Effect of culture conditions on recombinant protein production in *Chlamydomonas reinhardtii*

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PhD by Research

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Certificate of original authorship

I, Navpreet Kaur Walia declare that this thesis, is submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the Faculty of Science at the University of Technology Sydney. This research is supported by an Australian Government Research Training Program. This thesis is wholly my own work unless otherwise reference or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis. This document has not been submitted for qualifications at any other academic institution.

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Acknowledgement

I would first like to thank Waheguru for always being there with me. I would like to thank my supervisors: Audrey Commault and Mathieu Pernice for their guidance, support, patience and criticisms. I want to specially thank my co-supervisor Dr. Audrey Commault because without her support and cooperation this thesis would not have been possible. I would also like to thank my co-authors for their contributions, without which the work submitted for publication contained in this thesis would not have been possible. I'd also like to thank Paul Brooks and UTS technical staff for helping me with the various training and inductions. To all the people I worked with along the way, especially Algal bio-factory group, thank you all for your help throughout my PhD. Finally, most importantly, I would like to thank my parents and husband because this thesis would not have been possible without their unconditional support, encouragement, patience, and cooperation.

Preface

This thesis has been prepared for submission as a thesis by compilation, however the thesis contains a publishable work. As a result, there is a degree of repetition across chapters, particularly within the introductions and materials and methods sections of each chapter. Supplementary information for each chapter appears in the supplementary data and have been re-numbered accordingly. The referencing format used in this thesis is nature referencing style.

This thesis is dedicated to my loving parents

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Abstract

Microalgae are increasingly being used in recombinant protein production for a number of reasons including low cultivation costs, and the presence of post-translational modification mechanisms. There has been a lot of research on optimization of culture conditions in other expression systems like mammalian cell lines and plants to improve the yield of recombinant proteins. However, not much has been done on optimizing culture conditions to improve the yield of recombinant protein production from microalgae so far. The focus of this thesis was to investigate how different culture conditions affect recombinant protein production from nuclear transgenes in *C. reinhardtii,* with the ultimate goal to find suitable optimized culture strategies to increase production without significant negative side effect on the growth and health of algal cultures. More particularly, this thesis investigated the effect of a culturing strategy using two different growth phases (i.e. biphasic growth) on recombinant protein production in *C. reinhardtii*. The recombinant protein used in this study, interferon alpha 2 A (rIFN α2A) was chosen because of its high commercial value, anti-viral and anti-cancer properties.

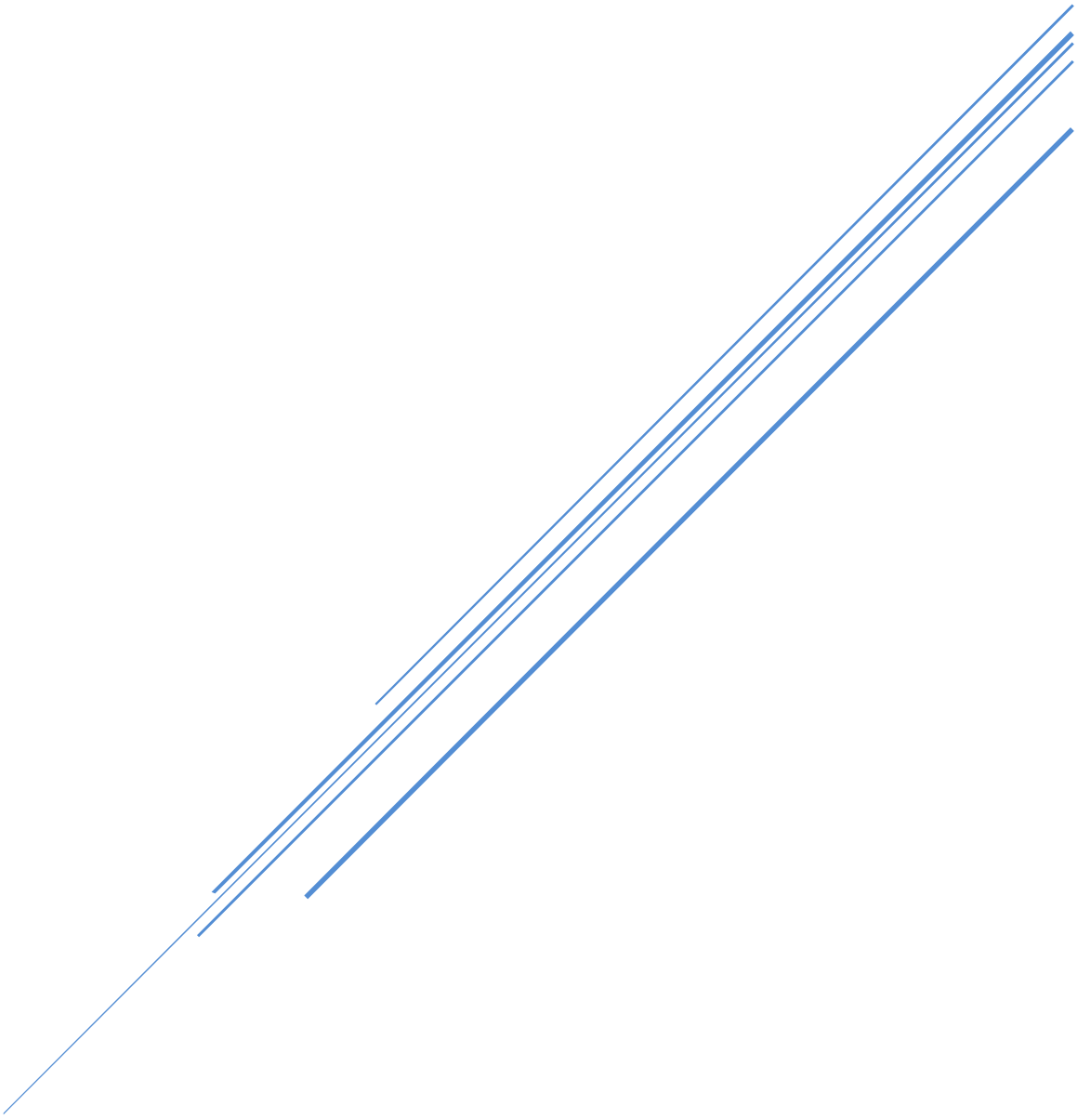
Overall, my results suggest that biphasic growth (temperature regime and carbon source) can have significant effect on recombinant protein production in *C. reinhardtii* and should definitively be considered to improve application of microalgae as a commercially viable platform for recombinant protein production.

Publications:

- Chapter 2 has been recently submitted to Biotechnology & Bioengineering and the manuscript is under review now: Navpreet Kaur Walia, Audrey S. Commault, Michele Fabris, Lorenzo Barolo, Jack Adriaans, Peter J. Ralph, Mathieu Pernice. Biphasic temperature regime increases the yield of a therapeutic recombinant protein in the green alga *Chlamydomonas reinhardtii*

- Chapter 3-4 will be submitted in the near future to peer-reviewed scientific journals.

Chapter 1: Introduction



1.1. Cell structure and life cycle of *Chlamydomonas reinhardtii*

Chlamydomonas was first discovered in 1838 by C.G. Ehrenberg. *Chlamys*, is a Greek word which means a cloak or mantle; while *Monas* means solitary, a term now generically used for certain unicellular flagellates. In 1976, Ettl characterised *Chlamydomonas reinhardtii* (*C. reinhardtii),* which then became widely used in laboratory work. The main features of the genus *Chlamydomonas* are two anterior flagella of equal length, a cell wall, and a single chloroplast or chromatophore having one or more pyrenoids (Fig. 1).

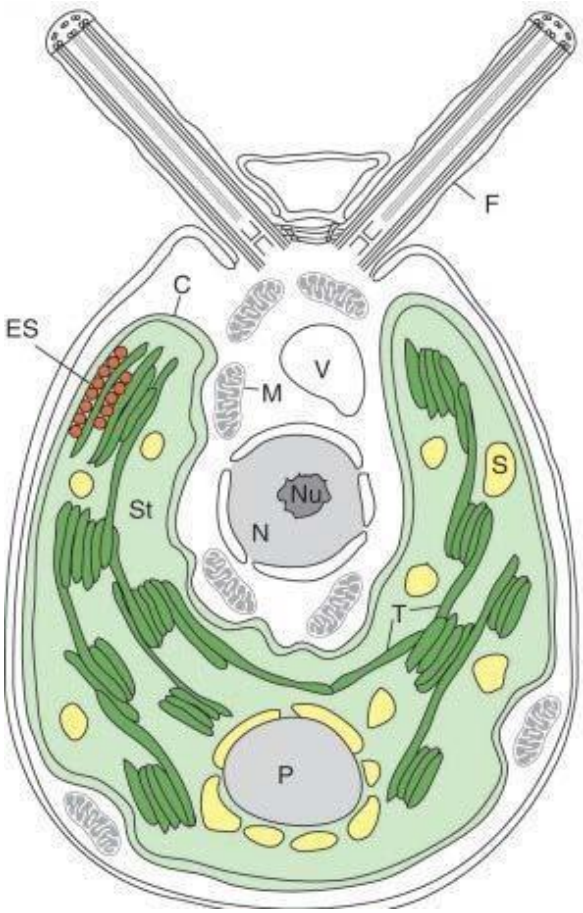


***Fig. 1 Cell Structure of Chlamydomonas reinhardtii*. Central nucleus (N); nucleolus (Nu); two isoform flagella (F); cup-shaped chloroplast (C) with eyespot (ES) and thylakoid membranes (T); pyrenoid (P) comprised of starch; mitochondria (M); starch grains (S); Golgi vesicles (G); stroma (St); and vacuoles(V). Sourced from [1].**

Chlamydomonas sp. possess a controlled sexual cycle[2]. Its photosynthetic apparatus resembles that of vascular plants and is encoded by both nuclear and chloroplast genomes. *Chlamydomonas* sp. possess a cell wall, have the ability to grow in heterotrophic conditions and perform phototaxis, as

a result of two flagella that allow moving away or towards light to protect its photosystem from photo-damage [2]. *Chlamydomonas* sp. can adapt to anaerobic metabolism and can produce hydrogen gas and metabolites such as ethanol and formate[2]. *Chlamydomonas reinhardtii* has a wide metabolic flexibility, which makes it possible to survive in varied nutrient conditions. There is extensive research to date using *Chlamydomonas* sp. to produce biofuels and bioremediation [3,4], and it is now emerging as a new expression system for recombinant protein production. Indeed, *Chlamydomonas* sp. is the only known eukaryote to date that can be transformed in all three genomes: nuclear, chloroplast and mitochondria [5], making it an organism of choice for genetic studies. The first evidence of mammalian protein expression in *C. reinhardtii* was HSV8-lsc, a single chain antibody, which was intended to protect against glycoprotein D of herpes simplex virus [6]. This PhD thesis focuses on the production of a recombinant human protein, Interferon alpha 2 A (rIFN α2A), in *C. reinhardtii*. The next section focuses on introducing what role interferons play in the human immune system and their importance as therapeutic drugs for the treatment of diverse diseases.

1.2 Interferons

1.2.1. Interferons structure andfunction

Interferons (IFNs) are a group of cytokines that have antiviral and anti-proliferative effects, being the first line of defense against viral infections [7]. Interferons trigger an immunogenic response and act as a signaling protein for communication between cells [8]. They are produced by infected cells as a warning signal to surrounding cells [8]. There are mainly two types of interferons: type I interferons, which includes interferons alpha (IFNα) and beta (IFNβ) and type II interferon, which includes interferon gamma (IFNγ) (Figure 2).

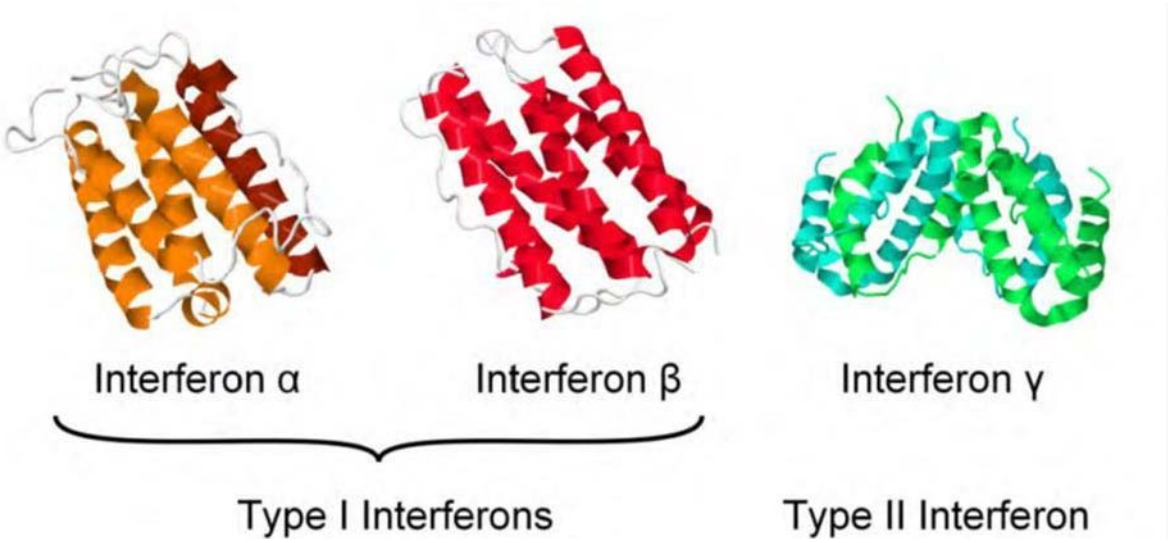


Fig. 2 Structures of interferon proteins. Type I interferons, interferons alpha and beta, possess a similar structure, which is comprised of five alpha helices. While both IFN α2A and IFNβ1 (shown here as illustration) have similar monomers, they dimerize in a different way resulting in different specific function. IFN α2A dimerizes alongside homologous surfaces whereas, IFNβ1 dimerizes towards the opposing side of the protein[9]. IFNγ is shown in its dimerized form, and the two colors represent two intertwined monomers. Sourced from [8].

All the three interferons have been reported to have different cellular origins, IFNα having a leukocyte origin, while IFNβ and IFNγ have fibroblast and lymphocyte origin, respectively [10]. Type I interferons includes a diversity of types, comprising IFN-α (IFN-α1, -α2, -α4, -α5, -α6, -α7, -α8, -α10, -α13, -α14, -α16, -α17 and -α21), IFN-β, IFN-δ, IFN-ε, IFN-κ, IFN-τ and IFN-ω1,2,3, but all of them have similar structure and function. IFN-α, IFN-β, IFN-e, IFN-κ and IFN-ω are found in humans, while IFN-δ and IFN-τ are designated only for pigs and cattle as there are no humans' homologues. Most of the human cells have receptors for type I IFNs [11] and share a common cell surface binder for all of type I IFNs (type I IFN receptor1,2,3). This PhD focuses on recombinant interferon alpha 2A (rIFN α2A), which comes under Type I IFNs.

1.2.2. Interferon alpha 2A as a recombinant protein of choice

Recombinant proteins can be defined as proteins encoded by an exogenous gene (i.e. DNA synthetically produced or originating from an organism that would normally be absent from the host genome). In this study, *Chlamydomonas reinhardtii* has been genetically engineered to produce a recombinant interferon alpha 2A (rIFN α2A), which is a human protein with anti-cancer

and anti-viral properties and is encoded by the *ifnα2A* gene [12]. It was selected as a target early in this thesis, mostly because of its high commercial value. It has been used in the treatment of chronic illness and cancers [12] and offers a cure for several human diseases, including: chronic viral hepatitis C (HCV), chronic myeloid leukemia (CML), Kaposi sarcoma, follicular lymphoma, renal cell carcinoma (RCC), melanoma, T cell lymphoma multiple myeloma and condylomata acuminate [12,13].

Fig. 3 An example of a commercially available recombinant IFN α2A sold as an injectable from bacteria (source- Sinosource Biopharmaceuticals Inc.).

1.3 Advantages of microalgae for recombinant protein production over other expression systems

In pharmaceutical industry, recombinant proteins are mainly expressed in bacteria, yeast, plants and mammalian cells [14,15,16]. The most used production systems for recombinant proteins are bacterial systems [17]. However, the absence of post translational modification mechanisms and complexities in purification of proteins from inclusion bodies deems them less efficient for recombinant protein production [18]. Yeast are eukaryotes and are good alternative expression systems to prokaryotic expression systems, but yeast expression systems sustain specific post translation modifications that are not always suitable for therapeutic proteins designed for animal or human consumption due to the immunogenicity risk [19]. Recombinant proteins in yeast are usually hyper-glycosylated, which alters immunogenic epitopes, and the high-

mannose glycosylation performed in such systems results in low *in vivo* half-life of proteins. The combination of these two factors largely compromises the therapeutic activity of the products [19,20]. In addition, secretion mechanisms in yeast-based expression systems have resulted in poor protein yields [21]. Other production systems like insect and fungal-based systems also have some drawbacks to produce recombinant proteins. Insect-based systems, despite having post translational properties like glycosylation, result in high cost and many technical difficulties in scale-up [22]. High concentration of proteases has limited the use of fungal based systems for recombinant protein production [23]. Post-translational modifications, mainly N- and O- linked glycosylation, are important to produce recombinant protein due to their effect on *in vivo* immune and biological activity in humans [24]. Therefore, many therapeutic proteins have been produced in mammalian cell lines to ensure the development of human-like glycosylation patterns. Mammalian cell lines used for this purpose are Chinese Hamster Ovary (CHO) cells [25]; Human Embryonic Kidney (HEK 293) cells [26]; and Baby Hamster Kidney (BHK) cells [27]. The main limitations with these expression systems are the complex and expensive media for cultivation making the upstream process expensive. Plants can also be used as an expression system for recombinant protein production.

There are many advantages in using plants as a production platform for proteins, such as ─ being less prone to human pathogens contamination like virus as compared to mammalian cell cultures [28]. But stable transformed plants expression systems have very low yields i.e. typically 1% of total soluble protein (TSP) [29]. Algae offer advantages similar to transgenic plants but with less risk of contamination by airborne contaminants, because algae can be grown in sealed bioreactors, limiting the risk of environmental contamination by genetically engineered algae. In addition, microalgae have a faster doubling time compared to higher plants, which means that microalgal systems are faster to develop from initial transformation to large-scale production compared to plant expression systems, with an estimated time of 2-3 weeks for microalgae, compared to 4–6 months for plants[30]. Accumulation of recombinant protein is also more consistent in microalgae because they are single-celled organisms, which results in more homogenous material for downstream processing. Further, because many species of algae are generally regarded as safe (GRAS), meaning they are safe to eat and are possible source for oral delivery of therapeutic proteins with little or no purification steps [31,32], purification steps can be streamlined when using algae as a production platform for recombinant proteins. Therefore, algae hold more potential for industrial applications than any other

expression systems [33]. Among many other microalgae species, *Chlamydomonas reinhardtii* has been extensively studied due to the availability of genetic resources, its whole genome being sequenced, as well as being a model of choice in algal research.

Many studies have explored microalgae for their ability to produce biofuels, metabolites and nutraceuticals, but it is only recently that microalgae have been used as new tools for recombinant protein production [6,34]. Rochaix and Van dillewin were the first to report an expression of foreign DNA in *Chlamydomonas* in 1982[35]. In 1989, two laboratories successfully transformed *Chlamydomonas reinhardtii* nuclear genomes [35,36]. Since then, algae have been widely explored for their capability for recombinant protein expression. In some cases, algae can be preferred to using expression systems such as bacteria, yeast and mammalian cell cultures as a platform for recombinant protein production [37]. The reasons for this are the presence of chaperons and other cellular machinery that are available in algae to help fold complex human proteins, which are unavailable in bacteria [38].

Table 1.1: Comparison of microalgae expression system over other expression systems (adapted from[19])

Expression systems	Microalgae	Bacteria	Mammalian cells	Plants
Cost	**Low**	Medium	High	Low
Scalability	**High**	Limited	Limited	High
Product Quality	**High**	Low	High	High
Production Time	**Short**	Short	Long	Long
Protein Folding Accuracy	**High**	Low	High	High
Glycosylation	**Minor differences**	None	High	Minor differences
Safety	**High**	Low	High	High
Endotoxin level	**Low**	Medium	High	---
Protein Yield	**Low**	Medium	High	High

C. reinhardtii has been extensively used as a model eukaryotic alga, as it offers many advantages like rapid growth, as well as easy genetic and metabolic manipulability. Moreover, it can be grown axenically and has a rapid and controlled sexual cycle having stable and viable haploid state therefore, resulting in less variation in recombinant protein accumulation thus making downstream processing more uniform [5,30]. It is possible to transform all the three genomes i.e. chloroplast, mitochondria and nucleus in *C. reinhardtii* [38]. There is, therefore, an abundance of options for targeted gene manipulation[5]. Transgene insertion is commonly performed in chloroplast of *C. reinhardtii* [39] as it has been largely targeted for strategies of genetic manipulation. Engineering the nucleus of *C. reinhardtii* offers the capacity to secrete recombinant proteins. Another advantage of the green alga *C. reinhardtii* in general, is that it can be grown under heterotrophic, phototrophic and mixotrophic culture conditions thus offering greater flexibility when it comes to available cultivation techniques [30]. Therefore, microalgae can be grown in open ponds, enclosed systems like PBRs or fermentation reactors [40]. At present, the main research focus of the researchers around the world is to develop, understand and improve genetic engineering and protein accumulation in the chloroplast and nucleus[5,6,27,31].

1.4 Recombinant protein expression in chloroplast versus nuclear genome of *C. reinhardtii*

Bioactive recombinant proteins have been successfully produced using both nuclear and chloroplast genomes of *C. reinhardtii*. Currently, recombinant protein production in *C. reinhardtii* is mostly focused on chloroplast transgene expression [41], mainly because of chloroplast genome ability for efficient homologous recombination [42]. In addition, a yield of 5% recombinant protein (RP) per total soluble protein (TSP) has been observed in chloroplast [43], whereas in the case of nuclear genome the yield drops to only 0.2 % of TSP [44]. One reason for this low yield in nuclear genome is probably linked to random integration of the transgene leading to nuclear gene silencing[5] and un-optimised growth conditions. Therefore, at present for commercial recombinant protein production, chloroplast system is considered more suitable than nuclear system for intracellular recombinant protein production. The chloroplast transformation techniques in *C. reinhardtii*, which are reproducible, rapid and combined with high-level expression systems has shown promising results to produce therapeutic and edible proteins devoid of any contamination [43]. However, there is a significant drawback in chloroplast

transformation techniques, which is the absence of key post-translational modifications such as glycosylation that are generally required for proper recombinant protein activity and stability. Therefore, depending on the product and market targeted, recombinant protein production from nucleus be a better alternative to chloroplast, especially for human therapeutics use.

Nuclear transformation in *C. reinhardtii* occurs by integration of exogenous DNA into the nuclear genome by non-homologous recombination. The first stable nuclear transformation in *C. reinhardtii* was reported in 1989 [35,45] Transgene expression from the nuclear genome of *C. reinhardtii* offers several advantages over chloroplast expression. These include glycosylation and other post-translational modifications and heterologous protein-targeting to sub-cellular locations or secretion mechanisms, which are not available in chloroplasts [31]. Glycosylation is a form of post-translational modification that provides greater proteomic diversity than other posttranslational modifications (PTMs). Protein glycosylation is defined as the addition of carbohydrate moieties to protein, thus helping properprotein folding, protein-protein interactions, stability and optimal pharmacokinetics [46]. It also helps to increase the half-life of the protein, making it more stable than non-glycosylated proteins [46]. The nuclear genome also allows protein secretion where the protein encoded by nuclear transgenes are targeted to the endoplasmic reticulum and Golgi where they are glycosylated. Secreted recombinant proteins can be purified directly from the culture medium without the need to lyse cells, enabling the production of soluble and biologically active proteins at a reduced process cost [47]. Further, given that the glycosylation pattern in *Chlamydomonas* is different from the one seen in human cells, enzymes involved in glycosylation have to be engineered for the product to have human-like glycosylation pattern and not to trigger immune reactions in humans treated with algal recombinant proteins[47].

A wide range of recombinant proteins have been successfully expressed in *C. reinhardtii* (**Table 2**)[5] from nuclear and chloroplast transgenes. The first recombinant protein expression was achieved in the chloroplast; it was a single chain antibody (HSV8-lsc) against the herpes simplex virus [6]. Several other human proteins have also been expressed in *C. reinhardtii* since. For example, a full length human IgG1 monoclonal antibody was expressed in the chloroplast of *Chlamydomonas* sp. [48] as well as a protein known to induce apoptosis in virus and tumour infected cells, which is related to the human tumour necrosis factor known as tumour related apoptosis-inducing ligand (TRAIL) was also produced in *Chlamydomonas* choloroplast [49]. Humanglutamic

acid decarboxylase (hGAD65) was also successfully expressed in the *Chlamydomonas* chloroplast[50]. While a human protein used for the treatment of anaemia i.e. Human erythropoietin (Epo) was produced from the nuclear genome of *Chlamydomonas*[51]. Other examples include a bovine mammary-associated serum amyloid (M-SAA), biologically active, which was also expressed in the chloroplast [43] and an expression vector pACTBVP1 (*C. reinhardtii* expression vector), which was used to express the fusion protein having foot and mouth disease virus (FMDV) VP1 gene and the cholera toxin B subunit (CTB) gene and subsequently to express CTBVP1 in *Chlamydomonas* chloroplast [52]. The first example of functional oral delivered vaccines from algae was done by successful engineering of chloroplast of *C. reinhardtii* for the expression of D2 fibronectin-binding domain of *Staphylococcus aureus* fused to cholera toxin B subunit [53]. In addition, in the same year four recombinant proteins (10NF3-14FN3, M-SAA-Interferon b1, Proinsulin and VEGF) were expressed in the algae chloroplast, which showed economic viability (3% TSP) [54]. There has been sufficient proof of concept data available for the production of recombinant proteins from microalgae, but microalgae-based expression systems are still in developmental stages.

