biology **15**, 5762–5769.

Nicolaou KC, Yang Z, Liu JJ, Ueno H, Nantermet PG, Guy RK, Claiborne CF, Renaud J, Couladouros EA, Paulvannan K, Sorensen EJ. 1994. Total synthesis of taxol. Nature **367**, 630-634.

Niederholtmeyer H, Wolfstädter BT, Savage DF, Silver PA, Way JC. 2010. Engineering cyanobacteria to synthesize and export hydrophilic products. Applied and Environmental Microbiology **76**, 3462-3466.

Nielsen AZ, Ziersen B, Jensen K, Lassen LM, Olsen CE, Møller BL, Jensen PE. 2013. Redirecting photosynthetic reducing power toward bioactive natural product synthesis. ACS Synthetic Biology **2**, 308-315.

Nielsen CH, Balachandran P, Christensen O, Pugh ND, Tamta H, Sufka KJ, Wu X, Walsted A, Schjørring-Thyssen M, Enevold C, Pasco DS. 2010. Enhancement of natural killer cell activity in healthy subjects by Immulina®, a *Spirulina* extract enriched for Braun-type lipoproteins. Planta Medica **76**, 1802-1808.

Ohresser M, Matagne RF, Loppes R. 1997. Expression of the arylsulphatase reporter gene under the control of the nit1 promoter in *Chlamydomonas reinhardtii*. Current Genetics **31**, 264-271.

Ortiz-Marquez JC, Do Nascimento M, Curatti L. 2014. Metabolic engineering of ammonium release for nitrogen-fixing multispecies microbial cell-factories. Metabolic Engineering **23**, 154-164.

Ortiz-Marquez JC, Do Nascimento M, Dublan Mde L, Curatti L. 2012. Association with an ammonium-excreting bacterium allows diazotrophic culture of oil-rich eukaryotic microalgae. Applied and Environmental Microbiology **78**, 2345-2352.

Ortiz-Marquez JCF, Do Nascimento M, Zehr JP, Curatti L. 2013. Genetic engineering of multispecies microbial cell factories as an alternative for bioenergy production. Trends in Biotechnology **31**, 521-529.

Pandhal J, Noirel J. 2014. Synthetic microbial ecosystems for biotechnology. Biotechnology Letters **36**, 1141-1151.

Park J, Kerner A, Burns MA, Lin XN. 2011. Microdroplet-enabled highly parallel co-cultivation of microbial communities. PLoS ONE **6**, e17019.

Pourmir A, Noor-Mohammadi S, Johannes TW. 2013. Production of xylitol by recombinant microalgae. Journal of Biotechnology **165**, 178-183.

Pragya N, Pandey KK, Sahoo PK. 2013. A review on harvesting, oil extraction and biofuels production technologies from microalgae. Renewable and Sustainable Energy Reviews **24**, 159-171.

Pratheesh PT, Vineetha M, Kurup GM. 2014. An efficient protocol for the *Agrobacterium*-mediated genetic transformation of microalga *Chlamydomonas reinhardtii*. Molecular Biotechnology **56**, 507-515.

Purton S. 2007. Tools and techniques for chloroplast transformation of *Chlamydomonas*. Advances in Experimental Medicine and Biology **616**, 34-45.

Purton S, Szaub JB, Wannathong T, Young R, Economou CK. 2013. Genetic engineering of algal chloroplasts: progress and prospects. Russian Journal of Plant Physiology **60**, 491-499.

Randolph-Anderson BL, Boynton JE, Gillham NW, Harris EH, Johnson AM, Dorthu MP, Matagne RF. 1993. Further characterization of the respiratory deficient *dum-1* mutation of *Chlamydomonas reinhardtii* and its use as a recipient for mitochondrial transformation. Molecular and General Genetics **236**, 235-244.

Rasala BA, Barrera DJ, Ng J, Plucinak TM, Rosenberg JN, Weeks DP, Oyler GA, Peterson TC, Haerizadeh F, Mayfield SP. 2013. Expanding the spectral palette of fluorescent proteins for the green microalga *Chlamydomonas reinhardtii*. Plant Journal **74**, 545-556.

