Transient heterologous protein expression in green microalgae

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Abstract

Introduction: Recombinant proteins are produced using recombinant DNA technology and are widely used in numerous industrial applications, as well as innovative therapeutics. As the requirements of industries and pharma grow more complex chemically, traditional chemical engineering is, at times, limited by sets of synthetic chemical reactions currently available. This drives the need for applying novel chemical biology.

When recombinant proteins are produced in a host organism other than the gene's original source, it is referred to as heterologous protein expression. The baculovirus-insect cell culture, yeast, and plants, such as *Nicotiana benthamiana*, are only a few of the commercial expression systems where this technology has been extensively used. These systems, however, often have disadvantages in terms of their cost, safety and scalability. The potential of green microalgae as an innovative heterologous expression system for the production of recombinant proteins is examined in this thesis. To enable this, more specifically, the preparation of algal biomass of various axenic isolates, and their incorporation into a packed-bed matrix mediated by diatomaceous earth was investigated as an approach to be exploited for the expression of various heterologous proteins, using transient gene insertion rather than stable, nuclear gene integration (GMO). Microalgae is ideally suited for this as they are some of the most diverse, complex cells found in nature.

Advantages of Green Microalgae: The various benefits of green microalgae become apparent when compared with conventional expression systems. These photosynthetic organisms grow quickly and can be cultivated in large numbers using very straightforward methods as well as simple inexpensive media compositions. Furthermore, unlike all other eukaryotic plants of higher order such as angiosperms, bryophytes and pteridophytes, green microalgae are able to shift their metabolism from light-mediated photosynthetic reduction of carbon dioxide to oxidative respiration and reduction of complex carbon (this includes glucose, acetate and glycerol) to fulfil their energy requirements for growth, reproduction and metabolism. Known as heterotrophy, this results in volumetric feeding of biomass rather than surface area-based growth profiles, as is the case with light-dependent photosynthesis; with a resultant increase in algal growth rates as well as exponential increases in biomass yield. Green microalgae have been used to make a variety of recombinant proteins in the past. *Chlamydomonas reinhardtii*, for example, has been thoroughly characterised and investigated as a host of heterologous protein expression in both the chloroplast and within the cell nucleus. By developing a more ubiquitous approach to establishing transient expression systems in green microalgae, their metabolic diversity can be harnessed.

Plant Expression Systems: Due to their affordability, scalability, and safety compared to conventional expression systems, plants are being investigated more and more as a source of heterologous protein production. While the potential of transgenesis has been studied for decades, *Rhizobium radiobacter*-mediated transient plant expression (formerly known as *Agrobacterium tumefaciens*-mediated plant expression before taxonomy revision), which involves the introduction of a recombinant plasmid into plant cells, is nowadays one of the most frequently used plant expression systems. In the present study,

harnessing the single-celled nature of eukaryotic microalgae, as well as their superior biodiversity to fill the gaps left by plant-based systems, *R. radiobacter*-mediated transient protein expression in green microalgae was investigated.

Development of a Microalgal Library: In the development of a microalgal library, which is described in this thesis, microalgae were grown axenically. The library was screened for native isolate resistance to various antibiotic agents, as well as for media preference for growth rate maximisation and their ability to be grown axenically and withstand cryogenesis at -80 ℃. Finally, applicable species were identified using 18s RNA sequencing. The collection is a useful tool for finding and selecting strains with high performance for heterologous protein production, with intention to further up-scale for implementation as a feasible industrial platform for the expression of various heterologous protein products.

Cell-Pack Column System Construction: A column system was conceptualised and built to enable intentional concentration of microalgae, their contacting with chemical and biological agents including transfection agents, growth, expression and product and biomass recovery or separation or both. Following design, it was used to investigate enhancing the growth, yield, and stability of green microalgae, which used photosynthetically growing biomass, but also looked at how this biomass could withstand the column environment which is light impermeable. Initially, the system development process looked at algal biomass harvest efficiency, formation of the packed bed or matrix-mediated algal cell pack within the column, and material selection, with a final investigation into up-scale of column formation. Thereafter a methodology was developed for the separation of an algal ecology by size as well as preliminary findings of the cell pack's ability to induce cryptic, latent viral sequences, harboured within the algal genome, into a state of cell lysis.

Microalgal biomass was able to grow and reproduce within the column, with additional demonstration of the matrix-mediated column's ability to separate microalgal ecologies by size for further library investigation and development of novel microalgal libraries. This offers a useful production platform for large-scale operations, as well as a novel tool for the generation of axenic algal libraries from ecological sources.

Investigation of Heterologous Protein Expression: Several heterologous proteins were selected for expression in the novel system using *R. radiobacter*-mediated transient expression in green microalgae through contacting in the matrix-mediated algal cell pack column. These included *nptII* stable expression with the plasmid vector pTRAkc ERH::rfp, β-glucuronidase (GUS) with the vector pCAMBIA1301, green fluorescent protein (GFP) with the vector pTRAc::eGFP, horseradish peroxidase (HRP) with the vectors pTRAc::HRPΔC and pRIC::HRPΔC, as well as HPV16 hL1, transient expression and virus-like particle (VLP) formation with the vector pTRAkc-CTP rbcs1:: HPV16 hL1. All were proven to be expressible within the Matrix-mediated Algal cell pack.

Conclusion: The power of dove-tailing various green microalgal isolates with their ideal transient expression vector, in a high throughput system was demonstrated by this study. In order to not only identify microalgal isolates amenable to industrial application, but in the case of heterologous protein expression, the vectors that allow for maximal protein expression yields, must rely on high throughput approaches, such as this novel

diatomaceous earth-based column approach. This firstly allows for cheap media removal and biomass concentration. Secondly, it allows for identification of ideal isolate- vector pairing for the expression of recombinant proteins. The creation of both a novel microalgal library and the development of a diatomaceous earth-based column expression system, as well as their application to the *R. radiobacter*-mediated transient expression of a range of heterologous proteins in microalgae, are significant advances within biotechnology, demonstrating the potential to harness the synthetic biology of novel microalgae and heterologous expression through novel process engineering. These advances will open the door for the use of green microalgae as a vehicle for commercial expression. The findings of this study serve to demonstrate the value of a novel column-based expression system, which allows for discovery and experimentation of novel microalgae for their application in a column system for biomass harvesting without centrifugation and growth. Furthermore, the findings demonstrate the value of an effective contacting system for microalgae and transfection agents or other chemicals. The results presented in this thesis regarding use of the algal cell pack column for expression of various heterologous proteins and centrifuge-free biomass harvesting and as well as the column system itself will prove invaluable for the expression-development and production of recombinant proteins in the future using planktonic organisms, as well as delivering a research tool that allows for greater study of novel microalgae from different aquatic ecologies.

Keywords: microalgae, virus like particle (VLP), matrix-mediated algal cell pack, heterologous protein, transient expression, diatomaceous earth, axenic culture, heterotrophic ability, microalgal cryogenesis, matrix-mediated cell culture system, Celite545.

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“When confronted by an aspect of light that engages you, drink deeply”

−Harold Speed

“There is a light in the forest. There’s a face in the trees...”

−Tom Waits

Kevin Roberts

Phillip Weideman

David Ashley Stevens

Mark Gerald Stevens

Prof. Ingrid (Muffin) Elise Stevens

This is for you.

…happy hunting.

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1 List of abbreviations

+ve Positive

-ve Negative

2D Two Dimensional

3N BBM 3xNitrogen Bold's Basal Medium

AOX Alcohol Oxidase

BAC Bacterial Artificial Chromosome

Ble Bleomycin

CFU Colony Forming Unit

CHO Chinese Hamster Ovary

CSIR Council for Industrial and Scientific Research

CTP Chloroplast Targeting Peptide

CaMV Cauliflower Mosaic Virus

Carb Carbendazim

CeBER Centre for Bioprocess Engineering Research

DNA Deoxyribose Nucleic Acid

Em Emission

Ex Excitation

FACS Fluorescence Activated Cell Sorting

FC Fragment Constant

FDA Federal Drug Administration

FMDV Foot and Mouth Disease Virus

FV Fragment Variable

G-418 Geneticin 418

GFP Green Fluorescent Protein

GHD Guard House Dam

GM Genetically Modified

GMO Genetically Modified Organism

GRAS Generally Regarded as Safe

Gpr Gene (production)

Gsm Gene (selection marker)

HA Haemagglutinin Antigen

HPV Human Papilloma Virus

HRP Horseradish Peroxidase

Hgr Hygromycin

IG Immunoglobulin

Kan Kanamycin

LBB Lysogeny Broth (as stated by Bertani)

MAC Mammalian Artificial Chromosome

MCS Multiple Cloning Site

MPA Mpumalanga

OD Optical Density (nm)

PCR Polymerase Chain Reaction

PE Positional Effect(s)

PTM Post-translational Modification

PrP Protein (product)

Psm Protein (selection marker)

RNA Ribose Nucleic Acid

Rpm revolutions per minute

SDS-PAGE Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SEM Scanning Electron Microscopy

TAP Tris acetate phosphate

TEM Transmission electron microscopy

TSP Total Soluble Protein

Ti Tumour inducing

UCT University of Cape Town

UTEX University of Texas

V_H Variable Heavy

V_L Variable Light

VLP Virus Like Protein

X-GLUC x-glucuronide

YAC Yeast Artificial Chromosome

cDNA complimentary DNA

ddH2O double distilled water

dsRED Discosoma striata red

eGFP enhanced GFP

kDA kilo Dalton

mRNA messenger RNA

nptII neomycin phosphotransferase 2

rDNA recombinant DNA

siRNA small interfering RNA

ssDNA single stranded DNA

2 Literature review

2.1 Introduction

With the advent of recombinant DNA technology (rDNA) in 1972 (Wright, 1986), it was first demonstrated that various segments of DNA could be ligated to one another, and inserted into a host cell. This not only allowed for the manipulation of the cell's genetics, but also enabled the possibility of making foreign proteins.

Recombinant DNA technology paved the way for the study of cell biology in a manner not previously possible, ultimately providing for the recombination of gene sequences from various organisms into a limitless number of novel arrangements (Jackson et al., 1972). It made it possible, for example, to produce the industrial enzyme, horseradish peroxidase (HRP) transiently in a tobacco plant, with its expression under the control of a plant viral promoter (Huddy *et al*, 2018; Walwyn, 2015). Furthermore, it became possible, in 1978, to express human insulin in the bacteria, *Escherichia coli,* under a bacterial promoter. Prior to this, both proteins would have had to be extracted from endogenous sources, as was the case in the manufacture of porcine insulin for the treatment of human diabetes mellitus sufferers (Ladisch, 1995).

Currently, the market value for the top ten selling recombinant proteins is worth 201.06 US$ billion per annum, with a compound interest rate predicted at 11.04% until 2030 (Grand View Research, 2023). These recombinant proteins include a monoclonal antibody (mAb) directed against the tumour necrosis factor (TNF) for use in the treatment of human autoimmune disease (Enbrel); a monoclonal antibody (mAb) directed toward leukaemia and lymphoma cells displaying the cell surface receptor CD20 (Rituxan); the PEGylated form of the human granulocyte colony stimulating hormone used to stimulate white blood cell growth following aggressive bone marrow chemotherapy (Neulasta); as well as human insulin itself, used to treat type I and type II diabetes (Kayser and Heribert, 2012).

2.1.1 Recombinant DNA technology

Recombinant DNA technology has enabled the development of the genetically modified organism (GMO) revolution, delivering crop plants and animals with increased production yields, better nutritional profiles, increased resistances to cold, desiccation and pest stresses (Boulter et al., 1990; Evenson, 2003; Sanghera et al., 2011). In a recent technoeconomic assessment of the impact of pest-resistant maize in Spain and Portugal since its introduction some 21 years ago, upward of 678 tonnes of pesticide was spared (-37%) (Brookes, 2019).

Indeed, all rDNA technologies act at the subcellular genomic level, essentially. Although, many may rely on organ-level or organism-level downstream effects resulting from DNA sequence changes, rDNA technologies fundamentally harness the molecular complexity of a host cell to produce proteins beyond the capability of

traditional chemical catalyst-based methods. This is known as heterologous protein expression.

As proposed by the so-called Central Dogma of molecular biology, rDNA is transcribed into messenger RNA (mRNA), which is in turn translated into an amino acid-based peptide. This peptide is then actively folded into a protein, the functional polymer of the cell. The amino acid sequence is directly transcribed from that of the genetic information via the intermediary of the mRNA. Though this view of molecular biology, as introduced by Crick, was erroneous and grossly simplifies the actual complexity of the cell, it is useful as a conceptual framework for discussing the often-complex nature of heterologous protein expression (**Figure 1**) (Crick, 1970).

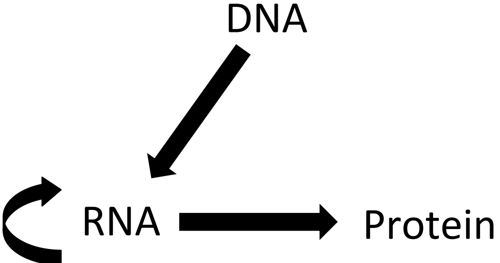


Figure 1. The Central Dogma of Molecular Biology. This paradigm suggests that heritable information, in the form of DNA in the nucleus, is transcribed into a mobile polymer, mRNA, which leaves the nucleus. This is then translated via coupling with various ribosomal subunits to allow for amino acid production. In this manner, heritable DNA always generates the sequence of amino acids via a messenger RNA intermediate (mRNA), with the reverse being rare. Changes to the DNA of a cell allow for changes to cell physiology, as well as allowing for the expression of recombinant proteins.

2.2 Heterologous protein expression and production

Production of heterologous proteins involves the introduction of foreign genetic material into a production cell.

Several steps are involved in the process to allow for foreign genetic material to be introduced into a host production cell. Firstly, genes that are to be expressed in the production cell are identified and isolated. Secondly, the genetics that best suit expression in the host organism are identified. Lastly, a suitable vector is also selected to allow for DNA insertion and gene expression in the host cell. The vector not only allows for the movement of the production gene(s) into the cell but also contains various control elements that facilitate gene expression. This is followed by construction of the expression vector by insertion of the production gene(s) into this vector, aligned with appropriate components to control expression, with the use of various molecular techniques. This then forms an rDNA construct that is compatible with the various cellular machinery of the production cell, to allow for the formation, folding and addition of tertiary structures resulting in maximal functionality, yield and ease of production (Rai and Padh, 2001).

Many expression vectors and host systems have been developed for the production of heterologous proteins. The host organism must be compatible not only with the vector that has been chosen for expression, but of fundamental importance, the production gene-vector system must be compatible with the various production processes, stresses and parameters involved in upscaling. Generally, heterologous protein value is directly relatable to its complexity and the technicalities of production and harvesting, resulting in the need for an ongoing development process for novel expression systems. When choosing a possible expression system to use, the following should be considered:

- **Expression of the production gene(s) may be stable or transient**. Stable gene integration into the host cell's genome allows for heritable gene expression but often suffers from lower production yields, gene silencing, increased metabolic burden and does not allow for expression of products that may cause cellular toxicity. Transient expression is not heritable; it often produces higher yields and allows for the expression of toxic products. Turnaround time, from initiation to production scale-up, is often much quicker, allowing for novel protein product expression at batch scale in as little as four weeks (see **Figure 4**). It does however rely on very efficient gene introduction into the host population as a whole. Only certain systems allow for this modality of heterologous protein expression, which may not be amenable to all products (Büssow, 2015; Vink et al., 2014).
- **The molecular complexity of the heterologous protein product that can be produced by the host organism is directly related to the cellular complexity of the host**. Bacterial-based production systems, though very productive, are not able to express complex multimeric proteins that may require post-translational modification or complex tertiary sidechain additions often required for functionality by proteins originating from higher organisms. These include those of eukaryotic origin, as is often the case with mammalian proteins (Tate et al., 2003).
- **Heterologous peptides are mobile inside the host cell**. Certain vectors allow for the localization of peptides into various subcellular compartments/organelles and may also allow for export and secretion of the protein product from the cell. This may not only directly influence how the protein is processed, affecting production yields, but also allows for the possibility of direct product harvesting from growth media, if the production system is planktonic. This has a direct influence upon process design (Rasala et al., 2012; Weisheit et al., 2009).
- **As DNA codon usage by various organisms may be disparate, the codons of the production gene(s) may be incompatible with that of the host cell**. This may cause low/no production yields (Gustafsson et al., 2004). In this case, production gene sequences are often codon optimized for the host cell. In certain cases, this may not increase yields

because of peptide instability, similarly resulting in lower yields. Thus, there is often the need to design various vectors that are then assessed stochastically.

- **Lastly, prospective commercial value must be considered**. As the cost of biomass production, process development, product harvesting and downstream processing vary considerably from production system to production system; heterologous protein product value must inform the production system used (Tate et al., 2003).

Currently, numerous rDNA expression vectors are in existence, with more continually being developed, allowing for the expression of a diverse array of heterologous protein products in a diverse array of hosts. These vectors fall into three classes. Firstly, circular DNA constructs known as plasmids for expression in bacteria or yeasts. Secondly, artificial chromosomes which include mammalian artificial chromosomes (MACs), yeast artificial chromosomes (YACs) or bacterial artificial chromosomes (BACs). Thirdly, virus-based constructs such as lambda phages, phagemids and cosmids, as well as eukaryote virus-derived vectors. (Collins and Hohntt, 1978; Faye and Gomord, 2009; Ikeno et al., 1998; Terpe, 2006).

These groupings have enabled a best-of-fit options to be explored, allowing cost-effective production without sacrificing protein yield, or their molecular and structural integrity or quality.

All plasmid-based expression vectors share certain elements. These include:

- An **origin of replication (O_E)**, that allows for the copying of the DNA of the plasmid inside a eukaryotic cell.
- A **promoter element (P_E)**, that acts as a transcription initiator inside eukaryotic cells. These are often of viral origin, such as the cauliflower mosaic virus (CaMV) 35S promoter. Viral promoters are strong initiators of transcription, and are generally limited to host cell type, such that plant, mammalian and bacterial promoters are constrained to each group respectively. Additionally, the P_E can be constitutive, such as the CaMV 35S promoter, i.e. they enable continuous active expression, or inducible i.e. they are activated selectively when production gene transcription is required. This is achieved by modulating various environmental parameters such as the addition of a chemical stress, pH change or temperature change.
- A **multiple cloning site (MCS)** that contains several restriction enzymes (RE) binding sequences, that allows for the cloning and integration of expression gene(s). The MCS must be downstream of the P_E, as its effect is unidirectional.
- A **selection marker (SM)**, which allows for the selection of host cells containing and expressing the plasmid vector. These are in and of themselves genes, but their expression produces an enzyme that, if active, will catalyse the breakdown of an otherwise cellularly toxic

substance. SM are not generally present in transiently expressing heterologous systems but are fundamental to stable expression systems. Plasmids often contain two selection markers. The first allows for selection of eukaryotic cells for production **(M_E)**, the second allows for bacterial selection to allow for bacterial-mediated plasmid proliferation **(M_P).**

- A **transcription terminator (T_E) element**. This element is located downstream of the MCS, and ensures that the mRNA formed after transcription of the production gene(s) disengages efficiently from the plasmid.

The sequences of all elements making up a recombinant plasmid may be of natural origin, isolated from a highly diverse array of species, or they may be synthetic. Unquestionably, this is the power of rDNA technology!

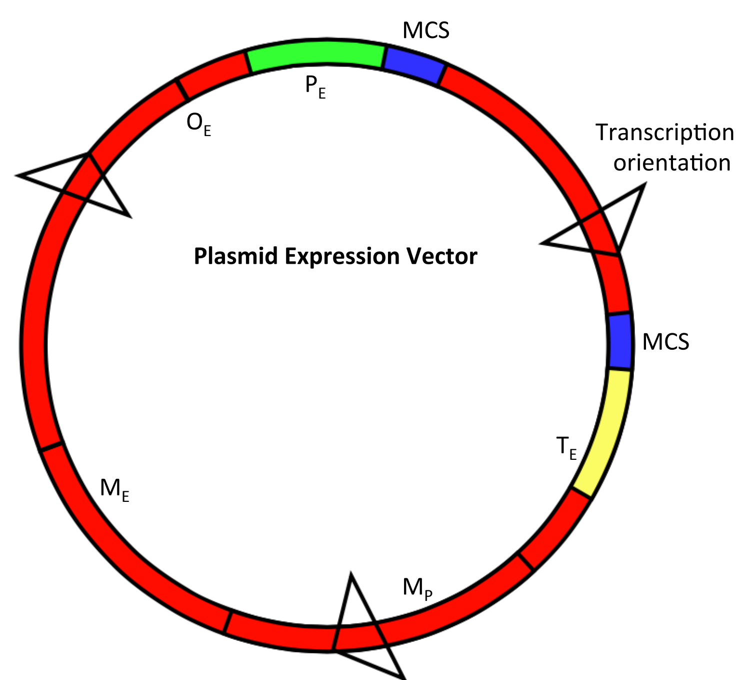


Figure 2. The general elements of a plasmid. This circular segment of DNA, constructed of various recombinant elements for the expression of recombinant proteins contains various functional units. OE allows for copying of the plasmid in the eukaryotic host cell. PE allows for transcription initiation. MCS contains various restriction sites to allow for cloning of recombinant DNA into the expression vector. TE facilitates decoupling, ending RNA transcription. Op is the bacterial origin of replication, allowing for DNA copying and proliferation in a bacterial host. Mp is a bacterial selection gene. ME is a eukaryotic selection gene. These must convey resistance to an otherwise toxic substance in either cell-type.

2.3 The nature of heterologous protein expression

Expression systems can be categorised in two basic classes; namely, stable expression systems and transient expression systems.

Stable expression systems rely upon heritable rDNA integration into the genome of the host organism, and the formation of GMOs. As the process of genomic production-gene integration and its subsequent heritability by the progeny of the initial GMO is a rare event, this method of heterologous protein expression necessarily relies upon the ability to screen for stably expressing individual organisms in a very efficient manner. All vectors that aim to create GMOs contain some resistance gene or metabolic mechanism that prevents the growth of non-transformed or transiently transformed hosts, but promotes that of stably expressing ones. As the resistance gene is located on the same rDNA segment as the production gene, gain-of-function resistance by the host organism to an otherwise lethal parameter ensures only the survival of continuously expressing host-rDNA outcomes. As this selection coefficient is modulated, either by extending exposure time, or selection intensity, so can heritability and/or productivity be selected for, respectively (Rasala et al., 2013). In general, selection genes allow for the transformed host to differentially metabolise an otherwise toxic substance, such as an antibiotic, but several methods are now applied that allow the transformed organism to differentially metabolise an otherwise non-native metabolic substrate, as is the case with the selection of microalgal transformants by a gain-of-function ability to use phosphite as a sole phosphate source (Sanchez-calderón et al., 2016). Additionally, negative selection markers may be employed, such that transformants are sensitive to an extracellular compound, as is the case with thymidine kinase expression and the resultant susceptibility of transformants to ganciclovir negative selection, where a cellular toxin is produced from the otherwise non-toxic compound (Beck et al., 1995).

Stable expression systems all share similar attributes:

- Stable expression systems may be batch-based or continuous systems. Batch-based systems involve initial small populations of production organisms, which are inoculated, allowed to proliferate and are then harvested. Continuous systems, once at an optimized biomass, allow for continual harvesting of product, which is balanced by culture feeding and dilution. This does only apply however, to planktonic systems, and not generally to whole plant systems, though if expression is undertaken in fruit or flowers this to a degree is then continuous. This is the case with expression in the fluidic exudates of the pitcher plant, *Nepenthes mirabilis* (Buch et al., 2014).
- The process of selecting and maintaining the selection for stably expressing individuals is time consuming, taking as much as 12 weeks for stable cell line generation; and costly, requiring a constant input of selection to maintain cell line product expression.
- Because the process of stable gene introduction is a rare event, this system is always started by a small number of initial organisms. Through many generations, the organisms give rise to production-scale populations. This not

only necessitates the need for a large production footprint due to the necessity for prevention of escape of GMOs, but as many generations are required for production at scale, there is a high likelihood of heterologous gene mutation and selection of sub optimally producing sub-populations making up the final production population. Additionally, such selections may dramatically alter final amino acid compositions, with direct impact on safety and batch-to-batch consistency.

- Vectors that allow for gene delivery are selected upon the criteria of allowing for favourable, controllable and higher yielding integration events into the genome of the host cell. Commonly used gene delivery methods for stable gene introduction include the following:

  Biolistic Bombardment: high density nanoparticles, often made of gold or tungsten, are coated with rDNA and introduced into cells using high velocity bombardment in a random process.

  Microinjection: rDNA is directly inserted into the nucleus with the use of nanosyringes under a microscope.

  Electroporation: electric pulses are used to porate cellular membranes, such that naked rDNA can pass randomly into cells.

  DNA agitation: cells are shaken in a liquid culture medium in the presence of rDNA, which often includes a mechanical shearing agent, such as silicon carbide whiskers or glass beads (Gangl et al., 2015; Nguyen, 2016; Run et al., 2016; Ten Lohuis and Miller, 1998).

Transient expression systems do not rely upon rDNA gene integration with the genome of the production organism. Instead, the vector interacts with the cell's DNA transcription machinery as an extra-chromosomal or extra-nuclear entity (episome), such that the vector may indeed be translocated to the nucleus and may also integrate into the genome. Moreover, this mode of heterologous protein production relies not upon the genomic location or the heritability of the rDNA. Instead, transient expression systems rely upon the insertion of the production gene(s) into the unmodified host population in a highly efficient manner (Malla et al., 2021).

Transient systems all share similar attributes within the production of heterologous protein production.

- Transient systems are necessarily batch-based systems, demonstrating ramp up, ramp down expression kinetics. This is due to the initial production of wild-type biomass, lacking any transgenes. Transgenesis is then undertaken on this population at large, directing much of the metabolic energies of this biomass to heterologous protein expression. At the point of peak expression, this biomass is destroyed to allow for protein harvesting (Fischer et al., 2004). This too necessitates the need for the production of the transforming agent i.e. *R. radiobacter* culture, recombinant virus.

- The function of the rDNA expression vector does not hinge upon the selection of a subclade of individual organisms of the host population that contain rDNA. These vectors do not contain a host-specific selection mechanism for production. This being said, often for rDNA maintenance and proliferation, these vectors may contain a bacterial selection mechanism (Terpe, 2006).
- Transient heterologous protein production does not result in or necessitate the development of GMO organisms that heritably contain rDNA. Instead, rDNA is delivered to wild-type host species in a highly efficient manner, such that host species' biomass may be produced without concern for GMO escape. The production footprint only needs to encapsulate biomass post transgene delivery, but also the transforming vector system.
- Gene delivery methods are necessarily highly efficient regarding the host population. These can rely upon a genetically modified pathogen to allow for such highly efficient rDNA delivery. This is the case with *Rhizobium radiobacter*, used for transient expression in plants, as well as viral systems, such as recombinant baculoviruses, for transient expression in insect and mammalian cell lines. Although this requires the need for batch-based production, batch-to-batch variation is constrained leading to higher indexes of safety, protein quality and reproducibility (Yamamoto et al, 2018).

2.4 Industrial heterologous protein expression systems

Although in theory any organism can be utilized for the production of heterologous protein, all expression systems fall into two general classes according to their cellular complexity. These are bacterial systems and eukaryotic systems. Bacterial systems are all planktonic and so are grown in various bioreactors, while eukaryotic systems may be whole organisms, parts of an organism, planktonic, sessile or derived synthetic cell cultures (Demain and Vaishnav, 2011)

Bacterial systems generally produce large amounts of protein product (<2% of total cellular protein (Pryor and Leiting, 1997)) very quickly, grow on relatively simple and inexpensive media, and are genetically modified with relative ease. These systems do, however, have several shortcomings, which relate to the nature of the protein produced. Bacterial physiology does not allow for eukaryote-type post-translational modification of proteins, often fundamental for the functioning of eukaryotic proteins, which are more complex. Overexpression of the protein product often results in the formation of insoluble, non-folded agglomerates of peptide, known as inclusion bodies. Cell populations will often discard rDNA if maintained in media free of the selective agent i.e., typically antibiotic-free media. These subpopulations that have discarded inserted DNA typically have higher growth rates relative to the transformed population, and are thus selected for, resulting in sub optimal protein yields at scale (Baneyx, 1999; Terpe, 2006).

Eukaryotic systems may be unicellular or multicellular. Multicellular organisms may be plants, fungi or animals. Although the ethical considerations when working with plants are fewer, GMO escape into wild-type populations or into natural ecologies must be

prevented. This has been recently legislated in the US, with the passing of House Bill 2739, in which Oregon farmers will have legal recourse should Monsanto-derived GMOs/GMO genetics be found within their crop plants. Additionally, although animals produce high quality products, housing and maintenance is very costly and genetic modification is time consuming and technically complex (Demain and Vaishnav, 2011; Houdebine, 2009).

Unicellular systems are metabolically complex organisms, such as yeast, green microalgae or synthetic cell cultures of plant, insect or mammalian origin. Not only do they have the ability to produce complex multimeric heterologous products, but they also have the ability to be grown quickly, producing high biomass yields due their volumetric growth. As a result, this allows for minimal risk of GMO escape because they are necessarily enclosed in sealed GMP-compliant bioreactors.

2.5 Overview of common commercialized expression systems

2.5.1 Bacterial expression systems

The gram-negative bacterium *Escherichia coli* is the workhorse of modern heterologous protein production. It currently acts as the host for half of the heterologous protein products in the biopharmaceutical industry. Since it was first isolated from the gut microbiome of infants in 1881, it has played a major role in such diverse fields as experimental bacterial evolution, the life cycle of lytic and lysogenic bacterial viruses, elucidation of the genetic code, and more recently in the biotechnology field of heterologous protein production. The reasons for its popularity include its high growth rate, being generally non-pathogenic, and its robust growth characteristics.

Following the completion of its genome sequence in 1997, this has provided a comprehensive and robust molecular toolkit for facilitating the development and expression of new products (Blount, 2015).

This being said, there are some disadvantages specifically associated with this microbe in terms of its ability to act as a heterologous protein host. As this is a relatively simple organism, many attempts to express more complex proteins have failed. It also produces lipopolysaccharides, known as endotoxins, which are pyrogenic to human physiology. These must be removed in a second, costly, downstream purification unit operation if the end-user application is of human or animal origin. For this reason, other microbial expression systems have been looked to that are non-pyrogenic (Terpe, 2006).

A few other microbial expression systems include members of the *Bacillus* genus, such as *B. megaterium, B. subtilis* and *B. brevis*. This family does not produce lipopolysaccharides; thus, these microbes have been granted GRAS status (Generally Regarded as Safe) by the American based Federal Drug Administration (FDA), allowing for their consumption and application without reservation. They are also an attractive option as they was demonstrated by this study secrete protein products directly into their growth medium very efficiently. They are, however, not very well characterized and therefore the cellular mechanisms of peptide disulphide bridge

formation have not been well studied, which is essential for complex tertiary peptide folding (Terpe, 2006).

2.5.2 Baculovirus-insect cell culture system

During wild-type infection of a living cell by a metabolically inert virus particle, the protein-based viral coat interacts mechanistically and in a highly specific manner with the cell's surface. This interaction is generally mediated through specific cell-surface receptors which act in a lock and key fashion directing viral binding and entry of viral hereditary material into the cell. This hereditary material, which may be DNA or RNA-based, is then taken up by the cell where it co-opts the cell's protein transcription machinery into producing not the peptides of host cell origin, but rather the various peptides making up the original virus. Simultaneously, while viral peptides are folded and combined into virus particles, viral DNA/RNA is replicated and uses the cell to produce functional virus particles capable of infecting new cells. During the final stage of intracellular exponential production of virus particles, known as viral lysis, the metabolic energies of the cell are directed toward viral protein expression. The cell is destroyed, along with its membrane, releasing the newly formed viral particles (D'Amico and Murhammer, 2009).

In this regard, not only are viral promoters extremely strong initiators of host transcription, but the lock and key process of gene introduction into the cell by the viral coat is a discrete process. Even though gene insertion is strictly dependent upon viral coat conformation, the genes inserted can be of any origin. More specifically, recombinant viruses allow for cell surface recognition, as would occur with the native strain, but now genes of non-native viral origin are inserted into the host cell. Thus, recombinant viral technologies are pivotal in facilitating industrial heterologous protein production. Members of the *Baculovirus* genus are now routinely used together with cultured insect cells or even larvae as their primary host cell type (Berger et al., 2004; Kost et al., 2013).

The family *Baculoviridae* comprises 68 species, falling into 4 genera. These viruses have been shown to natively infect some 600 hosts of the phylum Arthropoda, but specifically of class Insecta. This relatively broad host range, resulting from a conserved clathrin-mediated endocytosis mechanism, allows for an ideal vector for rDNA (Wang et al., 2014). It has been demonstrated that mammalian cells can also be transfected with baculovirus rDNA, although viral replication is not possible, and requires a low pH (Kost et al., 2013). Additionally, this highly conserved clathrin residue permits not only whole larvae infection and transient expression, but also infection of de-differentiated insect cell cultures, such that the production process can be done in sealed bioreactors.

A major limitation of this expression system is the need for the production of infectious recombinant viruses. Though this is a safer process than working with mammalian viruses, this is technically demanding and requires the infection and lysis of relatively expensive insect cell cultures to produce the recombinant virus prior to transient transfection for heterologous protein production (Wang et al., 2014).

2.5.3 Yeast expression systems

Yeasts are some of the most successfully used organisms for heterologous protein expression. The most efficacious yeast-based expression system is the single celled species, *Pichia pastoris* (recently renamed *Komagataella phaffii),* with more than 500 proteins having been expressed in this system. *P. pastoris*, closely related to Brewer's Yeast (*Saccharomyces cerevisiae*), is one of the most scientifically characterized species, allowing for easy and predictable genetic manipulation (Cos et al., 2006). *P. pastoris* can be grown in continuous fermenters at high biomass concentrations with the addition of methanol as the sole carbon source. This gain-of-function methanotrophic anabolism by GMO *P. pastoris* acts as a highly efficient continuous selection mechanism in maintaining heterologous expressing individuals, and also prevents contamination of the culture medium by exogenous biology, due to methanol's general toxicity. As the gene alcohol oxidase (AOX) is highly upregulated during methanol metabolism, oftentimes making up 30% of total soluble protein (TSP), this can be used as a transcription tether to drive heterologous gene transcription rates (Cregg, 1999) .

Further benefits of the use of *P. pastoris* as an expression host are its ability to produce high levels of foreign proteins either intracellularly, or as secreted proteins. This species is also able to perform various post-translational modifications (PTM) necessary for eukaryotic protein function. These include glycosylation, disulphide bridge formation and proteolytic cleavage. Of major concern however, this system tends to hyper glycosylate peptide products (Lowe, 2001).

2.6 Existing and prospective commercial value of plants as heterologous expression systems

All sectors of the US$ 10 billion dollar protein market have reported growth in larger more complex molecules compared to smaller ones (Goodman, 2009). This is due in part to two developments. Firstly, as researchers gain a greater understanding of human biology, so does the need arise for the development and production of personalized small run therapeutics, known as pharmacogenomics; produced for the treatment of individualized pathophysiology. This will become increasingly common as expression systems become more efficient and disease mechanisms are better correlated to their underlying genetic and metabolic causes. Secondly, as industry accesses more complex molecules, it is being revolutionized. Currently, only a few platforms have been developed sufficiently for their implementation as industrial heterologous protein expression platforms. Though these platforms perform very well with certain applications, the limitations intrinsic to each system and the often unique requirements for production of each new heterologous protein warrants the development of new heterologous expression systems (Merlin et al., 2014).

Exploiting the power of plants delivers many solutions to the challenges hindering the production of cheap, high quality heterologous proteins quickly and with a high degree of genetic control, as well as a high intrinsic safety index.

The complex nature of the eukaryotic plant cell can perform all the necessary requirements for producing even the most complex of protein structures. For example and of specific importance here as VLP expression was demonstrated later, plants have been used to produce highly immunogenic virus-like particles (VLPs) in the application of mammalian vaccines. To elucidate, this involves construction of a rDNA vector for plant expression that contains only the viral genes responsible for viral protein coat formation. Once expressed, the plant cell assembles these viral protein monomers into fully formed virus particles. However, although these VLPs lack any virulence due to their lack of any heritable genetic material, they are structurally identical to the native form of the virus, thus eliciting a complete immune cascade, as well as B-cell maturation and humoral memory (Naupu et al., 2020; Scotti and Rybicki, 2013).

Several plant-produced viral vaccines are now in various stages of development and release. These include the Norwalk virus, foot and mouth disease virus, human influenza virus and the human papillomaviruses, as well as a VLP-based vaccine for SARS-CoV-2, produced by Medicargo Inc. (Franconi, 2010; Merlin et al., 2014; Moon et al., 2022; Scotti and Rybicki, 2013) The benefit of using plants to produce VLPs (or any mammalian therapeutic protein) is not only their ability to process large highly complex protein structures in the sub-micron range, but also, there are no risks of the inadvertent transmission of other viruses to the mammalian recipient, as is the case with vaccine production in mammalian or insect systems. Additionally, plant-based systems, unlike bacterial systems, do not produce lipid-based endotoxins.

In production, when a small number of platforms are primarily used for industrial applications, it is often prohibitively challenging to fulfil the requirements of either once-off production runs - as is the case with single-patient pharmacogenomic applications, or for applications requiring rapid scaling or protein volumes in the kg range. Plant systems may prove invaluable to solving this. Firstly, as higher plants can be induced into a de-differentiated state, known as synthetic plant cell culture, small scale volumetric production is possible, allowing for quick batch turnaround time and single-use product development. To add to this, one of the most diverse plant taxa, microalgae, exist natively in this single celled state, allowing for high throughput screening for industrially relevant species. In terms of requirements for large protein quantities, stably expressing crop plants can be grown using traditional means of cultivation, producing metric tons of recombinant protein at highly competitive prices.

2.7 *R. radiobacter*: a bacterial plant pathogen

Crown gall disease is a plant pathology that is of major concern to many agricultural industries, especially ones that produce stone fruit, nuts and grapes (*Vitis vinifera*). It is pervasive across numerous agricultural species. This plant disease presents as the formation of tumours, directly affecting product quality and yield. Much work was done to pinpoint the causative agent, identified as the bacteria, *R. radiobacter* in 1907 (Smith and Townsend, 1907). Although this discovery allowed for the management of this pathogen, it soon prompted interest into the study of molecular and environmental factors that may relate to tumorigenesis in higher animals. It was hoped the study of *R. radiobacter* as a causative agent of tumour formation in plants, could be used as a

model for elucidation of the complex molecular processes of tumourigenesis in *Homo sapiens* (Levin, 1919). While this was not realized, it drew much attention to *R. radiobacter* and set the foundations for the field of Agrobiology, a cornerstone methodology of heterologous protein production in plants.

The pathophysiology of gall formation in the host is characterized by induction of quiescent cells into a metabolically active state. This shift in metabolism coincides with the production, by the host, of a class of non-native atypical amino acid-like compounds, known as opines. Each strain of *R. radiobacter* induces its host to anabolise a specific opine, such that each strain ensures it is the sole recipient of this anabolic shift, securing its carbon and nitrogen source (Zupan et al., 2000). As this shift in the host's metabolic focus is both a complex process and detrimental to the host, this necessitates the need by *R. radiobacter* to elicit tight control of the host's genome and proteome. This is done by the physical insertion of a single stranded linear segment of DNA (ssDNA) into the host cell. This is known as the Tumour Inducing (Ti) plasmid, and is mediated by the recognition of the host cell via the T-complex, which leads to the structural formation of the pilli, and Ti plasmid DNA translocation (Binns and Thomashow, 1988). It was speculated soon after it was discovered that crown gall disease was actually related to the physical transfer of genetic material from *R. radiobacter*, that this very closely resembled another bacteria-bacteria interaction, known as conjugation.

This, however, was found to not be the case, with the broad host range of *R. radiobacter* being subsequently demonstrated, known as interkingdom conjugal gene transfer. This has been observed not only in higher plants, but also in mammalian cells, macroalgae and planktonic microalgae (Kunik et al., 2001; Soltani et al., 2008).

The Ti plasmid is ~250 kb in length. Only a segment of DNA of Ti plasmid that is flanked by two elements, known as the left and right border, and known as the T-DNA is inserted into the host cell. T-DNA in its native pathogenic form contains genes responsible for opine anabolism as well as factors that promote metabolic shifts in the host. As shown in Figure 3, (Costa, 1990) genes outside the left and right boarder are responsible for agrobacterial virulence allowing for generation, translocation, protection and integration of the T-DNA, as well as those that allow opine-specific catabolism by the *R. radiobacter* cell.

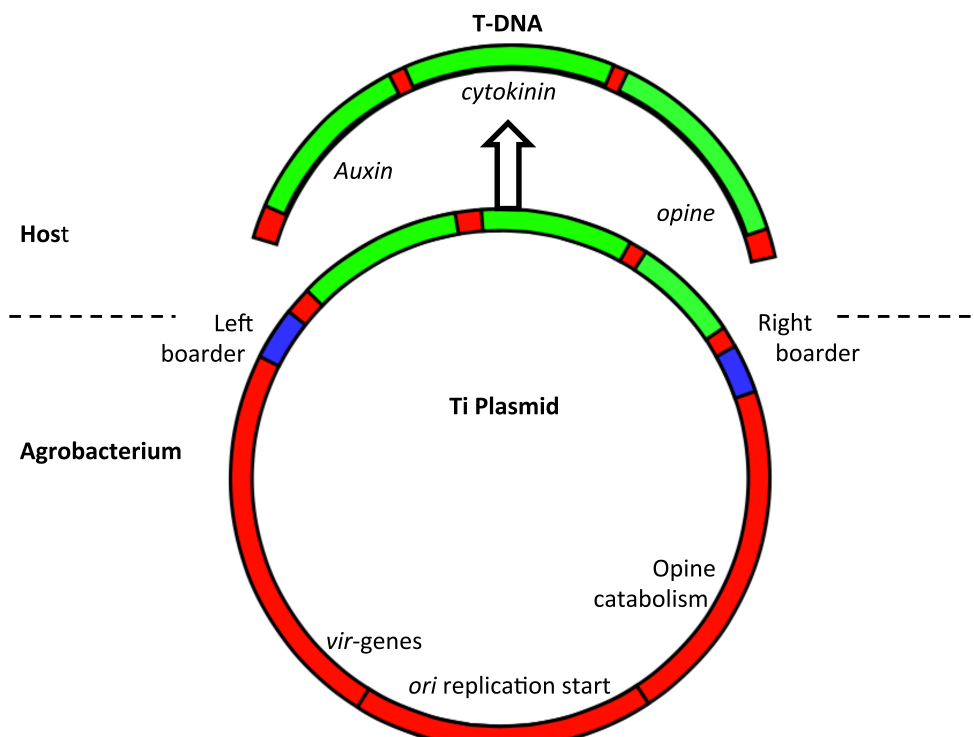


Figure 3. The Ti-Plasmid and constituent elements. The left and right boarder regions demark DNA that is translocated to the host cell versus that which is not. The T-DNA contains genes responsible for opine catalysis are fundamental to the native pathogen. Gene sequences that lie outside the left and right boarders allow for anabolism of the plant produced opines, following T-DNA translocation. Vir-Genes are responsible for initiating the process of agrobacterial virulence in preparation for transfection. These are induced via various phenolic compounds that are released by the host during wounding, and include acetosyringone, vanillin, coumarin and cinnamic acid (Cha et al., 2011).

2.7.1 R. radiobacter-mediated expression in plants

Soon after the discovery that movement of the T-DNA into the host cell was responsible for virulence, it was understood that modification of the Ti plasmid could be used as a natural vector for expression of heterologous proteins in plants. This was done by firstly removing the genetic elements that cause agrobacterial-associated pathology in the host. These are all the genes found between the left and right border regions on the Ti plasmid. These could now be replaced with rDNA from any source that encodes for heterologous protein expression.

The benefits of this system for heterologous expression include:

- The relatively large size of DNA that can be translocated, allows for expression of proteins from DNA segment lengths of up to 2kb.
- Multimeric proteins, consisting of several genes can be expressed simultaneously. Additionally, different strains of *R. radiobacter* containing different segments of rDNA can be simultaneously co-infiltrated into the same host thus expressing more than one heterologous protein in the same cell.