Further research is required to take algae-based expression systems from bench to commercial scale. This includes (i) optimisation of algal growth conditions to increase recombinant protein yield, (ii) optimisation of promoters for algae expression systems, (iii) better understanding protein degradation pathways in algal cells and (iv) identification of genes involved in the degradation of the protein of interest (e.g. genes coding for proteases). There is a considerable amount of research that still needs to be undertaken on *C. reinhardtii* to make it as an economically viable platform for recombinant protein production from nuclear transgenes. Some of these strategies are described in the section below.

Table 1.2: Recombinant protein expression in *C. reinhardtii* (Adapted from [5])

Name of recombinant protein	Function	*C. reinhardtii* strains	Chloroplast / Nucleus	Intracellular / Secreted	Yield	Reference
HSV8-lsc	Treatment of herpes simplex virus	*C. reinhardtii* strain 137c	Chloroplast	Intracellular	detectable	[6]
Monoclonal antibody IgG1	Murine and human antibodies (LC and HC)	*C. reinhardtii*	Chloroplast	Intracellular	detectable	[48]
Tumour related apoptosis-inducing ligand (TRAIL	Human tumor necrosis factor-related apoptosis-inducing ligand (TRAIL	*C. reinhardtii* strain 137c	Chloroplast	Intracellular	0.67% TSP	[49]
hGAD65	Diabetes-associated anutoantigen human glutamic acid decarboxylase 65	*C. reinhardtii* strain 137c	Chloroplast	Intracellular	0.3% TSP	[50]
Lolium perenne ice binding protein (LpIBP)	Food texturing and cryopreservation additive	*C. reinhardtii* UVM4	Nucleus	Secreted	12mg/L	[46]
Human erythropoietin (Epo) (ARS2-crEpo-his6)	Human erythropoietin fused to ARS2	*C. reinhardtii* strain *cw15arg*	Nucleus	Secreted	100µg/L culture	[51]

	export sequence w/6xhis tag					
M-SAA	Bovine mammary-associated serum amyloid	*C. reinhardtii* strain 137c	Chloroplast	Intracellular	detectable	[43]
CTB-VP1	Cholera toxin B subunit fused to foot and mouth disease VP1	*C. reinhardtii* strain 137c	Chloroplast	Intracellular	3%TSP	[52]
CTB-D2	D2 fibronectin-binding domain of Staphylococcus aureus fused with the	*C. reinhardtii* strain CC-125 (mt+)	Chloroplast	Intracellular	0.7% TSP	[53]
1. 10NF3, 14FN3 2. M-SAA-Interferon b1 3. Proinsulin 4. VEGF	1. Domains 10 and 14 of human fibronectin, potential antibody mimics. 2. Multiple sclerosis treatment fused to M-SAA 3. Treatment for type I diabetes 4. Human vascular endothelial growth factor isoform 121	*C. reinhardtii* wt strain 137c (mt+)	Chloroplast	Intracellular	1. 10NF3-detectable, 14FN3- 3% TSP 2.Detectable 3.Detectable 4. 2% TSP	[55]

Pal endolysin	Anti-bacterial	*C. reinhardtii* cell wall-reduced strain TN72	Chloroplast	Intracellular	6.77 mg/L	[89]
SSVenus-$(SP)_n$	Molecular tool kit	*C. reinhardtii* cell-wall-deficient UVM4 strain	Nucleus	Secreted	15 mg/L	[90]

1.5 Factors affecting recombinant protein expression and strategies for optimising yields of nucleartransgene

C. reinhardtii is an emerging platform for recombinant protein production, but many factors are yet to be optimized. Many challenges have been faced by researchers to express transgenes in *Chlamydomonas* including codon usage, promoters and culture conditions optimisation.

1.5.1 Codon optimization

Among the many factors affecting recombinant protein expression in *C. reinhardtii*, a major one is codon optimisation [56]. The *Chlamydomonas* genome is GC rich. However, most of the transgenes, specifically the antibiotic selection markers that were trial initially for selection in *Chlamydomonas* were AT rich as they were sourced from bacterial work [57]. The quantitative method used for calculating the level of expression of native and heterologous genes based on their codon usage is Codon adaptation Index (COI) [56]. A reference set of highly expressed genes should be used to compute this parameter to get the most out of the heterologous gene expression. In *C. reinhardtii*, all the three genomes (chloroplast, mitochondrial and nuclear) have different codon bias, thereby a genome specific CAI values should be used for an optimal translation in each of these compartments [58]. *Chlamydomonas* nuclear genome has been observed to have a significant bias towards GC rich codon usage, with 66%, 48% and 86% of GC content at first, second and third nucleotide positions of codons respectively. *gfp* was the first codon optimized gene to be expressed in the nucleus of *Chlamydomonas* as a fusion protein with *ble*. Mayfield et al. (2003) found that quantity of GFP accumulation was 80-fold more in codon-optimized nuclear *gfp* gene as compared to non-optimized *gfp* gene [6]. Therefore, the increased recombinant protein production in these studies demonstrated codon optimization as an effective and necessary step for optimization of gene sequence and increasing recombinant protein yield from microalgae.

1.5.2. Promoters, introns and UTRs

The promoter is a region in DNA responsible for the initiation of the transcription ofgenes. Thus, in addition to codon optimization, promoters and regulatory mRNA untranslated region (UTR) are another very important factor to maximize transgene expression [5]. Since it was reported that the expression of the *ble* gene was driven by the regulatory elements of the *RBCS2* gene from *Chlamydomonas*, a lot of efforts have been made to identify more efficient and stronger *Chlamydomonas* promoters [57]. The three most used promoters for the transgene expression in nucleus of *C. reinhardtii* are *HSP70A, psaD*, and *rbcS2* promoters [59,60,61]. The promoter of 70A

heat shock protein functions as an activator when it is placed upstream of RbcS2, beta 2-Tub, and Hsp70B by enhancing their transcriptional efficiency [59]. In addition, transgene expression from nucleus was also increased by putting endogenous intronic sequences in the transgenes regardless of their orientation or position [57].

It has been observed that the transgene expression in plants has been increased by the use of introns. Henceforth, many researchers have explored the potentials of improving transgene expression in *Chlamydomonas* by employing introns [57]. A significant increase in *ble* expression and in the number of transformants was observed by introducing first intron of RBCS2 genefrom *Chlamydomonas* [62]. The most likely explanation for this is that there is an enhancer element in the RBCS2 intron1 and its splicing possibly stabilizes the mRNA as observed in plants [62]. Another study reported an increased in transgene expression from 1.4 to 1.8-fold by the introduction of more than one RBCS2 introns as compared to insertion of only intron1 [51]. They also observed a 4.5-fold increase in transgene expression by introducing three RBCS2 introns in their natural order. While these results clearly indicated that the number and order of introns have a significant impact on nucleus gene expression in *Chlamydomonas* [51 63, 64] more extensive research is clearly needed to better understand and identify the mechanisms driving recombinant proteins expression in microalgae.

1.5.3. Culture conditions

Although the effects of culture conditions on algal biomass composition (protein, lipids, carbohydrate) are well documented, little is known about how they affect recombinant protein production in *C. reinhardtii*. Based on the knowledge available in the literature, some culture strategies need to be further tested to increase the production of recombinant proteins. Below is an overview of the different culture conditions that could be optimized to increase recombinant protein accumulation, such as light, nitrogen, sulphur, phosphate, and carbon. There are three main types of culture that can be defined by the carbon source utilized by algae. Photoautotrophic or phototrophic growth culture can be defined as, CO_2 assimilated from photosynthesis as a sole carbon source. Heterotrophic growth represents cultures grown in darkness and in presence of an organic carbon source, usually acetate in the case of *Chlamydomonas* sp. Mixotrophic growth defines a culture which can use both photosynthesis and heterotrophy as a carbon source for growth. Some algae grow well phototrophically [78,79] while some grow well under heterotrophic culture conditions [80, 81].

Previous study investigating culture parameters for *C. reinhardtii* producing a secretory ice binding protein, Lolium perenne (LpIBP) found that mixotrophy, i.e. the combination of acetate and carbon dioxide feeding with illumination, resulted in the highest recombinant protein accumulation with up to 10 mg/ L in the culture medium [82] .

Carbon fixation is utilised by photosynthetic organisms to generate carbohydrates, which later combine to form monosaccharide or disaccharide sugars such as glucose or sucrose. Light intensity in plants affects the N-linked glycosylation, which is crucial for the folding of therapeutic proteins, while lack of glucose can completely inhibit the glycosylation mechanism [83]. A study conducted by Schroda et al. (2000) showed that the light-sensitive reporter gene *hsp70* can be used as transcriptional activator when placed upstream of the promoters R8CS2, 2TU8 and Hsp708. When the activation of these promoters was observed under light induction and basal conditions, it was found that the transgene expression could be further increased when cells were heat shocked at 40ºC for 1 hour [84]. Therefore, suitable light condition should be assured if recombinant glycoprotein is to be expressed with high yield in transgenic photosynthetic organisms [83,85]. Temperature is another environmental factor which affects the growth and recombinant protein yield in photosynthetic expression systems.

High temperature can induce biochemical, physiological and genetic changes in plant metabolism including protein denaturation and lipid liquefaction[83]. High stressful temperature is observed to initiate the expression of heat shock protein/chaperone cascades that helps to prevent misfolding, denaturation and aberrant aggregation of cellular proteins [83]. A study conducted by Steven et al (2000) showed that the combination of 25 °C with high light increased plant biomass and total soluble protein content, while the combination of 15 °C/high light conditions favored the generation of recombinant monoclonal antibody in transgenic tobacco plants [86]. Another study conducted in Chinese hamster ovary cells, one of the most popular system for recombinant protein expression, by Nam et al (2008) showed that the drop in temperature from 37 °C to 33 °C at the start of stationary phase increased the production of recombinant protein by 133% [87]. Many different expression systems like insect and mammalian cell systems have shown to be affected by temperature [83]. Thus, these results support temperature as an essential factor to produce recombinant proteins in eukaryotic systems.

1.6 Aims and objectives of this thesis

The main focus of this thesis is to investigate how different culture conditions affect recombinant protein production from nuclear transgenes in *C. reinhardtii*. As mentioned above there has been a lot of research on optimization of culture conditions in other expression systems like mammalian cell lines and plants to improve the yield of recombinant proteins. However, not much has been studied on optimizing culture conditions to improve the yield of recombinant protein production from microalgae. Therefore, the goal of this research is to find suitable optimized culture strategies to increase the yield of recombinant protein production in *C. reinhardtii* without any negative effect on the growth and health of *C. reinhardtii* cultures. To achieve this aim three scientific questions were formulated and addressed in three separate chapters:

1- Does biphasic temperature regime affect the yield of recombinant rIFN α2A production in *C. reinhardtii*?
2- How carbon source affects the yield of rIFN α2A recombinant production in *C. reinhardtii*?
3- Is rIFN α2A recombinant protein produced in *C. reinhardtii* functionally active?

In order to address the first question, in Chapter 2, three biological transformants of *C. reinhardtii* were screened. The main objective of this study was to observe the effect of biphasic temperature regime on the production of rIFN α2A from *C. reinhardtii.* Biphasic growth can be defined as a culturing strategy in which cultures are grown in two different growth phases, in this case, optimal temperature (25 °C) followed by sub-optimal growth temperature (15 °C). It has been observed that biphasic temperature regime (lowering temperature) increased the recombinant protein production in mammalian cell lines (CHO cells) [88]. However, it has not been tested on alternative expression systems such as microalgae.

As explained above, researchers have reported that nutrients can affect recombinant protein production in other host systems. However, there has not been many studies about the effect of culture conditions on the production of recombinant protein in microalgae, which have an increased metabolic repertoire, being able to assimilate carbon by Heterotrophy, Autotrophy or mixotropy. Based on the knowledge available in the literature on protein accumulation in algae, some culture strategies need to be further tested to increase the production of recombinant proteins. Therefore, in this chapter, effect of different culture conditions on recombinant IFN α2A production such as phototrophy, heterotrophy and mixotrophy are investigated.

In the last chapter, the functional activity of recombinant IFN α2A protein was further assessed when produced in three *C. reinhardtii* transformants. The three transformants were grown in large volume (1 L) to allow purification of recombinant IFN α2A protein using two step chromatography. Purification of recombinant IFN α2A protein is crucial to analyse its functional activity and compare it with the other commercially available sources.

Overall this thesis aims to generate new knowledge regarding the functional activity of rIFN α2A protein produced in cell lines of *C. reinhardtii* and identify optimal culture conditions for producing rIFN α2A in this alternative expression system by testing different light, carbon sources and temperatures.

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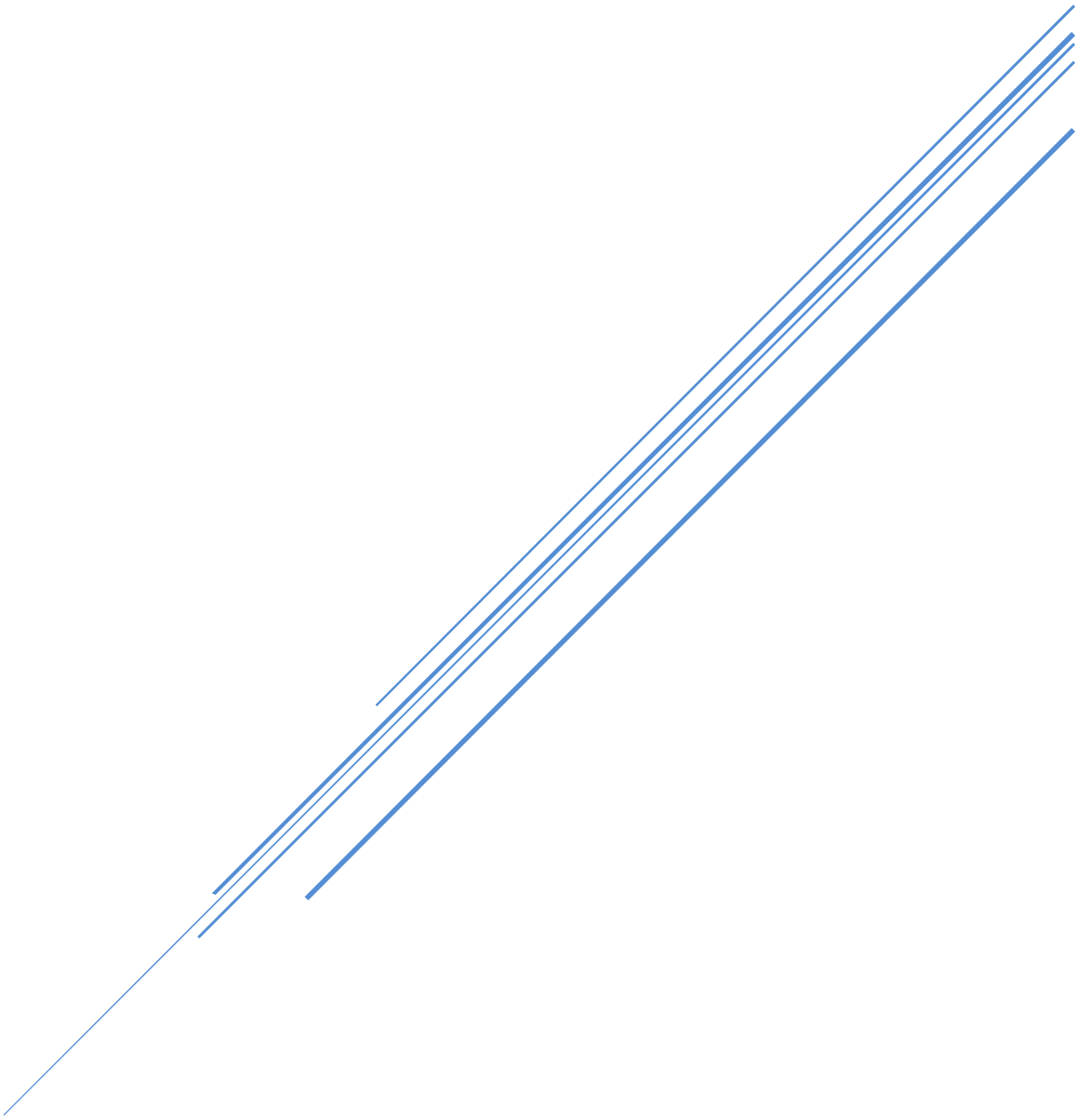
Chapter 2- Biphasic temperature regime increases the yield of a therapeutic recombinant protein in the green alga *Chlamydomonas reinhardtii*



2.1 Introduction

The green alga *Chlamydomonas reinhardtii* has recently been gaining interest as an attractive novel platform for the production of human therapeutic proteins[1]. It is suggested to offer low production costs, scalability and is free of endotoxins or human viral agents[2]. In addition, it has the ability to perform eukaryotic post-translational modifications such as glycosylation, which is often necessary for the production of biologically active and stable biopharmaceuticals[3]. However, the expression of recombinant proteins in *C. reinhardtii* is still in its infancy and the yield of recombinant protein still has to be greatly improved for this system to be economically viable[4]. Large knowledge gaps surrounding the optimization of this new expression platform remain, and in particular the effects of culture conditions on the production of recombinant proteins.

This study focuses on implementing a biphasic temperature regime, whereby cells are first grown under optimal temperature conditions for maximal cell proliferation and to initiate the production of the recombinant protein, this is then followed by a second phase whereby temperature is shifted to enhance the recombinant protein production phase. Studies on Chinese hamster ovary (CHO) cells, cell lines commonly used for commercial production of therapeutic proteins, have shown that a temperature shift from 37 °C to 31 °C can increase recombinant protein yield by prolonging the protein production phase, maintaining cell viability and reducing apoptosis and protease activity[5]. This technique has been widely used in the cultivation of heterologous mammalian cell cultures to increase recombinant protein yields, while maintaining or improving post-translational modifications[6,5,7,8]. The success of biphasic temperature culturing of mammalian cells in improving production and quality of recombinant protein forms the basis of this present study.

The recombinant protein used in this study is the human interferon-alpha 2A (rIFN α2A), recommended for the treatment of chronic viral hepatitis B, chronic viral hepatitis C, chronic myeloid leukemia, Kaposi sarcoma, follicular lymphoma, renal cell carcinoma, melanoma, T-cell lymphoma, multiple myeloma and condylomata acuminate[9,10.] Since its initial FDA approval, IFN α2A has established itself as a powerful anti-viral and anti-tumor treatment, effectiveness in treating the aforementioned conditions has made recombinant IFN α2A a highly valuable therapeutic. IFN α2A is currently produced recombinantly in *E. coli* (non-glycosylated form, ROFERON-A®, Roche)[11]. However, the ability to produce rIFN α2A in microalgae could prove useful in mitigating both the downstream refolding required for *E. coli* expressed proteins as well

as the high costs associated with mammalian expression systems (CHO and HEK). Furthermore, IFN α2A is a relatively small soluble protein, consisting of 165 amino acids (~19 kDa), making it appropriate for the optimisation of a new recombinant expression system such as microalgae.

In *C. reinhardtii*, recombinant proteins can be produced either by homologous recombination between the expression vector and the chloroplast genome or by random insertion into the nuclear genome[14,1.] IFN α2A being O-glycosylated[15,16] it is necessary to insert the recombinant DNA into the nucleus of *C. reinhardtii* to ensure proper export of the recombinant IFN α2A to the Golgi apparatus for correct glycosylation. However, the production of recombinant proteins in nucleus-transformed *C. reinhardtii* is generally associated with low transgene expression levels. Possible reasons for poor transgene expression include (i) weak promoters; (ii) genome integration 'position effects', where the genomic region surrounding the transgene influence its level of expression[17]. (iii) and transgene silencing at both the transcriptional and post-transcriptional levels[18,19]. Recent improvements have been made on the molecular toolkit of *C. reinhardtii*, with for instance the implementation of introns and the foot-and-mouth-disease-virus 2A self-cleavage peptide[20,21], together with the development of new MoClo kit, which provides more than 100 domesticated gene parts to allow advanced synthetic biology in microalgae [40]. However, knowledge on the effect of culture conditions on the yield of recombinant proteins in transgenic strains of *C. reinhardtii* is needed. This study investigates the effect of different temperature regimes on recombinant IFN α2A (rIFN α2A) production and transgene expression. Using three independent cell lines of *C. reinhardtii* producing rIFN α2A cultivated at optimal temperature (25°C), we first identified the growth stage at which the accumulation of rIFN α2A was the highest. Once the production of rIFN α2A was reported to be maximal, the temperature was either dropped to 15 °C or increased to 35 °C to prolong or even increase the production of rIFN α2A. This is the first time that a biphasic cultivation protocol has been tested on *C. reinhardtii*.

2.2 Material and Methods

2.2.1 *C. reinhardtii* strain and cultureconditions

The CC-125 wild type mt+ [137c] *C. reinhardtii* strain used in this study was obtained from the GeneArt® *Chlamydomonas* protein expression kit (Thermo Fisher Scientific). Strain 137c was axenically cultivated in Tris-acetate-phosphate (TAP) liquid medium[22] or TAP-agar plates. The transformed cell lines were maintained on TAP plates supplemented with Zeocin™ (5 µg/mL) to maintain a selective pressure. Liquid cultivation was performed in baffled flasks under continuous light (50 µmol photons m^{-2} s^{-1}) at 100 rpm. The temperature was set to 25 °C unless otherwise noted. The experiment described in this study was carried out on three independent biological replicates (n = 3) grown at 25 °C for 48 hours after inoculation (cell density of 3-4 x 10^4 cells/mL) before changing the temperature to either 15°C or 35°C, while keeping the controls at 25 °C. Cells were sampled every 24 hours for cell density (microscopic cell count), protein quantification (Immunoblot) and mRNA analysis (qPCR).

2.2.2 Vector design

The pChlamy_4 backbone (GeneArt® *Chlamydomonas* protein expression kit, Thermo Fisher Scientific), designed to engineer *C. reinhardtii* nuclear genome, was used in this study. The transcript sequence of the human *ifnα-2A* gene (Accession: DB00034) was optimised to match *C. reinhardtii* codon usage and then cloned into the pChlamy_4 vector between the EcoRI and XbaI restriction sites, resulting in *ifnα-2A* gene to be fused, via a foot and mouth disease virus (FMDV) 2A self-cleavage peptide, to the bleomycin (*she ble)* gene from *Streptoalloteic hushindustanus,* which provides resistance to Zeocin™[21]. (Fig. 1). Expression of the recombinant *ifnα-2A* gene was mediated by the chimeric constitutive Hsp70A-Rbc S2 promoter with the 3'untranslated region (3'-UTR) from the ribulose bisphosphate carboxylate oxygenase small subunit 2 (RbcS2)[21]. The produced recombinant IFN α2A protein had a 6xHis tag at its C-terminus for immunoblot detection and purification purposes. The assembled plasmid was replicated in One Shot® TOP10 Chemically Competent *Escherichia coli* cultivated in Luria broth (LB) with 100 µg mL^{-1} of ampicillin and purified for transformation in *C. reinhardtii*. The assembled plasmid was confirmed by Sanger sequencing, prior transformation of *C. reinhardtii*.

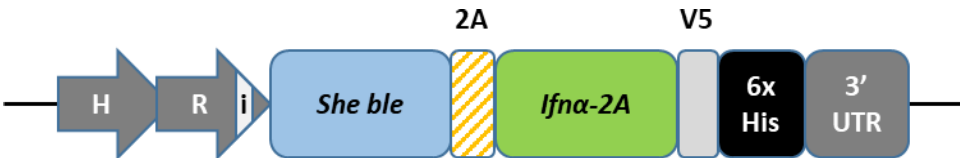


Fig. 1 Vector pChlamy_4_Zeo_ifnα2A with the HSP70/RBCS2i1 promoter (H R i), Zeocin resistance gene (She ble), FMDV 2A self-cleavage peptide (2A), human interferon-alpha 2A gene (ifnα-2A), V5 epitope, 6xHis tag and 3' untranslated region (UTR) of the rbcS2 gene.

