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Genetic Engineering Studies of Escherichia coli and Microalgae for Expression of Hydrolytic Enzymes and Development of High Throughput Screening Technique

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Abstract

The field of biochemical engineering has made substantial progress through major advances in genetic and metabolic engineering with applications in various sectors such as energy, food science, pharmaceuticals, etc. The hosts used for this work are constantly broadening. A host particularly important for energy applications are microalgae. The potential to enhance microalgae genetically for energy applications is not well explored and was therefore investigated in this thesis. Non-photosynthetic micro-organisms and photosynthetic microalgae offer a potential approach to enhance sustainable biochemical production. In this study expression vectors for *Escherichia coli* (*E. coli*) and *Chlorella vulgaris* (*C. vulgaris*) were constructed for the expression of two enzymes: exo-inulinase enzyme and β-glucuronidase (GUS reporter) reporter protein. The expressed enzymes were characterized for their activity. The study involved three phases.

In the first phase of the research, the inulin hydrolyzing enzyme exo-inulinase was expressed in *E. coli* strain Rosetta-gami B(DE3). For this purpose, first the exo-inulinase gene from *Aspergillus niger* 12 (*A. niger* 12) was isolated by reverse transcription polymerase chain reaction (RT-PCR) and further an expression vector pET32a+EX-INU was constructed. The recombinant exo-inulinase was then expressed in *E. coli* strain Rosetta-gami B(DE3). The recombinant exo-inulinase was purified, and characterized for its activity. The molecular weight of the recombinant exo-inulinase was 81 Da (similar to native exo-inulinase). The K_m and V_{max} toward inulin were 5.3 ± 1.1 mM and 402.1 ± 53.1 µmol min^{-1} mg^{-1} protein respectively and the optimum temperature and pH for maximum enzyme activity were 55 ^{0}C and 5.0 respectively.

In the second phase of the research, the 4-Methylumbelliferyl-β-D-glucuronide (4-MUG) hydrolyzing enzyme β-glucuronidase was expressed in the photosynthetic microalgae *Chlorella vulgaris* (*C. vulgaris*). The expression vector pBIN+TetR+TetO was first constructed and transformed into *C. vulgaris* (UTEX 2714) by co-cultivation with *Agrobacterium tumefaciens* LBA 4404 (*A. tumefaciens*). For intact cell screening of high expression level of β-glucuronidase in transgenic *C. vulgaris,* a new high throughput screening (HTS) method was proposed, developed and evaluated. A recombinant micro-algal

isolate (1 of 32 transgenic *C. vulgaris*) gave the highest florescence intensity of 16,988 ± 1168 by expression of the GUS reporter enzyme. In a lysed cell study, enzyme kinetic analysis for the expressed phenotype was also carried out. The values of K_m and V_{max} of the recombinant GUS enzyme were 0.1304 ± 0.0101 mM and 0.35 ± 0.004 pmol 4-MU/min/ml of crude cell lysate respectively.

In the third and final phase of the research, the proposed HTS method developed in the previous study was applied to a second microalgal system. First, 4-MUG hydrolyzing GUS enzyme was expressed in *Chlamydomonas reinhardtii* CC1690 (*C. reinhardtii*). The transgenic *C. reinhardtii* expressing GUS enzyme was developed by *A. tumefaciens* transformation techniques. The expression vector used in this work was pBIN-Hyg-Tx–GUS-INT. High throughput screening of transgenic colonies expressing β-glucuronidase (GUS activity) was carried out directly from agar plates. 1 out of 126 transgenic *C. reinhardtii* colony, showed the highest fluorescence intensity 1,113. This study confirmed the application of the HTS method to microalgal systems. This is a new tool which can be applied for fast screening of genetic transformations and expression in microalgal systems.

Keywords – *E. coli*, exo-inulinase, *Chlorella vulgaris, Chlamydomonas reinhardtii,* 4-Methylumbelliferyl-β-D-glucuronide, and High Throughput Screening

Co-Authorship Statement

This thesis was completed under the supervision of Dr. Amarjeet Bassi and Dr. Lars Rehmann. Four articles were written and co-authored. For all four articles the first author is Shreyas Yedahalli. The co-author and corresponding author are Dr. Lars Rehmann and Dr. Amarjeet Bassi respectively.

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Finally, my warmest thanks must be to my family. I would like to thank my sisters Dr. Ashwini, and Dr. Shalini. Thanks to my parents, Mr. Somegowda, and Mrs. Navamani, who never doubted, constantly supported, and always loved.

Dedicated to

To my parents Some Gowda Yedahalli Namadaru and Navamani Chandagal

Kunne Gowda

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Abbreviations

Chlamydomonas reinhardtii	**C. reinhardtii**
Aspergillus niger	**A. niger**
Agrobacterium tumefaciens	**A. tumefaciens**
Parachlorella kessleri	**P. kessleri**
Chlorella variabilis	**C. variabilis**
Chlorella protothecoides	**C. protothecoides**
Botryococcus braunii	**B. braunii**
Coccomyxa subellipsoidea	**C. subellipsoidea**
Monoraphidium neglectum	**M. neglectum**
Thalassiosira pseudonana	**T. pseudonana**
Fistulifera solaris	**F. solaris**
Nannochloropsis oceanica	**N. oceanica**
Nannochloropsis gaditana	**N. gaditana**
Nannochloropsis granulate	**N. granulate**
Nannochloropsis oculata	**N. oculata**

Phaeodactylum tricornutum   ***P. tricornutum***

Ostreococcus tauri   ***O. tauri***

Cyclotella cryptica   ***C. cryptica***

Dunaliella salina   ***D. salina***

Pleurochrysis carterae   ***P. carterae***

Pseudochoricystis ellipsoidea   ***P. ellipsoidea***

Nannochloropsis salina   ***N. salina***

Lobosphaera incisa   ***L. incisa***

Amphora coffeaeformis   ***A. coffeaeformis***

Chlorella zofingiensis   ***C. zofingiensis***

Phaeodactylum tricornutum   ***P. tricornutum***

Dunaliella tertiolecta   ***D. tertiolecta***

Tetraselmis chuii   ***T. chuii***

Thalassiosira pseudonana   ***T. pseudonana***

Isochrysis galbana   ***I. galbana***

Parietochloris incisa   ***P. incisa***

Phaeodactylum tricornutum Bohlin   ***P. tricornutum* Bohlin**

Chlorella ellipsoidea	**C. ellipsoidea**
Symbiodinium microadriaticum	**S. microadriaticum**
Haematococcus pluvialis	**H. pluvialis**
Ankistrodesmus convolutus	**A. convolutes**
Arabidopsis thaliana	**A. thaliana**
Neochloris oleoabundans	**N. oleoabundans**
Chlorella vulgaris	**C. vulgaris**
Escherichia coli	**E. coli**
New england biolabs	**NEB**
Super Optimal broth with Catabolite repression	**SOC**
Yeast Extract Broth	**YEB**
Yeast Mannitol Broth	**YM broth**
Polymerase chain reaction	**PCR**
Reverse transcription polymerase chain reaction	**RT-PCR**
Messenger RNA	**mRNA**
Deoxyribonucleic acid	**DNA**
Rotation per minute	**RPM**

Deionized water   **DI**

Phosphate-buffered saline   **PBS**

Isopropyl β-D-1-thiogalactopyranoside   **IPTG**

Dinitrosalicylic acid   **DNS**

Bicinchoninic acid assay   **BCA**

Sodium dodecyl sulfate polyacrylamide gel electrophoresis   **SDS-PAGE**

Laemmli Tris-HCl gels   **TGX gels**

Nominal molecular weight limit   **NMWL**

transfer RNA   **tRNA**

Kilo dalton   **kDA**

Kanamycin   **K_n**

Hygromycin B   **HygB**

Ampicillin   **A_p**

Streptomycin   **S_t**

Rifampicin   **R_f**

Geneticin selective antibiotic   **G418 sulfate**

Tris-Acetate-Phosphate   **TAP**

Michaelis–Menten kinetic constant	K_m
Maximum enzyme activity	V_{max}
β-glucuronidase	**GUS**
University of Texas	**UTEX**
Optical density	**OD**
Regression coefficient	R^2
4-Methylumbelliferyl-β-D-glucuronide	**4-MUG**
4-Methylumbelliferone	**4-MU**
5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid	**X-Gluc**
Exo-inulinase	**EX-INU**

Chapter 1

1   Introduction

Bio-chemicals from microbes are an attractive source of sustainable production. They are produced by non-photosynthetic and photosynthetic microbes. However, for non-photosynthetic heterotrophic microbes, carbohydrates are essential for their growth and biochemical production. The development of new tools and techniques in biotechnology has been the main driving force for many advances. Many prokaryotic and eukaryotic systems are being applied for a myriad of biotechnological applications including biofuel production.

