Microalgae as Solar-Powered Protein Factories

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Abstract

Microalgae have an enormous ecological relevance as they contribute significantly to global carbon fixation. But also for biotechnology microalgae became increasingly interesting during the last decades as many algae provide valuable natural products. Especially the high lipid content of some species currently attracts much attention in the biodiesel industry. A further application that emerged some years ago is the use of microalgae as expression platform for recombinant proteins. Several projects on the production of therapeutics, vaccines and feed supplements demonstrated the great potential of using microalgae as novel low-cost expression platform. This review provides an overview on the prospects and advantages of microalgal protein expression systems and gives an outlook on potential future applications.

Keywords

Bioreactor • Microalgae • Protein expression platform • Recombinant proteins

16.1 Introduction

In 1982 human insulin was the first protein that was produced in a microbial system and approved for pharmaceutical use. Today recombinant proteins are indispensible in daily life, as they became essential instruments in many industrial sectors like food, fuel, textile and pharma industry. Especially the medical sector is a fast growing market and complex eukaryotic proteins like monoclonal antibodies, hormones and growth factors are needed in high quantity and dominated the biotech field for the last years reaching US

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sales of more than 40 billion dollar in 2011 [1]. Unfortunately, the production costs for most of these proteins are still very high limiting a broad therapeutic use, hence present expression systems need to be improved and novel production platforms should be explored.

Bacteria were the first expression systems for recombinant proteins established already in the 1970s and today still represent the basic workhorse in white biotechnology. They are first choice for production of most industrial enzymes and also 30 % of pharmaceutical proteins are still produced in bacteria as growth rates and expression levels are very high and overall production costs are relatively low [2]. For production of most complex eukaryotic proteins, however, bacterial systems are not feasible as proteins lack eukaryotic post-translational modifications critical for folding, stability and biological activity e.g. disulfide bond formation, phosphorylation and glycosylation being most important modifications [3]. Furthermore, many eukaryotic proteins accumulate in bacteria as insoluble aggregates and need costly downstream processing for purification and refolding. Some of these problems can be overcome by the use of yeasts like *S. cerevisiae*. These fungi are able to perform eukaryotic post-translational modifications and are as cost-effective as bacteria exhibiting high growth rates, high productivity and easy scalability [4]. However, a major problem concerning production of pharmaceutical proteins in yeast is linked to N-linked glycosylation, which differs from mammalian systems and hinders expression of correctly modified complex human therapeutics. Inefficient secretion and proteolysis are further critical issues for high scale expression of complex therapeutics like monoclonal antibodies [5]. Nevertheless, advances in metabolic engineering e.g. towards humanized glycosylation patterns might make yeast more interesting for pharmaceutical protein production in future [6–9]. Other eukaryotic expression systems like insect cells exist but 50 % of licensed pharmaceutical proteins are currently produced in mammalian cells, i.e. hamster cell lines (CHO), human cell lines or hybridoma cell lines in case of monoclonal antibodies [2, 10]. The major advantage of these expression systems is that recombinant proteins produced in mammalian cell lines exhibit correct post-translational modifications needed for therapeutic applications. On the other side, however, mammalian systems are very limited in scale up options and the production process is very cost-intense due to expensive media and complex cultivation processes representing serious bottlenecks for high scale production pipelines [11]. The contamination with human pathogens is a further critical issue necessitating thorough checks on biosafety [12].

To overcome high production costs and reduce the risk of pathogenic contaminations the idea of using plants as expression platform for recombinant proteins became very popular in the 1990s and is often referred to as *molecular farming* [13–16]. Plant-based production is fueled by sunlight, thus the production process itself should be very cheap and agricultural cultivation is well established. Ideally, plants might be used as production platform for edible vaccines making such therapeutics available for large parts of the population and especially in developing countries where they are needed most [17, 18]. In the last 25 years a lot of effort was put into that field of research and trials with engineered plant systems producing humanized glycosylation patterns look promising [19, 20]. However, major hurdles for using whole plants as expression system are low production levels and costly purification processes for products with no oral application. Furthermore, ethical concerns as well as the risk of contaminating food crops make the cultivation of transgenic plants a highly controversial issue [21]. Plant cell culture based systems appear more promising as higher production levels and protein secretion can be achieved and contained reactors allow production with good manufacturing practice (GMP) [22, 23]. In 2012 human glucocerebrosidase produced in a carrot cell culture system was the first plant made pharmaceutical approved by the Food and Drug Administration (FDA) [1]. However, cultivation costs in plant cell cultures are still significant.

16.2 Algae as Bioreactor for Recombinant Protein Production

Algae are solar-powered like plants and especially unicellular microalgae that possess high photosynthesis rates and yield biomass much faster than plants are interesting for diverse biotechnological applications. Many microalgae species provide valuable natural compounds such as vitamins, pigments, proteins and lipids and have been used in cosmetic, food and veterinary industry for many years [24, 25]. Microalgae attract currently much attention in the biofuel sector as many species possess high lipid content and might provide a sustainable and cheap source for biodiesel in future [26–29]. Still underestimated though, is the idea of using microalgae as expression platform for recombinant proteins. Microalgae can be cultivated with low costs needing basically water and sunlight and combine rapid growth rates, easy handling and high scale up capacity with the advantages of eukaryotic expression systems [30–33]. The genome sequence of different microalgae species became available within the last years and basic genetic tools like stable transfection of the nucleus and chloroplast genome and inducible promoter systems were established to express recombinant proteins within different cellular compartments or target proteins for secretion into the culture medium. Compared to complex systems like plants or mammalian cells, microalgae are very robust and easily accessible for genetic manipulation making rapid high-throughput analysis possible. Many microalgae are used as food source and are regarded as safe as they contain no harmful components and are no host for human pathogens. Hence, the expression of therapeutic proteins and also oral application of whole cell extracts represent a promising option. Within the last years different therapeutic proteins like monoclonal antibodies, immunotoxins, subunit vaccines and feed supplements have been expressed in microalgae [31]. Most of these studies focused so far on the green alga *Chlamydomonas reinhardtii*, which is the model alga in basic research and was the first microalga to be sequenced and accessible for genetic engineering. At present *C. reinhardtii* is mainly used for recombinant protein expression in the chloroplast, but also other algal systems like *Dunaliella* and *Chlorella* species and the diatom *Phaeodactylum tricornutum* are now explored and especially *P. tricornutum* reveals great potential in using alga for expressing recombinant proteins from the nucleus genome. This review highlights recent progress in using microalgae as expression system for recombinant proteins and gives an overview on general concepts and practical considerations.

