Investigating the dynamics of recombinant protein secretion from a microalgal host

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Author contributions

- Kyle Lauersen was responsible for manuscript writing, figure design, collaboration organization, the development of strain UV*c*CA, secreted protein preparation for ice recrystallization analysis, bioluminescence analysis in the laboratory of Frank Gudermann and Dirk Lütkemeyer, as well as dot-blotting.

- Isabel Huber and Julian Wichmann were responsible for cultivation and media screening experiments as well as culture parameter data collection.

- Thomas Baier was responsible for cloning and transformation of pOpt_cCA_gLuc_Paro and pOpt_cCA_gLuc_LpIBP_Paro vectors into strain UVM4.

- Andreas Leiter and Volker Gaukel were responsible for ice recrystallization inhibition analysis.

- Viktor Kartushin, Anke Rattenholl, Frank Gudermann, and Dirk Lütkemeyer were responsible for wave-bag cultivation of strain UV*c*CA and daily sample collection / experimental organization.

- Christian Steinweg, Lena von Riesen, and Clemens Posten were responsible for the cultivation of strain UV*c*CA and daily culture parameter sampling of the flat-panel photobioreactor cultivation.

- Jan Mussgnug was involved in manuscript preparation and writing as well as experimental design.

- Work by Kyle Lauersen was conducted in the laboratory of Prof. Dr. Olaf Kruse, who was involved in experimental design and manuscript preparations.

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. Purely 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 litre 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 seven days, demonstrating the potential of secreted products from microalgae for use as food additives.

Keywords: Microalgae, *Lolium perenne* ice-binding protein, recombinant protein secretion, Flat panel photobioreactor, Wave bag culture, *Chlamydomonas reinhardtii*.

Abbreviations:

*g*Luc – *Gaussia princeps* luciferase

*c*CA – secretion signal of *C. reinhardtii* carbonic anhydrase 1

IRI – ice recrystallization inhibition

*Lp*IBP – *Lolium perenne* ice binding protein

HiT – High-Tris media

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 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. 2013; Rasala et al. 2012). 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; Lauersen et al. 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; Lauersen et al. 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; Donhowe and Hartel 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 *Lolium 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*CA*g*Luc*Lp*IBP vector (Lauersen et al. 2013a; Lauersen et al. 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 (5x96 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 normalised to cell density. Measurements were conducted in technical triplicate, from three biological cultivation replicates.

2.2 Investigations of culture pre-conditions for *g*Luc*Lp*IBP secretion and UV*c*CA growth

For all media investigations, precultures were centrifuged for 3 min at 1000xg 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 analysed 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 *g*Luc*Lp*IBP

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 bubbling) levels. Strict photoautotrophic cultivation (3% CO_2) and RP production dynamics were investigated in Sueoka's high salt medium (HSM) (Sueoka 1960), and an in-house 'High-Tris' medium (designated HiT) containing 12 g L^{-1} Tris (for recipe see Table S1). All cultivations were conducted in three biological replicates of 400 mL stirred glass flasks, bubbled with either air or air plus 3% CO_2 at 50 L h^{-1} and 350 µmol photons $m^{-2}s^{-1}$, unless cultivated in the dark. Culture parameters including cell density and dry biomass were recorded. In addition, daily media samples were taken and bioluminescence readings were performed (not shown) as previously described (Lauersen et al. 2013a). Absence of bacterial contamination was controlled for by plating culture aliquots on TAP media containing yeast extract, as well as analysis of supernatant clarity following centrifugation. The best performing biological replicate of each cultivation in bioluminescence assays was analysed by dot-blot using the α-*g*Luc antibody as previously described (Lauersen et al. 2013b).

2.4 Cultivation of UV*c*CA in 10 L flat panel photobioreactor

Cultivation scale-up was conducted with a custom built 10 L flat panel bioreactor using TAP medium. To avoid photoinhibitory effects, illumination was set to ~50 µmol photons $m^{-2}s^{-1}$ for the first day after inoculum and then increased to ~100 µmol photons $m^{-2}s^{-1}$ for the remainder of the cultivation. The culture was inoculated to an initial density of $4x10^6$ cells mL^{-1} from a TAP grown preculture, cultivation was

conducted for 144 hours prior to termination. Cultivation temperature was regulated between 27-29 ˚C with an internal cooling system. Aeration and mixing was accomplished by bubbling with 800 mL min^{-1} 3% CO_2. The total cultivation volume was ~9 L. Due to formation of foam on top of the culture, approximately 10 mL of antifoam was added to the culture (Antifoam A, Sigma). Formation of sediment was observed which could not be resuspended as an increase of the airflow led to a deformation of the Plexiglas walls. Daily sampling included cell density and dry biomass. Quantification of *g*Luc in culture medium was conducted as above.

2.5 Cultivation of UV*c*CA in an illuminated 10 L wave bag bioreactor

The BIOSTAT CultiBag RM system from Sartorius Stedim Biotech GmbH (Göttingen, Germany) was used with a CultiBag RM 20L optical bag (together: Wave bag) for cultivation of UV*c*CA. The system was set to 13 rocks min^{-1}, at an angle of 8.5˚ and the process run at room temperature. Cultivation was conducted in TAP medium with 3% CO_2 surface aeration and given white light from four fluorescence bulbs in a hanging ballast to between ~150-200 µmol photons $m^{-2}s^{-1}$ depending on the angle of rocking. The bag was filled with 10 L TAP medium through a 0.2 µm sterile filter and inoculated to OD_{750} 0.1 from a TAP grown preculture. Cells were counted automatically using the Cedex HiRes System (Roche Diagnostics, Mannheim, Germany) daily in addition to cell dry biomass measurements. Samples were taken daily until termination of cultivation at 144 hours. For quantification of *g*Luc in culture medium, samples were subjected to dot-blotting as well as bioluminescence analysis as above.