Even though *Escherichia coli* is one of the most well studied microbes, the expression of eukaryotic proteins in *E. coli* is still difficult due to factors like codon bias, and the environment inside the cytoplasm is reducing, which does not aid in di-sulphide bond formation. Over the past decade, microalgae have emerged as microbes of increasing interest due to their ability to produces compounds ranging from renewable fuels to monoclonal antibodies. To this day, very few tools are available for the transformation of microalgae. Most of the transformation techniques are based on heterologous transformation. The transgenic mRNA and recombinant protein expression varies from strain to strain. Thus, challenges still exist in finding new tools and systems for the transformation of eukaryotic genes.

In this study first, the exo-inulinase gene from *Aspergillus niger* 12 was expressed in *E. coli* strain Rosetta-gami B (DE3) which can make active eukaryotic proteins. The exo-inulinase enzyme was purified and its biochemical characterization and kinetics studies

were carried out. Next, the transformation of *Chlorella vulgaris* by *Agrobacterium tumefaciens* for expression of GUS (β-glucuronidase) reporter was demonstrated (**Figure 1.1**). A new method of isolating high level of GUS enzyme, i.e., high-throughput screening, was developed based on the recombinant system's ability to hydrolyze 4-MUG to 4-MU. The transformation and high through screening of a second microalgal species was then demonstrated using the methods developed in the previous study. *Chlamydomonas reinhardtii transformed* by *A. tumefaciens* were able to express the GUS reporter.
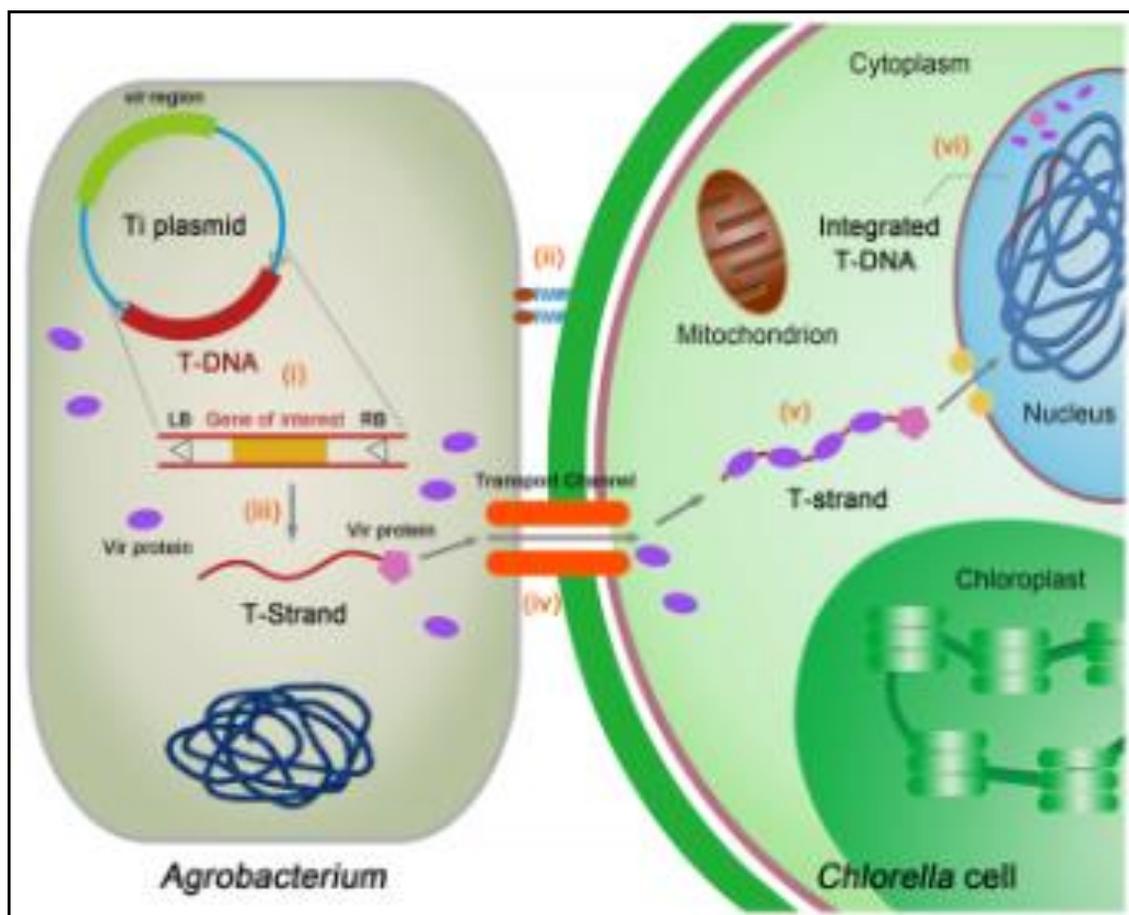
Figure 1.1 - Schematic representation of *Chlorella vulgaris* transformation by *Agrobacterium tumefaciens* [1]. Adapted from "*Chlorella* species as hosts for genetic

engineering and expression of heterologous proteins: Progress, challenge and perspective", by B. Yang, J. Liu, Y. Jiang, and F. Chen. *Biotechnol. J.*, vol. 11, no. 10, pp. 1244–1261, Oct. 2016. Adapted with permission.

1.1 Research structure

The research study was divided into three main phases. The first phase involved an investigation on expression of exo-inulinase and its characterization. The second phase involved the expression of GUS enzyme in *Chlorella vulgaris,* development of a high throughput screening technique for intact transgenic *C. vulgaris* expressing GUS enzyme and its characterization. The third phase was the expression of GUS enzyme in *Chlamydomonas reinhardtii* and development of high throughput screening technique for transgenic *C. reinhardtii* expressing GUS enzyme using colonies directly from agar plates.

In the first phase, the exo-inulinase cDNA from *Aspergillus niger* 12 was isolated by RT-PCR. The isolated exo-inulinase gene was ligated into *Escherichia coli* expression vector pET 32a(+) to get final expression vector pET 32a+EX-INU. The expression vector was transformed into *E. coli* strain Rosetta-gami B (DE3). The recombinant exo-inulinase expressed has a N-terminal histidine tag. Exo-inulinase was purified by affinity chromatography. The purified enzyme was quantified. The purity and molecular weight of recombinant exo-inulinase was analyzed by SDS-PAGE and Western blot. This was followed by an exo-inulinase activity study using inulin and sucrose. The exo-inulinase characteristics like stability, optimum pH, optimum temperature, and effect of metal ions on enzyme activity was carried out for inulin and sucrose.

In the second phase, the binary vectors pBIN-HYG-TX-GUS-INT and pBIN-Tet R were used for the construction of expression vector pBIN+TetR-TetO. The expression vector was transformed into *Agrobacterium tumefaciens* LBA 4404 by electroporation. *C. vulgaris* was transformed by *A. tumefaciens* LBA 4404 carrying expression vector pBIN+TetR-TetO. The transgenic *Chlorella vulgaris* were grown on G418 antibiotic containing agar plates and each colony were subsequently grown in liquid culture containing G418 sulfate antibiotic. Screening was carried out using a GUS histochemical assay. The same strains were subjected to GUS high throughput screening. In the GUS activity assay, 4-MUG was hydrolyzed to 4-MU. The 4-MU has an excitation/emission wavelength 365/455 nm. The GUS activity study was carried out for cell lysate and intact cells. Further, for cell lysate, enzyme kinetics were further assessed. Also, the effect of Triton X-100 on release of 4-MU into the buffer was carried out. Overall, this work led to development of a high throughput screening technique for intact transgenic *C. vulgaris.*

In the third phase, the binary vector pBIN-HYG-TX-GUS-INT was transformed into *A. tumefaciens* LBA 4404 by electroporation. *C. reinhardtii* was transformed by *A. tumefaciens* LBA 4404 carrying expression vector pBIN-HYG-TX-GUS-INT. The transgenic *C. reinhardtii* were grown on Hygromycin B antibiotic containing agar plates and single colonies were re-streaked on fresh agar plate containing Hygromycin B. The re-streaked colonies were directly used for high throughput screening in a 96 well black microplate by measuring GUS activity through florescence reading.

1.2   Objectives

Towards the completion of this study, one overall objective and several sub-objectives were proposed.

1.2.1   Overall objective

The overall objective of this study was to demonstrate the expression of recombinant proteins in *E. coli*, *C. vulgaris* and *C. reinhardtii*. The recombinant proteins expressed were characterized and activity was measured. For microalgae, a new high throughput screening was developed.

1.2.2   Specific objectives

The following were specific sub-objectives or milestones of this study.

Objective 1: Investigation of exo-inulinase enzyme expression in *E. coli* strain Rosetta-gami B (DE3)

The expression vector pET 32a+EX-INU was constructed by molecular cloning techniques. The expression vector was transformed into *E. coli* strain Rosetta-gami B (DE3). The recombinant exo-inulinase was successfully expressed. The recombinant exo-inulinase was analyzed for molecular weight and purity by western blot and SDS-PAGE. The exo-inulinase was of expected size and the enzyme was soluble and active.