16.3 Pro- and Eukaryotic Expression Traits Within One Cell

In contrast to most other expression systems microalgae provide the opportunity to express recombinant proteins either from the nucleus genome in a eukaryotic environment or in an "advanced" prokaryotic milieu within the chloroplast. Most research so far focused on expression in the chloroplast as in the model alga *C. reinhardtii* higher expression levels were observed for the chloroplast than for the nucleus genome ranging from 0.1 to 5 % of total soluble protein and even 21 % in one study (Table 16.1). Another interesting feature for protein expression within the chloroplast is the fact that this originally prokaryotic organelle, unlike bacteria, harbors an advanced set of chaperons and enzymes to form disulfide bonds [73, 74]. Complete IgG antibodies and special variants like dimeric single chain antibodies can be produced in the chloroplast of *C. reinhardtii* and are fully assembled and able to bind to the target antigens [36, 39]. Recently, it was shown that the chloroplast is also interesting for the production of immunotoxins, chimeric proteins that consist of an antigen-binding domain fused to a eukaryotic toxin like PE40 or gelonin [37, 38]. As these components are toxic for eukaryotes that kind of therapeutics normally have to be produced in bacterial systems with the drawback that protein complexity is very limited. In the chloroplast, however, complex divalent

Table 16.1 Overview of algal produced therapeutics, feed supplements and other recombinant proteins (Modified and updated from Rasala et al. [31])

Recombinant protein		Algae species	Expression site	Expression level	Genome, promoter	Comments	Reference
Antibodies							
Human IgG αHBsAg	Antibody against Hepatitis B Virus surface protein	*P. tricornutum*	Intracellular (ER)	~9 % TSP, 22 mg/g dw	Nucleus, NR	IgG antibodies are fully assembled and recognize the target antigen; high levels of recombinant protein were obtained without large scale screening or genetic engineering of wild type cells	[34]
			Secreted	2.5 mg/L		Fully assembled and functional IgG antibodies are secreted efficiently into the culture medium; rarely other proteins were secreted hence the antibody in the media was already relatively pure	[35]
Human IgG αPA83	Antibody against Anthrax	*C. reinhardtii*	Intracellular (chloroplast)	100 µg/g dw	Chloroplast, *psbA*	Assembled and functional IgG antibodies accumulate in the chloroplast demonstrating that this prokaryotic organelle is able to fold also complex eukaryotic molecules	[36]
Immunotoxin αCD22-PE40	scFv antibodies against CD22 coupled to PE40	*C. reinhardtii*	Intracellular (chloroplast)	0.2–0.4 % TSP	Chloroplast, *psbA*	Mono- and dimeric single chain immunotoxins were able to bind and kill B cell lymphoma cells in vitro; anti-tumor activity was observed in mice	[37]
Immunotoxins αCD22-Gelonin	scFv antibodies against CD22 coupled to Gelonin	*C. reinhardtii*	Intracellular (chloroplast)	0.1–0.7 % TSP	Chloroplast, *psbA*	Mono- and dimeric single chain immunotoxins were able to bind and prevent proliferation of B-cell lymphomas; the inhibitory effect of dimeric immunotoxins was 15 to 25-fold higher compared to the monomeric form	[38]
lsc αHSV glycoprotein D	Large single-chain antibody against Herpes simplex virus glycoprotein D	*C. reinhardtii*	Intracellular (chloroplast)	n.s.	Chloroplast, *atpA*, *rbcL*	First report on antibody production in a microalga; Large single chain antibodies were expressed in the chloroplast and were proven to assemble as dimer that binds to target antigen	[39]

Nanobodies αBoNT/A	Variable domains of camelid heavy chain antibodies against botulinum neurotoxin A	*C. reinhardtii*	Intracellular (chloroplast)	5 % TSP	Chloroplast, *psbA*	Nanobodies bound to BoNT/A in ELISA and were capable of protecting rat neurons from BoNT/A inactivation; nanobodies stayed intact within the stomach and small intestine of mice after oral delivery of transgenic algae	[40]
Vaccines							
D2-CTB	D2 fibronectin binding domain of S. aureus fused to ß-subunit of Cholera toxin	*C. reinhardtii*	Intracellular (chloroplast)	0.7 % TSP 1.6 mg/g dw	Chloroplast, *rbcL*	Oral vaccinated mice show mucosal and systemic immune response and are protected from lethal *S. aureus* infections; lyophilized vaccine is active for at least 1.5 years at room temperature	[41]
AMA1- and MSP1-GBSS	*P. berghei* Apical Major Antigen 1 and Major Surface Protein 1 fused to the algal granule bound starch synthase	*C. reinhardtii*	Intracellular (chloroplast)	0.2–1 µg/mg starch	Nucleus, *hsp70A-rbcS2*	Malaria vaccines were targeted to starch granules; oral as well as intraperitoneally vaccination led to reduced parasitemia in mice; MSP1 elicited IgG antibodies inhibiting intra-erythrocytic asexual development of different Plasmodium species *in vitro*	[42]
Pfs25, Pfs28	*P. falciparum* surface proteins 25 and 28	*C. reinhardtii*	Intracellular (chloroplast)	0.5 %, 0.2 % TSP	Chloroplast, *psbA*	Both antigens are soluble and correctly folded; algal produced Pfs25 is shown to elicit antibodies with transmission blocking activity in mice	[43]
Psf25-CTB	*P. falciparum* surface protein 25 fused to the β-subunit of cholera toxin	*C. reinhardtii*	Intracellular (chloroplast)	0.09 % TSP	Chloroplast, *psbA*	Oral vaccination of mice elicited CTB and Pfs25 specific IgA antibodies but no transmission blocking IgG antibodies; CTB-Pfs25 is stable in freeze-dried algae at 4–22 °C for at least 6 month	[44]
Pfs48/45 C-term	*P. falciparum* surface protein 48/45, C-terminal domain	*C. reinhardtii*	Intracellular (chloroplast)	n.s.	Chloroplast, *psbA*, *psbD*	C-terminal part of Pfs 48/45 is recognized by transmission blocking antibodies	[45]
Bovine LFB-dsRed	Bovine lactoferricin, anti-microbial peptide	*N. oculata*	n.s.	n.s.	Nucleus, *hsp70A-rbcs2* (*C. reinhardtii*)	Oral vaccination of medaka fish resulted in significant survival rate after *Vibrio parahaemo-lyticus* infection	[46]