Rasala BA, Lee PA, Shen Z, Briggs SP, Mendez M, Mayfield SP. 2012. Robust expression and secretion of Xylanase1 in *Chlamydomonas reinhardtii* by fusion to a selection gene and processing with the FMDV 2A peptide. PLoS ONE **7**, e43349.

Rasala BA, Mayfield SP. 2014. Photosynthetic biomanufacturing in green algae; production of recombinant proteins for industrial, nutritional, and medical uses. Photosynthesis Research **3**, 227-239.

Rasala BA, Muto M, Lee PA Jager M, Cardoso RM, Behnke CA, Kirk P, Hokanson CA, Crea R, Mendez M, Mayfield SP. 2010. Production of therapeutic proteins in algae, analysis of expression

of seven human proteins in the chloroplast of *Chlamydomonas reinhardtii*. Plant Biotechnology Journal **8**, 719-733.

Rawat I, Kumar RR, Mutanda T, Bux F. 2013. Biodiesel from microalgae: A critical evaluation from laboratory to large scale production. Applied Energy **103**, 444-467.

Ríos SD, Torres CM, Torras C, Salvadó J, Mateo-Sanz JM, Jiménez L. 2013. Microalgae-based biodiesel: Economic analysis of downstream process realistic scenarios. Bioresource Technology **136**, 617-625.

Robert FO, Pandhal J, Wright PC. 2010. Exploiting cyanobacterial P450 pathways. Current Opinion in Microbiology **13**, 301-306.

Ruffing AM. 2014. Improved free fatty acid production in cyanobacteria with *Synechococcus* sp. PCC 7002 as host. Frontiers in Bioengineering and Biotechnology **2**, 17.

Ruecker O, Zillner K, Groebner-Ferreira R, Heitzer, M. 2008. Gaussia-luciferase as a sensitive reporter gene for monitoring promoter activity in the nucleus of the green alga Chlamydomonas reinhardtii. Molecular Genetics and Genomics **280**, 153-162.

Ryckebosch E, Bruneel C, Termote-Verhalle R, Goiris K, Muylaert K, Foubert I. 2014. Nutritional evaluation of microalgae oils rich in omega-3 long chain polyunsaturated fatty acids as an alternative for fish oil. Food Chemistry **160**, 393-400.

Sabra W, Dietz D, Tjahjasari D, Zeng A-P. 2010. Biosystems analysis and engineering of microbial consortia for industrial biotechnology. Engineering in Life Sciences **10**, 407-421.

Schmidt FR. 2004. Recombinant expression systems in the pharmaceutical industry. Applied Microbiology and Biotechnology **65**, 363-372.

Scoma A, Giannelli L, Faraloni C, Torzillo G. 2012. Outdoor H_2 production in a 50-L tubular photobioreactor by means of a sulfur-deprived culture of the microalga *Chlamydomonas reinhardtii*. Journal of Biotechnology **157**, 620-627.

Scott SA, Davey MP, Dennis JS, Horst I, Howe CJ, Lea-Smith DJ, Smith AG. 2010. Biodiesel from algae: challenges and prospects. Current Opinion in Biotechnology **21**, 277-286.

Serafim LS, Lemos PC, Albuquerque MGE, Reis MAM. 2008. Strategies for PHA production by mixed cultures and renewable waste materials. Applied Microbiology and Biotechnology **81**, 615-628.

Seyedsayamdost MR, Carr G, Kolter R, Clardy J. 2011. Roseobacticides: small molecule modulators of an algal-bacterial symbiosis. Journal of the American Chemical Society **133**, 18343-18349.

Shimogawara K, Fujiwara S, Grossmann A, Usuda H. 1998. High-efficiency transformation of *Chlamydomonas reinhardtii* by electroporation. Genetics **148**, 1821-1828.

Sizova IA, Lapina TV, Frolova ON, Alexandrova NN, Akopiants KE, Danilenko VN. 1996. Stable nuclear transformation of *Chlamydomonas reinhardtii* with a *Streptomyces rimosus* gene as the selective marker. Gene **181**, 13-18.