Objective 2: Transformation of *C. vulgaris* and *C. reinhardtii* by *A. tumefaciens* LBA 4404

For *C. vulgaris*, expression vector pBIN+TetR-TetO was constructed by molecular cloning technique. The expression vector pBIN+TetR-TetO was transformed into *A.*

tumefaciens LBA 4404 by electroporation. Transformation of *C. vulgaris* was carried out by co-cultivation of *A. tumefaciens* LBA 4404 carrying pBIN+TetR-TetO with *C. vulgaris.* Transgenic *C. vulgaris* grew on G418 antibiotic TAP agar plate.

For *C. reinhardtii,* expression vector pBIN-HYG-TX-GUS-INT was used. The expression vector pBIN-HYG-TX-GUS-INT was transformed into *A. tumefaciens* LBA 4404 by electroporation. Transformation of *C. reinhardtii* was carried out by co-cultivation of *A. tumefaciens* LBA 4404 carrying pBIN-HYG-TX-GUS-INT with *C. reinhardtii.* Transgenic *C. reinhardtii* grew on Hygromycin B antibiotic TAP agar plate.

Objective 3: Development and evaluation of a new high throughput screening technique for transgenic *C. vulgaris* and *C. reinhardtii* expressing GUS gene

In the *C. vulgaris* study*,* transgenic *C. vulgaris* colonies from G418 sulfate antibiotic TAP agar plates were grown in G418 antibiotic TAP liquid culture. The screening was carried out with small volumes of transgenic (150 µl) *C. vulgaris*. The screening was carried out in 96 well black microplates. 32 samples in triplicate were screened at once. From this study, the highest GUS expressing strain was isolated without cell lysis (intact cell). Stability over long period of incubation was carried out. The optimum concentration of Triton X-100 necessary in the assay buffer for intact cell study and its stability over long duration was carried out.

In the *C. reinhardtii* study, transgenic *C. reinhardtii* from G418 antibiotic agar plates were directly added into assay buffer containing 4-MUG. From this study, transgenic *C. reinhardtii* expressing highest GUS activity was isolated. 126 strains were screened in 96 well black microplates.

Objective 4: Characterization and enzyme kinetic study of recombinant exo-inulinase from *E. coli* and recombinant β-glucuronidase (GUS) enzyme from *C. vulgaris*

The recombinant exo-inulinase expressed in *E. coli* was purified and characterized. Enzyme kinetics were carried out to find the K_m and V_{max} towards inulin and sucrose. This was followed by optimum pH, optimum temperature, thermal stability, effect of metal ion was carried out for exo-inulinase and invertase activity.

For *C. vulgaris,* lysed cell study was carried out on crude GUS cell lysate to find the K_m and V_{max} towards 4-MUG. Small volume (75 µl) of cell lysate was sufficient to carry out the kinetics for each point. Stability of crude cell lysate over long period of incubation was carried out. Finally, effect of different concentration of Triton X-100 and incubation time on crude GUS cell lysate was carried out.

1.3   Outline of the Thesis

This thesis is structured as follows:

Chapter 1 – Introduction

Introduces the thesis, including an overview of genetic engineering, transformation techniques, recombinant protein expression systems used in *E. coli, C. vulgaris, C. reinhardtii*.

Chapter 2 – Literature review of microalgae

Reviews the progress on development of recombinant microalgae. The review looks into the different components involved in genetic engineering of microalgae. The components are genome sequence, gene transformation techniques, selectable markers, photosynthetic markers, fluorescent proteins expressed in microalgae, promoters and terminators, inducible systems, affinity tags, and export signals.

Chapter 3 – Materials and Methods

This chapter provides details of the materials and methods that are common to all the following chapters (chapter 4, chapter 5, and chapter 6). Its gives details of molecular biology techniques, strains, plasmids, and growth media. Materials and methods pertinent to individual chapters are presented accordingly in those chapters.

Chapter 4 - Expression of exo-inulinase gene from *Aspergillus niger* 12 in *E. coli*

This chapter investigates the expression of recombinant exo-inulinase enzyme in *E. coli* strain Rosetta-gami B (DE3). The prokaryotic gene isolated from *Aspergillus niger* 12 was expressed successfully. The Rosetta-gami B (DE3) is a transgenic *E. coli* which aids in expression of active eukaryotic proteins. Also, the recombinant exo-inulinase was purified, enzyme kinetics, and characterization were carried out.

Chapter 5 - Expression of β-glucuronidase in *C. vulgaris* and development of novel high throughput screening from liquid culture

This chapter investigates the expression of β-glucuronidase reporter enzyme in *C. vulgaris* by using *A. tumefaciens* as a transformation tool. Also, a new method of

screening transgenic *C. vulgaris* expressing β-glucuronidase reporter enzyme without cell lysis was developed. The expression of β-glucuronidase reporter enzyme can be quantified without the cell lysis.

Chapter 6 - Application of high throughput screening technique for transgenic *Chlamydomonas reinhardtii* colonies on agar plate

This chapter investigates the expression of β-glucuronidase reporter enzyme in *C. reinhardtii* by using *A. tumefaciens* as a transformation tool. Also, a new method of screening transgenic *C. reinhardtii* expressing β-glucuronidase reporter enzyme without cell lysis was developed. The expression of β-glucuronidase reporter enzyme can be quantified without the cell lysis.

Chapter 7 - Concluding remarks and recommendations

Based on the experimental results acquired in this thesis, final concluding remarks and pertinent recommendations are provided.

1.4   References

[1]   B. Yang, J. Liu, Y. Jiang, and F. Chen, "Chlorella species as hosts for genetic engineering and expression of heterologous proteins: Progress, challenge and perspective," *Biotechnol. J.*, vol. 11, no. 10, pp. 1244–1261, Oct. 2016.

Chapter 2

2 Molecular Biology Tools and Approaches for Genetic Modification of Microalgae – A Review of Recent Developments

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Abstract

Microalgae are micro-organisms which thrive in diverse environmental conditions by utilizing sunlight, CO_2, and inorganic nutrients. Microalgae produce various bio-chemicals which can be converted to biofuel, biopharmaceuticals, animal feed, cosmetics, health, specialty chemicals that have a potential impact on many biotechnological industries. However, such applications are currently hampered by high costs of production due to low yield of bio-chemicals. Many of these issues can be addressed by genetic and metabolic engineering of microalgae. A few genetic engineering tools have been developed successfully for a few commercially important microalgae. But there are many microalgae for which genetic engineering tools need to be developed. Many factors contribute to the successful gene transformation required for transgenic microalgae development. The literature review reports the recent developments in genetic

engineering approaches and techniques that have been carried out in microalgae. The objectives of this study are to assist researchers in this field by providing state-of-the art developments in genetic engineering over the past decade of trangenic microalgae.

Keywords – microalgae, nuclear transformation, chloroplast transformation, promoters, selection marker, fluorescent protein, inducible system, export signal.

2.1   Introduction

Microalgae are a diverse group of oxygenic, photosynthetic eukaryotic microorganisms. The number of species of eukaryotic microalgae can range from 30,000 to 70,000 species in the world [1]. Microalgae thrive in a wide range of environments like fresh water, sea water, land, plants, acidic ponds, cold environment, hot environment, and volcanic locations. Microalgae can grow in low concentration of nitrates, phosphates, and carbon dioxide. Most of these nutrients are produced as waste from industry, farms, and fertilizer run offs. This makes microalgae ideal candidates for biotechnological and industrial applications. Many microalgae species produce unique bioactive compounds of commercial interest [2] like a few microalgae e.g., *Dunaliella,* and *Monoraphidium,* can grow in more extreme environments such as highly saline and highly caustic condition [3] [4]. *Trebouxiophyceae* sp. MX-AZ01 is a fresh water microalga but it can grow in extremely low pH of 2.3 and in high concentration of toxic heavy metals [5]. These characteristics make certain species of microalgae ideal for outdoor cultivation in diverse environments.