(continued)

Table 16.1 (continued)

Recombinant protein		Algae species	Expression site	Expression level	Genome, promoter	Comments	Reference
VP28 (WSSV)	Vaccine against White Spot Syndrome Virus	*D. salina*	Intracellular (n.s.)	0.0003 % TSP	Nucleus, Ω TMV	Oral vaccination of crayfish elicited significant survival rate after WSSV infection	[47]
HBsAg	Hepatitis B Virus surface antigen	*P. tricornutum*	Intracellular (ER)	0.7 % TSP	Nucleus, NR	Algal produced HBsAg is recognized by αHBsAg antibodies in inhibitory ELISA	[34]
		D. salina	Intracellular (n.s.)	1.6–3.1 ng/mg TSP	Nucleus, ubil-Ω	First report on recombinant protein expression in *D. salina*; no assays on functionality provided	[48]
E7 HPV-16	E7 oncogene of Human Papilloma Virus 16	*C. reinhardtii*	Intracellular (chloroplast)	0.12 % TSP	Chloroplast, *psbD*	Subcutaneous vaccination of mice elicits specific IgG response, T cell proliferation and tumor protection	[49]
VP1 (FMDV)-CTB	Vaccine against Foot-and-Mouth-Disease Virus fused to ß-subunit of Cholera toxin	*C. reinhardtii*	Intracellular (chloroplast)	3 % TSP	Chloroplast *chlL*	Algal produced protein was shown to bind to GM1 ganglioside receptors; no in vivo studies for oral vaccination were performed	[50]
VP28 (WSSV)	Vaccine against White Spot Syndrome Virus	*C. reinhardtii*	Intracellular (chloroplast)	0.2–21 % TCP	Chloroplast, *psbA*, *atpA*	Highest expression level for microalgal chloroplast transfection reported so far; large scale screens revealed highly variable expression levels with same transfection parameters	[51]
E2 (CSFV)	Vaccine against Classical Swine Fever Virus	*C. reinhardtii*	Intracellular (chloroplast)	1.5–2 % TSP	Chloroplast, *rbcL*	Subcutaneous immunization with algal extract elicits serum specific antibodies in mice	[52]
Angiotensin II-HBcAg	Vaccine against hypertension	*C. reinhardtii*	Intracellular (n.s.)	0.05 % TSP	Nucleus, CaMV35S	Angiotensin II was expressed in fusion with Hepatitis B Virus nucleocapsid antigen	[53]
Other therapeutic proteins							
hGH	Human growth hormone	*C. vulgaris*, *C. sorokiniana*	Secreted	200–600 ng/mL	Nucleus, CaMV35S, *rbcS2*	One of the first reports on expression of a therapeutic protein in micro-algae; no stable transformants	[54]

M-SAA	Bovine mammary-associated serum amyloid, gut active therapeutic	*C. reinhardtii*	Intracellular (chloroplast)	5 % TSP	Chloroplast, *psbD*, *psbA*	Purified algal produced protein stimulated mucin expression in cell culture demonstrating potential as edible gut active therapeutic acting in prophylaxis of bacterial and viral infections	[55]
Human Epo	Erythropoietin, therapeutic for anemia treatment	*C. reinhardtii*	Secreted	~100 µg/L	Nucleus, *hsp70A-rbcS2*	Epo is secreted into the culture medium when fused with an endogenous signal peptide; intronic sequences enhance the expression of recombinant proteins	[56]
Epo, interferon-ß, proinsulin, VEGF, HMGB1, 10FN3, 14FN3	Therapeutics for diverse treatments	*C. reinhardtii*	Intracellular (chloroplast)	Up to 3 % TSP	Chloroplast, *psbA*, *atpA*	All algal produced proteins were soluble and showed biological activity; expression level was enhanced under psbA promoter	[57]
Rabbit NP-1	Rabbit neutrophil peptide 1, anti-microbial peptide	*C. ellipsoidea*	Intracellular (n.s.)	n.s.	Nucleus, ubiquitin-Ω	Anti-microbial activity of algal extract against different bacteria and fungi was confirmed	[58]
		C. ellipsoidea	Intracellular (n.s.)	11.4 mg/L culture	Nucleus, ubiquitin-Ω	Purified NP-1 protein exhibited strong anti-microbial activity against *E. coli*	[59]
Human TRAIL	TNF-related apoptosis-inducing ligand induces apoptosis in various tumor cells	*C. reinhardtii*	Intracellular (chloroplast)	0.43–0.67 % TSP	Chloroplast, *atpA*	TRAIL is expressed as soluble protein	[60]
hGAD65	Autoantigen human glutamic acid decarboxylase 65; marker for diabetes I diagnostics	*C. reinhardtii*	Intracellular (chloroplast)	0.25–0.3 % TSP	Chloroplast, *rbcL*	Purified algal produced hGAD65 induced proliferation of spleen cells from NOD mice and showed immunoreactivity to diabetic sera	[61]
SKTI	Soybean kunitz trypsin inhibitor, therapeutic protein with anti-viral and anti-cancer activity	*D. salina*	Intracellular (n.s.)	0.68 % TSP	Nucleus, 35S	Stable expression; no assays on functionality	[62]

(continued)

Table 16.1 (continued)