Smith VH, Crews T. 2014. Applying ecological principles of crop cultivation in large-scale algal biomass production. Algal Research **4**, 23-34.

Sørensen L, Hantke A, Eriksen NT. 2013. Purification of the photosynthetic pigment C-phycocyanin from heterotrophic *Galdieria sulphuraria*. Journal of the Science of Food and Agriculture **93**, 2933-2938.

Sorokin C. 1967. New high-temperature *Chlorella*. Science **158**, 1204-1205.

Spadiut O, Capone S, Krainer F, Glieder A, Herwig C. 2014. Microbials for the production of monoclonal antibodies and antibody fragments. Trends in Biotechnology **32**, 54-60.

Specht EA, Mayfield SP. 2013. Synthetic oligonucleotide libraries reveal novel regulatory elements in *Chlamydomonas* Chloroplast mRNAs. ACS Synthetic Biology **2**, 34-46.

Specht EA, Mayfield SP. 2014. Algae-based oral recombinant vaccines. Frontiers in Microbiology **5**, 60.

Stephanopoulos G. 2012. Synthetic biology and metabolic engineering. ACS Synthetic Biology **1**, 514-525.

Stevens DR, Purton S. 1997. Genetic engineering of eukaryotic algae: Progress and prospects. Journal of Phycology **33**, 713-722.

Stevens DR, Rochaix JD, Purton S. 1996. The bacterial phleomycin resistance gene *ble* as a dominant selectable marker in *Chlamydomonas*. Molecular and General Genetics **251**, 23-30.

Sun M, Qian K, Su N, Chang H, Liu J, Shen G. 2003. Foot-and-mouth disease virus VP1 protein fused with cholera toxin B subunit expressed in *Chlamydomonas reinhardtii* chloroplast. Biotechnology Letters **25**, 1087-1092.

Tan LT. 2013. Pharmaceutical agents from filamentous marine cyanobacteria. Drug Discovery Today **18**, 863-871.

Tang DK, Qiao SY, Wu M. 1995. Insertion mutagenesis of *Chlamydomonas reinhardtii* by electroporation and heterologous DNA. International Journal of Biochemistry and Molecular Biology **36,** 1025–1035.

Thana P, Machmudah S, Goto M, Sasaki M, Pavasant P, Shotipruk A. 2008. Response surface methodology to supercritical carbon dioxide extraction of astaxanthin from *Haematococcus pluvialis*. Bioresource Technology **99**, 3110-3115.

Thompson AW, Foster RA, Krupke A, Carter BJ, Musat N, Vaulot D, Kuypers MMM, Zehr JP. 2012. Unicellular cyanobacterium symbiotic with a single-celled eukaryotic alga. Science **337**, 1546-1550.

Tran M, Henry RE, Siefker D, Van C, Newkirk G, Kim J, Bui J, Mayfield SP. 2013. Production of anti-cancer immunotoxins in algae: ribosome inactivating proteins as fusion partners. Biotechnology and Bioengineering **110**, 2826-2835.

Tran M, Van C, Barrera DJ, Pettersson PL, Peinado CD, Bui J, Mayfield, SP. 2012. Production of unique immunotoxin cancer therapeutics in algal chloroplasts. Proceedings of the National Academy of Sciences **110**, E15-22.

Tran M, Zhou B, Pettersson PL, Gonzalez MJ, Mayfield SP. 2009. Synthesis and assembly of a full-length human monoclonal antibody in algal chloroplasts. Biotechnology and Bioengineering **104**, 663-673.

Ungerer J, Tao L, Davis M, Ghiradi M, Maness P-C, Yu J. 2012. Sustained photosynthetic conversion of CO_2 to ethylene in recombinant cyanobacterium *Synechocystis* 6803. Energy & Environmental Science **5**, 8998-9006.

Walker TL, Collet C, Purton S. 2005. Algal transgenics in the genomic era. Journal of Phycology **41**, 1077-1093.