2.6 Simulated food product IRI analysis

IRI activity of secreted *g*Luc*Lp*IBP using total concentrated extracellular protein (CEP) samples from *C. reinhardtii* strain UV*c*CA was demonstrated in a simplified ice cream model solution (49% sucrose (w/w)). Due to the sugar content water is only frozen partly which leads to the concurrent presence of ice crystals and unfrozen solution during storage. This is a characteristic situation not only for ice cream but for many food and food like systems in which recrystallization occurs. Regand and Goff (2005) used a similar solution with less sucrose (23%) for recrystallization analytics. We decided to use a higher sucrose content because this reveals a more realistic ice content for ice cream as the sucrose represents all solutes in the simplified system and the typical dry mass of ice cream is around 40%. In addition a slightly higher sucrose content simplifies the ice crystal analytics due to the lower ice content during storage without changing the principal RI

mechanism (Gaukel et al., 2014). CEP was prepared by cultivation of UV*c*CA and parental strain UVM4 (WT) in TAP medium bubbled with 3% CO_2 to late logarithmic phase under standard conditions in 10 L stirred flasks with 300 µmol photons $m^{-2}s^{-1}$, followed by centrifugation, microfiltration, and concentration by tangential crossflow filtration of medium as previously described (Lauersen et al. 2013b). IRI activity of *g*Luc*Lp*IBP in UV*c*CA CEP without purification was compared with the activity of equimolar amounts (0.154 µM) of purified fish ice binding protein (also known as antifreeze protein, or ice structuring protein) AFP III, isolated from ocean pout (*Macrozoarces americanus*) (Hew et al. 1984; Hew et al. 1988) (purchased from A/F Protein (Waltham, USA)), and parental strain (WT) total CEP. Sucrose Solutions (49% (w/w)) were prepared with a final concentration of 1 mg L^{-1} of AFP III, total UVcCA CEP to a final concentration of 5 mg L^{-1} *g*Luc*Lp*IBP, and an equivalent concentration of CEP for the parental strain (WT).

Ice crystal growth analysis was performed as previously described (Gaukel et al. 2014). An amount of 18 µl of the sample solution was placed between two microscope cover slips on an object slide, then covered with another cover slip and sealed with silicone. Three object slides of each solution were prepared and analysed. The samples were subjected to a fast freezing process by immersion in liquid nitrogen for a few seconds to transform the aqueous solution into a glassy state. After freezing, the samples were stored at a constant temperature of -12 ˚C, +/- 0.1 ˚C, in a small storage chamber, placed in a deep-freeze room (also -12 ˚C). This procedure allows the system to crystallize in a uniform way by heating up from the glassy state. For the principal investigation of the recrystallization mechanism it is a reproducible method for the initial formation of small ice crystals, however, differs from industrial frozen food preparation (Gaukel et al., 2014). The temperature inside the chamber was recorded by a thermocouple during the storage time of 1 week. During storage, pictures of ice crystals were taken at 5 h, 24 h, 49 h, 96 h and 168 h after freezing by a camera (altra SIS20, Olympus, Japan) attached to a polarization microscope (BX41, Olympus, Japan) installed in the deep-freeze room. For evaluation of the pictures, the contours of the ice crystals were manually circumscribed on a computer with the software ImagePro Plus 5.0 (Media Cybernetics, USA). From the defined areas of each crystal, the equivalent diameter was calculated as the diameter of a circle with the same area. 300 to 400 ice crystals were analysed from each slide and the mean equivalent diameter was determined. The mean crystal size and standard deviation of the three object slides were then calculated for each sample time point.

3. Results

3.1 Fusion of *Lp*IBP to the C-terminus of *Gaussia* Luciferase (*g*Luc) enhances protein secretion efficiency

In previous experiments, we demonstrated that the recombinant protein *g*Luc*Lp*IBP, a synthetic fusion protein of *Gaussia* Luciferase and *Lolium perenne* ice binding protein, expressed from the p*c*CA*g*Luc*Lp*IBP vector, was secreted from *C. reinhardtii* and accumulated to a maximum of ~10 mg L^{-1} in standard TAP medium cultivations (Lauersen et al. 2013a; Lauersen et al. 2013b). We were interested to directly compare this with the secretion of *g*Luc alone, as recent analysis of this reporter from the pOpt_cCA_gLuc_Paro vector resulted in a maximum expression of only ~0.5 mg L^{-1} culture under the same conditions (Lauersen et al. 2015). In order to directly compare *g*Luc*Lp*IBP secretion to *g*Luc, we constructed a *Lp*IBP containing vector, pOpt_cCA_gLuc_LpIBP_Paro (Fig. 1A), and transformed this or the vector pOpt_cCA_gLuc_Paro into parental strain UVM4. Interestingly, transformants expressing *g*Luc*Lp*IBP resulted in higher secreted recombinant protein titres than transformants expressing the *g*Luc marker alone (Fig. 1B) indicating that fusion of *Lp*IBP to the C-terminus of *g*Luc resulted in greater secretion into culture medium, although the recombinant protein is of significantly higher molecular weight. The results clearly indicate that molecular factors related to the amino acid sequence must exist which can promote or inhibit recombinant protein production and secretion. Although this is a subject of on-going investigations, these factors currently are not known.

3.2 Screening cultivation conditions and media that promote efficient production and secretion of recombinant proteins

Mixing of cell cultures is an important factor influencing biomass and recombinant protein production. In our setup, we tested three possible methods, shaking, baffled shaking, or stirring, and compared the respective cell culture growth and secreted recombinant protein production of *g*Luc*Lp*IBP in UV*c*CA medium. As shown in Figure 2, stirred cultures generated significantly higher cell densities in early stages of cultivation, up to 48 h, and exhibited a more rapid accumulation of the secreted *g*Luc*Lp*IBP in culture medium (Fig. 2A,B). This lead us to use stir-mixed flasks for all further medium investigations, including pre-screening of photoautotrophic cultivations with various in-house medium recipes.

Photoautotrophic media screening for growth of UV*c*CA resulted in the identification of one medium with robust culture performance and secreted *g*Luc*Lp*IBP accumulation (see

Supporting Information for medium recipe). This medium, called HiT (for High-Tris), was used in subsequent comparative culture performance analysis with common *C. reinhardtii* media (HSM and TAP). Media and growth strategies were then directly compared in standardized triplicate 400 mL batch cultures with the strain UV*c*CA, and used to determine suitable cultivation styles for the secretion of *g*Luc*Lp*IBP into culture media (Fig. 3).

Both HSM and HiT media were used to investigate growth under strictly photoautotrophic conditions, with 3%CO_2 bubbling as a sole carbon source and illumination as the energy source. TAP medium was used for investigation of strictly heterotrophic cultivation in the dark with acetate as a sole carbon source, as well as for mixotrophic conditions in the light with either acetate and low (air)- or high (3%)-CO_2 (TAP(air) and TAP(CO_2), respectively). Culture performance was assessed by recording cell density and dry biomass (Fig. 3A, upper and lower panels, respectively) and secreted *g*Luc*Lp*IBP titres were quantified by dot-blot of media samples (Fig. 3B). Heterotrophic growth in TAP medium resulted in the lowest performance of all investigated culture set-ups, while mixotrophic cultivation in TAP(CO_2), exhibited the highest performance (Fig. 3A).