Recombinant protein		Algae species	Expression site	Expression level	Genome, promoter	Comments	Reference
apcA + apcB	Allophyocyanin, potential therapeutic against S-180 carcinoma	*C. reinhardtii*	Intracellular (chloroplast)	2–3 % TSP	Chloroplast, *atpA*	Both subunits were expressed; assembly of the protein complex not verified	[63]
Feed supplements							
fGH	Flounder growth hormone, feed additive for aquaculture	*C. ellipsoidea*	Intracellular, (n.s.)	400 µg/L culture	Nucleus, CaMV35S	Flounder fry fed on transformed *Chlorella* revealed 25 % growth increase after 30 days	[64]
ypGH	Yellowfin porgy growth hormone, feed additive for aquaculture	*N. oculata*	Intracellular (n.s.)	0.27–0.41 µg/mL culture	Nucleus, *hsp70A-rbcS2* (*C. reinhardtii*)	Red tilapia fry fed on transformed *N. oculata* showed 212 % weight increase and 71 % length increase after 4 weeks	[65]
Phytase AppA	Feed additive to increase phytate phosphorus utilization	*C. reinhardtii*	Intracellular (chloroplast)	n.s.	Chloroplast, *atpA*	Orally delivered algae extract reduced fecal phytate excretion in broiler chicks	[66]
Endo-β-1,4-xylanase	Feed additive for hemicellulose breakdown	*C. reinhardtii*	Intracellular (cytosol) Secreted	0.25 % TSP n.s.	Nucleus, AR4	Expression of xyn1 as fusion protein with the selection marker led to 100-fold increase of xyn1 levels; self-cleaving peptide allowed production and secretion of autonomous xyn1 functional in activity assays	[67]
Xylanase, α-Galactohydrolase, phytase	Div. feed additives	*D. tertiolecta*	Intracellular (chloroplast)	n.s.	Chloroplast, *psbD*	Algal produced enzymes were functional in activity assays and expression was compared to *C. reinhardtii* expression; first description of *D. tertiolecta* chloroplast transformation protocol	[68]

hu Sep15	Selenoprotein, nutritional supplement	*C. reinhardtii*	Intracellular (n.s.)	n.s.	Nucleus, *hsp70-rbcS2*	Cell viability was not affected	[69]
Bioremediation and environmental control							
Metallothionein-like protein (*F. rubra*)	Metal binding	*C. reinhardtii*	Intracellular (chloroplast)	n.s.	Chloroplast, *atpA*	Transgenic algae showed higher cadmium binding capacity and tolerance; IC_{50} of Cd^{2+} was 55.43 % enhanced compared to wild type strain	[70]
TMOF	Trypsin-modulating oostatic factor, insecticide against mosquito larvae	*Chlorella* sp.	Intracellular	?	?	Feeding to mosquito larvae caused larval mortality	[71]
hMT-2	Human metallothionein-2, protection against UV-radiation	*C. reinhardtii*	Intracellular (chloroplast)	n.s.	Chloroplast, *psbA*	Expression of hMT-2 conferred enhanced resistance to UV-B exposure	[72]

CTB cholera toxin subunit B, *dw* dry weight, *ER* endoplasmic reticulum, *NR* nitrate reductase, *n.s.* not specified, *TCP* total chloroplast protein, *TSP* total soluble protein

immunotoxins can be produced showing enhanced cytotoxicity compared to the monovalent form [37]. Beside antibodies, further complex structured eukaryotic proteins like *Plasmodium falciparum* peptides interesting as malaria transmission-blocking vaccine were shown to be expressed as soluble and correctly folded proteins within the chloroplast [43–45]. Most proteins of *P. falciparum* are not glycosylated and expression in classical eukaryotic expression systems is problematic [75]. As chloroplasts harbor no glycosylation machinery, proteins expressed within the chloroplast remain aglycosylated, which represents a further advantage in this special case preventing allergic reactions to foreign glycoprofiles. Feeding mice with freeze-dried algae expressing *P. falciparum* surface protein 25 elicited specific IgA and IgG antibodies demonstrating that algae are like plants highly interesting in terms of oral vaccination [44]. Protein storage within the chloroplast might come along with enhanced protein protection as in another study a chloroplast expressed *Staphylococcus aureus* protein was shown to be potent in lyophilized algae for at least 20 month at room temperature and protected against proteolysis within a stomach-like pepsin environment [41]. In summary, the algal chloroplast is of special interest for the expression of complex aglycosylated or toxic proteins that cannot be produced in bacterial systems. Oral delivery might represent an ideal application form for different subunit vaccines as well as for some feed supplements [31, 76] (Fig. 16.1).

Protein expression from the nucleus genome provides the advantage of eukaryotic post-translational modifications, which are important for conformation, stability and activity of most eukaryotic proteins. Nevertheless expression from algal nucleus genomes was so far mostly disregarded as most studies in *C. reinhardtii* showed rather low expression levels ranging from 0.05 to 0.25 % of total soluble protein (Table 16.1). Research especially from the last years, however, revealed that this topic deserves more attention and that it is worth to test other algal species as well. In 2011 a human IgG antibody against the Hepatitis B Virus Surface protein was expressed in the diatom *P. tricornutum* and accumulated to ~9 % of total soluble protein—corresponding to about 22 mg antibody per 1 g dry weight [34]. Interestingly, further analyses revealed that the deletion of an initially used ER-retention signal even led to secretion of the fully assembled and functional IgG antibodies into the culture medium [35]. As *P. tricornutum* does not seem to secrete many proteins by natural means, the antibodies were remarkably pure and accumulated in this assay without further engineering to about 2.5 mg/L medium [35]. The secretion of proteins into the culture medium is of course a great benefit simplifying downstream processes and reducing cost-intense purification steps incredibly. Recent work demonstrated that also engineered *C. reinhardtii* cells can secrete high amounts of recombinant protein into the medium as shown exemplarily for the reporter protein gLuc (*Gaussia princeps* luciferase) yielding approximately 10 mg per liter culture medium in case of an engineered cell wall deficient strain [77]. Altogether, research especially from the last years reveals the potential of microalgae as solar-driven expression system capable to produce complex eukaryotic proteins that can be secreted efficiently into the culture media. The chloroplast as an advanced prokaryotic compartment represents a further expression site within the algal cell interesting for special products like complex aglycosylated proteins or eukaryotic toxins (Fig. 16.1).