- Due to the large size of T-DNA, other genetic elements can be co-expressed with the production gene(s). This allows for overcoming possible hurdles to expression or low expression yields. These genetic elements include silencing suppressors, such as p19, to ameliorate the effects of post-translational gene silencing by the host, or viral-based elements that allow for T-DNA self-replication inside the host cell to increase template copy number and yield (Regnard et al., 2010; Voinnet et al., 2003)
- Through Ti plasmid modifications, T-DNA can be randomly integrated into the host genome for stable expression or inserted into areas of the genome with high transcription rates.
- Modification of the Ti plasmid allows for harnessing of the high transfection rates of this system, but without high rates of genomic integration, such that T-DNA does not integrate to a high degree, but rather translocates to the nucleus and remains as an extrachromosomal element called a nucleosome. This allows for high-level expression, but in a transient fashion.
- The turn-around time from initial plant and *R. radiobacter* biomass production to product harvesting and purification is very rapid, taking less than 5 weeks.
- The process is extremely scalable, allowing for a negligible increase of resources used in the transfection of one plant versus 10 000 plants.
- The process delivers good accuracy. Because the eventual plant biomass is stored as seeds, the genotypic, molecular and phenotypic variability can be controlled, thus maintaining homogeneity and minimizing batch variability. This also prevents any unwanted mutations from building up in the production gene(s) as is often the case within transgenic plants. Additionally, agrobacterial strains are cryogenically preserved from master stocks, such that there exists negligible strain or sequence variance in *R. radiobacter* stocks and Ti plasmids across production batches.

2.7.2 Methodological overview of transient R. radiobacter-mediated heterologous protein expression in plants

Since 1989, when a functional monoclonal antibody was expressed in transgenic tobacco to a total concentration of 1.3% of TSP (Hiatt et al., 1989), many lessons have been learnt regarding the use plants and agrobacteria for the expression of heterologous proteins. More specifically, the use of a species of tobacco, known as *Nicotiana benthamiana*, which was isolated in an arid region of Australia, has proven most useful as a transient expression host. It had been earlier discovered that *Nicotiana benthamiana* was susceptible to infection by a notably large number of viral species because resource-limited adaptive natural selection had caused it to discard much of its innate immune system. This, in combination with its ability to produce biomass relatively quickly, growing from seed to application in 4-5 weeks, and having a high leaf surface area: biomass ratio has allowed it to overshadow even *Arabidopsis thaliana*—the model organism for plant molecular biology - as a transient production host (Goodin et al., 2008). Indeed, Medicago Inc., one of the first companies to build

production-scale transient expression facilities, moved from using alfalfa (*Medicago sativa*) to using *N. benthamiana* for their transient expression production.

R. radiobacter-mediated transient expression of heterologous proteins in any system, including that of *N. benthamiana,* regardless of scaling, follows the same principal methodology. Biomass that is to be transfected is grown up to an optimally transfectable life-stage, which is determined by maximal net biomass as well as optimal cellular age for protein expression. As older plants, although larger, are less metabolically and proteomically plastic, this forms a multi-objective optimizable parameter (Wixom et al., 2018).

N. benthamiana plants are transfected when these parameters are co-optimal, typically just before the plants are 4-5 weeks old. *R. radiobacter* is grown up from cryopreserved stock cultures, and sub-cultured until a desired volume is reached depending on the mass of plant material to be transfected. Although optical density (OD) is used to determine when cultures have reached sufficient turbidity for transfection. Cell density (OD) is an optimizable parameter for transfection maximisation that is directly related to the heterologous protein being expressed (Kayser and Heribert, 2012).

Prior to infiltration, *R. radiobacter* may require induction of virulence genes that allow for pilli formation and plant cell transfection. This is achieved by the addition of various phenolic compounds; acetosyringone, a hormone released during plant wounding, is commonly used (Gelvin, 2018; Tzfira and Citovsky, 2006).

During *N. benthamiana* transfection, force is required to allow for agrobacterial entry into intracellular interstitial airspaces in leaves to promote bacterial-plant cell contact, pilli formation and T-DNA translocation. At lab scale, the force required to do this is achieved by syringe infiltrating each leaf separately. As the scale increases, this is achieved by vacuum infiltration, where whole plants are suspended in an agrobacterial solution under vacuum. When the vacuum is released, the agrobacterial solution is pulled into leaves.

T-DNA movement occurs during the first 24-36 hours after infiltration. Subsequent mobilization of the plant's protein transcription machinery is dependent on the protein being expressed along with the cell type being used. *N. benthamiana* biomass is generally harvested between 3-7 days post-infiltration.

Once predetermined maximal heterologous protein yields have been expressed, leaves are harvested and homogenized with a buffered solution at which stage downstream processing may take many forms depending on final product identity, specification and application (**Figure 4**).

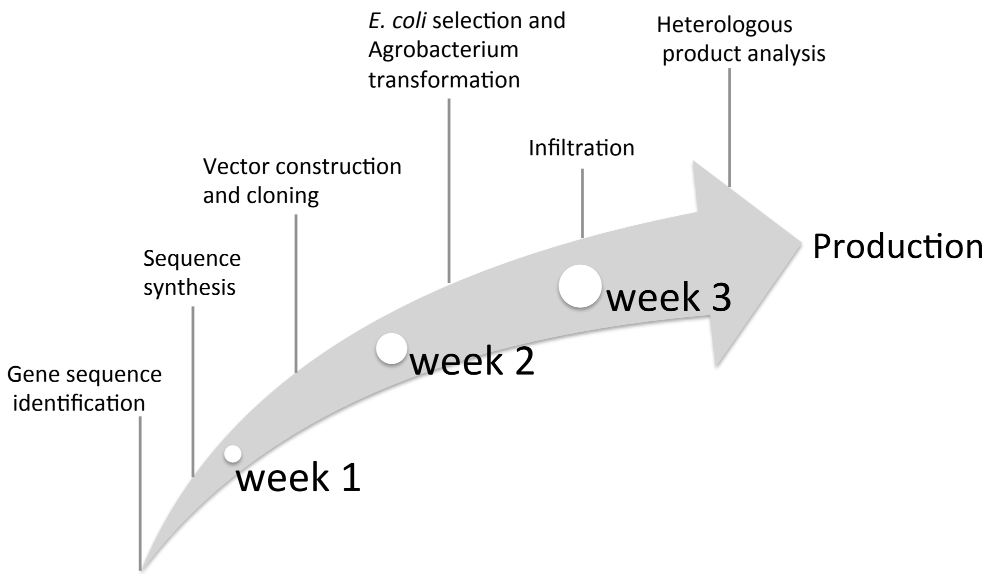


Figure 4. Product development pipeline of transiently expressed proteins in plants. (Adapted from Kayser and Heribert, 2012).

2.7.3 Overview of relevant plasmid vector backbones for transient heterologous protein expression

The transient expression of desired genes in plants is made possible by a class of plant expression vectors called pTRA binary vectors. These vectors were developed from earlier binary vectors, which were initially applied to stable genetic engineering of plants. Outside of the circular plasmid's left and right border regions derived from the *R. radiobacter* Ti plasmid, all the vectors in this collection have similar components in common. These areas enable plasmid vector replication in an *E. coli* carrier and, as mentioned previously in this thesis, are not introduced into the plant cell.

The cloning site, which includes the restriction enzyme recognition sites AFL III and Xho I that permit the cloning of an expression gene into the plasmid, is one crucial component of pTRA vectors. The fundamental pTRA vector also includes an *E. coli* origin of replication and a gene for bleomycin phosphotransferase resistance. This enables the choice of transformed *E. coli* and the plasmid's duplication before being transferred to *R. radiobacter*. In contrast, the pTRAkc and pTRAkc ERH vectors have a neomycin phosphotransferase gene that is located within the left and right borders, enabling transformed plant cells to break down kanamycin and geneticin G418 and produce GMO plants and/or cell lines (**Figure 5**)(Rybicki, 2014, 2009; Williamson et al., 2013). Additionally, they feature a histidine tag that adds six histidine residues to expressed proteins. If a primary antibody is not available, this tag can be utilized for western blotting. The ERH vector also includes a P35S element that directs the expressed protein mRNA to the endoplasmic reticulum.

Neomycin phosphotranspherase is likewise included in the pTRA KC RBCs1 CTP vector to allow for stable GMO transformant selection. The RBCs1 region allows for directing of the mRNA to the chloroplast for translation. Each of these vectors carries a single copy of the plasmid into the cell of the host plant (**Figure 5**)(Williamson et al., 2013).

Based on the pTRA vectors, the pRIC vector has a SAR area that enables insertion of the pRIC replicon. This replicon permits self-replication, which raises the number of gene copes, creating more templates for increased mRNA transcription and thus greater protein yields (**Figure 6**)(Chen and Lai, 2015; Regnard et al., 2010).

The pCAMBIA 1301 vector is part of the pCAMBIA open access vector suite. This vector contains the kanamycin resistance gene for *E. coli* selection, as well as two genes: hygromycin B phosphotransferase (HygR) for selection of genetically modified plant strains via gain-of-function resistance to the eukaryotic toxin, hygromycin, as well as the GUS (β-glucuronidase) gene. This allows for the conversion of X-Gal (a colourless substrate) to a blue product for detection of expression. Both of these genes are under the control of the cauliflower mosaic virus promoter 35S. The pCAMBIA vector suite is commonly used for transient expression investigations in various plant and algal species, with the presence of blue cells in relation to the total population allowing for efficient gauging of the degree of transient expression and parameter optimization (**Figure 7**).

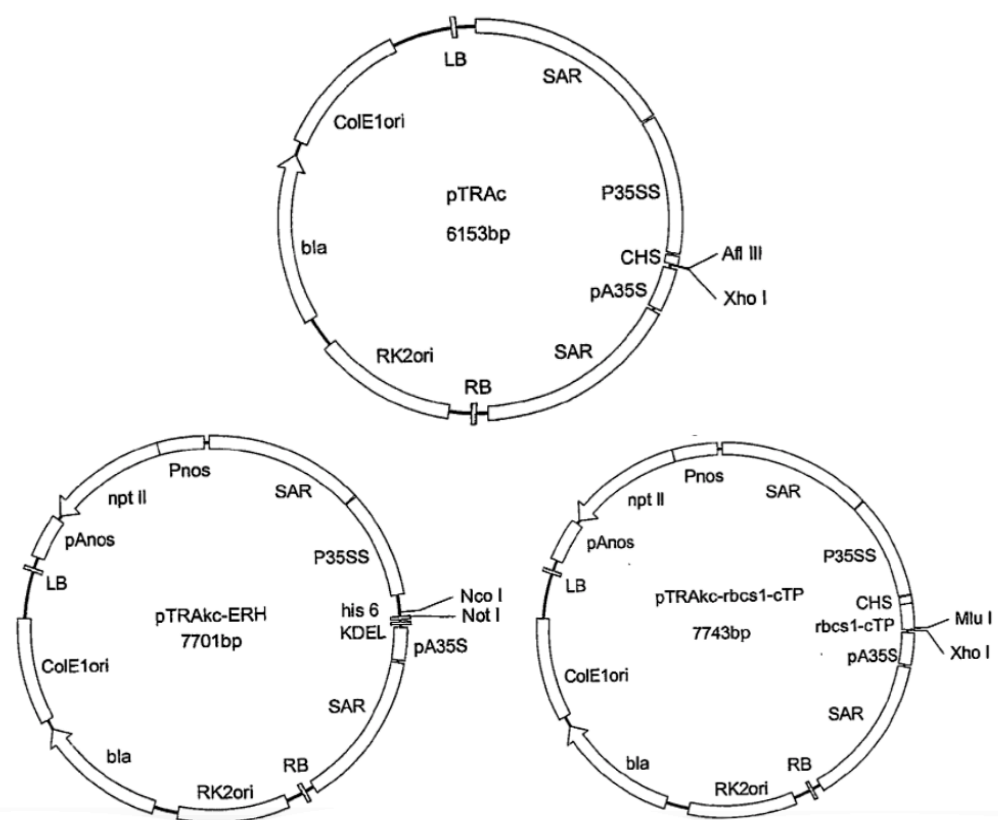


Figure 5. Binary vector maps; pTRAc, pTRAkc-ERH and pTRAkc-rbcs1-cTP (Williamson et al., 2013)**.**

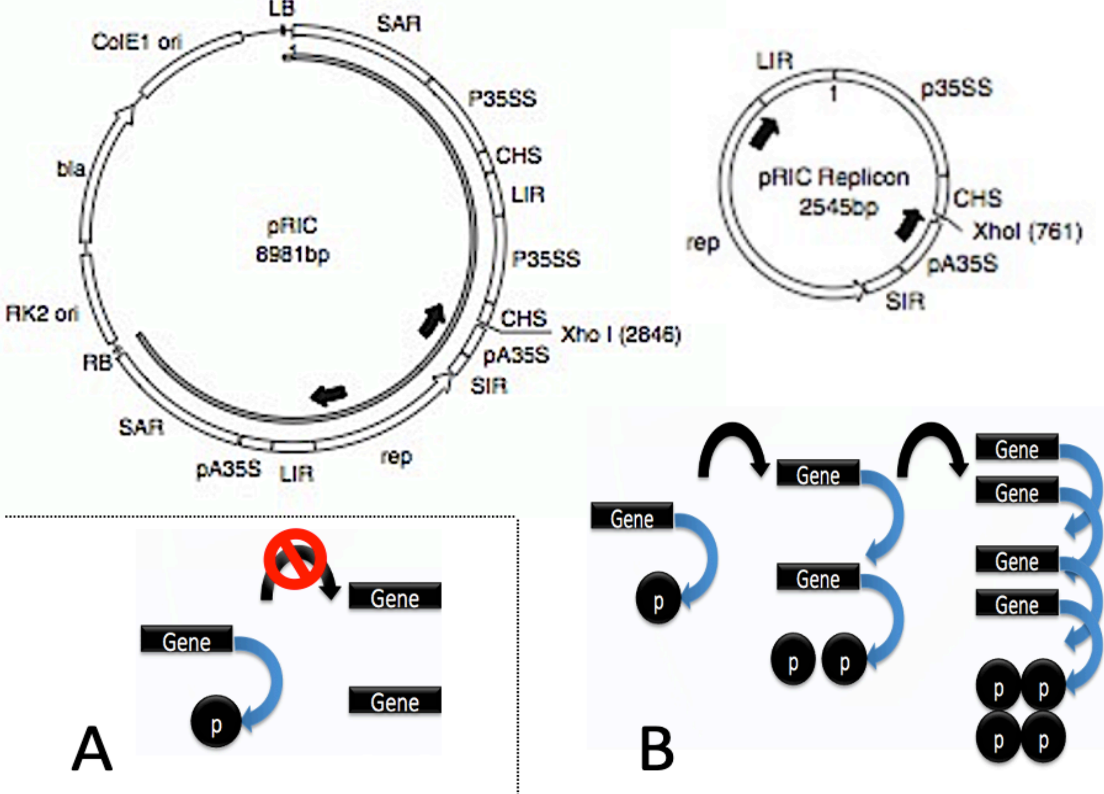


Figure 6. pRIC vector map and diagrammatic representation of self-replication for increased gene copy number(B), resulting in greater protein (P) expression, relative to non-replicating vectors, such as pCAMBIA and pTRAc (A) (Regnard et al., 2010)**.**

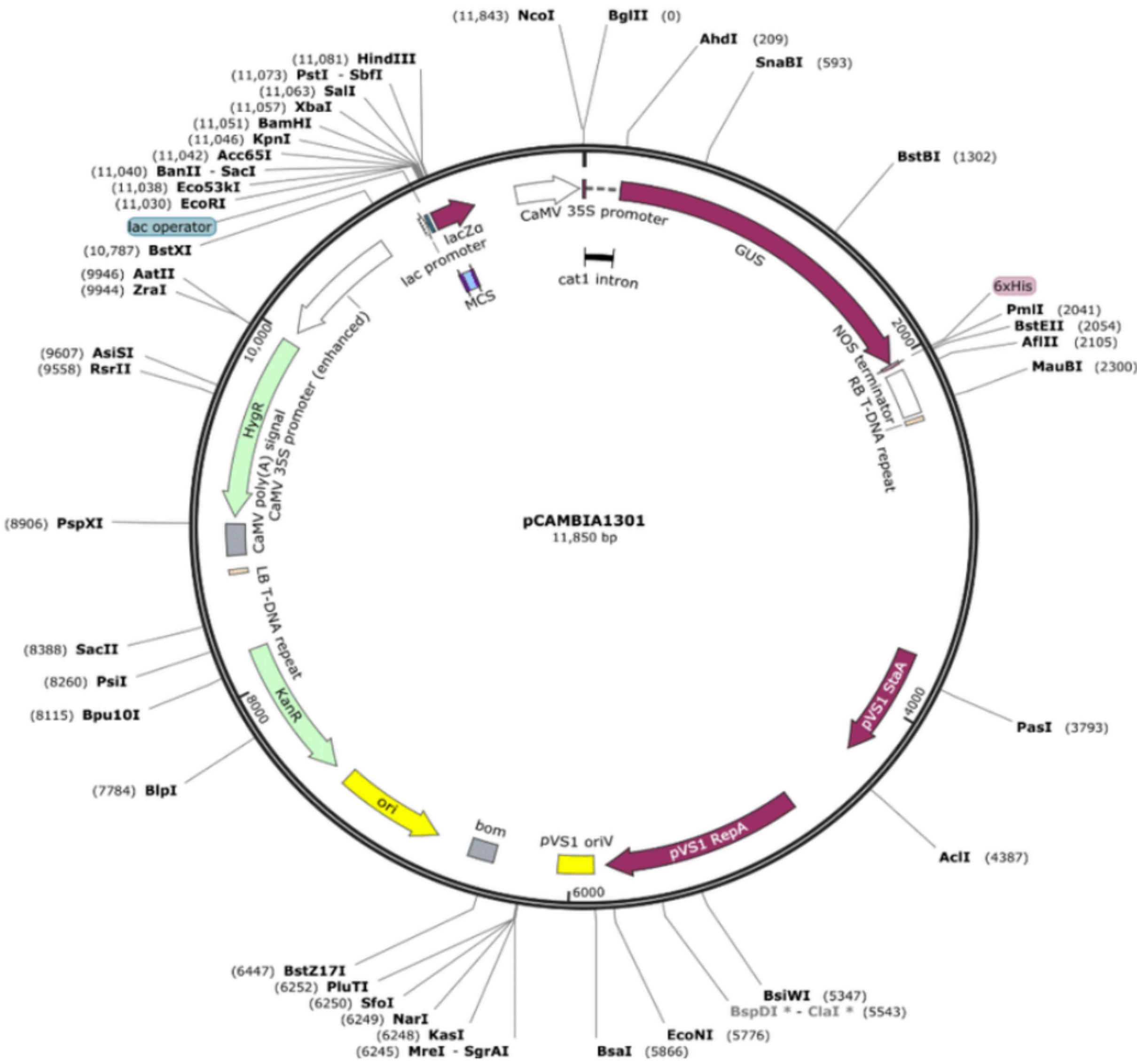


Figure 7. pCAMBIA1301 vector map. Gene products in transcriptional directional arrows (protein products). Labels indicate restriction enzyme binding sites. (created using SnapGene www.snapgene.com).

2.7.4 Large-scale *R. radiobacter*-mediated heterologous protein production in plants

The US Defence Advanced Research Projects Agency (DARPA) and the Blue Angel Program funded four programmes to develop agrobacterial-mediated expression of proteins in transient plant systems; namely, Medicago, Fraunhofer CMB, Caliber Biotherapeutics and Kentucky BioProcessing. Each of these facilities has had a specific mandate and was tasked with demonstrating key aspects of the application of agrobacterial-mediated transient plant expression to manufacture vaccines and biotherapeutics rapidly in response to epidemics, outbreaks and seasonal infections (Holtz et al., 2015) Of special importance regarding the recent emergence of three global novel coronavirus outbreaks, are radiobacter-mediated transiently expressed

Covid vaccines showing great promise in preventing and controlling this novel coronavirus (Shanmugaraj et al., 2021).

Medicago Inc. was established in 1999. Since then, the company was listed publicly, securing its financial capacity that has allowed for the successful large-scale production of three monoclonal anti-Ebola Zaire antibodies (ZMapp) in plants. This antibody cocktail was originally produced by Merck, using Vero cells of African green monkey (*Chlorocebus sp*.) origin. Medicago Inc. has also demonstrated its ability to produce 10 million doses a month of the H5N1 influenza virus VLP vaccine. Its quadrivalent seasonal influenza vaccine is licenced by Canadian authorities, as was its SARS-CoV-2 VLP-based vaccine (Laere et al., 2016; Rybicki, 2014; Ward et al., 2021). Medicago Inc. however ceased operations due to funding cuts in 2023 by their parent holding company, Mitsubishi (Benvenuto et al., 2023).

Fraunhofer CMB (Newark, Germany) hosted a pilot facility to demonstrate initial process feasibility of protein expression in plants. The scale of their process is 100 kg raw plant biomass per batch.

Caliber Biotherapeutics (Bryan Texas, USA) and **Kentucky BioProcessing** (Owensboro Kentucky, USA) have built commercial facilities, with a focus on automation and containment, to implement a process known as G-Con. G-Con allows for rapid batch changeover, such that multiple downstream process trains could be safely implemented. These facilities utilize hydroponics to produce plant biomass. This minimizes any possibility of contamination by soil and is built to withstand unforeseen events such as earthquakes and hurricanes. Process scale is 3500 kg of plant biomass per week.

At **Caliber Biotherapeutics**, G-Con manufacture in its cGMP facility follows the hazard analysis and critical control points (HACCP) protocol to minimize the risk of back-contamination of the facility. Additionally, modular or pod-based design and construction of this facility allows for expansion of various pods as the need arises. The plant growth and germination rooms, which currently allow for the processing of 3500 kg of biomass per week, can expand to allow for the processing of 7000 kg of plant biomass per week. Each concrete pod was constructed off-site, and once on site were then fitted with the various requirements for each in a pod-by-pod fashion (**Figure 8**). This allowed for the implementation of the whole facility, from initiation to functionality in which it produces 3500kg of plant biomass or 1 kg of purified product (haemagglutinin antigen, HA) per week, to be achieved in less than18 months. This encapsulates the core objectives set out by the Blue Angel Programme (Holtz et al., 2015), which in general terms involve, commercial therapeutic protein production of 1kg/week, to be achieved in less than 18 months, using a plant system with a production facility that is hardened, self-sufficient and provides a high degree of containment.

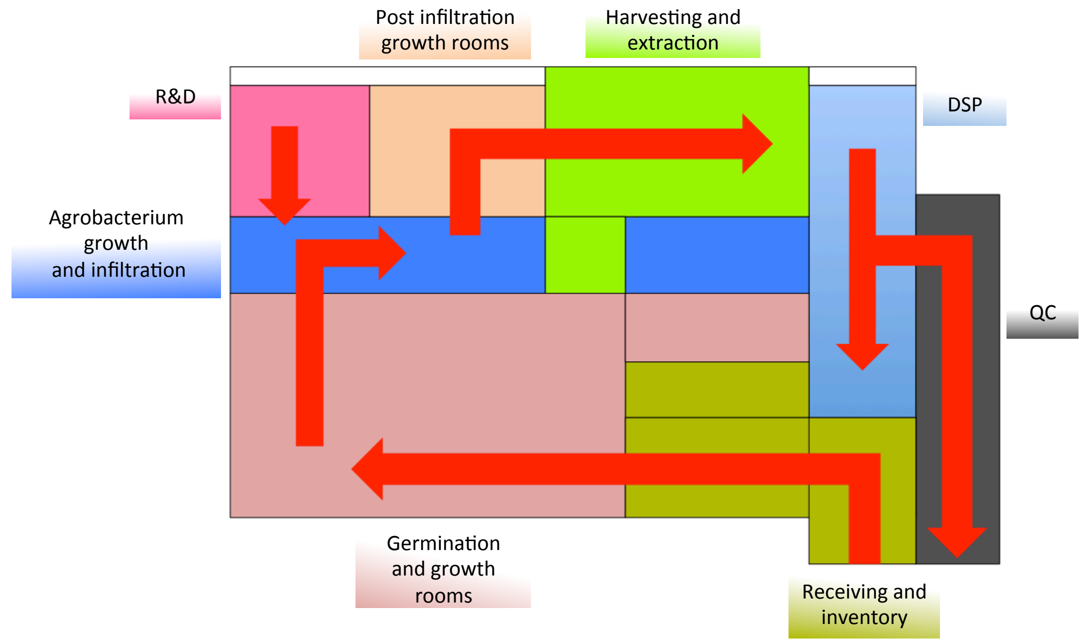


Figure 8. Caliber process flow and floor plan.

2.8 Microalgae

2.8.1 Introduction

Microalgae (Microphylae) are a group of highly diverse protists, that inhabit diverse ecological niches ranging from marine to freshwater to terrestrial environments, and even display pathogenic or symbiotic associations with other species, such as molluscs, protozoa, flatworms and fungi (Corliss, 2002; Van Etten et al., 1991). Humans have not escaped this evolutionary trajectory. The pathogenic microbe responsible for malaria, *Plasmodium falciparum*, has its evolutionary origins as a microalga (Waller, 2000). Although several thousand microalgal species have been cultured to date, this is a small fraction of the proposed several million species that exist in nature (Pulz and Gross, 2004). Microalgae also possess the highest genetic and biochemical diversities of all eukaryotic organisms. This makes microalgae a priority target for research and industrial investigation. They are also at high risk from anthropogenic climate change and habitat loss, with several microalgae species having now been declared extinct (Brodie et al., 2009).

Microalgae are split into various taxa. These include Cyanobacteria, Chlorophyta, Dinoflagellates, Rhodophyta, Bacillariophyta and the Diatoms, amongst others, with more than 50 species having had their genomes sequenced. They are likely to provide solutions to allow for the progression of humanity through the vehement exploitation of their very diverse properties and products.

2.8.2 Microalgal cultivation

Many microalgal species have only simple substrate requirements for growth and reproduction. These include a carbon source, (CO_2), a light source, a nitrate and

phosphate source and trace elements in the form of simple salts. Although most species are photosynthetic, many may be grown in the absence of light, heterotrophically or may grow mixotrophically. *Chlamydomonas reinhardtii,* for example, can be grown in the absence of light with the addition of tris-acetate-phosphate (TAP) to the growth medium; this provides enhanced growth rates compared to autotrophic conditions. Similar results were found in the growth rates of *Spirulina sp.* when grown with the addition of glucose to the medium (Chojnacka and Noworyta, 2004; Kumar et al., 2004). These simple requirements for growth make microalgae a very attractive option for the production of numerous economically relevant and naturally produced chemicals, such as β-carotene, alginic acid, carrageenans; oil for use in biofuels, as well as proteins for the food and pharmaceutical industries.

Although photosynthetic growth is easily accessed, this means of production has some critical limitations. Biomass productivity in enclosed photobioreactors is directly related to light exposure, which quickly becomes a self-limiting process as scaling or cell densities are increased. This is firstly due to cell shadowing, where cells proximal to the culture surface prevent photonic penetration to distal cells, preventing photosynthesis in those cells. Secondly, as culture volumes are increased, typically the ratio of surface area to volume decrease. Maximal microalgal biomass concentrations using photobioreactors are often economically prohibitively low for low value products, with upper limits of <4 $g.L^{-1}$ (Ugwu et al., 2008). Additionally, past a critical level, extreme photon densities inhibit the growth of all microalgae, thus adding a further confounding parameter (Wang et al., 2012).

Other options include growing the biomass in open ponds or in raceways. Due to a lack of containment, this allows for the risk of environmental contamination. Predatory organisms that reduce biomass, and toxin-producing microbes, as was found in commercially available *Spirulina platensis,* are examples of such contamination.

Prospects for economically viable yields of recombinant proteins from microalgal biomass rely upon their production in closed bioreactors, both for containment and to avoid contamination. Owing to constraints on light provision due to surface area to volume ratios decreasing on scale as well as the need already in place to use axenic culture methods for heterologous protein production, ability to grow in the absence of light is considered a key parameter. Here, a metabolic shift from CO_2-dependent photosynthesis to oxygen-dependent respiration must take place. The shift to oxygen-dependent respiration has been found to increase biomass concentrations by as much as 25-fold (Morales-Sánchez et al., 2017). Indeed, *Crypthecodinium cohnii*, a dinoflagellate microalga, was grown to a biomass concentration of 109 g. L^{-1} (dry biomass) in continuous culture on a feedstock of ethanol (De Swaaf et al., 2003a; De Swaaf et al., 2003b).

Currently, glucose, glycerol and acetate are the most widely used heterotrophic feedstocks. However, it is a reasonable conclusion that all microalgae, due to their passivity in their environments and so not always able to photosynthesize, must have some sort of dormancy mechanism or heterotrophic ability. As more microalgal species are sequenced and so-called molecular toolkit become more complete, so too

are heterotrophic pathways being revealed (Perez-garcia et al., 2010)(Lin, 2005), but also the ability to engineer, through transgenesis, novel pathways into industrially relevant species that lack obvious respiratory mechanisms. It was recently discovered that giant viruses of the *Mimiviridae* family that infect the marine algae, *Tetraselmis sp.* (*Chlorodendrophyceae*), encode key fermentation gene cassettes that allow for oxidative respiration of infected algae which results in a massive increase in growth rates and the development of blooms (Schvarcz and Steward, 2018a).

This being said, there is an application for both photosynthetic and respirative growth. At lab scale, photosynthetic growth allows for strong positive selection and senescence of microalgae, thus cultures can be maintained for long periods of time without reculturing, and without a high risk of bacterial contamination. This form of culture maintenance is however often performed using semi sterile techniques, which come with their own set of risks. It has been found that several microalgal species are capable of becoming readily airborne while retaining viability. This poses a major risk for cross-contamination of microalgal cultures in research facilities that lack sufficient storage or have proper culture maintenance procedures (Schlichting, 2012).

Additionally, longer generation times result in a decrease in the effects of natural selection, thus model strains in different labs will have fewer genetic branches. Although highly productive, respirative growth can be highly contaminable due to its enriched media requirements. Currently, this makes the use of heterotrophic growth of microalgae only viable for high value compounds and urgently requires novel as-yet undeveloped cost reduction strategies. Its application however, is demonstrated by the world's leading algal-based omega-3 fatty acid manufacturers (Martins et al., 2013).

2.8.3 Microalgae and recombinant proteins

Proteins produced in plants are cheaper, safer and generally easier to produce than in any other organisms. Proteins produced in plants are as much as four orders of magnitude cheaper than those produced in mammalian systems (Miyake-stoner and Mayfield, 2010). Additionally, as microalgae do not contain mammalian prions, transposons or viruses, use of this system is safer than mammalian or insect cells. Several species have been granted GRAS (Generally Recognized as Safe) status by the Federal Drug Administration (FDA). Microalgal species with GRAS fall under the genera *Chlorella, Scenedesmus* and *Dunaliella,* as well as *Chlamydomonas*.

Although having GRAS status is a clear metric of safety, it does have knock-on effects relating to cost (Khosroushahi, 2014; Miyake-stoner and Mayfield, 2010). The initial product development pipeline is much shorter, and clinical trials are much cheaper, but this also has a direct impact on the requirements for downstream processing. Several studies have demonstrated the efficacy of oral vaccinations. Transgenic microalgae expressing a vaccine are administered orally as food for people. This type of delivery elicits a powerful immune response and also effectively eliminates the need for any downstream processing because protein purification, removal of unwanted harmful proteins or vaccine formulation is not necessary. Furthermore, food administered vaccines remove the need for trained medical personal to handle and

administer vaccines which could have far reaching implications in resource limited places (Specht and Mayfield, 2014). All this being said, it is often very difficult to predict expression, uptake and antigenicity of recombinant vaccine antigens. Microalgae, which allow for the possibility for high-throughput screening, are ideal for rapid efficacy determination (Rybicki, 2009).

Although microalgae, and especially *Chlamydomonas reinhardtii* which is the algal species the most explored for transgenic protein production, show much promise for heterologous protein production, several hurdles still exist. These drawbacks related to low heterologous protein yield include:

- Positional Effects (PE) associated with random insertion of the transgenic sequence into the genome of the cell (Potvin and Zhang, 2010). In this random process, either genes are insufficiently translated or may integrate into gene sequences that are essential for cellular function, thus creating a selection bias for low producers.
- Post-Translational Silencing (PTS) associated with the protection of algal cells from viral infection, specifically target heterologous gene sequences, preventing their translation into protein products (San et al., 2012; Zhao et al., 2007).
- Epigenetic gene modification, whereby genes are either transcriptionally silenced or upregulated. Here the specific sequence of addition of methyl groups to a gene sequence influence whether the gene is recognized as part of the cells genetic material or of foreign origin (Kong et al., 2014; Neupert et al., 2009).
- Transgenic protein-associated cellular toxicity, where cells that do produce large quantities of transgenic protein are killed through metabolic stresses, selecting for lines that are less efficient producers but are viable (Surzycki et al., 2009).

2.8.4 *Chlamydomonas reinhardtii* and heterologous protein expression

C. reinhardtii, the best studied microalgal species to date (Rasala and Mayfield, 2014), has had all three genomes fully sequenced – that is, the nuclear, chloroplastic and mitochondrial genomes (Mayfield and Franklin, 2005). Indeed, more than 100 microalgal species have now had their genomes sequenced (Nelson et al., 2021). This is a pioneering step toward unlocking the potential of using microalgae for producing heterologous proteins as it allows for the development of much needed molecular tools to overcome the challenges of comparably low protein yield.

Chlamydomonas reinhardtii has been worked on extensively with regard to the modification of its genetic makeup, resulting in the most comprehensive molecular toolkit available for any microalga. This is in part due to it having become the model organism for microalgal gene expression and also for the study of the mechanisms of photosynthesis. *C. reinhardtii* also demonstrates several other advantages as a biomass for transgenic protein production. These advantages include the following:

- its relatively short doubling-time, producing biomass quickly (Abreu. et al., 2022).
- the nuclear, mitochondrial and chloroplastic genomes are relatively easy to transform, containing a wide range of promoter options for the selective control of gene expression either via light as with chloroplast-associated genes or with nutrient, chemical or physiological induction of nuclear-associated genes (Rasala and Mayfield, 2014);
- the flexibility of being autotrophically or heterotrophically culturable (Abreu. et al., 2022);
- strains are easily maintained, and due to their haploid genomes, outcrossing of modified strains is done with ease, facilitating the creation of double or multiple mutant strains(Doron et al., 2016);
- the ability to produce secretable proteins (Rasala et al., 2012) and
- the ability to quickly and cost effectively upscale from a few litres in the laboratory to ponds or bioreactors for industrial application (Gómez et al., 2013).

The methods used all rely upon the formation of stably expressing GMO cell lines, with transiently expressing cell lines and/or colonies being seen as a methodological failure and/or by-product.

The microalga consists of a single large chloroplast that contributes some 40% to each cell’s volume. This organelle consists of a single circular DNA molecule containing approximately 200kb, with as many as 80 copies of each gene. Of high importance in the expression of heterologous proteins from the chloroplast, is codon bias, where more than 80% of nucleotides in the third position have been found to be an Adenine or Thymine which, when sequence optimised, has been shown to result in an 80-fold increase in heterologous protein production (Mayfield and Franklin, 2005).

Several methods now exist for the genetic modification of *C. reinhardtii.* These are split into two groups: those that modify the nuclear genome and those that modify the chloroplastic genome, with discrete advantages and disadvantages to both detailed in **Figure 9**.

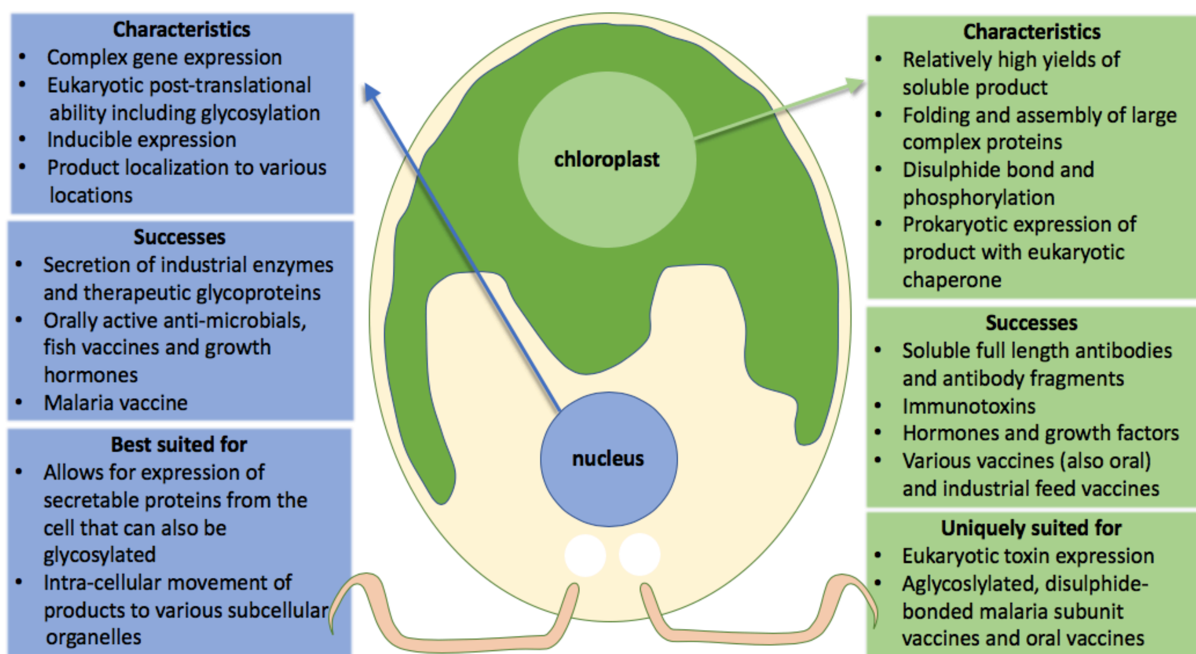


Figure 9. *C. Reinhardtii* and Heterologous Proteins. A comparison of transformation of the nuclear and plastid genomes of *C. reinhardtii, and* their benefits, drawbacks and successes already achieved with each (Mayfield et al., 2007; Miyake-stoner and Mayfield, 2010; Rasala et al., 2012)**.**

2.8.5 Chloroplast genome modification in *C. reinhardtii*

Various methods have now been developed for the genetic modification of the chloroplastic genome of *C. reinhardtii.* These include biolistic bombardment, naked rDNA agitation with glass beads and silicon carbide whiskers as well as electroporation. The last two methods often rely upon algal strains deficient in a cell wall to allow for DNA insertion. All three of these methods rely upon the process of homologous recombination (Miyake-stoner and Mayfield, 2010). During homologous recombination, transgenes are flanked by sequences identical to ones already present in the chloroplast, such that these are aligned, and replace endogenous DNA. This process can be accomplished in as little as 6 weeks and benefits from having a high protein transcription rate due to the chloroplast having low levels of proteases, which degrade protein resulting in decreased productivities. This method does not however allow for cellular export of heterologous proteins due to there being no plastid export machinery. Proteins are transcribed in a prokaryotic manner, but the chaperone system is eukaryotic in nature. Thus disulphide bond formation, phosphorylation and eukaryotic protein folding is possible (Rasala and Mayfield, 2014).

2.9 Chloroplast-expressed heterologous protein products in C. reinhardtii: key advances

2.9.1 Vaccines

There are several types of vaccines, including whole virus or bacteria- based vaccines, protein-based vaccines that simulate a viral coat protein, or mRNA vaccines that provide the potential for the cell to produce these antigens itself (Schlake et al., 2012). The fundamental function of a vaccine is to stimulate an immune response in the immunized organism that is preferably stable and long lasting. More traditional vaccines contained either killed or attenuated whole viruses or bacteria, which would be recognized by the host's immune system similarly to the live organisms, as well as an adjuvant to enhance the immune response if the killed or attenuated pathogen did not stimulate a sufficiently strong immune response (Mak et al., 2014).

Modern protein-based vaccines, where possible, rather contain coat or subunit proteins that stimulate an immune response: salient examples are the hepatitis B and human papillomavirus vaccines produced as virus-like particles (VLPs) in yeast. These proteins are produced not in the native pathogenic organism but instead in a heterologous protein transcription system, as discussed above, where they are transfected with the gene responsible for the production of the protein. The protein can then be harvested and purified for vaccine formulation and immunization, or in the case of oral vaccines, whole cells that contain the immune-active protein of interest may be administered orally (Specht and Mayfield, 2014).

Oral vaccines have several benefits over traditional vaccines, in that they do not require subcutaneous injection and the associated risk of the spread of infection, alleviate stresses during immunization, especially in livestock, they are less intensive and more cost effective to produce, though a lower degree of humoral immunity is elicited, favouring instead a mucosal, epithelial or cellular type response.

As an example of production of a heterologous protein-based vaccine in a microalgal system, *C. reinhardtii* was transfected with the gene p57 from the bacterium *Renibacterium salmoninarum,* one of the most devastating farmed salmonid pathogens, causing Bacterial Kidney Disease. Juvenile fish were then either immersed in a medium containing the p57-transformed *C. reinhardtii* or fed the freeze-dried transgenic microalgae. It was found that depending on the route of administration of the antigen, different immune responses were elicited. Feeding of antigen formed blood-activated circulating antibodies to the pathogen, while individuals that were immersed in the live algae produced mucousal salivary and epithelial IgM (Siripornadulsil et al., 2007).

2.9.2 Immunotoxin antibody-conjugates as cancer therapeutics

A very appealing direction for the development of novel therapies for the treatment of human disease, namely cancer, is the possibility of highly selective delivery of apoptosis-inducing toxins to specific cells. This selection, easily achieved by the recognition and binding of an antibody to a cancer-associated cell surface receptor,

could foreseeably allow for the destruction of very specific cell lines that display target cell surface receptors, while leaving all other cell lines unaffected. This results in a “silver bullet” approach for the selective destruction of tumours, for instance. This also results in minimal or no toxicity to cells or tissues not bound by the immunotoxin-antibody conjugate. A similar approach is used for the targeting of cells for administration of therapeutic rather than apoptotic agents. With immunotoxins, this form of highly directed cellular destruction utilizes the ability of antibodies to recognize extremely diverse epitopes in a highly selective manner, with it being possible to design and direct antibody recognition and binding to very nearly every protein domain and therefore any human cell type, provided that the specific epitope is available to the antibody.

Although the idea of using targeted protein recognition of tumour cells to deliver eukaryotic toxins is not a new one, with its feasibility having been established by Shen et al. (1988) some twenty years ago, there remained some physiological hurdles to their production. Due to their complex tertiary structure, heterologous proteins for targeted protein recognition of tumour cells could not be produced in *E. coli*, which cannot fold multiple domains or introduce protein disulphide bonds into proteins. Further, these proteins could not be produced in *S. cerevisiae* or in insect and mammalian cell lines such as Chinese Hamster Ovary (CHO) cells due to the intracellular accumulation of eukaryotic toxins, often targeting eukaryotic transcription.

To overcome this, CHO lines were used to produce the antibody domain, which was then fused to the toxin domain produced in a pathogen line, such as Exotoxin A from *Pseudomonas aeruginosa,* or Gelonin from *Gelonium multiflorm.* However, this method is very costly as there are added processing steps, and there is more of a risk of introducing a pathogen into the immunotoxin.

It has now been found that, while the plastid transcription machinery of *C. reinhardtii* is prokaryotic in nature and therefore unaffected by eukaryotic toxins, the eukaryotic nature of the folding machinery of the microalgae could adequately fold the immunotoxins into their functional tertiary structure, as the microalgae have eukaryotic chaperones, protein disulphide isomerase and peptidylprolyl isomerase (Tran et al., 2013). This provides a preferred route to functional immunotoxins,

For immunotoxin production in *C. reinhardtii* several approaches have been investigated which are explored below.

2.9.3 Gelonin CD22 immunotoxin therapeutic protein: optimisation of structure for maximisation of therapeutic effect

This method involves the production of a chimeric protein. A single chain antibody is produced that is fused to the eukaryotic toxin, Gelonin. The single chain antibody recognizes and binds the B-cell epitope CD22, which once bound, acts as a strong signal for cellular uptake of the antibody (endocytosis)-gelonin conjugate, which is also known as αCD22Gel. Although this is a dosage dependent process, with the effects of gelonin on the arrest of CD22 cells increasing with more delivery of αCD22Gel to each cell, the effects of this toxin are pronounced, requiring very small dosages to inhibit the translational machinery in eukaryotic cells by cleavage of the 28siRNA (Tran

et al., 2013). It was found that if the constant fragment (Fc domain) of human immunoglobulin G (IgG) was inserted between the singe chain antibody and the gelonin-encoding region, *C. reinhardtii* could be induced to produce dimeric forms of αCD22Gel, known as αCD22CH23Gel (**Figure 7**). This had the dual advantage of delivering twice the dosage of toxin per CD22-antibody recognition event, as well as increasing the shelf life of the protein extensively.