TAP(CO_2) cultures grew to a cell density of 5.1±0.25 x10^7 cells mL^{-1} and a dry biomass of 1.33 ±0.10 g L^{-1} in 96 hours of cultivation (Fig. 3A). Without the additional CO_2 (TAP(air)), the cultures grew to approximately half the cell density and biomass (2.3±0.17 x10^7 cells mL^{-1} and 0.56 ±0.01 g L^{-1}) in the same period of cultivation time. This result demonstrates that although a reduced carbon source is present in the form of acetate in TAP medium, additional application of CO_2 lead to a significant boost of cell growth.

Heterotrophically cultivated cell cultures in the dark (TAP dark) only reached 0.78±0.2 x10^7 cells mL^{-1} and 0.19 ±0.02 g L^{-1} biomass, indicating that additional light energy was an important factor for optimal cell growth (Fig. 3A). Strictly photoautotrophic cultivation in either HSM or HiT media resulted in cultures with up to 1.4±0.06 x10^7 cells mL^{-1} and 1.28 ±0.10 g L^{-1}, or 2.3±0.48 x10^7 cells mL^{-1} and 1.13±0.06 g L^{-1}, respectively (Fig. 3A).

Cell density in TAP(CO_2) cultures was more than three times higher compared to photoautotrophic HSM cultures. However, a similar dry biomass at the end of cultivation was observed for both, indicating that the reduced cell division rate was compensated for by increased intracellular biomass accumulation in photoautotrophic HSM cultivations.

Accumulation of secreted *g*Luc*Lp*IBP in culture media correlated with relative culture cell densities in each trial up to 72 hours of cultivation (Fig. 3A,B). Strictly photoautotrophic

production of *g*Luc*Lp*IBP was achieved to less than 2 mg L^{-1} in HSM, however, HiT medium cultures accumulated ~5-6 mg L^{-1} *g*Luc*Lp*IBP without the addition of an organic carbon source (Fig. 3B). Purely heterotrophic TAP cultivations produced ~2 mg L^{-1} *g*Luc*Lp*IBP from the 1 g L^{-1} acetate present in this medium (Fig. 3B). TAP(air) and TAP(CO_2) photomixotrophic cultivations accumulated ~10 mg L^{-1} of this protein by 96 h (Fig 3B), however, accumulation of *g*Luc*Lp*IBP in TAP(CO_2) cultivations occurred earlier than TAP(air) cultivations, correlated with the higher cell densities achieved in these time points (Fig. 3A, upper panel).

3.3 Cultivation of UV*c*CA in flat panel and wave bag photobioreactor systems

The potential for culture scale-up is of crucial importance for any biotechnological production system. Therefore, after the establishment of optimal nutrition conditions in 400 mL small scale batch cultivations, two medium scale cultivation strategies were compared in terms of culture growth parameters and secreted *g*Luc*Lp*IBP titres, a 10 L flat panel bioreactor (Fig. 4A, left), designed to optimize light penetration into algal culture, and a 10 L wave-bag system designed for the gentle cultivation of various cell types (Fig. 4A, right), including Chinese Hamster Ovary (CHO) and insect cell culture (Baldi et al. 2007; Ikonomou et al. 2003). The flat-panel system has been described to produce high biomass titres from microalgal strains due to optimized light penetrance into the culture volume, a limiting factor for microalgal culture scale up (Posten 2009). The wave-bag system represents a certified good manufacturing practice (cGMP) grade system which has been adapted to tissue culture of another photosynthetic organism, the moss *Physcomitrella patens* (Gitzinger et al. 2009), but was, to our knowledge, not yet applied to eukaryotic microalgae.

Since the combination of TAP medium with 3% CO_2 gassing resulted in the best overall culture performance as well as titres of secreted *g*Luc*Lp*IBP up to 10 mg L^{-1} (Fig. 3), these conditions were chosen for the 10 L scale-up trials.

Growth parameters monitored from each cultivation are presented in Figure 4B. Measurements from 400 mL photoheterotrophic batch test are included for reference. Medium scale cultures were conducted for 6 days, and assessed for relative performance in terms of cell density and dry biomass (Fig. 4B, upper and lower panels, respectively).

As expected, the flat panel system clearly outperformed the wave bag in terms of early culture cell density, reaching ~6.0±0.4 x10^7 cells mL^{-1} within the first 48 h of cultivation. However, these values declined after this point (Fig. 4B), indicating onset of cell death.

The wave bag system exhibited a steady increase in cell density throughout the trial, reaching ~4.0±0.7 x10^7 cells mL^{-1} at the end of cultivation period (Fig. 4B, upper panel). Overall dry biomass of the flat panel system was up to 1.2±0.06 g L^{-1} which was similar to the 400 mL culture at 96 hours (1.3±0.10 g L^{-1}) and higher than the wave-bag system, ~0.9±0.10 g L^{-1} (Fig. 4B, lower panel).

In terms of algal biomass productivity, the flat panel system clearly outperformed the wave bag system, even with a lower light intensity (Fig. 4B). Interestingly, the opposite was observed for the amount of secreted *g*Luc*Lp*IBP in the culture medium. The wave bag system accumulated the recombinant protein to ~12 mg L^{-1} after 144 h of cultivation (Fig. 4C). Therefore, in comparison to 400 mL cultures, in which ~7.5-10 mg L^{-1} was produced, the wave bag reached this protein titre within 96 h cultivation and even surpassed this later (Fig. 4C). In contrast, the flat panel demonstrated only accumulation to a maximum of ~2 mg L^{-1} at 48 h cultivation, which was then even seemingly degraded (Fig. 4C), coinciding with the decline in culture cell density (Fig. 4B). These results show that despite slower biomass generation, the more gentle cultivation in the wave bag system lead to overall higher recombinant protein production.

3.4 IRI from algal produced *Lp*IBP in a simplified ice-cream model solution

The ice recrystallization inhibition activity of *g*Luc*Lp*IBP produced from UVcCA has been demonstrated previously in total extracellular protein containing culture medium solutions (Lauersen et al. 2013b). Since the primary commercial application for ice binding proteins is proposed as cryopreservation and texturing of frozen foods (Griffith and Ewart 1995; Hassas-Roudsari and Goff 2012), we intended to investigate if total concentrated extracellular proteins (CEP) from UV*c*CA could be used to inhibit ice recrystallization in a simplified ice cream model solution, consisting of 49% sucrose in water. As shown in Figure 5A and quantified in Fig. 5B, *g*Luc*Lp*IBP containing CEP added to sucrose solutions inhibited ice crystal growth as well as purified fish antifreeze protein, exhibiting smaller crystal sizes for up to 168 hours, while the same concentration of extracellular proteins from the parental strain (UVM4: WT) did not inhibit recrystallization and was quantitatively comparable to sucrose solution used as negative control (Fig. 5B).

