

1 **Biotechnological exploitation of microalgae**

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27 **Short statement**

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

31 **Abstract**

32

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

44

45

46 **1. Introduction**

47

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

65

66 **2. High-value natural products in microalgae**

67

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

74
75 Relatively few proteins have been purified from microalgae for commercial use, but *Spirulina* is
76 a rich source of phycocyanin, a protein that constitutes 14% of the dry weight of this
77 cyanobacterium (McCarty, 2007). The US Food and Drug Administration (FDA) has approved
78 phycocyanin from *Spirulina* as a blue food colorant. Moreover phycocyanobilin, the
79 tetrapyrrole chromophore of phycocyanin, manifests fluorescent properties that have been
80 exploited for labelling of antibodies in immunofluorescence and flow cytometry. In mammalian
81 tissues it can be enzymatically reduced to phycocyanorubin (a close homolog of bilirubin) and
82 inhibits the activity of NADPH oxidase, thus reducing the generation of reactive oxygen
83 species. It has been suggested that a regular intake of phycocyanobilin may provide protection
84 against cancer and other diseases (Sørensen *et al.*, 2013). Moreover, recent studies have proven
85 the beneficial health effect of microalgae (*Chlorella*, *Spirulina*) by increasing natural killer cell
86 levels and stimulating immune and anti-inflammatory system response in humans (Nielsen *et*
87 *al.*, 2010; Kwak *et al.*, 2012).

88
89 Carotenoids are important products that are extracted from microalgae, and indeed the first
90 commercialised product derived from algae was β -carotene. It is produced in very high amounts
91 by *Dunaliella salina*, a halophilic alga growing in saline habitats which makes the cultures less
92 susceptible to contamination. What differentiates *Dunaliella* β -carotene from the synthetic
93 product (present only in the form of all-*trans* isomer) is that it is rich in the 9-*cis* isomer, and a
94 negative effect of the use of all-*trans* isomer, such an effect on plasma cholesterol levels and
95 atherogenesis, has been reported from mice studies (Borowitzka, 2013; Harari *et al.*, 2008).
96 Another example of a carotenoid with a well-established and growing market in the
97 nutraceutical area is astaxanthin from the freshwater green alga *Haematococcus pluvialis*.
98 Astaxanthin is mainly used as a feed supplement and pigmentation source for salmon and
99 shrimp farming, but due to its high antioxidant properties (10-fold greater than other
100 carotenoids) and protective activities it has also many applications in the pharmaceutical and
101 cosmetic industries. Astaxanthin has also been shown to prevent bacterial infection, vascular
102 failure and cancer (Ambati *et al.*, 2014).

103
104 Fatty acids are the other natural components produced commercially from microalgae. Several
105 marine algal species are rich in omega-3 long chain polyunsaturated fatty acids (LC-PUFAs),

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

113

114 Other algal species such as *Dunaliella salina* and *Botryococcus braunii* can accumulate up to
115 60% of storage lipids as triacylglycerides (TAGs), potentially making them a valuable source of
116 oil for biodiesel production (Scott *et al.*, 2010). Compared to plants, algae exhibit higher
117 productivities and theoretically could give 10- to 100-fold higher yields of oil per acre, although
118 such capacities have not yet been achieved on a commercial scale (Greenwell *et al.*, 2010).
119 Nevertheless, algae appear to be a potential solution to the controversial food vs. fuel problem
120 that is associated with the use of fertile land to produce plant-derived biofuels. TAGs are not the
121 only way in which microalgae could be exploited for biofuels. Under anaerobic conditions and
122 sulphur depletion, some microalgae produce hydrogen gas, which could be used as an
123 alternative to fossil fuels in the future (Melis *et al.*, 2000; Zhang *et al.*, 2002). Recently, Scoma
124 *et al.* have attempted to produce hydrogen from *Chlamydomonas reinhardtii* using natural
125 sunlight. Although successful, the overall yields did not exceed those obtained in lab-scale
126 settings (Scoma *et al.*, 2012) and so this technology is at an early stage.