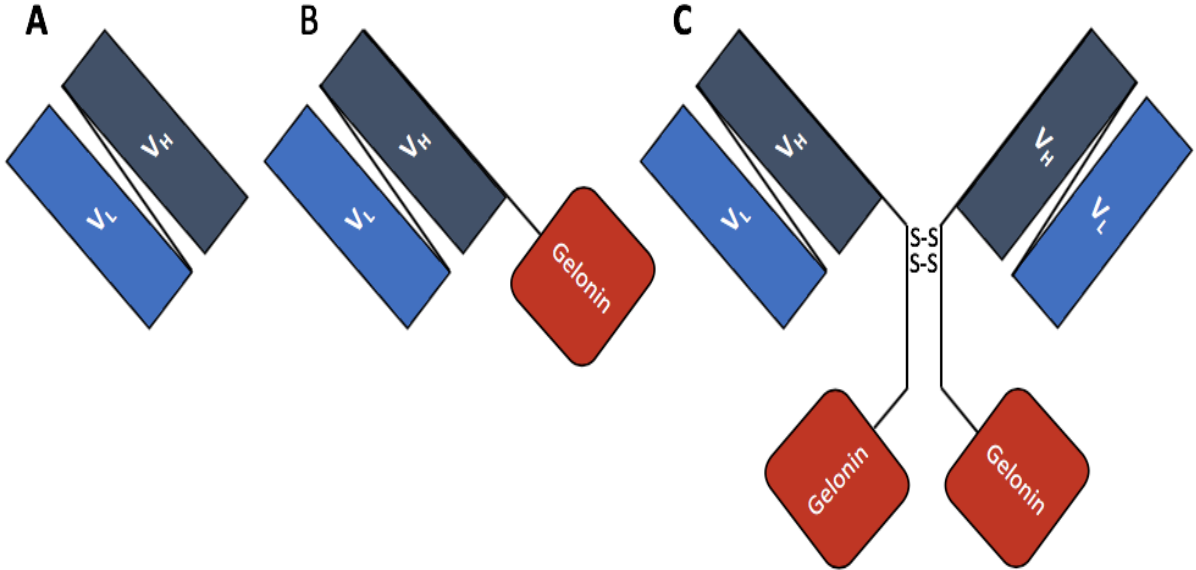


Figure 10. Three transcripts produced in C. reinhardtii. A) αCD22 Single chain antibody responsible for the recognition of CD22 B-cell epitopes. It is composed of a Variable light and heavy chain. **B**) αCD22Gel is the monomeric version of the Gelonin containing immunotoxin. **C**) αCD22CH23Gel is the dimeric version of αCD22Gel, with the inserted CH2 and CH3 domains between the Gelonin toxin domain and the antibody domain. This insertion of CH2 and CH2 allows for the monomeric association and their dimerization through disulphide bond formation (adapted from Tran et al., 2013).

The production of the αCD22Gel in the *C. reinhardtii* plastid was accomplished by transformation with biolistic bombardment of cells and the homologous recombination of transgenes into the plastid genome that are responsible for the production of either αCD22Gel or αCD22CH23Gel. In this investigation, it was found that the binding strength and specificity of the single chain antibody produced in a microalgal expression system was comparable with that of antibodies produced in live rabbits. Additionally, it was found that all plastid-produced Gelonin was sequestered to the chloroplast, with none leaving this organelle and so not causing microalgal cell death via disruption of the eukaryotic nuclear cellular machinery (Tran et al., 2013).

A similarly constructed immunotoxin has been created that combines a single chain antibody recognizing CD22, with a fusion protein derived from domains II and III from *Pseudomonas aeruginosa* Exotoxin A. This toxin, once endocytosed into eukaryotic cells, prevents the formation and elongation of polypeptide chains in the nucleus by inhibition of the eukaryotic elongation factor 2. It was also found that the creation of a dimeric form significantly enhanced protein stability (Tran et al., 2012). Of real interest,

is, not only the exciting use of a combinatorial expression approach to a bacterial-eucaryotic fusion protein, such that the eukaryotic toxin does not result in algal cell toxicity during expression, but that algal cells are able to express mammalian antibodies, with biosimilar or even bio-better binding efficiencies.

2.10 Nuclear-expressed heterologous protein expression in microalgae: key advances

2.10.1 Selection 2A product-gene tethering

The process of expression of heterologous proteins from the nucleus of microalgae relies on selection and screening of antibiotic resistant colonies for those highly expressing lines that produce the heterologous protein product, maximally. This has two major disadvantages. Firstly, the screening process is tedious. As is the case with *C. reinhardtii*, where a large proportion of resistant colonies are generated. Secondly, very little heterologous protein can be produced even though the selected lines had a high degree of resistance. This is the result of the resistance marker mRNA and the heterologous product mRNA that are transcribed as separate RNA transcripts - the product of which is differential silencing of these transcripts. And so, as cell survival in the presence of chemical selection is only reliant on selection for those lines that efficiently express selection marker mRNA, heterologous protein mRNA may be completely silenced, resulting in lines that may show a high degree of resistance to the selection antibiotic, but produce no product.

At the forefront of circumventing this differential silencing hurdle, is a novel mechanism that allows cell selection via antibiotic resistance to be directly tethered to heterologous protein production.

In this process, the selection marker gene and the heterologous product gene are located adjacent to one another, separated by a short ~60bp rDNA linker. Once the linker is translated into a 20 amino acid sequence, it has the ability to self-cleave, resulting in two functional peptides: the selection marker and the heterologous product. Thus, differential heterologous protein product mRNA silencing is bypassed. Furthermore, several selection markers, such as those conferring resistance to Hygromycin and Bleomycin, bind to their targets in a covalent manner. For every unit of antibiotic selection that a cell is exposed to, the equivalent unit of resistant marker peptide must be expressed, which is necessarily equivalent to the expression rate of the heterologous protein. Therefore, cellular silencing can be limited and, as the concentration of the selection antibiotic is increased, so are cell lines highly expressing of the heterologous protein product selected (**Figure 11**). This mechanism has been used to express several fluorescent proteins simultaneously in single lines of transgenic *C. reinhardtii* (Rasala et al., 2013), as well as the production of cell lines capable of excretion of the industrially relevant enzyme, Xylanase, into the culture media (Rasala et al., 2012) to great effect.

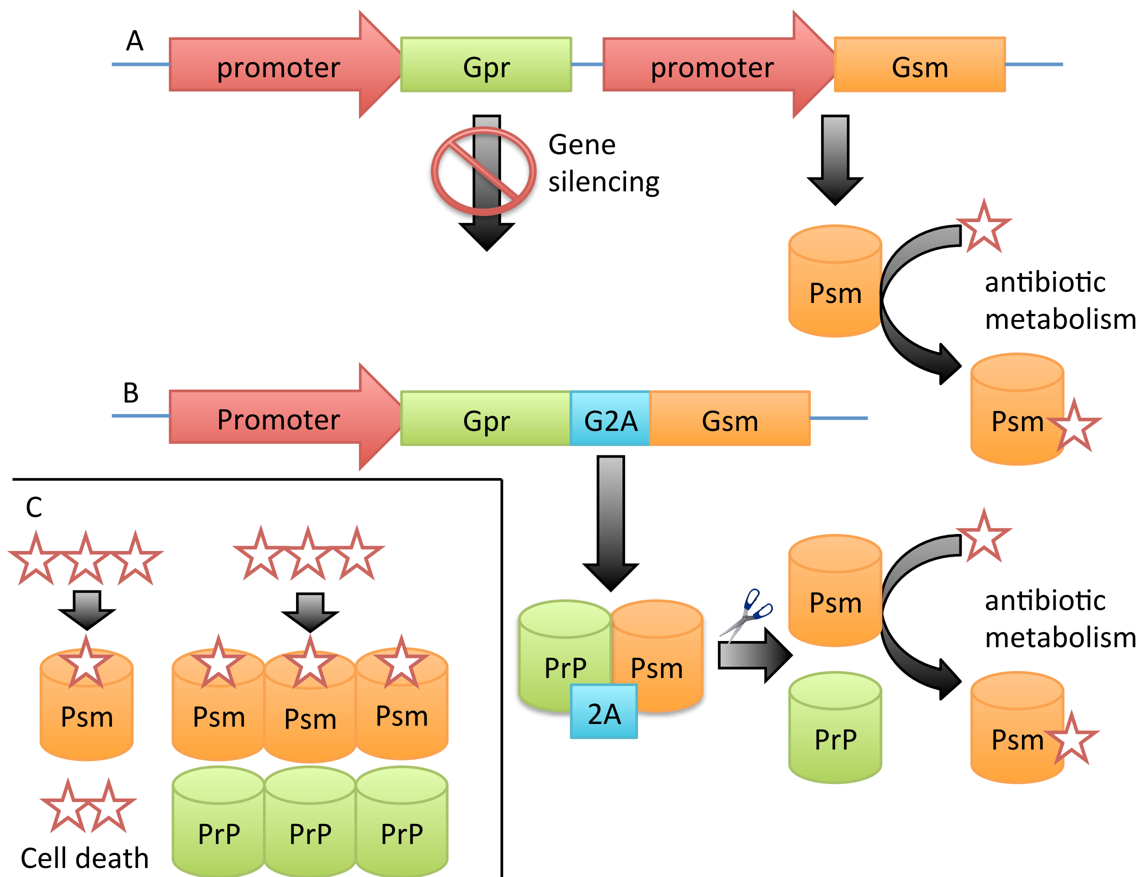


Figure 11. Mechanism of 2A-mediated tethering of product and selection marker expression. A) Most selection vectors have the product gene (Gpr) and the selection marker gene (Gsm) under different promoters (promoter). In algae, this often leads to cell lines that gain resistance to an antibiotic via selection marker protein (Psm) expression, but do not produce product (PPr). B) This can be circumvented when these two genes are linked by the 2A sequence, under a single promoter element. Here, a singe peptide is produced which auto-cleaves itself at the 2A region. This then leads to parity in terms of the concentration of each protein, such that the degree of selection on a population of differential expressers allows for indirect selection of high product producers. C) When this is combined with certain selection genes (Hygromycin and Bleomycin), that detoxify antibiotics via covalent binding, such that each selection protein produced deactivates a single molecule of antibiotic, as antibiotic concentration is increased so too can high product producers be selected from low producers. (Rasala et al., 2012; Ryan and Drew, 1994)

Although this method has proven invaluable in circumventing low heterologous protein yields in microalgae that can result from silencing, it does have one major shortcoming. At industrial scales, where numerous generations are required, as well as large production volumes, either large amounts of eukaryotic selection agents are needed, which are highly toxic and very costly, or silencing may reoccur.

Thus, other methods have been investigated to prevent heterologous gene silencing in microalgae. The Nit1 sequence, for instance, acts as a transcription repressor in

the presence of ammonia, but as a transcription enhancer when cells are grown in the presence of a nitrite-containing medium (Miyake-stoner and Mayfield, 2010). This is however curtailed by lower transcription rates of protein due to more post-translational controls being exerted upon the nuclear transcription machinery compared to that of the plastid, this includes nuclear gene silencing. Some success has been achieved in reducing transgene silencing in microalgae through the use of UV-screening and selection for strains that exhibit more antibiotic resistance with greater impairment of transgene silencing (Neupert et al., 2009). Another method that has been successfully used to increase protein production is a method of inserting the intron from the endogenous rbcS2 into the transgene, which increases mRNA along with recombinant protein transcription and accumulation. Several endogenous microalgal promoters have also been found to enhance transcription. These include HSP70a, psaD and rbcS2 sequences.

2.11 Agrobacterial-mediated expression in green microalgae

Since *C. reinhardtii* was first transformed with the use of *R. radiobacter* (Kumar et al., 2004), this method has been demonstrated in several other microalgal species. These transformations follow a similar process; firstly, algal cultures are grown to mid-exponential phase, then, biomass is harvested and plated onto solidified agar media, which is then allowed to grow until a lawn of cells is formed. Once this lawn has been established, the cells are harvested and suspended in a pre-grown agrobacterial culture. This mixture is then plated onto solidified media that can maintain both the agrobacterial and algal populations. Following this, one of two discrete routes may be undertaken. Either, algal cells can be investigated for transient gene expression, or cells can be plated onto selection media, with the subsequent generation of stable expressing colonies. In the latter, a known number of cells are plated such that the number of colonies formed gives an indication of the degree to which stable transgene integration occurs.

As with higher order plants, it has been found that the transformation rates vary across microalgae. Some species are more transfectable than others. Acknowledgement of this is required for the protection of unexplored or vulnerable aquatic ecosystems and the development of legislation and high-throughput methodologies for industrially relevant isolate discovery.

Transfection rates are also linked to other physical parameters, enabling the optimizing of physical parameters to be used to reduce the impact of the genetic limitations of certain microalgal species that demonstrate low rates of transfection. Key physical parameters include:

- **Duration of Preculture**. the number of days that algal cells are allowed to form a lawn prior to harvesting and the addition of *R. radiobacter*.
- **Agrobacterial Concentration**. the ratio of *R. radiobacter* to algal cells.
- **pH of co-cultivation media**. the pH of the solidified media following plating of the agrobacterial-algal suspension on solidified co-cultivation media.

- **Duration of co-cultivation**. This is the time that agrobacterial-algal co-culture is allowed to proceed
- **Co-cultivation temperature**. *R. radiobacter* transfection ability and algal cell surface permeability to that transfection is highly affected by temperature.
- **Acetosyringone concentration**. as mentioned previously, acetosyringone is released during plant wounding, and allows for T-DNA movement from *R. radiobacter* to the host cell, acting as an inducer of *R. radiobacter* virulence.

Acetosyringone is generally used to induce virulence in *R. radiobacter* before transfection. Similar phenolic compounds, such as cinnamic acid, coumarin and vanillin, have been shown to be better inducers of virulence and thus, transfection rates. Cinnamic acid was the most effective, delivering a two-fold increase in transfection (Cha et al., 2011).

Other parameters affecting the degree to which transfection efficiency occurs include the strain of *R. radiobacter*, and the elements that make up the T-DNA vector. It was found that with the use of more highly virulent strains of *R. radiobacter*, a two-fold increase in transfection could be achieved (Dehghani et al., 2017; Pratheesh et al., 2012). Additionally, as molecular biology advances our understanding of the highly diverse genetics of algal species, so too does our ability to truly harness the power of microalgae.

2.11.1 Stable nuclear agrobacterial-mediated expression in microalgae

The process of developing stable lines of transgenic microalgae using *R. radiobacter* has been achieved in several microalgal species with varying degrees of cell forming unit (CFU) efficiency post antibiotic selection (**Figure 12**).

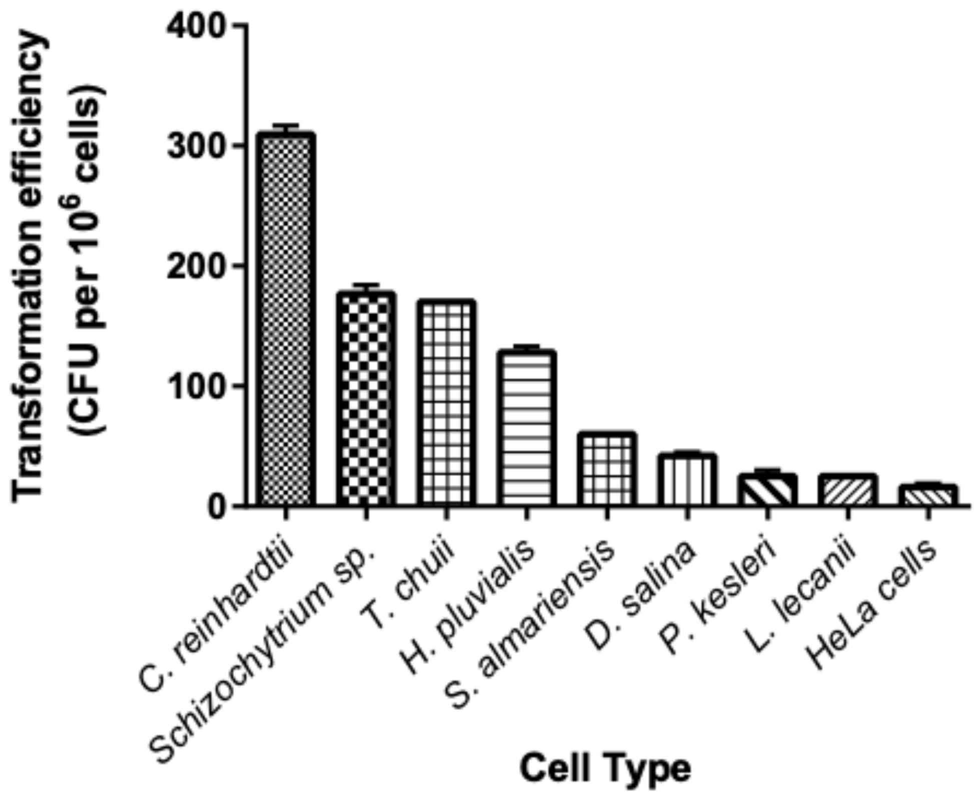


Figure 12. *R. radiobacter* efficiency in producing stable transformants in various planktonic systems. (Cha et al., 2011; Cheng et al., 2012; Guo et al., 2013; Kathiresan et al., 2009; Khatiwada et al., 2019; Kumar et al., 2004; Prasad et al., 2014; Rathod et al., 2013; Reddy et al., 2017; San et al., 2012; Sharif et al., 2015) (Graph compiled from above research papers).

Importantly, following transfection of T-DNA into microalgal cells, special attention must be paid to the removal of *R. radiobacter* from the microalgal culture to remove any possibility of false positive identification of the transgene in resistant colonies (Kumar et al., 2004). For this reason, aside from the eukaryotic selection antibiotic that is used, the antibiotic cefotaxime, which has high *R. radiobacter* toxicity and low microalgal toxicity, is also generally used.

2.11.2 Transient R. radiobacter-mediated expression in microalgae

Several species of microalgae have demonstrated *R. radiobacter*-mediated transient expression. These include *Scenedesmid* spp. (*S. obliquus S. falcatus S. acutus*), *Dunaliella salina* and *D. bardawil*, *Ankistrodesmus gracialis*, the *Chlorellids* (*C.*

ellipsoidea C. vulgaris C pyrenoidosa), *Nannochloropsis sp*., *Isochrysis galbana*, *Euglena gracialis*, *Haematococcus pluvialis*, *Schizochytrium sp*. *Parachlorella kessleri* and of course, *C. reinhardtii* (Cha et al., 2011; Cheng et al., 2012; Guo et al., 2013; Kathiresan et al., 2009; Khatiwada et al., 2019; Kumar et al., 2004; Prasad et al., 2014; Rathod et al., 2013; Reddy et al., 2017; San et al., 2012; Sharif et al., 2015). This is a developing area, with species being added to the list continually.

Although transient expression of marker genes has been demonstrated in various microalgal species, these have only been used as a rudimentary measure to allow for optimization of the factors affecting expression efficiency, with these conditions allowing for transient expression studies to allow for maximal gene insertion and subsequent GMO cell line development. In this regard the reporter gene, β-glucuronidase (GUS) has been extensively investigated. When individual algal cells express this transgene, cells develop a blue colour in the presence of the substrate, X-Gal, allowing for methodological and parametric quantification i.e., by counting blue individuals versus non-blue individuals. This has yielded numerous research publications but very little real progress in terms of harnessing microalgae for agrobacterial-based heterologous protein production. Investigations of transient expression of this nature have yielded significant insight into how physical parameters have vastly variant transfection outcomes from species to species. For instance, the cyanobacteria, *Spirulina (Arthrospira) platensis,* showed a seven-fold expression **reduction** in the presence of acetosyringone (200µM- 0µM), which relates to *R. radiobacter* virulence, although this was done in this bacterium (Dehghani et al., 2018), Contrasting this, however, *Nannochloropsis sp. and Chlorella vulgaris* were found to have optimal expression profiles when exposed to acetosyringone concentrations of 100µM (14% of total cell population transiently transformed) and 300µM (9% of total cell population transiently transformed), respectively. Non-optimised versus multi-parametric optimised transient transfection outcomes in *Nannochloropsis sp.* were 12.5% and 24.5%, and 8.91% to 25.0% in *Chlorella vulgaris* (San et al., 2012; Yee et al., 2012). This is remarkable, considering that, after optimization, expression with the use of other vector systems, such as carbide whisker agitation delivered only 15- 24 transformants per 10^7 cells (Ten Lohuis and Miller, 1998).

Investigations into transient expression in microalgae are largely directed toward a single species, with no methods for high-throughput screening of efficient expressers being available. Additionally, a vast majority of *R. radiobacter*-algal transfection research looks to demonstrate transfectability. This approach, though highly publishable, often relies on demonstrating marker gene expression, rather than developing these algal species as platforms for *R. radiobacter*-mediated heterologous protein production. Let alone, transient expression analysis relies on standard microbiological methodologies, where biomass growth is limited to that which can be cultured via agar plating. Thus far all investigations into microalgal transient expression have been solely for research purposes, with transient expression not yet having been considered as a novel means of heterologous protein production.

2.12 Non-tissue multilayer cell pack technology

Recent work describes enhancement of the transfection and transient expression of heterologous proteins of synthetic plant cell cultures with *R. radiobacter* in a non-tissue multilayer cell pack, and not in co-culture as is the general method (Patent no. WO2013/1134504 A1; Rademacher et al., 2019).

This cell pack is created by vacuum and the removal of the liquid growth medium from the cell culture, distributing cells on a filter or in a column. Once the liquid medium is completely removed, the cell pack is maintained at a density of between 0.1 and 0.9 $g.cm^{-3}$ and a relative humidity of 50 - 100%. Following creation of this cell pack, *R. radiobacter* liquid culture is then vacuum infiltrated through the cell mass. It was found that *R. radiobacter* transfection of *Nicotiana tabacum* cells was far more efficient in the cell pack compared to that observed in liquid co-culture. Remarkably, transfection rates in the cell pack were found to be as high as 80% of the total cell population (Rademacher, 2013).

This was determined by comparing red fluorescence in each sample as well as comparing the degree of expression of the heterologous protein, the anti-HIV Env 2G12 antibody in each sample. Here neither red fluorescence nor 2G12 antibody could be detected in the co-cultured sample (Rademacher, 2013), but was readily detected in the cell pack.

Aside from expression and production of various heterologous proteins, including vaccine candidate proteins from *Malarium fulciparium*, as well as the use of cell pack columns for high-throughput parametric optimization for transfection efficiency, it was also demonstrated that it is possible to non-destructively harvest cellular products from the cell pack. This was demonstrated with the use of various strains of *R. radiobacter* harbouring several different plasmid vectors.

These strains/vectors included:

- Secretory antibody M12 and an endoplasmic reticulum sequestered dsRED
- Secretory dsRED
- Endoplasmic reticulum sequestered dsRED
- Protein body-forming dsRED

Secretory proteins were detected by washing cell packs with elution buffer post-transfection, then collected secreted protein products from the interstitial column space.

The large endoplasmic reticulum-sequestered dsRed, which is intracellular, is a good indication of cellular rupture during cell pack formation or during the elution process. Following centrifugation of the elute, heterologous proteins were quantified by Coomassie staining and SDS-page gel. It was found that the intensity of bands corresponded almost exactly between intracellular and secretory heterologous protein levels, with some 50% of the total protein that was transcribed being secreted into the medium, while the endoplasmic reticulum-retained dsRED variant showed only a slight

presence of the protein in the cell pack protein elution, indicating that vary little cell damage occurred, and no protein was secreted.

This method allows for a high degree of simplicity and very little downstream processing for recovery of excretable proteins of interest, which may often contribute as much as 80% to the cost of producing heterologous proteins (Corchero et al., 2013).

2.13 Literature review: core conclusions

- Plant expression systems are safe, having low probabilities of toxic carryover for human and animal therapeutic application, while also having the cellular complexity required for correct folding of these proteins.
- R. radiobacter-mediated heterologous protein expression has been demonstrated in many plant species, some of which have been highly investigated for industrial scale production.
- Microalgae also are able to express heterologous proteins, but have several advantages over plant expression systems. These include being in a highly culturable and productive single celled state, able to grow autotrophically or heterotrophically, orders of magnitude more genetically and biochemically diverse than higher plants and being amenable to biomass movement through pumping of media containing cells in an industrial setting.
- Microalgae, though highly diverse have not been extensively studied for genetic manipulation, with the model species, *C. reinhardtii* being most investigated for application with the various methodologies for heterologous protein expression.
- Transient heterologous protein expression is less demanding with regard to infrastructure than stable GMO development, maintenance and escape prevention.
- Transient transfection and heterologous protein expression has been demonstrated in microalgae using *R. radiobacter,* though this has not been investigated as a possible means of heterologous protein production.
- The cell pack has demonstrated an exciting potential for transient heterologous protein expression in plant cell cultures. This process does however suffer from scaling issues.
- Microalgae, with their intrinsic cellular state being planktonic, are an exciting novel avenue for implementation within a column system for transient heterologous protein expression with *R. radiobacter.*

Hence, through integrating and synthesising these knowledge components, we see transient transfection of microalgae with *R. radiobacter* for heterologous protein expression as a prospective avenue for the development of a novel column-based approach. We seek to exploit this across wide-ranging algal species, to enable effective and rapid screening for exceptional algal hosts as well as R. radiobacter strains or plasmid vectors. Lastly, we plan to explore the concept of the cell pack and its useability with microalgae.

3 Research aspect

3.1 Microalgae: new problems require new solutions

It is now accepted that the total number of species of cellular life on our planet is in the order of 10^7, though some upper estimates are as high as 3.10^7 species (Stork, 1993). Worryingly, it is estimated that the current extinction rate is in the range of 17 500-35 000 per year, making it twice as likely for a species to become extinct rather than be described by science.

Only 20 types of plants are cultivated to feed the majority of the vast human population on the planet, with the total number of plant species eaten regularly being in the range of 5 000, globally. This, in combination with the looming threat of irreversible climate change, necessitates urgent investigation and development of food security redundancies, as well as conservation and exploration of novel biology for industrial applications. Thus, with international legislation viewing biodiversity as that which is owned by the nation in which it is convened, countries may partner with multinational corporations in search of organisms that enable novel and sustainable industrial and agricultural solutions, as is the case with the partnership between Costa Rica's national Biodiversity Institute and Merck & Co. which serves as a depiction for preserving biodiversity (Stork, 1993). Although partnerships such as this are making progress, without new methodologies that allow, not only for high throughput screening of organisms for industrial applicability, but also for development of industrial processes that enable efficient production of complex molecules, the success of these solutions will be limited. To add to this, with increased frequency of human society's abutment onto nature, and the associated increase in the probability of viral species jump; as is the case with zoonotic viruses, this necessitates treatment action plans with minimal turnaround time and quick mobilisation (Johnson et al., 2015) .

There exists a vast, underutilised resource for biotechnology, and to meet the various needs of global society in general, in the form of microalgal biodiversity. Of the six 2050 United Nations development goals (Griggs, 2015),

which include:

- "Thriving lives and livelihoods. End poverty and improve well-being through access to education, employment and information, better health and housing, and reduced inequality while moving towards sustainable consumption and production."

- "Sustainable food security. End hunger and achieve long-term food security — including better nutrition — through sustainable systems of production, distribution."

- "Sustainable water security. Achieve universal access to clean water and basic sanitation, and ensure efficient allocation through integrated water- resource."

- “Universal clean energy. Improve universal, affordable access to clean energy that minimizes local pollution and health impacts.”

- “Healthy and productive ecosystems. Sustain biodiversity and ecosystem services through better management, valuation, measurement, conservation and restoration.”

- “Governance for sustainable societies. Transform governance and institutions at all levels to address the other five sustainable development goals.”

Each of the above objectives can be achieved should microalgae become a priority in terms of conservation, industrialisation, identification and commercialisation.

3.2 General study scope: the non-tissue multilayer cell pack, transient expression of heterologous proteins and microalgae

This thesis work, part of which has been patented (“Matrix-mediated cell culture system”. Global accession no. WO2020110083A1. US accession no. US20220041977A1), is an investigation into harnessing the superior productivity and genetic diversity of microalgae as a transient expression biomass for heterologous protein production using *R. radiobacter,* and a novel matrix-mediated column-based approach, referred to as the algal cell pack.

This thesis and the related research consider each of the fundamental concepts of microalgal research i.e., conservation, industrialisation, identification and commercialisation, while exploring specific hypotheses and objectives as well as acting as a guide for future work in the molecular and heterologous protein expression sphere of microalgal research. This was done by creating various resources in a step-by-step manner, which are then utilised in later research stages. Inasmuch as the development of each stage was fundamental to following stages, each stage has been developed and investigated so as to have relevance to microalgal research in general, that is not necessarily geared toward transient expression of heterologous proteins in microalgae. Wherever possible, experimental design was done to allow for methodological implementation in other systems or with other organisms.

The overarching research aims of this study include microalgal library development, expression column system development, microalgal screening for stable heterologous protein expression and transient heterologous protein expression within the column.

All three of the above overarching research aims were carried out sequentially, and within each of these components, various investigations were done. These are described below.

Microalgal library development had several core objectives. These allow for the generation of a stable, highly diverse and non-contaminated working algal library of species that can be supported through both autotrophic photosynthetic growth and heterotrophic growth to allow for later investigation.

The working collection of microalgal species was developed for screening for protein expression and ready for other research application. This was collected partly by the Council for Scientific and Industrial Research's (CSIR) microalgal sampling drive conducted in Mpumalanga and the Western Cape, and from the CeBER algal culture collection. As an initial departure point, it was required that as many as possible of these isolates be coaxed to grow in an axenic state, with no bacterial or fungal contamination. This allows not only for investigation of heterotrophic ability, which is of core importance if biomass is to be incorporated into a light-impervious diatomaceous earth/Celite®545 column, but also ensures single strain investigation, reproducibility, and good scientific practice. For this, several methods were used, some were selected from the literature, and some devised *de novo*. Once axenia was achieved, these isolates were observed for their ability to withstand various antibiotics, their ability to be revived post cryogenic storage, along with their ability to be grown heterotrophically in the absence of light.

Development of the expression column system, also known as the algal cell pack was undertaken to achieve three system requirements.

These include:

Providing a vehicle in which algal biomass was rapidly concentrated, while still forming a porous column to allow for effective contacting of a liquid R. radiobacter culture for transfection, or other biological or chemical agents of interest, to allow for maximal contacting.

Ideally a system in which expression of the transformed algae could take place.

A system for algal biomass harvesting and protein recovery.

This involved the combining of axenic isolates, from the microalgal library, with diatomaceous earth/Celite®545 which was packed using crude vacuum, forming a porous high-humidity column. The expression column system was then investigated in terms of algal cell harvesting efficiency relative to column size, the ability of microalgae to survive column incorporation, and then also, the ability of the system to separate aquatic ecosystems by size in a self-organising manner while simultaneously allowing for selection of algal species suitable for use in the column.

Lastly, the study investigated whether the matrix-mediated Celite545-based column system could be used to stimulate viral lysis in microalgal isolates. It is well understood that some viruses may be passed on across generations in a number of cell systems, both pro- and eukaryotic, without being lysed or expressing proteins. The production of viral peptides and several other viral proteins from the inherited viral sequences, however, may take place under specific circumstances, such as favourable climates or harsh environments. When parasitic wasps lay their eggs inside the host, polydnaviruses are incorporated into their genome and subsequently expressed as active viruses. This phenomenon is observed in the co-opting of polydnaviruses by parasitoid wasps (Fleming and Summers, 1991). These hereditary viruses ensure the survival and growth of the parasitic progeny by preventing the host's immune system from identifying the parasitoid wasp larva. Here virions express from inherited viral sequences and are injected with the wasp larvae into the host, where virions then

target the host's immune system, allowing for wasp larvae to grow to maturity, undetected.

Similarly with sequences derived from plant viruses, such as the cauliflower mosaic virus promoter (CaMV) sequence, when this is incorporated into a plasmid expression vector, it allows for evasion of the host cell's defence mechanisms against foreign gene sequences. This allows for maximal expression of transgenes.

It is essential to sequence viral genomes in order to uncover sequences that particularly target the evasion of endogenous cell responses before finding viral sequences in order to begin transgenes expression. Identifying viruses that target algae has proved difficult but is fundamental to identifying algal-targeting sequences of viral origin to allow for maximal transgene expression.

Heterologous protein expression, mediated by *R. radiobacter,* was investigated across a range of algal species by first looking at whether stable GM microalgae could be produced in the column.

Firstly, various strains of *R. radiobacter* harbouring various expression vectors were studied. It was not within the scope of this project to design new vectors, but a series of tests was done to check sequence fidelity and functionality of the transforming bacteria. Then, genetic modification was investigated in various axenic microalgal strains/ species. This was done by investigating cell survival and heritable gene introduction of the selection marker, neomycin phosphotransferase II, representing stable GMO development within the column. Thereafter, a system for transient expression of heterologous proteins was developed. Initial protein expression studies used two reporter proteins, green fluorescent protein (GFP) and ß-glucuronidase (GUS). This was followed by investigations into expression of the industrially relevant protein horse radish peroxidase (HRP), as well as a VLP-based vaccine for human papillomavirus 16 (HPV-16).

3.3 Research section structure and methodological approach

In the development of a transient heterologous protein expression system from green microalgae, using a matrix-based column, various resources were required in a stepwise fashion. The research section is structured such that each chapter is split according to its own Introduction and Chapter description, Materials and Methodology, Results and Conclusion, with sub-sectioning of each, as needed, to expound novel methodologies and results. The three core research sections are then followed by an overarching conclusion which covers problems encountered during the research, as well as future prospects and novel developments and applications of the system. The last section of this thesis briefly details findings that do not have direct bearing on the core objectives themselves although they are of scientific interest.

Regarding the use of controls, experiments were designed such that (wherever possible) various investigative methodologies were implemented so as to validate results. These included DNA sequence fidelity and presence, protein fidelity and presence, cell survival post antibiotic exposure, as well as the implementation of existing systems such as transient expression in *N. benthamiana*. This was combined

with comparisons of on-the-market drugs such as the human papilloma virus (HPV) virus like protein-based (VLP-based) vaccine, Gardasil®. With consideration to system efficiency, reporter proteins were also co-opted to allow for this novel research to have relevant bridging into other heterologous expression investigations as well as other, disparate avenues of microalgal research.

3.4 Research focus and constraints

Due to its the size, this research investigation was constrained on some levels.

It was decided that the model organism *C.* vulgaris UTEX 395 as well as *Scenedesmus sp*. would be utilised as model organisms. To add to this, it was decided that the CSIR's Mpumalanga microalgal collection would provide the focus in terms of algal library development. During the various investigations, if a species could not pass various hurdles required for industrialization in the system, they were discarded. This included: the inability to be cryogenically preserved, the inability to be grown axenically, mismatch of response to either antibiotic selection or resistance, and the ability to demonstrate *R. radiobacter*-mediated gene transfer.

R. radiobacter strains and vectors were all obtained from UCT's Biopharming Research Unit or from the University of Stellenbosch. It was not within the scope of this research project to develop novel vectors and novel gene sequences.

In terms of demonstration of transient expression in the system, the core objectives were to look at detectable reporter protein expression, expression of a protein of industrial relevance and then a VLP-based vaccine. This was a proof-of-concept investigation. And so, once these three aspects were demonstrated, project findings were disclosed for patenting, culminating in the finalization of this thesis project, but forming just the beginning of this novel avenue of research, which is in its infancy.

4 Microalgal library development

4.1 Chapter introduction, overview and aims

In overview, this research section looks to create a biodiverse algal library that is of maximal application in later research endeavours. This library must allow for variable heterotrophic ability assessment across the library. Allow for a high degree of experimental reproducibility, so each isolate must be axenic; containing only one algal isolate without any bacterial or fungal contamination. Also, the library must have a low degree of evolvability, which was achieved by developing this library to withstand cryogenic storage, so that in the various later experiments a genetically identical cryogenic aliquot could be used per experiment per isolate.

As outlined in Chapter two, this research to a large degree focused on species isolated by the CSIR's microalgal sampling drive, and specifically those originating from the Mpumalanga province in South Africa. This library contained 37 initial isolates, with each one denoted by 'MPA', followed by a number. Although there is a possibility that some of these isolates were duplicate species, because they had not yet been sequenced, each isolate was treated as a separate species. Aside from this, two model algal species were also used. *Chlorella vulgaris* UTEX 395 was acquired from the University of Texas' algal library collection. The second algal species, *Scenedesmus sp*. was made available as a working stock as part of the algal collection. Several trips were also undertaken to various streams flowing off Cape Town's Table Mountain to gather algal samples, though of interest to ecological work, these were not extensively explored in the later investigations.

The algal collections from which all algal isolates and species were taken were not prepared as axenic, hence they were contaminated with a vast array of unidentified fungal and bacterial species. To carry out molecular or protein expression work on these isolates, it was a prerequisite that each isolate be induced to grow under axenic conditions to ensure that biological contaminants would interfere with transfection experiments or introduce endotoxins, which would make final application for human or animal therapeutic use unsuitable. Moreover, bacterial and fungal contaminants would change the algal growth profile or lead to overgrowth of said contaminant. This is especially true in enriched heterotrophic media used in this study in preference to the autotrophic environments under which most species had been isolated. Previous research has also shown that certain algal species cannot grow without a bacterial symbiote that provides vitamin B12 (Ramanan et al., 2016; Thi Vu et al., 2010). In that case, any algal species that relied upon such a symbiotic relationship would not necessarily be amenable to industrialization, as this system would then require a co-culture, which is not only more complex but also would not be granted GRAS.

To prepare axenic cultures for experimentation, several purification methods were used. In the first method, the contaminated cultures were exposed to various antibiotics and antifungal agents. The antifungal agent used initially was amphotericin B; this was found to have an inadequately short half-life when plated and fungal overgrowth was pronounced. Consequently, a second antifungal known as

carbendazim was identified and combined with differential slant plating of ampicillin and kanamycin.

Secondly, a method for mechanical separation of newly formed axenic algal colonies from contaminating species was developed. It was found that individual axenic colonies could be observed under the microscope on plates that were still contaminated. A very precise method of picking individual colonies needed to be developed. To achieve this, a light microscope was sterilised with ethanol, then put into a laminar flow hood. Newly growing axenic colonies were then identified and marked under the light microscope. This was then defocused, and a sterilised Pasteur pipette was secured to a Helping-hands clamp which was placed precisely over the colony and used to pick out the colony of interest. The chosen colony was then plated onto a clean heterotrophic plate that did not contain any antibiotic agents.

These methods resulted in the preparation of 18 axenic isolates out of the initial 44 studied. These isolates were confirmed to be axenic by streak plating and grown in an antibiotic-free high-nutrient liquid media followed by an investigation into cryopreservation ability of each isolate.

To assess how the various isolates comprising the library respond to various chemical selection agents, the 18 isolates were exposed to antibiotics, one of which included geneticin G418, that allows for stable GMO algal formation after transfection with the binary vector, pTRAc, which is broken down by stably transfected algal cells due to the nuclear incorporation and expression of the bleomycin phosphotransferase gene. Bleomycin phosphotransferase not only breaks down both bleomycin, but also geneticin G418.

In addition to this, axenic algal cultures were exposed to cefotaxime, which clears *R. radiobacter* from transfected algal cultures, post transfection, but does not affect algal growth.

Heterotrophic ability was also investigated, and a cryogenically stable algal library was developed. Here various isolates were roughly assessed for their ability to withstand storage at -80°C and then be revived, allowing for halting of isolate evolution as well as acting as working stocks for future experimentation, reducing the risk of contamination and allowing for amelioration of batch variation from experiment to experiment. Lastly, isolates that demonstrated favourable outcomes of these parameters were speciated using 18s ribosomal subunit sequencing. These isolates included *Scenedesmus sp.,* MPA 16.1 and MPA 49.1.

4.2 Materials and methodology

4.2.1 Heterotrophic investigation and axenic development of CSIR's Mpumalanga algae library

$5\mu l$ of liquid culture from all 37 isolates (grown in 3N BBM at ambient temperature, 135rpm) were drop plated onto one of four 0.8% 3N BBM agar plate types, containing a reduced carbon source and antibiotics. Specifically, the first plate-type contained 4 g.L^{-1} glucose and 100 mg.L^{-1} of each, kanamycin sulphate, ampicillin and streptomycin

sulphate. The second plate-type also contained 4 g. L^{-1} glucose, but only 10 mg. L^{-1} of each antibiotic. The third and fourth plate-types contained both contained 100 mg. L^{-1} of each of the antibiotics, but instead of glucose, these contained either 8 g. L^{-1} glycerol or 4 g.L^{-1} sodium acetate (see **Appendix Supplementary Data**).

Plates were sealed with Parafilm and incubated under cool fluorescent lighting and at ambient temperature (20-24 ℃) over-night, before being placed in the dark to start forming colonies to allow the heterotrophic capability of each isolate with regard to carbon source to be assessed along with their relative antibiotic tolerances. Ten days after being placed in the dark, relative growth was scored by either the presence or absence of green colony formation per carbon source.

At this point it was also discovered that all isolates were contaminated with an unknown array of fungal species. However only some isolates were contaminated with unknown bacterial species.

In order to reduce the bacterial and fungal contamination and prepare axenic isolates, drop plating was repeated as outlined above, with each isolate plated onto carbon sources with antibiotic concentration combinations that lead to best growth per individual isolate. In addition to that 2.5 mg. L^{-1} of the antimycotic, amphotericin B was added to each of the prepared plates. However, the effective lifespan of amphotericin B was lower than the time taken for algal colonies to form hence fungal overgrowth occurred. A second antimycotic agent, Carbendazim (Carb), was investigated. It was additionally realised that for an algal isolate to be amenable to later, transient agrobacterial-mediated protein expression, they must necessarily demonstrate native resistance to ampicillin (Amp) and kanamycin (Kan), as the *R. radiobacter* strain, GV3101 used in the majority of later experiments, was resistant to these antibiotics. To determine the optimum concentration of these agents to use, a 3N BBM 2D gel gradient selection plate was devised, such that 100 mg. L^{-1} of both kanamycin and ampicillin at their maximum concentration oppose, 40 mg. L^{-1} carbendazim at its maximum concentration (Figure 13), resulting in a reverse concentration gradient across the plate.

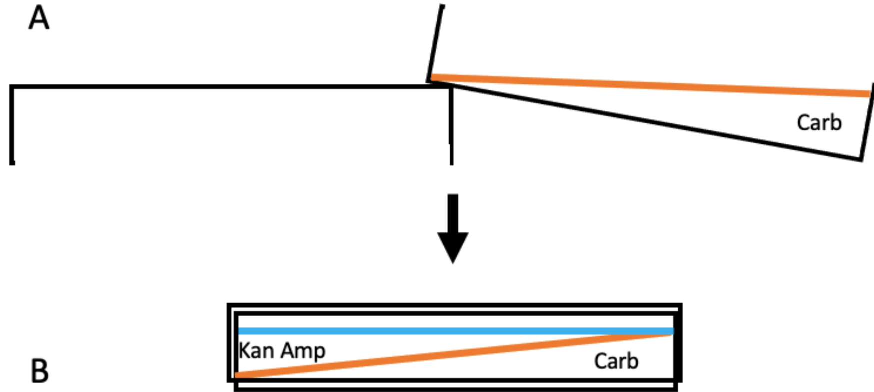


Figure 13. Diagram of 2D selection plate pouring. In A, the carbendazim-containing agar was first poured, with the plate at an angle, by resting it on its lid. This is allowed to solidify, before the kanamycin and ampicillin-containing agar solution is poured on top of the carbendazim-containing agar (B). This results in an opposed dual concentration gradient across the plate.

Once algal colonies began to form, they were assessed for either fungal or bacterial contamination with the use of a mobile stage light microscope and were picked with sterile filter tip in a laminar flow hood into 1 mL sterile 3N BBM liquid culture and allowed to grow for two days at room temperature under lights. This initial 2D plate resulted in several axenic cultures. The plates that still presented with fungal contamination were re-plated onto a 2D selection plate. This plate only contained carbendazim (40 mg. L^{-1}), and no opposing antibiotic gradient, as these cultures had no bacterial contamination but did have fungal contamination. This resulted in a decreasing carbendazim concentration across the plate. Once colonies had formed, they were marked under the light microscope, and were very precisely picked up with a Pasteur pipette secured to a Helping hands clamp. The Helping Hands is a clamp device more commonly used in soldering. It allows for small objects to be clamped, with their ease of movement in 3D space, but was used here to place the Pasteur pipette tip precisely above a newly formed agal colony that did not show any contamination when viewed under the microscope.