 Many high value bio-chemicals like carotenoids, astaxanthin, β-Carotene, lutein, and vitamins can be extracted from microalgae which have applications in food, medicine,

and cosmetic industry. Also, microalgae have application in low value high volume

bioproducts in biofuel, animal feed, fish feed, bio-remediation, and waste water

treatment. Microalgae can also be used to produce many biopharmaceutical products like

monoclonal antibodies [6]. Microalgae have potential for production of the above-

mentioned bio-products but further improvements in microalgae are necessary for them

to be industrially feasible. Genetic engineering offers many tools to realize the full

potential of microalgae.
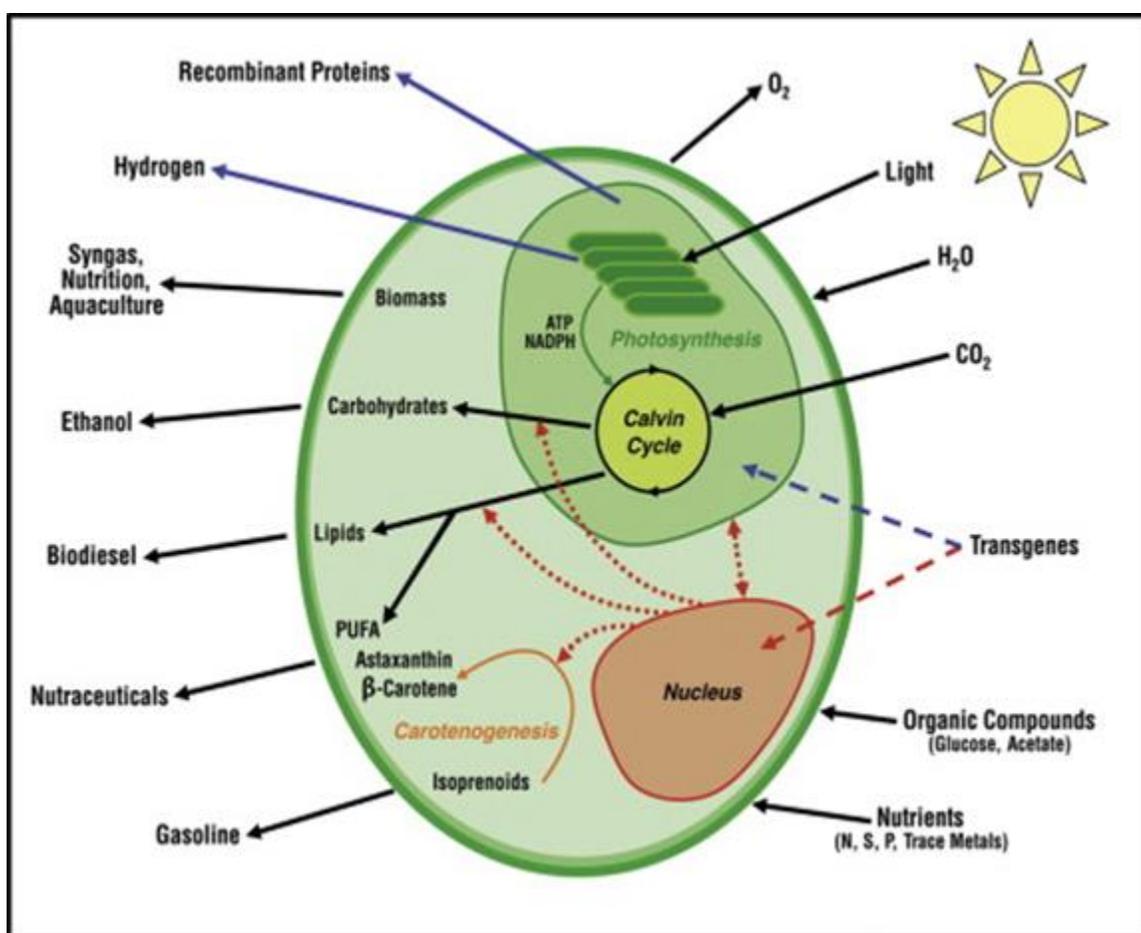
Figure 2.1 – Schematic representation of microalgae. The figure shows the expression of transgene in nucleus and chloroplast. The potential enhancement of commercially important bioproducts [103]. Adapted from "A green light for engineered algae: redirecting metabolism to fuel a biotechnology revolution.," by J. N. Rosenberg, G. A. Oyler, L. Wilkinson, and M. J. Betenbaugh. *Curr. Opin. Biotechnol.*, vol. 19, no. 5, pp. 430–6, Oct. 2008. Adapted with permission.

To date, only one commercial gene expression kit is reported for *C. reinhardtii* (https://www.thermofisher.com/ca/en/home/life-science/protein-biology/protein-expression/algal-protein-expression/chlamydomonas-protein-expression-kit.html).   This indicates that more work on genetic engineering of microalgae is necessary. Several commercially important microalgae genome sequences are currently available. In this review, the latest developments in this field over the past decade are discussed. The important components of genetic engineering like genome sequence, transformation methods, selection markers, marker free selection system, fluorescent proteins, promoters and terminators, inducible systems, affinity tags, and export signals have been reviewed.

2.1.1   Genome sequence

Due to the diverse applications of microalgae in biotechnology, genome sequences play an important role in understanding genome wide expression, identification and genetic engineering of microalgae for further exploitation. Several microalgae genes which have been sequenced are maintained by different websites. A comprehensive list of all the microalgae that have been isolated is maintained by AlgaeBase (http://www.algaebase.org/). The first major genomic effort was directed towards human genome and human pathogen genomes; however, the cost of sequencing was very high. With the development of new technologies like high-throughput sequencing the cost of has been substantially reduced. In microalgae, genes are in three organelles. These are the nucleus, chloroplast, and in mitochondria. **Table 2.1** shows the gene sequencing that has been carried out over the past decade for various microalgae systems. These are discussed below.

C. reinhardtii, a fresh water species was the first microalgae to be sequenced and is considered a model for genetic engineering work in microalgae. The nuclear genome size for this species is 121 Mbp and genetic engineering techniques have been developed for mithochondria, chloroplast, and nucleus. *C. reinhardtii* has main application for recombinant protein production like cellulose, therapeutic proteins and monoclonal antibodies [7]. *C. reinhardtii* genome sequence is available (http://genome.jgi.doe.gov/Chlre3/Chlre3.home.html). *Cyclotella cryptica* (*C. cryptica*), a brackish water microalgae and is promising microalgae for large scale omega-3 fatty acid production. The size of the nuclear genome is 161.7 Mbp [8]. *C. cryptica* genome sequence is available (http://genomes.mcdb.ucla.edu/Cyclotella/download.html). *Thalassiosira pseudonana* (*T. pseudonana*) is a marine microalge and it has shown promising application as drug delivery vehicle. The nuclear gene size is 34 Mbp, chloroplast is 1290 Kbp and mitochondria is 440 Kbp [9][10]. *T. pseudonana* genome sequence is available (http://genome.jgi.doe.gov/Thaps3/Thaps3.home.html).

Parachlorella kessleri, is a fresh water microalga that has shown promising application for biofuel production. The whole transcriptome and genome sequence for this system is available and the nuclear geneome size is 62.5 Mbp [11]. Another species, *Chlorella variabilis* NC64A is a fresh water microalgae and it is a suitable model microalgae for studying virus and algal interactions. The genome sequence is available and the nuclear genome size is 46 Mbp, while chloroplast genome size is 124,793 bp and in the mitochondria genome size is 78,500 bp [12][13]. *Picochlorum* SENEW3 is a marine microalge and it is developed as model system for studying the salinity stress in marine microalgae. The nuclear genome size is 13.5 Mbp [14]. *Fistulifera solaris* JPCC DA0580

is a marine microalge and has application in biofuel production. The nuclear genome size is 49.7 Mbp, while the chloroplast genome size is 134.9 Kbp and in the mitochondria the genome size is 38.6 Kbp [15][16].

Chlorella protothecoides sp. 0710 is a fresh water microalge and has application in biofuel production. The nuclear genome size is 22.9 Mbp, while the chloroplast is 84.5 Kbp and in the mitochondria the genome size is 57.2 Kbp [17] [18]. *Nannochloropsis oceanica* IMET1 is a marine microalge and has application in biofuel production. The nuclear gene size is 31.36 Mbp, chloroplast is 117.5 Kbp and mitochondria is 38 Kbp [19].

Nannochloropsis gaditana CCMP526 is a marine microalga and has application in biofuel production. The nuclear gene size is 29 Mbp, chloroplast is 114.9 Kbp and mitochondria is 38.9 Kbp [20]. *Nannochloropsis oceanica* CCMP531 is a marine microalga and has application in biofuel production. The nuclear gene size is 35.5 Mbp, mitochondria is 38 Kbp, and chloroplast is 117.6 Kbp [19]. *Nannochloropsis granulate* CCMP529 is a marine microalga and has application in biofuel production. The nuclear gene size is 30.1 Mbp, mitochondria is 38.7 Kbp, and chloroplast is 117.6 Kbp [19]. *Nannochloropsis oculata* CCMP525 is a marine microalga and has application in biofuel production. The nuclear gene size is 34.5 Mbp, mitochondria is 38.4 Kbp, and chloroplast is 117.4 Kbp [19]. *Nannochloropsis salina* CCMP537 is a marine microalga and has application in biofuel production. The nuclear gene size is 26.9 Mbp, mitochondria is 41.9 Kbp, and chloroplast is 114.8 Kbp [19].

Phaeodactylum tricornutum is a marine microalga and has application in biofuel production. It is a model system for studying diatoms. The nuclear gene size is 27.4 Mbp, and chloroplast is 117.4 Kbp [21] [22] [23]. *Ostreococcus tauri* is a marine microalga and it is an interesting microalge as they are the smallest free living photosynthetic eukaryotes. The gene sequence is available and the nuclear gene size is 13 Mbp [24] [25]. *Coccomyxa subellipsoidea* C-169 is fresh water psychrotolerant microalgae. It is used as a model system to study the chromosome repair from irradiation. The nuclear gene size is 48.8 Mbp [12].