127

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

140

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

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

148

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

155

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

157

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

170

171 In recent years there have been rapid developments of genetic tools for microalgae in an attempt
172 to generate more effective microalgal cell factories. Microalgae have several distinct advantages
173 compared with plant-based production and some other bioreactor systems. Firstly, many
174 microalgae can double their biomass in less than 24 hours, with some species such as *Chlorella*
175 *sorokiniana* having a doubling time under optimum conditions of less than three hours (Sorokin,
176 1967). Therefore only a short period of time is required for large-scale production compared
177 with plants. Furthermore, the time from creation of a new transgenic line to industrial scale up
178 can be as short as two months (Mayfield *et al.*, 2007; Schmidt, 2004). Secondly, genes for
179 recombinant proteins can be expressed from the nuclear or chloroplast genomes (León-Bañares
180 *et al.*, 2004), and eukaryotic microalgae possess post-translational modification pathways which
181 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.

189

190 **3.1. DNA Delivery Method.**

191 A variety of different methods have been employed for delivering transgenes into algal cells
192 (**Table 1**), and the method of choice is very much determined by the cell size and nature of the
193 cell wall of the chosen algal species (Stevens and Purton, 1997). The first demonstrations of
194 stable transformation of an alga involved the bombardment of *C. reinhardtii* cells with DNA-
195 coated microprojectiles (so-called biolistics) and proved successful for the delivery of DNA into
196 both the chloroplast (Boynton *et al.*, 1988) and nucleus (Debuchy *et al.*, 1989). Biolistics was
197 also used to transform the mitochondrial genome of *C. reinhardtii* (Randolph-Anderson *et al.*,
198 1993).

199

200 Subsequently, the nuclear genome of *C. reinhardtii* has been genetically transformed using
201 other delivery methods including agitation in the presence of glass beads or silicon carbide
202 whiskers, electroporation, *Agrobacterium*-mediated transformation, and, more recently,
203 positively-charged aminoclay nanoparticles (Brown *et al.*, 1991; Dunahay, 1993; Kim *et al.*,
204 2014; Kindle, 1990; Kumar *et al.*, 2004). Unlike biolistics or electroporation, methods using
205 glass beads, silicon carbide whiskers or *Agrobacterium* do not involve any specialised
206 equipment and can be done in any laboratory at low cost, although silicon carbide whiskers can
207 be difficult to obtain and may potentially be a health hazard (León-Bañares *et al.*, 2004). The
208 glass bead method remains popular, and has also been employed for chloroplast transformation
209 as a simple alternative to biolistics (Economou *et al.*, 2014; Kindle *et al.*, 1991). For nuclear
210 transformation by electroporation, cell wall-less strains (either mutants or cells treated with
211 autolysin) are normally used (Brown *et al.*, 1991; Shimogawara *et al.*, 1998). However, *C.*
212 *reinhardtii* with an intact cell wall was recently transformed using a series of multi-
213 electroporation pulses (Yamano *et al.*, 2013). The cheaper *Agrobacterium*-mediated gene
214 transfer technique can also be used with walled strains and has been reported to give high
215 transformation frequency compared to glass bead transformation (Kumar *et al.*, 2004), and this
216 can be increased further by inducing the *Agrobacterium* prior to infection using acetosyringone
217 and glycine betaine (Pratheesh *et al.*, 2014).

218

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

228

229 The nuclear genome of the diatom *P. tricornutum* is most commonly transformed via particle
230 bombardment (Apt *et al.*, 1996; Falciatore *et al.*, 1999). Diatoms are generally quite difficult to
231 transform due to challenges such as controlling and manipulating their life cycles, as well as
232 penetrating their rigid cells walls (Falciatore *et al.*, 1999). Nonetheless, recently the *P.*
233 *tricornutum* nuclear genome was successfully transformed by electroporation (Miyahara *et al.*,
234 2013; Zhang and Hu, 2014). More remarkable, given the four membranes that surround a
235 diatom chloroplast, is a recent demonstration that electroporation can be used to deliver DNA
236 into the chloroplast genome (Xie *et al.*, 2014).

