Algal Research 33 (2018) 109–117

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Fed-batch mixotrophic cultivation of *Chlamydomonas reinhardtii* for high-density cultures

Francis J. Fields*, Joseph T. Ostrand, Stephen P. Mayfield

The California Center for Algae Biotechnology, Department of Biological Sciences, University of California San Diego, 9500 Gilman Dr., La Jolla, California 92093, United States

ARTICLE INFO

Keywords:
Chlamydomonas reinhardtii
Heterotrophic
Fed-batch
High density
Recombinant protein production
Primary metabolites

ABSTRACT

The green algae *Chlamydomonas reinhardtii* is a model organism that has been proposed as a potential production platform for high-value recombinant products, but to date, there has been little development of a commercially viable cultivation process. Here we demonstrate a novel mixotrophic fed-batch cultivation strategy in which average biomass density, productivity, and total amount of recombinant GFP significantly increases. Systematic feeding of acetic acid and nutrients into a bioreactor resulted in culture densities increasing from an initial 0.45 ± 0.03 to $23.69 \pm 0.5\ g\ L^{-1}$ AFDW after 168 h at an average productivity of $181.01 \pm 8.8\ mg\ L^{-1}\ h^{-1}$, a 10-fold increase in comparison to traditional batch cultures. GFP expression was low under both conditions, but fed-batch cultivation resulted in a 2.5-fold increase in total GFP upon culture termination. The effect of fed-batch cultivation on lipid composition and primary metabolites was investigated and elevated levels of the osmoregulatory molecules proline and glycerol were found, suggesting that salt accumulation may have increased over time in fed-batch cultures and eventually limited growth.

1. Introduction

Chlamydomonas reinhardtii is a eukaryotic single-celled soil-dwelling green microalgae (Chlorophyta). For over fifty years it has been a premier model organism used to understand the biology, physiology, and genetics of algae, as well as a model organism to understand photosynthesis in both plants and algae [1,2]. One fundamental reason this species became a model organism is its ability to heterotrophically metabolize acetate, allowing for growth in complete darkness and the isolation of non-photosynthetic mutants. The cumulative research into this organism has resulted in a significant understanding of the algal cell cycle, metabolism, genomics, and genetic circuits [3]; making *C. reinhardtii* potentially the best studied and well characterized species of algae. Due to this extensive research, *C. reinhardtii* has been targeted for use in biotechnology, most frequently proposed as a potential production platform for high-value recombinant products for medical, nutritional, and industrial use [4,5]. Additionally, a recently published toxicological evaluation demonstrated that the consumption of *C. reinhardtii* biomass presents no health concerns, thus opening the door for *C. reinhardtii* biomass to be used as a nutritional additive in feeds and foods [6].

The molecular tools developed for *C. reinhardtii* have allowed for facile transformation of both the chloroplast and nuclear genomes [5], and to date, over one hundred high-value recombinant proteins have been expressed in *C. reinhardtii*, including multiple therapeutic proteins, such as antibodies and immunotoxins, vaccines, industrial enzymes, animal feeds, and nutritional supplements [4,7–11]. While the techniques for nuclear transformation are well established in *C. reinhardtii*, the lack of directed homologous recombination for transgene integration results recombinant protein expression being strongly influenced by positional effects in the genome, leading to low and inconsistent protein accumulation levels from nuclear transformants [5,10]. Unlike gene integration in the nuclear genome, chloroplast transformation proceeds by homologous recombination. Coupled with the sophisticated protein-folding machinery found in the chloroplast, the plastid genome is a more practical expression platform for transgene integration and recombinant protein expression. Even with these advantages in the chloroplast, expression remains low and available genetic constructs are light-regulated, resulting in variable expression as light penetration into the culture changes [12]. The accumulation of recombinant protein expressed in the *C. reinhardtii* chloroplast rarely exceeds 5% total protein and is more commonly < 1% [13]. In comparison, the expression of intracellular recombinant proteins in bacteria, yeasts, and mammalian cells can be much higher, reaching above 10% of total protein content in some cases. However, these platforms can face difficulties producing many sought-after high-value proteins, often due to

* Corresponding author.
E-mail address: ffields@ucsd.edu (F.J. Fields).

https://doi.org/10.1016/j.algal.2018.05.006
Received 20 March 2017; Received in revised form 8 May 2018; Accepted 10 May 2018

Table 1
Maximum observed biomass density and productivity of *C. reinhardtii* strains grown mixotrophically in TAP media.

CC-strain	Biomass density (DW g L^{-1})	Cell density (cells × 10^7 mL^{-1})	Productivity (DW g L^{-1} d^{-1})
125	1.76	1.9	0.25
1009	1.74	1.75	0.24
1373	1.8	2.58	0.25
1690	1.76	2.21	0.25
2290	1.78	1.07	0.27
2343	1.82	1.88	0.27
2344	1.9	3.44	0.24
2931	1.64	1.62	0.27
2935	1.84	4.62	0.28
2937	2.08	4.5	0.29
2938	1.64	3.24	0.26
4147	0.33	0.34	0.1

challenges involving protein folding, post-translational modification, or production costs [14].

A practical consequence of low expression in *C. reinhardtii* is an inability to produce the minimum quantities of heterologous products needed for characterization of post-translational modifications, bioactivity assays, or in vivo experiments (e.g., animal trials). In order to generate meaningful quantities of recombinant products and make *C. reinhardtii* a realistic production platform, there are two main developments that must occur: (1) development of robust and controllable gene expression systems that are comparable to alternative production platforms and (2) development of cultivation processes that allow for rapid growth and maximize biomass concentration.