This mechanical method allows for the very precise picking out of colonies, where the colony itself is axenic, but where some fungal contamination may be present elsewhere on the plate. It is necessary that colony picking be undertaken prior to fungal sporulation.

Initially, individual axenic colonies are identified with the use of a light microscope. These are marked on the underside of the plate. Plates are then moved to a laminar flow hood, containing a light microscope that has been wiped down with ethanol, as well as the “helping hands” clamp that has also been sterilised. In the laminar flow hood, plates are opened and marked colonies are reidentified, and the helping hands clamp, holding a sterilised fine Pasteur pipette is placed above the colony to be picked. This is all done under magnification. Once everything is in place, the microscope is gently defocused, picking the colony of interest, which is quickly removed to either another plate or liquid culture.

Following 2D plate selection and mechanical colony separation (Helping hands picking), all isolates were confirmed to be axenic. To test for this, the isolates were plated on heterotrophic media containing no antibiotics, and viewed under the microscope.

The above process was repeated for all other isolates - including the model organism *C. vulgaris* UTEX 395.

Apart from having access to the Mpumalanga algal library and the University of Texas algal library (one species), only one environmental isolate was developed for the experiments detailed in Chapter four and Chapter five of this thesis. In brief, water and sediment were sampled from a stream flowing off Table Mountain in the Western Cape province denoted “Guard House Dam” (GHD). The sample was filtered through Miracloth (Merck Millipore. 475855) to remove debris. This was followed by filtration through 20- 25 $\mu\mathrm{m}$ (Whatman; Grade 4 filter discs) (to remove larger planktonic species. Retentate was then caught on a 0.45 $\mu\mathrm{m}$ filter (Sigma-Aldrich; single use disposable syringe filter) and washed well with sterile PBS (see Appendix recipes) to remove smaller contaminants, such as fungal spores and bacterial contamination. A

$100\mu L$ aliquot of PBS-suspended retentate was then plated onto 3N BBM plates containing 4 g.L^{-1} glucose and sodium acetate, 100 mg. L^{-1} kanamycin and ampicillin, and 40 mg. L^{-1} carbendazim. These plates were then placed in the dark, to allow for selection of microalgal species that grow heterotrophically.

4.2.2 Algal library screening

Vitamin requirements

As an initial investigation into possible parameters that may affect an isolate's applicability to industrialisation, isolates were plated onto 3N BBM agar plates containing carbendazim (40 mg. L^{-1}), that did not contain additional antibiotics. However, following plate pouring and before they could set, the vitamins B1 and B12 were spot pipetted (see **Appendix recipes** 3N BBM, for concentrations) onto agar plates and allowed to solidify. The algal library was then spread-plated onto the spot-plated vitamin plates, and growth was assessed for each isolate, of the spot that contained the vitamin and this growth was assessed relative to the rest of the other plates.

Antibiotic screening

Initially, all isolates were plated onto 3N BBM agar plates containing various antibiotics, to assess their growth response. The antibiotics that were applied included streptomycin (50 mg. L^{-1}), tetracycline (10 mg. L^{-1}), erythromycin (50 mg. L^{-1}), chloramphenicol (34 mg. L^{-1}) and gentamycin (50 mg. L^{-1}) (Valgas et al., 2007).

To add to this, isolates were assessed for their ability to grow in the presence of cefotaxime (200 mg. L^{-1}), and at various concentrations of kanamycin (40 mg. L^{-1}, 60 mg. L^{-1} and 80 mg. L^{-1}) as well as geneticin G-418 (20 mg. L^{-1}, 40 mg. L^{-1}, 60 mg. L^{-1} and 80 mg. L^{-1}).

Lastly, the model organism, *C. vulgaris* UTEX395 was exposed to increasing concentrations of the eukaryotic toxin, hygromycin (1 mg. L^{-1}, 2 mg. L^{-1}, 3 mg. L^{-1}, 5 mg. L^{-1}, 10 mg. L^{-1}, 15 mg. L^{-1}, 20 mg. L^{-1} and 40 mg. L^{-1}). This was done in triplicate on 3N BBM agar plates, with 10^5 cells plated on each one. After algal growth was observed, cells were collected by rinsing off the agar plates with sterile PBS, and the cells were counted with a counting chamber, and normalised relative to negative control plates that did not contain hygromycin.

Cryogenic storage and revival

All axenic isolates were grown up in LB for 6 days, and dense cultures were observed. These were then aliquoted into 2 mL Eppendorf tubes and frozen down at -80°C, after which, cell viability was inspected at three months, six months and one year, by plating onto 3N BBM media, as well as LB broth. Plating the culture was achieved by removing a section of the frozen culture with a sterilised scalpel blade, under strict sterile conditions with the use of a laminar flow hood.

Heterotrophic and mixotrophic growth

Heterotrophy was initially investigated across the various isolates by either plating them on 3N BBM agar plates containing 4 $g.L^{-1}$ glucose and 4 $g.L^{-1}$ sodium acetate, or by inoculation into similar 2 mL Eppendorf tubes containing liquid media, and then incubated with shaking (70 rpm). Both sets of agar plates were placed in the dark. Finally, relative growth was qualified after 10 days in each of these sets (**Table 1**).

Following this, it was of special interest to investigate whether isolates would demonstrate, intra-growth variation within various enriched media mixotrophically, but also inter-growth variation across the various isolates.

For this investigation, the isolates, MPA5.1, *Scenedesmus sp.* and MPA49.1 were selected, with 10^6 cells of each being inoculated (in triplicate in shake flasks) into 20 mL of 3N BBM containing either no enrichment, 10mM glucose and glycerol, or 10mM glucose and glycerol, 5 $g.L^{-1}$ yeast extract and 10 $g.L^{-1}$ tryptone, mixed together as a single formulation (here after referred to as **Heterotrophic media**). Cell density was then assessed after seven days, via cell counting, with a student t-test being employed to demonstrate significance of treatment effect.

As the previous investigation had been done using liquid culture, it was of interest to develop a quick method to assess growth response of an isolate, relative to media composition, by qualifying colony size. Here the isolate, MPA16.1, was plated onto either 3N BBM or heterotrophic media, with colony size being assessed after seven days.

Following this, all four isolates from the investigations detailed above were assessed according to their growth response to various media, under mixotrophic and heterotrophic conditions. 100 μL of each isolate (MPA5.1, MPA 16.1, MPA49.1 and *Scenedesmus* sp.) were drop plated onto either heterotrophic plates, TAP plates (see **Appendix recipes**), or LB plates (see **Appendix recipes**), in duplicate. The heterotrophic plates were placed in the dark and the mixotrophic plates were incubated under light (63 Lm/W; 24 hr)

The final investigation involved the assessment of microalgal growth under mixotrophic and heterotrophic conditions in the presence of an antibiotic. From the literature, and from previous investigations, it seemed that the presence of kanamycin could improve growth response. To test this, *C. vulgaris* UTEX395, was grown in liquid TAP media, either mixotrophically of heterotrophically, with the addition of 30 mg. L^{-1} kanamycin. Growth was assessed via cell count on days one, three, five, seven, and nine. Cell dry weight was quantified at day nine as well.

4.2.3 Species identification via 18s ribosomal subunit sequencing

The isolates, with working designations: *Scenedesmus sp.,* MPA16.1 and MPA49.1 were selected for species identification via 18S ribosomal subunit gene sequencing, as these showed favourable growth under both mixotrophic and heterotrophic conditions, and in combination with the various antibiotics. Additionally, these isolates were viable post cryogenic storage.

Colonies from each of these isolates were picked into 10 μL ddH_2O, thereafter the cells were lysed by incubation for 10 min at 95°C. DNA extractions were done using Extract-N-Amp™ Plant Tissue PCR Kits (Sigma-Aldrich, St Louis, MO, USA) from algae species: *Scenedesmus* sp., MPA49.1 and MPA16.1. (Work done by H. Els).

The 18s regions were amplified using KAPA HiFi HotStart ReadyMix PCR kit (Roche. Inc).

PCR conditions:

18sForward primer: CCTGGTTGATCCTGCCAG

18sReverse primer: TTGATCCTTCTGCAGGTTCA

The PCR protocol (manufacturer defined) comprises an initial denaturation step at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s (denaturation step), 50°C for 1 min (annealing step) and 72°C for 1 min (extension), and final extension at 72°C for 7 min.

PCR reaction was run on an 1% TAE agarose gel, the fragment was excised, cleaned and cloned into the linearized pJET1.2 plasmid and sent away for sequencing at GenScript Biotech inc., where sequence data was returned following Sequence Blast and speciation.

4.3 Results and discussion

4.3.1 Heterotrophic investigation and axenic development of the CSIR Mpumalanga algal library

From the investigation of the CSIR Mpumalanga algal library's ability to grow on solidified media containing carbon sources, it was discovered that individual isolates demonstrated a marked diversity in their ability to utilise various carbon sources for heterotrophy (See **Appendix**; **Supplementary Data**).

Eighteen isolates demonstrated an inability to grow on carbendazim, while thirteen isolates demonstrated intermediate growth and 13 isolates demonstrated good growth. Axenia in 5 isolates from the "intermediate growers" was developed, while 4 isolates from the "good growers" was developed using the carbendazim—kanamycin, ampicillin 2D plate technique.

Following this, precise colony picking using the "helping hands method" allowed for the development of a further eight axenic isolates.

Of note to the development of axenia, algal isolates that demonstrate rigorous growth accomplish axenia much more readily. This is due to several factors, including their culminating in a higher ratio of algal cells to contaminating ones, and also allowing for early algal colony picking while selective agents are still active and contaminants less developed. It must be kept in mind that this does introduce bias regarding axenic library development, where fast growing isolates that display innate resistance to various antibiotic and antimycotic agents, may have any number of underlying phenotypic, genetic or metabolic similarities.

4.3.2 Algal library screening

Vitamin requirements

From the library, it was found that 14 isolates (MPA 38.3, MPA 34.2, MPA 18.2, MPA 34.1, MPA 29.1, MPA 38.1, MPA 39.2, MPA 53.1, MPA 33.1, MPA 38.2, MPA 7.1, MPA 27.1, MPA 16.1, MPA 20.2) demonstrated better colony formation in the presence of vitamin B6 and B12. While these all demonstrated growth without these vitamins, there was a marked increase in colony density formation of vitamin spots relative to plate regions not containing vitamins. The remaining isolates did not show any variance in growth. Of interest, no isolate demonstrated worse growth in the presence of antibiotics.

The relevance to algal culturing hinges on the scale at which this is being attempted. At laboratory scale, all media should contain these antibiotics, however, when large-scale biomass production is being investigated, antibiotic requirement profiling should be performed, to optimise media cost: biomass productivity.

Antibiotic screening and heterotrophic ability

From the initial axenic algal library, variance across isolates was found in terms of their ability to not only grow heterotrophically, but also in their ability to metabolise various antibiotics (**Table 1**). For an isolate to be applicable to further investigation, it must not only demonstrate heterotrophic ability, but also have a certain antibiotic resistance/susceptibility profile. Here, specifically, isolates must demonstrate no growth inhibition in the presence of cefotaxime, as this is necessary for clearing of *R. radiobacter* following transfection should stable gene insertion be a prospect. Additionally, isolates should demonstrate susceptibility and a differential in growth inhibition to the antibiotic G-418, as is applicable here, to allow for selection of those cells demonstrating stable gene insertion, following G-418 selection. Additionally, isolates must demonstrate good growth when exposed to kanamycin, as this promotes selection of *R. radiobacter,* while acting as a broad-spectrum preventative measure against contamination by foreign bacterial species, mitigating the risk of contamination in future experimentation in which enriched heterotrophic media is used for algal culturing. As, such, several isolates demonstrated correct responses to these prerequisites. These isolates include MPA 40.1, MPA 53.1, MPA 5.1, MPA 18.2, MPA 16.1, *Scenedesmus sp*., MPA 49.1. All of these were then focused on in future experimentation.

Table 1. Algal library screen for heterotrophy and native antibiotic resistance.

	Heterotrophic plate	Cefotaxime (200mg.L^{-1})	Kanamycin (Kan') (40mg.L^{-1})	Kan' (60mg.L^{-1})	Kan' (80mg.L^{-1}	G-418 (20mg.L^{-1})	G-418 (40mg.L^{-1})	G-418 (60mg.L^{-1})	G-418 (80mg.L^{-1})
MPA 46.1	★★	★★	★★	★★	★★	★★	★★	★★	★
MPA 40.1	★★	★★	★★	★★	★★	★★	★★	★	0
MPA 53.1	★★	★★	★★	★★	★★	★★	★★	★	0
MPA 11.1	★★	★★	★★	★★	★★	0	0	0	0
MPA 5.1	★★	★★	★★	★★	★★	★★	0	0	0
$B_3\alpha_2$	★★	★★	★★	★★	★	0	0	0	0
MPA 18.2	★★	★★	★★	★★	★★	★★	★	0	0
MPA 16.1	★★	★★	★★	★★	★★	★★	★★	★★	0
Sc'. Sp	★★	★★	★	★	0	0	0	0	0
MPA 15.1	★★	★★	★★	★★	★★	★★	★★	★★	★
MPA 49.1	★★	★★	★★	★★	★★	★	0	0	0
MPA 23.1	★★	★★	★★	★★	★★	★★	★★	★	★
MPA 33.1	★	★	0	0	0	0	0	0	0
MPA 10.2	★	★	★	★	0	★	0	0	0
MPA 39.2	0	0	0	0	★	0	0	0	0
$B_1\beta$	★★	★★	★★	★	0	0	0	0	0

Figure 14 legend. Relative growth. 0 indicates no green. 2 stars indicate pronounced green. 1 star indicates slight green.

It was of interest to assess whether *C. vulgaris* UTEX395 would respond to growth inhibition by the selective antibiotic, hygromycin, as this would be selectively metabolised by transformed cells post transfection with the pCAMBIA1301 vector. Here, complete growth inhibition was found to occur at a hygromycin concentration of 15 mg. L^{-1} (**Figure 14**). Interestingly, growth inhibition displayed some degree of variance across plates up to the point of total inhibition, where total CFU formation was completely inhibited. This, however, was not seen when complete growth inhibition was achieved. Should this method be attempted for the generation of stable genetically modified strains of microalgae, this region of selection variance may require further investigation via plating on subsect concentrations of the selective antibiotic, should transformants not be generated at the initial complete inhibition concentration. This is due to the relative rate of resistance enzyme expression, where catalytic breakdown of the selecting agent—here hygromycin—relates to enzyme transcription and cell survival in transformed cells. Indeed, in the majority of transformants generated, expression levels are still too low to allow for sufficient resistance due to low level of selective agent catalysis due to low levels of enzyme transcription.

As a further consideration, selection coefficients may be strongly affected when native resistance is investigated with different growth media compositions or even solidified versus liquid media (data not shown).

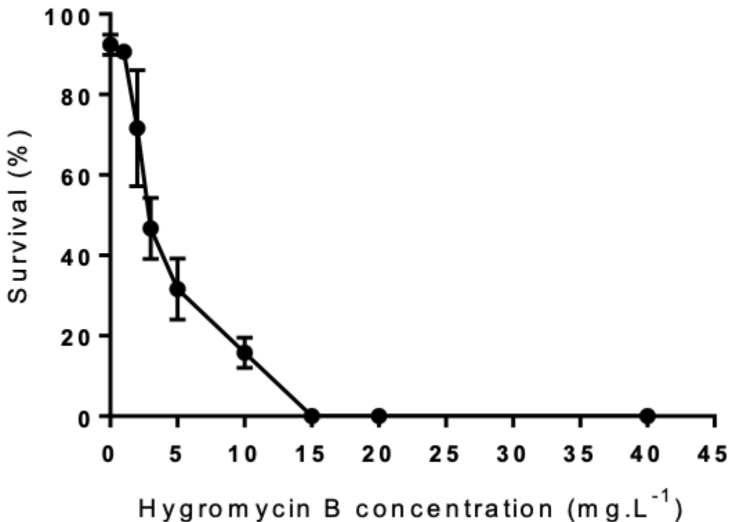


Figure 15. Colony survival of *C. vulgaris* UTEX395 relative to increasing concentrations of hygromycin B

Cryogenic storage and revival

Seventeen Mpumalanga algal library isolates that were revivable (MPA 15.1, MPA 6.1, MPA 50.2, MPA 5.1, MPA 39.2, MPA 23.1, MPA 28.1, MPA 16.1, MPA 46.1, MPA 40.1, MPA 49.1, MPA 33.1, MPA 10.2, MPA 11.1, MPA 18.2) as well as *Scenedesmus sp. and C. vulgaris* UTEX 395 and the isolate originating from Cape Town's Table Mountain; (GHD1). This was assessed qualitatively; the high degree of revivability across the library as a whole may in part be due to the high cell numbers that were placed in cryogenic storage, due to having been grown in enriched LB medium. This highly enriched media may in part also have a direct influence on individual algal cell integrity, allowing for enhanced resistance to freeze thaw stresses encountered during cryogenesis.

The ability of an algal species to be cryogenically stored and revived is highly significant should a species be employed for batch production at scale, as this allows for negligible batch-to-batch genetic variation in the algal production strain to be generated, through aliquot storage and initial biomass generation from identical cryogenic stocks. This is not possible in other eukaryotic heterologous protein production systems, but is highly beneficial when safety, batch-to-batch variation and repeatability are of the highest concern, as is the case in the manufacture of highly complex protein-based therapeutics, where any process variation can have far-reaching impact on product fidelity, functionality and safety.

Heterotrophic and mixotrophic growth

Following from isolate growth profiling of the axenic library, isolates MPA 16.1, MPA 5.1, MPA 49.1, *Scenedesmus sp*. and *C. vulgaris* UTEX 395 were selected for further investigation. It was found initially that when the isolates MPA 5.1, MPA 49.1 and *Scenedesmus sp.* were grown mixotrophically on various media.

These included:

- 3N BBM minimal media
- 3N BBM minimal media containing 10 mM glucose and glycerol
- 3N BBM minimal media containing 10 mM glucose and glycerol, yeast extract and tryptone

Here, each isolate demonstrated significant growth variation after 7 days to each media type (**Figure 15**).

Interestingly, both MPA 5.1 and the *Scenedesmus* sp. demonstrated a marked increase in growth in the highly enriched media containing the addition of yeast extract and tryptone, while MPA 49.1 showed a slight decrease in cell numbers in this media. This growth decrease, though still statistically significant, was not as marked as that demonstrated for the other isolates.

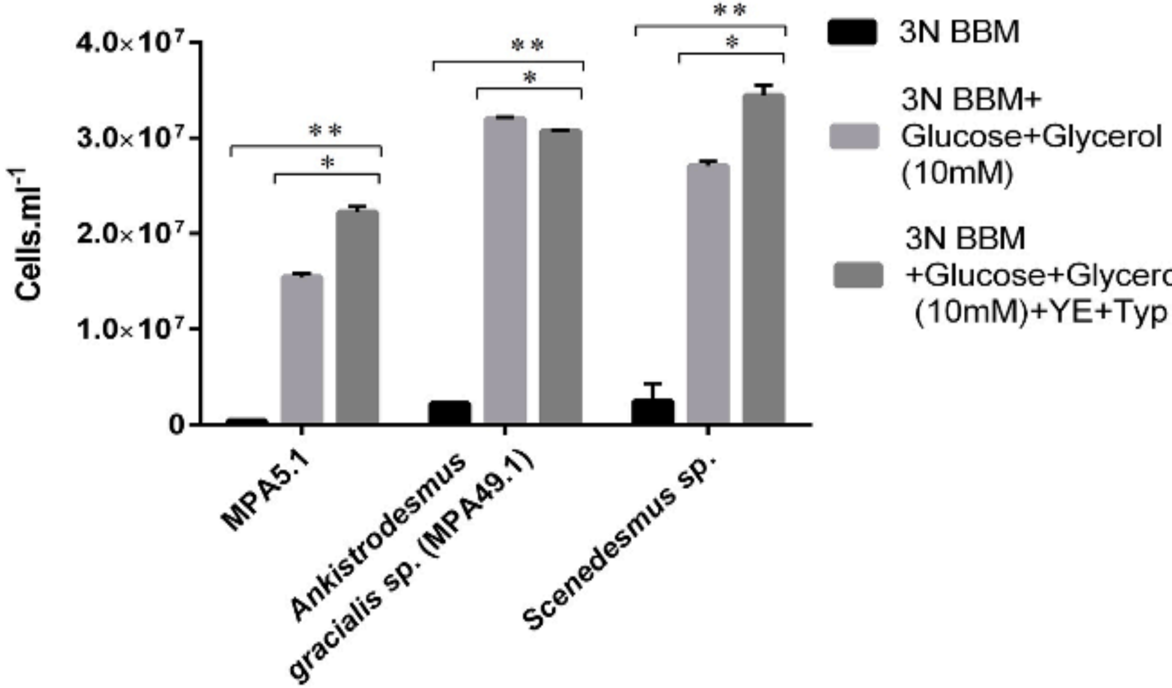


Figure 16. Cell densities of various microalgal species comparing growth in autotropic and heterotrophic media as well as media supplementation after 7 days. Stars indicate highly significant variation using Student's T test at p=0.001

Following this the isolate MPA 16.1 was investigated for the relative ability to produce colonies of different sizes on different solidified media (3N BBM versus heterotrophic media; as previously defined).This experiment was also done using mixotrophic conditions, and though cell colony survival was the same (CFU = 47), colony size formation was markedly different on enriched heterotrophic media relative to 3N BBN (**Figure 16**), where colonies are often several orders of magnitude larger and therefore faster growing than on 3N BBM minimal media.

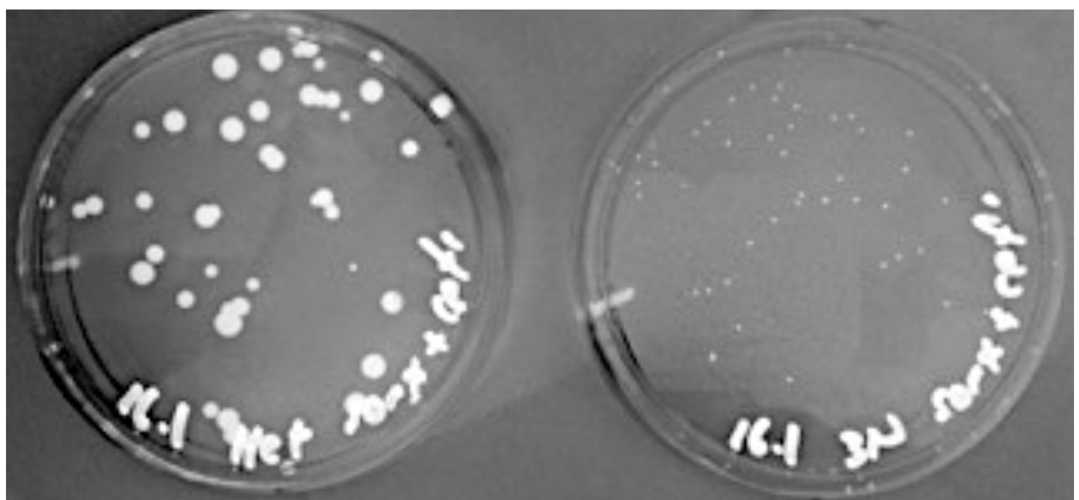


Figure 17. Colony survival vs. growth response of MPA16.1 (*Desmodesmus sp.)* plated on Het' media (left) and 3N BBM media (right) with 200mg. L^{-1} cefotaxime in each (see **Appendix recipes**)

As a last investigation into media requirements of these algal species and isolates, to allow for them to grow and produce proteins in the non-photosynthetic internal environment of the column, MPA 16.1, MPA 5.1 and MPA 49.1 as well as the *Scenedesmus sp.* were drop plated into solidified heterotrophic media, TAP media and LBB media, all of which contain a carbon source to allow for heterotrophic growth. One plate of each type was then placed in the dark, to assess heterotrophic growth, and one was kept under light, to assess mixotrophic growth of each species or isolate (**Figure 17**).

Here, it was found that the heterotrophic plates placed in the dark demonstrated good growth across all isolates, with comparable growth, mixotrophically. Interestingly, MPA 49.1 demonstrated worse growth, here. As a postulate, this could possibly be explained by this isolate having respirative oxidative phosphorylation pathways incompatible with those employed during photosynthesis.

In contrast to the above, all cultures demonstrated better mixotrophic growth on solidified LBB media relative to heterotrophic growth, in the dark.

Lastly, cultures plated onto the TAP plates showed very limited growth, with *Scenedesmus sp.* as well as MPA 49.1 having no mixotrophic growth, but limited growth, heterotrophically.

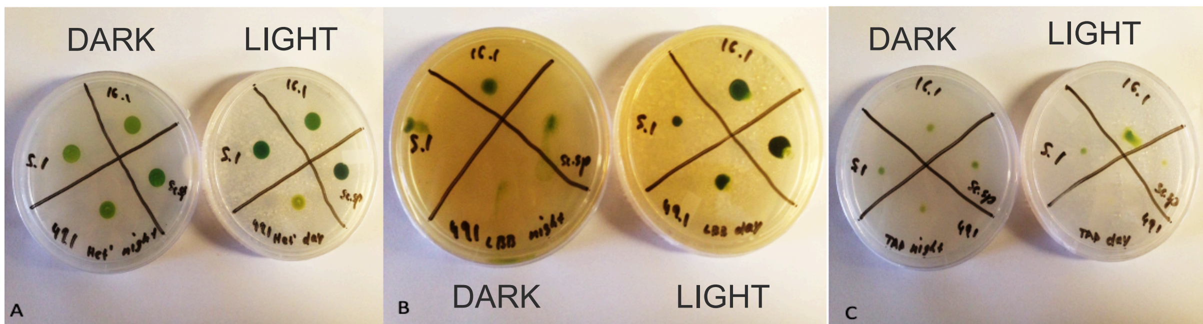


Figure 18. Mixotrophic vs. heterotrophic microalgal growth variation on various media. In A, heterotrophic solidified media, in B, LBB solidified media and in C, TAP media. Top quadrant, MPA 16.1 (*Desmodesmus. sp*.), Left quadrant MPA 5.1, Right quadrant, *Scenedesmus sp.* (QUCCCM63) and bottom quadrant MPA 49.1 (*Ankystrodesmus gracilis*). Plate on left kept in dark, plate on right, kept in light.

These observations could be explained by the relative ease with which metabolic shifting can occur relative to different carbon sources. As a postulate, as all these cultures were originally grown in 3N BBM, lacking a carbon source, when they were drop plated onto enriched solidified media, selection coefficients for certain plate types may be too strong, and require more generations for real assessment of isolate growth ability, such as with the TAP plates.

As a last initial investigation into growth rates, it was of interest to combine heterotrophy with antibiotics. This was done using the algal species, *C. vulgaris UTEX 395,* using TAP media and 30mg.L^{-1} kanamycin (**Figure 18**). This was only investigated with *C. vulgaris UTEX395,* as this isolate was employed as the model organism for this research.

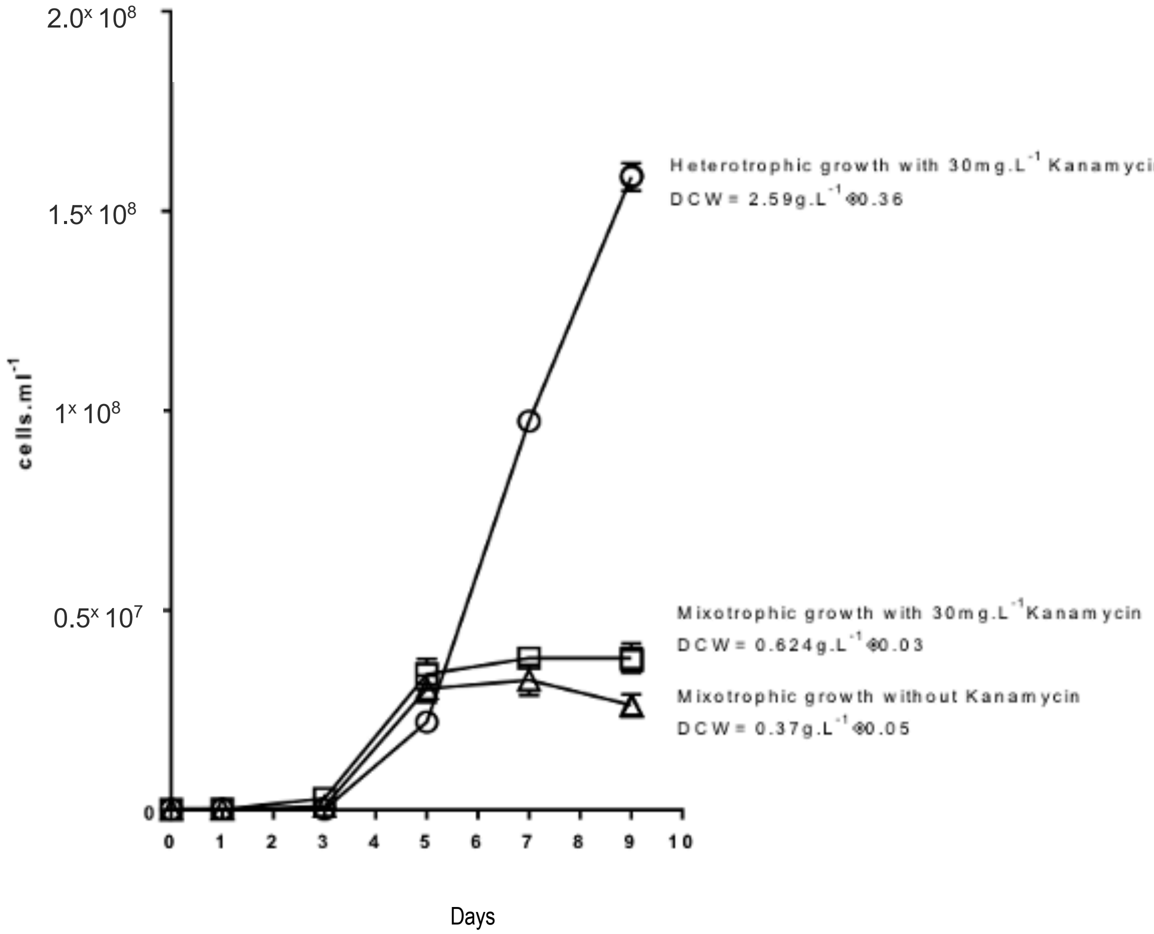


Figure 18. *C. vulgaris* UTEX 395 mixotrophic vs. heterotrophic growth (cells. mL^{-1} and corresponding DCW per data point/ sample)

A marked increase in cell density was observed post day 3 in the heterotrophic culture, containing 30mg.L^{-1} relative to the other two cultures that were grown mixotrophically, in the presence of light. Interestingly, these two cultures had comparable growth, regardless of whether kanamycin was present or not. This observation was further reinforced by the measure of DCW in the three cultures at day 9 (heterotrophic culture with kanamycin = 2.59 $\pm$ 0.3g.L^{-1}/ mixotrophic culture with kanamycin = 0.624 $\pm$ 0.3 g.L^{-1} / mixotrophic culture without kanamycin = 0.37 $\pm$ 0.05 g.L^{-1}).

This kanamycin-related, heterotrophy (complete dark growth) relative to mixotrophic growth (complete light exposure) increase in growth response is explainable when one considers that it has been established that algal blooms may be induced by external factors, such as other organisms present in the environment, such as viruses, fungi and bacteria, as well as quorum-type sensing of biologics released by these organisms, such as kanamycin (Schvarcz and Steward, 2018b). This becomes especially plausible when it is realised that antimicrobial agents commonly originate in members of the fungal family. The relevance of this in terms of heterotrophic growth is that it may be an evolved algal response to evade fungal overgrowth when in environments containing a high degree of reduced carbon. This is not a newly

observed phenomenon, however, where algal growth has been observed to improve with increasing concentrations of antibiotic compounds (Jones et al., 1973). An interesting study would be to look at gene expression in these cultures, following bloom induction, though this was not within the scope of this investigation.

4.3.3 Species identification via 18s ribosomal subunit sequencing

After sequencing and species Blast of the algal isolate sequences, MPA16.1, MPA49.1 and *Scenedesmus sp.* were identified as follows: MPA16.1 was identified as *Desmodesmus sp*. MPA49.1 was identified as *Ankystrodesmus gracilis,* and *Scenedesmus sp.* was identified as Scenedesmus sp. (QUCCCM63). Of the region sequenced, all showed an E value of 0.0 however, a good reverse read was not obtained for *Desmodesmus* sp., though forward and reverse reads were obtained for the other two species.

4.4 Conclusion

The algae library isolated by the South Africa Council for Scientific and Industrial Research (CSIR) from the province of Mpumalanga was selected for this research due to its suspected high degree of biodiversity and elevated growth rates due to coming from a warm region. Mpumalanga is located close to the equator and experiences very little temperature fluctuation, making it conducive to the supposed evolution of algal species that can tolerate high temperatures and exhibit higher rates of biomass production. This is an autotrophic algal library and as such had to be made axenic to explore heterotrophy. As such, it was necessary to induce individual isolates into axenic conditions, which was essential for heterotrophic assessment; a necessary requirement for later investigations into the impact of isolates when incorporated into a light-impervious column system and the necessity of heterotrophy.

The diatomaceous earth-mediated cell pack is a dark internal space, and algal species must swap from photosynthesis to oxidative phosphorylation, which requires the presence of enriched media. However, this media can facilitate overgrowth of the fungal and bacterial contaminant species present, which was overcome by physically and chemically separating individual colonies through helping hands colony picking and 2D plate selection methods. This resulted in an axenic library consisting of 18 species, all of which were amenable to cryogenic preservation.

Furthermore, 18S sequencing of the most favourable isolates for further investigation resulted in the sequence identification of *Ankystrodesmus gracilis* (MPA 49.1), and the *Scenedesmus sp*. QUCCCM63, as well as the *Desmodesmus sp*. (MPA16.1). Additionally, *Chlorella vulgaris* UTEX 395, which was also coaxed into an axenic state, exhibited pronounced heterotrophic growth profiles, especially in the presence of the antibiotic, kanamycin.

From the above investigation into properties possessed by various microalgal species, it is of utmost importance that species be selected for a library as quickly as possible, so as to limit the number of species worked with. Here, a comprehensive understanding of the most important parameters required by those species to be worked with as well as the specific application that they are to be employed in, must

inform species choice. These parameters may often be resource and labour intensive to investigate but are often unavoidable. Specifically, it would have been a prudent choice to first screen the library for multivariate antibiotic resistances, this however could not be attempted first as the presence of contaminating biology would skew results when investigated in concert with enriched media, which has an additional effect on antibiotic efficacy.

Additionally, as heterotrophy exists to an unknown quantity in novel algal libraries, axenic conditions were not developed initially, but developed post hoc, by fitting heterotrophic media compositions for specific isolates, which may be applicable, but was not an option here, as the most important parameter for an isolate to be included in the study was its ability to demonstrate growth in the dark environment of the column.

5 Diatomaceous earth-based column development and microalgal biomass interactions

5.1 Chapter introduction, overview and aims

Having developed various South African microalgal isolates and model species into a functional and working cryogenically preservable library (all species were revivable after 3 months on Het' plates; -80℃ storage in 2 mL Eppendorf tubes, straight freezing after being grown up in Het' media to confluence), as well as demonstrating the numerous availabilities of these isolates and species to withstand exposure to antibiotics and varied other conditions for growth, this library was ultimately investigated for the application in a novel transformation and expression system; that is, the diatomaceous earth-mediated algal cell pack. This application sought to draw parallels with what has already been implemented in *N. benthamiana* whole plant transgene expression, as well as with induced plant cell cultures, but using microalgal biomass, with diatomaceous earth as a matrix for support of these smaller cells. This chapter investigates the formation of a porous diatomaceous earth-based column, where microalgal biomass is interspersed throughout. This column was easily created using an initial suspension of microalgal cells and diatomaceous earth, which when passed through a crude filter removes media, leaving behind the matrix-mediated algal cell pack.

The system detailed and developed in this thesis aims to allow for a similar cell distribution as would occur in the leaves of *N. benthamiana*, with the exception of using numerous microalgal cells interspersed within the porous diatomaceous earth-mediated column. Regarding this novel system, important questions included whether these porous columns, with interspersed microalgal cells, mimic a plant leaf, and to what degree scaling in x, y and z dimensions can be achieved. Also, to what degree can heterotrophic microalgae survive inside the column environment relative to microalgae that are not heterotrophic, requiring photosynthesis for survival?

It was also important to investigate the particle size distribution of diatomaceous earth Celite®545, and how this particle size distribution relates to that of the different microalgal species. As an aside to this, it was also important to investigate the degree to which column formation could be used to harvest various densities of microalgae for the application of harvesting wild-type microalgae for other biotechnology applications. Lastly, it was also important to investigate how column formation could be used to investigate ecological samples containing microalgae and then split them up according to cell size. This has multiple applications but will be discussed in later in the chapter conclusion.

This chapter will give detail to the following concepts:

- Investigating various methodologies used to create these columns.

- How different algal isolates and species respond to the column environment over time.
- How the column harvest efficiency is affected by cultures of decreasing cell density.
- How smaller lab-scale columns can be created in a highly efficient manner for the screening of, for instance, isolates from an algal library for their ability to produce a specific protein of interest.

5.2 Materials and methodology

5.2.1 General system and setup

The requirements and final outcome of the system are to form a porous column, with microalgal cells interspersed throughout this porous column. The column is supported with diatomaceous earth, which not only supports the biomass but also acts as reservoirs for media replenishment. These porous columns, named the algal cell pack, once formed allow for vacuuming of fresh heterotrophic media to maintain algal biomass, but also for the replacement of media with various possible other solutions. Media containing transforming *R. radiobacter* is explored later.

For the formation of the matrix-mediated cell culture system, all work was done in a laminar flow hood. The system itself comprises a pneumatic electric vacuum pump, a vacuum tube connected to a collection vessel, which was connected to a primary vacuum tube into which was placed a modified blue 1 mL pipette tip, of which the end was cut off to fit the BioRad chromatography tubes. This end could be modified as needed, for various applications (**Figure 20**). This was the general setup for most of the investigations for the purpose of this thesis. Two clamps were put onto a retort stand. The first clamp held the collection vessel, while the second clamp was used to affix the chromatography tubes. Into each chromatography tube was placed 0.75g of diatomaceous earth Celite®545, which provided the particle uniformity and settling rate required for experimentation relative to other brands and types, which were investigated but didn't prove their utility, as well as 5 mL of an actively growing axenic algal culture, which are suspended with this suspension vacuumed onto a filter. This filter varies depending on the nature of the column being formed (**see later**). Once porous algal-Celite®545 columns were formed, the various experiments and investigations were performed.

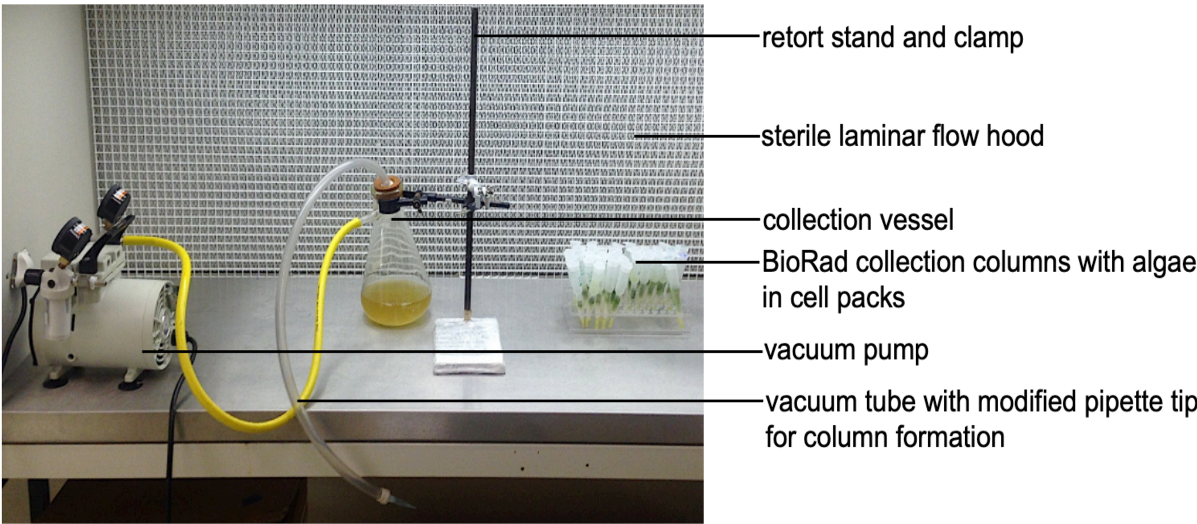


Figure 19. System Setup.

5.2.2 Diatomaceous earth Celite®545 analysis

The various requirements of the matrix support for formation of the algal cell pack are as follows:

- Non-toxic to microalgae.
- Have a narrow particle size distribution that exceeds that of microalgal cell size distribution.
- A sedimentation rate that is faster than that of microalgal biomass, but not so fast that column formation cannot progress quickly enough without it sedimenting out of suspension.

Particle size distribution of diatomaceous earth Celite®545 was investigated with the use of a Malvern Mastersizer 2000, which works by red and blue laser diffraction as well as forward and side laser light scatter to assess percentages of a sample that fall into different size categories. This is also output as a particle size distribution based on particle volume. This apparatus allows for total particle size discrimination across a sample of various mass. Transformations can be carried out to convert this to a diameter basis based on assumption of spherical particles.

5.2.3 Laboratory-scale column investigation

Following initial investigations into algal column formation without matrix support, it was found that these were too dense to be useful due to small cell-size, and high packing density. At this point it was necessary to investigate diatomaceous earth, to be used to form a porous matrix.

For laboratory-scale columns that allowed for the workability of many algal cultures simultaneously, consecutive preliminary testing of a number of columns resulted in BioRad Poly-Prep Chromatography columns being chosen. This is the ideal column set up because BioRad Poly-Prep Chromatography columns provide a 12 mL working

volume and have a 30 µm polypropylene bed support for fine particulate retention. This acts as the bed support for the various small-scale algal cell packs.

To determine whether the columns could maintain algal growth, two species were selected that demonstrated an ability, or lack thereof respectively, to grow in the dark, heterotrophically. The selected algae species were *Desmodesmus* sp. (MPA16.1) and MPA33.1.

A 6 mL aliquot of axenic cultures of *Desmodesmus* sp. (MPA16.1) and MPA33.1 were grown to mid-exponential phase (OD_{600}=1). To each was added 0.7 g autoclaved Celite®545, which filled the working chambre of the BioRad tubes. All work was done under sterile conditions using sterile techniques in a laminar flow hood. This cell-Celite®545 suspension was then poured with shaking into a BioRad Poly-Prep column under vacuum, retaining cell-Celite®545 in the form of a porous matrix, while simultaneously removing excess culture liquid. Then, columns were capped to retain a humid environment. The columns were pulse fed every day with 1 mL of heterotrophic medium under vacuum. After 10 days algal cell packs were removed from chromatography columns and were cut transversely at which point, algal growth was assessed qualitatively.

5.2.4 Large-scale column investigation

A 200 mL aliquot of axenic algal isolate, *Desmodesmus sp.* (MPA16.1) was grown to a cell density of $2x10^7$ cells. mL^{-1}. To this, 80 mL (188.8 g) of sterile autoclaved Celite®545 diatomaceous earth was added. While shaking, this algal cell-celite suspension was poured into a 250 mL graduated polypropylene measuring cylinder. This previously had a hole drilled in the bottom. This cylinder was plugged with a crude filter prior to culture-diatomaceous earth suspension addition, consisting of cottonwool, for moisture retention, and Miracloth (pore size: 20-25 µm. Merck Millipore; 475855). As the culture was added, the addition of vacuum was used to allow for porous algal-celite column formation.

5.2.5 Column harvest efficiency of algal biomass

Here constant Celite545 and constant algal biomass was diluted in increasing volumes of diluent, with cells then being counted to assess if media concentration affects the efficiency that algal cell packs allow for biomass harvesting.

One culture of *Chlorella vulgaris* UTEX395 (OD_{600}=1), grown in axenic heterotrophic media was divided into 15 cultures of 10mL each. These were added to 2g sterile Celite®545, and diluted thus: 0 mL dH_20 (no dilution), 10 mL dH_20, 70 mL dH_20, 310 mL dH_20 and 1270 mL dH_20. Each of these cultures were then formed into algal cell packs using BioRad Poly-Prep columns (as above), under vacuum.

Each one was then resuspended in 15mL dH_20 and cells were counted using a counting chamber.

