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Optimising light conditions increases recombinant protein production in *Chlamydomonas reinhardtii* chloroplasts

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ABSTRACT

The green alga *Chlamydomonas reinhardtii* provides a platform for cheap, scalable and safe production of complex proteins. Despite the fact that chloroplast gene expression in photosynthetic organisms is tightly regulated by light, most expression studies have analysed chloroplast recombinant protein production under constant light. Here, the influence of light period and intensity on expression of green fluorescent protein (GFP) and a GFP-bacterial-lysin (PlyGBS) fusion protein was analysed. Protein yields were strongly influenced by the light period (6–24 h d^{-1}), the light intensity (0–450 μE m^{-2} s^{-1}) and trophic condition. Heterotrophic conditions showed low yields of both recombinant proteins due to low growth rates, despite high protein accumulation per cell. Mixotrophic conditions exhibited the highest yields for GFP (4 mg·L^{-1}·d^{-1}) under constant light at 35 μE m^{-2} s^{-1} and GFP-PlyGBS (0.4 mg·L^{-1}·d^{-1}) under a light period of 15 h d^{-1} and 35 μE m^{-2} s^{-1}. This is due to the high growth rates and cellular protein content. For GFP-PlyGBS the maximum increase in cellular protein accumulation was ~24-fold, and in total protein yield ~10-fold, in comparison to constant light conditions (~200 μE m^{-2} s^{-1}). The highest yields under photoautrophic conditions were obtained under a 9 h d^{-1} light period. GFP yielded 1.2 mg·L^{-1}·d^{-1} and GFP-PlyGBS 0.42 mg·L^{-1}·d^{-1}. This represented a ~5-fold increase in cellular protein accumulation for GFP-PlyGBS in comparison to constant light conditions (~200 μE m^{-2} s^{-1}). Optimising light conditions to balance growth and protein expression can significantly enhance overall recombinant protein production in *C. reinhardtii* cultures.

1. Introduction

Photosynthetic single-cell green algae (microalgae) provide a platform for the production of a wide range of complex proteins. They are increasingly recognised as being cheap, scalable and safe and able to complement bacterial, yeast, mammalian, insect, viral as well as higher plant systems in a number of ways. Bacterial and yeast systems offer well established low cost protein expression platforms but are limited in their ability to conduct sophisticated post-translational modifications essential for many complex proteins [1,2]. Mammalian and insect cell cultures are capable of correctly folding and post-translationally modifying many proteins, but typically have lower expression yields and are generally significantly more costly and difficult to handle and scale. In addition, mammalian systems are also subject to contamination by human pathogens. Plant expression systems have advanced significantly, but the production of transformants can still require 6–12 months and transgene containment remains an issue [3].

In contrast, microalgae offer significant advantages. Transgenic expression cell lines can typically be generated in ~2–4 weeks [3] and support high rates of biomass production (~350 t algae biomass fresh weight ha^{-1} yr^{-1} vs. ~1.75 t ha^{-1} yr^{-1} for tobacco (Food and Agriculture Organisation, United Nations) [4]. These speed and yield factors offer significant cost advantages for scale up [5]. Microalgae show high growth rates (similar to yeast), can be grown with simple inexpensive growth media consisting mainly of inorganic salts without any mammalian derived compounds (e.g. BSA), and require only simple and low-cost scalable bioreactors to enable controlled and contained cultivation suitable for Good Manufacturing Processes (GMP). A range of algal products have also been granted FDA approval on the basis that the production strains are classified as '*Generally Recognised As Safe*' (GRAS). This GRAS classification was simplified by the fact that microalgae are generally free of human, bacterial or viral pathogens [6], bacterial endotoxins [7] and prions [8]. Purification is simplified by the absence of pyrogenic contaminants (e.g. bacterial lipopolysaccharide) and the use of CO_2 rather than organic carbon sources under photoautrophic conditions, which supports the maintenance of axenic cultures (inhibits yeast, bacterial and fungal contamination). Finally secretion or cell rupture release recombinant proteins from the cell [9].

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The optimisation of expressed protein release is dependent on the strain and expression location and remains an active area of research [10,11].

Chlamydomonas reinhardtii is one of the best established microalgal model systems, for both nuclear and chloroplast expression, with a wide variety of molecular tools already developed. The *C. reinhardtii* chloroplast makes up ~70% of the cell volume and is of particular interest for recombinant protein expression as it can effectively fold and disulfide-link proteins. A range of complex proteins have already been produced in the chloroplast. Examples include full-length monoclonal antibodies with 16 disulfide bonds [12], chimeric anti-cancer immunotoxins that could not be produced in *E. coli* or eukaryotic systems [13], and a wide variety of other therapeutic proteins such as erythropoietin, human fibronectin, interferon, pro-insulin, human vascular endothelial growth factor, wound healing high mobility group protein B1 [14,15], a White Spot Syndrome Virus vaccine-like protein for tiger prawns [16], and an orally applicable cholera vaccine [17]. Microalgal chloroplast expression therefore opens up the potential for new protein therapeutic development and low cost production. Currently, in the chloroplast, expression yields are typically in the 0.02–5% of total soluble protein (TSP) range, except for a few notable exceptions (e.g. 42% of TSP of VP28 [16]). Therefore production efficiencies can still be significantly improved.

Chloroplast gene expression is highly regulated by light, both in terms of quality and quantity [18,19]. Given the obvious importance of light for photoautotrophic growth, it is surprising that most microalgal protein expression trials have been conducted under continuous illumination rather than natural diurnal, or otherwise altered light-dark cycles [13,16,20]. While continuous light is expected to support the highest rates of growth, recent literature has also identified effects of incident light on protein expression [18,21,22].