2.2.3 *C. reinhardtii* transformation andscreening

Transformation of 137c was performed using electroporation. Cells were grown to 1-2 x 10^6 cells/mL in TAP medium, harvested by centrifugation, rinsed and resuspended in GeneArt® MAX Efficiency® Transformation Reagent (Invitrogen) to a final concentration of 2-3 x 10^8 cells/mL. 250 µL of cells were incubated with 4 µg of circular plasmid for 5 min on ice in a 4 mm cuvette. An exponential electric pulse of 500 V was applied to the sample for 30 ms using a Gene Pulser Xcell™ (Bio-Rad) electroporation apparatus. The capacitance was set at 50 µF with a shunt resistor of 800 Ω. Cells recovered for 16 hours in 10 mL of TAP-40 mM sucrose solution, before being plated onto TAP-agar-Zeocin™ (5 µg/mL) and grown for 7-10 days (constant light, 50 µmol photons m^{-2} s^{-1} and 25 °C). The colonies grew under the selective pressure of Zeocin™ were assumed to have the *she ble* antibiotic gene correctly inserted and therefore had potentially been successfully transformed to produce rIFN α2A. This was confirmed by screening the colonies using polymerase chain reaction (PCR) and rIFN α2A specific primers (forward-ATGTGCGACCTGCCCCAGACCCA / reverse- TTACTCCTTGCTGCGCAGGC). The resulting amplicons were purified from the agarose gel using a gel extraction kit (Thermofisher Scientific) and sequenced (Macrogen). The strains positive by PCR were further screened for production of the recombinant IFN α2A protein by western blotting as described in section 2.2.6.

2.2.4 Automated cell counts

Samples (500 µL) were taken every 24 hours from each biological replicate. Cells were fixed with 0.22 µm filtered glutaraldehyde (final concentration of 2%) and stored at 4 °C. The automated microscopic cell count was performed as described by Tran et al. (2016)[23].

2.2.5 Gene expression analysis (RT-qPCR)

Reverse Transcription Quantitative PCR (RT-qPCR) was performed to gain information about the effect of temperature on the *ifnα-2A* gene expression. Samples (40 mL) were collected before temperature shift (48 hours) and after temperature shift (72 hours and 96 hours). *C. reinhardtii* cells were pelleted at 3,000 x g for 3 min at 4 °C, rinsed once with phosphate-buffered saline (PBS) 1x and flash frozen in liquid nitrogen. The frozen cell pellets were stored at -80 °C until further analysis. RNA extraction and data acquisition were performed as previously described[24] with 1:10 dilutions of cDNA from each sample. To ensure that the cDNA samples were devoid of genomic DNA contamination a "no template" control, as well as a "no reverse transcription" control were included. The primers were designed using the software Primer3 version 4.1.0 (http://bioinfo.ut.ee/primer3/; Table 2)[25]. Expression levels of the target gene (*ifnα-2A*) were normalized against the three reference genes (*cblp*, *rpl13*, *rpl10a*; Table 2) to obtain Normalized Relative Quantities (NRQ). Gene names, primers sequences, amplicons length, melting temperatures, and RT-qPCR efficiencies are indicated. *ifn-α 2A-6xHis*: codon optimized human interferon alpha 2A gene (including 6x His-tag); *cblp*: *Chlamydomonas* β subunit-like polypeptide gene; *rpl13*: ribosomal protein L13 gene. The RT-qPCR efficiency for each primer set was determined from a cDNA dilution gradient of 27, 9, 3 and 1 ng and a linear regression model[26,27,28].

Table 2.1: Sequence-specific primers used in this study for RT-qPCR analysis.

Name	Reference	Primer forward sequence	Primer reverse sequence	Amplicon length (bp)	Tm (°C)	Efficiency (%)
ifnα-2A-6xhis	This study	AGGAGGGTCTAGAGGGCAAG	GTGGTGGTGGTGGTGGTG	83	60	93
cblp	[29]	TGCTGTGGGACCTGGCTGA	GCCTTCTTGCTGGTGATGTTG	193	61.5	98
Rpl13	[30]	AGCACGGCTAGAGACAGATG	TAGTGCGTGGCTGTTTGTTG	115	57.0	92

2.2.6 Cell lysis and western blot analysis

Prior to western blot, 20 mL of sample were collected and centrifuged immediately at 3,000 x*g* for 3 min. The cell pellets were resuspended in 200 µL of lysis buffer (50 mM Tris-HCl, 400 mM NaCl, 0.5% Tween 20, and protease inhibitor cocktail 1x (Sigma-Aldrich), pH 8.0), snap frozen in liquid nitrogen, and stored at -80 °C for further analysis. The cells were lysed using a TissueLyser LT (Qiagen) at 50 Hz for 5 minutes, by adding 250 µL of glass beads (0.5 mm) to the frozen cell pellet. Cell debris were removed by centrifugation (10,000 x *g*, 10 min at 4 °C) and the

supernatants containing the soluble proteins were stored at -80 °C for further western blot analysis. The samples (30 µL) were then incubated with 1 x Laemmli sample buffer (Bio-Rad) containing 2-Mercaptoethanol (1:10) for 10 minutes at 95 °C for denaturation. Protein samples (15 µL) were separated according to size by denaturing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) at 300 V, 290 A, for 20 min, using 4-20 % Mini-PROTEAN® TGX Stain-Free™ pre-cast gels (Bio-Rad) and transferred on to a PVDF membrane using Trans-Blot® Turbo™ Midi PVDF Transfer Packs (Bio-Rad) and a Trans-Blot® Turbo™ Transfer system (Bio-Rad). After the transfer, the stain-free image of the membrane was taken using ChemiDoc MP System (Bio-Rad) and the membrane was incubated for 1 hour at room temperature in blocking solution (5% skim milk power, 1 x PBS containing 0.05% Tween 20 (PBS-T)). The membrane was then washed in PBST three times (5 min) and incubated with mouse anti-6X His tag® antibody (Abcam, 1: 1,000 dilution in blocking solution) for one hour. The membrane was then washed three more times (5 min) with PBST before being incubated for one hour with anti-mouse horseradish peroxidase (HRP) conjugated secondary antibody (Abcam, 1:3,000 dilution in blocking solution). After a final wash with PBST (3 x 5 min), the membrane was incubated for five minutes with 5 mL of Clarity™ Western Enhanced Chemiluminescence (ECL) blotting substrate (Bio-Rad) and the final blot was imaged using ChemiDoc MP System (Bio-Rad). Relative quantitation western blot data was obtained by normalising the intensity of the band corresponding to the rIFNα-2A to the total amount of proteins on the stain-free blot image using the Image Lab Software version 6.0.1 (Bio-Rad) as described by Taylor et al. (2013)[31].

2.2.7 Statistical analysis

Statistical analyses were performed using GraphPad Prism 8.0. Shapiro-wilk test and Levene'test were used first to confirm normality and homoscedasticity of the data respectively, a two-tailed unpaired *t*-test and a two-way ANOVA were applied to test the effect of temperature regimes on *ifnα-2A* transcript levels (RT-qPCR data) and protein production data, respectively. Significant effects were then analysed using Fisher's LSD test for the cell density, maximum quantum yield and bands relative intensity data. The results were considered significant at $P < 0.05$ (Table S2, S3, S4). Throughout the paper, values given are mean ± SEM (n = 3 biological replicates).

2.3 Results

2.3.1 Growth and photosynthetic activity of rIFNα2A transformed *C. reinhardtii* cells under a biphasic temperature regime

The successful insertion of the full *ifnα-2A* gene into three *C. reinhardtii* transformants was confirmed by PCR and sequencing (Table. S1 (supplementary data chapter 2). The production of rIFN α2A from these three strains was then confirmed by western blot and monitored overtime (Fig. 2). Although the HSP70/RBCS2i1 is a constitutive promoter, the production of rIFN α2A varied depending on the growth phase in the three *C. reinhardtii* transformants (Fig. 2). Peak production of rIFN α2A was observed at 24 and 48 hours of cultivation, which corresponded to the early exponential phase of growth (Fig. 2). Based on these results, temperature was shifted at 48 hours of cultivation for the rest of the study in order to maximize both biomass and rIFN α2A production. The growth of transgenic *C. reinhardtii* producing rIFN α2A was compared with WT *C. reinhardtii* and empty vector (Fig. 3). It was found that gene insertion did not affect the growth of transgenic *C. reinhardtii* (Fig. 3).

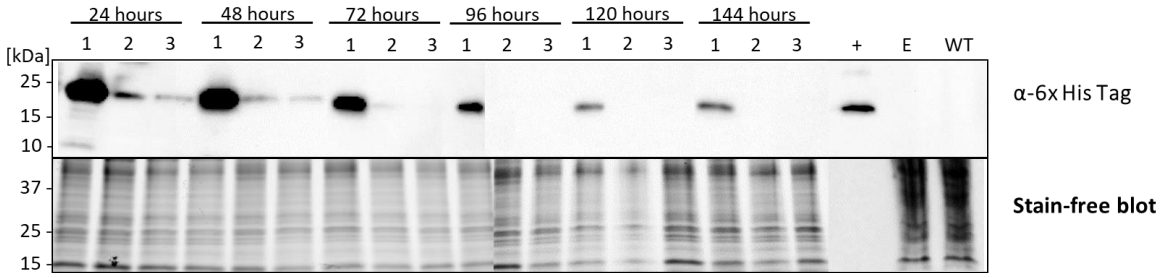


Fig. 2 Western blot analysis of rIFN α2A production in three independent *C. reinhardtii* cell lines grown at 25 °C. rIFN α2A production decreases in the three biological replicates (1 to 3) after 48 hours. Immunodetection using raw cell extracts. Equal amounts of total proteins (2 mg/ml) was loaded for the three transformants.

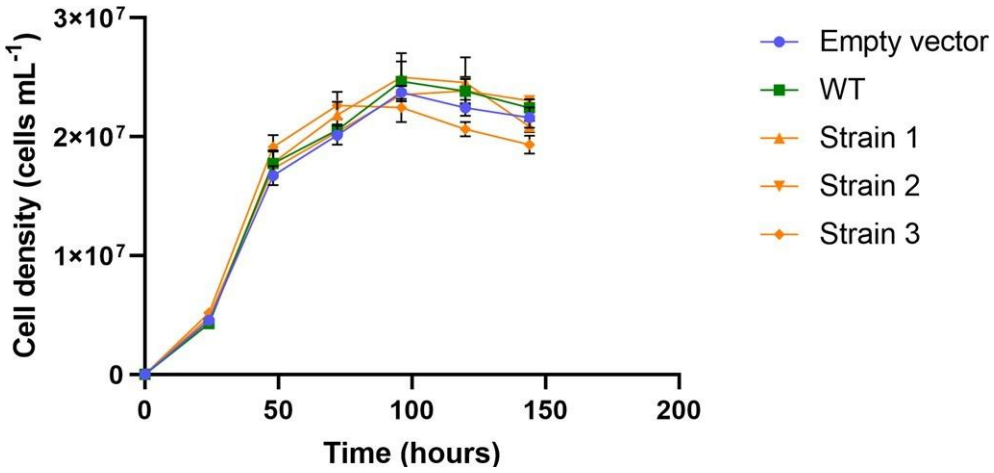


Fig. 3 Cell density at 25 °C of three independent rIFN α2a transformed *C. reinhardtii* cell lines (described as Strain 1, Strain 2 and Strain 3) compared to the wild-type strain (WT) and one *C. reinhardtii* cell line transformed with the empty vector (E). Error bars: Mean ± SEM (n = 3, for each independent cell line). No significant differences were found (Tukey's multiple comparisons test, P>0.05) between the different cell lines

The shift in temperature affected the transformants cell density, with faster growth reported at higher temperature (35 °C), and slower growth at lower temperature (15 °C) (Fig. 4). Significant differences (Fisher's LSD, $P<0.05$) in growth were observed only 24 hours after the change in temperature. All the cultures reached stationary phase at 120 hours independently of the temperature and no difference in cell densities were observed at 144 hours, showing that the cells completely recovered from the stress inflicted from the temperature shift.

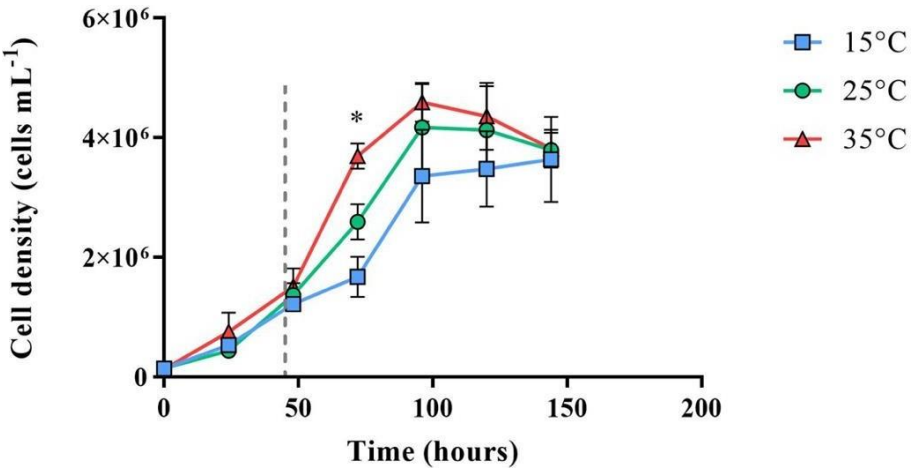


Fig. 4 Cell density of rIFN α2A transformed *C. reinhardtii* cells grown at different temperatures. The grey dashed line represents the time at which the temperature was shifted from 25 °C to either 15 °C or 35 °C. Error bars: Mean ± SEM (n = 3, biological replicates). *Significant difference between 25 °C vs 15 °C and 25 °C vs 35 °C at 72 hours (Fisher's LSD, P<0.05).

2.3.2 Analysis of gene expression and protein production

Although significant only at the protein level, lowering the temperature seemed to have a positive effect on the relative abundance of *ifnα-2A* transcripts (Fig. 5A). The quantity of *ifnα-2A* mRNA kept increasing up to 96 hours at 15 °C, while it was declining at 25 °C and 35 °C in the same time period. The quantity of recombinant IFN α2A protein followed the same trend with 3.3 and 3.5 times more rIFN α2A protein being produced at 15 °C than at 25 °C and 35 °C, respectively at 96 hours (Fisher's LSD, P<0.05) (Fig. 5B, C). The results were consistent in all three independent biological replicates. At 15 °C, high levels of protein were observed up to 144 hours, whereas at 25 °C and 35 °C protein levels decreased to almost negligible amounts after 96 hours.

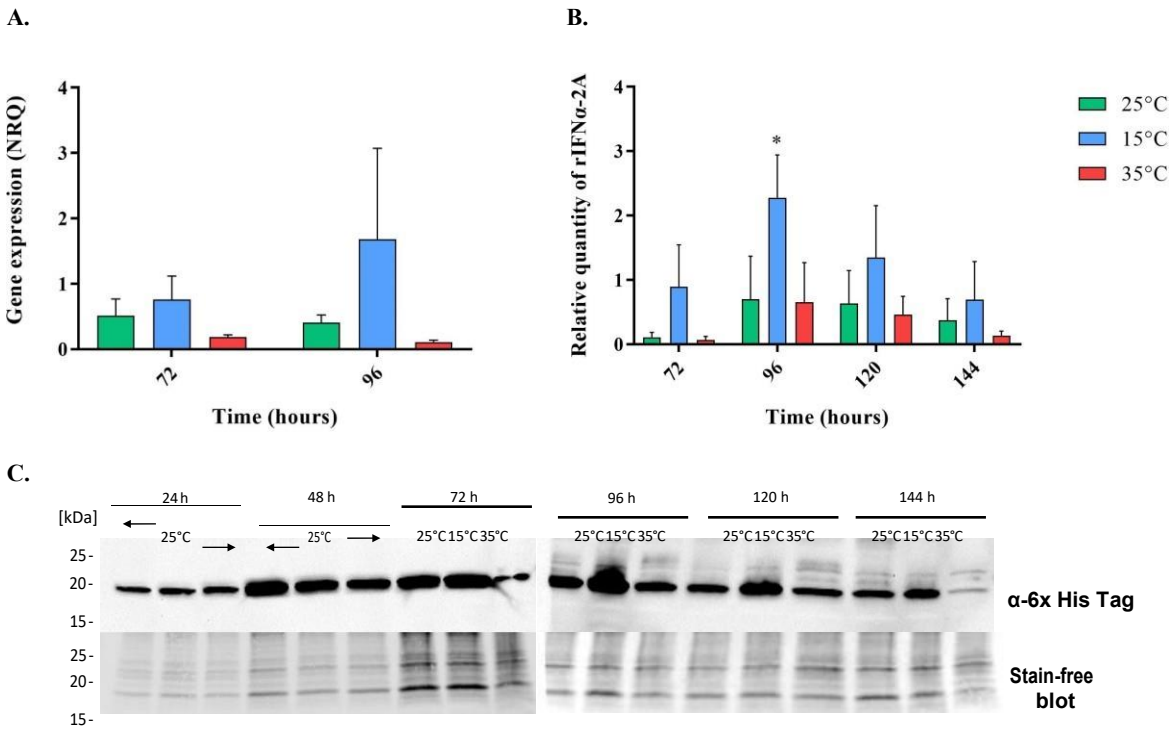


Fig. 5 (A) Relative expression level (Normalized Relative Quantity, NRQ) of the rIFN α2A transcript and (B) relative quantity of recombinant IFN α2A protein after the temperature was changed from 25 °C to 15 °C and 35 °C. The latter is based on relative band intensities from western blot images after normalization to the total amount of protein loaded per lane (stain-free blot) as shown in (C) for one representative *C. reinhardtii* transformant. Error bars: Mean ± SEM, n=3. *Significant difference between 25 °C, 15 °C and 35 °C at 96 hours (Fisher's LSD, P<0.05).

2.4 Discussion

A biphasic temperature regime was applied to three biological replicates of rIFN α2A transformed *C. reinhardtii* cells to determine whether a lower temperature could increase rIFN α2A production in *C. reinhardtii* cells as seen previously in CHO cells[6,5,7,32]. Given that *C. reinhardtii* can grow at temperatures ranging from 15 °C – 37 °C[33,34], we selected the following temperature regimes: 15 °C, 25 °C (optimum) and 35 °C. Our results showed that temperature shifts only temporarily affected the growth of the transformed cell lines without any long-term detrimental effect. After an initial period of stress caused by the temperature shift, the cells recovered quickly and no significant difference in final cell density was observed at 144 hours of growth. Vitova et al. (2011) reported that at temperatures below 20 °C, the growth of *C. reinhardtii* decreased and cell cycle

lengthened, while growth rate increased at temperatures higher than 25 °C[35], as observed in this study (Fig. 4).

Lowering the temperature from 25 °C to 15 °C resulted in a 3.3-fold increase in relative recombinant protein (rIFN α2A) abundance in *C. reinhardtii* at 96 hours (Fig. 5B), while increasing the culture temperature to 35 °C negatively impacted recombinant protein production (Fig. 5B,C). The biphasic temperature regime also prolonged rIFN α2A production up to 144 hours. Protein and gene expression followed the same trend as the relative expression of *ifnα-2A* increased by lowering culture temperature, suggesting that the regulation for rIFN α2A production and accumulation was occurring at the transcriptional level. The HSP70/RBCS2 promotor used in this study can be induced by both heat shock and light[36], but no evidence of it being responsive to low temperatures has ever been reported. The increase in rIFN α2A production is therefore likely due to the slowdown of the cellular machinery leading to reduced protein degradation at both transcripts and protein levels. Interestingly, the effect of temperature was more obvious 48 hours after the temperature shift (at 96 hours), which could reflect the time needed for the cellular machinery to reorganize and adapt to the new temperature conditions.

Cultivation of mammalian cells at low temperatures is an established method carried out to increase recombinant protein productivity, cell viability, reduce growth rate and decrease cell metabolism[37,6,5]. It has been shown that at lower culture temperatures apoptosis and protease abundance are reduced, while post-translational modifications such as glycosylation are retained and even enhanced[37,5,8]. Lowering the culture temperature of CHO cells from 37 °C to 33 °C resulted in a 4-fold increase of the productivity of recombinant erythropoietin (EPO)[8], a 6-fold increase in recombinant human granulocyte macrophage colony stimulating factor (rhGM-CSF) production[37] and a 1.4-fold increase in antibody (IgG) production[7]. Yoon et al. (2003) explained this productivity increase as being partially due to extended culture longevity resulting in the decreased release of proteolytic enzymes from dead cells. They also observed an increase in the relative EPO mRNA content at low culture temperature[8]. Bollati-Fogolin et al. (2005) showed that the quality and glycosylation profile of the rhGM-CSF was unaffected by the lower temperature. Comparative proteome and transcriptome analyses of CHO cells suggested that vesicle trafficking, endocytosis, apoptosis, glycoprotein quality control and cytoskeletal elements are involved in increased recombinant proteins productivity upon reduction of the culture temperature (from

37 °C to 31-33 °C) [5,7]. With a low culture temperature of 15 °C inducing an immediate decrease in growth in *C. reinhardtii*, it is legitimate to assume that similar mechanisms could take place inside the microalgal cell.

The effect of altered temperature regimes on recombinant proteins production has been previously investigated in *C. reinhardtii* by Braun-Galleani et al. (2015)[35]. However, the experimental design used by these authors exhibited a few important differences compared to the present study, (i) the chloroplast genome was engineered instead of the nuclear genome; (ii) native atpA promoter/5'UTR element was used instead of HSP70/RBCS2i1; and (iii) the temperatures tested were higher than 25 °C (30 °C and 37 °C). Their results indicated that vivid Verde Fluorescent Protein (VFP) co-expressed with a gene encoding the *Escherichia coli* Spy chaperone was slightly more abundant at 30 °C, while accumulation of a recombinant antimicrobial endolysin (Cpl-1) was more pronounced at 25 °C under mixotrophic conditions. The authors concluded that culture optimisation of chloroplast transgenes is product-specific and needs to be optimized individually[35]. While the study from Braun-Galleani et al. (2015) was the first to demonstrate an effect of temperature on protein production for chloroplast transgene in *C. reinhardtii*, our study suggests that their findings are not transferable to nuclear transgenes.

Interestingly, the production of rIFN α2A did not vary only based on the culture temperature, but the growth phase also seemed to have an influence. Indeed, the production of rIFN α2A at 25 °C peaked at 48 hours (early-log phase) and then gradually decreased in the three independent transformants studied. Lauersen et al. (2016) also observed that the rates of protein production (relative to biomass) from an intracellular recombinant patchoulol synthase coded by a nuclear transgene in *C. reinhardtii* exhibited peak productivity between 24 and 48 h of cultivation in the early logarithmic phase, after which the production rates steadily decreased[38]. The same observation was made for a recombinant bisabolene synthase, with the productivity of (E)-α-bisabolene peaking sharply between 48 and 72 h in mixotrophy conditions (under constant light)[20]. Unlike intracellular recombinant proteins, the productivity of secreted proteins seems dissociated from the growth phase as they accumulate in the medium[39]. When screening *C. reinhardtii* transformants (from nuclear transgenes) for intracellular recombinant proteins, it is important to consider the growth phase as the "window of expression" might be missed. Similarly, the yield of

intracellular recombinant proteins can be largely underestimated, if measured during the wrong growth phase.

Lowering the temperature is a good strategy to further increase the yield of intracellular recombinant proteins in *C. reinhardtii*. The same results could potentially be obtained for secreted recombinant proteins, but it has yet to be tested. Undoubtedly, *C. reinhardtii* has proved itself as an impending source for recombinant protein production. However, despite the extensive research already achieved, various optimization steps are still needed to improve the molecularmachinery, but also importantly, the culture conditions of *C. reinhardtii* in order to reach its full potential as a commercial platform for recombinant protein production.

2.5 Conclusion

This study concluded that the biphasic temperature regime (lowering temperature) is a valid strategy to increase the yield of recombinant protein IFN α2A from transgenic *C. reinhardtii* cultures. Cells were first grown under an optimal temperature (25 °C) in order to increase cell biomass prior to lowering the temperature to 15 °C to slow down recombinant protein degradation and lengthen the production phase. Decreasing temperature to 15 °C was beneficial for rIFN α2A production with a 3.3-fold increase in protein production relative to control conditions (25 °C). By demonstrating that a temperature shift can significantly affect transcript levels and protein production of a nuclear transgene in *C. reinhardtii*, our results not only advanced the field of recombinant protein production in microalgae, but also suggest that the dynamic modulation of temperature regimes should definitively be considered when optimising culture conditions of microalgae transgenic lines.