237

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

243

244 **3.2. Selectable Markers, Reporter Genes and Promoters**

245 Different types of selectable marker and reporter genes can be used in identifying putative
246 transformants from the population of untransformed cells (**Table 2**). Endogenous selectable
247 markers for nuclear transformation of the haploid *C. reinhardtii* allow the rescue of auxotrophic
248 or non-photosynthetic mutants with examples for nuclear transformation being *ARG7*, *NIT1* and
249 *OEE1* that allow rescue of recessive mutants defective in arginine biosynthesis, nitrate
250 metabolism and photosynthesis, respectively (Stevens and Purton, 1997). A wide range of
251 dominant markers are commonly used in *C. reinhardtii* and confer resistance to various
252 antibiotics or herbicides, as detailed in **Table 2**. For chloroplast transformation, several
253 dominant markers (e.g. *aadA* and *aphA6*) have been developed (**Table 2**). In addition,
254 endogenous chloroplast genes have been used as selectable markers for phototrophic rescue of
255 the corresponding chloroplast mutant: for example the *atpB* gene rescues an *atpB* mutant
256 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 (Zaslavskaja *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; Zaslavskaja *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 biphosphate 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,

295 efforts have also been directed towards producing them in bacteria and yeast (Spadiut *et al.*,
296 2014). The first expression of a functional antibody in microalgae was achieved in the
297 chloroplast of *C. reinhardtii*. A large single chain antibody directed against the herpes simplex
298 virus (HSV) glycoprotein D was successfully expressed in this green alga. The genetic construct
299 was integrated into the chloroplast genome via homologous recombination and expression was
300 driven by *atpA* or *rbcL* promoters and 5' UTRs. The antibodies were shown to accumulate as
301 soluble proteins in the chloroplast and could bind to HSV proteins in ELISA experiments.
302 Heavy and light chains assembled by forming disulphide bonds, however, no other post-
303 translational modifications were detected (Mayfield *et al.*, 2003). In another study, a fully
304 functional monoclonal antibody against anthrax protective antigen 83 (PA83) was expressed in
305 the *C. reinhardtii* chloroplast. Separate constructs for the heavy and light chain were introduced
306 and the antibody was shown to assemble spontaneously by forming the necessary 16 disulphide
307 bonds (Tran *et al.*, 2009).

308

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

317

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

331

332 Several vaccine antigens have been produced in the chloroplast of *C. reinhardtii* (Demurtas et
333 al., 2013; He et al., 2007; Sun et al., 2003). The GRAS status of the alga makes the production
334 of antigens without costly purification from residual toxins or impurities attractive and could
335 even be exploited for oral delivery of vaccines. An algal produced *Staphylococcus aureus*
336 vaccine was shown to protect orally vaccinated mice against sub-lethal and lethal doses of *S.*
337 *aureus* infection (Dreesen et al., 2010). Malaria transmission blocking vaccines were also
338 successfully expressed in the *C. reinhardtii* chloroplast (Gregory et al., 2012; Gregory et al.,
339 2013; Jones et al., 2013). In addition, Bayne et al. showed that it is possible to produce
340 influenza haemagglutinin (HA) in the heterotrophic alga *Schizochytrium sp.* thereby presenting
341 an alternative to current egg-based vaccine production platforms (Bayne et al., 2013).

342

343 Other proteins expressed in microalgae include mammary-associated serum amyloid (M-SAA)
344 which could be used as viral and bacterial prophylaxis in new born mammals (Manuell et al.,
345 2007); domains 10 and 14 of human fibronectin which have potential as antibody mimics;
346 proinsulin and vascular endothelial growth factor, as well as the high mobility group protein B1
347 involved in wound healing (Rasala et al., 2010). Furthermore, a human selenoprotein, Sep15
348 (Hou et al., 2013) and antitoxins against botulinum neurotoxin have been successfully produced
349 (Barrera et al., 2014).

350

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

361

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

369

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

371

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

382

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

393

394 **5.1 Towards engineering metabolic pathways**

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

404

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

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

412

413 P450s are monooxygenases performing stereospecific hydroxylations and they are anchored in
414 the plant endoplasmic reticulum (ER). In order to function they need to be powered by single
415 electron transfers from NADPH-dependent cytochrome P450 reductase (CPR). The expression
416 level of these proteins is relatively low and the activity is often limited by NADPH and
417 substrate pool (Jensen *et al.*, 2011). It has been demonstrated that these energy-consuming
418 reactions can be bypassed by relocating P450-dependent metabolic pathways to the thylakoid
419 membranes of tobacco chloroplasts, where the reducing power generated by photosystem I
420 (PSI) in the form of reduced ferredoxin (Fd) is a cheap and essentially unlimited source of
421 electrons for the P450s (Nielsen *et al.*, 2013) (Fig. 1). More recently, it has been shown that a
422 P450 can be expressed in the chloroplasts of transgenic *C. reinhardtii* and it was furthermore
423 shown that the enzyme was targeted into the chloroplast membranes and highly active (Gangl *et al.*,
424 2014). This strongly suggests that it will be possible to produce pharmacologically
425 important terpenoids not only in plant chloroplasts (Bock and Warzecha, 2010), but also in
426 microalgal chloroplasts.