Due to the lack of exogenous expression signals functional in *C. reinhardtii* chloroplasts, most recombinant protein expression is performed using endogenous regulatory elements. Consequently, it is likely that recombinant protein expression and accumulation is subjected to similar light regulation as the endogenous genes, at transcriptional, translational and protein degradation levels. The use of often unnaturally combined expression signals (e.g. promoter/UTRs from different genes), however, makes predictions of regulation effects difficult.

The focus of this study was to examine the effect of continuous light versus light/dark cycles, as well as light intensity, on the expression of two recombinant proteins; the Green Fluorescent Protein (GFP) reporter, and a bacterial lysin-GFP fusion protein (GFP-PlyGBS). Optimised protein production conditions were determined both on a per cell and per culture volume ($mg\,L^{-1}$) basis for photoautotrophic, mixotrophic and heterotrophic conditions.

2. Results

2.1. GFP and GFP-PlyGBS expression

To confirm the ability to produce GFP and the fusion protein GFP-PlyGBS in *C. reinhardtii*, expression constructs for each were transformed into wild-type CC125 and CC124 cells. PCR analysis confirmed that all putative positive transformants were homoplasmic. Successful production of GFP and GFP-PlyGBS under standard mixotrophic production conditions (TAP medium, 180–200 $\mu E\,m^{-2}\,s^{-1}$ constant light) was confirmed using native polyacrylamide gel electrophoresis (PAGE, Fig. 1a) and mass spectrometric protein identification. Each sample loading was normalised to the same optical density at 750 nm (OD_{750}), which was used as a proxy for cell number (pre-harvest and protein extraction); consequently the band intensity (Fig. 1a) corresponds to cellular accumulation levels of these recombinant proteins.

2.2. Growth cycle does not influence recombinant protein accumulation

To test whether protein yields could be increased by harvesting at specific time points in the growth cycle, synchronised GFP mutants were freshly inoculated into TAP medium and grown at 180–200 $\mu E\,m^{-2}\,s^{-1}$ under constant light for 4 days. To see if cellular protein content varied at different stages, protein levels were analysed by native-PAGE in duplicate at daily time points during the four day experiment. Cell samples were normalised based on OD_{750}. Fig. 1b shows no noticeable change in fluorescence, suggesting a constant recombinant protein accumulation in the chloroplast under the conditions tested. Consequently, in subsequent analyses, samples were harvested at the end of mid-log phase; 4 days for mixotrophic cultivation and 5 days for photoautotrophic cultivation conditions.

2.3. Effect of light period and trophic condition at saturating light on cellular protein expression and growth

Microalgae can utilise inorganic CO_2 when grown photoautotrophically and mixotrophically, or organic carbon (e.g. acetate in TAP), when grown mixotrophically and heterotrophically, to support cellular metabolism and growth. This, in turn, can affect recombinant protein production. Fig. 1c, in agreement with previous reports [21], suggests that heterotrophic growth can accumulate higher amounts of protein per cell than mixotrophically grown cells exposed to constant light at saturating intensities (180–200 $\mu E\,m^{-2}\,s^{-1}$).

Little information is available on the influence of light/dark cycles on recombinant protein expression. Consequently, the effect of varying the light period (0 h d^{-1} = dark (D), and 6, 9, 12 and 15 h d^{-1}) against a constant light control (CL, 24 h d^{-1}), was analysed on the expression of GFP and GFP-PlyGBS under photoautotrophic and mixotrophic conditions at saturating light intensity (180–200 $\mu E\,m^{-2}\,s^{-1}$) and heterotrophic conditions. Recombinant protein production was quantified using the relative change in fluorescence signal to that under constant light and normalised on a cellular basis via OD_{750}.

2.3.1. Relative cellular protein production under photoautotrophic conditions

Native-PAGE analysis shows the effect of light period under photoautotrophic conditions (180–200 $\mu E\,m^{-2}\,s^{-1}$ light intensity) on recombinant GFP and GFP-PlyGBS accumulation, normalised to the same cell density (OD_{750}) (Fig. 2a). Fig. 2b quantifies the expression levels relative to constant light (control) on a cellular basis. For both proteins, cellular yields peaked at a light period of 9 h d^{-1} (Fig. 2a & b). Here, GFP yields were ~2-fold those obtained under constant light, while GFP-PlyGBS accumulation showed an increase of ~5-fold over those exposed to constant light. This highlights the importance of light period in terms of recombinant protein production.

Interestingly, cellular GFP-PlyGBS levels (Fig. 2b right) are nearly constant for all light periods except under constant light, which were extremely low. The GFP-PlyGBS native-PAGE gels (Fig. 2a right) also showed a second lower molecular weight band similar in size to GFP alone, thought to be a degradation product. This suggests that GFP-PlyGBS accumulation could be increased further by preventing protein degradation.

The effect of light on cell growth rate (Fig. 2b blue line) was also determined for each algal strain and light condition. As expected, the constant 24 h d^{-1} light period yielded the highest growth rates for both strains. Overall, growth rates were reduced with decreasing illumination time (Fig. 2b blue line). Under a 9 h d^{-1} light period, the growth rate of the GFP and GFP-PlyGBS mutants dropped by about 30% compared to that of the 24 h light period control. Consequently a clear opposing effect of light period on protein accumulation and cell growth was observed (Fig. 2b).

2.3.2. Relative cellular protein production under mixotrophic and heterotrophic conditions

A similar pattern was observed under mixotrophic conditions (Fig. 2c & d), except that the highest cellular recombinant protein levels

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