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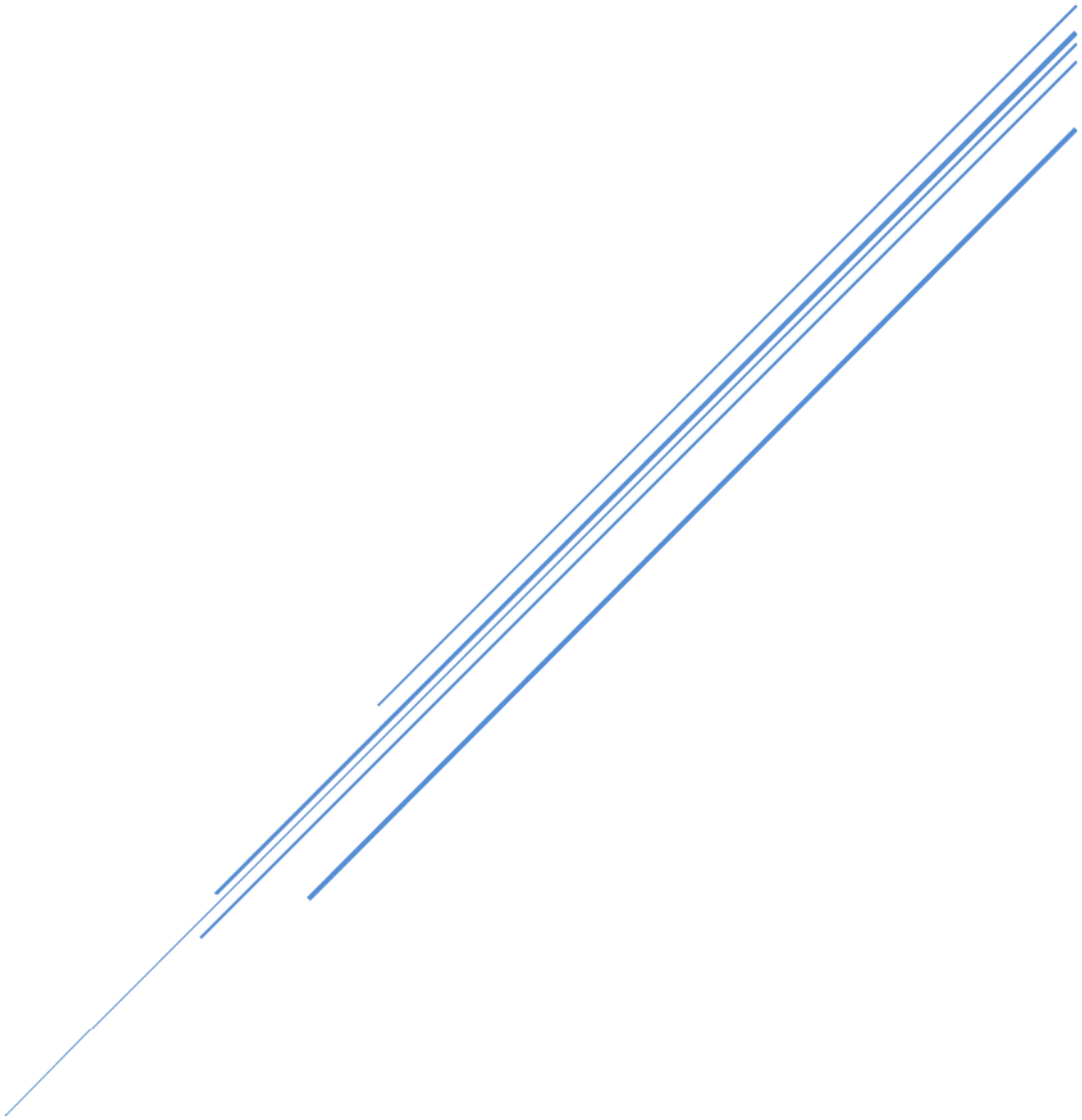
Chapter 3: Effect of different culture conditions on rIFN α2A production from nuclear transgenes in *C. reinhardtii*



3.1 Introduction

Recombinant proteins are of high therapeutic and industrial importance [1]. Recombinant interferon alpha-2A (IFN α2A) (a human protein encoded by the *ifn-α2a* gene), for instance, is a valuable pharmaceutical, with a global market of approximately USD 2.5 billion[2], mainly thanks to its anti-cancer and anti-viral properties [3]. It offers a cure for chronic viral hepatitis C (HCV), chronic myeloid leukemia (CML), Kaposi sarcoma, follicular lymphoma, renal cell carcinoma (RCC), melanoma, T cell lymphoma multiple myeloma and condylomata acuminate[3,4]. It was chosen as product of interest in this thesis, because it has been produced in other expression systems (bacteria and CHO cells etc), making it possible to compare the activity and stability of algal interferon to others. The production of recombinant proteins by common expression systems can be improved by optimizing the main parameters of culture conditions such as the carbon source, pH and feed regime- like feeding the cultures when the nutrient is depleted in the medium. These growth requirements are different depending on the type of expression systems and product targeted[5].

The impact of carbon source on recombinant protein production was studied in yeast *Pichia pastoris* with the use of adaptive laboratory evolution technique (ALE)[6]. The data from this study revealed that the transformants adapted to YPM (Yeast, Peptone) medium (pH 7.4, 1% methanol) showed decreased protein production in both shake flask and fed batch cultivation. On the contrary, recombinant protein production was improved when the transformants were grown in minimal methanol medium (BMM)[6]. In another study, changes in various environmental parameters (e.g. carbon and oxygen concentration) were suggested to improve recombinant protein production in combination with adjusted feed strategies [7].

Approaches like genetic engineering and medium supplementation with amino acids and pyrimidines have been used to improve acetate tolerance in *E .coli* for recombinant protein production[8]. An effect of medium pH was investigated on *E. coli* cells grown on high acetate concentration. It was found that at pH 7.5 and 300mM acetate, the cellular growth was improved and the expression of recombinant proteins- glutathione S-transferase (GST), green fluorescent protein (GFP) and cytochrome P450 monooxygenase (CYP) was reinstated, therefore supporting that cultivation of *E.coli* in alkaline pH can improve the expression of recombinant proteins[8].

Two carbon sources (glycerol and glucose) were tested for recombinant protein production in *E. coli.* It was found that the glycerol feeding showed more recombinant protein production than glucose by using lactose as an inducer [9]. This can be due to glucose and glycerol having different regulation of carbon catabolite repression. In addition, glycerol is stated to a different metabolic pathway ultimately increasing metabolic flux towards recombinant protein production[10]. However, in another study investigating the impact of glucose and methanol as a carbon source on the production of recombinant rabies virus glycoprotein (RABV-G) in yeast *Pichia pastoris* [11], the authors concluded that glucose is a better carbon source for recombinant protein production than methanol. Under glucose feed the transcript gene levels of the central metabolic pathway were upregulated with low antioxidative gene transcript levels[11], meaning that there is a correlation among upregulation of central metabolic genes, biomass concentration and recombinant protein production in *P. pastoris* when grown on glucose as compared to methanol[11].

The feed regime also has an impact on recombinant protein production. A two stage, cyclic fed-batch bioprocess was implemented to produce a recombinant α-amylase from the yeast *Yarrowia lipolytica*[12]. The first stage is the growth stage, and, in this stage, the growth medium is fed to the optimal feed rate to maintain cell growth. A portion of the culture was then transferred to the second stage, which is called production stage, once the predetermined and preprogramed value was achieved in the first stage. Fresh growth medium was supplied to the remaining cells in the growth stage, and the α-amylase production was continuing in the second stage. Thus, the α-amylase production achieved in two stage cyclic fed batch culture was two-fold higher than in fed-batch culture[12].

Similar to the bacterial and yeast expression systems described above, the production of recombinant proteins in microalgae can be maximized by optimising the growth conditions. Although, the effects of nutrients and light on algal biomass composition (protein, lipids, carbohydrate, etc.) are well documented, little is known on the effect of culture conditions on recombinant protein production in *Chlamydomonas reinhardtii*. There are three different culture conditions that can be defined by the type of organic carbon source utilised by algae: (i) photoautotrophic or phototrophic growth culture can be defined as the use of CO_2 assimilated from photosynthesis as a sole carbon source; (ii) heterotrophic growth represents cultures grown in darkness with an organic carbon source, usually acetate in the case of *Chlamydomonas* sp; (iii)

mixotrophic growth conditions involve light and an organic carbon source (e.g., acetate). Lauersen et al. 2015 optimised culture parameters of *Chlamydomonas reinhardtii* secreting an ice binding protein, Lolium perenne (LpIBP). They found that the combination of acetate at 1g/L and 3% carbon dioxide feeding with constant illumination at ~200 mol photons $m^{-2}s^{-1}$ resulted in the highest recombinant protein accumulation, with up to 10 mg/L of LpIBP in the culture medium[13]. While they have investigated the effect of *C. reinhardtii* culture conditions on recombinant protein production, they have not investigated the effect of fed batch cultivation. Therefore, we investigated the production of recombinant IFN α2A protein in the following three main different culture conditions: autotrophic, heterotrophic and mixotrophic growth conditions. We further elaborated the study to test the effect of fed batch cultivation on rIFN α2A production from nuclear transgenes in *C. reinhardtii* (i.e. feeding the *C. reinhardtii* cultures with addition of acetate as organic carbon source once it is depleted in the medium). The overarching aim of this research is to help identifying the best culture conditions in order to improve the production yield of recombinant protein from nuclear transgene in *C. reinhardtii*.

3.2 Materials and methods

3.2.1 *C. reinhardtii* strain and cultureconditions

The CC-125 wild type mt+ [137c] *C. reinhardtii* strain used in this study was obtained from the GeneArt® *Chlamydomonas* protein expression kit (Thermo Fisher Scientific). Three cell lines producing rIFN α2A were generated as explained in sections 2.3 and 2.4 and maintained on Tris-acetate-phosphate (TAP) agar plates supplemented with Zeocin™ (5-µg/mL) to keep a selective pressure. The experiment was performed in 2L tubular photobiorectors (Fig. 1) at 25 ºC and under continuous stirring at 100rpm on the three independent cell lines (n = 3). The cells were axenically grown in TAP medium[14] in three different growth conditions: mixotrophy, phototrophy and heterotrophy (Table 1). Mass flow meters (Omega Engineering) were used to control the bubbling of 1% CO_2 – air mixture at a rate of 100 mL/min in all the three photobioreactors. The starting density of transformed cells was 4.02 x 10^5. Cells were sampled every 24 hours for cell density (microscopic cell count), photosynthetic efficiency (pulse amplitude-modulated fluorometry), protein quantification (Immunoblot), cell size and acetate analysis.

Table 3.1: Different culture conditions used in the experiment for the *C. reinhardtii* strains producing rIFN α2A

	1% CO_2-air mixture	Constant light (150 µmole photon m^{-2} s^{-1}	Complete darkness	Acetate (1g/L)
Mixotrophy	✓	✓		✓
Phototrophy	✓	✓		
Heterotrophy	✓		✓	✓

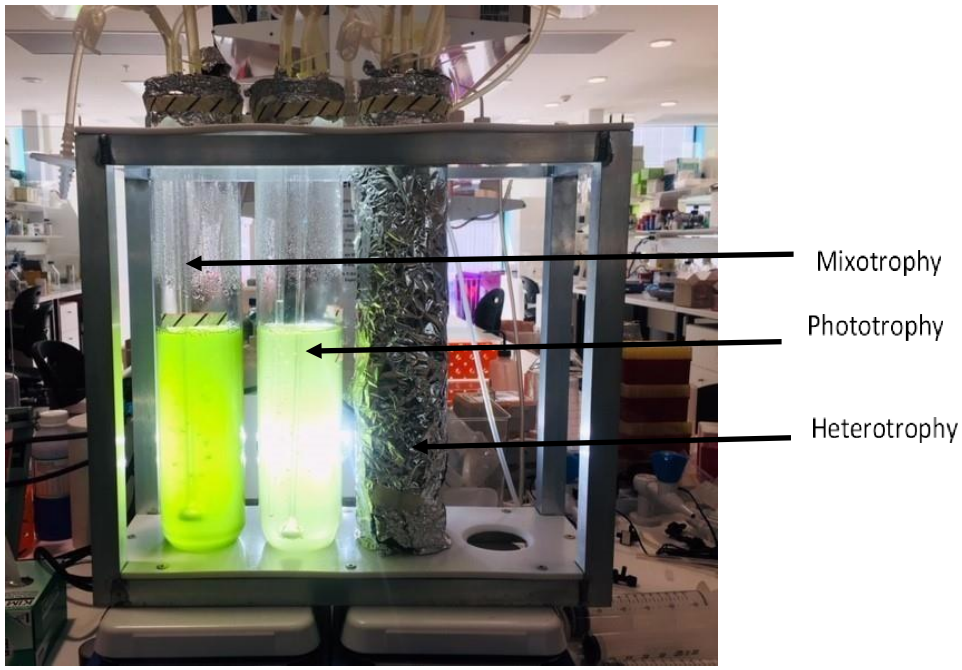


Fig. 1 Experimental set up: three 2L bubble column reactors were maintained under three different culture conditions. Sparger and sampling port are located in the middle of each vessel. Magnetic stirrer is placed at the bottom to ensure proper mixing. The light source for continuous illumination in phototrophy and mixotrophy is on the other side of the set up while the vessel under heterotrophy culture conditions is covered with aluminium foil to ensure darkness.

3.2.2 Vector design

Wild type *Chlamydomonas reinhardtii* 137c and pChlamy_4 vector was obtained from the GeneArt® Chlamydomonas Protein Expression Kit (Invitrogen). The vector pChlamy_4 hasbeen designed to enable efficient cloning of the gene of interest (*ifn-α2a*) for expression in the nucleus of *C. reinhardtii* 137c (please refer to Chapter 2 section 2.2.3 for the vector design and features).

3.2.3 *C. reinhardtii* transformation andscreening

Transformation was performed using an electroporator (Gene Pulser® II Biorad). Algae cells were grown to early log phase (1–2x10^6 cells /mL). The next steps were performed as described previously in chapter 2 section 2.2.6.

3.2.4 Automated cell counts

A 500 µL sample was taken every 24 hours from each biological replicate. Cells were fixed with 0.22 µm filtered glutaraldehyde (final concentration of 2%) and stored at 4 °C. The cell count was done using Nikon microscope. 10 µL of sample was loaded onto a haemocytometer and the cells were counted as described by Tran et al [15] using Image J software for imageanalysis.

3.2.5 Pulse-Amplitude Modulation Fluorometry

Pulse-amplitude modulated fluorometry (PAM) was used to measure the maximum quantum yield of photosystem II (Fv/Fm calculated as Fv = Fm – Fo, where Fo is the minimum fluorescence in the dark and Fm the maximum fluorescence) for rapid assessment of *C. reinhardtii* photosynthetic efficiency during the experiment, and to monitor for any photosystem stress caused by changes in culture conditions. The measurements were taken with a POCKET-PAM (Gademann Instruments GmbH, Germany) after 10 min of dark acclimation. These measurements were performed at room temperature with the following settings: blue light, measuring light intensity <0.2 mmol photons m^{-2} s^{-1} PAR, saturation pulse intensity of 2,700 µmol photons m^{-2} s^{-1} PAR, and saturation pulse width of 0.6 s.

3.2.6 Protein production analysis

To monitor the production of recombinant Interferon alpha 2 A proteins, western blot analysis was performed. 20 mL of sample were collected every 24 hours. Samples were harvested and lysed as explained in Chapter 2 section 2.2.5. Samples were then analysed by western blot as explained in Chapter 2 section 2.2.6.

3.2.7 Acetate analysis

Media samples were filtered through a 0.2 µM PTFE 13 mm syringe filters, tightly capped and stored in -20 °C until analysis. An Agilent 1290 HPLC system equipped with a binary pump with integrated vacuum degasser, thermostatic column compartment modules, Infinity 1290 autosampler and PDA detector was used for the analysis. Column separation was performed using a Zorbax Eclipse XDB HPLC 4.6 mm × 150 mm eluted with dilute sulfuric acid (0.05M) and monitoring with UV at 210 nm. Column temperature was maintained at 30° C. Prior to injection of analyte, the column was equilibrated for 30-40 min with mobile phase. The HPLC chromatograms were integrated for acetic acid peak and compared against a calibration curve plotted using known concentrations of standard acetic acid. The acetate depletion in the culture medium occurred after 96 hours. Following depletion, mixotrophic cultures were spiked with 1 g/L acetate after 96 hours, while the others were used as control (no acetate spiking). The impact of acetate spiking on the recombinant protein production was analysed using western blot as described in Chapter 2 section 2.2.6.

3.2.7 Statistical analysis

Statistical analyses were performed using GraphPad Prism 8.0. Shapiro-wilk test and Levene'test were used first to confirm normality and homoscedasticity of the data respectively. A two-way ANOVA was then applied to test the effect of culture conditions on the growth of rIFN α2A transformed *C.reinhardtii* cells, protein production data and a two-tailed unpaired *t*-test was applied on acetate spike cell density and effect of acetate on protein production respectively. Significant effects were then analysed using Tukey's test for the cell density, maximum quantum yield and bands relative intensity data. The results were considered significant at $P < 0.05$ and $P<0.01$ (Table S1, S2, S3). Throughout this Chapter, values given are mean ± SEM (n = 3 biological replicates).

3.3 Results

3.3.1 Growth of IFN α2A transformed *C. reinhardtii* cells under different culture conditions

The growth of rIFN α2A producing cells varied in the three different conditions. Cells grew much faster in mixotrophy than in the other two conditions. The cell density was significantly higher after 48 hours in mixotrophy (t-test, $P<0.001$) followed by heterotrophy and phototrophy (Fig. 2). The cells entered exponential phase after 24 hours in all the cultivation conditions. All the cells

reached stationary phase after 72 hours. The absence of stress was confirmed by a Fv/Fm (maximum quantum yield of photosystem II) value remaining between 0.7 to 0.8 for phototrophy and heterotrophy conditions. However, significant differences were observed between mixotrophy and heterotrophy at 24 hours and between mixotrophy and phototrophy from 48 hours (t-test, $P<0.001$; Fig. 2). The pH was similar and was between 7.2 to 6.8 in all the three conditions (Fig. 4). A slight drop in pH (6.8) was observed after 72 hours when the cultures entered the stationary phase (Fig. 5). This drop in pH is unlikely to have impacted the growth and recombinant protein production as *C. reinhardtii* can grow at pH ranging from pH 6-8[16].

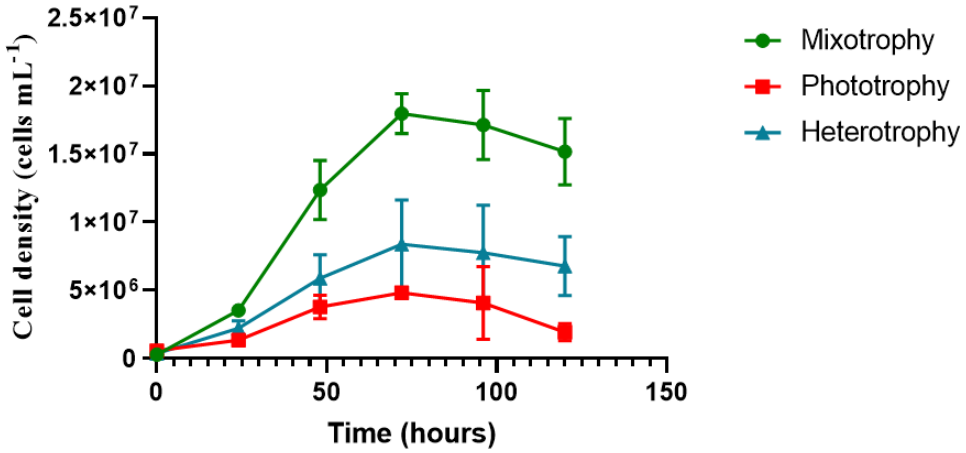


Fig. 2 Cell density of rIFN α2A transformed *C. reinhardtii* cells grown at different culture conditions. Error bars: Mean ± SEM (n = 3). *Significant difference between mixotrophy and phototrophy from 48 hours (t-test, P<0.01).

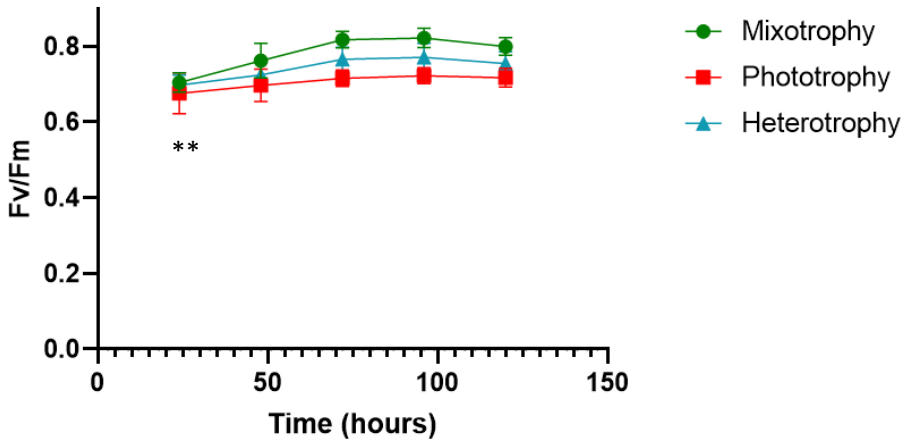


Fig. 3 Photosynthetic activity (Fv/Fm) of rIFN α2A transformed *C. reinhardtii* cells grown under different culture conditions. Error bars: Mean ± SEM (n = 3). *Significant difference between mixotrophy and phototrophy from 48 hours to 144hrs (t-test, P<0.05). ** Significant difference between mixotrophy and heterotrophy at 24 hours (t-test, P<0.05).

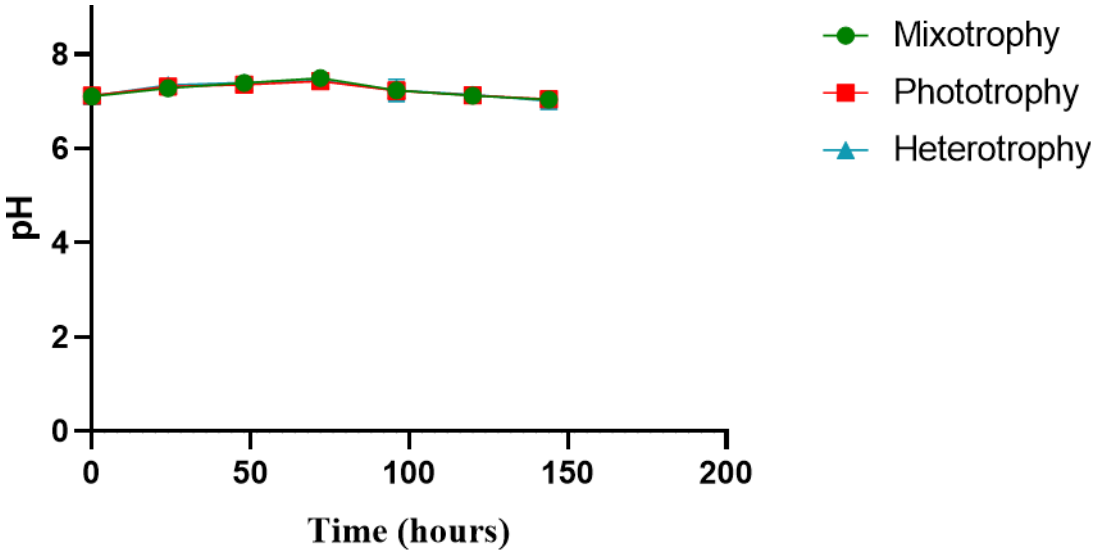
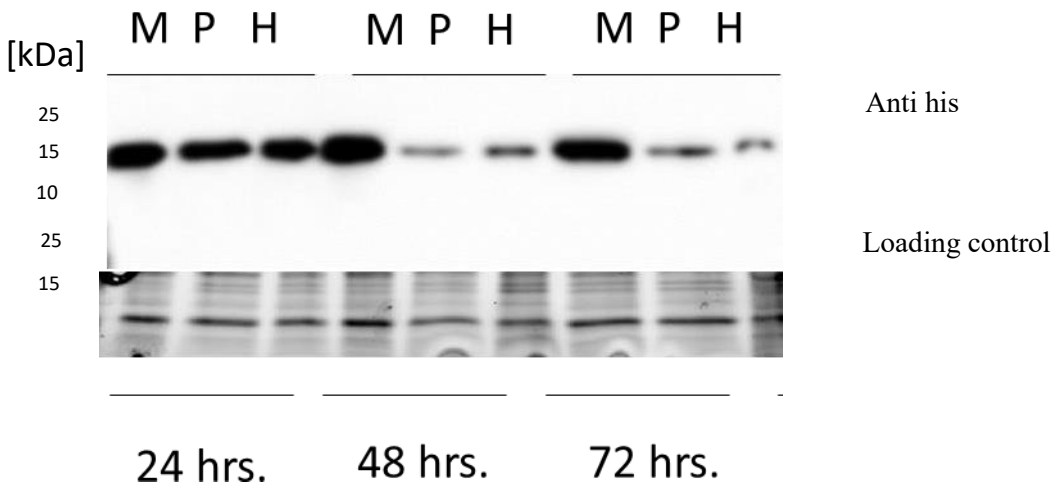


Fig. 4 pH of rIFN α2A transformed *C. reinhardtii* cells grown at different culture conditions. Error bars: Mean ± SEM (n = 3).

3.3.2 Effect of different culture conditions on rIFN α2A production

The production of rIFN α2A varied in three different conditions. The rIFN α2A production can be correlated to cell density as explained later in discussion section. Production of rIFN α2A, as measured by Western blot and relative intensity of corresponding bands followed the same trend as cell density with slightly more production of rIFN α2A in mixotrophy followed by heterotrophy and phototrophy (Fig.5A and 5B), although the differences were not statistically significant. In addition, the production of rIFN α2A was generally higher in early exponential phase (24 and 48 hours) in all the three conditions and decreased when the cultures reached the stationary phase after 72 hours (Fig. 5A), although, again these differences were not statistically significant.

A.