4. Discussion

Photosynthetic microalgae combine aspects of microbial growth, such as ease of containment compared to transgenic plant systems and the capacity for simple, photoautotrophic cultivation in inexpensive culture media. Therefore, these organisms represent potentially sustainable hosts for recombinant bio-product generation (Wijffels et al. 2013). Generally, bioprocesses seek to optimize for production of a single product, often found within the cell, the harvesting of which is at the expense of the cell biomass, or other valuable products found within. We previously demonstrated that, through secretion of a target recombinant product into the culture medium, the product could be harvested independently of the valuable algal biomass (Lauersen et al. 2013b). In this work, we intended to optimize cultivation parameters that result in an enhanced production of an industrially relevant secreted recombinant protein product concomitant with algal biomass production.

Dry biomass generated in photoautotrophic cultivation matched mixotrophic levels at 96 h, however, mixotrophic cultures had more than double cell density of all other cultures (Fig. 3). The discrepancy is likely due to cell size variations in the different cultivation media, as had been previously noted (Lauersen et al. 2013b). The differences in cell density were reflected in the total *g*Luc*Lp*IBP secreted into culture media, where TAP(air) or TAP(CO_2) cultivations again were the best performing (Fig. 3B). These results indicate that although photoautotrophic production, which is the hallmark of the algal system, is possible, optimization of cultivation media for secreted products is still necessary.

Heterotrophic cultivation is generally used for biotechnological systems based on bacteria yeast, or fungi as production hosts (Schmidt 2004). Since the green alga *C. reinhardtii* also offers the potential for strict heterotrophic growth, this option was tested in cultivations including acetate as energy and carbon source in the dark. As demonstrated, this cultivation strategy turned out to clearly be the worst of all options, given the low overall biomass productivity and also low secreted *g*Luc*Lp*IBP observed from this cultivation style (Fig. 3). In contrast, light-driven bioproduction was possible through photoautotrophic cultivation of this strain in HiT medium, where strict photoautotrophic production of secreted *g*Luc*Lp*IBP was possible up to ~5 mg L^{-1} (Fig. 3B). It has to be mentioned though that HiT medium contains 12 g L^{-1} Tris, which is economically unfavourable to scale up. Reduction of the Tris content even as little a 10 g L^{-1} with this medium resulted in reduced culture and secreted recombinant protein performance from modified HiT medium (not shown).

Mixotrophic cultivation with TAP(CO_2) demonstrated higher productivities than all other cultures as early as 24 h cultivation (Fig. 3A), likely due to the use of two carbon sources for cell growth. Final cell densities for these cultivations were similar to those of both mixotrophic TAP(air) and photoautotrophic HiT medium cultivations (Fig. 3), indicating that use of two carbon sources has an additive effect on the productive capacity of this algal system. The combination of acetate feeding with the photosynthetic capacity of *C. reinhardtii* enhanced its photo-bioproduction capacity, resulting in the highest rates of production observed in mixotrophic TAP(CO_2) and TAP(air) cultures. This mixotrophic growth effect of boosting cell cultivation by simultaneous CO_2 supply and acetate feeding has been recently shown in our laboratory as a mechanism regulated by the control of light harvesting efficiency (Berger et al. 2014). Elevated CO_2 supply under mixotrophic conditions causes the inhibition of translation repression of light harvesting proteins of photosystem II, resulting in larger antennas and improved photosynthetic growth.

Previous experiments in small volume shake flasks of cultures expressing other secreted RP targets, late logarithmic-early stationary phase was used as a harvesting point for these cultures as no increase in product was observed after stationary phase was reached. This was true for several fluorescent reporters in our laboratory, and used as the harvest point for chromatography attempts with recombinant human erythropoietin secreted from *C. reinhardtii* (Lauersen et al., 2015, Eichler-Stahlberg et al., 2009). We previously noted that in certain conditions, secreted *g*Luc*Lp*IBP was less stable in high density bubbled cultures after 72 h cultivation and it was proposed that repetitive batch cultures use a 72 h cycle, in late logarithmic growth, to avoid product loss (Lauersen et al. 2013b). RP instability by 96 hours in turbid high-density culture may explain why TAP(air) cultivations reached titers of *g*Luc*Lp*IBP comparable to TAP(CO_2) cultivations by 96 h.

The culture productivities observed from mixotrophic, TAP(CO_2), cultivations in small scale indicated that this cultivation style should be used for scale-up to medium volume systems. We chose to attempt cultivation of strain UV*c*CA in a medium-volume flat panel photobioreactor designed for optimal culture light penetrance for efficient photosynthetic growth (depicted in Fig. 4A, left panels). This system indeed resulted in biomass accumulation for strain UV*c*CA similar to 400 mL cultivations, as well as a rapid increase in cell density. However, in this culture, the rapid increase in cell density was not coupled with high yields of the *g*Luc*Lp*IBP, which seemingly degraded after 48 h cultivation (Fig. 4C). This was surprising, given in all previous cultivations higher cell densities coincided with higher secreted RP yield (Fig. 3), however, suggested that culture turbidity

had a significant influence on secreted products in the culture medium. Indeed, the culture within the flat panel reactor is exclusively mixed by gas flow aeration across the entire base of the culture. It is possible that at these cell densities in this turbid environment, some cell lysis occurs, resulting in protease release into culture medium. Although the flat panel reactor allowed robust biomass productivities in medium scale-up, concomitant *g*Luc*Lp*IBP accumulation within the medium in this cultivation set-up was significantly hindered, indicating the flat panel system was not optimal for the proposed RP secretion-production process.

For a secreted product, the balance between cell density and biomass productivity with the stability and production of the secreted product must be considered. Given the issues for *g*Luc*Lp*IBP production associated with high-density turbid cultivation in the flat panel bioreactor system, we looked to a more gentle cultivation strategy employed for sensitive cell cultures such as mammalian and insect cells, which had been previously used for cGMP grade photosynthetic tissue culture and recombinant protein production from the moss *P. patens* (Baldi et al. 2007; Gitzinger et al. 2009; Ikonomou et al. 2003).