5.2.6 Microalgal column survival

To assess microalgal cell survival in the inter-column environment, with or without the addition of *R. radiobacter*, dark-adapted, *C. vulgaris* UTEX395 was grown to mid-exponential phase (OD_{600}=0.569), and formed into 24 matrix-mediated cell packs (as previously described in Section 4.2.3). Half of these cell packs were then vacuum infiltrated with 1 mL of *R. radiobacter* (LBA4404 pCAMBIA1301, OD_{600}=0.75)(please see R. radiobacter preparation in Appendix recipes), with microalgal growth then being assessed in triplicate on days 3, 5, 7, 9. This was done by resuspending columns in sterile falcon tubes containing 15 mL sterile PBS solution. After column resuspension, Celite545 was allowed to sediment out for 10 min. Following this, 10 µL of the suspension was spread plated onto TAP plates containing 30 mg.L^{-1} kanamycin (to suppress growth of *R. radiobacter* and other bacteria), and allowed to grow for ten days, at which point microalgal colonies were counted.

5.2.7 Investigation of green microalgae separation by size

A 150 mL aliquot of water was sampled from a stream flowing off Cape Town's Table Mountain, making sure to avoid debris, in three 50 mL Falcon tubes. At the laboratory, 7.5 g.L^{-1} glucose was added to each of these sample and incubated at 4°C, to enable fungal spore germination and to prevent bacterial overgrowth respectively. Prior to this, dry diatomaceous earth (Merck, Celite®545) was packed into a plastic syringe with a filter (165mL, height 15.5mL, 3.37cm diameter) and sterilised in the autoclave. Following overnight incubation of the environmental samples, all of them were vacuum infiltrated through the diatomaceous earth column. After which, 100mL of algal heterotrophic nutrient medium was vacuumed through the column (1:1, 3N Basal Bold's Medium: LB + 7.5g.L^{-1} glucose). The column was exposed further to vacuuming until visible interstitial air spaces formed. After the nutrient media was removed from the column, both ends of it were sealed with parafilm and the column was left under fluorescent lights.

Ten days post column formation, green colonies could be seen on the surface of the column. At this point, the column was removed from the syringe, and the top two centimetres of the column were cut away with a scalpel and discarded (This region contained trapped germinated fungal mycelium and larger zooplankton). Moving down the column, two centimetres sections of the column were removed from the region where green algal colonies could be seen, as well as the bottom two centimetres of the column, which also contained algal colonies.

Each of these two column sections were then resuspended in 20 mL 3N BBM with vigorous vortexing which brought about column disintegration and dissociation of the algal cells from the diatomaceous earth matrix. Each of these two cultures were incubated at room temperature to allow for Celite®545 to settle out of suspension (20-30min). A 100 µL aliquot of each sample was plated onto minimal medium plates (2% agarose, 3N Bold's Basal Medium) and colonies were allowed to form (RT and 24 hr light). One week later, plates were visualised under light microscope and photographed.

5.2.8 Viral lysis induction via algal cell pack incorporation

Viral lysis was studied with the use of algae cell packs, which were made by constructing flat packs of five centimetres in diameter and one centimetre in height. Algal isolate MPA16.1 (*Desmodesmus spp*.) was added, in the hunt for viral lysis induction and viral particle discovery. White plaques could be seen on the flat columns after these packs had been allowed to dry for two weeks.

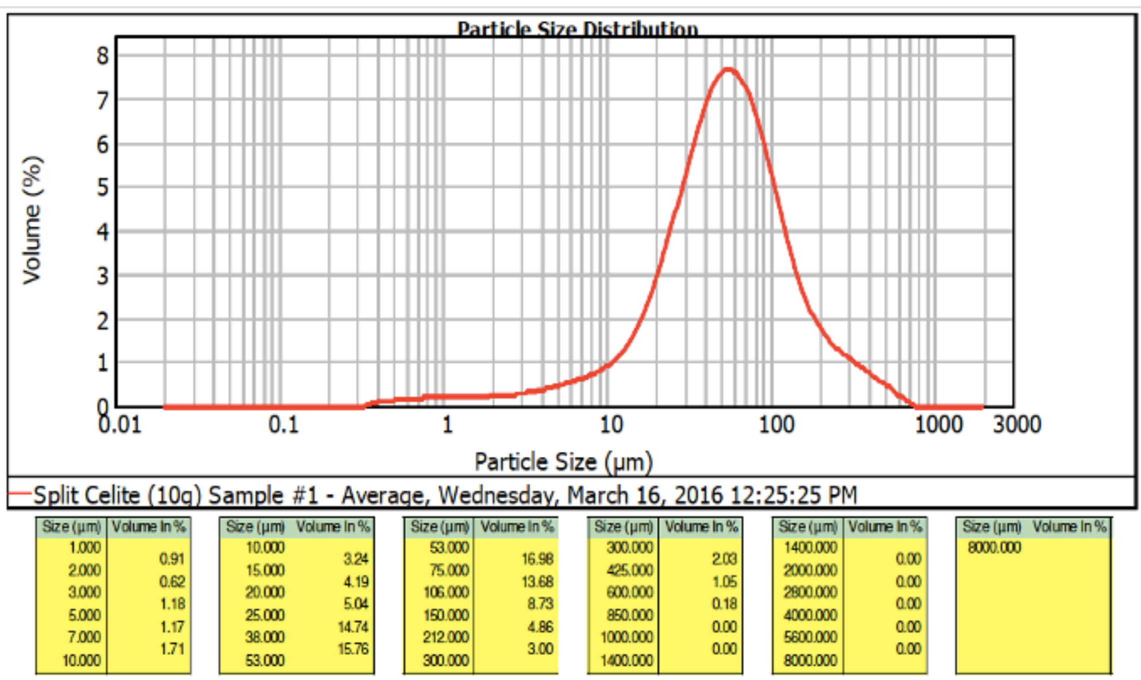
After purifying these plaques via resuspension in buffered solution and removing the suspended debris, a transmission electron microscope (TEM) was used to image the viruses. Understanding viral lysis in algae and whether the algal cell pack with its slow drying effect could elicit latent viral sequences into a lytic state, were the goals of this investigation.

5.3 Results and discussion

5.3.1 Diatomaceous earth Celite®545 analysis

Three diatomaceous earth types were investigated as matrix support for the algal cell pack. Two were low-grade non-laboratory formulations, but these were unworkable. The third, Celite®545 (Merck) was therefore investigated further.

The particle size distribution of Celite®545 was determined to favourably coincide with that of algal cells. Here, 73.63% by volume of a 10 g sample was found to be between 10 μm-106μm (**Figure 21**). Of special relevance to the microalgal species worked with, *A. gracilis* (MPA49.1) has a mean cell size of 23.8 by 3.3μm (Sipaúba-Tavares, 2008), *Desmodesmus sp*. (MPA16.1) has a cell length of between 9- 19 μm (Olenina,. 2006), *Scenedesmus sp.* (QUCCCM63) has a cell length of 11- 18 μm (Hegewald,. 1997) and *Chlorella vulgaris* has a cell length of 5- 10 μm (Scagg,. 2003), all of which fall strongly within the Celite®545 particle size distribution.



Size (µm)	Volume In %
1.000	0.91
2.000	0.62
3.000	1.18
5.000	1.17
7.000	1.71
10.000	

Size (µm)	Volume In %
10.000	3.24
15.000	4.19
20.000	5.04
25.000	14.74
38.000	15.76
53.000	

Size (µm)	Volume In %
53.000	16.98
75.000	13.68
106.000	8.73
150.000	4.86
212.000	3.00
300.000	

Size (µm)	Volume In %
300.000	2.03
425.000	1.05
600.000	0.18
850.000	0.00
1000.000	0.00
1400.000	

Size (µm)	Volume In %
1400.000	0.00
2000.000	0.00
2800.000	0.00
4000.000	0.00
5600.000	0.00
8000.000	

Size (µm)	Volume In %
8000.000	

Figure 20. Diatomaceous Earth Celite545 particle size distribution
(Malverne Mastersizer 2000 output data from 10g sample)

It must be remembered that column formation only occurs at the interface of column formation and that of the algal-diatomaceous earth liquid media suspension. An algal-diatomaceous earth size overlap is important here, though density becomes more relevant as algal cells are required to be separated from the denser Celite®545 mass. Diatomaceous earth types in general can have a huge diversity in particle size distribution, as they are of biological origin. Though several types of diatomaceous earth were looked into (data not shown), these either could not achieve porous column formation, sedimented too quickly or did not allow algal cell separation from the column after algal cell harvesting. It was of great importance to utilise diatomaceous earth that could fulfil all these requirements, thus Celite®545 was used for all further experiments.

5.3.2 Laboratory-scale column formation investigation

One of the laboratory-scale columns contained *Desmodesmus sp.,* the other laboratory-scale column contained the algal isolate MPA33.1. After ten days of feeding the two laboratory-scale columns, they were cut transversely (**Figure 22**).

It was found that the heterotrophic species, *Desmodesmus sp.,* had indeed been able to grow in the column, due to the notable green colour. Reassuringly, this column did not show differential growth on the column's exterior, indicating that *Desmodesmus sp.,* switched preferentially to oxidative respiration of the heterotrophic media, in favour of photosynthesis. However, this was, not the case with algal isolate MPA33.1, which showed pronounced die-off of algal biomass in the column's interior but demonstrated biomass maintenance on the column's exterior. The cause of this was the columns

having been incubated under lights, maintaining MPA33.1's metabolic autotrophy via photosynthesis.

These results demonstrate that the feeding of the algal biomass within the column's interior with heterotrophic media also removed dissolved waste products across the column's aspect with no notable variance in media penetration across the column's width or depth. This is important as effective contacting of *R. radiobacter* with algal biomass is crucial for high rates of plasmid delivery and maximisation of transient heterologous protein expression. Further, for maximal protein production, algal biomass must be in an actively growing state.

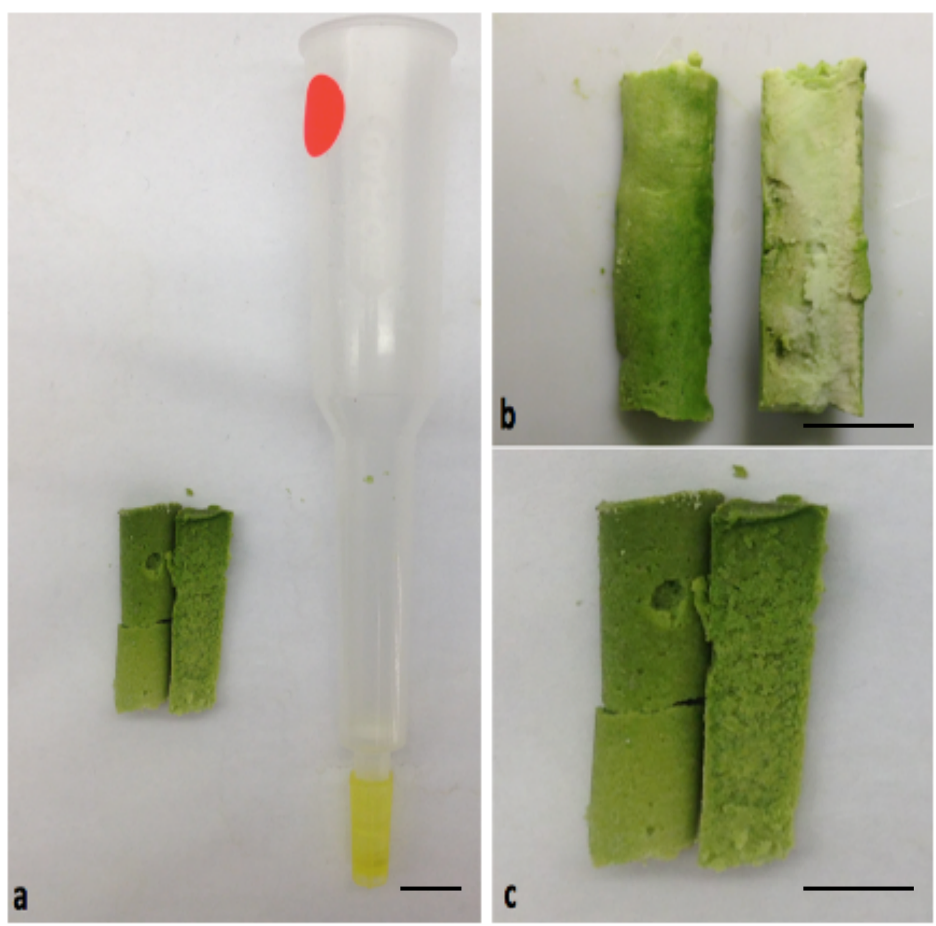


Figure 21. Small column demonstration. Algal cell maintenance post-feeding with the heterotrophic species Desmodesmus sp. (MPA16.1) (**b**) and with the non-heterotrophic isolate (MPA33.1) (**c**). Scale given in (**a**) (scale bars, 1cm).

5.3.3 Large-scale column formation investigation

The formation of a large-scale matrix-assisted algal column was accomplished, such that 80 ml of sterile diatomaceous earth Celite®454 and 200 ml of algal culture yielded a column of 110 ml (**Figure 23**). This was achieved by using heterotrophically growing *Desmodesmus sp.* (MPA16.1). What is noteworthy, is that column formation proceeds from the interface, so as long as the aqueous phase of algal-Celite®545 suspension

was kept suspended with slight agitation, then the interface and column formation progressed up through the collection vessel with little change in impedance, regardless of whether it was taking place at the beginning of column formation at the filter, or further up the collection vessel.

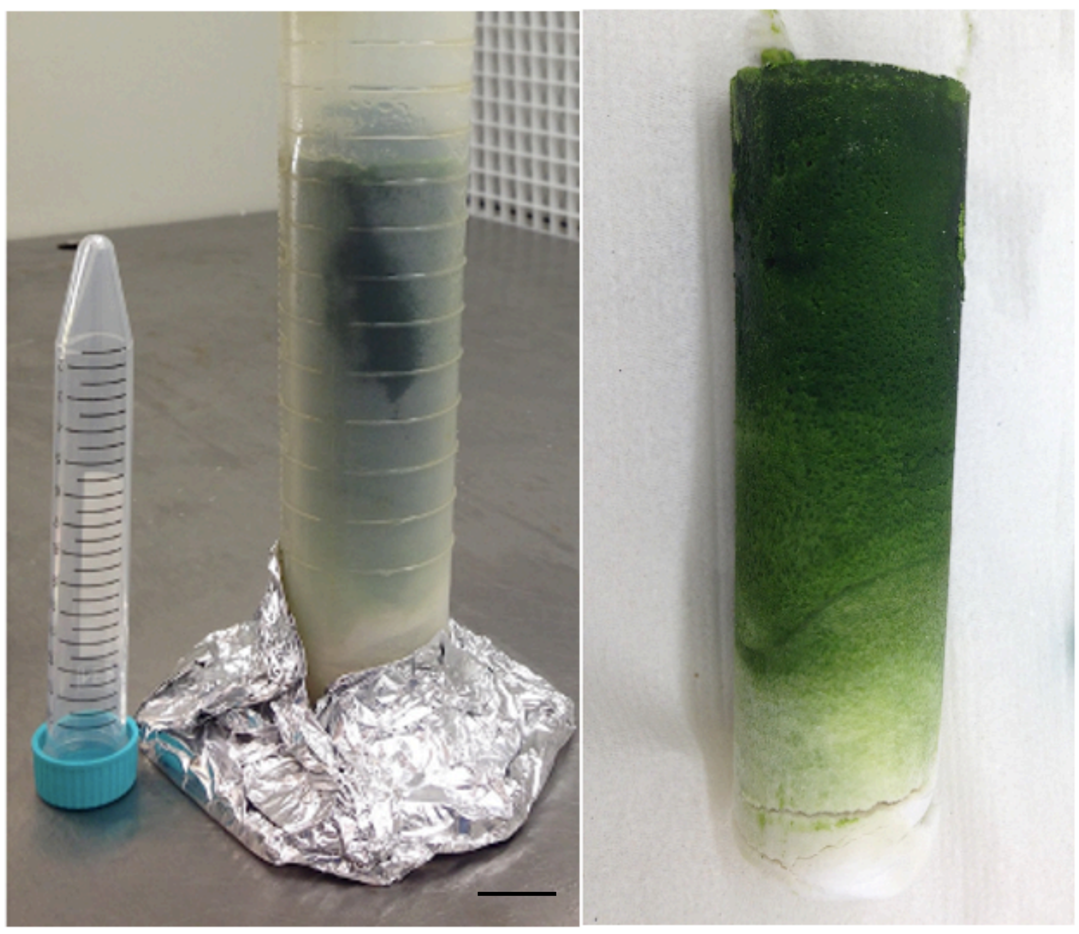


Figure 22. Large Column demonstration (scale bar, 2cm).

When observing the large column, it was observed that some variation in algal cell distribution across the column existed. This can however be ameliorated in industrial settings by incorporation of an impeller into the harvest vessel to maintain algal cell-Celite®545 suspension, minimising volumetric distribution variation of these. Additionally, Celite®545 could be added to the algal culture prior to collection, so that as the newly formed algal cell-Celite®545 composite suspension is delivered to the collection vessel, column formation proceeds immediately, maintaining homogeneity across the newly formed column.

5.3.4 Column harvest efficiency of algal biomass

To further investigate the biomass harvest efficiency of algal biomass during Celite®545 column formation, a constant volume of Celite®545 as well a constant algal biomass was combined with increasing volumes of liquid culture.

It was observed that there was an increase in the harvest efficiency when 320 ml of liquid culture was used, relative to that of 10 ml, 20 ml and 80 ml liquid culture (**Figure**

24). However, there was no increase in harvest efficiency when 1280 ml of culture was used. A possible explanation for this might be, when it is considered that the surface to volume ratio of 320ml column is lower than that of the other columns, so that interface fluid loss, occurring when column formation initially starts, has a decreased impact of cell trapping. However, this hypothesis, was not apparently held when biomass retention of 1280ml columns is considered.

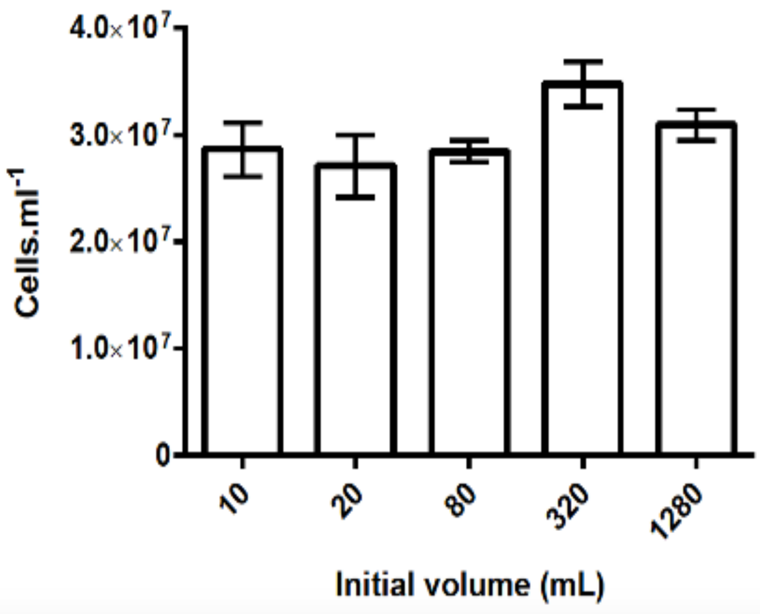


Figure 23. Column harvest efficiency. C. vulgaris UTEX395. Constant Celite545 and constant cell number but increased precolumn media volume

5.3.5 Microalgal column survival in the presence and absence of R. radiobacter

To study the transient *R. radiobacter*-mediated heterologous protein expression by green microalgae, it is necessary to have viable algae in an appropriate growth environment; hence, it was necessary to assess and ensure that microalgae could grow within the Celite®545 column with feeding of heterotrophic media, and that it could occur in the presence of *R. radiobacter.*

Interestingly, on day three it was observed that there was a variance in algal cell survival of columns that did not carry *R. radiobacter* relative to those that did (**Figure 25**). This is likely to indicate a direct influence of algal cell survival due to *R. radiobacter* interactions. Cell survival across column-types however was statistically non-variant by day five. This effect was likely explained by the algal biomass being newly incorporated into the column environment, leading to cellular attrition of susceptible cells and strong selection for those that can withstand not only the column internal environment but also the shift to oxidative respiration required to metabolise the enriched heterotrophic media.

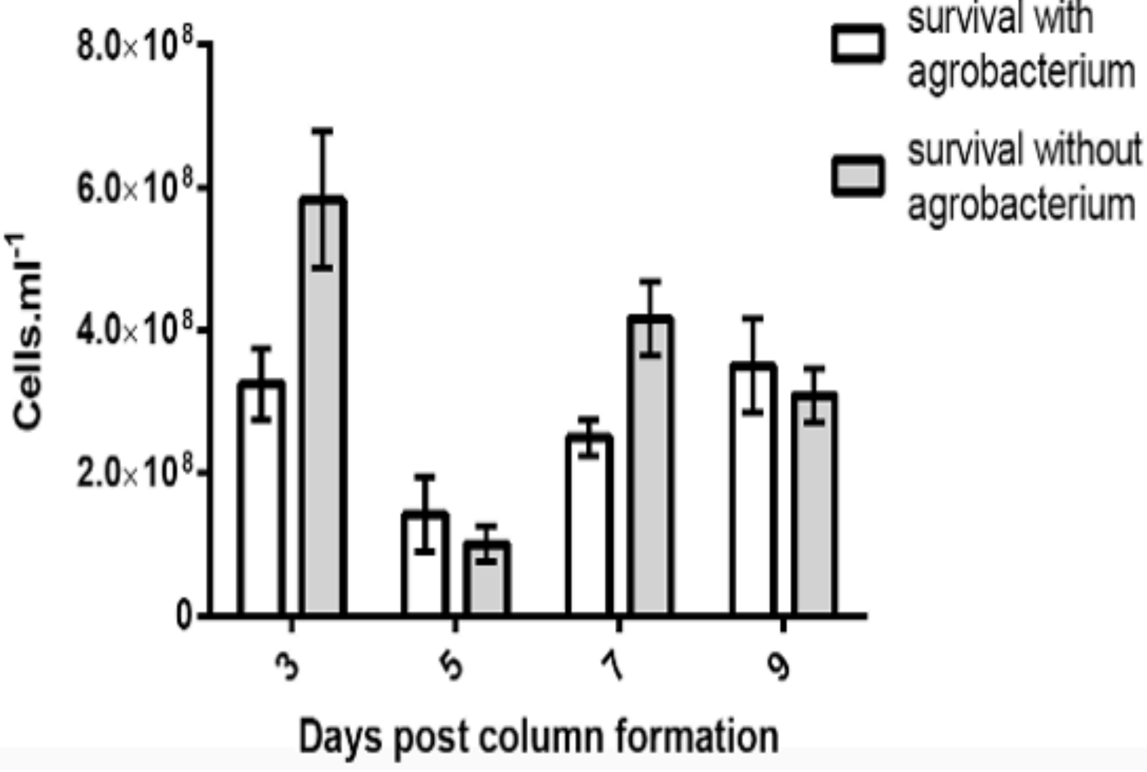


Figure 24. Within column cell survival of C. vulgaris UTEX395 with and without *R. radiobacter* (LBA4404) (D=3 and D=5 demonstrated significant difference. Student's T test, p=0.001)

By day seven, algal biomass in both column types had begun to recover, though there was still a significant variance in algal biomass concentration. This was then completely abolished by day nine, which may indicate why the *R. radiobacter* initially present in the column, had lost potency, or why its effect on the algal population has been reduced to such a degree that growth of the algal population was unhindered.

5.3.6 Separation of ecologically-derived green microalgae by size

The topmost section of the column yielded only a single algal species, which had a very large cell diameter of around 50µm; however, the bottom diatomaceous earth section yielded only small algal cells of around 10µm, but the plate contained well over ten species (**Figure 26**).

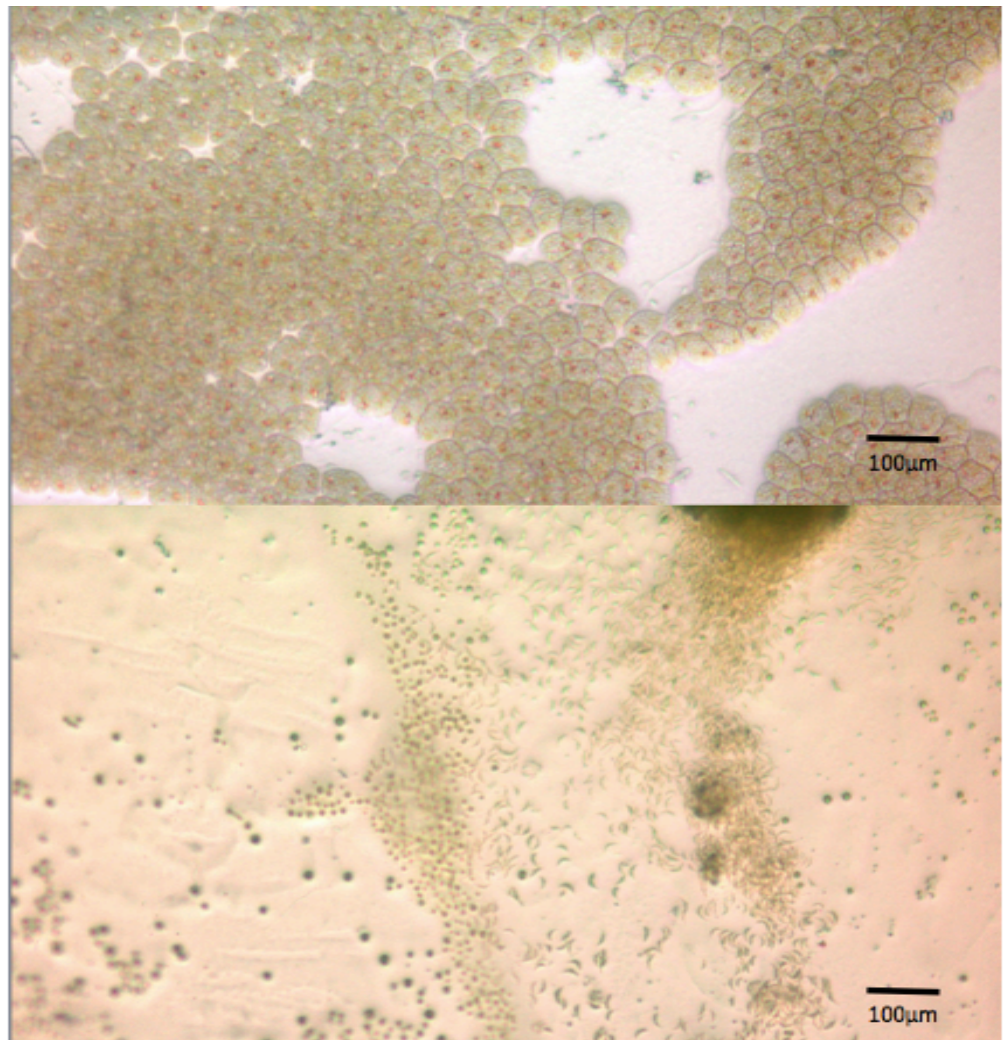


Figure 25. Algal species selection by size. (Top) Only one species of algae was visualized from the top section of the selection column. These were in the region of 50µm in diameter. (Bottom) The bottom section contained none of the large diameter microalgae present on the other plate, however, as many as ten species were present in this column section, but all were of a similar size.

5.3.7 Viral lysis induction via algal cell pack incorporation

White plaque development was noticed after two weeks after integrating the algae into the progressively drying algal cell column, signalling algal biomass die-off (**Figure 27 left**). The plaques were cleaned by resuspension in buffered aqueous solution, and suspended debris was removed by centrifugation in order to further explore this phenomenon. Transmission electron microscopy was then used to image the resultant liquid media for examination (**Figure 29 bottom**).

The micrograph produced by this study showed that there were multiple virus-like particles, that were remarkably similar to the well-known potyvirus, which are 470-1000nm in length, consisting of numerous coat protein monomers, and revealing terminal anti-sense RNA (**Figure 27 right**). In fact, potyviral derived sequences have

been expressed in *N. benthamiana* and used to trigger potyviral resistance in potatoes (Savenkov and Valkonen, 2001).

The discovery of viroid particles shows that viral infection was most likely the root cause of the development of the white plaques. Since viral sequences can be used to induce viral lysis and improve transgene expression through identification of novel promoter sequences, as well as maximal algal growth induction through bloom formation These findings are very encouraging.

Although more research is required to fully recognise the implications of these findings, they may prove very important for the isolation and characterisation of yet to be discovered viruses infecting microalgae.

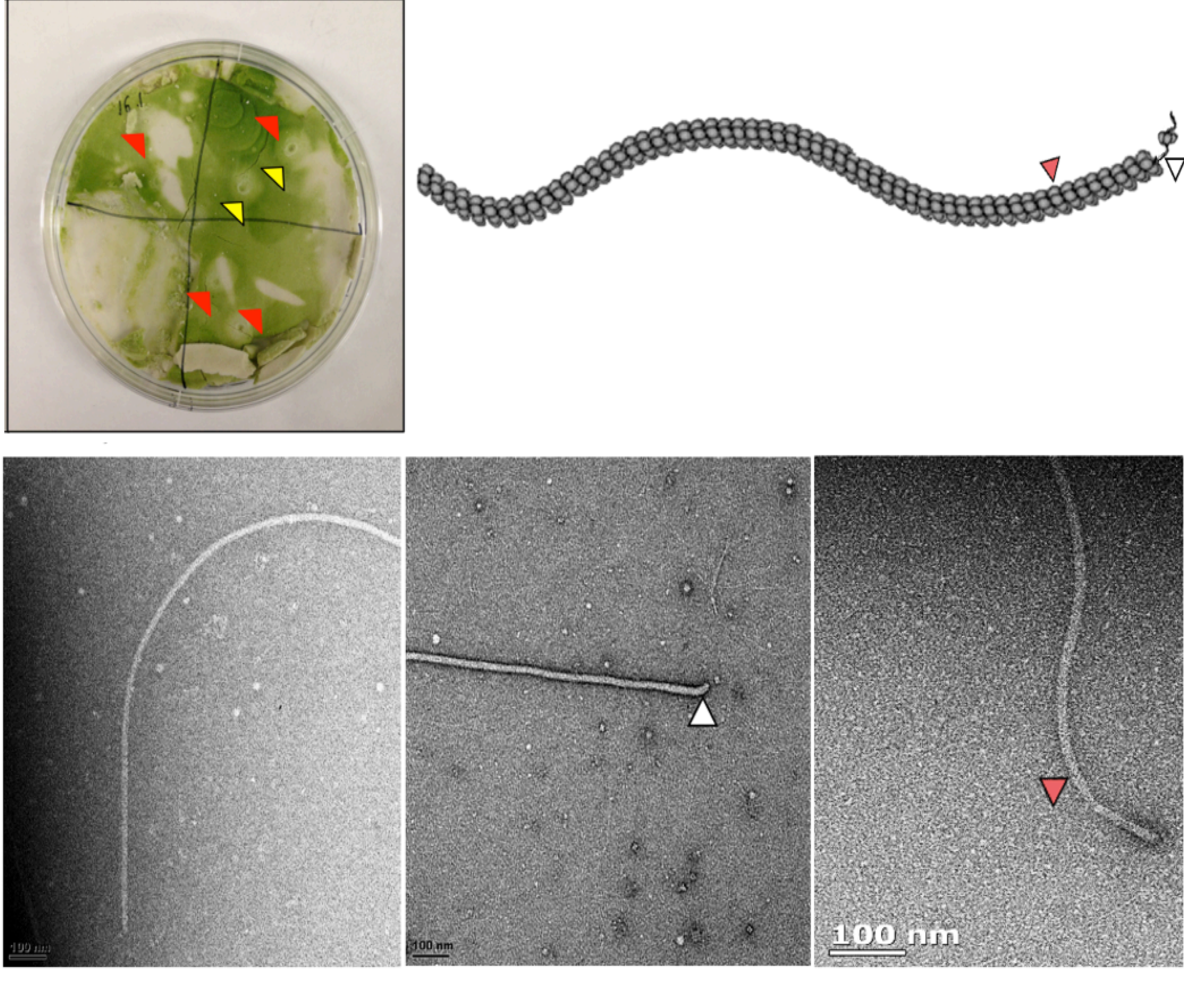


Figure 27. Top left. Plaque formation. Yellow arrows indicate *R. radiobacter* infiltration sites. Red arrows indicate white plaques**. Top right.** Diagrammatic representation of a potyvirus. Red arrows denote coat proteins (CP), white arrow denotes terminal anti-sense RNA (Mishra et al., 2014). **Bottom.** TEM images of purified virus isolated from white plaques. White arrow denotes terminal anti-sense RNA. Red arrow denotes CP.

5.4 Conclusion

This novel method - of incorporating algal biomass into a porous diatomaceous earth-based column allowed for algal biomass harvesting from liquid media in a highly efficient manner. It requires only a simple filter and vacuum pump.

It was successfully demonstrated that algal cell density, can be harvested, regardless of how low the algal cell density is. In cases where low concentrations are present, column formation can be undertaken, trapping algal biomass, this column can then be resuspended with all new algal culture, with the repeated removal of growth media, and the consequent doubling of algal biomass concentration in the column. In such cases where this column system is employed for algal biomass harvesting, the algal cell size must be matched with the diatomaceous earth particle size distribution, as was done in this case with that of Merck's Celite®545 (**Figure 28**).

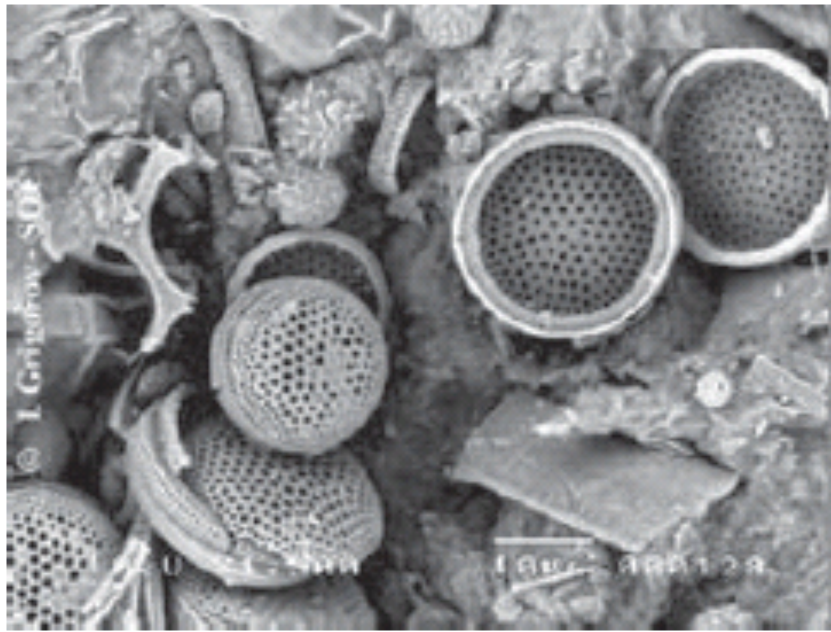


Figure 28. A TEM image of Diatomaceous earth (Celite®545).

When looking at the algal cell pack, it is useful to view this now as a proxy for a leaf, in which the algal biomass takes on the role of living cells that can be more easily utilised for biologic production of various endogenous products but can also be coaxed from photosynthesis-based biomass production, to oxidative respiration after being incorporated into the pseudo leaf space of the column (**Figure 29**). The diatomaceous earth fulfils the role of support, allowing for scaling of the column without the end result of column collapse and a loss of porosity should this support not be incorporated.

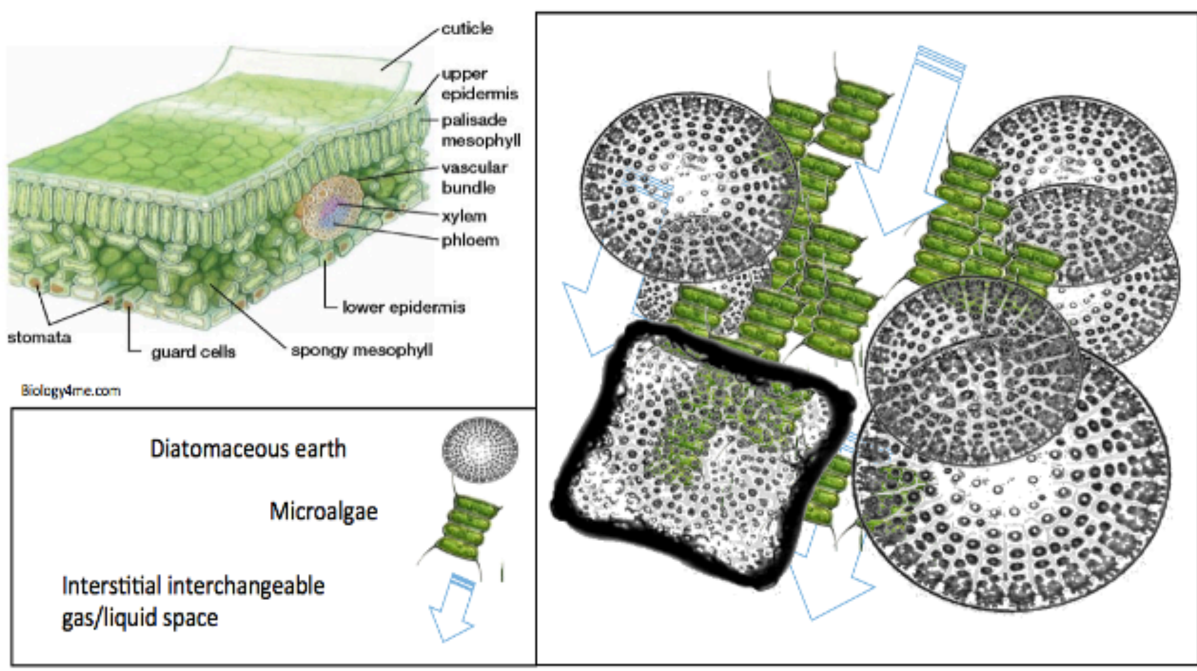


Figure 29. The pseudo-leaf space of the diatomaceous earth-mediated matrix-assisted cell culture column.

Although feeding of the biomass is required with heterotrophic media to allow for cell maintenance, algal biomass can still grow within this environment. This does however require not only renewal of nutrient media for respiration to function, but also removal of spent media containing waste products, as well as removal of gaseous waste products and replenishing of the oxygen replete microenvironment required for biomass maintenance and growth within the column. This is easily achieved with vacuum of new nutrient media into the column, maintaining a high humidity environment but still maintaining interstitial airspaces to allow for gaseous exchange. Of consideration here, the ability to vacuum various liquids through the algal column, allows not only for harvesting of algal bioproducts should a cell lysis solution be used, but also transient expression of heterologous proteins, should a gene delivery system be employed. Such is the case here, with the use of *R. radiobacter*.

From an industrial perspective, column size must inevitably be scalable. This is dependent on the degree to which column front formation can occur relative to diatomaceous earth settling rate. For this reason, Celite® 545 was the most suitable candidate, not settling too rapidly, but settling rapidly enough, so that should this process be used to concentrate algal biomass from low density cultures, it does settle out of suspension more rapidly than the algal biomass, which can now be pumped off for further processing, leaving the Celite® 545 behind, for further harvesting of low-density culture, in a continuous process.

As an aside to the ability of the column to be scaled in height, with the ability of the column to maintain algal biomass and promote growth, this environment is highly novel from an algal evolutionary perspective. As such, it would be prudent to first assess various species for their heterotrophic ability within the column, but once a species is

identified, this trait it could be selected for within this species, for maximal biomass production within the column.

Lastly, the column should be employed as an initial selection tool for identification of algal isolates, should the end application be algal biomass maintenance within the column, as would be the case for algal bioproduct production and eventual harvesting.

The column can be employed for size selection of algal isolates from ecological samples, which can then be further employed for initial assessing of isolate suitability to further industrial processes, without the need for labour intensive algal library generation and then assessment of individual isolate suitability to the column.

6 Transfection, protein expression and analysis

6.1 Chapter introduction, overview and aims

Having demonstrated that an axenic microalgae library could be developed from environmental water sources or autotrophic isolates which were highly contaminated with various fungal and bacterial species on culturing under heterotrophic conditions, the axenic microalgae were then assessed to establish whether the isolates would grow in various media, survive cryogenic storage, grow heterotrophically in the dark on enriched media, and how they responded to antibiotics and the presence of axenia itself. The algal biomass was also tested to ascertain the recovery of viable algal cells on processing through a packed bed of algal biomass and celite, and their subsequent ability to survive in the column with and without the addition of *R. radiobacter*.

The axenic microalgal library was used in the novel application within a three-dimensionally scalable matrix-mediated cell pack. This was to demonstrate the ability to form a pseudo plant leaf, where cells are maintainable through interstitial airspace flooding with nutrient media and then recreated, removing waste products and allowing for gaseous exchange, maintaining biomass in the system, as well as demonstrating the cell pack's ability to separate ecologically derived microalga according to cell size, but also the ability of the diatomaceous earth to efficiently capture algal biomass at varying cell densities.

Following this, it was decided to investigate one of the more challenging possible applications of this novel system i.e., could the system be employed, not just for cell maintenance, capture and for the investigation of ecological samples, but could it be employed as a system for *R. radiobacter*-mediated microalgal transfection, and the subsequent expression of various heterologous proteins (**Figure 30**). To this end, several methodologies and approaches were developed. These included the investigation of stable gene integration of the resistance gene *neomycin phosphotransferase II* (nptII) and subsequent selection of transformants using geneticin g418; transient expression of the reporter proteins, β −glucuronidase and GFP, as well as transient expression of the industrially relevant enzyme, horseradish peroxidase (HRP); and lastly the transient expression of a vaccine candidate, HPV-16 hL1, a self-assembling capsid protein monomer of viral origin that forms virus-like particles (VLPs).

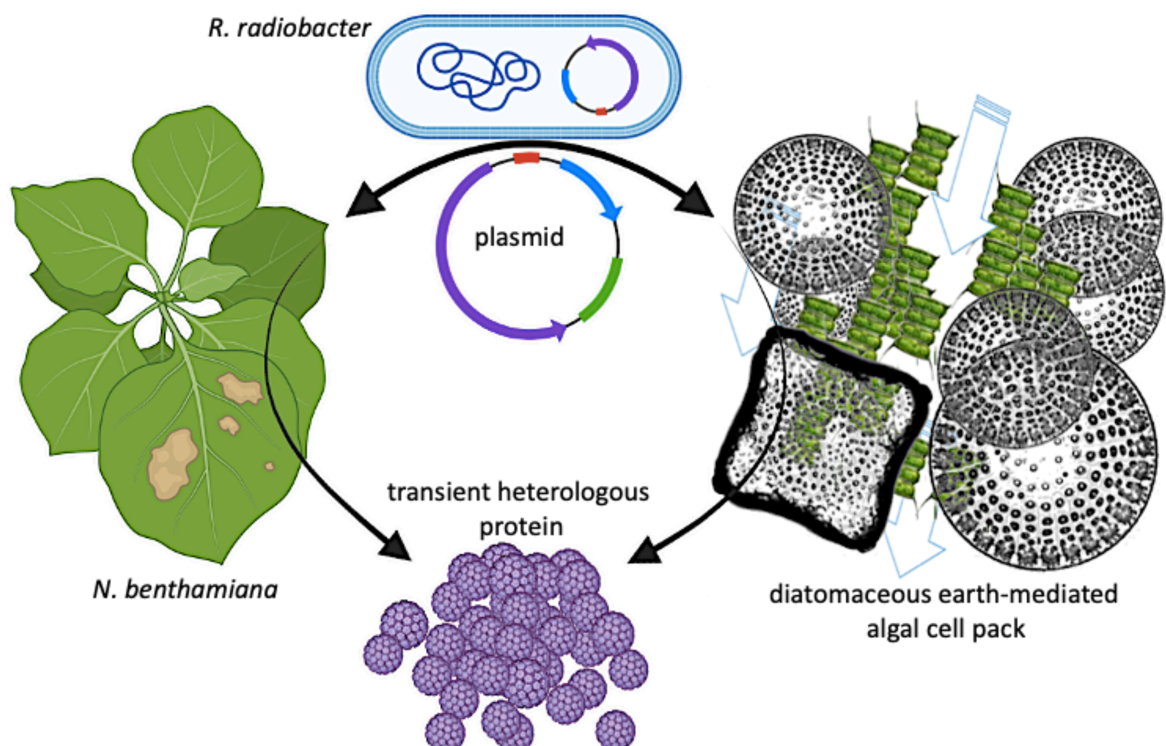


Figure 30. The Genetic Transformation Process. On contacting of *R. radiobacter*, a genetically modified plant pathogen, with microalgal cells, recombinant DNA is inserted into the algal cell population. This sequence is not of bacterial origin but may originate from any biological source, such as a reporter protein, an industrial enzyme or a vaccine candidate, such as a VLP. The diatomaceous matrix fulfils a similar role as would structural tissues in a plant leaf, such as vascular cellulosic tissues, giving support and allowing for through-flow of media, so replenishing column nutrients and simultaneously removing metabolic waste products.