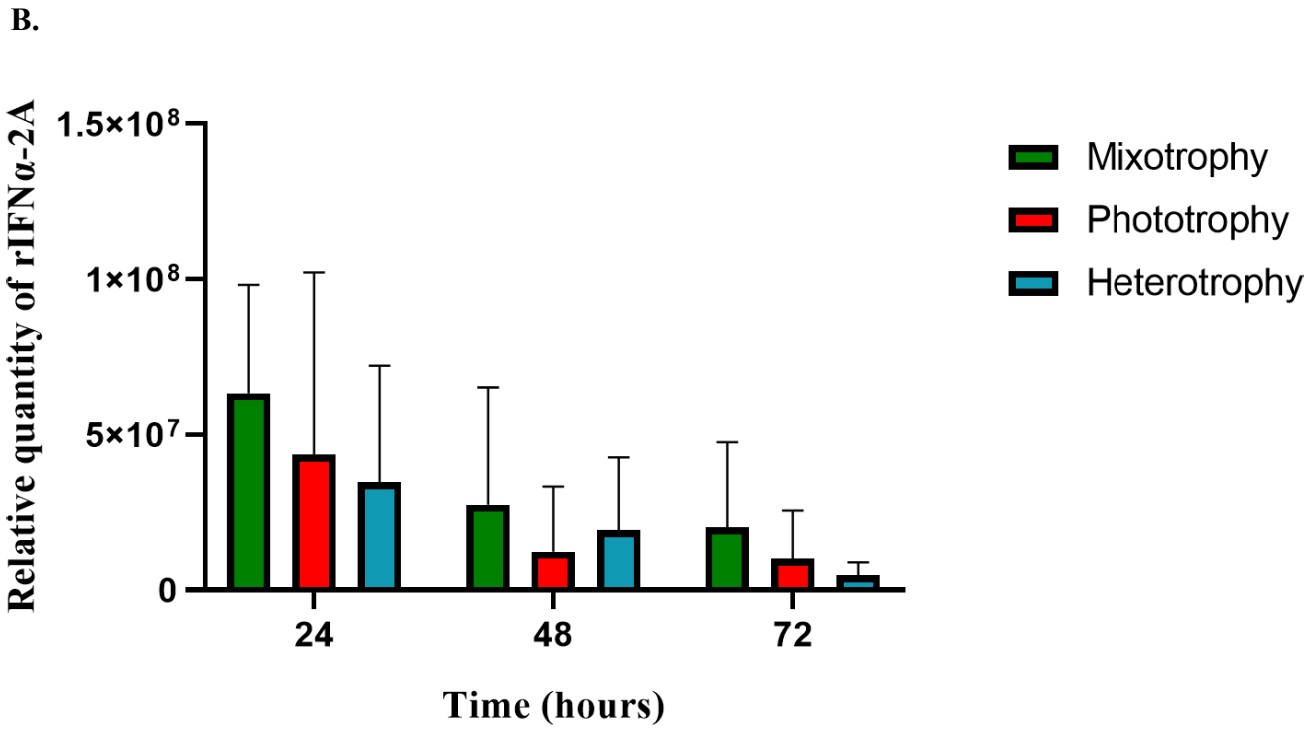


Fig. 5. (A) Western blot analysis of rIFN α2A production (as measured using anti-His antibody) in three independent *C. reinhardtii* cell lines grown at three different culture conditions. Higher accumulation of rIFN α2A band was observed in mixotrophy (M) followed by heterotrophy (H) and phototrophy (P). Equal amounts of total proteins (3 mg/ml) was loaded for the three transformants. (B) Relative quantity of recombinant IFN α2A protein in three different culture conditions, based on relative band intensities from western blot images after normalization to the total amount of protein loaded per lane (stain-free blot). Error bars: Mean ± SEM, n=3.

3.3.3 Effect of acetate spike on rIFN α2A production

Acetate consumption was measured in mixotrophic and heterotrophic cultures. Acetate started depleting in the medium after inoculation in both the culture conditions. Mixotrophic cultures consumed acetate significantly slower (T-test, $p<0.001$) than heterotrophic cultures (Fig. 6A). Further, cultures entered the stationary phase when the acetate was depleted in the medium (96 hours). In an additional experiment, cultures were therefore spiked with acetate after 96 hours and compared with control cultures (no acetate spike). While cultures that were spiked with acetate at 96 hours started growing again and did not enter the stationary phase till the end of the experiment, the cultures not spiked with acetate entered the stationary phase after 96 hours as illustrated by the

significant differences in cell densities observed after 96 hours (t-test, $p<0.001$; Fig. 6B.) The protein production was also analysed in the control and acetate spiked rIFN α2A transformed *C. reinhardtii* cultures. The cultures spiked with acetate continued to produce recombinant protein till 120 hours and at 120 hours the rIFN α2A production was 3.6-fold more than the cultures not spiked with acetate and the control cultures did not show much protein production after 96 hours (Fig. 7).

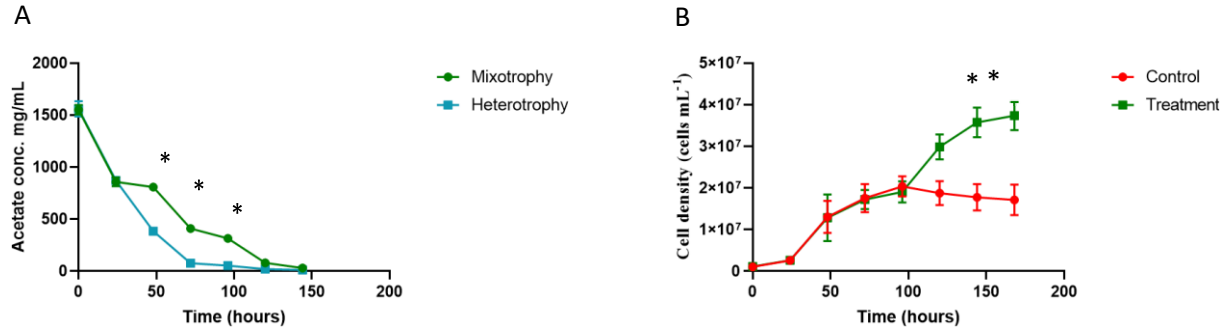


Fig. 6 (A) The concentration of acetate in mixotrophy and heterotrophy. * significant differences (t- test P<0.001) in acetate consumption from 48 to 96 hours. Error bars Mean ± SEM, n=3. (B) Cell density of rIFN α2A transformed *C. reinhardtii* in mixotrophy with (Treatment) and without (Control) acetate spike. * Significant differences in growth (T-test, P<0.05) after acetate spike at 120, 144 and 168 hrs. Error bars Mean ± SEM, n=3.

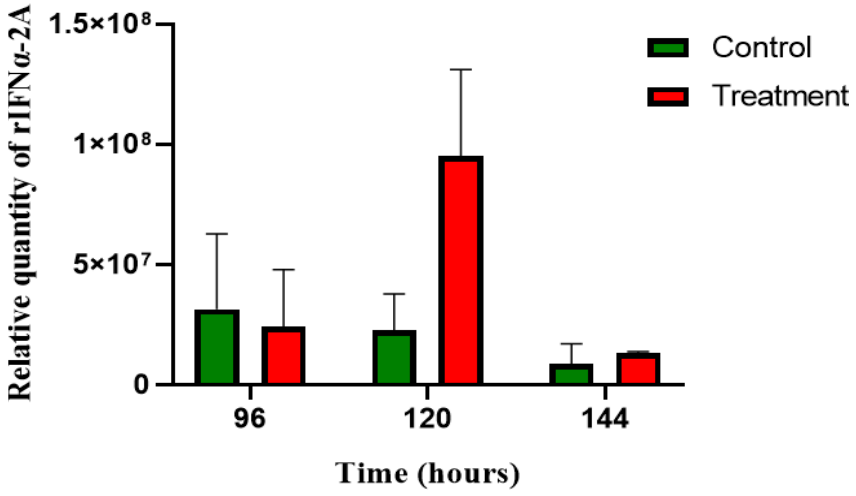


Fig. 7 Relative quantity of rIFNα-2A protein in mixotrophy based on relative band intensities from western blot images after normalization to the total amount of protein loaded per lane (stain-free blot). Error bars: Mean ± SEM, n=2.

3.4 Discussion

Three culture conditions (mixotrophy, phototrophy and heterotrophy) were tested to improve the production yield of rIFN α2A from nuclear transgenes in *C. reinhardtii.* The cells grew faster under mixotrophic conditions compared to heterotrophy and phototrophy. The cells in all conditions entered exponential phase after 24 hours (Fig. 1). Similar results were observed by Taghavi et al. 2016, where they found that mixotrophically grown *C. reinhardtii* cells grew faster compared to cells grown phototrophically and heterotrophically, likely due to simultaneous use of external carbon source and light[17]. *C. reinhardtii* cells grew faster and to a higher optical density in mixotrophy followed by phototrophy and heterotrophy[18]. Though the cell density was higher in mixotrophy, the Fv/Fm values were similar for all the conditions, which indicated that the microalgae cultures were healthy and not under any stress in different conditions (Fig. 2). Our results support these findings, indicating that the change in culture conditions were not detrimental to the photosynthetic health of the transformed cell lines, with values for all the transformants found to be between 0.6 to 0.8, which is considered optimal for *C. reinhardtii* cultures[19]. The rIFN α2A production was higher in mixotrophy as compared to phototrophy and heterotrophy (Fig. 5 A, B). These results are congruent with Lauerson et al. 2015, who also observed that mixotrophy is best cultivation condition for recombinant protein production from microalgae[22]. These authors suggest that it could be due to the usage of two carbon sources (i.e., acetate and CO_2), which has an additive effect on *C. reinhardtii* productive capacity[22]. They further indicated that the recombinant protein production in phototrophic cultivation (without acetate) is possible but the medium requires a high concentration of trisaminomethane (TRIS), which is not always economically viable.

High cell densities are related to high recombinant protein production in yeast *Pichia pastoris* because of the over expression of the genes involved in translation and expression[11]. It is important to achieve high cell density to get more recombinant protein production. However, there are some challenges related to high cell density cultivation strategies. Although most of the research related to medium optimisation in order to improve the recombinant protein production has been done in *E. coli* and yeast, other hosts also have the potential to be grown at “higher than usual” cell densities[5]. In this study, we implemented a fed batch technique to replenish the cultures with carbon source (i.e.,

acetate) when depleted in the medium, in order to increase the cell density and ultimately the yield of rIFN α2A from *C. reinhardtii.*

Acetate was depleted in the medium at 96 hours (Fig.6A). So, acetate was fed in the medium at 1 g/L concentration at 96 hours to achieve a high cell density and maintain the cultures in the exponential phase. The cultures which were fed with acetate continued to grow at higher cell densities and produced more rIFN α2A (Fig. 6B) as compared to the control cultures, which were not fed with acetate at 96 hours (Fig. 6B). We observed that the production of rIFN α2A continued after acetate feeding, while it stopped at 96 hours in control cultures. The cultures spiked with acetate produced 3.6-fold more rIFN α2A at 120 hours as compared to control cultures (Fig. 6,7). In studies, high cell densities have been achieved from fed-batch cultures by implementing different strategies like- exponential feeding, step wise increase of the feeding rate and constant rate feeding[5,23].

Fed batch culture offers the possibility of decoupling the growth and production phase, which offers better cellular activity and controlled nutrient uptake leading to more cell density and ultimately more recombinant protein production[24]. This technique has been applied in yeast and bacterial cultures; therefore, it would be interesting to apply this technique in microalgae systems. Also, cells should neither be starved nor over fed as it may alter the physical properties of culture medium and impair the protein production[25]. Fed-batch process either starts with carbon-source feeding or complete culture feeding mainly at the end of the batch when the main carbon source has been depleted in the medium, thus allowing the cell growth and metabolic activity to be retained, including recombinant protein production[23,5]. By affecting environmental and nutritional conditions, cultivation strategy is therefore crucial not only for the growth of cells but also for recombinant protein production[5].

In this study three *C. reinhardtii* cultivation strategies were tested to find the best conditions for rIFN α2A production. We found that mixotrophy is the best strategy for rIFN α2A production as already suggested by Lauerson et al, 2015[13] for the production of different recombinant proteins. We further observed that the rIFN α2A production was higher in exponential phase and decreased afterwards. Further, by applying fed batch technique in mixotrophic cultures (i.e. acetate being supplemented in the medium once it is depleted) the cells remained in the exponential phase and produce more rIFN α2A. This fed batch strategy of cultivation was beneficial on both cell density

and the production of recombinant protein.

3. 5 Conclusion

Overall, this study indicated that mixotrophy is the best condition for rIFN α2A production from nuclear transgenes in *C. reinhardtii.* Further, we observed that fed batch mode of cultivation proved to be beneficial in increasing not only the cell density but also the yield of rIFN α2A production in *C. reinhardtii.* However, optimisation of culture conditions remains virtually unexplored in the context of recombinant protein production in microalgae, and therefore holds great potential to make it a more commercially competitive source of recombinant protein in the future.

3.6 References

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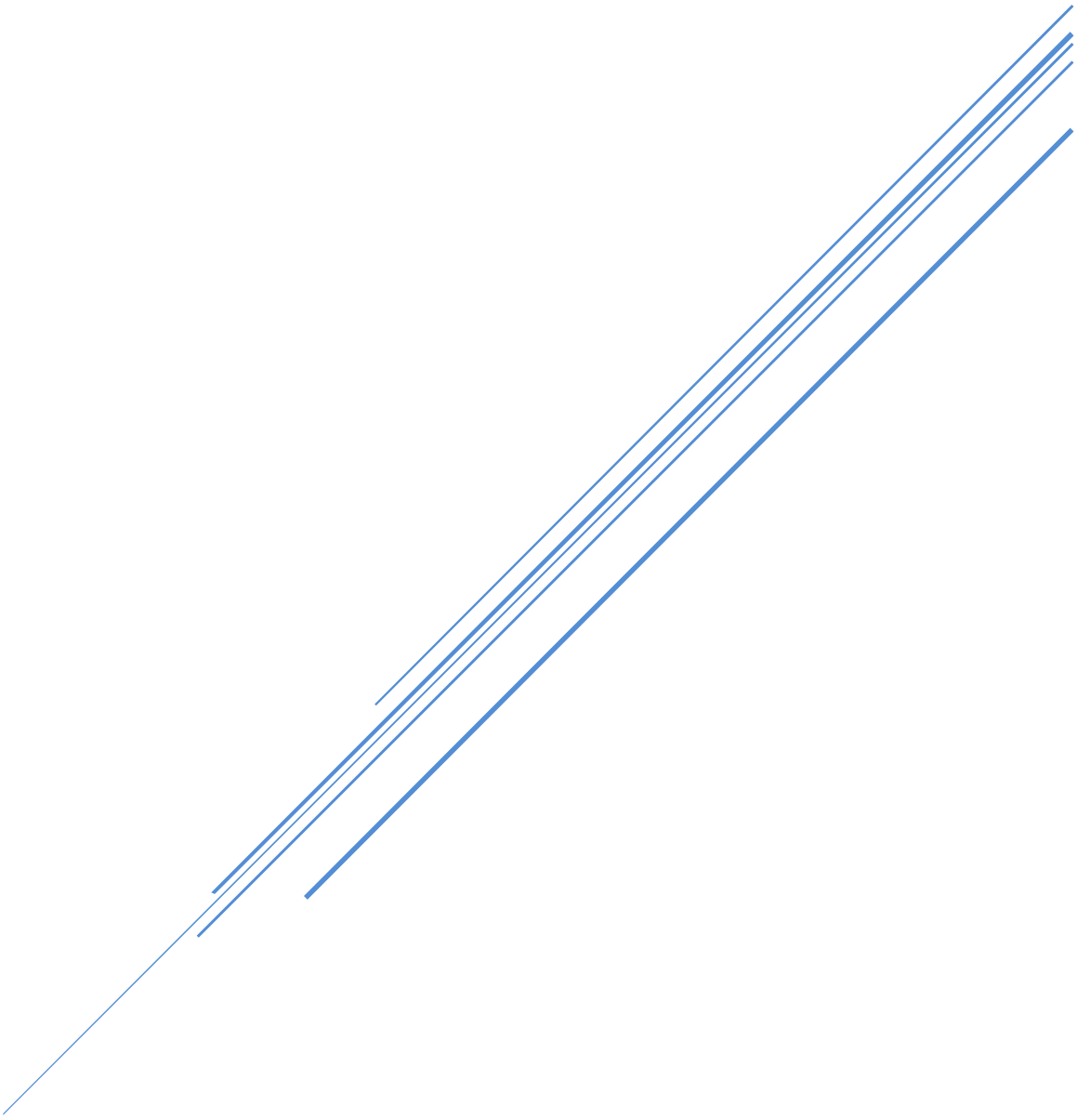
Chapter 4: Microalgal rIFN α2A purification and anti-viral potency



4.1 Introduction

Recombinant protein production came to light in 1980s, to overcome the limitations imposed from extraction of natural sources[1]. At present, recombinant protein production represents a multibillion-dollar market[2]. A recent report revealed that protein expression market was USD 1.6 billion in 2017 and is expected to reach USD 2.8 billion by 2022[3,1]. The first recombinant proteins produced as commercial pharmaceuticals (human insulin and growth hormone) were made in bacteria *E.coli*[1]. In 1982, human insulin became the first licensed drug created using genetic engineering techniques[1]. Recombinant proteins have potential applications in food, paper, detergent, chemical and cosmetic industries, however, its main usage is focused to biopharmaceuticals and industrial enzymes[1,2]. In the years 2011-2016 only, regulatory agencies of USA approved 62 new recombinant proteins[4]. As mentioned in previous chapters, this thesis is focused on recombinant interferon alpha 2A production. Interferons are cytokines that possess a strong antiviral, anti-proliferative and immunomodulatory effects. These cytokines are the first line of defense against viral infections. Interferons trigger an immunogenic response and act as a signaling protein for communication between cells[5]. They are produced by infected cells as a warning signal to surrounding cells[5].

In this study, *Chlamydomonas reinhardtii* has been genetically engineered to produce a human recombinant interferon alpha 2A (rIFN α2A) with anti-cancer and anti-viral properties [8]. It was selected as a target early in this thesis, because it has been used in the treatment of chronic illness and cancers[8] and offers a cure for several human diseases, including: chronic viral hepatitis C (HCV), chronic myeloid leukemia (CML), Kaposi sarcoma, follicular lymphoma, renal cell carcinoma (RCC), melanoma, T cell lymphoma multiple myeloma and condylomata acuminate[9].

Lavoi et al. studied the interaction of different subtypes of IFNs with their receptor components and found a correlation between antiproliferative activity and binding affinity for most of the IFN subtypes. However, there was a significant difference among EC50 (i.e. concentration of a drug that gives half-maximal response) values (1.5 nM versus 0.1 nM for IFN-alpha2 in WISH, which are heLa derivative versus Ovarian Carcinome cells[10]). They also observed that in anti-viral potency in several cases the relationships seem to be more complicated than simple binding. In IFN-α2, mutagenesis studies have shown that the increase in binding affinity to certain receptors, do not result in additional increase in anti-viral activity[10,11]. Overall, they found that IFNs showed

more anti-proliferative activity than anti-viral potency[10,11]. In another study, Yamaoka et al. investigated the activity of nine IFN-alpha subtypes purified from induced human Burkitt lymphoma cell lines. They observed after several tests of anti-viral and anti-proliferative assays that the most active interferon alpha sub-type was IFN-A10 followed by A14, with A1 being the least active[12]. IFNs are induced by dsRNAs, viruses, other microorganisms, cytokines and growth factors (Fig. 1). The production of IFNs is mainly facilitated by transcription factor complexes, (e.g. Nuclear factor kappa-B [NF-kB]), ATF (activating transcription factor) and interferon regulatory factors (IRFs)[13]. Further, the activation IRF-3 and IRF-7 takes place after interaction of Type I IFNs with their receptor complex. This leads to the activation of multiple signal transduction pathways including the Janus kinases (JAKs) signal transducer and activator of transcription proteins (STATs); JAK/STAT pathway. Eventually, these actions result in the biological functions of the Type I IFNs, including interferon alpha 2A (Fig. 1)[13]. It is interesting to note that the Type I IFN receptor complex has the ability to produce different biological responses depending upon which ligand is bound for instance- IFN-β selectively induces the association of tyrosine-phosphorylated IFNAR-1 and -2, while IFN-α1, -α2, -α6, -α7, -α8 and IFN-γ do not[13,14].

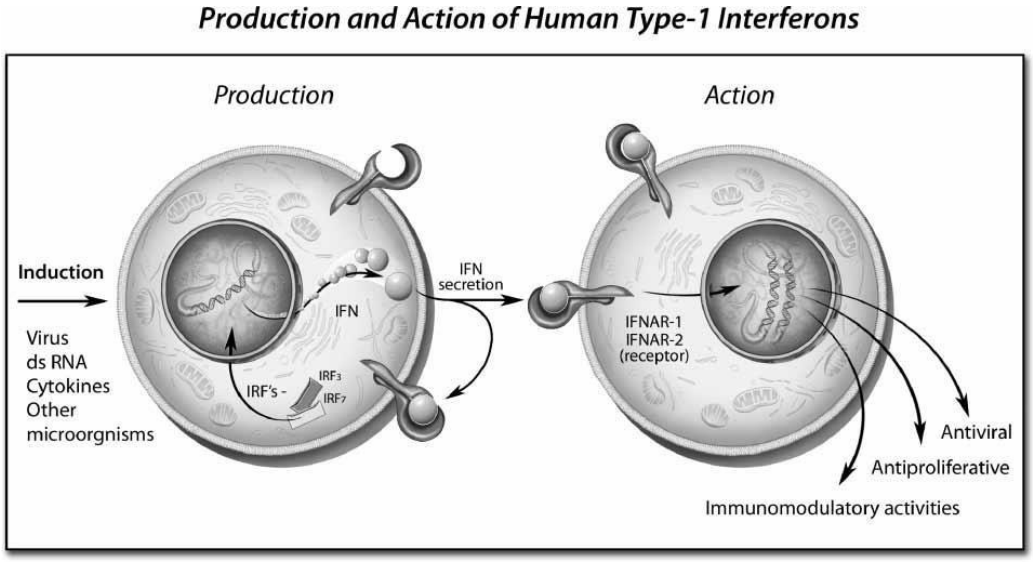


Fig.1 Human Type I Interferons production and mode of action- antiviral, antiproliferative and immunomodulatory effects are a result of secretion when interferon binds to the Type I receptor complex (IFNAR-1/IFNAR-2). Also, toll-like receptors are involved in the activation of interferon regulatory factors 3 and 7 (IRF-3 and IRF-7). Figure adapted from [13].

In this chapter, the anti-viral activity of the algal rIFN α2A has been investigated. The activity of recombinant IFN α2A from different hosts like bacteria and yeasts have already been examined but only one study has been conducted on microalgae so far [15]. In the present study, the activity of the rIFN α2A produced by the best producing strain (Strain 1, see Fig. 3, Chapter 1) was investigated. The transgenic *C. reinhardtii* cells were grown up to exponential phase (72 hours). The cells were then harvested and purified using two-step chromatography (affinity chromatography and ion exchange chromatography). The purity of rIFN α2A was calculated. After purification, mass spectrometry was done on purified rIFN α2A from three transformants to assess the presence of the complete rIFN α2A protein. Finally, anti-viral and anti-proliferative assays were performed to assess the functionality of the rIFN α2A produced from nuclear transgenes of *C. reinhardtii.*

4.2. Materials and methods

4.2.1 *C. reinhardtii* strain and cultureconditions

The CC-125 wild type mt+ [137c] *C. reinhardtii* strain used in this study was obtained from the GeneArt® *Chlamydomonas* protein expression kit (Thermo Fisher Scientific). Three cell lines producing rIFN α2A were generated as explained in chapter 2 sections 2.3 and 2.4 and maintained on Tris-acetate-phosphate (TAP) agar plates supplemented with Zeocin™ (5-µg mL^{-1}) to keep a selective pressure. The cultures were upscaled in 1L conical flasks in a stackable incubator at 25 ºC and under continuous stirring at 100rpm on the three independent cell lines (n = 3). The cells were axenically grown in TAP medium[16] in mixotrophy. The starting cell density of all the transformed cell was 4.02 x 10^5 cells /mL. Cells were harvested in mid-exponential phase, as it was previously determined to be the peak of rIFN α2A production (see Chapter 2 results), and subsequently lysed as explained in the following section.

4.2.2 Harvesting and lysing of rIFN α2A transformed *C. reinhardtii* cells

The workflow diagram is shown to explain the entire process (Fig. 2). The cells were harvested by centrifugation (2,500 x *g* for 5 min) at 72 hours to maximise both biomass production and rIFN α2A yield. The pellet was resuspended in lysis buffer containing imidazole (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 0.5% Tween20, pH 7.4) to a 5:1 ratio buffer-sample. The samples were then sonicated using QSonica probe sonicator (30% intensity, 5 minutes, 30 sec ON and 30 sec OFF) and subsequently centrifuged at 4˚C, 10,000 x g for 10 min to remove cell debris. The

rIFN α2A was separated from the whole soluble proteome by two-step chromatography on an ÄKTA™ Pure chromatography system (GE Healthcare) as explained in section 2.3.