Trebouxiophyceae sp. MX-AZ01 is a fresh water microalgae which grows in extremely low pH of 2.3 and in high concentration of toxic heavy metal. The nuclear gene is not available while the mitochondria gene size is 74.4 Kbp and chloroplast is 149.7 Kbp. The study of genes may unveil some of the proteins which codes for the microalgae to grow under extreme condition [5]. *Monoraphidium neglectum* is unique microalgae which grows in fresh water, marine water and in the pH range of 5-10. The nuclear gene size is 68 Mbp, mitochondria is 94 Kbp, and chloroplast is 135.3 Kbp [3]. *Botryococcus braunii* race B (Showa) is unique microalgae which secretes triterpene ($C_{30}H_{48}$) and has application in biofuel production. The nuclear gene size is 166.2 Mbp, mitochondria is 129.3 Kbp, and chloroplast is 156.4 Kbp [26] [27] [28].

Table 2.1 - Sequenced genes in microalgae (Nuclear – a, Mitochondria – b, and Chloroplast – c)

Species	Genome size (Mbp)	Genetically engineered part	Growth condition	Application	Reference
Chlamydomonas reinhardtii	121 [a]	Nuclear, chloroplast and mitochondria	Fresh water	Recombinant proteins production	[7]
Parachlorella kessleri	62.5 Mbp [a]	Nuclear	Fresh water	Biofuel	[11]
Chlorella variabilis NC64A	46 Mbp [a], 78.5 Kbp [b] and 124.7 Kbp [c]	N/A	Fresh water	Model system for studying virus/algal interactions	[12] and [13]
Chlorella protothecoides sp. 0710	22.9 Mbp [a], 57.2 Kbp [b], and 84.5 Kbp [c]	Nuclear	Fresh water	Biofuel	[17] and [18]
Trebouxiophyceae sp. MX-AZ01	74.4 Kbp [b], and 149.7 Kbp [c]	N/A	Fresh water and pH 2.3	Grows in extremely low pH, and toxic heavy metal concentrations	[5]
Botryococcus braunii race B (Showa)	166.2 Mbp [a], 129.3 Kbp [b], and 156.4. Kbp [c]	Nuclear	Fresh water	Biofuel	[26], [27], and [28]
Coccomyxa subellipsoidea C-169	48.8 Mbp [a]	N/A	Fresh water Psychrotolerant	Model to study chromosome repair from	[24]

				irradiation	
Monoraphidium neglectum	68 Mbp [a], 94 Kbp [b], and 135.3 Kbp [c]	N/A	Fresh water, Marine water, and pH 5-10	Grows in wide pH range and salt concentrations of up to 1.0% NaCl	[3]
Thalassiosira pseudonana	34 Mbp [a], 1.3 Kbp [b], and 440 Kbp [c]	Nuclear	Marine water	Drug delivery vehicle	[9] [10]
Picochlorum SENEW3	13.5 Mbp [a]	N/A	Marine water	Model system for studying salinity stress in microalgae	[14]
Fistulifera solaris JPCC DA0580	49.7 Mb [a], 38.6 Kbp [b], and 134.9 Kbp [c]	Nuclear	Marine water	Biofuel	[15], [16], and [29]
Nannochloropsis oceanica IMET1	31.36 Mbp [a], 38 Kbp [b], and 117.5 Kbp [c]	N/A	Marine water	Biofuel	[19]
Nannochloropsis gaditana CCMP526	29.0 Mbp [a], 38.9 Kbp [b], and114.9 Kbp [c]	Nuclear	Marine water	Biofuel	[20]
Nannochloropsis oceanica CCMP531	35.5 Mbp [a], 38 Kbp [b], and 117.6 Kbp [c]	N/A	Marine water	Biofuel	[19]
Nannochloropsis granulate CCMP529	30.1 Mbp [a], 38.7 Kbp [b], and 117.6	N/A	Marine water	Biofuel	[19]

	Kbp [c]				
Nannochloropsis oculata CCMP525	34.5 Mbp [a], 38.4 Kbp [b], and 117.4 Kbp [c]	N/A	Marine water	Biofuel	[19]
Nannochloropsis salina CCMP537	26.9 Mbp [a], 41.9 Kbp [b], and 114.8 Kbp [c]	N/A	Marine water	Biofuel	[19]
Phaeodactylum tricornutum	27.4 Mbp [a], and 117.4 Kbp [c]	Nuclear	Marine water	Model systems for studying diatoms	[21], [22], and [23]
Ostreococcus tauri	13 Mbp [a]	N/A	Marine water	Smallest free-living photosynthetic eukaryote	[30] and [25]
Cyclotella cryptica	161.7 [a]	Nuclear	Brackish water	Omega-3 fatty acid	[8]

2.1.2 Gene transformation techniques

Gene transformation is a method where new genes are incorporated into the genome of a cell. The introduction of a foreign gene into a host cell is an important step in genetic engineering. In biotechnology, genetic engineering has been exploited for improving host cell to produce a desired product or to contain particular traits. Gene transfer can be carried out by natural methods or physical methods. Natural methods include the use of *Agrobacterium* and conjugation method. Physical methods of gene transfer include electroporation, gene gun and glass bead methods. **Table 2.2** shows the gene transformation techniques that have been developed over the past decade. As mentioned earlier, *C. reinhardtii* is a model microalgae for genetic engineering work and one of the most extensively studied microalgae. It is also called "green yeast" due to its relative ease of handling and gene manipulation. Several gene transformation techniques have been developed in *C. reinhardtii.* In this section, new developments in *C. reinhardtii* and other microalgae are discussed. Kim et al. [31] developed a non-invasive method of nuclear transformation. They used positively charged amicoclay nanoparticles which coat negatively charged DNA. The aminoclay coated DNA mixture was plated on selective plates containg *C. reinhardtii* by high friction force applied during plating. The transformation efficieny was 5.03 x 10^2 transformants/µg DNA. The method is simple, efficient, safe, and reproducible technique [31].

Díaz-Santos et al. [32] carried out a glass bead method for nuclear transformation in *C. reinhardtii* and expressed ble and flocculin gene. The ble gene express bleomycin binding protein which inhibits in vitro DNA cleavage by the antibiotic bleomycin whereas, flocculin gene express specific cell surface lectin-like glycoproteins called

flocculins, which is responsible for the flocculation process in yeasts. The transformation efficiency was 2×10^{-7} transformants/µg DNA. The efficiency is low when compared to the non-invasive method recently developed by Kim et al [31]. Wannathong et al. [33] used a glass bead method for chloroplast transformation in *C. reinhardtii*. They have developed a marker free transformation system by using cell wall deficient and *psbH* deficient *C. reinhardtii* to express human growth hormone protein.
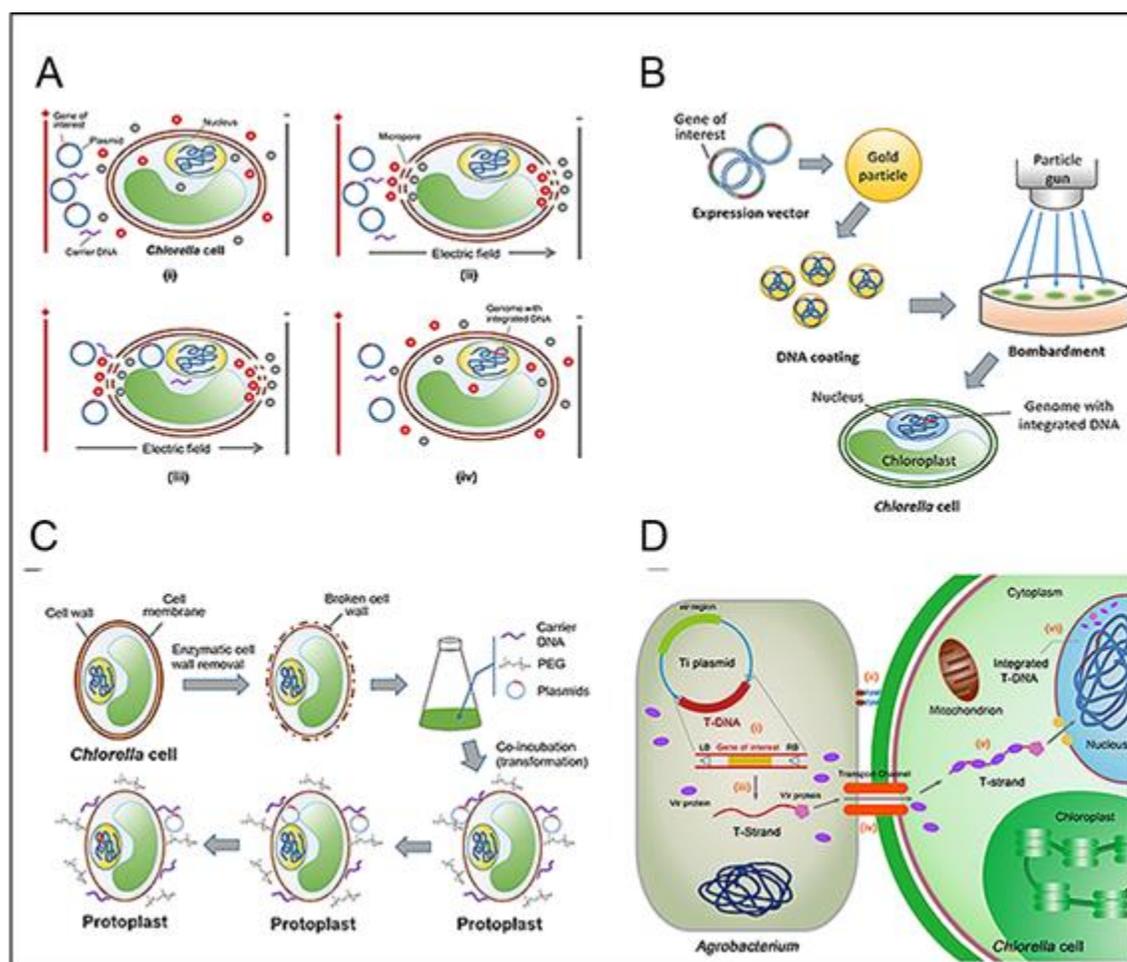
Figure 2.2 - Different transformation techniques of *Chlorella* sp. (A) Electroporation transformation. (B) Particle bombardment transformation. (C) PEG-mediated transformation. (D) *Agrobacterium*-mediated transformation [104]. Adapted from "*Chlorella* species as hosts for genetic engineering and expression of heterologous proteins: Progress, challenge and perspective", by B. Yang, J. Liu, Y. Jiang, and F. Chen. *Biotechnol. J.*, vol. 11, no. 10, pp. 1244–1261, Oct. 2016. Adapted with permission.