6.2 Materials and methodology

6.2.1 *NptII* stable expression

To demonstrate transfection and stable gene introduction, the vector pTRAkc ERH::rfp was used (Figure 2). This vector not only inserts the gene, Red Fluorescent Protein (rfp) gene into host cells, in this case microalgae, but also inserts the resistance gene *neomycin phosphotransferase II (nptII*). *NptII* allows for the production of an enzyme that can degrade various antibiotics, such as kanamycin as well as G-418. For this reason, several isolates of green microalgae were assessed for G-418 susceptibility, as described earlier in the thesis. To this end, it was found that *Ankystrodesmus gracilis* (MPA49.1) was susceptible to G-418 at 20 mg. L^{-1}, exhibiting complete growth inhibition. *A. gracilis* was also able to withstand exposure to 200 mg. L^{-1} cefotaxime, which is essential for the selective removal of possible *R. radiobacter* contamination post-transfection.

Diatomaceous earth-mediated algal cell packs were created as follows: diatomaceous earth (Celite® 545, Merck) was mixed into axenic *A. gracilis* culture, so that 0.75 g of diatomaceous earth was used per 5 ml of algal culture. Thereafter, 5 ml of algal-

diatomaceous earth suspension mixture was pipetted into clear plastic chromatography columns (Bio-Rad Laboratories, Inc), followed by excess media removal by drawing a vacuum. Columns were then transfected with *R. radiobacter* strain GV 3101 pMp90RK harbouring pTRAkc; ERH::rfp at an OD_{600} of 3.4 and left to incubate for an hour before excess *R. radiobacter* was introduced, so reinitiating interstitial airspaces. Simultaneously, *A. gracilis* / diatomaceous earth columns were formed and transfected with R. radiobacter strain containing only the Ti plasmid, but no transgene, thus acting as a negative control, denoted as, pTRAc::*empty*.

Three days following initial transfection, both transfected and non-transfected columns were harvested as follows: algal-diatomaceous earth was removed to sterile 15 ml falcon tubes containing 15 ml ddH_2O (autoclaved and filter sterilised, 0.22 μm) followed by vortexing. Diatomaceous earth was then allowed to settle for 15 min. Algal suspension was then removed to 2 ml Eppendorf tubes and a cell count was performed. Samples were then diluted accordingly and 200 μL containing $3x10^6$ cells of each sample were then plated onto heterotrophic media plates (2 % bacteriological agar containing: 3 N Bold's Basal Media with 20 mM Glucose and Glycerol as well as 20 $mg.L^{-1}$ G-418 and 200 $mg.L^{-1}$ Cefotaxime). Plates were allowed to incubate under lights (12hr:12hr; light: dark) until colony formation occurred.

No colonies formed on selection plates containing non-transfected *A. gracilis*, indicating a total inhibition of wild-type growth by G-418 (data not included as it is just a clear plate). However, on selection plates containing transfected *A. gracilis*, in excess of a thousand colonies formed (**Figure 31)**. From this initial plate, ten colonies were chosen randomly and plated onto a second selection plate (**Figure 31b**). What was indicated on these plates was that three of the randomly chosen colonies maintained G-418 resistance and had the ability to propagate in the presence of 20 mg. L^{-1} G-418. These colonies were then picked out and diluted in ddH_2O and plated onto three separate plates (**Figure 31c, 31d and 31e**). Interestingly, the last colony picked demonstrated a marked increase in growth ability relative to the other two colonies (**Figure 31e**). Colony polymerase chain reaction (PCR) was then done to amplify *nptII* to confirm that G-418 resistance in the above was indeed due to transfection of *nptII*. This was done from plate scrapings of the three plates shown in Figures 31c, 31d and 31e as follows: algal cells were collected from each plate and removed to sterile PCR tubes containing 50 μL 5% Chelex suspension. These were vortexed for 1 min, boiled at 98 °C for 5 min and then cooled on ice for 1 min, then vortexed for 1 min and centrifuged at 13000 rpm for 1 min.

PCR primers and conditions were as follows:

FWD 5' **GAGGCTATTCGGCTATGACTG** 3'

REV 5' **ATCGGGAGCGGCGATACCGTA** 3'

PCR reaction: 95 °C for 3 min (one cycle), 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min 30 sec (40 cycles), 72 °C for 10 min (one cycle) and then held at 4 °C.

6.2.2 β −glucuronidase (GUS) transient expression

E. coli strain JM109, carrying the open-access plant expression vector, pCAMBIA1301 (kindly provided by the University of Stellenbosch's Institute of Plant Biotechnology), was streak plated onto LB agar, with five colonies picked into 10 ml liquid growth media, at which point DNA concentration was quantified with Nanodrop spectrophotometry, following plasmid DNA extraction (QIAGEN plasmid mini prep kit). DNA was cut with the restriction enzyme, PagI1 (BspHI), recognising DNA sequence T^CATGA and proposed to cut the pCAMBIA3101 vector at two sites, yielding DNA segments of a length 6841bp and 5009bp, respectively. Following this, electrocompetent *R. radiobacter* strain LBA4404 was transformed via electroporation with pCAMBIA1301 plasmid DNA and plated out on solid LB agar containing the antibiotics, rifampicin (20 mg. L^{-1}) and kanamycin (50 mg. L^{-1}). After two days twenty colonies were picked to a grid, at which point a colony PCR was done on the first ten colonies to confirm the presence of pCAMBIA1301 in transformed *R. radiobacter* LBA4404 colonies. The presence of pCAMBIA1301 would yield a 150 bp band. This was done using the primer pair, M13, additionally, primer pair sequence alignment was performed with pCAMBIA1301 sequence. (Positive controls included pCAMBIA1301 plasmid DNA from original *E. coli* stocks as well as plasmid DNA from the PUC-57 vector also containing M-13 binding sites; amplicon of 650 bp).

PCR primers and conditions were as follows:

M13-F: 5' **CGC CAG GGT TTT CCC AGT CAC GAC**

M13-R: 5' **GAG CGG ATA ACA ATT TCA CAC AGG**

PCR reaction: 95 °C for 2min (one cycle), 95 °C for 30 sec, 59 °C for 30 sec, 72 °C for 30 sec (30 cycles), 72 °C for 2 min and then held at 4 °C.

Once the presence of pCAMBIA1301 was confirmed, two colonies were picked from the master plate, streaked out on selection media, then grown up overnight, at which point cryogenic glycerol stocks were frozen down at -80 °C.

Although, LBA4404 pCAMBIA1301, not only displayed vector presence with PCR but also via gain-of-function resistance to rifampicin, it was decided to determine real transient expression capability of this strain, to act as a positive control for further algal experiments. This was done by assessing whether this strain could produce transient expression of β −glucuronidase in *N. benthamiana* post syringe infiltration. This also allowed for fine-tuning of the X-glucuronide (X-GLUC) concentration (5 mg X-glucuronide in 100 μL dimethylformamide (DMF), dissolved in 10 mL sodium phosphate buffered solution at pH 7) (Kathiresan et al., 2009; Radha, 2014; Sharon-gojman et al., 2015) assay for implementation with algae later on.

Syringe infiltration was done in the following way: *R. radiobacter* strain LBA4404::pCAMBIA1301 cryogenic glycerol stocks were revived in 10 ml liquid LB media containing selective antibiotics (rifampicin 50 mg.L^{-1}, kanamycin 30 mg.L^{-1}) over night. A 2 mL aliquot of actively growing culture was then inoculated into infiltration media and allowed to grow overnight with shaking (200 rpm) at 27°C, along with LBA4404 containing no vector to act as a negative control. The following day,

infiltration cultures were diluted to an OD_{600} of 0.5 in similar medium and syringe infiltrated into *N. benthamiana*. On the third day post-infiltration, leaf discs were harvested and the X-GLUC assay was performed. This involved leaf disc incubation with agitation (200 rpm) at 25°C overnight in 2 mL x-glucuronide sodium phosphate-buffered solution (pH 7). Following colour development, leaf discs were washed in sterile PBS to stop reaction and chlorophyl was destained by washing in absolute ethanol, at which point cells were observed under the microscope.

To determine whether LBA4404 pCAMBIA1301 could be utilised for GUS transient expression in microalgae, an actively growing, axenic *C. vulgaris* UTEX395 culture (OD_{600} of 0.569), growing in TAP media with kanamycin (30 mg. L^{-1}) was used. Matrix-mediated algal columns were formed as previously described (**section 4.2.3**) and were infiltrated with 1 mL LBA4404 pCAMBIA1301 (OD_{600} of 0.75), as well as LBA4404 without pCAMBIA1301 to act as a negative control, in infiltration media. On the third day 3 post-infiltration columns were resuspended in 15 ml sterile PBS buffered solution in 15 ml Eppendorf tubes. Diatomaceous earth was allowed to sediment out for 10 minutes. Following this, 12 mL of the algal supernatant was carefully moved to a new sterile 15 ml Eppendorf tube and centrifuged for 10 minutes at 6000 rpm. Pellet was collected in 1 ml sterile PBS and washed once in PBS. Following this, pellet was resuspended in x-GLUC solution, and incubated overnight at 37°C with agitation at 200 rpm. The following day, cells were washed in ddH_2O to stop reaction, and chlorophyll was destained with absolute ethanol, re-pelleted and resuspended in sterile PBS, at which point, the cells were observed under the microscope.

6.2.3 GFP transient expression

GV3101 pMP90RK *R. radiobacter* strain carrying either the vector pTRAc::eGFP or pRIC::eGFP was streak plated onto LB agar containing the antibiotics, rifampicin (50µg/ml), carbenicillin (50µg/ml) and kanamycin (30µg/ml). Once colony formation had occurred, seven colonies from each of the pRIC and pTRAc plates were inoculated in LB liquid media with antibiotics, at which point a colony PCR was performed, such that the whole eGFP ORF was amplified.

PCR primers and conditions were as follows:

FWD 5’ **ATG GTG AGC AAG GGC GAG GAG**

REV 5’ **TTA CTT GTA CAG CTC CAT GTC CAT GCC GA**

PCR reaction: 95°C for 3minutes (one cycle), 95°C for 30 seconds, 51°C for 30 seconds, 72°C for 1 minute (25 cycles), 72°C for 1 minute and then held at 4°C. (GoTaq polymerase, www.promega.com). Samples were then analysed via gel electrophoresis (1%).

Following confirmation of the presence of the plasmid vectors within GV3101 mPR90Rk, samples were frozen down at -80 °C and stored as working stocks.

An algal cell pack was created using *Scenedesmus sp.* (QUCCCM63) and transfected with pTRAc::eGFP. On the third day post-transfection, this ACP was resuspended in

sterile PBS and viewed under confocal microscope, using bright field and a UV filter cube.

Following this, relative fluorescence across the axenic library over time was investigated. This was done using isolates, *Scenedesmus sp.* (QUCCCM63), *Desmodesmus sp*. (MPA16.1), *Ankystrodesmus gracilis* (MPA49.1), GHD.1 (heterotrophic isolate that was previously isolated on Table Mountain), MPA 34.1, MPA 28.1, MPA 46.1, MPA 33.1, MPA 39.1, MPA 10.1, MPA 18.2, MPA 29.1, MPA 40.1, MPA 53.1, MPA 25.1, MPA 51.1, MPA 7.1, MPA 27.1, MPA 13.1.

Each of these isolates were grown up axenically in 3N BBM. 1ml of each culture was then added to 1ml of 3N BBM in 24 well plates, in duplicate, and allowed to grow for two days under the lights (12:12 light: dark) with shaking. At this point 100μL of OD_{600}= 0.66 induced agrobacterial culture carrying the binary vector pTRAc::eGFP was added to one of each of the cultures.

Relative fluorescence was then measured on day one, three, five and seven. This was done by transfer of 100 μL of each culture to a black 96 well plate, where fluorescence was then measured with an ELISA spectral analyser (blue laser, Ex 495nm, Em read at 550nm. Thermo Multiscan FC Microplate Reader. Thermo Fisher). Relative fluorescence was then calculated by subtracting, agrobacterial-positive reading from negative reading for each isolate, which was then plotted on a graph. As an aside, both 3N BBM and 3N BBM with the added agrobacterial culture, was assessed for fluorescence signal, for baseline signal control.

It was of interest to assess GFP expression in the algal cell pack via western blot. Here preliminary evidence was found that the self-replicating, chloroplast targeting geminivirus-derived vector pRIC3.0-cTP::LALF-E7 had expressed well (blot run by R. Yanez, BRU), although no algal controls were scrutinised . For this reason, western blotting was performed using this vector with the addition of GFP i.e., pRIC3.0-cTP::LALF-E7;GFP.

Desmodesmus sp. (MPA16.1) was grown up axenically until mid-exponential phase in combination media (1 :1) 3N BBM :: LB broth, with the addition of 7.5 $g.L^{-1}$ glucose. The algal cell pack was formed and 2ml *R. radiobacter* culture carrying the above vector (OD_{600}=0.1) was used to resuspend the above column, and was then allowed to incubate for an hour, before the column was reformed by vacuuming. This was similarly repeated, but with pRIC3.0-cTP::LALF-E7, which does not encode GFP. This was to act a negative control. At day 8, columns were harvested, and protein was extracted using 8M urea, and run on a 10% polyacrylamide gel. The blot was then probed with the monoclonal antibody, anti-GFP (1:50 000 dilution, produced in rabbit) and secondary antibody, alkaline phosphatase anti-rabbit conjugate (1:5000 dilution). GFP produced in *N. benthamiana* using the plant vector peaQ::tmv,GFP, was used as a positive control (kindly provided by M. Baloyi of the BRU).

6.2.4 HRP transient expression

Twenty axenic algal species were isolated from various inland lakes around South Africa's Mpumalanga region. These were grown up in 15 ml of a combination medium (3:1, 3N Bold's Basal Medium (BBM): Lysogeny Broth (LB)) until stationary phase was reached in 50 ml shake flasks. Four days prior to cell pack formation, 500 µL LB broth was added to each shake flask. At this stage, three *R. radiobacter* strains: (1) GV3101 pMp90RK pTRAc::HRPΔC; (2) GV3101 pMp90RK pRIC::HRPΔC and (3) GV3101 pMp90RK pTRAc::empty (R. radiobacter strain with ti plasmid, but no transgene) were grown up overnight.

The first strain introduced a single copy of a gene derived from the horseradish *Armoracia rusticana*, which produces isotype C of horseradish peroxidase (HRP). The second strain introduced a self-replicating viral based construct that copies its own DNA intracellularly, so providing more HRP gene copies for protein production (Figure 6), while the third strain introduced a vector carrying no added gene, acting as a negative control (**Figure 5**). The HRP gene in the native horseradish or in transgenic organisms produces the industrially relevant enzyme HRP, which has a wide range of diagnostic and therapeutic applications, with a retail price of approximately £370 per 100 mg.

Cell packs were formed in the following way: diatomaceous earth was mixed into each algal culture, so that 0.75 g of diatomaceous earth was present per 5 ml of algal culture. A 5 ml aliquot of algal-diatomaceous earth mixture was then pipetted into clear plastic columns (BioRad), followed by excess media removal by vacuum. Each isolate was then transfected with each of the *R. radiobacter* strains, such that 1 ml (OD_{600}=0.2) of each was vacuumed into the cell pack and left to incubate for one hour before the liquid was removed from the cell packs to reform interstitial air spaces. The next day, 500 µl of 3 N BBM with 4 $g.l^{-1}$ glucose and MES, was vacuumed through each cell pack. After which, on the following day, liquid was collect into 2 ml Eppendorf tubes which were assayed for horseradish peroxidase activity. (TMB Microwell Peroxidase Substrate System, KPL, Gaitherburg MD 20878, USA) This was performed on days three, five and seven.

Furthermore, the isolate showing the greatest colour change using the HRP assay was harvested and analysed by a western blot to allow for HRP detection. This was done as follows: Following seven days post transfection, algal cell-packs were resuspended in 10 ml PBS buffer with vortexing. This dissociated algal biomass from the Celite® matrix. Celite® was then allowed to sediment for 30 minutes, and algal PBS suspension was removed to a 15 ml centrifuge tube. This was then centrifuged for 10 minutes at 10 000 rpm. Supernatant was poured off and pellet was resuspended in 1 ml PBS and placed in Eppendorf tubes. These were then centrifuged at 13 000 rpm for 3 minute and supernatant was removed. Algal pellet was then frozen with liquid nitrogen and cells lysed with a micropestle. This was followed by the addition of 150 µl PBS to cell lysate. This was centrifuged for 3 minutes at 13 000 rpm. 100 µl of supernatant was then removed to another Eppendorf tube to which 25 µl of Sample Application Buffer was added. Samples were then denatured at 94°C for 5 minutes and run on a 2 % SDS-PAGE gel. Following nitrocellulose transfer and immobilisation,

blots were incubated over night with primary anti-HRP antibody (ABCam ab2110: anti-HRP produced in mice; 1:5000 concentration). Primary antibody was then detected with anti-mouse alkaline phosphatase conjugated secondary antibody produced in goat. Each cell pack was maintained in this manner, and three weeks after initial *R. radiobacter* transfection, the highest producing isolate was then resuspended in 3 N BBM media, where a plate count of viable cells was performed.

Algal species that did not reproduce heterotrophically in the presence of glucose were not able to form colonies on the inside of the cell packs as they were light-impermeable (**Figure 22.b**), while algal species that did switch to heterotrophic glucose respiration were able to colonise the inside of the cell pack (**Figure 22.c**). Furthermore, heterotrophic metabolism is more productive. In addition, it is useful for protein production if the microalgae have the ability to withstand being stored at very low temperatures - halting the cell cycle - and then later on, revived. This enables the halting of strain evolution and prevents batch-to-batch variation. However, cryogenic preservation and heterotrophic growth are disparate abilities that may not be shared by a single isolate.

Thus, a model microalga (*Chlorella vulgaris* UTEX 395) was assessed for its ability to withstand cryogenesis and its ability to grow heterotrophically. The isolate was grown heterotrophically to assess media requirements for heterotrophic metabolism as well as initial growth investigations via cell counts. An axenic algal library, stored at -80°C, was then revived on solid heterotrophic media (in the dark). *Chlorella vulgaris* was successfully grown heterotrophically completely in the dark. From the initial library, thirteen isolates were cryogenically revived on autotrophic media and five were revived on heterotrophic media directly. The isolates were then investigated for demonstrated high biomass yields when grown heterotrophically. Algal survival in a column was investigated, both with and without *R. radiobacter*, after feeding with an enriched media that the specific isolate was selected for, to allow heterotrophic growth (in the absence of light). Thereafter, harvest efficiency was determined, where 10 ml of a dense, heterotrophically grown, algal culture was mixed with 2 g of Celite®. The total volume was increased via dilution which demonstrated the ability of the column to harvest high density, heterotrophic algal cultures as well as low density autotrophic algal cultures using Celite®.

Once column formation was completed, the same cell number was present, although the initial volume was highly variable (**Figure 24**). Twenty axenic isolates were used to form algal cell packs and transfected with *R. radiobacter* strains that introduce the HRP gene and a positive reaction was detected through colour change with the KPL Peroxidase Substrate System in the isolates. Of note, one of the isolates demonstrated a marked reaction with both the single gene inserting vector (pTRAc::HRPΔC) and the self-replicating vector (pRIC::HRPΔC) (**Figure 43**). This indicates that not only was HRP being produced by the transfected algal cell-pack but that the algae were exporting the HRP into the media.

6.2.5 HPV16 hL1 transient expression

The cryogenic *R. radiobacter* strain GV3101::pMp90RK carrying the binary vector pTRAkc-rbcs1 CTP::HPV16 ^{h}L1 was sourced from the Biopharming Research Unit's culture library. This was revived in liquid LB growth media containing the antibiotics, rifampicin (50μg/ml), carbenicillin (50μg/ml) and kanamycin (30μg/ml). This vector allows for the transient expression of the human codon-optimised L1 coat protein, derived from the human papillomavirus (HPV), to be expressed in *N. benthamiana.* Once L1 monomers are expressed in plant leaves, they spontaneously assemble into virus-like particles (VLPs) consisting of 360 monomers arranged into 72 pentameric capsomeres (Sapp and DiGiuseppe, 2017). These VLPs make for excellent vaccine candidates, due to their bio-similar structure relative to native, infective HPV virions, can thus elicit an appropriate immune response.

Scenedesmus sp. (QUCCCM63) was revived from senescent, axenic liquid 3N BBM cultures, via the addition of 100μL of each to 50ml LBB.

To act as a positive control, assessing the transfectability and the functionality of the newly acquired *R. radiobacter* strain, it was decided to syringe infiltrate *N. benthamiana.* As a secondary investigation, in this regard, it was of interest to assess whether infiltration of the bacteria would be affected if different infiltration media was used. Four leaves from four different plants were syringe infiltrated with this strain. The first leaf was infiltrated with induction medium containing 200mM aceto-syringone at a pH of 5.6, the second leaf was infiltrated with LBB media also containing aceto-syringone at a pH of 5.6, the third leaf was infiltrated with TAP media containing aceto-syringone at a pH of 5.6, and lastly, the fourth leaf was infiltrated with pTRAkc::*empty* in induction media with this last leaf acting as a negative control.

Three days post infiltration, leaf discs from each leaf, from the different plants that had been infiltrated with the same media, were harvested and combined into a single sample. These were then crushed with liquid nitrogen under micropestle, with the addition of 200μL 8M urea and protease inhibitor and then assessed via western blot. Total protein concentration loaded was controlled for via comparison of each sample using Bradford's assay (known albumin concentrations of 1mg.ml^{-1}, 0.5mg.ml^{-1} and 0.25mg.ml^{-1}, absorbance at 750nm). Blot was detected with anti-Gardasil primary antibody (1:2000 dilution produced in rabbit). Secondary antibody: anti-rabbit alkaline phosphatase conjugate, 1:5000 dilution. The anti-Gardasil® primary antibody used is an in-house produced antibody, used for the detection of L1 monomers, and is based on use of the on-the-market HPV treatment, Gardasil®, for antibody production).

Following this, each algal culture was doubled in volume by adding 50ml LBB media and two columns were formed for each. The first was then infiltrated with pTRAkc-rbcs1 CTP::HPV16 ^{h}L1 and the second was infiltrated with pTRAkc::*empty* to act as a negative control. These were allowed to incubate for four days before harvesting, at which point columns were resuspended in sterile PBS and algal biomass was removed from Celite®545 matrix, following Celite®545 sedimentation. Algal suspension was then inoculated into 30ml heterotrophic media containing kanamycin (80mg.L^{-1}) and concentrated via centrifugation, at which point samples were prepared for western blotting. Following this, western blotting was performed on the samples. A

commercially produced monoclonal primary antibody was used, known as CamVir (1:10000 dilution, produced in mice. Secondary antibody, anti-mouse alkaline phosphatase conjugate 1:10000 dilution). This blot included two negative controls i.e., untransfected *Scenedesmus sp.* (QUCCCM63) and also *Scenedesmus sp.* (QUCCCM63) transfected with pTRAc::HRPΔC.

Following this, *Scenedesmus sp*. (QUCMMM63) was prepared for transmission electron microscopy (TEM), to assess whether VLPs had been expressed in this sample.

6.3 Results and Discussion

6.3.1 NptII stable expression on solid media

Demonstrating the ability of the algal-celite column to produce stable lines of transgenic algae was accomplished by physiological maintenance of resistance to the algacidal agent, G418, as well as the cellular maintenance of the gene, *nptII*, that confers this resistance, with detection via PCR, in resistant cell lines (**Figure 31**). Seven colonies that were chosen from the initial selection plate containing 20 mg. L^{-1} G418 were not able to reproduce, having lost resistance. However, three colonies did demonstrate stable resistance. The resistance of these three colonies was variable, with one of the three resistant cell lines demonstrating elevated efficiency in G418 catabolism, relative to the other two cell lines. This was expected, considering that transgene introduction occurs in a random way, whereby any number of positional and/or silencing processes are present that may affect the ability of the *nptII* enzyme to fulfilling its function of G418 phosphorylation.

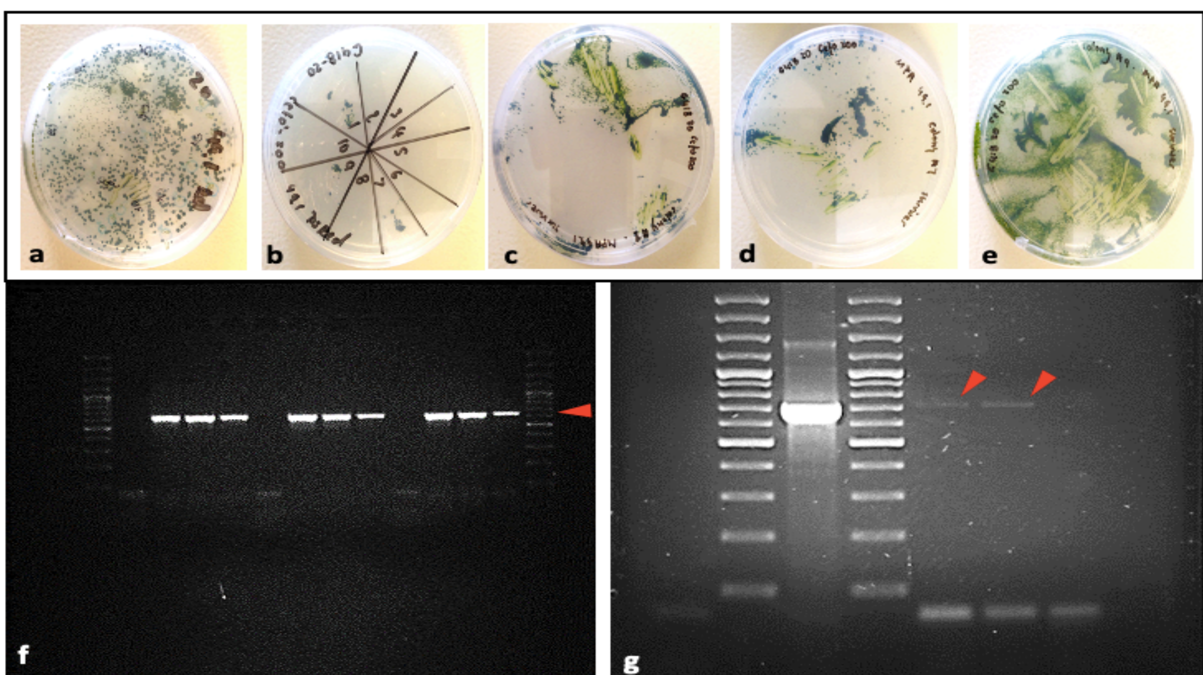


Figure 31. Stable transformant generation. Ten randomly picked *Ankystrodesmus gracilis* colonies able to survive on G418, Cefotaxime selection plates, post algal cell-pack mediated transfection were transferred to new selection plate. Three of these then continued to display growth. These

were removed to their own selection plates. Plate scrape was performed on each followed by Chelex-mediated DNA extraction and colony PCR for the resistance gene, *nptII*. This was repeated with a positive control consisting of pure plasmid DNA harbouring the *nptII*. Red arrows denote amplification of bands of correct size in algal samples. Negative controls contained no DNA.

To add to this, triplicate PCR demonstrated the presence of the *nptII* gene in all three lines. This was initially shown without a PCR positive control and was repeated with one. Although the samples were identical in each PCR, the addition of the highly positive control, may have resulted in a decreased signal of the test samples, which may be explained by the constant ethidium bromide concentration across both gels, with the positive control acting as a sink, so decreasing test binding and reducing signal strength.

6.3.2 NptII stable expression in liquid media

Following transfection of *Desmodesmus* sp., (MPA16.1) columns with the stably introduced transgene, *nptII*, it was found that no significant growth variation relative to negative control columns was found, when placed in liquid media that did not contain the selective agent, G418. Significant variation in growth ($p = 0.01$, Student's t-test) was, however observed when these columns were placed in media containing the selective agent G418, as well as relative to those grown in media not containing any selective agent (**Figure 32**).

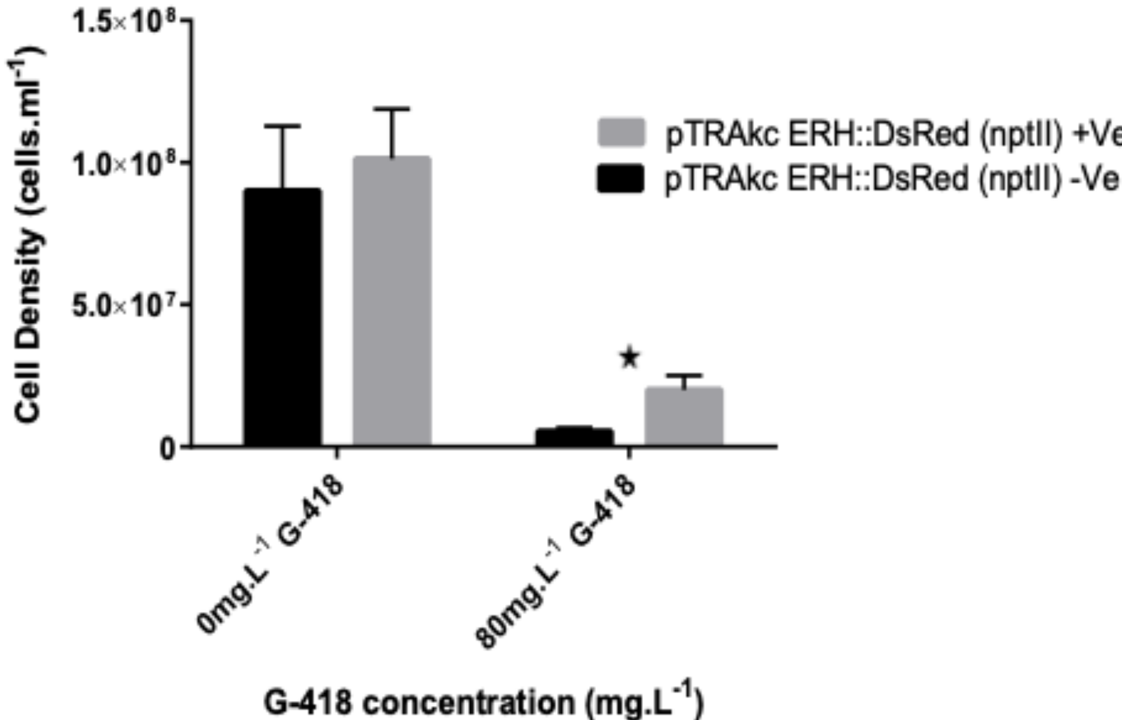


Figure 32. Stable transformant survival of *Desmodesmus spp.* (MPA16.1) with and without antibiotic selection. (Student's T test, p= 0.001)

This variance in inter-group growth demonstrates the degree to which selection by G418 still acts upon the transfected population, indicating that while transfection of the algal population is only an order of magnitude less than the total population, strong selection still occurs in the transfected population relative to that of the non-transfected one.

When intra-group growth variation is considered between the transfected versus the non-transfected population, growth variation is highly significant, indicating that the stably transfected population is still highly resistant to G418 selection relative to the population that does not have *nptII*-mediated resistance.

6.3.3 β −glucuronidase (GUS) transient expression

To demonstrate transient expression of the reporter protein, b -glucuronidase, pure plasmid DNA was first cut with the restriction enzyme, pagL1 (BspHI), which was run on a gel, yielding fragments of size 6841bp, 5009bp and uncut plasmid DNA (11849bp) (**Figure 33**) as was predicted from the plasmid map (results not shown).

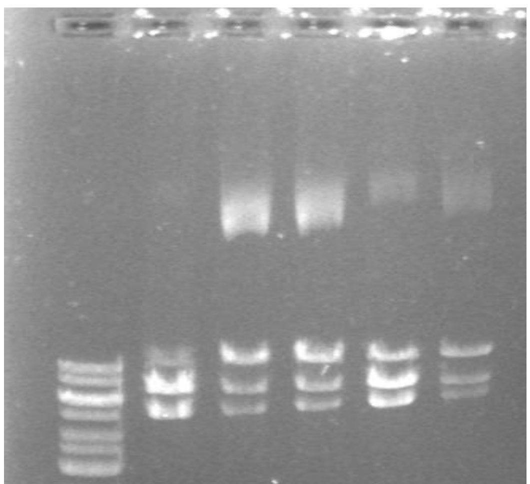


Figure 33. Restriction digest pCambia1301, pagl1 (BspHI). 1kb O'GeneRuler DNA marker. 6841bp and 5009bp fragment, and uncut plasmid (11849bp)

As a secondary confirmation of the fidelity of the pCAMBIA1301 plasmid, PCR was done using the M13 primer pair, which also gave bands of correct sizes in eleven of the thirteen colonies assessed (**Figure 34**).

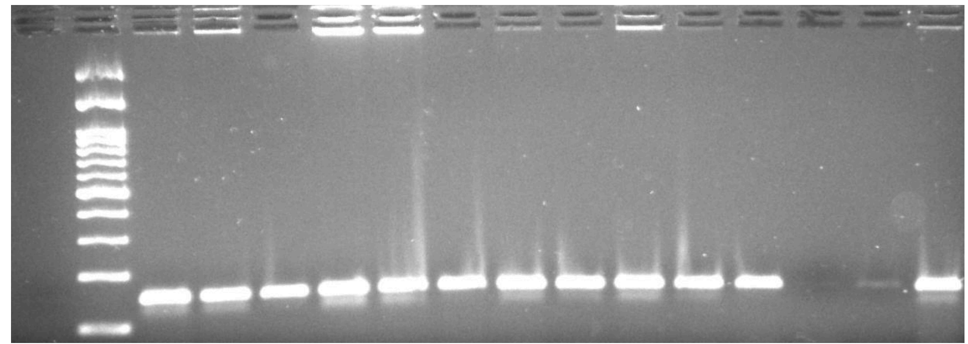


Figure 34. Colony PCR M13 pCambia1301.

When the M13 primer pair was sequence aligned graphically with that of the pCAMBIA1301 plasmid sequence, no alignment was initially found. Only after it was repeated using the sequence alignment tool, SnapGene, was it found that the “M13” annotated region of the pCAMBIA1301 plasmid was in very close proximity to the site of the M13 primer binding sites, but was not with that annotated region. When using annotated or auto-annotation of sequences, it is prudent to check that these elements are in fact annotated correctly (**Figure 35**).

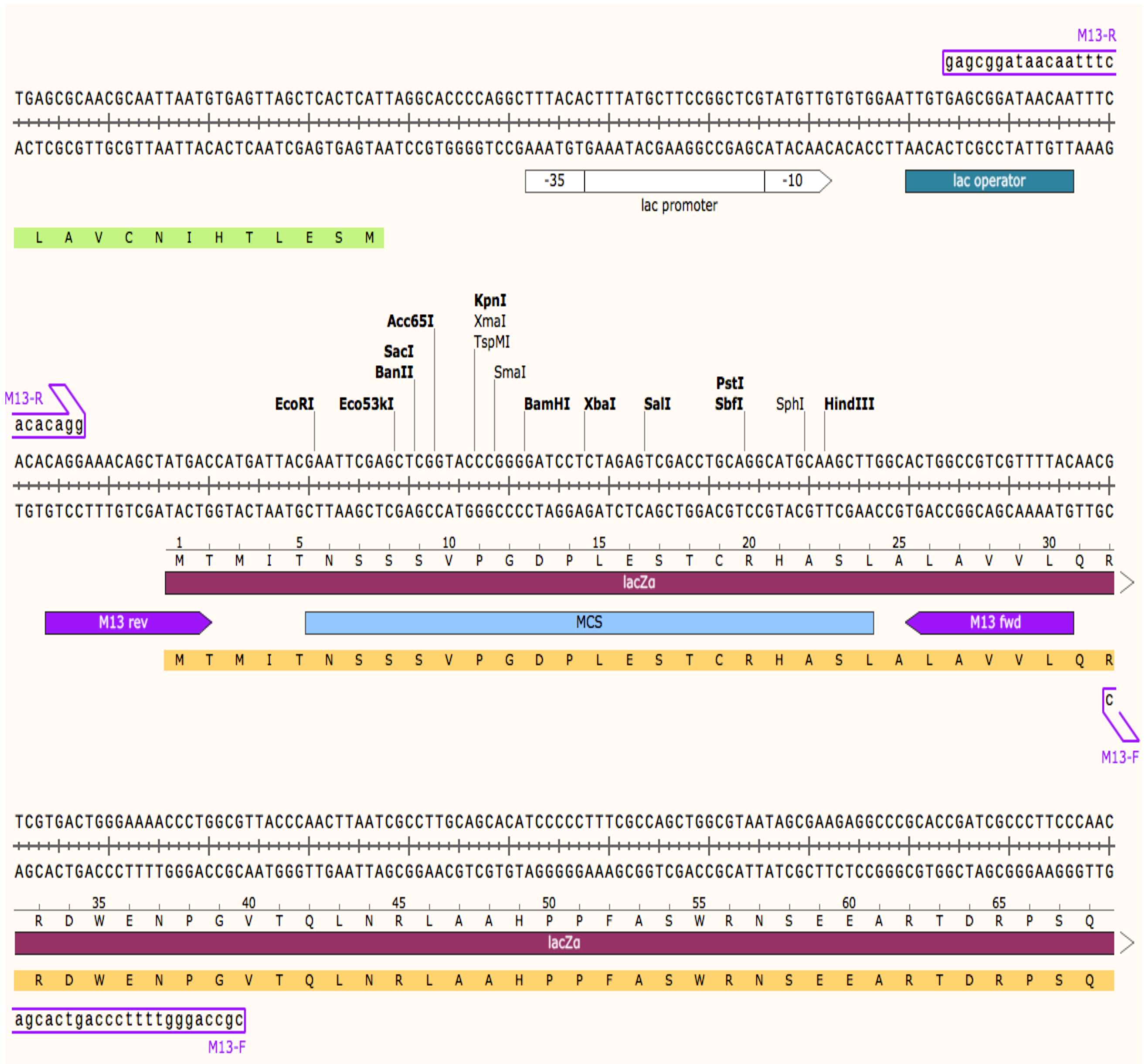


Figure 35. M13 sequence region, proposed location and actual location.

Plasmid incorporation into the *R. radiobacter* strain LBA4404 was confirmed with colony PCR (**Figure 36**). Two positive controls were used, the first control contained pCAMBIA plasmid DNA (lane3), the second control contained DNA originating from another M13 containing vector. The negative control contained no DNA.

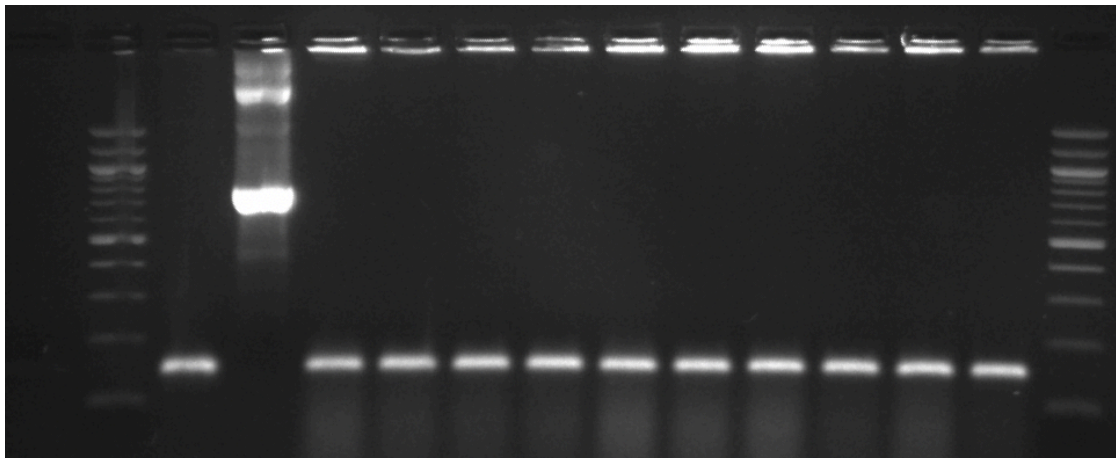


Figure 36. Colony PCR pCambia1301 LBA4404.

Once pCAMBIA1301 plasmid was confirmed in LBA4404 colonies, they were then grown up and cryogenically stored in glycerol (1:1) to act as working stocks.

To act as a further control for pCAMBIA1301 LBA4404 transfection ability *N. benthamiana* leaf discs were transfected with either LBA4404::pCAMBIA1301 or LBA4404::empty vector, and incubated in x-glucuronide, followed by the removal of chlorophyll by ethanol washing. In the test samples, a vivid blue colour signified the presence of $b-\mathrm{glucuronidase}$ and the metabolism of x-glucuronide, whereas in the negative control a vivid blue colour stain did not appear, so no metabolism of x-glucuronide had occurred. (**Figure 37**). In Figure37 it can also be seen that although colour formation is widespread across the leaf surface, cells near the midrib and leaf vasculature do not demonstrate this colour formation. This may indicate that these cells are either metabolically inactive or that LBA4404 cannot penetrate these cells to allow for transient gene expression.

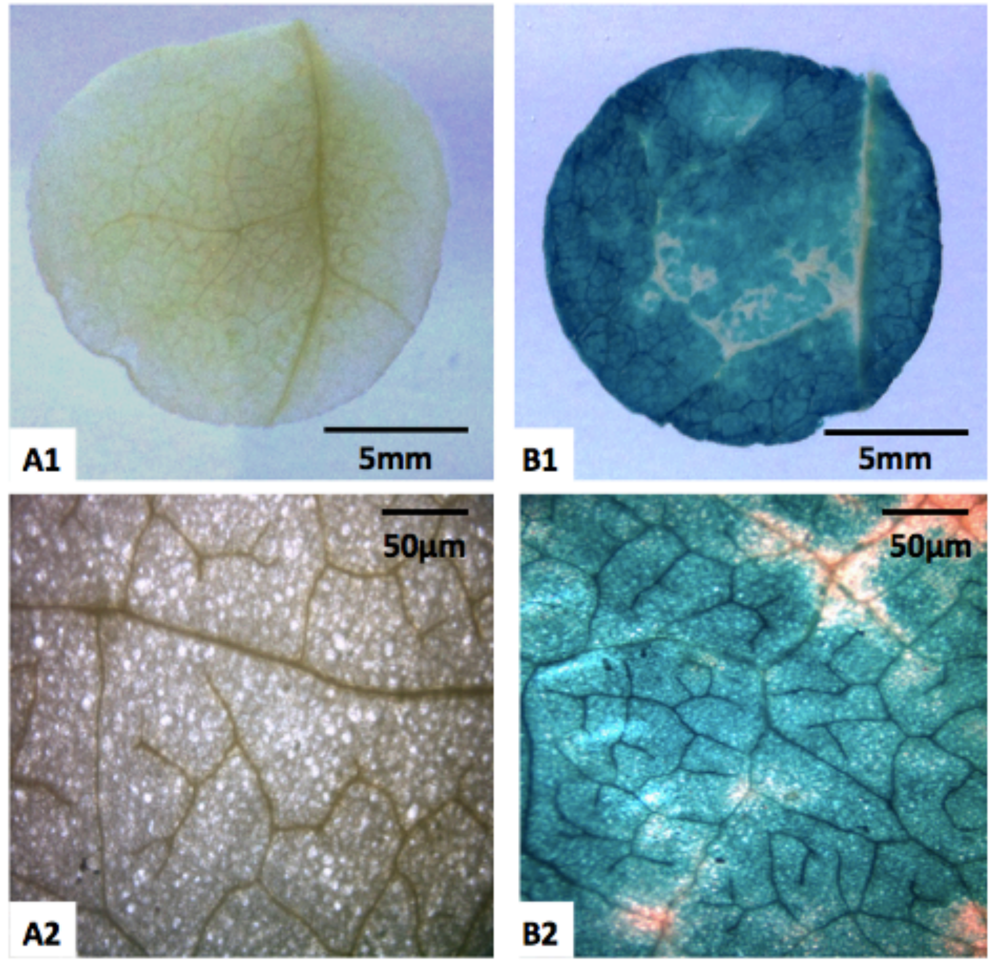


Figure 37. β −Glucuronidase expression in N. benthamiana. Leaf-discs and light microscope images from two plants exposed to the X-Glucuronide substrate and then ethanol-mediated chlorophyll extraction for better visualisation of colour change. In A1 and A2, plants were syringe infiltrated with vectorless A. tumifaciens strain; LBA4404. In B1 and B2, plants were syringe infiltrated with A. tumifaciens strain; LBA4404 harbouring the vector: pCAMBIA1301. Blue product formation denotes the presence of the heterologous marker enzyme, β −glucuronidase**.**

Following LBA4404::pCAMBIA1301 expression in *N. benthamiana*, and the assurance that not only was this plant able to allow for transient expression of β −glucuronidase, but also the equally important demonstration that the x-glucuronide assay was being done to allow for discrimination of cells either expressing this transgene or not, this process was repeated. This however was done using the algal-Celite column containing *C. vulgaris* UTEX395.