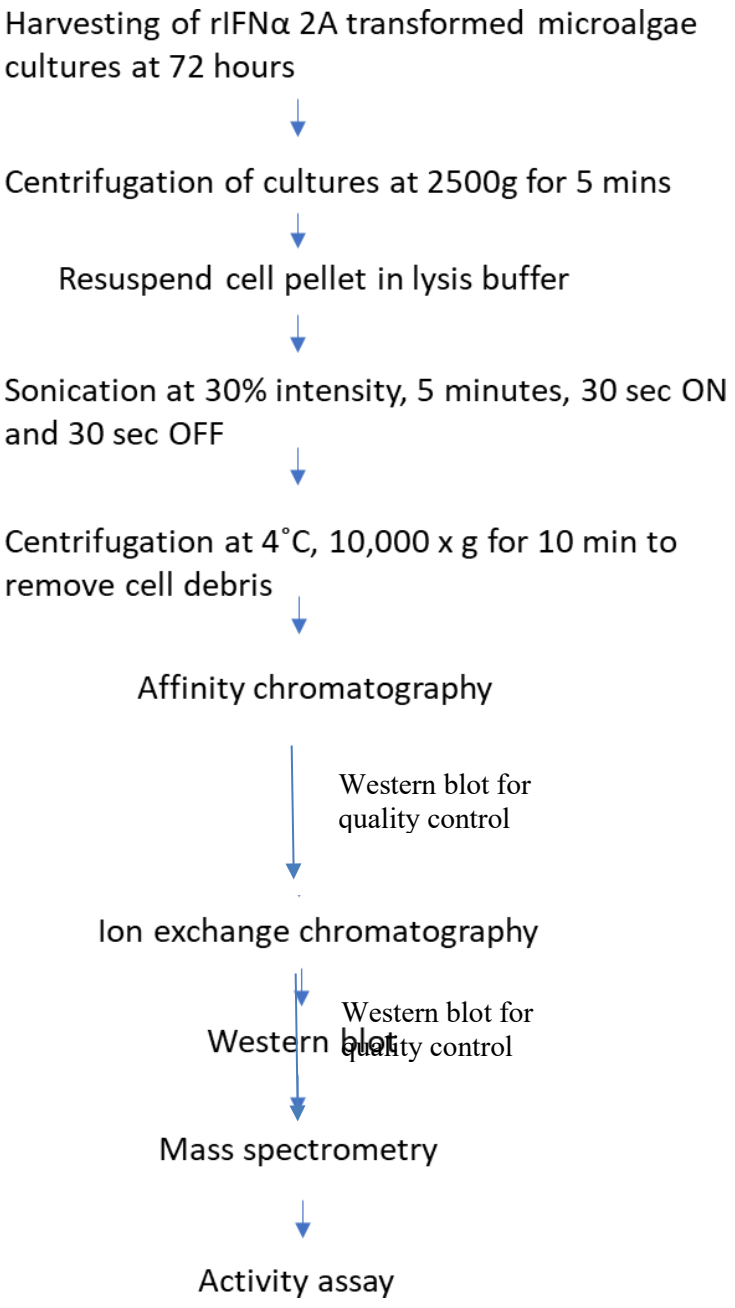


Fig. 2 Workflow diagram of the entire process from cell harvesting to activity assay.

4.2.3 Purification and yield estimation of rIFN α2A transformed *C. reinhardtii* cells

First, Immobilised Metal Affinity Chromatography (IMAC) was performed using a 1mL HisTrap™ FF Crude column (GE Healthcare). The column was washed with 10 column volumes (CV) of ultrapure water and then equilibrated with 10 CV of binding buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 7.4). The sample was loaded onto the column at a flow rate of 1 mL /min. The column was washed again with 20 CV of binding buffer. The elution was performed in 10 CV with a step gradient of imidazole (100 – 200 – 300 – 400 – 500 mM) in 50 mM NaH_2PO_4, 300 mM NaCl, pH 7.4. Fractions containing the protein of interest were identified via western blot. Prior to ion-exchange chromatography, the positive fractions were pooled together, and the buffer was changed to the ion exchange binding buffer (50 mM sodium acetate (CH_3COONa), pH 5) using crossflow filtration with a 3,000 MWCO PES membrane (Vivaspin Turbo 15, Sartorius). The ion-exchange chromatography was performed using a 1mL HiTrap™ Capto S (GE Healthcare). The column was washed with 10 CV of ultrapure water prior equilibration with 10 CV of binding buffer (50 mM CH_3COONa, pH 5). The sample was loaded onto the column at a 0.4 mL min^{-1} flow rate and pushed through by another 20 CV of binding buffer. The protein was eluted with a step gradient of NaCl (0 – 50 – 100 – 500 mM) in 50 mM sodium phosphate (Na3PO4) at pH 7.8 in 28 CV total. The fractions containing rIFN α2A were identified via western blot. The rIFN α2A was estimated to be 82.7% pure by SDS-PAGE (4-20% Mini-PROTEAN® TGX Stain-Free™ pre-cast gels, Bio-Rad) (Fig. S1). Recombinant rIFN α2A concentration was estimated by Pierce™ BCA Protein Assay Kit (Thermo Fisher).

4.2.4 Mass spectrometry on purified rIFN α2A

Using an Acquity M-class nanoLC system (Waters, USA), 5 µL of the sample was loaded at 15mL/min for 2 minutes onto a nanoEase Symmetry C18 trapping column (180mm x 20mm) before being washed onto a PicoFrit column (75 mmID x 300 mm; New Objective, Woburn, MA) packed with Magic C18AQ resin (3mm, Michrom Bioresources, Auburn, CA). Peptides were eluted from the column and into the source of a Q Exactive Plus mass spectrometer (Thermo Scientific) using the following program: 5-30% MS buffer B (98% Acetonitrile + 0.2% Formic Acid) over 90 minutes, 30-80% MS buffer B over 3 minutes, 80% MS buffer B for 2 minutes, 80-5% for 3 min. The eluting peptides were ionised at 2400V. A Data Dependant MS/MS (dd-MS2) experiment was performed, with a survey scan of 350-1500 Da performed at 70,000 resolution for peptides of charge state 2+ or higher with an AGC target of 3e6 and maximum Injection Time of

50ms. The Top 12 peptides were selected fragmented in the HCD cell using an isolation window of 1.4 m/z, an AGC target of 1e5 and maximum injection time of 100ms. Fragments were scanned in the Orbitrap analyser at 17,500 resolution and the product ion fragment masses measured over a mass range of 50-2000 Da. The mass of the precursor peptide was then excluded for 30 seconds.

4.2.5 Data Analysis of Massspectrometry

The MS/MS data files were searched using Peaks Studio X against human IFN amino acid sequence and a database of common contaminants with the following parameter settings. Fixed modifications: none. Variable modifications: propionamide, oxidised methionine, deamidated asparagine. Enzyme: semi-trypsin. Number of allowed missed cleavages: 3. Peptide mass tolerance: 10 ppm. MS/MS mass tolerance: 0.05 Da. The results of the search were then filtered to include peptides with a –log10P score that was determined by the False Discovery Rate (FDR) of <1%, the score being that where decoy database search matches were <1% of the total matches.

4.2.6 Anti- viral activity assay

A Cytopathic Effect Assay (CPE) was conducted by PBL assay science (NJ, USA). The purified algal rIFN α2A was run in triplicate alongside human IFN alpha reference material from bacterial source provided by PBL assay science in a viral challenge assay using EMC virus on A549 cells. The plate was stained with crystal violet, a visual CPE was performed, and the dye was then solubilized, followed by absorbance reading at 570nm. These data were then analysed in GraphPad Prism using a sigmoidal fit (variable slop).

4.2.7 Anti-Proliferation Assay

The purified algal rIFN α2A was run in triplicate alongside human IFN Alpha reference material from bacterial source provided by PBL assay science in an anti-proliferation assay using OVCAR-3 cells. Anti-proliferation was quantified using Promega MTS (Cat # G5430) and read at an absorbance of 490 nm. These data were then analyzed in GraphPad Prism using a sigmoidal fit (variable slope). Cell control (with no sample) and Media control (with no cells) were included in this study. Cell and media controls were supplemented with 50 mM sodium phosphate to check the effect of the sample buffer containing 50 mM sodium phosphate on the assay. A mock sample made of 50 mM sodium phosphate solution diluted by 3-fold was also added to the plate (Table 1).

Table 4.1: Cell anti-proliferation assay plate setup. Concentrations of sample and standard after final 2-fold assay dilution

	1	2	3	4	5	6	7	8	9	10	11	12	
A													
B	57000	19000	6333	2111	703.7	234.6	78.2	26.1	8.7	2.9	1.0	0.3	Sample (ng /mL)
C	57000	19000	6333	2111	703.7	234.6	78.2	26.1	8.7	2.9	1.0	0.3	
D	57000	19000	6333	2111	703.7	234.6	78.2	26.1	8.7	2.9	1.0	0.3	
E	18.8	9.4	4.7	2.3	1.2	0.59	0.29	0.15	0.07	0.04	0.02	0.01	Std (ng /mL)
F	CC (Cell control)						MC (Media control)						Controls
G	CC + 25 mM Sodium Phosphate						MC + 25 mM Sodium Phosphate						
H	25 mM sodium phosphate solution diluted by 3-fold												Mock Sample

4.3 Results

4.3.1 Purification of rIFN α2A and yield estimation

The affinity chromatography partially purified the protein with other impurities still present (Fig 3A). Fractions containing the protein of interest (~50%) were identified via western blot (Fig 3B). Prior to ion-exchange chromatography, the 10 fractions containing the rIFN α2A were pooled together and then were passed through a 1mL HiTrap™ Capto S column (GE Healthcare). After the cation exchange chromatography, the algal rIFN α2A was estimated to be 82.7% pure using SDS-PAGE gel and immunoblot images (Fig 4A, B), while the yield of rIFN α2A was estimated to be 140 µg/L.

Fig.3 A- SDS-PAGE gel (stain free) showing different fractions of rIFN α2A (transformant 1) after affinity chromatography. The expected size for rIFN α2A is shown by arrow. B- Immunodetection on PVDF membrane using raw cell extracts after binding with anti his antibody showing the protein of interest.

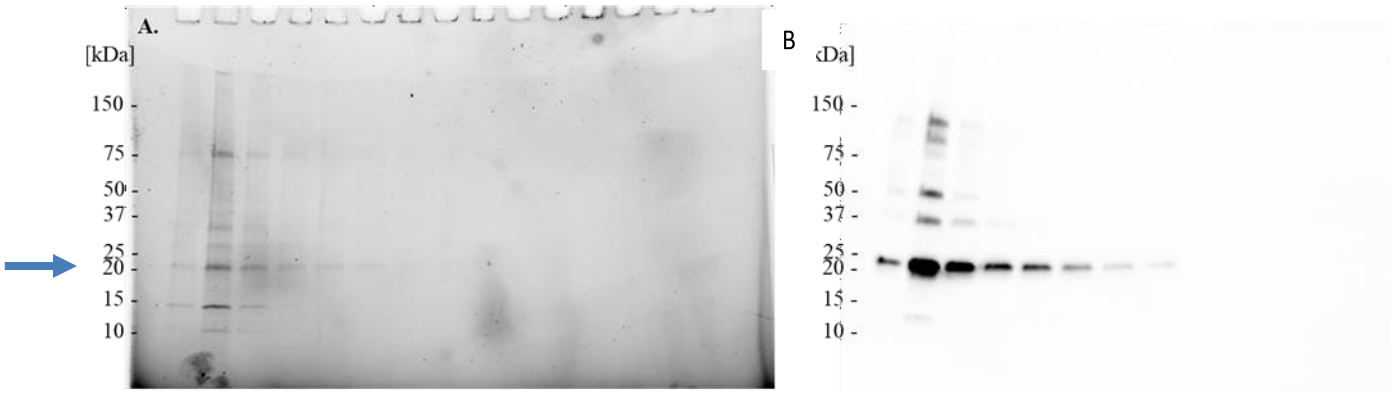


Fig. 4 A- SDS-PAGE gel showing different fractions of rIFN α2A (transformant 1) after ion exchange chromatography. The expected size for rIFN α2A (arrow) with less impurities as compared to affinity (Fig. 3A). B Immunodetection on PVDF membrane using the fractions obtained after affinity chromatography and subsequent binding with anti his antibody. rIFN α2A was estimated to be 82.7% pure by SDS-PAGE (4-20% Mini-PROTEAN® TGX Stain-Free™ pre-cast gels, Bio-Rad*).*

4.3.2 Mass spectrometry

Once purified, the rIFN α2A produced by Strain 1 was analysed using mass spectrometry. After trypsin digestion, the different fragments in the samples could cover 79% of the human IFN α2A amino acid sequence (Fig 5). The recombinant protein produced in this study was confirmed to be the human interferon alpha 2A.

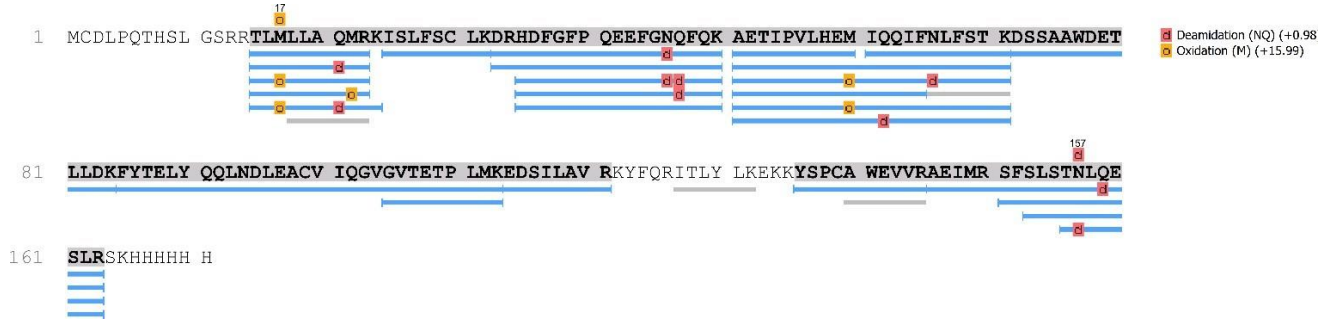


Fig. 5 Alignment of algal rIFN α2A peptides detected by mass spectrometry against the human IFN α2A amino acid sequence. The percentage of coverage was 79% for the rIFN α2A protein produced by Strain 1.

4.3.3 Anti-viral activity assay

The antiviral activity of the purified algal rIFN α2A was assessed using a CPE assay. It was observed that the algal rIFN α2A was active in suppressing the EMC virus on A549 cells. The specific activity of the sample calculated from the CPE assay was found to be 2.4E+03 U /mL which was 10-fold lower than the standard IFN α2A from bacterial source (2.78E+04 U /mL) (Fig. 6).

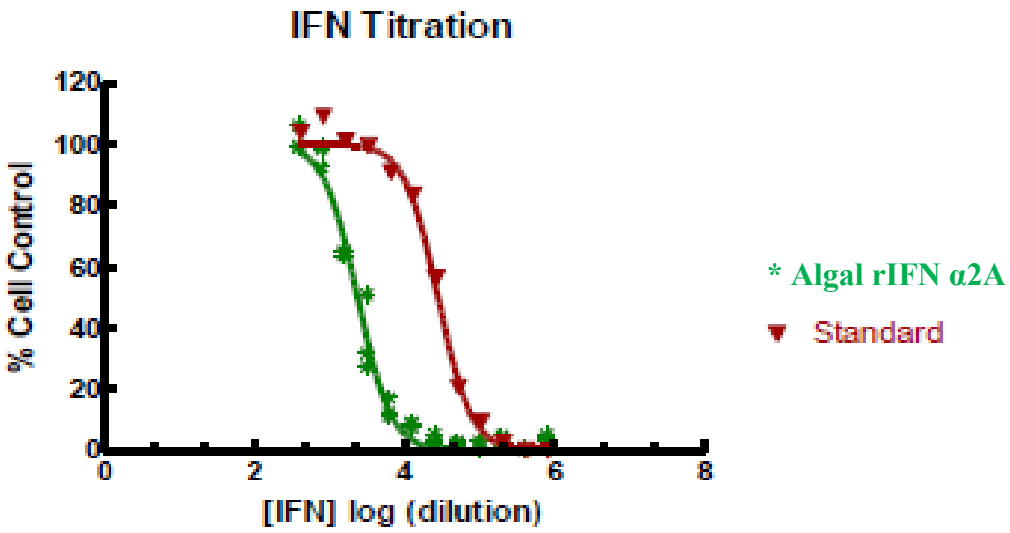


Fig. 6 Titration curves of the algal rIFN α2A versus the standard. The standard was active at higher dilution than for the algal rIFN α2A, revealing a lower activity of the algal rIFN α2A compared to the standard produced in *E. coli*.

4.3.4 Anti-proliferative activity assay

The sample failed to show a dose-response curve in the cell anti-proliferation assay (Fig. 7). Moreover, the mock sample resulted in almost similar O.D. values to the algal sample. The cell control with 50 mM sodium phosphate resulted in lower O.D. values in comparison to the cell control (without 50 mM sodium phosphate). For this assay, the algal sample was prepared based on the concentration calculated using BCA assay, which only gives an approximate value. We estimated the concentration of the algal rIFN α2A in the sample to be 140 µg /L, but it might be an overestimation. The lack anti-proliferative activity could be due to the sample not

matching the sensitivity limit of this assay, and it is therefore likely to be underestimated. Hence, we could not confirm any anti-proliferation activity of the algal rIFN α2A.

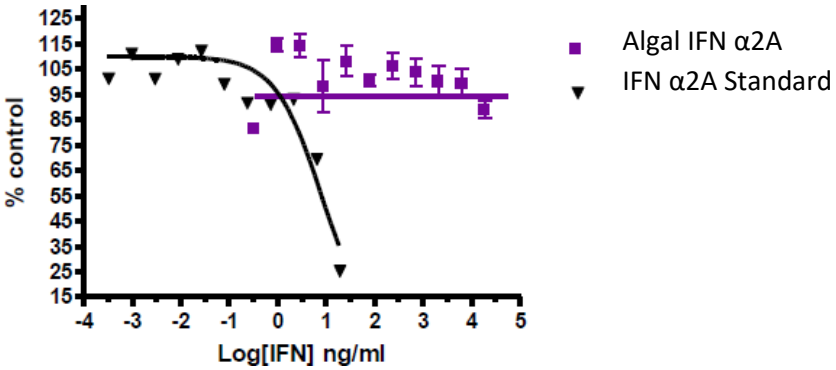


Fig. 7 Anti-Proliferation Assay Results EC50 (ng/ml)

4.5 Discussion

In this study, the activity of a recombinant human interferon alpha 2A protein (rIFN α2A) produced from a nuclear transgene in *C. reinhardtii* was analysed. Amongst the three transformants previously selected for having the full *ifn α2a* gene inserted in their nuclear genome (Chapter 2 section 2.3), one Strain seemed to express more protein than the others. This difference could be due to various levels of inactivation of the *ifn α2a* gene at transcription level. It was observedthat gene silencing is a major problem in *C. reinhardtii* nuclear gene transformation[17]. Theexpression

of a bacterial aadA in *C. reinhardtii* was transcriptionally suppressed by epigenetic mechanisms[18]. The changes which occurs in chromatin structure and affect transcription are called epigenetic changes. These epigenetic changes are the main cause of transcription gene silencing in almost all eukaryotes including yeast, fungi, Drosophila, plants and mammals[19]. In plants, even if the transgene expression is initially high, it can be impaired at later stages of development[20]. This is due to the position effect which consequently leads to low transgene expression levels thatare the result of homology-dependent gene silencing (HDGS)[21]. Additionally, the presence of a transgene with homologous sequences (co-suppression) can also be the reason of endogenous gene silencing[20], with transgene and endogenous gene interaction leading to post transcriptional gene silencing process, likely RNA degradation[22]. In transgenic plants, overproduction of a given RNA accelerate the turnover of that RNA[22]. However, it is unlikely to happen in this case as *C. reinhardtii* does not possess homolog of the human interferon alpha 2A gene. In a similar fashion as for plants, in *C. reinhardtii,* the transgenes are silenced by epigenetic gene silencing mechanism, which happens at both the transcriptional and posttranscriptional levels. Transcriptional silencing in case of single copy transgenes occurs without detectable cytosine methylation of the introduced DNA[23]. The genes introduced into the nuclear genome of *Chlamydomonas* by transformation are usually expressed at reduced levels when compared with the endogenous genes[24]. The reason for this reduced expression is not properly understood. However, it could be due to the position effects, inefficient transcription from heterologous promoters, gene silencing by methylation or other processes, inaccurate RNA processing or export from the nucleus and mRNA instability[25].

The strain which showed the highest expression of rIFN α2A was further investigated for its yield and corresponding activity. A yield of 140 µg /L was obtained after a two-step chromatography purification procedure. This yield is higher than previously reported for the secretory human erythropoietin fused to ARS2 export sequence w/6xhis tag (100 µg /L) protein from *C. reinhardtii* strain *cw15arg*[26]. However, this yield is lower than the one reported for the secretory (SP)n-fused Venus from *C. reinhardtii* that was found to be 15 mg /L, which is the highest yield reported from nuclear transgenes of *C. reinhardtii* so far [27]. This yield seems minimal as compared to the recombinant protein production from yeast (e.g. a yield of 30 mg /L of insulin precursor recombinant protein was secreted by yeast *Kluyveromyces lactis*[28]*)*. Merlin et al. compared the

production of four recombinant protein in different hosts and found that the production of recombinant human Glutamic Acid Decarboxylase (hGAD65) was the highest in *E.coli* (12.5 g /L) followed by *Saccharomyces cerevisiae* (0.46 g /L), *Spodoptera frugiperda cells* (0.02 g /L) and least in Mouse myeloma cells (1.67 mg /L)[29]. The same results were found for recombinant human interleukin- 6 (hIL-6), for which the highest production was reported in *E.coli* (7.5 g /L) followed by *Pichia pastoris* (0.28mg /L), *Spodoptera frugiperda cells* (1 mg /L)[29].

It is commonly admitted that about 50% of recombinant proteins are usually lost during a two-step purification. The yield obtained in this thesis could be therefore significantly increased by optimising the purification procedure. We observed that affinity chromatography only partially purified the protein as the SDS-PAGE gel clearly indicated the presence of other impurities (Fig 3 A, B). The histidine and cysteine-rich spots in superfluous proteins often compete with tagged proteins to bind to the column and interfere with IMAC, consequently resulting in contamination of the final product[30]. However, proteins eluted from IMAC retains their activity or native folding as they are eluted by mild elution conditions[31]. Despite of affinity chromatography being a useful method as a first step for the purification of crude extracts, it cannot be used independently for recombinant protein purification due to the resulting low purity[30]. Therefore, in this study we also applied cation exchange chromatography after affinity chromatography to achieve high purification of the rIFN α2A. After ion exchange chromatography, the rIFN α2A purity was calculated to be 82.7% (Fig 4 A, B). Ion exchange chromatography offers high resolution with high binding capacity under mild conditions[30,32]. Ion exchange chromatography has the capability to differentiate between the related protein variants and also separates the protein with the similar retention properties[33]. It was investigated that 1 g of pure IFN α2A was recovered using a single step Q- sepharose cation- exchange chromatography[34]. Thus, it is a powerful technique offering high performance purification. However, the more purification steps being added, the less recombinant proteins might be recovered, the difficulty relying on finding the right balance between yield and purity.

The algal rIFN α2A was tested for anti-viral activity. It showed activity against EMC virus on A549 cells at 2.4E+03 U /mL. However, the rIFN α2A produced from microalgae was less active than the standard rIFN α2A produced from bacteria, which showed an activity of 2.78E+04 U /mL

against EMC viral challenge on A549 cells. In a study conducted by El-Ayouty et al., the anti-viral activity of three recombinant IFN α2a produced by *C. reinhardtii* transgenic lines were observed to be 2 to 7-fold higher than the wild-type (WT) extracts[15]. They also showed comparable activity to a PEGylated IFN standard[15]. In the same study, the partially purified rIFNα2a proteins had an anti-tumor activity ~37 fold higher than the Cr.WT protein extract. However, it should be noted that in this study, the authors only compared the activity with Cr. WT protein extract, not with the pure IFNα2a as it was done in this study. In another study, a high specific activity of IFN α2A was reported by using a cytopathic effect inhibition assay with vesicular stomatitis virus after the last and fourth step of purification, which is ultrafiltration[35]. This high activity could be due to high purity level >95% of rIFN α2A while in the present study, only two steps purification was done. The algal rIFN α2A produced in this study has shown a significant antiviral activity though less potent than for the IFNs available from bacterial sources. One possible reason for the low anti-viral activity of the algal rIFN α2A could be the presence of aggregates. rIFN-α2a formed aggregates not dissociating under standard denaturing conditions. Aggregates reduced rIFN-α2a solubility and therefore potentially lowered its activity. Interferons are prone to protein aggregation due to their hydrophobic nature. IFN aggregation can be prevented by PEGylation, which consists in attaching a polyethylene glycol (PEG) polymer to a protein drug. Aggregates reduce the activity of interferon alpha based drugs and removing high molecular weight aggregates (approximately 650 kDa) as well as dimers, denatured and reduced variants can increase the potency of interferon alpha 2 by 87%[36]. Moreover, the presence of aggregates increases the risk to break immune tolerance in patient, therefore leading to immunogenic reactions and limiting the effectiveness of therapeutic treatment[36]. Contaminations related to host cells, protein modifications and aggregate formations are known to trigger the immunogenicity in several biopharmaceuticals including IFN α2A, insulin and growth hormone[36,37]. Glycosylation can also impart immunogenicity. It has been observed that the higher immunogenicity of IFN-ß derived from *E. coli* as compared with mammalian cell product is due to the reduced solubility of the non- glycosylated bacterial product[36,38]. IFN α2A being O- glycosylated and microalgae glycans are different from human glycans so it can trigger immune response when injected to human. At present existing analytical methods are unable to predict the biological and clinical properties of a protein. Thus, producing a new biopharmaceutical and comparing it to another product cannot be done without performing a wide range of clinical trials[36].