Cultivation at the 10 L scale in the wave bag system, proved to be a viable option for UV*c*CA cultivation and secreted *g*Luc*Lp*IBP production (Fig. 4). Biomass steadily accumulated in this system to ~0.9±0.10 g L^{-1} (Fig. 4B), and secreted *g*Luc*Lp*IBP accumulated to significant titres within 6 days of cultivation (Fig. 4C), surpassing that observed after 96 hours cultivation in 400 mL (Fig. 3B). Turbidity in the wave bag system was reduced, as gassing is injected to the bag on the culture surface, rather than bubbled through the medium, which likely resulted in reduced sheer stress to cells.

cGMP grade level cultivation in the wave bag system may be a valuable property for bio-production as described for other human-use products (Decker and Reski 2012; Gitzinger et al. 2009). Given the potential for the use of the *Lp*IBP for frozen food IRI, a safe, reliable cultivation strategy for production of this edible foodstuff is desirable (Griffith and Ewart 1995). However, the inherent costs of these bag systems makes them unreasonable for medium-value bulk food additive production, the list price for each bag can range from €240-350, without additional filters or tubing (Sartorius Stedim Biotech, Germany). Nevertheless, the concepts of surface gassing and gentle culture rocking to minimize sheer stress can be adapted to less expensive, food-grade plastic bag systems for microalgal cultivation. In addition, we have previously demonstrated that several cycles of repetitive batch cultivation of strain UVcCA is possible without inhibition of *g*Luc*Lp*IBP secretion

(Lauersen et al. 2013b). Therefore, bag systems could be re-used in a repetitive cultivation style, to limit process overhead costs.

Ice binding proteins with IRI activity are proposed as additives to increase storage time of frozen foods due to their ability to inhibit ice crystal growth at very low concentrations (Feeney and Yeh 1998; Griffith and Ewart 1995). It has been determined that IBPs pose no risk to human health, as these proteins are routinely consumed in the diets of people living in northern climates (Crevel et al. 2002). A prominent example where these proteins may be of use is as an additive to ice cream, in which recrystallization occurs within 24 hours of storage, and is intensified in varying temperature storage (Donhowe and Hartel 1996a; Donhowe and Hartel 1996b). Soluble protein extracts from cold-acclimated Winter Wheat, a frost tolerant plant (Regand and Goff 2006b; Regand and Goff 2006a), as well as different fish antifreeze proteins (Gaukel et al. 2014) have been shown to illicit IRI activity in sucrose solutions. Given that the *Lp*IBP can tolerate pasteurization (Pudney et al. 2003; Sidebottom et al. 2000), and demonstrates a strong IRI as low as 0.055 µM (Yu et al. 2010), it is a prime candidate for this purpose. Additionally, microalgae are generally regarded as safe for human consumption (GRAS) by the Food and Drug Administration of the United States of America (Gantar and Svirčev 2008; Rasala and Mayfield 2014). Therefore, we tested *Lp*IBP secreted from *C. reinhardtii* UV*c*CA, which had demonstrated IRI in pure media solutions previously (Lauersen et al. 2013b), in simplified ice-cream model solutions (Fig. 5). In order to limit the downstream processing costs associated with our algal product, total CEP from the algal culture was used, requiring only algal separation and concentration prior to use (Lauersen et al. 2013b). Clear IRI activity was detected in 49% sucrose after addition of total CEP samples from UV*c*CA cultures and lasted for up to 7 days, when the experimental trials were ended (Fig. 5). In this work, we did not study long term IRI, but since no signs for a decrease of IRI efficiency was detectable after 7 days, it is likely that the IRI would be effective for a significantly longer period of time. IRI did not occur for the equivalent CEP from the parental wild-type strain or sucrose solutions alone (Fig. 5A,B), demonstrating the specificity of this effect from the recombinant construct and indicating the possibility of using the CEP from transgenic *C. reinhardtii* as a potential food additive.

Currently, a recombinant fish IBP is industrially produced in yeasts and used to texture low-fat ice creams sold in the USA, Australia, and New Zealand (Penders 2011). AFP III has been shown to be produced to ~10-12 mg L^{-1} in *Escherichia coli* (Chao et al. 1993), and accumulates to ~20 g L^{-1} in fish blood (Fletcher et al. 1985). No data on this from

yeast is publically available, although RP titres from yeast systems can be up to several grams per litre culture (Porro et al. 2005). *E. coli* recombinant expression of the *Lp*IBP has been reported up to ~30 mg L^{-1} (Middleton et al. 2009), however, processing to yield a pure product requires several purification steps, including ice-affinity chromatography which would be costly to scale-up, highlighting the value of minimal processing as with CEP from GRAS algal culture. *Lp*IBP exhibits IRI at dilutions as low as 0.055 µM (Yu et al. 2010), for the 33-54 kDa *g*Luc*Lp*IBP species observed to be secreted from *C. reinhardtii* (Lauersen et al. 2013a), this equates to concentrations between 1.8 mg L^{-1} to 3 mg L^{-1} protein required for the IRI effect.

Approximately 12 mg L^{-1} *g*Luc*Lp*IBP was produced in 144 h from UV*c*CA in the Wave bag system, this titre equates to enough secreted product for up to ~67 L ice cream from a single photomixotrophically cultivated 10 L algal culture bag. In order to make this process cost effective, however, increased protein titres, process efficiency, perhaps through serial cultivation of multiple 10 L bags, the use of other inexpensive cultivation bags, and employing repetitive batch processes will be necessary.

5. Conclusions

Given the low media costs of algal cultivation, and the possibility of using the total concentrated extracellular proteins without target RP purification, *C. reinhardtii* based secretion of IBPs may represent a novel source for these food-texturing proteins. Scale-up of algal systems presents many technical hurdles, and the data presented here indicate that photobioreactors, which produce optimal culture biomass, may not necessarily be productive for secreted RPs. Although most protein targets will require individualized culture conditions, the secretion of *g*Luc*Lp*IBP presented here represents first insights into the interplay of RP secretion behaviour and microalgal cultivation. The results of this work suggest that traditionally secreted soluble recombinant products accumulate during cell doubling, therefore, cultivation conditions which allow high-cell densities should be used for production. In addition, sheer stress and turbidity should be reduced, in order to prevent secreted RP degradation and loss. A balance between culture density and cultivation parameters must exist to assist stable secreted RP in culture media. Photosynthetic production capacity of *C. reinhardtii* is greater than its heterotrophic capacity, and through addition of some organic carbon source, photo-bioproduction of a secreted RP was enhanced. The wave bag system, which is cGMP grade, seems to provide a gentle environment for both moderate cell growth and recombinant protein secretion, although

less expensive bag systems will need to be used to make this production style cost-effective. It is likely that the reduced turbidity of this system was a major factor to allow stable RP accumulation in culture medium. However, secreted recombinant protein titres will need to be improved in order to make microalgae viable as an alternative for the production of industrially relevant products for the food industry.

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7. Conflict of Interest

The authors declare that they have no conflict of interest.