Wang B, Pugh S, Nielsen DR, Zhang W Meldrum DR. 2013. Engineering cyanobacteria for photosynthetic production of 3-hydroxybutyrate directly from CO_2. Metabolic Engineering **16**, 68-77.

Watanabe J, Minami M, Kobayashi M. 2006. Antitumor activity of TZT-1027 (Soblidotin). Anticancer Research **26**, 1973-1981.

Xie W-H, Zhu C-C, Zhang N-S, Li D-W, Yang W-D, Liu J-S, Sathishkumar R, Li H-Y. 2014. Construction of novel chloroplast expression vector and development of an efficient transformation system for the diatom *Phaeodactylum tricornutum*. Marine Biotechnology **16**, 538-546.

Xu L, Brilman DWF, Withag JAM, Brem G, Kersten S. 2011. Assessment of a dry and a wet route for the production of biofuels from microalgae: Energy balance analysis. Bioresource Technology **102**, 5113-5122.

Xue Y, Zhang Y, Grace S, He Q. 2014. Functional expression of an *Arabidopsis* p450 enzyme, *p*-coumarate-3-hydroxylase, in the cyanobacterium *Synechocystis* PCC 6803 for the biosynthesis of caffeic acid. Journal of Applied Phycology **26**, 219-226.

Yaakob Z, Ali E, Zainal A, Mohamad M, Takriff MS. 2014. An overview: biomolecules from microalgae for animal feed and aquaculture. Journal of Biological Research – Thessaloniki **21,** 6.

Yamano T, Iguchi H, Fukuzawa H. 2013. Rapid transformation of *Chlamydomonas reinhardtii* without cell-wall removal. Journal of Biosciences and Bioengineering **115**, 691-694.

Yoon S-M, Kim SY, Li KF, Yoon BH, Choe S, Kuo MM-C. 2011. Transgenic microalgae expressing *Escherichia coli* AppA phytase as feed additive to reduce phytate excretion in the manure of young broiler chicks. Applied Microbiology and Biotechnology **91**, 553-563.

You L, Cox RS, Weiss R, Arnold FH. 2004. Programmed population control by cell-cell communication and regulated killing. Nature **428**, 868-871.

Zaslavskaia LA, Lippmeier JC, Kroth PG, Grossman AR, Apt KE. 2000. Transformation of the diatom *Phaeodactylum tricornutum* (Bacillariophyceae) with a variety of selectable marker and reporter genes. Journal of Phycology **36**, 379-386.

Zaslavskaia LA, Lippmeier JC, Shih C, Ehrhardt D, Grossman AR, Apt KE. 2001. Trophic obligate conversion of an photoautotrophic organism through metabolic engineering. Science **292**, 2073-2075.

Zedler JAZ, Gangl D, Hamberger B, Purton S, Robinson C. 2014. Stable Expression of a bifunctional diterpene synthase in the chloroplast of *Chlamydomonas reinhardtii*. Journal of Applied Phycology doi:10.1007/s10811-014-0504-2.

Zeng RJ, Saunders AM, Yuan Z, Blackall LL, Keller J. 2003. Identification and comparison of aerobic and denitrifying polyphosphate-accumulating organisms. Biotechnology and Bioengineering **83**, 140-148.

Zhang C, Hu H. 2014. High-efficiency nuclear transformation of the diatom *Phaeodactylum tricornutum* by electroporation. Marine Genomics **16**, 63-66.

Zhang L, Happe T, Melis A. 2002. Biochemical and morphological characterization of sulfur-deprived and H_2-producing *Chlamydomonas reinhardtii* (green alga). Planta **214**, 552-561.

Zheng K, Wang C, Xiao M, Chen J, Li J, Hu Z. 2014. Expression of *bkt* and *bch* genes from *Haematococcus pluvialis* in transgenic *Chlamydomonas*. Science China Life Sciences **57**, 1028-1033.