Following column resuspension and x-glucuronide exposure and ethanol washing, a vivid blue colour developed in the test but did not develop in the negative control (**Figure 38**).

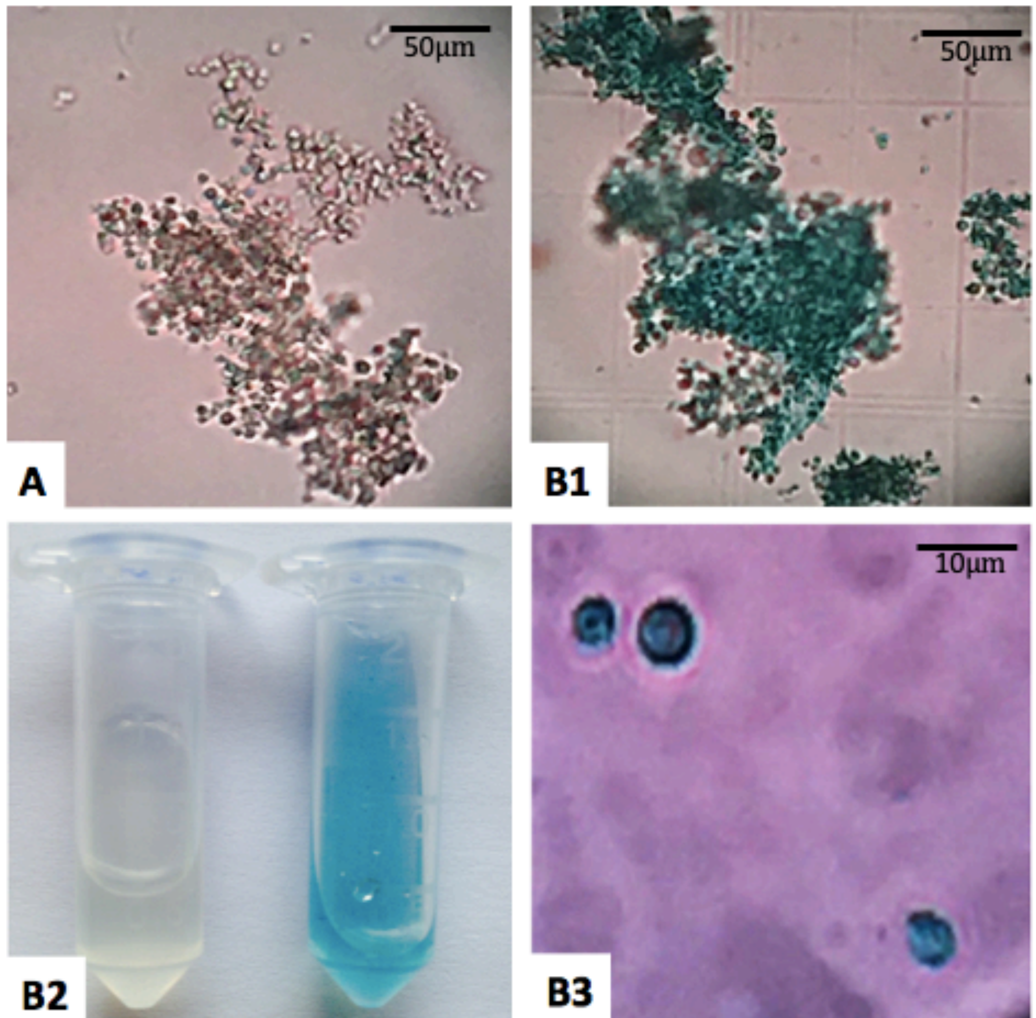


Figure 38. β −Glucuronidase expression in *Chlorella vulgaris* UTEX395 following algal cell pack-mediated transient expression. **A1**; negative control: *Chlorella vulgaris* UTEX395 algal cell pack transfected with vectorless *R. radiobacter* strain; LBA4404. In **B1, B2** and **B3** indicate *Chlorella vulgaris* UTEX395 transfected with agrobacterial strain; LBA4404 harbouring the vector pCAMBIA1301. Blue product formation indicates the presence of the marker enzyme; β −Glucuronidase with 100% of cells demonstrating expression.

6.3.4 GFP transient expression

Following confirmation of the presence of either pTRAc::eGFP or pRIC::eGFP in GV3101pMP90RK *R. radiobacter* cell lines with the use of colony PCR (**Figure 39**), lines containing plasmids were selected and cryogenically stored to act as working stocks.

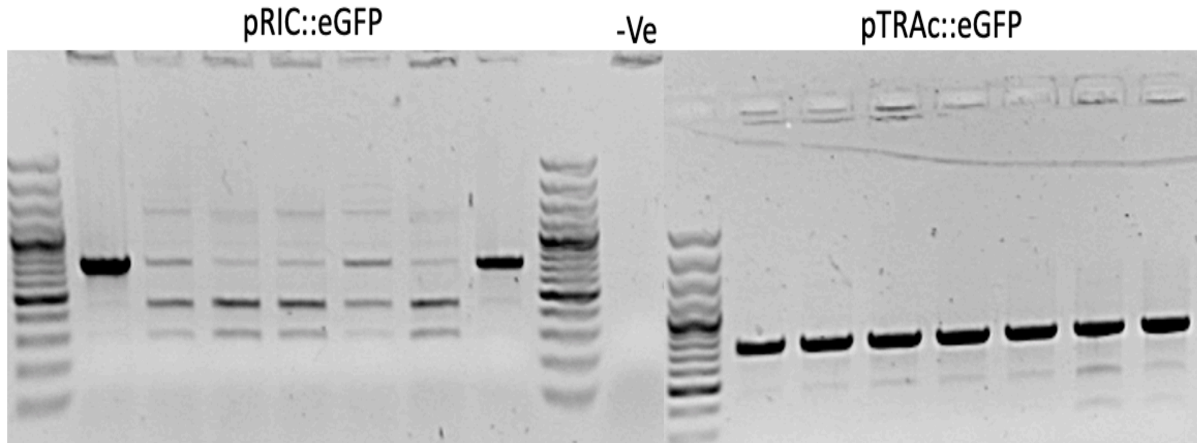


Figure 39. GV3101 pMP90RK:pTRAC::eGFP and GV3101 pMP90RK:pRIC::eGFP colony PCR.

After viewing the crude column, that had noCelite®545, a very limited number of GFP positive cells were observed (**Figure 40**).

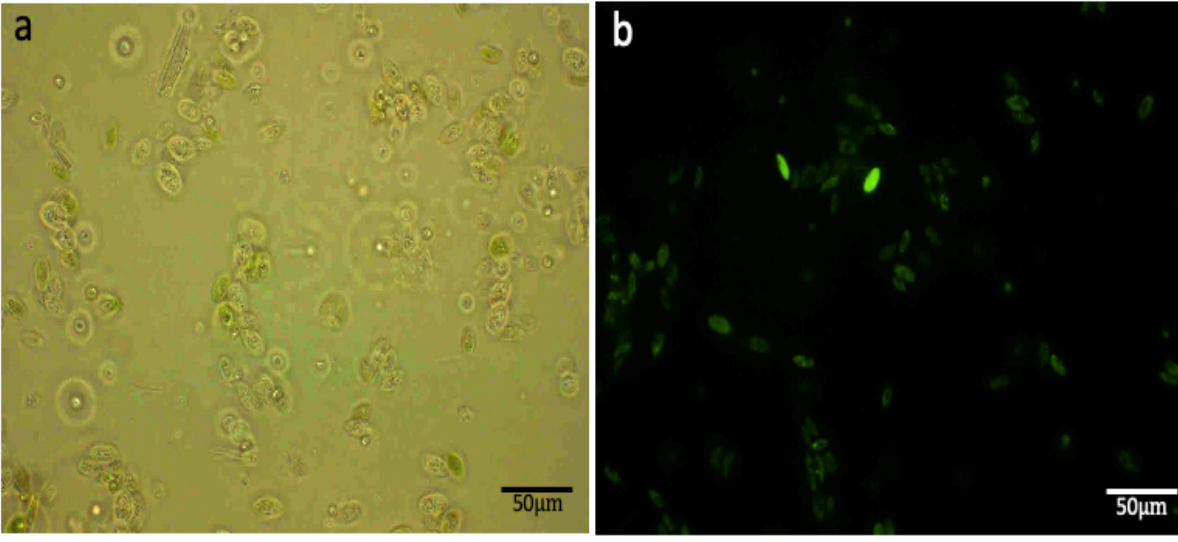


Figure 40. Brightfield vs. fluorescence micrograph of *Scenedesmus sp*. exhibiting *R. radiobacter*-mediated eGFP expression post-transient transfection.

For this reason, it was necessary to assess the entire axenic algal library for any variance in terms of highly fluorescing isolates. Over a seven-day period, green fluorescence signal of the microalgal- *R. radiobacter* co-culture was normalised against that of the negative control, containing only that of the same algal isolate. This resulted in a large variance in output across the algal library, with MPA46.1, MPA33.1, MPA10.2, MPA34.1, GHD.1, *Scenedesmus sp*. MPA16.1 and MPA49.1 demonstrating the greatest relative fluorescence (**Figure 41**).

As an additional control, an *R. radiobacter* culture was assessed for fluorescence signal, which was negligible (results not shown).

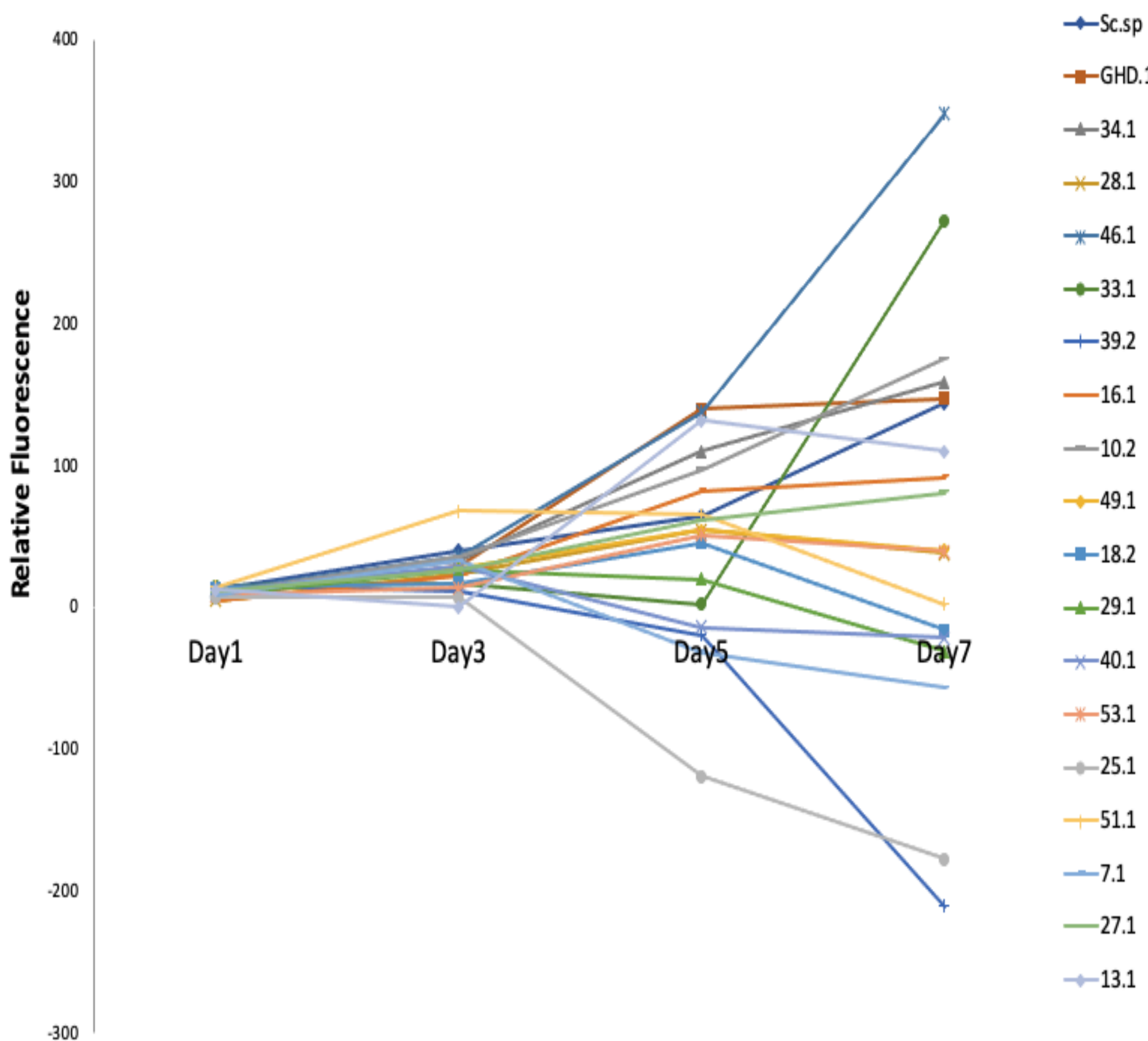


Figure 41. Relative fluorescence of axenic algal library in liquid culture. pTRAc::eGFP fluorescence in 19 axenic microalgal isolates grown in 3N BBM, relative to untransfected equivalent isolate

Interestingly, relative fluorescence varied markedly across the library at day seven. Isolates that demonstrated a marked increase in relative fluorescence between day one and day three (MPA51.1), had then decreased to negligible fluorescence by day seven, whereas some isolates (MPA46.1 and MPA33.1) did not demonstrate marked relative fluorescence by day 5, but showed a dramatic increase by day seven, contrary to the majority of the isolates that reached equilibrium.

Assessment of *Desmodesmus sp*. (MPA16.1) algal-Celite® column for transient expression of GFP was performed with western blot (**Figure 42**). Transient expression of GFP was done using the self-replicating vector pRIC::eGFP, while a second column, transfected with pRIC::LALF-E7 acted as a negative control.

Banding consistent with eGFP was observed on the blot (yellow arrow), with the negative control showing no banding, except for two bands that match that of the eGFP test.

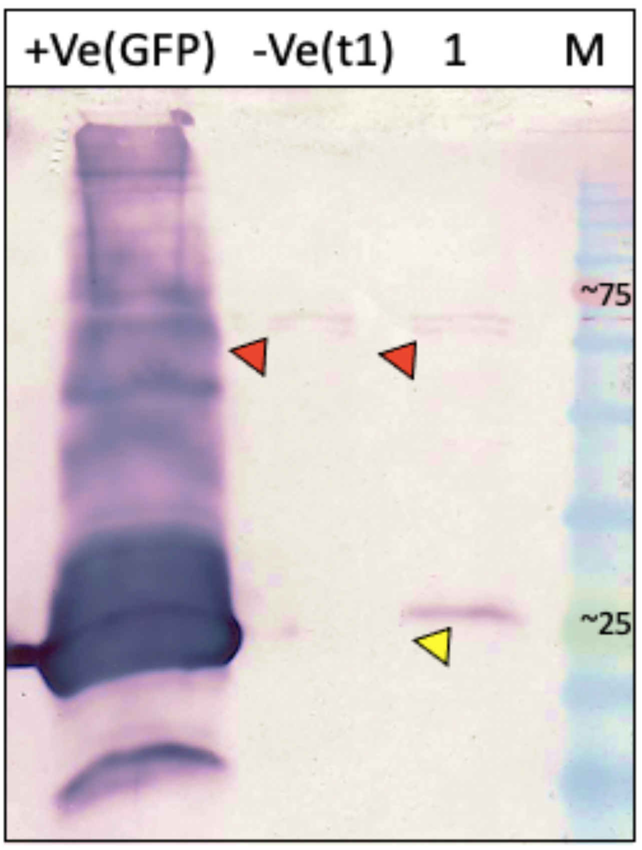


Figure 42. Western blot of GFP expression in Desmodesmus sp. (MPA16.1). Primary antibody: anti-GFP produced in rabbit. Secondary antibody: anti-rabbit alkaline phosphatase conjugate. Lane 1: GFP positive control (+-26.9kDa). -ve (GFP): algal isolate Desmodesmus sp. (MPA16.1) exposed to pRIC::LALF-E7. t1: algal isolate MPA16.1 exposed to pRIC::eGFP. Yellow arrow denotes corresponding band at t1.

6.3.5 HRP transient expression

The investigation into the presentation of HRP band pattern via western blot was performed to assess how this variably glycosylated protein may vary on the blot itself (**Figure 43**). This was done using *N. benthamiana*. The self-replicating vector, pRIC::HRPΔC produced several bands, while pTRAc::HRPΔC showed a single band.

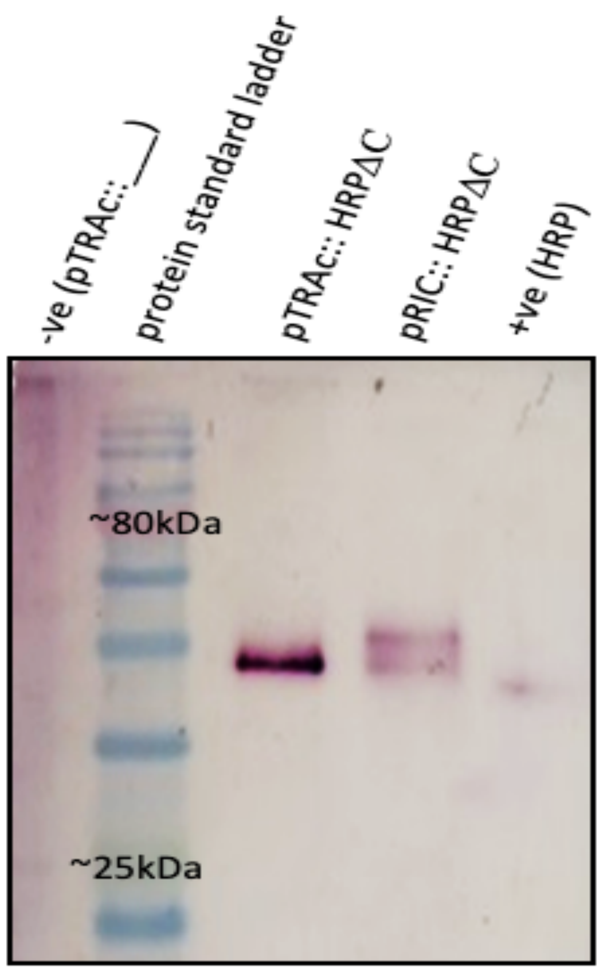


Figure 43. ***N. benthamiana*****-produced HRP from pTRAc and pRIC.**

The positive control band consisting of purified HRP, though faint, was slightly larger than the plant produced HRP. The difference in size of the control bands could be due to variable glycosylation of HRP.

Following this, two *Desmodesmus sp.* (MPA16.1) algal-Celite® columns were transfected with pTRAc::HRPΔC, and one was transfected with pTRAc::*empty*, that contained no transgene. This one acted as a negative control.

Prior to column resuspension and algal protein extraction for western blotting, PBS solution was vacuumed through each column, collected and then HRP substrate was added to each column. Both pTRAc::HRPΔC columns showed a blue colour change, while no colour change was observed for pTRAc::*empty* (**Figure 44**).

Western blotting showed the presence of hyper variable banding often associated with glycosylated proteins such as HRP, with no banding present in the negative control, except for some non-specific banding present in both the test samples.

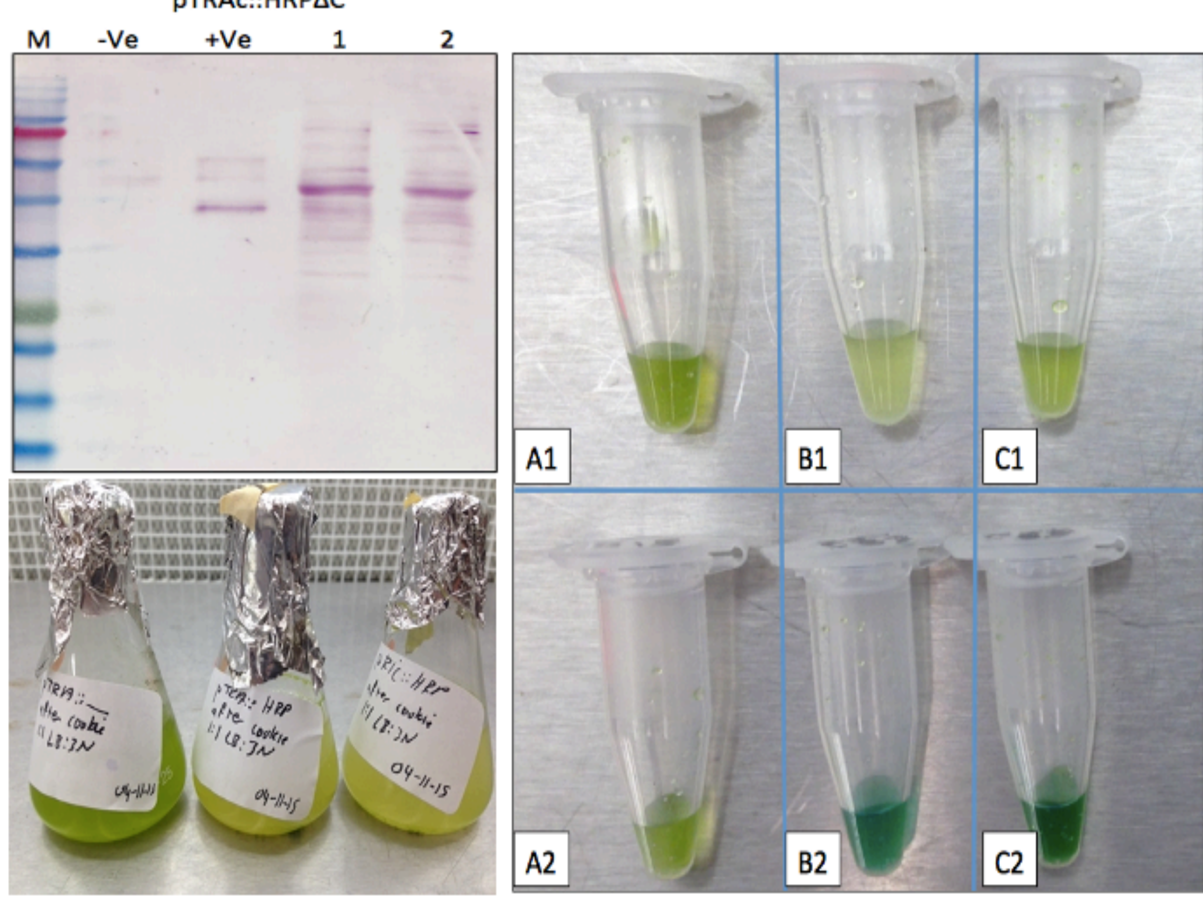


Figure 44. Cell pack mediated transient HRP expression in *Desmodesmus spp.* (MPA16.1). Western blot showing protein ladder, negative control (pTRAc:: empty vector), positive control (HRP produced in *N. benthamiana*), lane 4 and 5 showing HRP produced in *Desmodesmus spp*. (MPA 16.1) **(Top left).** Colour change reaction showing HRP activity with the KLP Blue system (**Right).** Gene expression impact on microalgal population, with all three containing *R. radiobacter*, but no expression gene present in right, while the other two contain the expression gene, HRP **(Bottom right).**

Interestingly, immediately after the columns were resuspended, the columns that contained the HRP transgene clearly showed metabolic stress in the algal biomass, while the column transfected with pTRAc::empty showed healthy algal biomass. This may be indicative, not that algal biomass is placed under metabolic stress by the presence of *R. radiobacter* itself, but rather by the actual transcription of transient heterologous proteins, such as HRP.

6.3.6 HPV16 hL1 transient expression

It was initially investigated to what degree infiltration media constituents may affect heterologous protein production in *N. benthamiana.* This is especially relevant to algal-Celite® column transfection, because although this media acts as a vehicle for *R. radiobacter* delivery, it may also allow for the simultaneous feeding of the algae within the column. Validating that nutrient media can allow for transient protein production

was therefore of high importance. This was done using traditional induction media, LBB and TAP media with the subsequent vacuum infiltration of leaves, protein extraction and quantification (TSP) and relative protein loading per lane on western blot (**Figure 45**). Relative protein loading was not done on the positive control, as this was purified HPV16hL1 subunit protein, and was loaded to merely act as a guide for western blot banding.

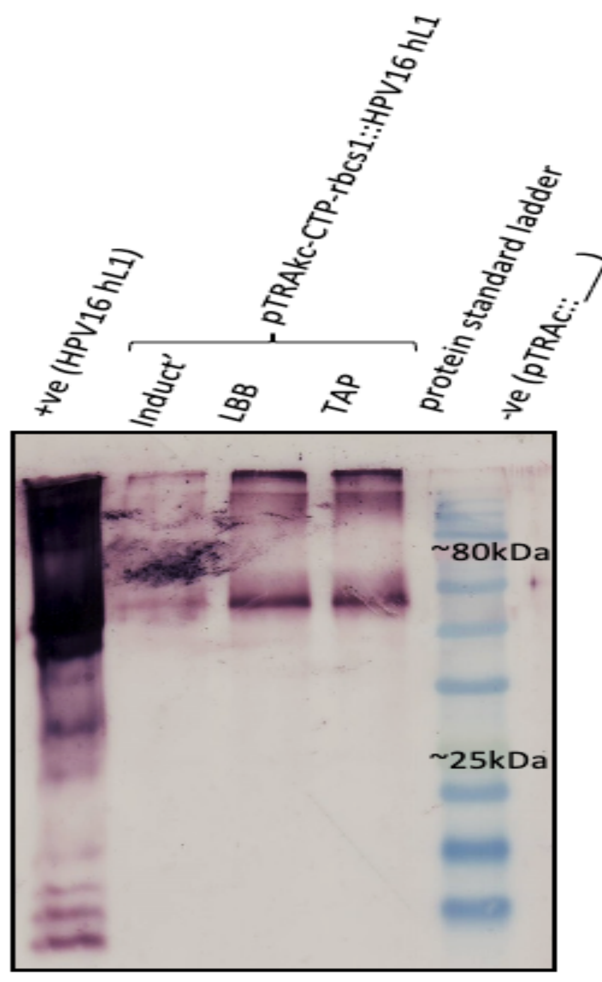


Figure 45. Western blot: pTRAkc CTP-rbcs1::HPV16hL1 transient expression in N. benthamiana; constant TSP but differential infiltration media. (Negative lane is cut in half as it was used concurrently as a negative lane for another blot. Not shown)

Interestingly, traditional induction media showed a marked decrease in band signal strength relative to the other two media compositions, which were of comparable intensity. This result is reassuring for investigations into the column later on, as it demonstrates that algal biomass feeding can be done in concert with transient gene delivery during vacuum infiltration.

Following this, *Scenedesmus spp. (QUCCCM63)* was transfected with pTRAkc CTP-rbcs1::HPV16hL1 (**Figure 46 a**). The positive controls consisted of 100ng purified hL1 as well as hL1 produced in *N. benthamiana.* It was clearly visible in these positive controls, that two bands appeared in the positive controls, which was also confirmed

through the work done by Naupu et al (2020) , that showed similar banding (**Figure 46 b**). In the *Scenedesmus sp*. (QUCCCM63) sample the +- 47 kDa band is present, but the +- 55 kDa is absent, now being found at +- 20 kDa. This difference can be explained, when it is considered that L1 has been shown to form truncated peptides when expressed in *N. benthamiana*, as is the case with HPV11 hL1, which was truncated from 55 kDa to 35.5 kDa (Kohl et al., 2007) (**Figure 46 c**).

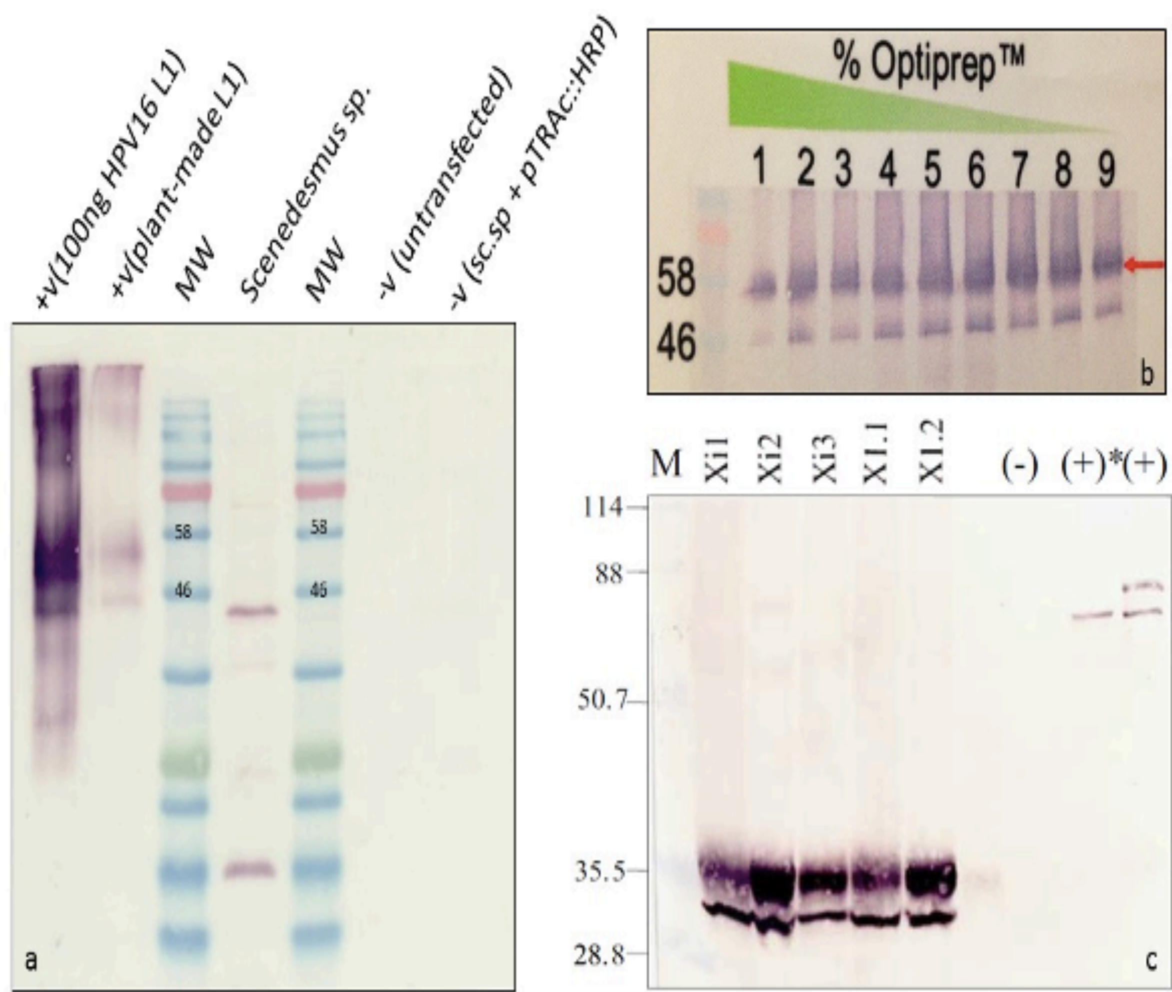


Figure 46. Western blots showing expression of various HPVhL1 heterologous proteins in various production systems. In **a** *Scenedesmus sp. QUCCCM63* transfected with pTRAkc-CTP, rbcs1::HPV16 ʰL1. This was done post ACP resuspension and selection (Kanamycin 80mg.L^{-1}). In **b**, a western blot with HPV16 L1 made in plants. In **c** is a western blot of HPV11 L1 made in plants. Though HPV16 L1 made in *Scenedesmus sp.* is forming a truncated form of the L1 protein at 46kDa, this band does correspond to a similar truncated L1 peptide form, present in plants (**1b**). Truncation events such as this have been known to occur even in plants, as evidenced by the truncated HPV11 L1, which was degraded to a size corresponding to 35.5kDa (**1c**) (western blot taken from Kohl et al., 2007)

Although hL1 was found to be seemingly truncated following western blot, samples were imaged via TEM to see whether hL1 monomers were able to form VLPs. From previous studies, SEM imaging has revealed VLP production in *N. benthamiana* that were +- 50 nm in diameter (**Figure 47a** and **b**). Though there does exist a rather

marked difference in the negative SEM image (**Figure 47c**) relative to the positive TEM image (**Figure 47d**). This difference is probably due to artifacts of the imaging process and do not represent VLP production. However, upon closer inspection, structures of the correct dimensions were observed which indicated limited VLP production They were seen as hollow spherical structures of the correct diameter that are remarkably similar to plant produced VLPs.

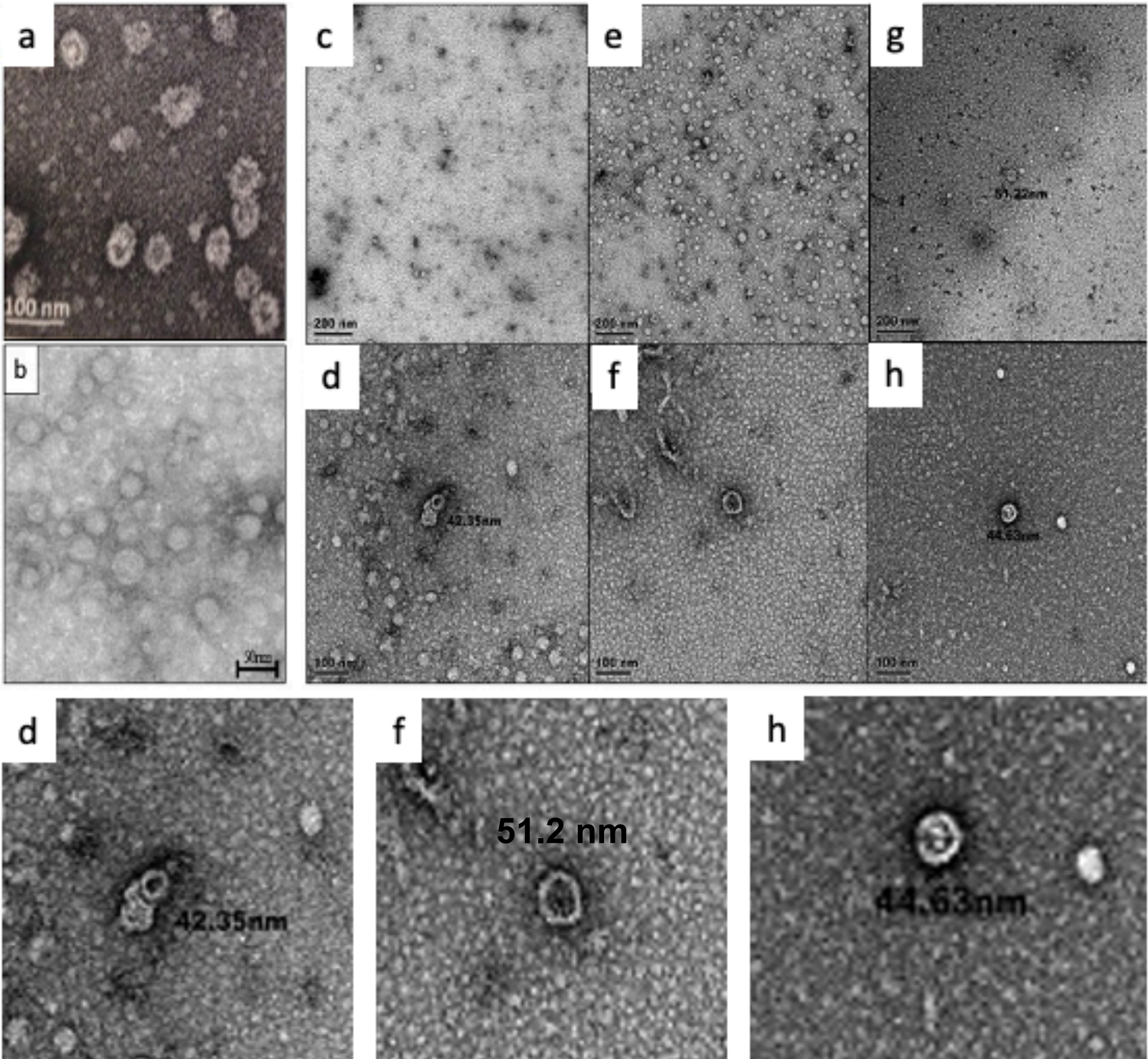


Figure 47. TEM images of proposed virus like particles (VLPs). In **a**, VLPs produced in plants (*N. benthamiana*), from HPV16 L1 (taken from Kohl et al., 2007). In **b**, VLPs produced from HPV11 L1 (taken from Naupu et al., 2020). In **c**, negative, non-transfected image of *Scenedesmus sp*.QUCMMM63. In **d-h**, possible VLP micrograph structures (42.35nm, 51.2nm and 44.63nm) produced in *Scenedesmus sp*. QUCCCM63 transfected with pTRAkc-CTP rbcs1:: HPV16 hL1.

6.4 Conclusion

This investigation focused on the expression and synthesis of heterologous proteins utilizing an algal cell pack. The final research outputs are intended to show the viability of this strategy and its potential as a research tool for the investigation of novel

planktonic organisms, that can simultaneously be put to use as a tool for the conservation of aquatic ecologies The other aim of researching an algal cell pack was to examine the possibility for future commercial application for the production of heterologous proteins.

6.4.1 Assessing stable integration of the transgene nptII

To achieve these aims, the research first evaluated the transgene *nptII* stable integration. This was done by assessing the effectiveness of the transfected versus untransfected populations in withstanding exposure to the algal eukaryotic toxin g418. After a few generations, PCR was used to further validate the stable nuclear incorporation of this gene into the genomes of transfected cell lines. The tests revealed that different resistant colonies contained a stable copy of the *nptII* gene sequence, with some colonies demonstrating higher levels of resistance to g418 relative to other colonies. This would be expected since random integration into the genome results in the integration into genomic regions of higher expression rates further resulting in higher resistance profiles of certain colonies relative to others.

In addition, it was discovered that certain colonies would lose their resistance profiles to g418 after multiple generations, though they still demonstrated the presence of the gene via PCR. This indicated that differential silencing does play a role in affecting resisting g418 of different algal colonies. The direct gene silencing either occurred at DNA level or possible downstream effects limited enzyme expression and their consequent loss of resistance. To investigate this further, cDNA could be used to elucidate this, though this was outside of the scope of this research. This, all-too-often encountered expression obstacle - associated with stable transgene integration into expression systems - is one of the core reasons for looking to transient expression. Transient expression oftentimes overcomes this hurdle of gene silencing and the nett loss of heterologous protein expression relative to the total population that carries transgenes.

6.4.2 Transient expression of two reporter proteins

The investigation of transient expression of two heterologous products, GFP and β-glucuronidase, followed after the two preceding investigations. These two reporter proteins, which are fundamental to the parametric investigation of factors that affect heterologous gene expression, allow for quantification of expressing individual cells versus those cells not expressing.

In the case of GFP, cells express a fluorescent protein derived from the jellyfish, *A. victoria,* enabling visualisation via fluorescent microscopy as well as assay via direct peptide detection using western blotting. Both of these methods were utilised in this investigation, and both methods subsequently demonstrated the presence of fluorescent cells after exposure to UV light, as well as showing banding patterns that confirmed the presence of the GFP peptide.

The presence of β-glucuronidase enzyme, in a functional conformation, allows for the metabolism of the colourless substrate, x-glucuronide, into a blue product. This colour change was first evident in the leaves of *N. benthamiana,* after removing the chlorophyll in an ethanol wash. The colour change was also evident in the microalgae *C. vulgaris* UTEX395, within the novel algal cell pack system. The chlorophyll in microalgae *C. vulgaris* UTEX395 was also removed in an ethanol wash, with clear visualisation of β-glucuronidase-expressing cells.

6.4.3 Expression of two commercially valuable heterologous proteins

Lastly, the expression of two heterologous proteins with high commercial value was investigated. The first was the expression of HRP, which was confirmed using western blot identification of HRP heterologous peptide, demonstrating HRP's characteristic differential glycosylation pattering, as well as proof-positive colour changes in transfected algal columns. The second investigation in this case, involved the expression of L1 from HPV16, which produced evidence of VLP formation. Although the expression level was low and the protein itself was truncated, the structures of the right size and composition were discovered. Truncation of this nature has been evident in *N. benthamiana,* previously with the expression of HPV11.

6.4.4 Differential proficiencies in the expression of different transgenes

Different microalgal isolates and species appear to exhibit disparate capability in the expression of various transgenes and their protein products. For instance, L1 from HPV16 was expressed in *Scenedesmus spp*. (QUCCCM63) but not in other investigated isolates this contrasts with the expression profiles of HRP and *nptII,* which were found to preferentially express in the *Desmodesmus spp*. (MPA16.1). For this reason, small-scale screening of algal isolates for their ability to produce heterologous protein product form a specific *R. radiobacter*- vector combination is essential for ideal pairing of these parameters.

6.4.5 Challenges in extracting heterologous protein products

The extraction of heterologous protein products from the expressing algal cells remains a challenge. Most assays required the algal biomass to be separated from the columns themselves, and then an extraction process to be undertaken. This often involves liquid nitrogen freezing and crushing to access transgenes and/or heterologous protein products, such as for western blot analysis. This hurdle needs to be overcome to make the algal cell pack valuable for industrial applications. Algal isolates should be sought that either allow for export of the protein product into the extracellular liquid space of the column, as was preliminarily demonstrated with the production of HRP and the algal isolate *Desmodesmus spp*. (MPA16.1).

Conversely, algal isolates should be sought that allow for ease of cell rupture in the presence of a lysis buffer. Once maximal heterologous product is being expressed, this lysis buffer could be vacuumed through the column, allowing for removal of soluble, stabilised product, and the retention within the column of cell debris. This would allow for simpler downstream processing of product, with cell debris then being

removed from the column via resuspension, diatomaceous earth settling and suspended debris removal via vacuum.

6.4.6 Valuable applications of algal cell pack

The algal cell pack is nevertheless beneficial for a variety of applications despite these difficulties. It may be used to first separate an algae ecosystem by size and examine which cultures are most suited for the transgene that is being expressed as well as the employed vector.

7 General conclusions and recommendations

The utilization of microalgae in a novel matrix-based column system as a new system for transient heterologous protein expression was the kernel research output of this investigation. Of specific relevance is the application of this system in quickly identifying microalgal isolates that are best suited to a specific application, such as specific protein expression, *a priori*.

During the development process, several other novel methodologies and resources were developed. These include:

- Development of an axenic algal library suitable for heterotrophic study from one originating from photosynthetic, autotrophic conditions with high associated contamination when grown autotrophically, from South Africa's Mpumalanga region.
- Development of cryogenic preservability of the axenic algal library at -80 ℃.
- Demonstration of the ability of metabolic shift of axenic microalgal isolates, from photosynthesis in minimal media to aerobic respiration of complex reduced carbon in enriched media, with increase in growth-rates as a function of volumetric feeding.
- Utilising the column system for centrifuge-free algal biomass harvesting, in a batch-based approach.
- Algal isolate selection via antibiotic exposure for later implementation utilising the algal cell pack column approach for transient heterologous protein expression. Here the algal cell pack enables iterative cell concentration by de-watering, followed by media replenishment and suspension for cell cultivation.
- Utilising the column system for teasing apart planktonic ecologies by size.
- Initial demonstration of endogenous microalgal viral lysis via column incorporation.
- Utilising the column system to allow for algal population growth, under antibiotic selection, within the column and thereby easier development of novel axenic isolates.
- Demonstration of the novel use of the pTRAc vector suite in transient expression of heterologous proteins in microalgae, specifically GFP and the industrially relevant enzyme, HRP.