Loera-Quezada et al. [34] used electroporation for nuclear transformation of *C. reinhardtii.* They expressed phosphite oxidoreductase which allows it to use phosphite for its growth. Since, most microbes lack phosphite digesting enzymes, transgenic *C. reinhardtii* can be grown outdoor without fear of being outcompeted by other microbes. The transformation efficiency was 156 transformants/µg DNA. Bae et al. [35] and their research group have come up with a new method for transformation. They developed a high throughput nanowire incorporated microdevice for nuclear transformation of *C. reinhardtii.* The transformation efficiency was 6.52×10^{-3} transformants/µg DNA, 6.52×10^4 -fold higher than conventional glass bead method, and 9.66×10^4-fold higher than electroporation. Below is transformation of few commercially important microalgae.

Dunaliella salina, is marine microalgae was transformed by *A. tumefaciens.* The transformation was carried out successfully however, it is interesting since, *A. tumefaciens* is a fresh water microbe which do not grow in saline condition. Simon et al. could carry out transformation by co-cultivation of *D. salina* with *A. tumefaciens* in 1 M NaCl. Since, *A. tumefaciens* growth in high saline water has not been reported till date. The transformation frequency was 40 ± 5 colonies/10^6 *D. salina* cells [4]. Fariba Akbari et al. [36] carried out a novel method of transformation of marine microalgae *D. salina* by using quaternary ammonium salt containing soybean oil. The transformation efficiency of 2.8 x 10^5 transformants/µg DNA was achieved which is quite high. Also, this new method is low cost, fast and can be used for marine microalgae.

Endo et al. [37] have carried out nuclear transformation *Pleurochrysis carterae* by using polyethylene glycol (PEG)-mediated transfer. This is the first reported transformation system for *P. carterae* algae. However, the transformation efficiency was very low (1

transformant/μg DNA). Kasai et al. [38] have carried out nuclear transformation of *P. ellipsoidea* by particle bombardment. The transformation efficiency was 57.3 ± 6.9 transformants/μg DNA. The *P. ellipsoidea* used for transformation was a uracil mutant and used uridine monophosphate synthase gene (PeUMPS) for selection. The technique developed here is useful for working with "self-cloning" systems in microalgae.

Kang et al. [39] carried out stable nuclear transformation of *Nannochloropsis salina* by particle bombardment. The transformation efficiency was 5.9 ± 1.6 and 4.7 ± 2.0 transformants/μg DNA for TUB and UEP promoters respectively. Transformation efficiency varied depending on the type of promoter used. The transformation efficiency was similar to that of electroporation. Zorin et al. [40] carried out nuclear transformation of *Lobosphaera incisa* by electroporation. Transformation was 1 transformant / μg DNA. The transformation efficiency was very low for electroporation and further development is needed for *L. incisa*.

Amphora coffeaeformis is a marine diatom. Buhmann et al. [41] has carried out nuclear transformation by particle bombardment. The transformation efficiency was 800 transformants/ 10^8 cells which is very low. The author have suggested using smaller particle size and acceleration pressure to improve efficiency. Liu et al. [42] have carried out nuclear transformation of *Chlorella zofingiensis* by electroporation and particle bombardment. The transformation efficiency was 14×10^{-6} transformants/μg DNA for electroporation and 3.7×10^{-6} transformants/μg DNA for particle bombardment. The electroporation transformation showed 3.7-fold higher transformation compared to particle bombardment. Also, it is shown the promoters influence transformation efficiency in the order of RBCS > PDS & NIT (different promoters tested).

Neochloris oleoabundans is a candidate for biofuel production. Chungjatupornchai et al. [43] have carried out nuclear transformation by electroporation. The transformation efficiency was highest with pAR-Hyg4 (codon optimized) vector. The transformation efficiency was 2.0×10^{-7} transformants/µg DNA. *Tetraselmis chuii* was nuclear transformed by using *A. tumefaciens*. The transformation frequency was $1.50 \pm 0.93 \times 10^{-4}$. Also, *A. tumefaciens* is shown to transform *T. chuii* successfully for the first time [44].

Karas et al. [45] transformed *P. tricornutum* and *T. pseudonana* by *E. coli*. The transformation was carried out by conjugation into the nucleus of microalgae. The transformation efficiency of both the transformation was 4.0×10^{-4} diatom cells. This is the first reported nuclear episomal vector for diatoms and stable episome replication of plasmid even in absence of antibiotic. The diatom *P. tricornutum* was plastid transformed by electroporation. The transformation efficiency was 12.5×10^{3} transformants/ µg DNA [46].

Table 2.2 – Gene transformation in microalgae

Strain	Type of Transformation	Gene / protein expressed	Efficiency of transformation	Remark	Reference
		Nuclear transformation			
Chlamydomonas reinhardtii	Non-Invasive Method	*HPT* gene	5.03×10^2 transformants/µg DNA	Simple, efficient, safe, and reproducible technique	[31]
	Glass bead method	*ble* gene and *flocculin* gene	2×10^{-7} transformants/µg DNA	Promoter less transformation technique (the technique can be used to isolate novel promoters in microalgae)	[32]
	Electroporation	*ptx D* gene (codon optimized)/ phosphite oxidoreductase	156 transformants/mg DNA	Utilized phosphite as a source for metabolic activity hence can be used in large scale outdoor cultivation	[34]
	Nanowire-Incorporated Microdevice	*Hyg3* gene	6.52×10^{-3} transformants/µg DNA	6.52×10^4 -fold higher than conventional glass bead beating method, and 9.66×10^4-fold higher than electroporation method	[35]
Dunaliella salina	*A. tumefaciens*	*cr-chy* gene/ β-carotene hydroxylase	40 ± 5 colonies/10^6 cells	At growth stage Violaxanthin increased by 2–3 fold, no change during stress	[4]

	Quaternary ammonium salt containing soybean oil	*GFP* gene	2.8×10^5 transformants/µg DNA	Low cost, fast, and can be used for transformation of Halophile microalgae	[36]
Pleurochrysis carterae	Polyethylene glycol (PEG)	*PyGUS* gene and *GFP* gene	1 transformants/µg DNA	First stable genetic transformation of a coccolithophore algae by PEG	[37]
Pseudochoricystis ellipsoidea	Particle bombardment	*Uridine monophosphate synthase* gene (PeUMPS)	57.3 ± 6.9 transformants/µg DNA	Useful for researchers working on self-cloning systems in microalgae	[38]
Nannochloropsis salina	Particle bombardment	sfCherry fluorescent protein	5.9 ± 1.6 and 4.7 ± 2.0 transformants/µg DNA for TUB and UEP promoters	Similar transformation efficiency as electroporation technique and transformation efficiency varies by promoter used	[39]
Lobosphaera incisa	Electroporation	*ble* gene and Δ5 desaturase gene	1 transformants / µg DNA	Technique can be used to further develop endogenous markers, inducible and constitutive promoters	[40]
Amphora coffeaeformis	Particle bombardment	*AC3362* gene (cell surface protein) and	800 transformants/ 10^8 cells	Particle size and acceleration pressure are important factors for transformation efficiency	[41]