4.5. Conclusion

The purity of the rIFN α2A produced from a nuclear transgene in *C. reinhardtii* was calculated to be 82.7 % with a yield of 140 µg/L of culture. Mass spectrometry confirmed that the recombinant protein produced was indeed a full-length human interferon alpha 2A. The recombinant algal rIFN α2A showed anti-viral activity, but it was less potent than the standard rIFN α2A from bacterial sources. Biological activity could be increased by improving the purification steps so that more recombinant protein is recovered. While there is no doubt that microalgae are a promising platform to produce recombinant proteins, research on algal recombinant protein production is still in its infancy. More optimisation in purification processes for aggregate removal and increased protein purity are clearly needed to improve the biological activity of recombinant protein from microalgae.

4.6 References

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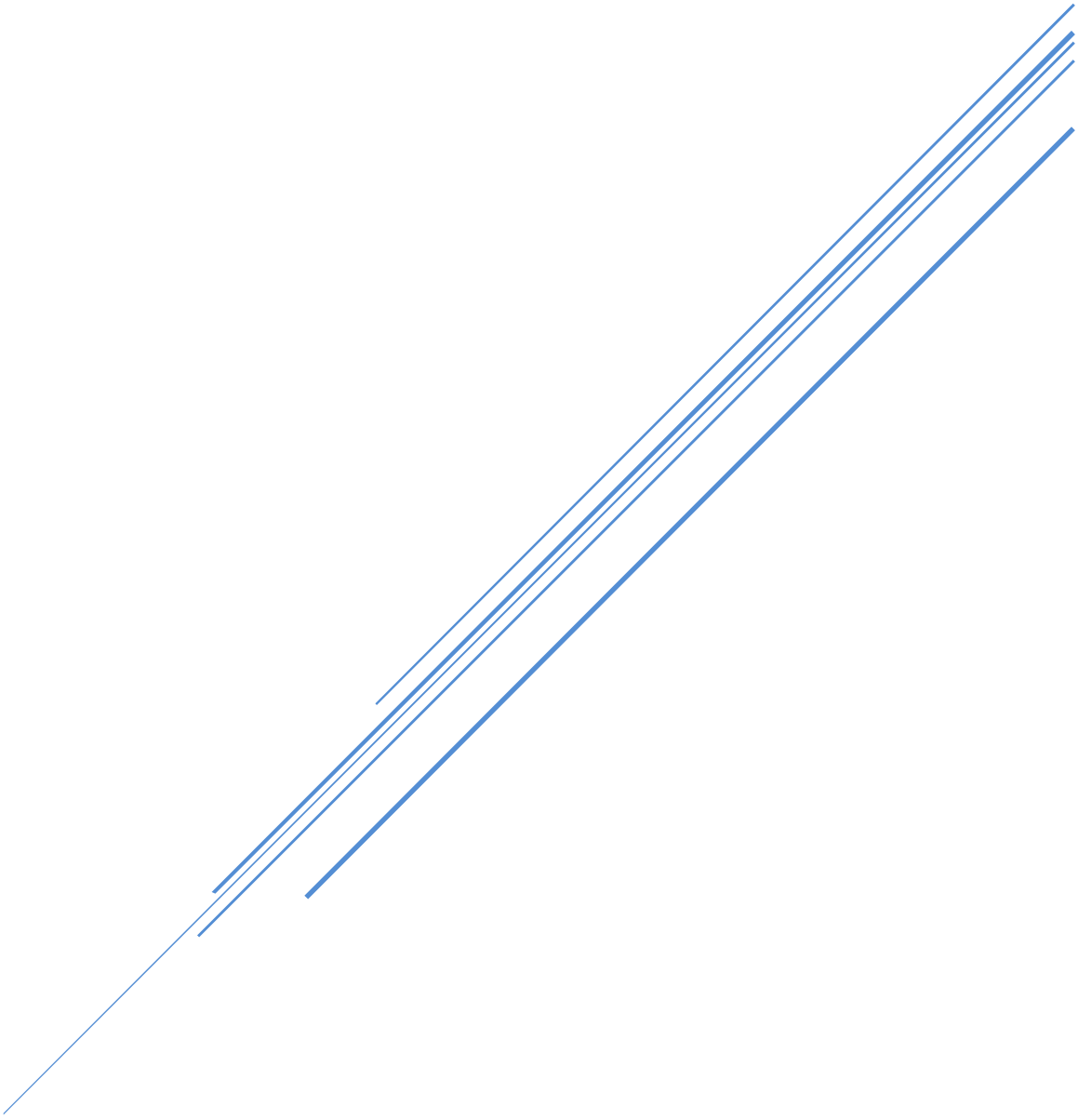
Chapter 5- Synthesis, limitations, perspective research and concluding remarks



5.1 Summary of thesis

The focus of this thesis was to investigate how different culture conditions affect recombinant protein production from nuclear transgenes in *C. reinhardtii*. As mentioned in chapter 1, there has been a lot of research on the optimization of culture conditions in other expression systems like mammalian cell lines and plants to improve the yield of recombinant proteins. However, not much has been done on optimizing culture conditions to improve the yield of recombinant protein from microalgae. Therefore, the goal of this research was to find suitable culture strategies to increase the yield of recombinant protein production in *C. reinhardtii* without any negative effect on the growth and health of *C. reinhardtii* cells.

5.2 Effect of culture conditions on rIFN α2A production

5.2.1. Effect of biphasic temperature regime (Chapter 2)

- Cultivation of mammalian cells at low temperatures is an established method to increase recombinant protein productivity and cell viability, but it also reduce growth rate and decrease cell metabolism generally [1,2]. Lowering the culture temperature of CHO cells from 37 °C to 33 °C resulted in a 4-fold increase of the productivity of recombinant erythropoietin (EPO)[3]. Bollati-Fogolin et al. 2005, observed a 6-fold increase in recombinant human granulocyte macrophage colony stimulating factor (rhGM-CSF) production[4] in CHO cells when the temperature was shifted from 37 °C to 30 °C. Comparative proteome and transcriptome analyses of CHO cells suggested that vesicle trafficking, endocytosis, apoptosis, glycoprotein quality control and cytoskeletal elements are involved in increased recombinant proteins productivity upon reduction of the culture temperature (from 37 °C to 31-33 °C)[1,5].

In the temperature experiment conducted in Chapter 2 of this thesis, it was observed that lowering temperature to 15 °C reduced the growth of *C. reinhardtii* but improved recombinant protein productivity, thus it can be assumed that similar mechanisms such as protease degradation, apoptosis reduction and proper protein folding could occur in *C. reinhardtii* cells in response to lower temperature. Braun-Galleani et al. 2015 studied the effect of changing temperature on recombinant protein production from *C. reinhardtii.* However, they used a different experimental plan – genetic engineering was done on chloroplast genome instead of nuclear genome, the

promotor used was the native atpA promoter/5'UTR element instead of HSP70/RBCS2i1 and they tested only (30 °C and 37 °C) temperatures which were higher than the optimal growth temperature of 25 °C for *C. reinhardtii*. They observed an increase in the production of Verde Fluorescent Protein (VFP) at 30 °C, the lowest temperature tested[6]. In this study, we transformed the nuclear genome and implemented a biphasic temperature regime, which was not implemented before on *C. reinhardtii* to test the effect on recombinant protein production. The key findings of this study were that we observed 3.3-fold increase in the production of rIFN α2A at low temperature (15 °C), while an increase in temperature to 35 °C negatively impacted the production of rIFN α2A. To conclude, our results showed that a biphasic temperature regime (lowering temperature) can be an effective strategy to increase the yield of recombinant rIFN α2A in *C. reinhardtii*.

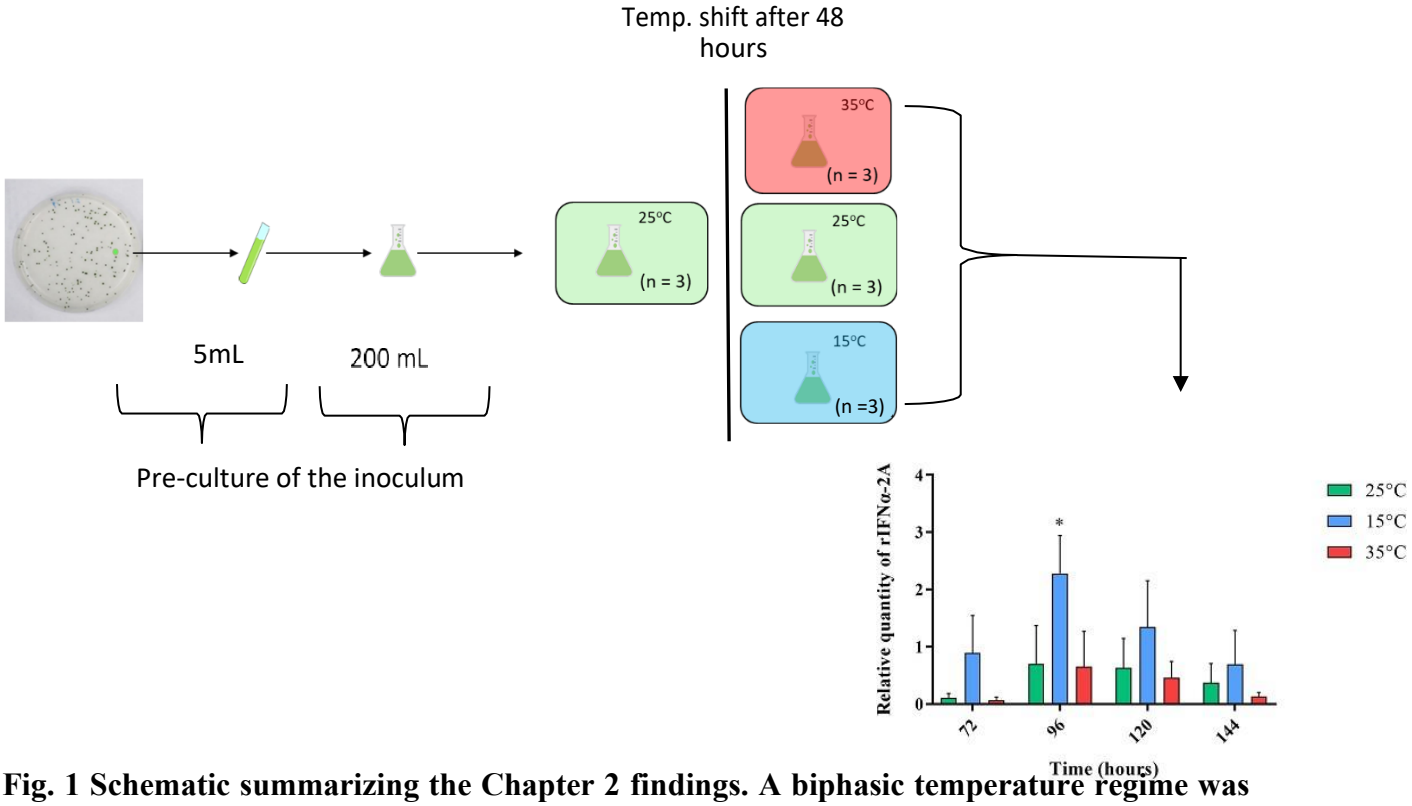


Fig. 1 Schematic summarizing the Chapter 2 findings. A biphasic temperature regime was applied after 48 hours of growth, resulting in more rIFN α2A production at 15°C followed by 25°C and 35°C.

5.2.2. Effect of bi phasic cultivation mode (Chapter 3)- Most of the culture optimisation studies have been carried on other expression systems for improving recombinant protein production. Culture conditions like carbon source and light are likely to also affect the recombinant protein production in *C. reinhardtii*. An effect of carbon source on recombinant protein production was studied in yeast *Pichia pastoris* with the use of adaptive laboratory evolution technique (ALE). The authors demonstrated an increase in recombinant protein production when supplemented with methanol in culture medium (BMM)[7] in both shake flask and fed batch cultivation. In another study, changes in various environmental parameters (e.g. carbon and oxygen concentration) were suggested to improve recombinant protein production in combination with adjusted feed strategies in yeast [8].

The impact of glucose and methanol as a carbon source on the production of recombinant rabies virus glycoprotein (RABV-G) in yeast *Pichia pastoris*[9] was investigated, the authors concluded that glucose is a better carbon source for recombinant protein production than methanol. Under glucose feed the transcript gene levels of the central metabolic pathway were upregulated with low antioxidative gene transcript levels[9], meaning that there is a correlation among upregulation of central metabolic genes, biomass concentration and recombinant protein production in *P.pastoris* when grown on glucose as compared to methanol[9].

Although the effects of nutrients and light on algal biomass composition (protein, lipids, carbohydrate, etc.) are well documented, little is known on the effect of culture conditions on recombinant protein production in *Chlamydomonas reinhardtii.* Lauersen et al. 2015, optimised culture parameters of *Chlamydomonas reinhardtii* secreting an ice binding protein, Lolium perenne (LpIBP). They found that the combination of acetate at 1g /L and 3 % carbon dioxide feeding with constant illumination at ~200 mol photons $m^{-2}s^{-1}$ resulted in the highest recombinant protein accumulation, with up to 10 mg/L of LpIBP in the culture medium[10]. However, they have not studied the effect of biphasic cultivation. Therefore, we investigated the production of recombinant IFN α2A protein in the following three main different culture conditions: autotrophic, heterotrophic and mixotrophic growth conditions. We further elaborated the study to test the effect of biphasic cultivation in mixotrophic growth conditions on rIFN α2A production from nuclear transgenes in *C. reinhardtii* (i.e. replenishing the *C. reinhardtii* cultures with 1 g /L of acetate once it is depleted in the medium). We investigated that the rIFN α2A production was higher in

mixotrophy. Further, addition of acetate improved the production of rIFN α2A as the cells continued to grow. This biphasic mode of cultivation was implemented before on other host systems to improve the yield of recombinant protein production[11,12]. Biphasic cultivation offers the possibility of decoupling the growth and production phase, which offers better cellular activity and controlled nutrient uptake leading to more cell density and ultimately more recombinant protein production[12]. This is the first time this kind of technique was implemented on *C. reinhardtii* for improving the yield of a recombinant protein from nuclear transgenes. The fed batch mode of cultivation proved to be beneficial in increasing not only the cell density but also the yield of rIFN α2A production in *C. reinhardtii*, supporting mixotrophy as the best strategy for rIFN α2A production. We demonstrated that the level of organic carbon in the medium affects the yield of recombinant protein. Further, by applying fed batch technique in mixotrophic cultures (i.e. acetate being supplemented in the medium once it is depleted) the cells remained in the exponential phase, resulting in higher rIFN α2A yield.

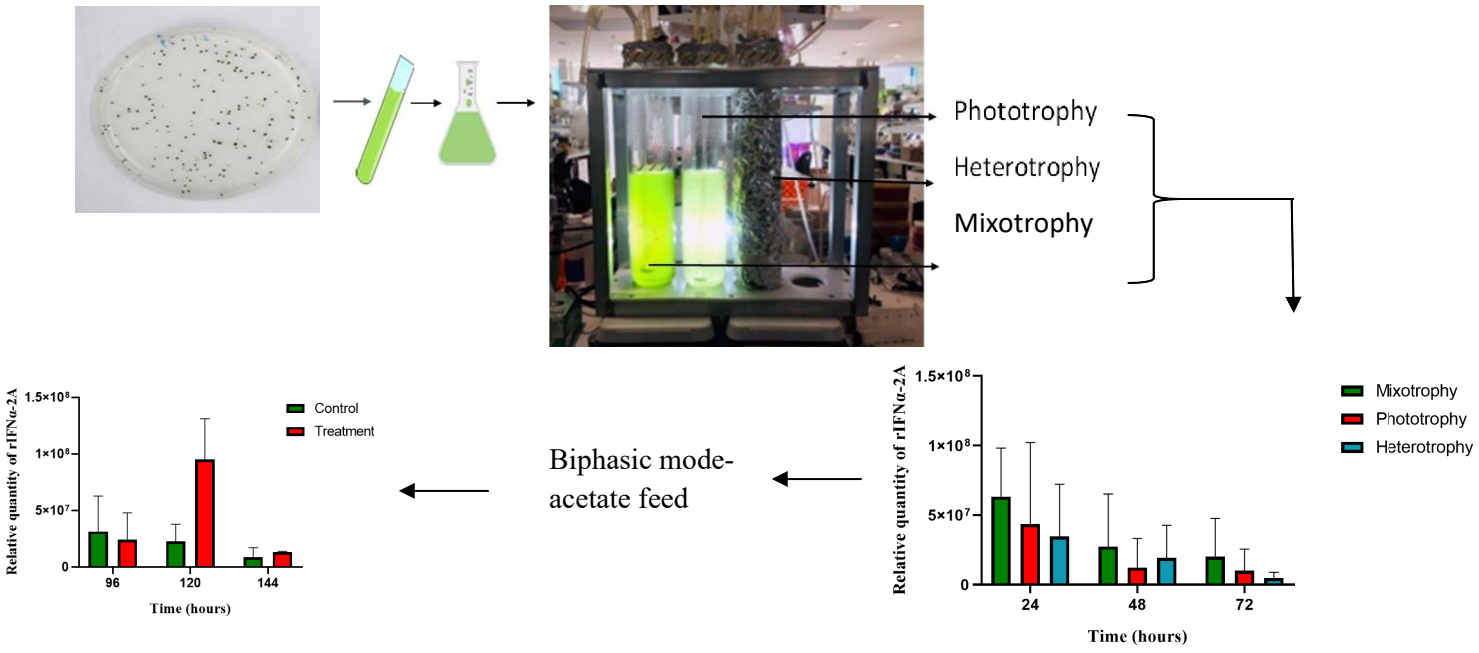


Fig. 2 Schematic of Chapter 3 results. Three conditions (mixotrophy, phototrophy and heterotrophy) were tested for rIFN α2A production. rIFN α2A production was higher in mixotrophy. Repletion of the medium with acetate further boosted the production of rIFN α2A.

5.3 Algal rIFN α2A purification, yield and activity

5.3.1 Comparison of yields

- Interferons are cytokines that possess strong antiviral, anti-proliferative and immunomodulatory effects. These cytokines are the first line of defense against viral infections. Interferons trigger an immunogenic response and act as a signaling protein for communication between cells[13]. As mentioned in previous chapters, this thesis is focused on recombinant interferon alpha 2A production. In this study, a yield of 140 µg/liter of culture was obtained after a two-step chromatography purification procedure.

This yield is similar to the yield of 100 µg/L of recombinant secretory human erythropoietin protein produced in *C. reinhardtii* strain *cw15arg*[14]. However, the yield reported in this thesis is less than previously reported for the secretory ice binding protein, Lolium perenne (LpIBP, 10 mg /L) which was also obtained via nuclear transgenes in *C. reinhardtii*[10]. Ramos-Martinez et al. (2017) have produced a maximum of 15 mg/L of recombinant yellow fluorescent protein Venus, when Venus was C-terminally fused with synthetic glycomodules comprised of tandem serine (Ser) and proline (Pro) repeats of 10 and 20 units and secreted into the culture media[15]. This later yield is still several orders of magnitude lower as compared to the yields reported in other expression systems, which can be as high as gram amounts of recombinant protein per litre of culture (Table 1). Approximate yields of recombinant protein in different hosts are mentioned in Table 1 for comparison. Generally, 50 % of recombinant protein is lost during the purification steps. Therefore, the yield obtained in this study could be increased by optimising the purification techniques. After ion exchange chromatography, the rIFN α2A purity was calculated to be 82.7%. No doubt, ion exchange chromatography is a powerful technique for purification of recombinant protein. However, the main hinderance is to find the right balance between yield and purity.

Table 5.1: Comparison of approximate yield of recombinant proteins in different expression systems

Expression systems	Strains	Average yield of RP
Microalgae	*Chlamydomonas reinhardtii*	10 mg/L[10]
Yeast	*Pischia pastoris*	30 mg/L[16]
Bacteria	*E. coli*	12.5 g/L[17]
Mammalian cells	CHO Cells	13 g/L[18]
Plants	Rice	9.24 mg/g[19]

5.3.2 Biological activity- In this thesis, the biological activity of a purified algal rIFN α2A was tested. It was observed that the rIFN α2A produced was active in suppressing the EMC virus on A549 cells. The specific activity of the algal rIFN α2A from the CPE assay was found to be 2.37 x 10^3 U /mL, which was 10-fold less than the standard IFN α2A (2.78 x 10^4 U /mL) from bacterial source. In another study, the high specific activity of IFN α2A produced from *E. coli* was observed by a cytopathic effect inhibition assay with vesicular stomatitis virus after the last and fourth step of purification performed by ultrafiltration[20]. This high activity could be due to high purity level >95% of rIFN α2A after five steps of purification (i.e., anion-exchange, cation-exchange, hydrophobic interaction, ultrafiltration and gel permeation chromatography) while in this thesis, only a two-step purification was performed. The rIFN α2A produced from *C. reinhardtii* has shown an antiviral activity though 10-fold less than the standard produced in *E. coli*. One possible reason for the low anti-viral activity of the algal rIFN α2A was the presence of aggregates. Aggregates reduce the activity of interferon alpha based drugs[21]. In addition, the risk to break immune tolerance in patient is increased with the presence of aggregates, which results in immunogenic reactions and limiting the effectiveness of therapeutic treatment. Also, the contaminations related to host cells, protein modifications and aggregate formations are known to be the source of immunogenicity of several biopharmaceuticals including IFN α2A, insulin and growth hormone[21,22]. Therefore, purification process needs to be optimized for increasing the purity of recombinant protein. One strategy is to add a last step of size exclusion chromatography or ultrafiltration at the end of the purification process to increase the purity and to remove the

aggregates leading to a recombinant protein, which is purer and biologically active. Size exclusion chromatography separates the molecules by size, consequently separating the larger aggregates from the monomers. Other reasons which could have led to low biological activity of rIFN α2A observed in this thesis is the absence of glycosylation. Glycosylation leads to protein stability, proper protein folding and increased protein activity[23]. In human, IFN α2A is O-glycosylated[24], however, in this study it was produced intracellularly preventing it to be glycosylated.

5.4 Drawbacks of the process

Producing recombinant proteins in microalgae is challenging for the different limitations discussed below.

5.4.1 Strain improvement-

In this thesis, the CC-125 wild type mt+ [137c] *C. reinhardtii* strain was used, which has less gene transformation capability than UVM4, an ultraviolet light-derived mutant of CC-4350. Screening for positive transformants is laborious with about two hundred transformants screened in this study for only 4 transgenic *C. reinhardtii* expressing rIFN α2A. Recent studies have focused on strain optimization for high recombinant protein production in *C. reinhardtii*. Ten years ago, Neupert et al. generated the strain UVM4, an ultraviolet light-derived mutant of CC-4350 (cw15 arg7-8 mt+), with a high transformation capability[26]. Since then, UVM4 has been used by many researchers around the world, and it could be used to produce IFN in future studies as it is likely to have reduced gene silencing mechanisms.

5.4.2 Transgene silencing-

Gene silencing is a major problem in *C. reinhardtii* nuclear gene transformation. Like plants, in *C. reinhardtii* the transgenes are silenced by epigenetic gene silencing mechanism which happen at both the transcriptional and posttranscriptional levels. These gene silencing mechanisms make the screening process for positive transformants extremely laborious. To go around this issue, researchers have been fusing their protein of interest with a report gene, often a fluorescent protein such as GFP or mVenus[27]. In this thesis, the rIFN α2A produced was not fused to a fluorescent protein as we did not want to impair the activity of the rIFN α2A, but it made it difficult to screen the clones. The presence of fluorescence tag makes the screening process easy and less time consuming. With the presence of fluorescence tag positive transformants can be identified rapidly by high-throughput plate-level screens or flow cytometry. Therefore, in future the addition of a cleavable fluorescence tag will be a good alternative to make screening process short and more efficient. However, the drawback of adding a fluorescent tag is

that it will add on another step of purification to cleave the fluorescent tag. This will ultimately lead to more protein being lost in the process and thus potentially in lower yield. A cold -inducible translation system was developed for the *C. reinhardtii* chloroplast by using a tRNA that can read through internal UGA codons at low temperatures only and this resulted in increased scope of the organism as a platform for heterologous protein production, metabolic engineering and the study of essential genes[29].

5.4.3 Purification- As mentioned above, only two step purification, immobilised metal ion affinity and cation exchange chromatography, were implemented in this study. Undoubtedly, these two are powerful techniques for recombinant protein purification. However, rIFN α2A is known to form aggregates which were observed in this study as well. Aggregates interfere with the biological activity of the recombinant protein and reduce its biological activity. So, to remove the aggregates, a size exclusion chromatography or an ultrafiltration step could be added as a third step to remove the aggregates and improve the functionality of rIFN α2A. Another alternative is to insert the secretion tag in the construct which allows the secretion of recombinant protein in the culture media. This will make the purification easier and is likely to lead to higher yields as more recombinant proteins are likely to accumulate in the medium than inside the cell. Secretion of protein will also help protein to fold properly which will result in more functionally active protein.