- Demonstration of transcription and translation of the self-replicating gemini viral vector, pRIC, during the expression of HRP.
- Expression of HPV16hL1 with the binary vector pTRAkc-CTP, rbcs1::HPV16 ^{h}L1in *Scenedesmus spp*. (QUCCCM63) as well as initial results demonstrating VLP production. This preliminary finding lies outside the original scope of this investigation. The possibility of utilising microalgal biomass for expression and self-assembly of human and or veterinary-originating viral peptides into VLPs for vaccine production may allow for the revolutionization of VLP production, not to mention using these structures to deliver highly targeted molecular payloads to specific cell types.

7.1 Recommendations for future study

As this column-based approach is novel, during the development process numerous applications were uncovered. All of these however require, to a greater or lesser extent, further development.

A primary consideration would be utilising already developed and optimised microalgal isolate, *R. radiobacter* and vector combinations, within the algal cell pack for investigation of transient heterologous protein production at scale. This requires the need for a reliable design of a novel combination bioreactor/ harvest system, allowing for column formation, biomass incorporation, *R. radiobacter* transfection, product elution and harvesting, and lastly algal debris separation from diatomaceous earth and repeat of this process.

The algal cell pack will however prove invaluable in future microalgal research and will not only allow for the filling of the gaps needed to solve humanities need for novel therapeutic proteins, but will allow for the investigation and study of novel planktonic microalgae and their conservation and protection. A fundamental future endeavour if for nothing other than to harness near-infinite biodiversity, both genetic and biochemically, of these important organisms.

From a biotechnological perspective, there exists a large degree of variance in the response of microalgal species to various selection parameters and parameters that are required for assessing microalgal isolate suitability to application.

These include:

- Diverse response variance in species selection to antibiotic selection.
- Variance of selective response of a single species when exposure occurs in the context of different media compositions.
- Single species protein expression variance in relation to the various plasmid vector systems possible
- Across species protein expression variance from a single plasmid vector.
- Possible protein expression variance relating to codon usage, silencing or any number of cryptic causalities.

7.1.1 Single species, multiple vector selection process

In the case where a specific species needs to be used for a protein expression application, various vectors would be pulse vacuum infiltrated through a single algal cell pack containing the specific species. If these vectors made use of fluorescent FMDV 2A expression tethering (**see Figure 11**), following transfection and resuspending algal biomass. Fluorescence signal could be selected for with the use of fluorescence activated cell sorting (FACS). Here, individual cell fluorescence signal then directly correlates to protein product translation.

7.1.2 Ecological sample, isolate selection process

In a similar process as described above, ecological samples could be transfected with single/ multiple vector combinations. Following FACS selection, isolates would be identified without *a priori* knowledge of vector suitability. This selection process would additionally work for selection of transiently transfectable isolates.

7.1.3 Synthetic evolution via selective iteration

Trait evolvability is related to the underlying nature of the organism experiencing the specific selection. Transient transfection ability is one such trait, which through iterative column formation/transfection/solubilisation/FACS selection for high fluorescence expressors, transient transfectability would be synthetically evolved. Whole genome sequencing of evolved versus non-evolved individuals could then be used to look at the underlying genetic mechanisms that allow for transfectability.

The correct perspective on microalgal species is that every single one has value.

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8 Appendix

8.1 Recipes

3N BBM

$NaNO_3$ 0.750 g/L

$CaCl_2 \cdot 3H_2O$ 0.025 g/L

$MgSO_4 \cdot 7H_2O$ 0.075 g/L

$K_2HPO_4 \cdot 3H_2O$ 0.075 g/L

KH_2PO_4 0.175 g/L

NaCl 0.025 g/L

PIV (Add 6 ml in 1 L 3N BBM)

$Na_2EDTA \cdot 2H_2O$ 0.750 g/L

$FeCl_3 \cdot 6H_2O$ 0.097 g/L

$MnCl_2 \cdot 4H_2O$ 0.041 g/L

$ZnCl_2$ 0.005 g/L

$CoCl_2 \cdot 6H_2O$ 0.002 g/L

$Na_2MoO_4 \cdot 2H_2O$ 0.004 g/L

Add vitamins after autoclaving.

Vit B1 (Thiamine/HCL) 0.0012 g/L

Vit B12 (cyanocobalamin) 1.10-6 g/L

Recipe found at Utex library (https://utex.org/products/modified-bold-3n-medium)

3N BBM Het media

2 x 3N BBM 500 mL/L

Tryptone 2 g/L

Glucose 4 g/L

Glycerol 100% 1.8 g/L

Yeast extract 1 g/L

dH2O to 1000 mL

Autoclave

Blocking Buffer

10X PBS 10 mL/L

10% Tween-20 1 mL/L

Skim-milk powder 5 g/L

dH2O to 1000 mL

Luria-Bertani (LB) Medium

Tryptone 10 g/L

NaCl 10 g/L

Yeast Extract 5 g/L

ddH2O to 1L

Autoclave

Luria-Bertani (LB) agar

Tryptone 10 g/L

NaCl 10 g/L

Yeast Extract 5 g/L

Bacteriological agar 1.5 g/L

ddH2O to 1L

Autoclave

Luria-Bertani Base (LBB)

Tryptone 2.5 g/L

Yeast extract 12.5 g/L

NaCl 5 g/L

MES 1M 10 mL/L

H2O up to 1 L

pH 5.6 with HCl.

Autoclave

10X Phosphate Buffered Saline (PBS)

NaCl 8 g/L

KCl 2 g/L

Na_2HPO_4 14.4 g/L

KH_2PO_4 2.4 g/L

Adjust to pH 7.4

ddH_2O to 1L

Autoclave

PBS was made from diluting 100 mL 10x PBS in 900 mL dH_2O

PBS +1% Triton X-100

10x PBS 100 mL/L

Triton X-100 10 mL/L

_ddH_2O to 1L

Resuspension solution

MES 0.975 g/L

$MgCl_2$ 2.03 g/L

H_2O up to 1 L

pH 5.6 with NaOH.

Running Buffer (1X)

Glycine 28.8 g/L

Tris base 6.04 g/L

Sodium dodecyl sulphate (SDS) 2 g/L

ddH_2O to 1L

5 x Sample application buffer

940 µL 10% SDS

470 µL 1M TrisCl

95 µL 100 mM Ethylenediaminetetraacetic acid (EDTA)

2.45 mL glycerol

545 µL ddH2O

205 µL β-mercaptoethanol

1 mg bromophenol blue

Transfer Buffer (1X)

Glycine 28.8 g/L

Tris Base 6.04 g/L

Methanol 200 mL/L

dH2O to 1L

Tris-Borate-EDTA (TBE) Electrophoresis Buffer

Tris base 10.8 g/L

Boric acid 5.5 g/L

EDTA 0.75 g/L

dH2O to 1 L

8.2 Supplementary data

8.2.1 Heterotrophic algal isolate growth

A:

G:

Gl:

G10:

Sample Name	Carbon Source	Algal Growth
5.1	A	no
	G	no
	Gl	no
	G10	maybe
6.1	A	slight green
	G	yes
	Gl	yes
	G10	yes (same as above)
7.1	A	maybe
	G	slight
	Gl	slight
	G10	same as above
8.1	A	good growth
	G	slight
	Gl	no
	G10	no
8.2	A	yes
	G	yes
	Gl	not really
	G10	yes
10.2	A	yes
	G	yes
	Gl	yes
	G10	yes

10.3	A	no
	G	white growth
	Gl	" "
	G10	" "
13.1	A	no
	G	no
	Gl	yes
	G10	good growth
16.1	A	good growth
	G	good growth
	Gl	good growth
	G10	good growth
19.2	A	white growth, maybe mould
	G	white growth, maybe mould
	Gl	slight green in white
	G10	slight green in white
19.3	A	no
	G	slight green in white
	Gl	no
	G10	no
25.1	A	very slight green
	G	yes
	Gl	yes, but less than glycerol
	G10	yes, but less than glycerol
27.1	A	very slight
	G	very slight
	Gl	maybe
	G10	more than G
27.3	A	no
	G	no
	Gl	no
	G10	yes

27.4	A	no
	G	white clump
	Gl	" "
	G10	yes
28.1	A	no
	G	no
	Gl	slight
	G10	yes
29.1	A	no
	G	maybe
	Gl	maybe
	G10	yes
31.1	A	no
	G	maybe
	Gl	no
	G10	yes
33.1	A	yes
	G	no
	Gl	no
	G10	no
34.1	A	yes
	G	no
	Gl	no
	G10	" "
34.2	A	yes
	G	maybe
	Gl	yes
	G10	yes
36.1	A	no
	G	no
	Gl	slightly
	G10	no
38.1	A	no
	G	very slightly
	Gl	maybe

	G10	yes
38.2	A	yes
	G	yes
	Gl	yes
	G10	yes
38.3	A	no
	G	yes
	Gl	yes
	G10	yes
39.1	A	no
	G	no
	Gl	no
	G10	no
39.2	A	no
	G	yes
	Gl	no
	G10	no
40.1	A	yes
	G	yes
	Gl	yes
	G10	yes
42.1	A	no
	G	yes
	Gl	yes
	G10	yes
46.1	A	no
	G	yes
	Gl	yes
	G10	yes
49.1	A	yes
	G	yes
	Gl	yes
	G10	yes
50.2	A	very slightly

	G	yes
	Gl	yes
	G10	yes
50.3	A	very sightly
	G	yes
	Gl	yes
	G10	yes
51.1	A	very slightly
	G	very slightly
	Gl	no
	G10	no
51.2	A	no
	G	no
	Gl	no
	G10	no
53.1	A	no
	G	no
	Gl	no
	G10	no
53.2	A	yes
	G	no
	Gl	no
	G10	no

8.2.2 18s sequence data

Desmodesmus sp. GM4a genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence

GenBank: AB917128.1

FASTA Graphics

Go to:

```
LOCUS       AB917128                2556 bp    DNA     linear   PLN 01-OCT-2014
DEFINITION  Desmodesmus sp. GM4a genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S
            rRNA, partial and complete sequence.
ACCESSION   AB917128
VERSION     AB917128.1
KEYWORDS    .
SOURCE      Desmodesmus sp. GM4a
  ORGANISM  Desmodesmus sp. GM4a
            Eukaryota; Viridiplantae; Chlorophyta; core chlorophytes;
            Chlorophyceae; CS clade; Sphaeropleales; Scenedesmaceae;
            Desmodesmus.
REFERENCE   1
  AUTHORS   Hoshina,R.
  TITLE     DNA analyses of a private collection of microbial green algae
            contribute to a better understanding of microbial diversity
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 2556)
  AUTHORS   Hoshina,R.
  TITLE     Direct Submission
  JOURNAL   Submitted (13-MAR-2014) Contact:Ryo Hoshina Nagahama Institute of
            Bioscience and Technology, Department of Bioscience; Tamura 1266,
            Nagahama, Shiga 526-0829, Japan URL
            :http://www.nagahama-i-bio.ac.jp/
FEATURES             Location/Qualifiers
     source          1..2556
                     /organism="Desmodesmus sp. GM4a"
                     /mol_type="genomic DNA"
                     /strain="GM4a"
                     /db_xref="taxon:1475466"
     rRNA            <1..1776
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     misc_RNA        1777..1934
                     /product="internal transcribed spacer 1"
     rRNA            1935..2088
                     /product="5.8S ribosomal RNA"
     misc_RNA        2089..2335
                     /product="internal transcribed spacer 2"
     rRNA            2336..>2556
                     /product="28S ribosomal RNA"
```

Scenedesmus sp. QUCCCM63 18S ribosomal RNA gene, partial sequence

GenBank: KM985413.1

FASTA Graphics

Go to:

```
LOCUS       KM985413                1293 bp    DNA     linear   PLN 30-DEC-2015
DEFINITION  Scenedesmus sp. QUCCCM63 18S ribosomal RNA gene, partial sequence.
ACCESSION   KM985413
VERSION     KM985413.1
KEYWORDS    .
SOURCE      Scenedesmus sp. QUCCCM63
  ORGANISM  Scenedesmus sp. QUCCCM63
            Eukaryota; Viridiplantae; Chlorophyta; core chlorophytes;
            Chlorophyceae; CS clade; Sphaeropleales; Scenedesmaceae;
            Scenedesmus.
REFERENCE   1  (bases 1 to 1293)
  AUTHORS   Saadaoui,I., Al Ghasal,G.S.H.S., Al Khelaifi,F.A., Al Jabri,H.M.
            and Potts,M.
  TITLE     Qatar Culture collection of Microalgae: a biological resource of
            biodiversity and Biotechnological study
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1293)
  AUTHORS   Saadaoui,I., Al Ghasal,G.S.H.S., Al Khelaifi,F.A., Al Jabri,H.M.
            and Potts,M.
  TITLE     Direct Submission
  JOURNAL   Submitted (11-OCT-2014) Algal Technologies Program, Centre for
            Sustainable Development, College of Arts and Sciences, Qatar
            University, Al Dafna, Doha, Al Jamaa Str. 2713, Qatar
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
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     source          1..1293
                     /organism="Scenedesmus sp. QUCCCM63"
                     /mol_type="genomic DNA"
                     /strain="QUCCCM63"
                     /isolation_source="desert soil"
                     /db_xref="taxon:1680532"
                     /country="Qatar"
     rRNA            <1..>1293
                     /product="18S ribosomal RNA"
ORIGIN
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Ankistrodesmus gracilis 18S ribosomal RNA gene, partial sequence

GenBank: KF574394.1

FASTA Graphics PopSet

Go to:

```
LOCUS       KF574394                1714 bp    DNA     linear   PLN 17-JAN-2015
DEFINITION  Ankistrodesmus gracilis 18S ribosomal RNA gene, partial sequence.
ACCESSION   KF574394
VERSION     KF574394.1
KEYWORDS    .
SOURCE      Messastrum gracile
  ORGANISM  Messastrum gracile
            Eukaryota; Viridiplantae; Chlorophyta; core chlorophytes;
            Chlorophyceae; CS clade; Sphaeropleales; Selenastraceae;
            Messastrum.
REFERENCE   1  (bases 1 to 1714)
  AUTHORS   Zhang,S.
  TITLE     Direct Submission
  JOURNAL   Submitted (22-AUG-2013) Ministry of Education Key Laboratory of
            Protection and Development Utilization, Hainan University, 58
            People Road, Haikou, Hainan 570228, China
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
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     source          1..1714
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                     /mol_type="genomic DNA"
                     /isolate="DL25"
                     /isolation_source="rivulet water"
                     /specimen_voucher="428200732DL25"
                     /db_xref="taxon:81816"
                     /country="China"
                     /lat_lon="18.66 N 109.92 E"
                     /collection_date="06-Jul-2011"
                     /PCR_primers="fwd_seq: acctggttgatcctgccagtag, rev_seq:
                     accttgttacgacttctccttcctc"
     rRNA            <1..>1714
                     /product="18S ribosomal RNA"
ORIGIN
```

8.3 Patent: Matrix-Mediated Cell Culture System

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
04 June 2020 (04.06.2020)

WIPO | PCT

(10) International Publication Number
WO 2020/110083 A1

(51) International Patent Classification:
A01G 33/00 (2006.01) *C12N 1/00* (2006.01)

(21) International Application Number:
PCT/IB2019/060330

(22) International Filing Date:
29 November 2019 (29.11.2019)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
2018/08076 29 November 2018 (29.11.2018) ZA

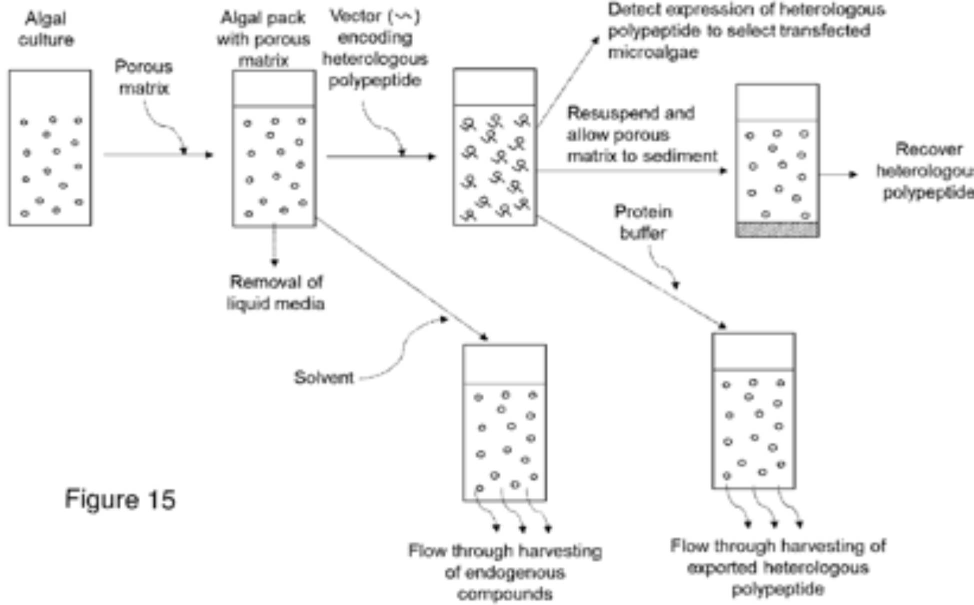
(71) Applicant: UNIVERSITY OF CAPE TOWN [ZA/ZA]; Lovers Walk, Rondebosch, 7701 Cape Town (ZA).

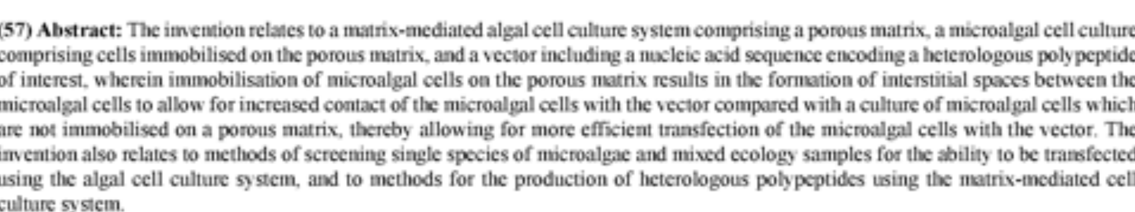
(72) Inventors: STEVENS, Dylan Troy; 34 Scott Road, Observatory, 7925 Cape Town (ZA). RYBICKI, Edward Peter; 34 Uitvlugt Rd, Pinelands, 7405 Cape Town (ZA). HARRISON, Susan T L; 34 Rathfelder Ave, Constantia, 7806 Cape Town (ZA).

(74) Agent: SPOOR & FISHER et al.; 11 Byls Bridge Boulevard, Building No. 14, Highveld Ext 73, 0157 Centurion (ZA).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA,

(54) Title: MATRIX-MEDIATED CELL CULTURE SYSTEM





(57) Abstract: The invention relates to a matrix-mediated algal cell culture system comprising a porous matrix, a microalgal cell culture comprising cells immobilised on the porous matrix, and a vector including a nucleic acid sequence encoding a heterologous polypeptide of interest, wherein immobilisation of microalgal cells on the porous matrix results in the formation of interstitial spaces between the microalgal cells to allow for increased contact of the microalgal cells with the vector compared with a culture of microalgal cells which are not immobilised on a porous matrix, thereby allowing for more efficient transfection of the microalgal cells with the vector. The invention also relates to methods of screening single species of microalgae and mixed ecology samples for the ability to be transfected using the algal cell culture system, and to methods for the production of heterologous polypeptides using the matrix-mediated cell culture system.

WO 2020/110083 A1

8.4 Material Safety Data Sheets

Supelco®

www.sigmaaldrich.com

SAFETY DATA SHEET

according to Regulation (EC) No. 1907/2006

Version 6.9
Revision Date 27.10.2023
Print Date 17.11.2023
GENERIC EU MSDS - NO COUNTRY SPECIFIC DATA - NO OEL DATA

SECTION 1: Identification of the substance/mixture and of the company/undertaking

1.1 Product identifiers

Product name	:	Celite® 545 particle size 0.02-0.1 mm
Product Number	:	1.02693
Catalogue No.	:	102693
Brand	:	Millipore
REACH No.	:	01-2119488518-22-XXXX
CAS-No.	:	68855-54-9

1.2 Relevant identified uses of the substance or mixture and uses advised against

Identified uses : Reagent for analysis, Chemical production

1.3 Details of the supplier of the safety data sheet

Company : Merck Pty Ltd.
259 Davidson Road, Corner Peddie Road
WADEVILLE, GERMISTON
1428
SOUTH AFRICA

Telephone : +27 (0) 8600 63725
Fax : +27 (0) 860 522 329

1.4 Emergency telephone

Emergency Phone # : 0-800-983-611 (CHEMTREC)

SECTION 2: Hazards identification

2.1 Classification of the substance or mixture

Classification according to Regulation (EC) No 1272/2008
Specific target organ toxicity - repeated exposure, Inhalation (Category 1), Lungs, H372

For the full text of the H-Statements mentioned in this Section, see Section 16.

2.2 Label elements

Labelling according Regulation (EC) No 1272/2008
Pictogram

Millipore- 1.02693 Page 1 of 12

The life science business of Merck operates as MilliporeSigma in the US and Canada

SAFETY DATA SHEET
Acetosyringone

Revision: 10/10/2017

according to Regulation (EC) No. 1907/2006 as amended by (EC) No. 1272/2008

Section 1. Identification of the Substance/Mixture and of the Company/Undertaking

1.1 Product Code: 23224

Product Name: Acetosyringone

Synonyms: 1-(4-hydroxy-3,5-dimethoxyphenyl)-ethanone; 3',5'-Dimethoxy-4'-hydroxyacetophenone;

1.2 Relevant identified uses of the substance or mixture and uses advised against:

Relevant identified uses: For research use only, not for human or veterinary use.

1.3 Details of the Supplier of the Safety Data Sheet:

Company Name: Cayman Chemical Company
1180 E. Ellsworth Rd.
Ann Arbor, MI 48108

Web site address: www.caymanchem.com

Information: Cayman Chemical Company +1 (734)971-3335

1.4 Emergency telephone number:

Emergency Contact: CHEMTREC Within USA and Canada: +1 (800)424-9300
CHEMTREC Outside USA and Canada: +1 (703)527-3887

Section 2. Hazards Identification

2.1 Classification of the Substance or Mixture:

Skin Corrosion/Irritation, Category 2

Serious Eye Damage/Eye Irritation, Category 2

Specific Target Organ Toxicity (single exposure), Category 3

2.2 Label Elements:

GHS Signal Word: **Warning**

GHS Hazard Phrases:

H315: Causes skin irritation.
H319: Causes serious eye irritation.
H335: May cause respiratory irritation.

GHS Precaution Phrases:

P261: Avoid breathing {dust/fume/gas/mist/vapors/spray}.
P264: Wash {hands} thoroughly after handling.
P280: Wear {protective gloves/protective clothing/eye protection/face protection}.

GHS Response Phrases:

P302+352: IF ON SKIN: Wash with plenty of soap and water.
P304+340: IF INHALED: Remove victim to fresh air and keep at rest in a position comfortable for breathing.
P305+351+338: IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P312: Call a POISON CENTER or doctor/physician if you feel unwell.
P332+313: If skin irritation occurs, get medical advice/attention.
P337+313: If eye irritation persists, get medical advice/attention.
P362+364: Take off contaminated clothing and wash it before reuse.

GHS Storage and Disposal Phrases:

Please refer to Section 7 for Storage and Section 13 for Disposal information.

Multi-region format

SECTION 1 - PRODUCT & COMPANY IDENTIFICATION

ARYSTA LifeScience South Africa (Pty) Ltd
Co. Reg. No.: 2009/019713/07
7 Sunbury Office Park,
Off Douglas Saunders Drive,
La Lucia Ridge, South Africa, 4019
Tel: 031 514 5600
Fax: 031 514 5611
e-mail: info@arysta.co.za
Web address: arystalifescience.co.za

Substance: epoxiconazole plus carbendazim
Product Name: **SOLO**
Product Use: Fungicide
Creation Date: November 2011
Revision Date: August 2019

24 Hr Emergency Number:
In case of Poisoning:
Poisons Helpline 0861 555 777
In case of Spillage:
Spill Tech Oil & Chemical Pollution Control 086 100 0366 / 083 253 6618

SECTION 2 - COMPOSITION / INFORMATION ON INGREDIENTS

Common name: 1) **Epoxiconazole** plus
2) **Carbendazim**
Chemical Name: 1) (2RS,3SR)-1-[3-(2-chlorophenyl)-2,3-epoxy-2-(4-fluorophenyl)propyl]-1H-1,2,4-triazole (**IUPAC**);
2) methyl benzimidazol-2-ylcarbamate (**IUPAC**)
CAS No.: 1) 133855-98-8
2) 790325
Chemical Family: 1) triazole;
2) benzimidazole carbamate
Chemical Formula: 1) $C_{17}H_{13}Cl\ FN_3O$; 2) C9H9N3O2
Molecular weight: 1) 329.8; 2) 191.2
Use: Combination product. Systemic fungicide for the control of diseases in plants. **Epoxiconazole** is a preventive and curative action fungicide inhibitor and ***Carbendazim*** is an inhibitor of germ tube development and mycelia growth.
Formulation: **Epoxiconazole** 125 g/ℓ plus
Carbendazim 125 g/ℓ
Suspension Concentrate

Symbol: N, Xn
Indication of danger: Environmentally Hazardous Substance; Harmful

SECTION 3 - HAZARD IDENTIFICATION

Likely routes of exposure:
Skin: May cause mild irritation.
Eye: May cause mild irritation.
Inhalation: Not a hazard under normal use conditions. Inhalation of excessive amounts of spray mist may cause respiratory irritation.
Swallowed: harmful when swallowed.

SECTION 4 - FIRST AID MEASURES AND PRECAUTIONS

Inhalation: If vapours or mists have been inhaled, and irritation has developed, remove the source of contamination or move victim to fresh air. The patient should be kept under observation and obtain medical attention if irritation persists.
Skin contact: Remove contaminated clothing, shoes and leather goods. Wash skin gently and thoroughly with cold water and nonabrasive soap. Obtain medical attention if irritation persists.
Eye contact: Immediately flush eyes with a stream of clean water for at least 20 minutes, holding the eyelid(s) open. Obtain medical attention if irritation persists.

SAFETY DATA SHEET
Cefotaxime (sodium salt)

Revision: 06/18/2018
Supersedes Revision: 10/28/2014

according to Regulation (EC) No. 1907/2006 as amended by (EC) No. 1272/2008

Section 1. Identification of the Substance/Mixture and of the Company/Undertaking

1.1 Product Code: 16040

Product Name: Cefotaxime (sodium salt)

Synonyms: (6R)-3-[(acetyloxy)methyl]-7R-[[(2Z)-2-(2-amino-4-thiazolyl)-2-(methoxyimino)acetyl]amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid, monosodium salt;

1.2 Relevant identified uses of the substance or mixture and uses advised against:

Relevant identified uses: For research use only, not for human or veterinary use.

1.3 Details of the Supplier of the Safety Data Sheet:

Company Name: Cayman Chemical Company
1180 E. Ellsworth Rd.
Ann Arbor, MI 48108

Web site address: www.caymanchem.com

Information: Cayman Chemical Company +1 (734)971-3335

1.4 Emergency telephone number:

Emergency Contact: CHEMTREC Within USA and Canada: +1 (800)424-9300
CHEMTREC Outside USA and Canada: +1 (703)527-3887

Section 2. Hazards Identification

2.1 Classification of the Substance or Mixture:

Respiratory Sensitization, Category 1

Skin Sensitization, Category 1

2.2 Label Elements:

GHS Signal Word: **Danger**

GHS Hazard Phrases:

H317: May cause an allergic skin reaction.
H334: May cause allergy or asthma symptoms or breathing difficulties if inhaled.

GHS Precaution Phrases:

P261: Avoid breathing {dust/fume/gas/mist/vapours/spray}.
P272: Contaminated work clothing should not be allowed out of the workplace.
P280: Wear {protective gloves/protective clothing/eye protection/face protection}.
P285: In case of inadequate ventilation wear respiratory protection.

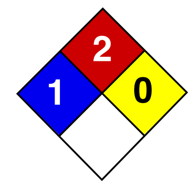
GHS Response Phrases:

P302+352: IF ON SKIN: Wash with plenty of soap and water.
P304+340: IF INHALED: Remove victim to fresh air and keep at rest in a position comfortable for breathing.
P333+313: If skin irritation or rash occurs, seek medical advice/attention.
P342+311: If experiencing respiratory symptoms call a POISON CENTER or doctor/physician.
P362+364: Take off contaminated clothing and wash it before reuse.

GHS Storage and Disposal Phrases:

Please refer to Section 7 for Storage and Section 13 for Disposal information.

Multi-region format



Material Safety Data Sheet
N,N-Dimethylformamide MSDS

Section 1: Chemical Product and Company Identification

Product Name: N,N-Dimethylformamide

Catalog Codes: SLD4261, SLD3331

CAS#: 68-12-2

RTECS: LQ2100000

TSCA: TSCA 8(b) inventory: N,N-Dimethylformamide

CI#: Not applicable.

Synonym: DMF; Dimethyl Formamide

Chemical Name: N,N-Dimethylformamide

Chemical Formula: HCON(CH3)2

Contact Information:

Sciencelab.com, Inc.
14025 Smith Rd.
Houston, Texas 77396

US Sales: **1-800-901-7247**
International Sales: **1-281-441-4400**

Order Online: ScienceLab.com

CHEMTREC (24HR Emergency Telephone), call:
1-800-424-9300

International CHEMTREC, call: 1-703-527-3887

For non-emergency assistance, call: 1-281-441-4400

Section 2: Composition and Information on Ingredients

Composition:

Name	CAS #	% by Weight
{N,N-}Dimethylformamide	68-12-2	100

Toxicological Data on Ingredients: N,N-Dimethylformamide: ORAL (LD50): Acute: 2800 mg/kg [Rat]. 2900 mg/kg [Mouse]. 5000 mg/kg [Rabbit]. DERMAL (LD50): Acute: 4720 mg/kg [Rabbit].

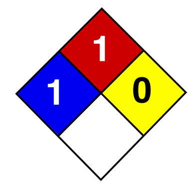
Section 3: Hazards Identification

Potential Acute Health Effects: Hazardous in case of skin contact (irritant, permeator), of eye contact (irritant), of ingestion, of inhalation.

Potential Chronic Health Effects:
CARCINOGENIC EFFECTS: 3 (Not classifiable for human.) by IARC. MUTAGENIC EFFECTS: Mutagenic for mammalian somatic cells. Mutagenic for bacteria and/or yeast. TERATOGENIC EFFECTS: Classified POSSIBLE for human. DEVELOPMENTAL TOXICITY: Classified Reproductive system/toxin/female, Reproductive system/toxin/male [POSSIBLE]. The substance is toxic to kidneys, liver, central nervous system (CNS). The substance may be toxic to blood, the nervous system. Repeated or prolonged exposure to the substance can produce target organs damage.

Section 4: First Aid Measures

Eye Contact:



Material Safety Data Sheet
Ethidium bromide MSDS

Section 1: Chemical Product and Company Identification

Product Name: Ethidium bromide

Catalog Codes: SLE1144

CAS#: 1239-45-8

RTECS: SF7950000

TSCA: TSCA 8(b) inventory: No products were found.

CI#: Not available.

Synonym: Homidium bromide

Chemical Formula: C21H20BrN3

Contact Information:

Sciencelab.com, Inc.
14025 Smith Rd.
Houston, Texas 77396

US Sales: **1-800-901-7247**
International Sales: **1-281-441-4400**

Order Online: ScienceLab.com

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1-800-424-9300

International CHEMTREC, call: 1-703-527-3887

For non-emergency assistance, call: 1-281-441-4400

Section 2: Composition and Information on Ingredients

Composition:

Name	CAS #	% by Weight
Ethidium bromide	1239-45-8	100

Toxicological Data on Ingredients: Not applicable.

Section 3: Hazards Identification

Potential Acute Health Effects:
Hazardous in case of ingestion. Slightly hazardous in case of skin contact (irritant), of eye contact (irritant), of inhalation.

Potential Chronic Health Effects:
Hazardous in case of ingestion. Slightly hazardous in case of skin contact (irritant), of eye contact (irritant), of inhalation.
CARCINOGENIC EFFECTS: Not available. MUTAGENIC EFFECTS: Not available. TERATOGENIC EFFECTS: Not available.
DEVELOPMENTAL TOXICITY: Not available.

Section 4: First Aid Measures

Eye Contact: Immediately flush eyes with running water for at least 15 minutes, keeping eyelids open. Cold water may be used.

Skin Contact:

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SAFETY DATA SHEET
Hygromycin B

Revision: 04/29/2017
Supersedes Revision: 03/09/2017

according to Regulation (EC) No. 1907/2006 as amended by (EC) No. 1272/2008

Section 1. Identification of the Substance/Mixture and of the Company/Undertaking

1.1 Product Code: 14291

Product Name: Hygromycin B

Synonyms: O-6-amino-6-deoxy-L-glycero-D-galacto-heptopyranosylidene-(1->2-3)-O-.beta.-D-talopyranosyl-(1->5)-2-deoxy-N3-methyl-D-streptamine;

1.2 Relevant identified uses of the substance or mixture and uses advised against:

Relevant identified uses: For research use only, not for human or veterinary use.

1.3 Details of the Supplier of the Safety Data Sheet:

Company Name: Cayman Chemical Company
1180 E. Ellsworth Rd.
Ann Arbor, MI 48108

Web site address: www.caymanchem.com

Information: Cayman Chemical Company +1 (734)971-3335

1.4 Emergency telephone number:

Emergency Contact: CHEMTREC Within USA and Canada: +1 (800)424-9300
CHEMTREC Outside USA and Canada: +1 (703)527-3887

Section 2. Hazards Identification

2.1 Classification of the Substance or Mixture:

Acute Toxicity: Inhalation, Category 2
Acute Toxicity: Oral, Category 2
Acute Toxicity: Skin, Category 2
Serious Eye Damage/Eye Irritation, Category 1
Respiratory Sensitization, Category 1

2.2 Label Elements:

GHS Signal Word: **Danger**

GHS Hazard Phrases:

H330: Fatal if inhaled.
H300: Fatal if swallowed.
H310: Fatal in contact with skin.
H318: Causes serious eye damage.
H334: May cause allergy or asthma symptoms or breathing difficulties if inhaled.

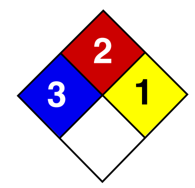
GHS Precaution Phrases:

P260: Do not breathe {dust/fume/gas/mist/vapours/spray}.
P284: {In case of inadequate ventilation, }wear respiratory protection {}.
P264: Wash {hands} thoroughly after handling.
P262: Do not get in eyes, on skin, or on clothing.
P280: Wear {protective gloves/protective clothing/eye protection/face protection}.
P361+364: Take off immediately all contaminated clothing and wash it before reuse.
P261: Avoid breathing {dust/fume/gas/mist/vapours/spray}.

GHS Response Phrases:

P304+340: IF INHALED: Remove victim to fresh air and keep at rest in a position comfortable for breathing.
P310: Immediately call a {POISON CENTER/doctor/...}.

Multi-region format



Material Safety Data Sheet
2-Mercaptoethanol MSDS

Section 1: Chemical Product and Company Identification

Product Name: 2-Mercaptoethanol

Catalog Codes: SLM1342

CAS#: 60-24-2

RTECS: KL5600000

TSCA: TSCA 8(b) inventory: 2-Mercaptoethanol

CI#: Not available.

Synonym: 1-Ethanol-2-thiol; 2-Hydroxy-1-ethanethiol; 2-Hydroxyethyl mercaptan; 2-Thioethanol; beta-Mercaptoethanol; Mercaptoethanol; Monothioethyleneglycol; Thiomonoglycol; Ethanol, 2-mercapto-; Thioglycol

Chemical Name: 2-Mercaptoethanol

Chemical Formula: HSC2H4OH

Contact Information:

Sciencelab.com, Inc.
14025 Smith Rd.
Houston, Texas 77396

US Sales: **1-800-901-7247**
International Sales: **1-281-441-4400**

Order Online: ScienceLab.com

CHEMTREC (24HR Emergency Telephone), call:
1-800-424-9300

International CHEMTREC, call: 1-703-527-3887

For non-emergency assistance, call: 1-281-441-4400

Section 2: Composition and Information on Ingredients

Composition:

Name	CAS #	% by Weight
{2-}Mercaptoethanol	60-24-2	100

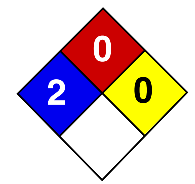
Toxicological Data on Ingredients: 2-Mercaptoethanol: ORAL (LD50): Acute: 244 mg/kg [Rat]. 190 mg/kg [Mouse]. DERMAL (LD50): Acute: 300 ul/kg [Guinea pig Rabbit]. 150 ul/kg [Rabbit].

Section 3: Hazards Identification

Potential Acute Health Effects:
Very hazardous in case of skin contact (permeator), of ingestion. Hazardous in case of skin contact (irritant), of eye contact (irritant), of inhalation. Severe over-exposure can result in death.

Potential Chronic Health Effects:
CARCINOGENIC EFFECTS: Not available. MUTAGENIC EFFECTS: Not available. TERATOGENIC EFFECTS: Not available. DEVELOPMENTAL TOXICITY: Not available. The substance is toxic to the nervous system, mucous membranes. The substance may be toxic to upper respiratory tract, eyes, central nervous system (CNS). Repeated or prolonged exposure to the substance can produce target organs damage. Repeated exposure to a highly toxic material may produce general deterioration of health by an accumulation in one or many human organs.

p. 1



Material Safety Data Sheet
Potassium ferricyanide MSDS

Section 1: Chemical Product and Company Identification

Product Name: Potassium ferricyanide

Catalog Codes: SLP4000, SLP1183

CAS#: 13746-66-2

RTECS: LJ8225000

TSCA: TSCA 8(b) inventory: Potassium ferricyanide

CI#: Not available.

Synonym: Potassium hexacyanoferrate (III)

Chemical Name: potassium ferricyanide

Chemical Formula: K3Fe(CN)6

Contact Information:

Sciencelab.com, Inc.
14025 Smith Rd.
Houston, Texas 77396

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International Sales: **1-281-441-4400**

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International CHEMTREC, call: 1-703-527-3887

For non-emergency assistance, call: 1-281-441-4400

Section 2: Composition and Information on Ingredients

Composition:

Name	CAS #	% by Weight
Potassium ferricyanide	13746-66-2	100

Toxicological Data on Ingredients: Potassium ferricyanide: ORAL (LD50): Acute: 2970 mg/kg [Mouse].

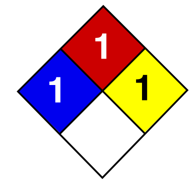
Section 3: Hazards Identification

Potential Acute Health Effects:
Hazardous in case of skin contact (irritant), of eye contact (irritant), of ingestion, of inhalation. Slightly hazardous in case of skin contact (permeator).

Potential Chronic Health Effects:
CARCINOGENIC EFFECTS: Not available. MUTAGENIC EFFECTS: Mutagenic for bacteria and/or yeast. TERATOGENIC EFFECTS: Not available. DEVELOPMENTAL TOXICITY: Not available. Repeated or prolonged exposure is not known to aggravate medical condition.

Section 4: First Aid Measures

Eye Contact:
Check for and remove any contact lenses. In case of contact, immediately flush eyes with plenty of water for at least 15 minutes. Cold water may be used. Get medical attention.



Material Safety Data Sheet
Rifampicin MSDS

Section 1: Chemical Product and Company Identification

Product Name: Rifampicin

Catalog Codes: SLR1366, SLR1036

CAS#: 13292-46-1

RTECS: VJ7000000

TSCA: TSCA 8(b) inventory: No products were found.

CI#: Not available.

Synonym: Rifampin Rifamycin AMP; 3-[[(4-Methyl-1-piperazinyl)imino]methyl]rifamycin

Chemical Name: Rifampicin

Chemical Formula: C43H58N4O12

Contact Information:

Sciencelab.com, Inc.
14025 Smith Rd.
Houston, Texas 77396

US Sales: **1-800-901-7247**
International Sales: **1-281-441-4400**

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CHEMTREC (24HR Emergency Telephone), call:
1-800-424-9300

International CHEMTREC, call: 1-703-527-3887

For non-emergency assistance, call: 1-281-441-4400

Section 2: Composition and Information on Ingredients

Composition:

Name	CAS #	% by Weight
Rifampicin	13292-46-1	100

Toxicological Data on Ingredients: Rifampicin: ORAL (LD50): Acute: 1570 mg/kg [Rat]. 500 mg/kg [Mouse]. 2120 mg/kg [Rabbit].

Section 3: Hazards Identification

Potential Acute Health Effects: Slightly hazardous in case of skin contact (irritant), of eye contact (irritant), of ingestion, of inhalation.

Potential Chronic Health Effects:
CARCINOGENIC EFFECTS: Not available. MUTAGENIC EFFECTS: Mutagenic for bacteria and/or yeast. TERATOGENIC EFFECTS: Not available. DEVELOPMENTAL TOXICITY: Not available. The substance may be toxic to blood, liver, heart, gastrointestinal tract, upper respiratory tract, eyes. Repeated or prolonged exposure to the substance can produce target organs damage.

Section 4: First Aid Measures

Eye Contact:

SAFETY DATA SHEET

(In accordance with COMMISSION REGULATION (EU) No 453/2010)

SECTION 1: Identification of the substance/mixture and of the company/undertaking

Product identifier

Product code R0851
Product name X-Gluc

Chemical Name Not applicable
REACH registration number No registration number is given yet for this substance / substances in this mixture since the annual import quantity is less than one tonnage per annum or the transition period for its registration according to Article 23 of REACH has not yet expired.

Relevant identified uses of the substance or mixture and uses advised against

Relevant identified uses Use as laboratory reagent, Scientific research and development
Use Description Code Not Available
Uses advised against Not for consumer use.

Details of the supplier of the safety data sheet

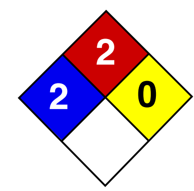
Manufacturer/Supplier

LIFE TECHNOLOGIES EUROPE BV
KWARTSWEG 2
2665 NN BLEISWIJK
NETHERLANDS
31-(0)180 392 400
Email: MSDS@lifetech.com

Thermo Fisher Scientific Baltics UAB
V.Graiciuno 8
LT-02241 Vilnius
Lithuania
Tel.: +370 5 2602131
Fax.: +370 5 2602142

24 hour Emergency Response: 866-536-0631
301-431-8585
Outside of the U.S. ++1-301-431-8585

Country specific Emergency Number (if available):
CHEMTREC Ireland (Dublin) +(353)-19014670 (Greeting Language: English and Irish)
CHEMTREC UK (London) +(44)-870-8200418 (Greeting Language: English)



Material Safety Data Sheet
Dimethyl sulfoxide MSDS

Section 1: Chemical Product and Company Identification

Product Name: Dimethyl sulfoxide

Catalog Codes: SLD3139, SLD1015

CAS#: 67-68-5

RTECS: PV6210000

TSCA: TSCA 8(b) inventory: Dimethyl sulfoxide

CI#: Not applicable.

Synonym: Methyl Sulfoxide; DMSO

Chemical Name: Dimethyl Sulfoxide

Chemical Formula: (CH3)2SO

Contact Information:

Sciencelab.com, Inc.
14025 Smith Rd.
Houston, Texas 77396

US Sales: **1-800-901-7247**
International Sales: **1-281-441-4400**

Order Online: ScienceLab.com

CHEMTREC (24HR Emergency Telephone), call:
1-800-424-9300

International CHEMTREC, call: 1-703-527-3887

For non-emergency assistance, call: 1-281-441-4400

Section 2: Composition and Information on Ingredients

Composition:

Name	CAS #	% by Weight
Dimethyl sulfoxide	67-68-5	100

Toxicological Data on Ingredients: Dimethyl sulfoxide: ORAL (LD50): Acute: 14500 mg/kg [Rat]. 7920 mg/kg [Mouse]. DERMAL (LD50): Acute: 40000 mg/kg [Rat].

Section 3: Hazards Identification

Potential Acute Health Effects:
Slightly hazardous in case of inhalation (lung irritant). Slightly hazardous in case of skin contact (irritant, permeator), of eye contact (irritant), of ingestion, .

Potential Chronic Health Effects:
Slightly hazardous in case of skin contact (irritant, sensitizer, permeator), of ingestion. CARCINOGENIC EFFECTS: Not available. MUTAGENIC EFFECTS: Mutagenic for mammalian somatic cells. Mutagenic for bacteria and/or yeast. TERATOGENIC EFFECTS: Not available. DEVELOPMENTAL TOXICITY: Not available. The substance may be toxic to blood, kidneys, liver, mucous membranes, skin, eyes. Repeated or prolonged exposure to the substance can produce target organs damage.

Section 4: First Aid Measures