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12. Tables

Table 1 DNA delivery methods for *Chlamydomonas reinhardtii* and *Phaeodactylum tricornutum*

Organelle	Species	Delivery Methods	References
Nucleus	*Chlamydomonas reinhardtii*	Microparticle bombardment, electroporation, *Agrobacterium tumefaciens*, glass beads, silicon carbide whiskers and aminoclay nanoparticles	Boynton *et al.*, 1988; Brown *et al.*, 1991; Dunahay, 1993; Kim *et al.*, 2014; Kindle, 1990; Kumar *et al.*, 2004
Nucleus	*Phaeodactylum tricornutum*	Microparticle bombardment, electroporation	Apt *et al.*, 1996; Falciatore *et al.*, 1999 Miyahara *et al.*, 2013; Zhang and Hu, 2014
Chloroplast	*Chlamydomonas reinhardtii*	Microparticle bombardment, glass beads	Boynton *et al.*, 1988 Kindle *et al.*, 1991
Chloroplast	*Phaeodactylum tricornutum*	Electroporation	Xie *et al.*, 2014
Mitochondria	*Chlamydomonas reinhardtii*	Microparticle bombardment	Randolph-Anderson *et al.*, 1993

Table 2 Common marker and reporter genes used in *Chlamydomonas reinhardtii* and *Phaeodactylum tricornutum*

Species	Marker/Reporter Genes	Description	Reference
Chlamydomonas reinhardtii	***Marker Genes*** **Chloroplast**		
	atpB	ATP synthase subunit	Boynton *et al.*, 1988
	psbH	Thylakoid membrane protein (PSII subunit)	Cullen *et al.*, 2007
	Nucleus		
	ARG7	Argininosuccinate lyase	Debuchy *et al.*, 1989
	NIT1	Nitrate reductase	Kindle *et al.* (1989)
	ble	Protein conferring resistance to bleomycin, phleomycin and zeomycin	Stevens *et al.*, 1996
	aadA	Adenylyl transferase (resistance to spectinomycin)	Goldschmidt-Clermont, 1991
	aph7"	*Streptomyces hygroscopicus* aminoglycoside phosphotransferase	Berthold *et al.*, 2002
	bptII	Neomycin phosphotransferase III	Hall *et al.*, 1993
	aphVIII	Aminoglycoside 3'phosphotransferase (resistance to paromomycin, kanamycin and neomycin)	Sizova *et al.*, 1996
	als	Acetolactate synthase (resistance to sulphonylurea herbicides)	Kovar *et al.*, 2002
	cry1-1	Ribosomal protein S14	Nelson and Lefebvre, 1995
	oee-1	Oxygen evolving enhance protein	Chang *et al.*, 2003
	cat	Chloramphenicol acetyltransferase (resistance to chloramphenicol)	Tang *et al.*, 1995

	Reporter genes		
	gus	β-Glucuronidase	Kumar *et al.*, 2004
	gfp	Green fluorescent protein	Kumar *et al.*, 2004
	luc	Luciferase	Fuhrmann *et al.*, 2004
	chgfp	Modified green fluorescent protein	Fuhrmann *et al.*, 1999
	ars	Arylsulphatase	Davies *et al.*, 1992
Phaeodactylum tricornutum	***Marker genes***		
	<u>*Chloroplast*</u>		
	cat	Chloramphenicol acetyltransferase	Xie *et al.* 2014
	<u>*Nuclear*</u>		
	ble	Resistance to bleomycin, phleomycin and zeomycin	(Apt *et al.*, 1996; Zaslavskaia *et al.*, 2001)
	nptII	Neomycin phosphotransferase III	
	hpt	Hygromycin B phosphotransferase	
	nat/sat1	Nourseothricin resistance	
	cat	Chloroamphenicol acetyltransferase (resistance to chloroamphenicol)	
	Reporter genes		
	egfp	Modified green fluorescent protein	(Zaslavskaia *et al.*, 2001; 2000)
	glut1	Glucose Transporter	
	hup1	Hexose Transporter	

Table 3 Key benefits of engineering algal communities for biotechnological exploitation.