16.4 Genetic Engineering for Enhanced Protein Expression in Microalgae

Transformation of the green alga *C. reinhardtii* was established about 25 years ago [78, 79] and today many other algae, *e.g.*, the green algae species *Dunaliella* and *Chlorella* as well as Heterokontophytes like *P. tricornutum*, *Thalassiosira pseudonana* and *Nannochloropsis* species can be routinely transformed. Microparticle bombardment is widely used for introducing new genes into the nucleus or chloroplast genome but also electroporation can be applied for most species

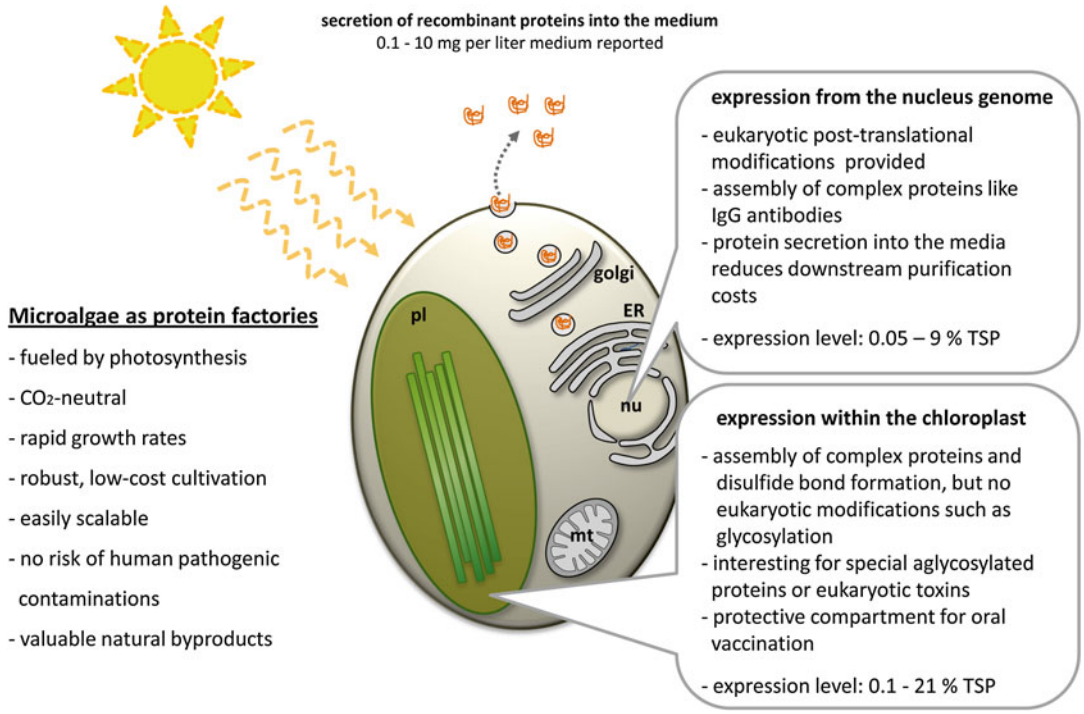

Fig. 16.1 Microalgae as solar powered expression system for recombinant proteins. Microalgae possess rapid growth rates, are very robust as well as easily scalable and might provide low-cost production of different therapeutic proteins as well as feed supplements in future. Proteins can be expressed either from the nucleus genome or within the chloroplast. Both options provide advantages depending on the application and the protein of interest. Most studies concentrated on the green alga *C. reinhardtii* so far but also other systems like *P. tricornutum*, *Chlorella* and *Dunaliella* species start to get explored and might provide some advantages. *ER* endoplasmatic reticulum, *mt* mitochondrion, *nu* nucleus, *pl* plastid

(Table 16.2) representing a more economical approach for large-scale assays. For detailed reviews on algal transformation techniques and selectable marker genes see Gong et al. [100], Leon-Banares et al. [101], and Potvin and Zhang [102]. In general, chloroplast transformation techniques take advantage of homologous recombination to integrate gene constructs into a specific genomic context [68, 78, 84]. Integration into the nucleus genome occurs in case of most transformation techniques random via non-homologous end joining. Only for *N. oceanica* it was shown so far that homologous recombination works also for the nucleus genome [86] representing a great benefit as the integration context can influence gene expression considerably. Targeted gene knock out is a further great advantage as it offers many engineering options possible in other algae like *C. reinhardtii* and *P. tricornutum* only via RNA interference at the moment [103, 104]. Very recently, however, also in these organisms some progress has been made for targeted insertion and gene knock out regarding the nuclear genome as engineered zinc-finger nucleases have been shown to allow specific DNA insertion in *C. reinhardtii* [105] and also for *P. tricornutum* genomic modifications via meganucleases and TALE nucleases have been reported [106, 107].

Like in other expression systems also for microalgae the adaption of DNA-sequences to the host specific codon-usage is beneficial and turned out to be very important at least for *C. reinhardtii* (with a GC-content of 61 %) in order to obtain detectable protein levels [108–110]. For the diatom *P. tricornutum*, which possesses a

typical eukaryotic GC-content of 48 % this might be less critical. Foreign genes can also be expressed without codon optimization and even bacterial enzymes can be expressed from the nucleus genome of *P. tricornutum* as shown in a study on bioplastic production [111].

