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Investigating the dynamics of recombinant protein secretion from a microalgal host

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ABSTRACT

Production of recombinant proteins with microalgae represents an alternative platform over plant- or bacterial-based expression systems for certain target proteins. Secretion of recombinant proteins allows accumulation of the target product physically separate from the valuable algal biomass. To date, there has been little investigation into the dynamics of recombinant protein secretion from microalgal hosts—the culture parameters that encourage secreted product accumulation and stability, while encouraging biomass production. In this work, the efficiency of recombinant protein production was optimized by adjusting cultivation parameters for a strain of *Chlamydomonas reinhardtii* previously engineered to secrete a functional recombinant *Lolium perenne* ice binding protein (*Lp*IBP), which has applications as a frozen food texturing and cryopreservation additive, into its culture medium. Three media and several cultivation styles were investigated for effects on secreted *Lp*IBP titres and culture growth. A combination of acetate and carbon dioxide feeding with illumination resulted in the highest overall biomass and recombinant protein titres up to 10 mg L^{-1} in the culture medium. Pure photoautotrophic production was possible using two media types, with recombinant protein accumulation in all cultivations correlating to culture cell density. Two different cultivation systems were used for scale-up to 10 L cultivations, one of which produced yields of secreted recombinant protein up to 12 mg L^{-1} within six cultivation days. Functional ice recrystallization inhibition (IRI) of the *Lp*IBP from total concentrated extracellular protein extracts was demonstrated in a sucrose solution used as a simplified ice cream model. IRI lasted up to 7 days, demonstrating the potential of secreted products from microalgae for use as food additives.

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1. Introduction

The Chlorophyte microalgae *Chlamydomonas reinhardtii* has served as a valuable model organism for fundamental photosynthetic and biological analysis for many years (Rochaix, 1995). Currently this alga has the most well-developed molecular toolkit of any eukaryotic microalgae, and transformation of nuclear, chloroplast, and mitochondrial genomes is possible (Bateman and Purton, 2000; Kindle, 1990; Remacle et al., 2006). Chloroplast-based recombinant protein (RP) expression in this organism has been shown to achieve titres up to 21% total soluble protein (TSP) (Surzycki et al., 2009). This capacity, in addition to the generally regarded as safe (GRAS) status of *C. reinhardtii*, has led to its proposed use for molecular farming of high value RPs, both as purified products, and as whole-cell edible gut-active

Abbreviations: gLuc, *Gaussia princeps* luciferase; cCA, secretion signal of *C. reinhardtii* carbonic anhydrase 1; IRI, ice recrystallization inhibition; LpIBP, *Lolium perenne* ice binding protein; HiT, high-Tris media.

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therapeutics (Franklin and Mayfield, 2004; Rasala and Mayfield, 2014; Rosales-Mendoza et al., 2012).

In contrast, nuclear transgene expression has resulted in significantly lower titres of RP, with a maximum reported of 0.25% TSP (Lauersen et al., 2015; Rasala et al., 2012, 2013). Nuclear transgene expression is mediated by eukaryotic translational machinery and is inherently more regulated than its plastid counterparts (Mayfield et al., 2007; Rasala and Mayfield, 2014). However, nuclear-based gene expression presents the possibility of subcellular targeting of RPs to various cellular compartments, posttranslational modifications, and the capacity for secretion of RPs into culture medium (Lauersen et al., 2013a, 2013b; Rasala et al., 2012).

The capacity of microalgae for growth driven by photosynthesis presents potentially sustainable production through these hosts, using only water, (sun)light energy and carbon dioxide as inputs (Wijffels et al., 2013). However, to date, technical limitations in large-scale photosynthetic algal cultivation prevent the widespread use of these organisms for many industrial concepts. Indeed, the first publication of greenhouse-style cultivation of transgenic *C. reinhardtii*, which expressed a target edible therapeutic in the chloroplast, was published only recently (Gimpel et al., 2014).

In light of the difficulties of engineering algal production systems, secretion of recombinant products from the algal host presents the potential for a new layer of production value for algal cultivation concepts, allowing the recombinant product to be harvested independently of the valuable algal biomass. Although therapeutic RPs have dominated research in *C. reinhardtii* transgenics, two examples of industrially relevant RP production have been demonstrated via expression from the nuclear genome and secretion into culture medium: a xylanase (Rasala et al., 2012), and recently in our laboratory, an active ice binding protein (IBP) (also known as ice structuring, antifreeze, or IRI protein) from the perennial ryegrass *Lolium perenne* (*Lp*IBP) with *C. reinhardtii* (Lauersen et al., 2013b). The latter was accomplished as a fusion protein made from a codon-optimized *Gaussia princeps* luciferase (*g*Luc) gene, synthetically modified to contain a *C. reinhardtii* carbonic anhydrase secretion signal (*c*CA), which allowed rapid identification of transformants exhibiting robust expression and secretion of the *g*Luc*Lp*IBP fusion (Lauersen et al., 2013a, 2013b).