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9. Figures

Fig. 1 Addition of the *Lp*IBP to the C-terminus of *g*Luc results in increased recombinant protein secretion. Presented are average bioluminescence signals of pre-stationary phase TAP grown cultures for four strains isolated from transformant populations generated with either pOpt_cCA_gLuc_Paro or pOpt_cCA_gLuc_LpIBP_Paro vectors (**A**). Bioluminescence signals from TAP grown cultures were normalized to cell density for four strains selected as the highest producers amongst 480 randomly picked colonies generated from either vector in triplicate transformations (**B**). Error bars indicate standard deviation. **H**: heat shock protein 70A promoter; **R**: ribulose bisphosphate carboxylase/oxygenase small subunit 2 (RBCS2) promoter; **i1/2** intron 1/2 of *C. reinhardtii* RBCS2; ***c*CA** carbonic anhydrase secretion signal; **3'** RBCS2 3' untranslated region.

Fig. 2 Precondition growth and secreted recombinant protein expression analysis of UV*c*CA in TAP medium. **A,B** 1 L shake, baffled shake, or stirred TAP medium cultures were used to determine appropriate cultivation styles for *g*Luc*Lp*IBP production. Culture cell density (**A**) and productivities of *g*Luc*Lp*IBP for each style, analyzed by dot blot of 1µl culture medium with α-*g*Luc antibody (**B**), are presented over 144 h cultivation.

Fig. 3 Analysis of strain UV*c*CA in 3 media and different cultivation conditions at the 400 mL scale with stir mixing and gas bubbling. TAP media, with 1 g L^{-1} acetate, was used for cultivation in three different conditions, with addition of 3% CO_2, with only air, or in the dark with air. HSM and HiT media were used for strictly photoautotrophic cultivations with only 3% CO_2 as a carbon source. **A** Cell density and dry biomass (upper and lower panels, respectively) were recorded and secreted *g*Luc*Lp*IBP was quantified from daily culture samples by dot-blot against the *g*Luc portion of the fusion protein (**B**). Recombinant *g*Luc produced by secretion from in *K. lactis* was used as a standard.

Fig. 4 UV*c*CA cultivation in medium volume scale-up. **A** Left panel, the 10 L flat panel reactor at 96 hours of cultivation, right panels depict the rocking 10 L wave bag reactor at 96 hours cultivation. **B** Culture growth parameters in 10 L flat panel and 10 L wave bag bioreactors. Values recorded from 400 mL TAP with 3% CO_2 cultivation are added for comparison. Cell density and dry biomass (upper and lower graphs, respectively) are

presented. **C** Accumulated *g*Luc*Lp*IBP in culture media from each system. Samples from 400 mL TAP 3% CO_2 cultures, 10 L flat panel, and 10 L wave bag are compared in dot blot of 1 µl culture media with an anti-*g*Luc antibody. WT, indicates culture medium from a 10 L cultivation of parental strain at 96 h and a 10X concentration of this protein extract to demonstrate the antibody specificity for *g*Luc and minimal background from native C. reinhardtii secreted proteins. The standard dilution series (right) was produced using recombinant *g*Luc from *K. lactis*.

Fig. 5 Demonstration of ice recrystallization inhibition activity using concentrated UV*c*CA culture supernatant in simplified ice cream model solutions. **A** Recrystallization occurs readily in 49% sucrose solution containing concentrated parental strain (WT) extracellular proteins while secreted *g*Luc*Lp*IBP from UV*c*CA or a purified fish antifreeze protein (AFP type III, from *M. americanus*) demonstrate effective ice recrystallization inhibition activities up to 168 h. Bar represents 100 µm. **B** Quantitative measurements of mean crystal sized in the four tested solutions including sucrose control.

10. Supplemental Data

Table S1 In house High-Tris medium

1. First prepare 100mM Tris (60.57 g in 3L), then pH to 7.3 (with an abundance of 37% HCl)
2. Then add stock solutions as follows:

Solution	mL of stock solution used for 5L medium	mL of stock solution used for 1L medium
NH_4NO_3	50	10
$CaCl_2.2H_2O$	10	2
$MgSO_4.7H_2O$	100	20
KH_2PO_4	10	2
$FeSO_4.7H_2O$	5	1
$CuSO_4.5H_2O$	5	1
$MnCl_2.4H_2O$	5	1
$ZnSO_4.7H_2O$	5	1
H_3BO_3	5	1
$(NH_4)6Mo7O_{24}.4H_2O)$	5	1
$CoCl_2.6H_2O$	5	1
Na_2SeO_3	5	1
$Na_2SiO_3.5H_2O$	5	1
EDTA Disodium Salt, pH8	5	1
	Fill to 5 L with ddH2O	

To prepare 500 mL of each stock solution

Stock solutions	g 500 mL^{-1}	Molecular weight (g mol^{-1})	Concentration factor	conc. in culture medium (mM)
NH_4NO_3	60.03	80.04	100	15
$CaCl_2.2H_2O$	31.24	147.01	1000	0.425
$MgSO_4.7H_2O$	9.24	246.48	100	0.750
KH_2PO_4	68.05	136.09	100	10
$FeSO_4.7H_2O$	0.14	278.01	1000	0.001
$CuSO_4.5H_2O$	0.80	249.68	1000	0.0064
$MnCl_2.4H_2O$	2.55	197.90	1000	0.0258
$ZnSO_4.7H_2O$	11.07	287.56	1000	0.077
H_3BO_3	5.69	61.83	1000	0.184
$(NH_4)6Mo7O_{24}.4H_2O)$	0.55	1235.87	1000	0.0009
$CoCl_2.6H_2O$	0.80	237.93	1000	0.0067
Na_2SeO_3	0.01	172.94	1000	0.0001
$Na_2SiO_3.5H_2O$	28.96	212.14	1000	0.273
EDTA Disodium Salt, pH8	100.00	372.24	1000	0.5373