427

428 **5.2. Cyanobacteria as a tool for synthetic biology**

429 During the last decade, cyanobacteria have been gaining renewed interest as a chassis for
430 metabolic engineering and synthetic biology approaches. Currently many efforts are being
431 undertaken to use genetically modified cyanobacteria as a production platform for biofuels such
432 as isoprene (Zhou and Li, 2010), bioplastics like polyhydroxybutyrate (Wang *et al.*, 2013) and
433 polylactic acid (Angermayr *et al.*, 2014), fatty acids (Ruffing, 2014), ethylene (Guerrero *et al.*,
434 2012; Ungerer *et al.*, 2012) and sugars (Jacobsen and Frigaard, 2014). In many cases reported
435 yields are not yet satisfactory, however, the ability to perform photosynthesis and the idea of
436 converting sunlight and carbon dioxide into high value chemicals are undoubtedly attractive.

437

438 As indicated above, expression of foreign pathways involving P450s in *E. coli* often causes
439 problems and is ineffective, due to difficulties with proper folding, posttranslational
440 modifications and targeting to the membranes. Unlike most native bacterial P450 enzymes that
441 are soluble, cyanobacterial P450s are membrane bound, exactly like plant P450s (Robert *et al.*,
442 2010). All the above considerations indicate that cyanobacteria and eukaryotic microalgae can
443 be suitable candidates for expression of foreign P450s.

444

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

461

462 **6. Techniques to engineer algal communities**

463

464 In their natural environment, algae live intimately with many other organisms. These
465 communities exhibit complex interactions including metabolite exchange, cell aggregation and
466 biofilm formation (Jagmann and Philipp, 2014). For example, at least half of all algal species
467 require an exogenous supply of vitamin B₁₂, and bacteria can be a source for this co-factor in
468 return for a carbon source, thus forming a tightly regulated symbiosis (Croft *et al.*, 2005;
469 Kazamia *et al.*, 2012). Further examples include the symbiosis between a unicellular
470 prymnesiophyte alga and a cyanobacterium (UCYN-A) with the former providing a carbon
471 source in return for fixed nitrogen (Thompson *et al.*, 2012), and the dynamic symbiosis between
472 the haptophyte alga *Emiliania huxleyi* and the bacterium *Phaeobacter gallaeciensis* BS107,
473 which can be mutualistic or parasitic according to environmental conditions (Seyedsayamdost *et*
474 *al.*, 2011).

475

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

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

487

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

491

492 **6.1. Genetic engineering for symbiosis**

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

509

510 **6.2. Screening symbioses**

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

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

533

534 **6.3. Environmental selection for symbiosis**

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

551

552 **6.4. Trait-based engineering**

553 Trait-based engineering is the creation of communities by organisms with unique but
554 complementary growth requirements. This is based on the principle of resource-use
555 complementarity, which allows, in principle, individuals to cohabit and lead to an increase in
556 productivity. Examples of trait-based complementarity include the co-culturing of algal species
557 with varying accessory light harvesting pigments maximising the utility of the visible light
558 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 11⁰C to *Chlorella pyrenoidosa*, with a
562 temperature optimum of 40⁰C (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

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

599

600 Algal community research has made great progress in recent years and a number of additional
601 technologies will be utilised alongside the engineering approaches discussed above. Some of
602 these emerging technologies include the monitoring of algal populations through the use of
603 qPCR; crop protection using traditional chemicals such as pesticides and herbicides (McBride *et al.*,
604 2014); the highly parallel screening of beneficial and novel microbial symbiotic interactions
605 through the use of microdroplet technology (Park *et al.*, 2011); development of online
606 databanks or libraries detailing functional traits of bacteria and algae so communities can be
607 designed for optimum trait functionality (Guiry *et al.*, 2014); and accurate modelling of
608 communities to help predict environments that induce and stabilise microbial interactions
609 (Grant *et al.*, 2014; Kim *et al.*, 2008; Klitgord and Segre, 2010).