The choice of the promoter is a further critical feature for efficient protein expression and regarding expression from the chloroplast genome 5′UTRs of *psbA*, *psbD*, *atpA* and *rbcL* are most frequently used (Table 16.2). The *psbA* promoter is one of the most efficient in *C. reinhardtii*, at least when the endogenous *psbA* gene is deleted [51, 55, 112], and expression levels of 0.1–21 % TSP were observed (Table 16.1). Photosynthesis can be restored in these strains by reintroducing an attenuated *psbA* gene in a different gene context [55]—however production rates decreased and strains were still unviable for commercial scale. Recently, *psbA* complementation was optimized, though, allowing high phototrophic growth rates while maintaining high production levels [113]. For recombinant protein expression from the nucleus genome of *C. reinhardtii* the 5′UTRs of *hsp70A*, *psaD*, *rbcS2* or the fusion *hsp70A-rbcS2* have been used in most assays (Table 16.1), however expression levels are very low, which might be a problem of gene silencing. Best expression levels were observed in a genetic screen of a mutant library accumulating recombinant protein to 0.2 % of TSP [114]. For a more detailed review on chloroplast and nuclear promoter studies in *C. reinhardtii* see Specht et al. [115]. The diatom *P. tricornutum* came into focus as expression system for recombinant proteins only very recently and therefore less data is available, but much higher nuclear expression levels than in *C. reinhardtii* were observed in initial tests when using the inducible promoter of the endogenous nitrate reductase (8.7 % of TSP and 0.7 %) [34, 35]. This promoter was established previously in the diatom *T. pseudonana* and can be tightly controlled via ammonia/nitrate in the culture medium [116]. The light inducible promoters of *fcpA* and *fcpB* of *P. tricornutum* are frequently used in basic research but no quantifications on expression levels are available so far.

Further strategies that have shown to enhance nuclear expression of recombinant proteins in microalgae include the insertion of introns from native genes [56, 117] and the expression as transcriptional fusion to an antibiotic resistance gene [67, 109]. Both strategies were applied in *C. reinhardtii* and might help to counteract transgene silencing. For the industrial enzyme xylanase it was exemplarily shown that expression levels could increase to 100-fold when expressing this protein in fusion with the coding region for the selection marker. The insertion of a viral self-cleavable peptide guaranteed a discrete protein as final product, which could also be secreted when including an endogenous signal peptide [67].

Altogether, the molecular toolbox for microalgae was more and more extended within the last years and not only the model alga *C. reinhardtii* but also other species like *P. tricornutum* and *N. oceanica* start to get explored revealing beneficial traits like higher expression levels from the nucleus genome or targeted gene insertion, respectively. In future, additional engineering might help to increase expression levels and provide modifications such as humanized glycosylation profiles—a critical quality attribute with the basics just being about to get investigated in microalgae [118–120]. In future, also other algal species like *Chlorella* might become interesting as expression platform as *Chlorella* possesses very rapid growth rates and provides valuable natural compounds interesting for food, cosmetic and biodiesel industry (Table 16.2). Compared to other microalgae, however, molecular tools for the expression of recombinant proteins in *Chlorella* are still very limited and studies on recombinant protein expression are still in a very early stage.

16.5 Expression of Protein Complexes

Multi-protein complexes are essential for many cellular processes and for their structural and biochemical analyses as well as for therapeutic applications large-scale production is necessary. However, the heterologous expression of protein

Table 16.2 Selected microalgal species interesting for the production of recombinant proteins

Microalga	Natural habitat	Relevant products	Genome sequence	Transformation techniques	Molecular tools
Phaeodactylum tricornutum (Heterokontophyta)	Sea water	Omega-3-fatty acids (nutrition); Lipids (biofuel)	+ [80]	Nucleus: biolistic [81]; electroporation [82, 83]; chloroplast: electroporation [84]	Inducible promoters, gene knock-down via antisense RNA. Gene knockout via TALEN, secretion of recombinant proteins confirmed
Nannocloropsis oceanica (Heterokontophyta)	Sea water	Polysaccharides, proteins, vitamins (nutrition); Lipids (biofuel)	+ [85]	Nucleus: electroporation [86]; electroporation [65]; electroporation [87]	Inducible promoters, gene knockout via homologous recombination
N. oculata			In progress		
N. gaditana			+ [87]		
Thalassiosira pseudonana (Heterokontophyta)	Sea water	Silica (nanotechnology)	+ [88]	Nucleus: biolistic [89]; biolistic [79]; glass beads [91]; electroporation [92]; *Agrobacterium* [93]; chloroplasts: biolistic [78]	Inducible promoters, gene knock-down via antisense RNA
Chlamydomonas reinhardtii (Chlorophyta)	Fresh water		+ [90]		Inducible promoters, gene knock-down via antisense RNA, Gene knockout via ZFN, secretion of recombinant proteins confirmed
Chlorella vulgaris (Chlorophyta)	Fresh water	Polysaccharides, proteins, vitamins (cosmetics, nutrition); Lipids (biofuel)	In progress *C. variabilis* [94]	Nucleus: electroporation [95]	Limited tools available, in progress, secretion of recombinant proteins confirmed
C. ellipsoidea			–	Nucleus: electroporation [58]; biolistic [96]	
C. sorokiniana			–		
Dunaliella salina (Chlorophyta)	Sea water	β-carotene and other carotenoids (cosmetics, nutrition); Lipids (biofuel)	In progress	Nucleus: electroporation [48], biolistic [97], glass beads [98]	Limited tools available, in progress
D. tertiolecta			–	Nucleus: electroporation [99]; chloroplast: biolistic [68]	

complexes consisting of multiple protein subunits still represents a great challenge. Historically, protein subunits were initially expressed separately, purified and reconstituted *in vitro*, but of course this is problematic in many cases as proteins form aggregates, have to be refolded and additional factors that might be needed for the assembly process are not present. Today co-expression and complex formation within the cell is favored and different complexes have been successfully expressed in bacteria, yeast, insect cells, plants as well as some mammalian cell lines. Different strategies are applied like the use of multiple expression vectors, plasmids with multiple cloning sites or polycistronic units in case of bacteria, as well as the expression of fusion proteins (see Kerrigan et al. [121] for a review). Data on the expression of protein complexes in microalgae is so far very limited but as mentioned previously the efficient expression of completely assembled IgG complexes consisting of two heavy and two light chains was shown to be feasible in *P. tricornutum* [34, 35]. The complex is assembled within the ER and can be secreted into the culture medium. Also in the chloroplast of *C. reinhardtii* completely assembled IgG complexes can be produced. The expression of other protein complexes has yet not been tested in microalgae; however, the molecular tools for the co-expression of multiple protein subunits are basically available. In *P. tricornutum* multiple plasmids can be co-transformed [122]; this technique was applied for example to introduce three bacterial enzymes for production of the bioplastic PHB [111]. As different resistance markers are available for many algae sequential transfections can be performed as well. Also plasmids with multiple cloning sites like the plasmid pPha-DUAL-[2xNR], which contains two multiple cloning sites, both under the control of a nitrate-inducible promoter, are available and have been used for the expression of IgG antibodies in *P. tricornutum* [34]. In *C. reinhardtii* it was shown very recently that also fusion proteins separated by viral self-cleaving sequences can be expressed from the nucleus genome leading to separate gene products [67, 123]. Hence, basic tools for the expression of multiple protein subunits in microalgae are available and it will be highly interesting to start expression assays of multi-subunit complexes in future.