5.5 Conclusion of the thesis

In this thesis, firstly, a biphasic temperature regime was implemented on the transgenic *C. reinhardtii*. This is the first study to test the effect of biphasic temperature regime on recombinant protein production from *C. reinhardtii.* We found that lowering the temperature to 15 °C increased the rIFN α2A production while increasing the temperature to 35 °C negatively impacted the rIFN α2A production. Secondly, the effect of three culture conditions (mixotrophy, phototrophy and heterotrophy) on transgenic *C. reinhardtii* were tested. Mixotrophy came out to be the best condition for rIFN α2A production from *C. reinhardtii* nuclear transgenes. Further, a biphasic approach was implemented in mixotrophy by adding acetate when depleted in the growth medium. Transgenic *C. reinhardtii* continued to produce rIFN α2A till 144 hours from the cultures fed with acetate. The algal rIFN α2A was further purified and its biological activity was tested. The rIFN α2A showed an anti-viral activity but 10 times lower than a recombinant IFN α2A produced in bacteria. It should be noted that at present existing analytical methods used for testing the biological activity are not very accurate[21]. Thus, new biopharmaceutical cannot be compared to

another product without performing a wide range of clinical comparisons[21]. Only one study has been published recently which showed the production of rIFN α2A from *C. reinhardtii*[28]. In the same study the recombinant Cr.IFNα2a showed an anti-tumour activity ~37 fold higher than Cr.WT protein extract. However, it should be noted that in this study, the authors only compared the activity with Cr.WT protein extract, not with the pure IFNα2a as it was done in this study. In addition, they have partially purified the Cr.IFNα2a with anion exchange and size-exclusion chromatography. The latter likely removed most of the aggregates, potentially explaining their higher anti-viral activity compared to the one observed in this study. While we have demonstrated that microalgae have the potential to produce recombinant proteins to a higher yield in optimized culture conditions, it is still early days. As mentioned in chapter 4 that the existing analytical methods are unable to predict the biological and clinical properties of a protein. Thus, producing a new biopharmaceutical and comparing it to another product cannot be done without performing a wide range of clinical trials. Researchers are mostly focusing on genetic studies to improve recombinant proteins production from microalgae, however, only few researchers have focused on culture optimisations. Culture optimisation can improve recombinant protein production to high level as observed in other expression systems. Additionally, optimisation of growth medium and purification processes is needed to increase recombinant proteins yields and purity. Undoubtedly, microalgae offer many advantages compare to other expression systems for recombinant protein production, such as being cost effective and easy to scale up. Therefore, our results suggest that optimizing temperature regime and culture conditions could bring microalgae closer to a commercially viable platform for recombinant protein production.

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6. Supplementary data

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CLUSTAL multiple sequence alignment by MUSCLE (3.8)

ifn           ATGTGCGACCTGCCCCAGACCCACAGCCTGGGCAGCCGCCGCACCCTGATGCTGCTGGCC
62 -------------------------- CCCAGACCCACAGCCTGGGCAGCCGCCGCACCCTGATGCTGCTGGCC
                               *********************************************

ifn             CAGATGCGCAAGATCAGCCTGTTCAGCTGCCTGAAGGACCGCCACGACTTCGGCTTCCCG
62              CAGATGCGCAAGATCAGCCTGTTCAGCTGCCTGAAGGACCGCCACGACTTCGGCTTCCCG
                  ************************************************************

ifn             CAGGAGGAGTTCGGCAACCAGTTCCAGAAGGCCGAGACCATCCCCGTGCTGCACGAGATG
62              CAGGAGGAGTTCGGCAACCAGTTCCAGAAGGCCGAGACCATCCCCGTGCTGCACGAGATG
                 ************************************************************

ifn             ATCCAGCAGATCTTCAACCTGTTCAGCACCAAGGACAGCAGCGCCGCCTGGGACGAGACC
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                 ************************************************************

ifn             CTGCTGGACAAGTTCTACACCGAGCTGTACCAGCAGCTGAACGACCTGGAGGCCTGCGTG
62              CTGCTGGACAAGTTCTACACCGAGCTGTACCAGCAGCTGAACGACCTGGAGGCCTGCGTG
                 ************************************************************

ifn             ATCCAGGGCGTGGGCGTGACCGAGACCCCCCTGATGAAGGAGGACAGCATCCTGGCCGTG
62              ATCCAGGGCGTGGGCGTGACCGAGACCCCCCTGATGAAGGAGGACAGCATCCTGGCCGTG
                 ************************************************************

ifn             CGCAAGTACTTCCAGCGCATCACCCTGTACCTGAAGGAGAAGAAGTACAGCCCCTGCGCC
62              CGCAAGTACTTCCAGCGCATCACCCTGTACCTGAAGGAGAAGAAGTACAGCCCCTGCGCC
                  ************************************************************

ifn             TGGGAGGTGGTGCGCGCCGAGATCATGCGCAGCTTCAGCCTGAGCACCAACCTGCAGGAG
62              TGGGAGGTGGTGCGCGCCGAGATCATGCGCAGCTTCAGCCTGAGCACCAACCTGCAGGAG
                ************************************************************

ifn             AGCCTGCGCAGCAAGGAG
62              AGCCTGC-----------
                *******

CLUSTAL multiple sequence alignment by MUSCLE (3.8)


ifn             ATGTGCGACCTGCCCCAGACCCACAGCCTGGGCAGCCGCCGCACCCTGATGCTGCTGGCC
63CCCCAGACCCACAGCCTGGGCAGCCGCCGCACCCTGATGCTGCTGGCC
                              *********************************************

ifn             CAGATGCGCAAGATCAGCCTGTTCAGCTGCCTGAAGGACCGCCACGACTTCGGCTTCCCG
63              CAGATGCGCAAGATCAGCCTGTTCAGCTGCCTGAAGGACCGCCACGACTTCGGCTTCCCG
                  ************************************************************

ifn             CAGGAGGAGTTCGGCAACCAGTTCCAGAAGGCCGAGACCATCCCCGTGCTGCACGAGATG
63              CAGGAGGAGTTCGGCAACCAGTTCCAGAAGGCCGAGACCATCCCCGTGCTGCACGAGATG
                  ************************************************************

ifn             ATCCAGCAGATCTTCAACCTGTTCAGCACCAAGGACAGCAGCGCCGCCTGGGACGAGACC
63              ATCCAGCAGATCTTCAACCTGTTCAGCACCAAGGACAGCAGCGCCGCCTGGGACGAGACC
                  ************************************************************

ifn             CTGCTGGACAAGTTCTACACCGAGCTGTACCAGCAGCTGAACGACCTGGAGGCCTGCGTG
63              CTGCTGGACAAGTTCTACACCGAGCTGTACCAGCAGCTGAACGACCTGGAGGCCTGCGTG
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                 ************************************************************

ifn              ATCCAGGGCGTGGGCGTGACCGAGACCCCCCTGATGAAGGAGGACAGCATCCTGGCCGTG
63               ATCCAGGGCGTGGGCGTGACCGAGACCCCCCTGATGAAGGAGGACAGCATCCTGGCCGTG
                 ************************************************************

ifn              CGCAAGTACTTCCAGCGCATCACCCTGTACCTGAAGGAGAAGAAGTACAGCCCCTGCGCC
63               CGCAAGTACTTCCAGCGCATCACCCTGTACCTGAAGGAGAAGAAGTACAGCCCCTGCGCC
                 ************************************************************

ifn              TGGGAGGTGGTGCGCGCCGAGATCATGCGCAGCTTCAGCCTGAGCACCAACCTGCAGGAG
63               TGGGAGGTGGTGCGCGCCGAGATCATGCGCAGCTTCAGCCTGAGCACCAACCTGCAGGAG
                 ************************************************************

ifn              AGCCTGCGCAGCAAGGAG
63               AGCCTGCGCA--------

CLUSTAL multiple sequence alignment by MUSCLE (3.8)

ifn              ATGTGCGACCTGCCCCAGACCCACAGCCTGGGCAGCCGCCGCACCCTGATGCTGCTGGCC
I2               ATGTGCGACCTGCCCCAGACCCACAGCCTGGGCAGCCGCCGCACCCTGATGCTGCTGGCC
                 ************************************************************

ifn              CAGATGCGCAAGATCAGCCTGTTCAGCTGCCTGAAGGACCGCCACGACTTCGGCTTCCCG
I2               CAGATGCGCAAGATCAGCCTGTTCAGCTGCCTGAAGGACCGCCACGACTTCGGCTTCCCG
                 ************************************************************

ifn              CAGGAGGAGTTCGGCAACCAGTTCCAGAAGGCCGAGACCATCCCCGTGCTGCACGAGATG
I2               CAGGAGGAGTTCGGCAACCAGTTCCAGAAGGCCGAGACCATCCCCGTGCTGCACGAGATG
                 ************************************************************

ifn              ATCCAGCAGATCTTCAACCTGTTCAGCACCAAGGACAGCAGCGCCGCCTGGGACGAGACC
I2               ATCCAGCAGATCTTCAACCTGTTCAGCACCAAGGACAGCAGCGCCGCCTGGGACGAGACC
                 ************************************************************

ifn              CTGCTGGACAAGTTCTACACCGAGCTGTACCAGCAGCTGAACGACCTGGAGGCCTGCGTG
I2               CTGCTGGACAAGTTCTACACCGAGCTGTACCAGCAGCTGAACGACCTGGAGGCCTGCGTG
                 ************************************************************

ifn              ATCCAGGGCGTGGGCGTGACCGAGACCCCCCTGATGAAGGAGGACAGCATCCTGGCCGTG
I2               ATCCAGGGCGTGGGCGTGACCGAGACCCCCCTGATGAAGGAGGACAGCATCCTGGCCGTG
                 ************************************************************

ifn              CGCAAGTACTTCCAGCGCATCACCCTGTACCTGAAGGAGAAGAAGTACAGCCCCTGCGCC
I2               CGCAAGTACTTCCAGCGCATCACCCTGTACCTGAAGGAGAAGAAGTACAGCCCCTGCGCC
                 ************************************************************

ifn              TGGGAGGTGGTGCGCGCCGAGATCATGCGCAGCTTCAGCCTGAGCACCAACCTGCAGGAG
I2               TGGGAGGTGGTGCGCGCCGAGATCATGCGCAGCTTCAGCCTGAGCACCAACCTGCAGGAG
                 ************************************************************

ifn              AGCCTGCGCAGCAAGGAG
I2               AGCCTGCGCAGCAAGGAG
                 ******************
```

Table S1. Confirmation by Sanger sequencing of the complete insertion of the transgene (ifn for *ifn-α2a*) into the genomes of the three *C. reinhardtii* transformants (62, 63 and I2, called strain 1, strain 2 and strain 3 in this study). Forward and reverse primers used are highlighted in blue and yellow, respectively.

Chapter 2- Supplementary figures

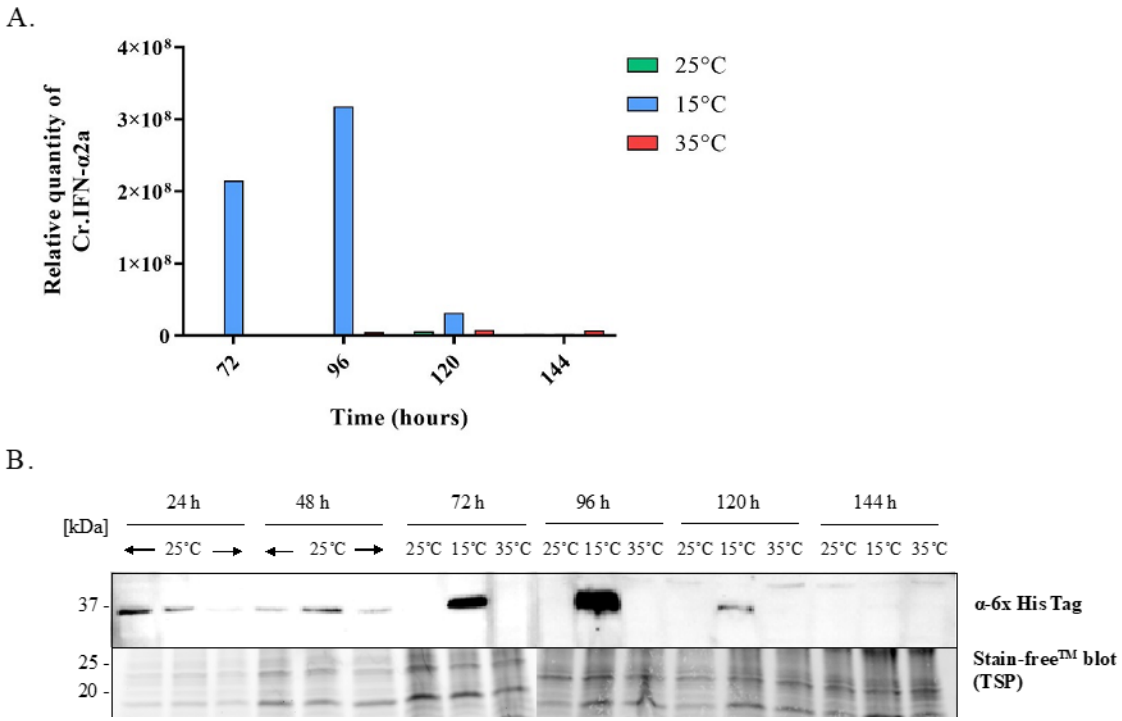

Fig. S1. (A) Relative quantity of recombinant IFN-α2a protein after temperature shift from 25°C to 15°C and from 25°C to 35°C in Cell line 2. Quantification is based on band intensities. from the western blot image (B) after normalization to the total amount of protein loaded per lane (stain-free™ blot). The figure was assembled from different images.

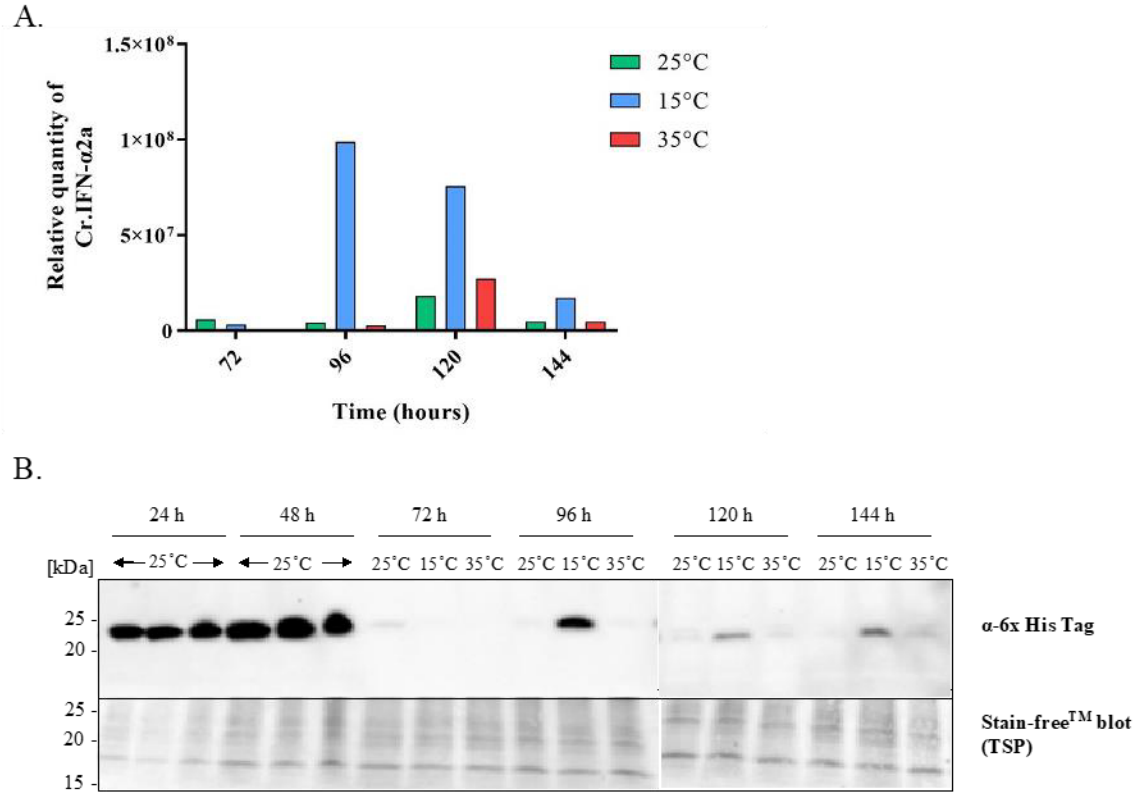

Fig. S2. (A) Relative quantity of recombinant IFN-α2a protein after temperature shift from 25°C to 15°C and from 25°C to 35°C in Cell line 3. Quantification is based on band intensities from the western blot image (B) after normalization to the total amount of protein loaded per lane (stain-free™ blot). The figure was assembled from different images.

Chapter 2- Statistical analysis

Table S1-two-way ANOVA of cell count

Assume sphericity?	Yes					
Alpha	0.05					
Source of Variation	% of total variation	P value	P value summary	Significant?		
Time x treatment	2.815	0.4363	ns	No		
Time	82.2	<0.0001	****	Yes		
treatment	2.734	0.2177	ns	No		
Subject	4.127	0.0163	*	Yes		
ANOVA table	SS	DF	MS	F (DFn	DFd)	P value

Time x treatment	5.11771E+12	12	4.26E+11	F (12	36)= 1.039	P=0.4363
Time	1.50E+14	6	2.49E+13	F (6	36)= 60.71	P<0.0001
treatment	4.9707E+12	2	2.49E+12	F (2	6) = 1.987	P=0.2177
Subject	7.50389E+12	6	1.25E+12	F (6	36)= 3.048	P=0.0163
Residual	1.47718E+13	36	4.1E+11			
Data summary						
Number of columns (treatment)	3					
Numberof rows(Time)	7					
Number of subjects (Subject)	9					
Numberofmissingvalues	0					

Table S2- Two-way ANOVA of relative band intensity of rIFN α2A production

Assume sphericity?	Yes					
Alpha	0.05					
Source of Variation	% of total variation	P value	P value summary	Significant?		
Time x Treatment	4.718	0.8056	ns	No		
Time	13.72	0.0655	ns	No		
Treatment	21.41	0.2102	ns	No		
Subject	31.4	0.0234	*	Yes		
ANOVA table	SS	DF	MS	F (DFn	DFd)	P value
Time x Treatment	1.49	6	0.2483	F (6	18) = 0.4924	P=0.8056
Time	4.334	3	1.445	F (3	18)= 2.865	P=0.0655
Treatment	6.761	2	3.38	F (2	6) = 2.045	P=0.2102
Subject	9.916	6	1.653	F (6	18)= 3.278	P=0.0234
Residual	9.076	18	0.5042			
Data summary						
Number of columns (Treatment)	3					
Number of rows (Time)	4					
Number of subjects (Subject)	9					
Number of missing values	0					

Chapter 3- Statistical analysis

Analyse of variance using GraphPad Prism 5.0 software to test the dissimilarities in cell density, quantum yield and relative intensity of rIFN α2A transformed *C.reinhardtii* cells in different culture conditions. Df = degrees of freedom; MS = mean sum of squares. Bold face indicates statistical significance (P < 0.05).

Table S1. Two-way ANOVA and post-hoc Tukey's HSD test results for the cell density of rIFN α2A transformed *C. reinhardtii* cells grown in different culture conditions

Tukey's multiple comparisons test

Source of Variation	DF	MS	P value	F
Time	**5**	**1.477e+014**	**P=0.0369**	**F= 21.36**
Treatment	**2**	**3.289e+014**	**P=0.0048**	**F=152.8**
Interaction	**10**	**28854554018519**	**P=0.0204**	**F=20.62**

Tukey's multiple comparisons test	Mean Diff.	Significant	Adjusted P Value
0 hours			
Mixotrophy vs. Phototrophy	-277667	No	0.5953
Mixotrophy vs. Heterotrophy	-74333	No	0.1106
Phototrophy vs. Heterotrophy	203333	No	0.7018
24 hours			
Mixotrophy vs. Phototrophy	2193333	Yes	<0.0001
Mixotrophy vs. Heterotrophy	1316667	Yes	0.0354
Phototrophy vs. Heterotrophy	-876667	No	0.0669
48 hours			
Mixotrophy vs. Phototrophy	8590000	Yes	0.0216
Mixotrophy vs. Heterotrophy	6476667	Yes	0.0063
Phototrophy vs. Heterotrophy	-2113333	No	0.2214
72 hours			
Mixotrophy vs. Phototrophy	13150000	Yes	0.0074
Mixotrophy vs. Heterotrophy	9590000	No	0.1132
Phototrophy vs. Heterotrophy	-3560000	No	0.3166
96 hours			
Mixotrophy vs. Phototrophy	13056667	Yes	0.0113
Mixotrophy vs. Heterotrophy	9376667	Yes	0.0433
Phototrophy vs. Heterotrophy	-3680000	Yes	0.0347
120 hours			
Mixotrophy vs. Phototrophy	13240000	Yes	0.0128
Mixotrophy vs. Heterotrophy	8386667	Yes	0.0118
Phototrophy vs. Heterotrophy	-4853333	No	0.0586

Table S2. Two-way ANOVA and post-hoc Tukey's HSD test results for the Fv/Fm of rIFN α2A transformed C. *reinhardtii* cells grown in different culture conditions

Source of Variation	DF	MS	F	P value
Treatment	4	0.009805	F = 6.979	P=0.1159
Time	2	0.02151	F = 8.819	P=0.0933
Interaction	8	0.0007202	F = 4.997	P=0.1247

Tukey's multiple comparisons test

Tukey's multiple comparisons test	Mean Diff.	Significant?	Adjusted P Value
24 hours			
Mixotrophy vs. Phototrophy	0.02833	No	0.3912
Mixotrophy vs. Heterotrophy	0.006667	Yes	0.0310
Phototrophy vs. Heterotrophy	-0.02167	No	0.5085
48 hours			
Mixotrophy vs. Phototrophy	0.06533	Yes	0.0177
Mixotrophy vs. Heterotrophy	0.03800	No	0.3777
Phototrophy vs. Heterotrophy	-0.02733	No	0.4623
72 hours			
Mixotrophy vs. Phototrophy	0.1023	Yes	0.0057
Mixotrophy vs. Heterotrophy	0.05167	No	0.3522
Phototrophy vs. Heterotrophy	-0.05067	No	0.3947
96 hours			
Mixotrophy vs. Phototrophy	0.1003	Yes	<0.0001
Mixotrophy vs. Heterotrophy	0.05167	No	0.3772
Phototrophy vs. Heterotrophy	-0.04867	No	0.3716
120 hours			
Mixotrophy vs. Phototrophy	0.08233	Yes	0.0044
Mixotrophy vs. Heterotrophy	0.04467	No	0.3721
Phototrophy vs. Heterotrophy	-0.03767	No	0.4041

Table S3. Two-way ANOVA and post-hoc Tukey's HSD test results for the relative intensity of rIFN α2A production in different culture conditions

Source of Variation	DF	MS	F	"P value"
Time	2	3.117e+015	F=3.909	P=0.1736
Treatment	2	7.984e+014	F=6.588	P=0.1218
Interaction	4	1.008e+014	F=0.4801	P=0.5603

Tukey's multiple comparisons test

Tukey's multiple comparisons test	Mean Diff.	Significant?	Adjusted P Value
24 hours			
Mixotrophy vs. Phototrophy	19714111	No	0.6321
Mixotrophy vs. Heterotrophy	28626419	No	0.1274
Phototrophy vs. Heterotrophy	8912308	No	0.7867
48 hours			
Mixotrophy vs. Phototrophy	15123656	No	0.4416
Mixotrophy vs. Heterotrophy	8100612	No	0.6710
Phototrophy vs. Heterotrophy	-7023043	No	0.3204
72 hours			
Mixotrophy vs. Phototrophy	10064672	No	0.4646
Mixotrophy vs. Heterotrophy	15442016	No	0.6018
Phototrophy vs. Heterotrophy	5377343	No	0.7785

Table S4. Multiple t-tests to test the significant differences in cell density of rIFN α2A transformed *C.reinhardtii* cells between control and acetate treated cells after 96 hours

Time in Hours	Significant?	P value	Mean of Treatment	SE of difference	df	Adjusted P Value
0	No	0.830255	1109667	327814	4.000	0.999018
24	No	0.822970	2583333	97696	4.000	0.999018
48	No	0.961130	12830000	3921141	4.000	0.999018
72	No	0.894894	17200000	2368778	4.000	0.999018
96	No	0.558334	19033333	2038518	4.000	0.983194
120	No	0.009719	29866667	2398379	4.000	0.056917
144	Yes	0.002830	35733333	2753584	4.000	0.019642
168	Yes	0.002286	37333333	2923088	4.000	0.018144

Table S5. Multiple t-tests to test the significant differences in acetate concentration of rIFN α2A transformed *C.reinhardtii* cells in mixotrophy and heterotrophy

Time in Hours	Significant?	P value	SE of difference	df	Adjusted P Value
0	No	0.892967	50.32	4.000	0.894639
24	No	0.675406	28.81	4.000	0.894639
48	Yes	0.000058	23.81	4.000	0.000408
72	Yes	0.000084	20.55	4.000	0.000506
96	Yes	0.000159	18.93	4.000	0.000797
120	Yes	0.000354	5.377	4.000	0.001416
144	Yes	0.010439	3.946	4.000	0.030991

Table S6. Multiple t-tests to test the significant differences in relative intensity of rIFN α2A production in control and acetate and acetate treated cells after 96 hours

Time in Hours	Significant?	P value	SE of difference	df	Adjusted P Value
96	No	0.867326	39406762	2.000	0.876457
120	No	0.205539	39140696	2.000	0.498561
144	No	0.648513	8436744	2.000	0.876457