Figure 1
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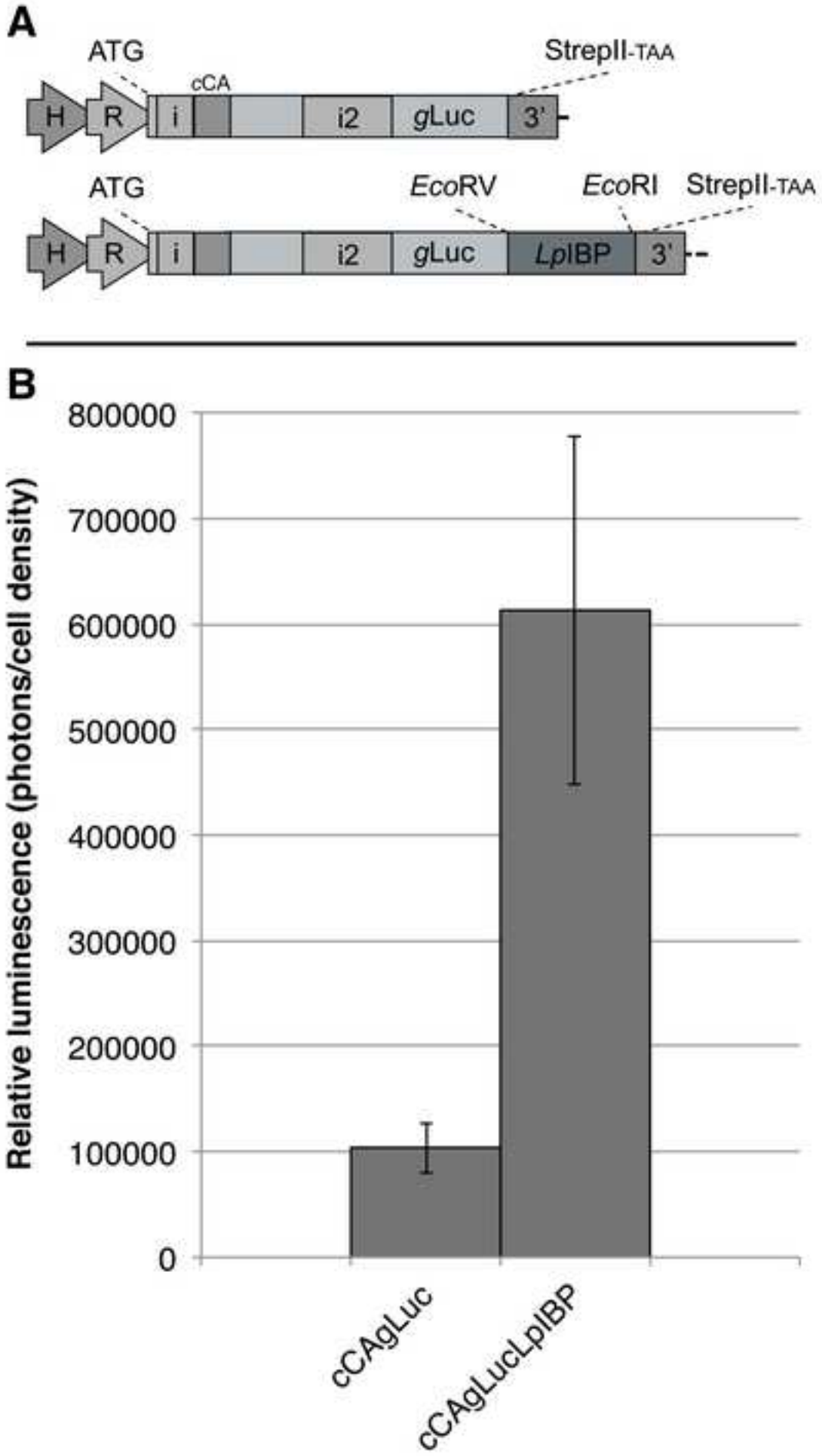

Figure 2
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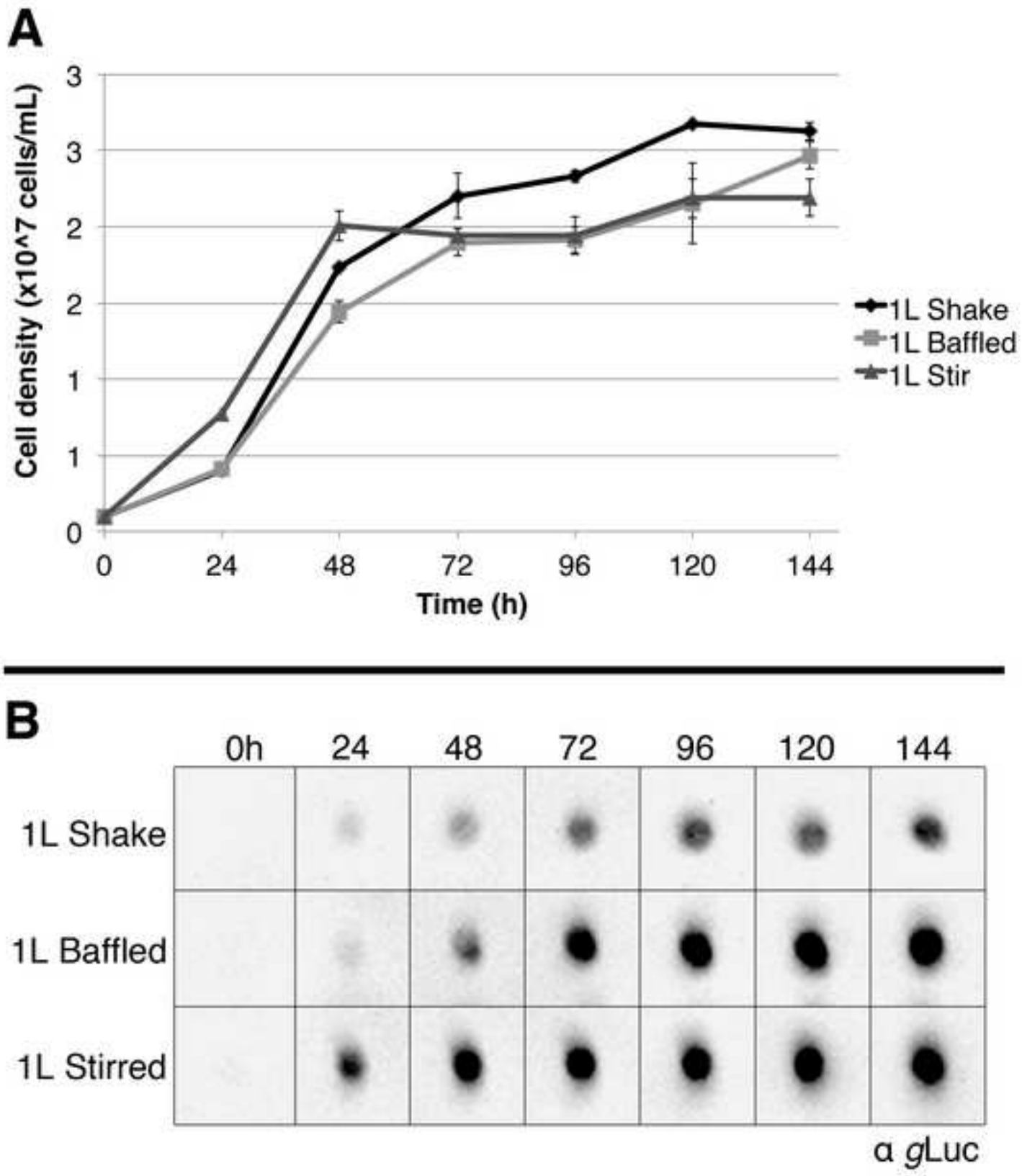