16.6 Algal Produced Therapeutics, Feed Supplements and Other Proteins

Within the last 15 years a broad spectra of recombinant proteins has been produced in different microalgae ranging from therapeutic proteins like antibodies, vaccines, hormones to feed supplements and industrial relevant enzymes (Table 16.1). The following sections provide an overview on different studies in the field.

16.6.1 Antibodies

Therapeutic antibodies currently represent the best-selling class of biologics with US sales reaching 20.3 billion dollar in 2011 [1]. Antibody production is based on mammalian cell culture, which is very expensive and therefore alternative expression systems are highly desirable. In 2003, a large single-chain antibody against Herpes simplex virus glycoprotein D was the first antibody to be expressed in an algal system [39]. This antibody was produced in the chloroplast of *C. reinhardtii* and was proven to assemble as dimer that binds to its target antigen. Further studies on antibody expression include the production of a complete human IgG antibody against anthrax in the chloroplast of *C. reinhardtii* [36] and the expression of a human IgG antibody against the Hepatitis B Virus surface protein. In contrast to previous studies, the latter was expressed from the nucleus genome in the diatom *P. tricornutum* and was produced very efficiently with 9 % of total soluble protein [34]. The deletion of a retention signal led to efficient secretion of the fully assembled and functional antibodies into the culture medium [35]. As rarely other proteins were detected in the media, the antibody was relatively pure without further treatment. In 2013, the production of mono and dimeric single chain immunotoxins was shown to be feasible in

the chloroplast of *C. reinhardtii*. These algal produced antibody variants were able to bind and kill B cell lymphoma cells *in vitro* and showed anti tumor activity in mice [37, 38]. Very recently, also camelid antitoxins were expressed in the chloroplast of *C. reinhardtii*. The algal produced nanobodies against botulinum neurotoxin A showed *in vitro* activity in toxin protection assays and remained intact in the gastrointestinal tract of mice fed with antitoxin-producing microalgae [40]. Altogether, these studies demonstrate the great potential of using microalgae as expression system for antibodies and further engineering concerning productivity and glycopatterns might make microalgae a low-cost production platform with little risk for pathogenic contaminations in future.

16.6.2 Vaccines

Protein based vaccines represent a further important class of therapeutics and microalgae might be of interest especially for the production of oral subunit vaccines. Many algal species are used as nutritional supply in food industry, hence complete cell extracts could be administered directly thereby bypassing costly purification steps and facilitating production as well as needle-free application [76]. Different reports on the expression of oral vaccines in microalgae have been published within the last years. In 2010, the D2 fibronectin-binding domain of *S. aureus* was expressed in fusion with the adjuvants CTB (cholera toxin B subunit) in the chloroplast of *C. reinhardtii*. Orally vaccinated mice showed mucosal as well as systemic immune response resulting in protection from lethal *S. aureus* infections [41]. The vaccine was stable within lyophilized algae for at least 20 month at room temperature. Furthermore, stability assays demonstrated that the protein was protected against proteolysis within a stomach-like pepsin environment representing a critical point for absorption within the intestine. The study demonstrates that the algal chloroplast could represent an ideal compartment for the expression of oral vaccines resulting in enhanced antigen stability and protection. In another study *Plasmodium berghei* antigens were targeted to chloroplast starch granules in *C. reinhardtii* [42]. Oral vaccination of mice led to reduced parasitemia and specific IgG antibodies could be detected inhibiting intraerythrocytic asexual development of different Plasmodium species *in vitro*. A further study on the production of a malaria transmission blocking vaccine in the chloroplast of *C. reinhardtii*, the *Plasmodium falciparum* surface protein 25 (Psf25-CTB), demonstrated that the protein is correctly folded and elicits transmission blocking antibodies in mice when injected intraperitoneally [43]. Orally vaccinated mice produced specific mucosal IgA antibodies but no systemic IgG antibody production was observed [44]. Of course, not every vaccine is suitable for oral administration to generate a systemic immune response and also the adjuvants used in this study might not be ideal for stimulating IgG production. But even though oral vaccination is still very limited due to the complexity of the mucosal system, ongoing research will certainly help to overcome present challenges with microalgae representing promising edible, low-cost expression systems [75]. Also in veterinary medicine and especially in aqua culture using microalgae as vaccination vehicle might be of great interest. One of the first reports on the production of oral therapeutics in microalgae is the expression of the anti-microbial peptide bovine lactoferricin in *Nannochloropsis oculata* [46]. Feeding experiments with medaka fish revealed a survival rate of 85 % after *Vibrio parahaemolyticus* infection. In another study the protein VP28 of the White Spot Syndrome Virus was expressed in the green alga *Dunaliella salina*. Even though expression levels were only very low, oral vaccination of crayfish resulted in significant survival rates after white spot syndrome virus (WSSV) infection [47].

Other vaccines that were produced in microalgae but not tested for oral application are the hepatitis B virus surface antigen (HBsAg) produced in the chloroplast of *D. salina* as well as in the endoplasmic reticulum of *P. tricornutum* [34, 48]. Additionally, different *Plasmodium* antigens interesting for malaria control [43, 45] and

proteins of different viruses, *i.e.*, human papilloma virus 16 [49], white spot syndrome virus [51], foot and mouth disease virus [50] and classical swine fever virus [52], were expressed within the chloroplast of *C. reinhardtii* with expression levels of 0.12–21 % of total soluble protein (Table 16.1).