Benefit	Reasoning	Reference
Improved algal productivity	Resource-use complementarity is when algal species with different growth requirements are grown together; the competition for resources between the organisms is lowered allowing the individual species to cohabit thus increasing net biomass of the culture.	(Kazamia *et al.*, 2014; Nalley *et al.*, 2014 and Smith and Crews 2014)
Enhanced crop protection	The competitive exclusion principle is when a culture contains a diverse array of organisms. Resources or niches are therefore occupied leading to an increased ability to competitively exclude invaders.	(Kazamia *et al.*, 2012; Nalley *et al.*, 2014 and Smith and Crews 2014)
Reduction of energy inputs	The use of organisms to provide scarce or expensive resources such as vitamin B_{12} or fixed nitrogen into the media.	(Kazamia *et al.*, 2014 and Ortiz-Marquez *et al.*, 2013)
Increased stability and resilience	Stability of communities can be defined as the minimal population fluctuation despite a disturbance and resilience can be defined as the ability for a community to revive after a disturbance. Increasing algal diversity and richness has been shown to improve the stability and resilience of algal cultures.	(Nalley *et al.*, 2014)
Increasingly broad and complex engineering of metabolic pathways	Microbial communities engineered to produce valuable products have many advantages due to the division of labour. The ability to compartmentalise complex pathways into a number of strains could lead to optimised functionality and lowering metabolic burden on any one cell.	(Jagmann and Phillip 2014)

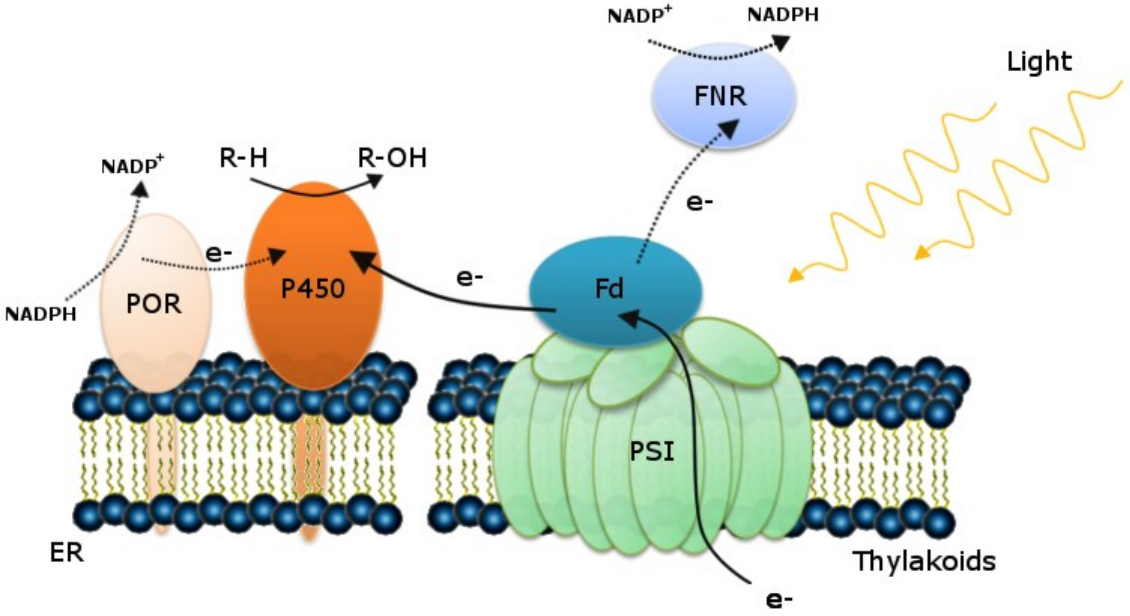
Table 4. **Secretion of recombinant proteins from *Chlamydomonas reinhardtii***