610

611 **7. Downstream processing of microalgal products**

612

613 As described above, microalgae are light-driven cell factories that can produce a wide spectrum
614 of natural products, or which can be engineered to produce diverse high-value compounds.
615 Extraction and purification of these products are critical processes that can contribute to up to
616 60% of the total production cost (Molina Grima *et al.*, 2003). Research to date has focused on
617 developing methods for downstream processing of algal biomass and for oil extraction and
618 biofuel production. Many have reviewed downstream processing for biofuel production from
619 different algae including *Chlorella*, *Dunaliella*, *Nannochloris*, *Nannochloropsis*, *Porphyridium*,
620 *Schizochytrium* and *Tetraselmis* (Ahmad *et al.*, 2014; Chen *et al.*, 2011; Halim *et al.*, 2012; Kim
621 *et al.*, 2013; Lee *et al.*, 2012; Mata *et al.*, 2010; Pragya *et al.*, 2013; Rawat *et al.*, 2013; Rios *et al.*,
622 2013). In this section, we review the common steps for processing microalgae in order to
623 prepare biofuels, recombinant proteins and other high-value products.

624

625 **7.1 Downstream processing methods for biofuels production**

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

634 to separate biomass from the media and the operating cost that can fluctuate between 20 and
635 30% of the total production cost (Gudin and Therpenier, 1986).

636

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

643

644 An alternative to the dry route is the wet route, in which wet biomass needs to be disrupted first
645 to release the intracellular products. Cell wall composition plays an important role in this route;
646 cell walls from microalgae are typically a thick and rigid matrix of polysaccharides and
647 glycoproteins that require costly downstream processing steps during the production of bio-
648 products (Kim *et al.*, 2013). For example, *Haematococcus pluvialis* possesses a thick cell wall
649 that makes this alga highly resistant to chemical and physical disruption, thus significantly
650 increasing astaxanthin production costs (Hagen *et al.*, 2002). The unit operations for the wet
651 route can be: mechanical (ultra-sonication, high pressure homogenisation, microwave, bead
652 beating, and electroporation); chemical (acids, alkalis and organic solvents); biological
653 (enzymes), or osmotic shock. Selection of methods depends again on the biomass
654 characteristics. Assessment of the dry and wet routes have demonstrated that both have a
655 positive energy balance for production of biofuels. This evidence also shows that the wet route
656 has more potential for high valuable biofuels whereas the dry route seems more attractive for
657 short term biofuel productions (Xu *et al.*, 2011). Whether the biomass goes into the dry or wet
658 route, the physical state of the output is a dried biomass or disrupted concentrate; this stream is
659 processed for lipid extraction. Different methods are available including: (i). Organic solvents:
660 algal oil can be recovered by using solvent such as chloroform, methanol, benzene, diethyl ether
661 and n-hexane. Other metabolites such as β -carotene and astaxanthin are also extracted by
662 solvents (Molina Grima *et al.*, 2003). However, solvent extraction can result in high toxicity if
663 the product is used for animal or human consumption. (ii). Supercritical fluid extraction: this is
664 a green technology based on CO₂ at supercritical conditions used as a non-toxic extracting
665 solvent in order to separate the lipids from matrix (dried biomass or disrupted concentrate). It
666 has been applied to extract lipids and other high valuable compounds from algae, however, the
667 operational cost is high (Mendes *et al.*, 1995; Thana *et al.*, 2008).

668

669 **7.2. Strategies to facilitate downstream processing of recombinant protein produced in**
670 **microalgae**

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

685

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

699

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

709

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

723

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

733

734 **8. Conclusions**

735

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

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

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12. Tables

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

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

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

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

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

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 <i>et al.</i> , 2014; Nalley <i>et al.</i> , 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 <i>et al.</i> , 2012; Nalley <i>et al.</i> , 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 <i>et al.</i> , 2014 and Ortiz-Marquez <i>et al.</i> , 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 <i>et al.</i> , 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 <i>et al.</i> , 2009
POXA1b (from <i>Pleurotus ostreatus</i>)	Laccase	Chiaiese <i>et al.</i> , 2011
Arylsulphatase (ARS1)	Xylanase	Rasala <i>et al.</i> , 2012
carbonic anhydrase 1 (CAH1)	Gaussia luciferase	Lauersen <i>et al.</i> , 2013a
Luciferase	Luciferase	Ruecker <i>et al.</i> , 2008
Carbonic anhydrase 1 (CAH1)	Ice-binding protein	Lauersen <i>et al.</i> , 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.