16.6.3 Other Therapeutic Proteins

Human growth hormone (hGH) was one of the first therapeutic proteins that were tested for expression in an algal system. In 1999, when molecular engineering of microalgae was still in the very beginning, Hawkins and colleagues expressed hGH in *Chlorella vulgaris* as well as in *Chlorella sorokiniana*, and showed that the hormone is expressed and secreted into the culture medium, even though transfection was only transient in these initial studies [54]. The bovine mammary-associated serum amyloid protein (M-SAA) is an anti-microbial protein that is found in the colostrum (first milk) of mammals and prevents bacterial infections by stimulating mucin synthesis in the small intestines. M-SAA was shown to be expressed efficiently in the chloroplast of *C. reinhardtii* as bioactive molecule stimulating mucin production in epithelial cell culture [55]. The study demonstrates the great potential of using microalgae for the production of edible gut active therapeutics and only recently, the US company Triton Health and Nutrition started the microalgal production of M-SAA. Further therapeutics that were expressed in algal systems include human erythropoietin, which was produced in the chloroplast of *C. reinhardtii* [57] as well as shown to be secreted when expressed from the nucleus genome [56]. Furthermore, diverse therapeutics such as interferon-β, proinsulin [57] and a marker for diabetes I diagnostics [61] were expressed in the chloroplast of *C. reinhardtii*. All proteins were shown to be biologically active and accumulated to up to 3 % of total soluble protein. The production of anti-microbial peptides was also tested in *D. salina* and *C. ellipsoidea* [58, 59, 62], which might become interesting as an expression system when more molecular tools become available in future.

16.6.4 Animal Feed Supplements

Many algae are used as feed additives since many species are rich in valuable natural compounds like long-chain polyunsaturated fatty acids (PUFAs), carotenoids, vitamins and high quality protein and carbohydrate. The opportunity to combine nutritional supply and direct delivery of recombinant feed additives like growth hormones or dietary enzymes for fiber break down makes microalgae an interesting low-cost expression system in that field. In 2002 the flounder growth hormone (fGH) was expressed in *C. ellipsoidea* and it was shown that flounder fry fed on transformed algae exhibit a 25 % growth increase after 1 month [64]. Promising results were also obtained when feeding tilapia fry with transgenic *N. oculata* cells expressing yellowfin porgy growth hormone (ypGH) [65]. After 4 weeks, a 212 % increase in weight and 71 % increase in length were observed. In another study, a bacterial phytase was expressed in *C. reinhardtii* to facilitate phytate digestion in monogastric animals. Feeding experiments on broiler chicks revealed a reduced excretion of fecal phytate [66]. The production of other phytases and further enzymes used as dietary supplements like α-galactohydrolases and a xylanase was also shown to be feasible in *C. reinhardtii* and *D. tertiolecta* [26].

16.6.5 Bioremediation and Environmental Control

Algae are used in wastewater treatment to provide oxygen supply for bacterial biodegradation and remove inorganic nitrogen and phosphorus [124]. In addition, the removal of heavy metals like cadmium, nickel and zinc was shown for some species [125, 126] and might be enhanced by genetic engineering. In two studies the expression of metallothioneins was assayed in *C. reinhardtii* demonstrating that transgenic cells exhibit

higher cadmium binding capacity and can grow to higher densities at toxic cadmium concentrations [70, 127]. Another study presents an algal-based approach on mosquito control. An insecticide against mosquito larvae acting on trypsin biosynthesis in the mosquito gut was produced in *Chlorella* sp. [71]. Feeding of transgenic algae to mosquito larvae caused larval mortality.

Altogether, a broad repertoire of recombinant proteins like many different therapeutic proteins, feed supplements for animal welfare and proteins interesting for environmental control have been expressed in microalgae (Table 16.1) demonstrating the great potential of microalgal expression systems.

16.7 Conclusions and Perspectives

The demand for recombinant protein therapeutics and industrial enzymes is enormous nowadays and the market for biologics is constantly growing. In future, it will be essential to improve existing expression systems but also to establish novel low-cost production platforms to guarantee affordable products. Microalgae are powered by sunlight, possess rapid growth rates and are genetically well accessible. In recent years significant progress has been made in recombinant protein expression in microalgae and many different projects on the production of therapeutics, vaccines and feed supplements demonstrate the great potential of using microalgae as novel low-cost expression platform. Microalgae are of special interest for the production of complex eukaryotic proteins that currently have to be produced in mammalian cell lines involving high production costs and the risk of human pathogenic contaminations. Especially the production of IgG antibodies that were shown to be secreted into the algae culture medium could offer an attractive option in future. The expression of oral vaccines and feed supplements represent a further promising approach. As many microalgae are edible and harbor valuable vitamins, proteins and fatty acids the complete cell extract could be administered directly saving expensive purification costs. Furthermore, the algal chloroplast represents an interesting expression platform especially for the production of complex aglycosylated proteins or eukaryotic toxins coupled to complex proteins like bivalent immunotoxins that are difficult to produce in other expression systems.

Concerning commercial applications, the production of recombinant proteins in microalgae is still at an early stage. Comparable to the establishment of previous expression systems it will be necessary to enhance productivity and secretion efficiency and establish glycoengineering approaches to provide therapeutics with humanized glycoprofiles in future. In addition to *C. reinhardtii* other microalgal strains like *P. tricornutum*, *N. oceanica* and *Chlorella* should be included for detailed expression studies as it was started in some projects only recently. Higher expression levels, exclusive molecular tools and higher growth rates could be some of the advantages. As microalgae biotechnology became very popular within the last years, a lot of effort has also been put into the development of algae photobioreactors. Promising solutions for large-scale cultivation can be provided by now [128] representing a further important aspect concerning technology transfer to industrial scale in future. Triton Health and Nutrition (USA) and Algenics (France) belong to the first companies that started to use microalgae as expression platform for recombinant proteins. But certainly other companies will follow soon considering the great potential of using microalgae as solar fueled, low-cost expression system.

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