Figure 3
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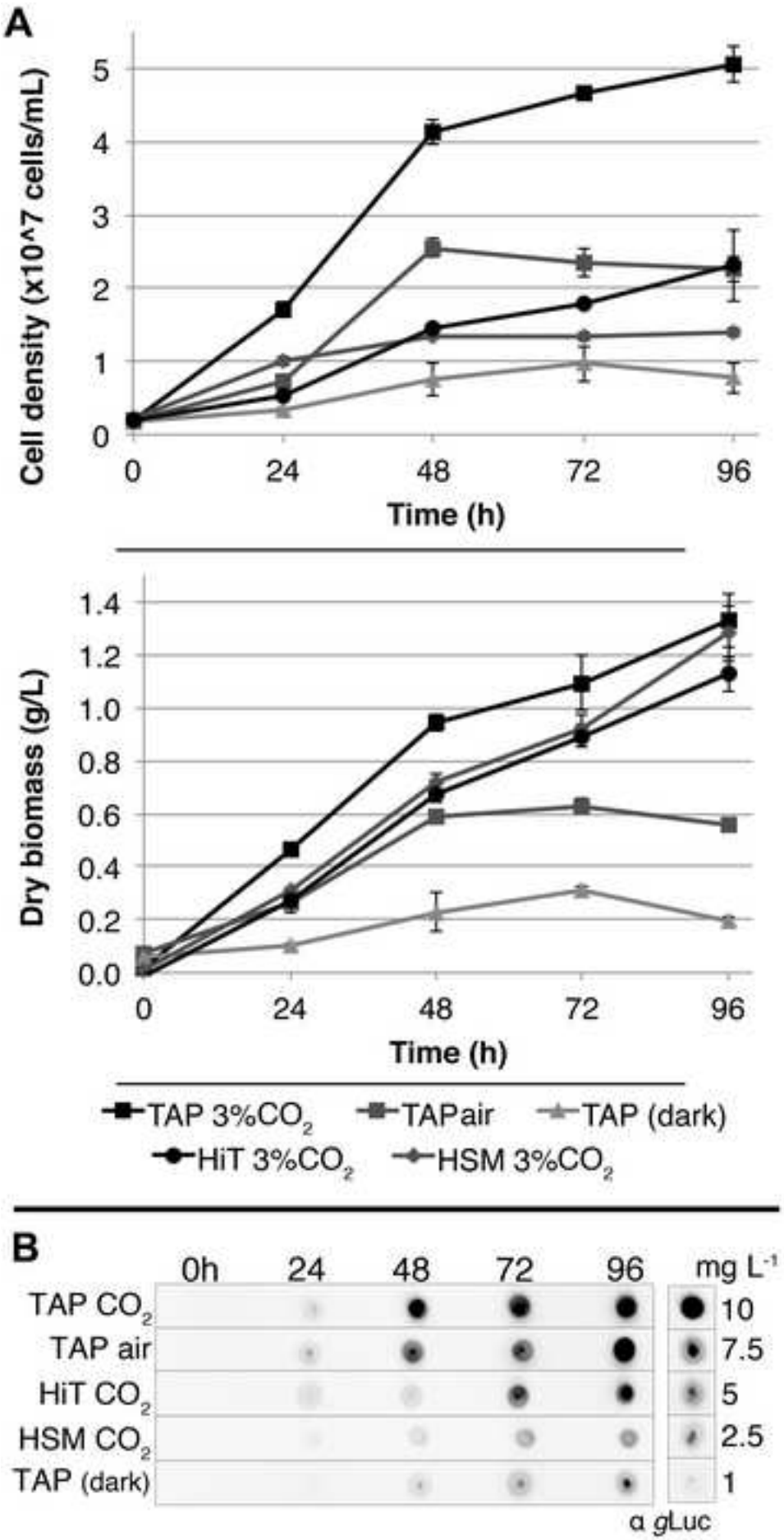

Figure 4

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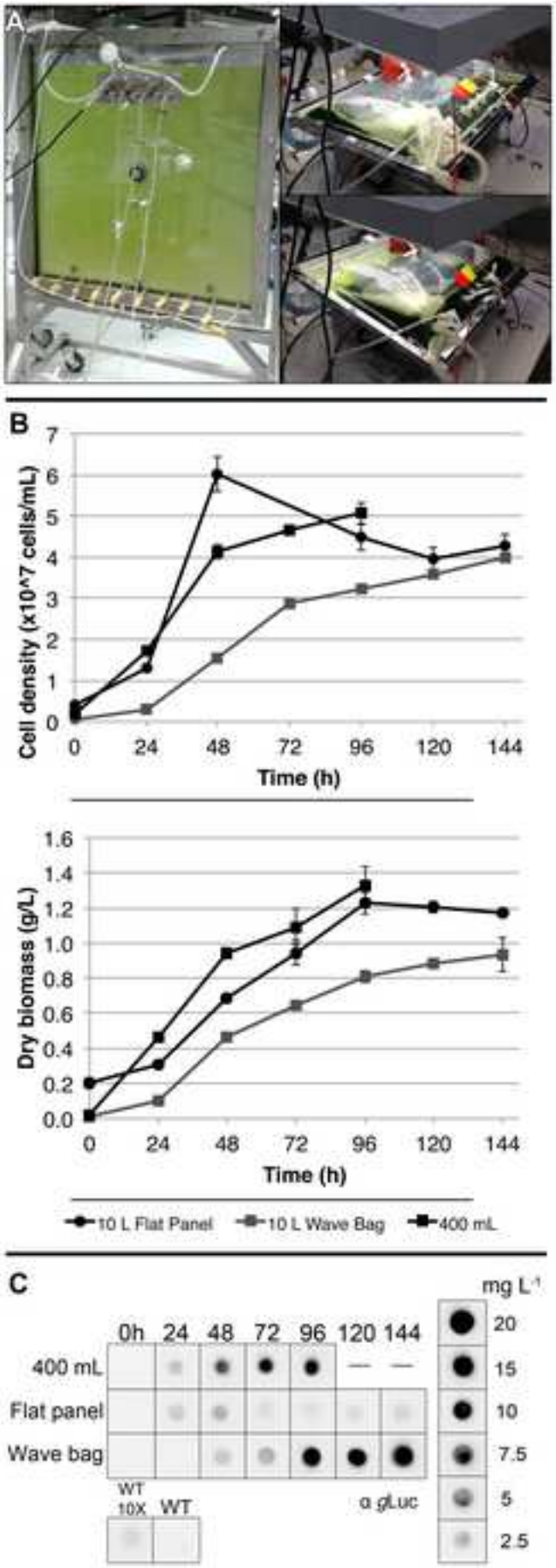

Figure 5
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