		Yellow fluorescent protein		by particle bombardment	
Chlorella zofingiensis	Electroporation	PDS-L516F/ norflurazon-resistant marker and phytoene desaturase protein	14×10^{-6} transformants/µg DNA	3.7-fold higher transformation compared to particle bombardment, and promoter's effects on transformation efficiency (RBCS > PDS & NIT) (different promoters tested)	[42]
	Particle bombardment	PDS-L516F/ norflurazon-resistant marker and phytoene desaturase protein	3.7×10^{-6} transformants/µg DNA	3.7-fold lower transformation compared to electroporation, promoter's effects on transformation efficiency (RBCS > PDS & NIT) (different promoters tested)	[42]
Neochloris oleoabundans	Electroporation	*Hyg3*, *Hyg4*, and *ChGfp* gene	5.2×10^{-4}, 2.0×10^{-4}, and 3.0×10^{-4} transformants/mg DNA	Development of transformation and co-transformation system in *N. oleoabundans*	[43]
Phaeodactylum tricornutum	Conjugation of *P. tricornutum* by *E. coli*	*p0521* and *p0521s* vector	4.0×10^{-4} diatom cells	First reported nuclear episomal vector for diatoms and stable episome replication of plasmid even in absence of antibiotic	[45]
Tetraselmis chuii	*A. tumefaciens*	*Shble* gene and GUS	Transformation	*A. tumefaciens*	[44]

		gene	frequency was $1.50 \pm 0.93 \times 10^{-4}$	transformation was successfully developed in *T. chuii*	
Thalassiosira pseudonana	Conjugation of *T. pseudonana* by *E. coli*	*p0521* and *p0521s* vector	4.0×10^{-4} diatom cells	First reported nuclear episomal vector for diatoms and stable episome replication of plasmid even in absence of antibiotic	[45]
Chloroplast transformation					
Chlamydomonas reinhardtii	Glass bead method	*ereB*/ human growth hormone	N/A	Generated marker free transformants using cell wall deficient and *psbH* deficient strain	[33]
Phaeodactylum tricornutum	Electroporation	*CAT* reporter gene and green fluorescent protein	12.5×10^3 transformants/ µg DNA	Green fluorescent protein was 0.12 % of the total soluble protein and transformation efficiency is very high	[46]

2.1.3   Selectable markers

A selectable marker is a major genetic engineering tool used for isolation of transformed cells. The transformed cells take up the gene and express recombinant proteins which blocks the negative effects of toxic chemicals and hence allow microbes to grow on them. This technique has been widely exploited in gene modification of microorganisms.

Markers are classified based on the mode of action by which it affects the microbes. Markers are classified into 4 types - herbicide, antibiotics, photosynthetic and metabolic marker. **Table 2.3** shows the antibiotics resistance gene which have been used in the microalgae over the past decade.

A new tetracycline marker TETX has been developed in *C. reinhardtii* by Garcia Echauri et al. [47]. Tetracycline binds to the 30S ribosomal subunit in microalgae thus inhibiting the protein synthesis. The *APHVIII* gene expressed in *C. reinhardtii* confers resistance to paromomycin [48]. The *nptII* gene used in *C. vulgaris,* confers resistance to both kanamycin and G418 [49]. Both these antibiotics act by blocking protein synthesis of microalgae. The RPL44 gene expressed in *Aurantiochytrium* sp., confers resistance to cycloheximide [50]. Cycloheximide should be avoided in microalgae which grow in alkaline condition since, cycloheximide degrades at pH > 7.

The Hyg3 gene expressed in *N. oleoabundans* conferred resistance to hygromycin B [43]. Hygromycin B acts by blocking the protein synthesis in eukaryotes and is extensively used in plant transformation. The Sh*ble* gene expressed in *N. salina* conferred resistance to zeocin [39] and zeocin is found to be an effective marker for

marine microalgae. Zeocin acts by binding to DNA and breaking the double strand. The *Nat* gene expressed in *T. pseudonana* conferred resistance to nourseothricin [45].

The *CAT* gene expressed in *P. tricornutum* conferred resistance to chloramphenicol [46]. Chloramphenicol acetyltransferase enzyme inactivates chloramphenicol by acetylation thus the antibiotic does not bind to 50S subunit. Paromomycin binds to 16 S ribosomal RNA, which inhibits protein synthesis in nonresistant cells. The ereB gene expressed in *D. tertiolecta* conferred resistance to erythromycin [51]. Erythromycin inhibits the protein biosynthesis by interfering with amino translocation.

In this section, the herbicide markers that have been used in microalgae transformation over the past decade are discussed. **Table 2.3** shows the list. The *pds*-L540R gene expressed in *I. galbana* conferred resistance to norflurazon [52]. Norflurazon acts as a microalgae pigment inhibitor and found to be effective against marine microalgae. *PimAHAS* gene expressed in *P. incisa* conferred resistance to sulfometuron methyl [53]. Sulfometuron methyl inhibits the biosynthesis of amino acid by inhibiting acetolactate synthase enzyme, which is important in biosynthesis of amino acid. The *bar* gene expressed in *Symbiodinium* sp. conferred resistance to basta but the concentration required is high [54]. Basta inhibits glutamine synthetase by binding to the glutamate site on the enzyme. Three genes GAT, protox rs-3, and PDS (R268T) expressed in *C. reinhardtii* allowed resistance to glyphosate, oxadiazon/oxyfluorfen, and norflurazon respectively. Glyphosate blocks the synthesis of aromatic amino acids in plant systems while Oxadiazon/oxyfluorfen blocks the photosynthesis process of the plant and microalgae, and norflurazon is a pigment inhibitor in microalgae.

Table 2.3 - Selection markers in microalge

Marker gene	Marker protein expressed	Microalgae expressed	Chemical used	Selection concentration	Remark	Reference
Antibiotic marker						
TETX	NADP-requiring oxidoreductase hydroxylates enzyme	*Chlamydomonas reinhardtii*	Tetracycline	15 μg/mL is sufficient but upto 100 μg/mL can be used	First reported use of tetracycline, TETX can degrade chlortetracycline, demeclocycline, doxycycline, minocycline, oxytetracycline, tetracycline and tigecycline	[47]
APHVIII	Aminoglycoside phosphotransferase VIII	*Chlamydomonas reinhardtii* 704	Paromomycin	30 μg/mL	The technique developed in this work can be used for isolate novel promoter in microalgae	[48]
nptII	Aminoglycoside 3'-phosphotransferase enzyme	*Chlorella vulgaris* CBS 15-2075	G418 sulphate	LC50 was 11.74 μg/mL but used 30 μg/ml of G418 sulphate for selection	Can use either kanamycin and G418 sulphate for microalgae selection. Kanamycin was ineffective even upto 500 μg/mL	[49]

RPL44 (P56Q)	Ribosomal protein (imparts cycloheximide resistance)	*Aurantiochytrium* sp. KRS101	Cycloheximide	1–50 μg/ml	Proline 56 was replaced with glutamine (point mutation) and this served as selection marker against cycloheximide	[50]
Hyg3 (aph7" gene + intron1 in rbcS2 gene)	Aminoglycoside phosphotransferase	*Neochloris oleoabundans*	Hygromycin B	5 μg/mL	up to 90% of the transformants exhibited green fluorescent protein (GFP) activity	[43]
Shble gene	Bleomycin resistance protein	*Nannochloropsis salina*	Zeocin	2.5 μg/mL	Expressed sfCherry fluorescent protein successfully	[39]
Nat gene (pTpPuc3 vector)	Nourseothricin N-acetyl transferase	*Thalassiosira pseudonana*	Nourseothricin	50 μg/ml	First reported nuclear episomal vector for diatoms and stable episome replication of plasmid even in absence of antibiotic	[45]
CAT gene	Chloramphenicol acetyl transferase	*Phaeodactylum tricornutum*	Chloramphenic-ol	300 μg/ml	Green fluorescent protein was 0.12 % of the total soluble protein and transformation efficiency was very high	[46]

ereB	Erythromycin esterase	*Dunaliella tertiolecta*	Erythromycin	100 µg/ml	*D. tertiolecta* is sensitive to erythromycin hence can be tried in other marine microalgae	[55]
Herbicide marker						
pds-L540R (carries a point mutation)	Mutated norflurazon-resistant phytoene desaturase	*Isochrysis* sp.	Norflurazon	7.5 µM	Effective selection marker for marine microalgae with full strength artificial sea water (growth medium), and low concentration of norflurazon is effective and increase the astaxanthin production	[52]
pds-L540R (carries a point mutation)	Mutated norflurazon-resistant phytoene desaturase	*Isochrysis galbana*	Norflurazon	0.2 µM	Effective selection marker for marine microalgae with full strength Artificial sea water (growth medium), and low concentration of norflurazon is effective and increase the astaxanthin production	[52]
PimAHAS	Acetohydroxyacid	*Parietochloris*	Sulfometuron -	N/A	Mutated protein had	[53]

	synthase	*incisa*	methyl		7-fold higher resistance than wild type protein	
bar gene	Phosphinothricin N-acetyltransferase	*Symbiodinium* sp.	Basta	1 mg/mL	Achieved better transformation efficiency compared to using other herbicide	[54]
GAT	Glyphosate acetyltransferase	*Chlamydomonas reinhardtii* CC124, CC1010, CC503, and CC3491	Glyphosate	20 mM	Transgenic cell lines are able to survive upto 40 mM glyphosate in liquid and agar media	[56]
protox rs-3	Protoporphyrinoge-n oxidase		Oxadiazon/oxy-fluorfen	0.17 μM	Transgenic cell lines are able to survive upto 15 μM oxadiazon/oxyfluor-fen in liquid and agar media	
PDS (R268T)	Phytoene desaturase		Norflurazon	4 μM	Transgenic cell lines were able to survive upto 60 μM norflurazon in liquid media and120 μM norflurazon in agar media	