The *Lp*IBP limits the thermodynamically favoured growth of ice crystals at high sub-zero temperatures, a phenomenon known as ice recrystallization (IR), which this protein controls in its native plant to assist overwintering (Lauersen et al., 2011; Middleton et al., 2009; Yu et al., 2010). However, IR is also a common cause of frozen food spoilage, the most pertinent example of IR is the unpleasant texture of ice cream stored for long periods (Donhowe and Hartel, 1996a, 1996b). Given the robust IRI activity of the *Lp*IBP, it has been proposed for use as a frozen food additive to limit frost damage over increased storage time (Griffith and Ewart, 1995; Hassas-Roudsari and Goff, 2012).

In both published examples of industrially relevant RP secretion from *C. reinhardtii*, only minimal efforts to investigate the culture parameters for stable protein production via secretion from the algal system were conducted (Lauersen et al., 2013b; Rasala et al., 2012). However, secreted RPs pose additional challenges for scale-up of cultivation systems, as the stability requirements of proteins in the culture medium may be different than those of the expression host. Therefore, we investigated culture parameters which would allow and optimize the efficiency of concomitant biomass and secreted RP production from *C. reinhardtii* using the *g*Luc*Lp*IBP as a model-secreted RP. Different culture media as well as growth regimes were investigated, and production up to 10 L scale was compared for two selected culture systems.

2. Materials and methods

2.1. Cultivation conditions, plasmids, transformation, and screening of transgenic C. reinhardtii

All precultures in this work were grown in TAP medium (Gorman and Levine, 1965) under standard conditions with ~150 μmol photons m^{-2} s^{-1} on a standard rotary shaker. UVM4 (graciously provided by Prof. Dr. Ralph Bock) and the *g*Luc*Lp*IBP secretion strain UV*c*CA (Lauersen et al., 2013a) cultures were routinely grown in TAP medium with 150 μmol photons m^{-2} s^{-1} light intensity in shake flasks or on TAP (agar) plates. *C. reinhardtii* UVM4 is a ultraviolet light-derived mutant of CC-4350 (cw15 arg7-8 mt+ [Matagne 302]) which was transformed with the emetine resistance cassette CRY1 as well as the ARG7 argininosuccinate lyase complementation vector and subsequently demonstrated nuclear transgene expression with high efficiency (Neupert et al., 2009). CC-4350 is available from the Chlamydomonas Resource Center (http://chlamycollection.org).

UVM4 was transformed with plasmid pOpt_cCA_gLuc_Paro (Lauersen et al., 2015), and a variation which has the codon optimized *L. perenne* ice binding protein (NCBI Access. No.: KF475785) cloned between *Eco*RV and *Eco*RI sites as a C-terminal fusion to the *g*Luc as was originally demonstrated for the p*c*CAg*Luc*Lp*IBP vector (Lauersen et al., 2013a, 2013b). Transformations were performed with glass bead agitation as previously described (Kindle, 1990). Transformants were recovered on TAP (agar) plates containing paromomycin at 10 mg L^{-1} with 150 μmol photons m^{-2} s^{-1} light intensity, and maintained on TAP (agar) plates by colony stamping.

Mutants were screened in the same way in which UV*c*CA was originally isolated, using plate-level bioluminescence assays as previously described (Lauersen et al., 2013a) from a population of 480 mutants (5 × 96 colony plates) per construct. Four mutants exhibiting the most robust bioluminescence signal from each vector construct were selected for cultivation in liquid culture. The relative bioluminescence of culture medium resulting from secretion of either the *g*Luc alone or *g*Luc*Lp*IBP, in late logarithmic phase was assessed in a Tecan infinite M200 plate reader (Männedorf, Switzerland) using black microtitre plates. Analysis of bioluminescence signal was conducted immediately after addition of 0.01 mM coelenterazine (PJK shop) with 2000 ms integration time and normalized to cell density. Measurements were conducted in technical triplicate, from three biological cultivation replicates.

2.2. Investigations of culture pre-conditions for gLucLpIBP secretion and UVcCA growth

For all media investigations, precultures were centrifuged for 3 min at 1000 × *g* followed by resuspension with target medium, this step was repeated two times in order to remove unwanted residual medium components from the cells.

Three styles of cultivation at the 1 L scale were investigated, UV*c*CA was grown in TAP medium without gassing in shake, baffled shake, or stirred 1 L volumes at ~200 μmol photons m^{-2} s^{-1}. The relative *g*Luc*Lp*IBP secretion from UV*c*CA in these cultures was analyzed by dot-blot of medium samples using the α-*g*Luc antibody with a secreted recombinant *g*Luc produced in *Kluyveromyces lactis* as standard (available commercially from Avidity) as previously described (Lauersen et al., 2013a).

2.3. Comparisons of media and cultivation strategies for the secreted gLucLpIBP

TAP medium was used to cultivate strain UV*c*CA heterotrophically (acetate, dark, air bubbling) and photo-mixotrophically with low (acetate, light, air bubbling) or high CO_2 (acetate, light, 3% CO_2

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