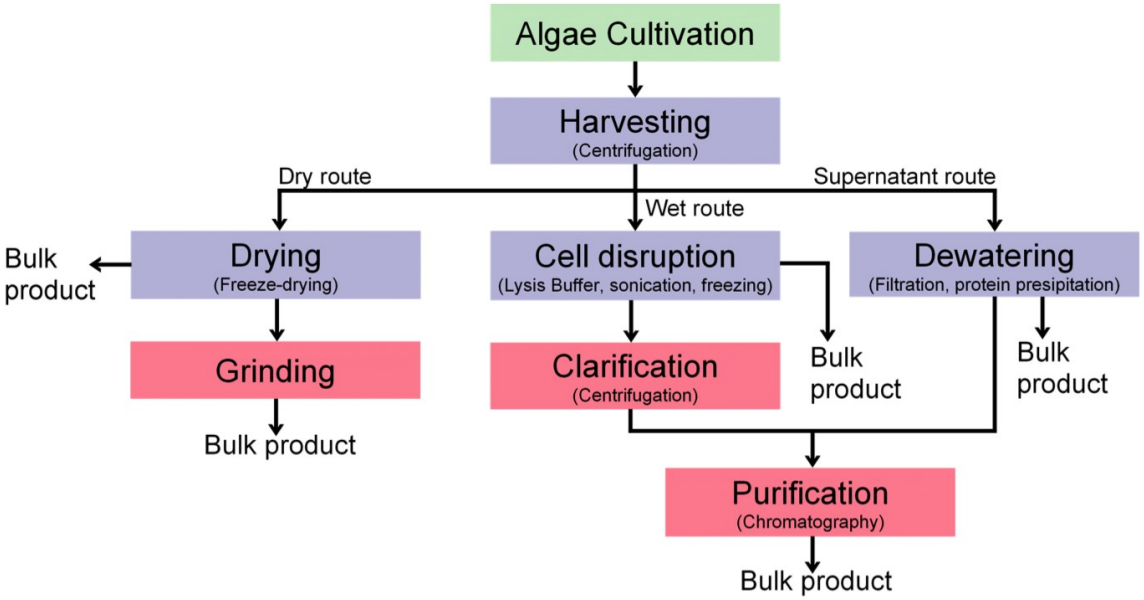
Signal peptide	**Protein secreted**	**Reference**
Arylsulphatase (ARS2)	Luciferase and erythropoietin	Eichler-Stahlberg *et al.*, 2009
POXA1b (from *Pleurotus ostreatus*)	Laccase	Chiaiese *et al.*, 2011
Arylsulphatase (ARS1)	Xylanase	Rasala *et al.*, 2012
carbonic anhydrase 1 (CAH1)	Gaussia luciferase	Lauersen *et al.*, 2013a
Luciferase	Luciferase	Ruecker *et al*., 2008
Carbonic anhydrase 1 (CAH1)	Ice-binding protein	Lauersen *et al.*, 2013b

13. Figure legends

Figure 1: Relocation of Cytochrome P450s from the Endoplasmic Reticulum (ER) into the thylakoid membranes for coupling with photosynthetic electron flow. Most cytochromes P450 function in the endoplasmic reticulum where they interact with specific enzymes (cytochrome P450 reductases) that provide the reducing power to drive complex substrate modifications. It has now been shown that these key enzymes can be expressed in the chloroplasts of plants, algae and cyanobacteria, where the P450s are active and able to use reducing power from ferredoxin. This provides a novel route for the formation of complex metabolites involving P450 enzymes.

Figure 2. Downstream processing of recombinant proteins extracted from transgenic algae systems. The Figure shows a flow-chart for the downstream processing of algal extracts by the 'dry route' involving freeze-drying or the 'wet route' involving cell disruption and fractionation.


NADP+
NADPH
FNR
Light
NADP+
R-H
R-OH
e-
e-
NADPH
POR
P450
e-
Fd
PSI
ER
Thylakoids
e-


Algae Cultivation
Harvesting
(Centrifugation)
Dry route
Wet route
Supernatant route
Bulk
product
Drying
(Freeze-drying)
Cell disruption
(Lysis Buffer, sonication, freezing)
Dewatering
(Filtration, protein presipitation)
Grinding
Clarification
(Centrifugation)
Bulk
product
Bulk
product
Bulk product
Purification
(Chromatography)
Bulk product