Research to improve transgene integration and expression in *C. reinhardtii* is ongoing and new technologies, like synthetic promoters and CRISPR, show great promise in this area [15,16]. Overlapping these technologies with classic host improvement strategies, like new strain discovery, breeding, and mutagenesis, will potentially lead to the necessary advancements in gene expression and ultimately product production.

However, improving gene expression and protein accumulation is not sufficient in itself to allow *C. reinhardtii* to become a commercially viable production platform. To be cost competitive with other microbial systems, greatly improved biomass production processes must be developed to enable the rapid generation of large amounts of algal biomass. Today, large volume cultures of algae are commonly grown under phototrophic conditions in ponds and specialized photobioreactors [17,18]. However, low biomass productivity of *C. reinhardtii* in open ponds [19], coupled with regulatory obstacles faced with producing genetically modified strains outdoors [20], has directed the cultivation of *C. reinhardtii* into closed systems operating under either mixotrophic or heterotrophic conditions. These systems (i.e., bioreactors and fermenters) allow for highly controlled commercial-scale alternatives and have been successfully implemented in the cultivation of numerous microbial hosts [14,21,22]. However, these systems come at a much higher capital equipment cost, and can require extensive optimization.

Most commonly, *C. reinhardtii* is cultivated in batches of media containing acetate as a carbon source [23], resulting in dry cell mass concentrations of 1–2 g L^{-1}. In the most notable attempt to achieve high-density *C. reinhardtii* cultures, Chen & Johns [24] conducted comparative studies using sodium acetate as a carbon source in batch, fed-batch, chemostat, and perfusion culture. Using a combination of fed-batch and hollow-fiber cell-recycle systems, biomass densities of 9 g L^{-1} dry weight were achieved. They theorized the culture growth became limited over time due to buildup of sodium ions as a consequence of using sodium acetate as the primary carbon source in the feed [25].

In the present study, we present an alternative method for generating high-density cultures of *C. reinhardtii* by using fed-batch mixotrophic growth and examine the effects it has on growth, biomass composition, and accumulation of a reporter recombinant protein. A fed-batch cultivation strategy was chosen in order to maximize final density of biomass and recombinant protein so as to simplify downstream processing. A strain of *C. reinhardtii* was engineered to produce a recombinant green fluorescent protein (GFP) in the chloroplast, used as a general model for recombinant protein production, and cultured under both batch and fed-batch conditions, in which the latter was operated as a pH-stat with a concentrated feed of acetic acid and nutrients pumped into the bioreactor. The primary goals of this study were to provide a cultivation strategy that would allow the generation of increased quantities of algal biomass and recombinant protein and to advance the development of *C. reinhardtii* towards cultivation in scalable industrial heterotrophic bioreactors.

2. Materials and methods

2.1. Strain selection

A preliminary study was conducted to characterize maximum biomass density, cell density, and productivity of our laboratory's *C. reinhardtii* collection of strains obtained from www.chlamycollection.org (Table 1). Strains were grown in Erlenmeyer flasks containing 50 mL of TAP media on a shaker under constant light (75 µMol m^{-2} s^{-1}) and sampled every day for one week. Among the strains included in this analysis, the strain CC-2937, a wild isolate from Quebec, Canada [26], had the highest maximum biomass density and productivity and was therefore chosen for further experimentation.

2.2. Vector construction and transformation

A construct (Fig. 1) was devised for chloroplast expression of a codon-optimized GFP [27] using the *C. reinhardtii psbD* promoter and 3′ and 5′ UTR regulatory elements [28]. This cassette was cloned into the 3HB vector [13], which contains 5′ and 3′ homologous chloroplast genomic sequence that confers transformation into the 3HB silent site. The *aphA-6* gene with the *atpA* promoter and 5′UTR and *rbcL* 3′UTR was subcloned into the upstream *BamHI* site. CC-2937 cells were cultured as described in Rasala et al. [29] and the vector was transformed via particle bombardment as described in Rasala et al. [28]. Transformants were selected by plating on TAP-agar plates containing 150 mg L^{-1} kanamycin. Strains that showed resistance to kanamycin were put through rounds of selection on kanamycin, and PCR screened for homoplasmy as described in Rasala et al. [29] using the forward primer CGTCCACTAAAATTTATTTACCCGAAGGGG and the reverse primer GTTAAGGCTAGCTGCTAAGTCTTCTTTTCGC which bind in the homology sequence of *psbH*. A homoplasmic colony was eventually identified after several weeks of growth on kanamycin-containing TAP-agar plates. This transformed strain was maintained and used in subsequent experiments.

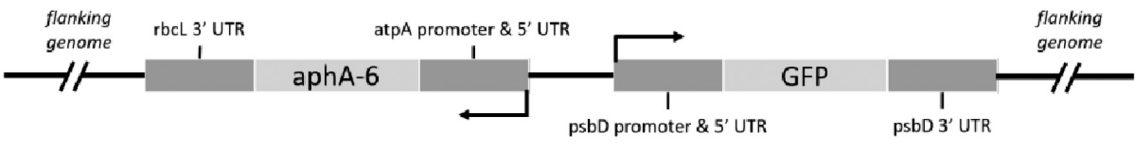

Fig. 1. Construct design for expression of kanamycin resistance (*aphA-6*) and of green fluorescent protein (*GFP*) in the *C. reinhardtii* chloroplast.