2.1.4 Photosynthetic markers

In photosynthetic markers, light is used as a selection method to isolate the transgenic microalgae. Microalgae use light as a source of energy during photosynthesis. In few mutant strains the photosynthesis system is disrupted. These mutant strains can restore their photosynthesis by using photosynthesis markers. This can be exploited for development of transgenic microalgae using photosynthetic marker. The advantage of this technique is that there is no need for any antibiotics or herbicide for selection of transgenic microalgae. *C. reinhardtii* has been used as a model system for development of photosynthetic markers. **Table 2.4** shows some of the marker free selection systems developed in *C. reinhardtii.*

C. reinhardtii Fud7 strain is a mutant which cannot grow under photoautotrophic condition. The recombinant strain was selected by adding the *psbA* gene which encodes for a protein that allows photoautotrophic growth [57]. *C. reinhardtii* TN72 is unable to grow under light. However, when this strain is transformed with the *psbH* gene, the transformed cells can be selected under light [33]. *C. reinhardtii* CC2653 is another mutant strain unable to grow under light. When this strain was transformed with *rbcL*90 gene, they were able to survive and grow under light [58]. *C. reinhardtii* FUD50 is a mutant strain unable to grow under light but when the mutant was transformed with the *atpB* gene, the transformed cells were able to grow under light [59]. *C. reinhardtii* Fud7 strain is a mutant which grows on acetate but not under photoautotrophic condition. The strain was transformed with *psbA* gene were able to grow under light [59]. *C. reinhardtii* TN72 and *C. reinhardtii* Bst are mutant strains which grow on acetate but not in the presence of light. However, when the mutant strain was transformed with *psbH* gene, the transgenic microalgae which has taken up *psbH* gene were isolated under light [60].

Few mutant strains which are unable to grow in the presence of few nutrients due to mutation has been exploited for development of marker free selection system. Below are few marker free system developed.

Phosphite cannot be utilized as a sole source of phosphorous for microalgae or microbial growth [61]. However, phosphite converted to phosphate can be utilized by microalgae for its growth. The *ptxD* gene transformed into *C. reinhardtii* CC-125 was able to oxidize phosphite to phosphate [34]. *C. reinhardtii arg*9-2 is a mutant strain which lacks synthesis of *N*-acetyl ornithine aminotransferase and cannot grow without arginine. However, when the mutant was transformed with the *ARG9* gene, they were able to grow without arginine addition [62]. *C. reinhardtii* cw15 mutant strain are resistant to 5-Fluorocytosine. However, when the *crCD* gene was transformed into *C. reinhardtii* cw15 became sensitive to 5-Fluorocytosine and hence, enabled isolation of mutant strains [63].

Table 2.4 - Photosynthetic markers in microalgae

Strain	Place of transformation	Mutant strain properties	Gene inserted	Recombinant protein expressed	Comments	Reference
Chlamydomonas reinhardtii Fud7	Chloroplast	Fud7 mutant strain	*psbA*	MPT64 (used for diagnostic purpose)	Mutant strain utilizes acetate as carbon source but can't grow under photoautotrophic condition	[57]
Chlamydomonas reinhardtii TN72	Chloroplast	Cell wall-deficient Δ*psbH* strain (deletion of photo system II)	*psbH*	Human growth hormone	Mutant strain cannot grow in presence of light but mutant strain can grow if the *psbH* gene is integrated into chloroplast	[33]
Chlamydomonas reinhardtii CC2653	Chloroplast	*rbcL* of CC2653 strain was replaced with *rbcL*90+ *ADH1* by homologous recombination	*rbcL*90	Alcohol dehydrogenase (*ADH1* gene)	Photoautotrophic selection, heterologous expression of *rbcL*90 gene with gene of interest (*ADH1* gene)	[64]
Chlamydomonas reinhardtii FUD50	Chloroplast	Strain lacking *atpB* gene	*atpB*	*acrV* or *vapA* (antigens from the fish pathogen *Aeromonas salmonicida*)	Photoautotrophic selection, heterologous expression of *atpB* gene with gene of interest (atpB-int vectors)	[59]

Chlamydomonas reinhardtii Fud7	Chloroplast	Strain lacking *psbA* gene	*aadA*	*acrV* or *vapA* (antigens from the fish pathogen *Aeromonas salmonicida*)	Repeated sub-culturing of transgene to obtain homoplasmic strain with no antibiotic selection pressure (*aadA* gene)	[59]
Chlamydomonas reinhardtii TN72	Chloroplast	Strain originates from the cell-wall deficient strain cw15	*psbH*	vivid Verde fluorescent Protein	Photoautotrophic selection, psbH gene was disrupted by the *aadA* cassette	[65]
Chlamydomonas reinhardtii Bst	Chloroplast	Strain originates from the wild-type strain CC-1021	*psbH*	vivid Verde fluorescent Protein	Photoautotrophic selection, psbH gene was disrupted by the *aadA* cassette	[65]
Chlamydomonas reinhardtii CC-125 (mt +)	Nuclear	N/A	*ptxD*	Phosphite oxidoreductase	Oxidize phosphite to phosphate (can be used in photobioreactors and open pond systems)	[61]
Chlamydomonas reinhardtii arg9-2	Nuclear	Strain lacks synthesis of N-acetyl ornithine aminotransferase (cannot grow without arginine)	*ARG9*	*N*-acetyl ornithine aminotransferase	Integration of *ARG9* gene into mutant strain can grow without arginine	[62]
Chlamydomonas reinhardtii cw15	Chloroplast	Wild type *cw15* have resistance to 5-Fluorocytosine	*crCD*	Cytosine deaminase	The UV mutated *cw15* become sensitive to 5-Fluorocytosine	[63]

2.1.5   Fluorescent proteins expressed in microalgae

Fluorescent proteins are a class of proteins with unique properties in that they exhibit florescence when exposed to appropriate light wavelength. The green fluorescent protein was the first fluorescent protein to be sequenced, cloned and expressed. The green fluorescent protein was isolated from the jellyfish Aequorea victoria. Fluorescent proteins are used in protein labeling, photobleaching, subcellular localization, promoter tracking, timers, cell and tissue labeling, DNA and RNA labeling, and protein-protein interaction studies. **Table 2.5** shows some of the fluorescent protein that have been expressed over the past decade in microalgae.

A new selection marker shCP is isolated from a water dewelling animal Stichodactyla haddoni. The shCP is made up of 227 amino acid residues and it is 96 % identical to green fluorescent protein. The shCP gene expressed in marine microalgae *N. oculata* expressed a purple chromophore protein which was visible to the naked eye and it can be quantified using a wavelength of 574 nm [66]. The vivid Verde fluorescent protein (VFP) is a newly isolated from coral Cyphastrea microphthalma found in the Australian Great Barrier Reef [67]. The VFP is a 26 kDa protein and has an excitation/emission of 491/503 nm. The VFP gene expressed in chloroplast of *C. reinhardtii* TN72 and *C. reinhardtii* Bst. However, the fluorescence level was very low and was only detectable by Western blot [65]. The VFP protein has been well expressed in *E. coli* but not in microalgae. This could be overcome by using codon optimized gene.

Rasala et al. [68] expressed 6 different fluorescent protein in *C. reinhardtii* CC1690. The mTagBFP gene expressed blue fluorescent protein with an excitation/emission of

402/457 nm. The blue fluorescent protein has high auto-fluorescence and is an excellent candidate for protein–protein interaction studies. The mCerulean gene expressed cerulean fluorescent protein with an excitation/emission of 433/475 nm. The cerulean fluorescent protein has high auto-fluorescence and is an excellent candidate for protein–protein interaction studies [68]. The venus gene expressed yellow fluorescent protein with an excitation/emission of 515/528 nm. The yellow fluorescent protein has good signal to noise ratio, excellent candidate for protein tagging, reporter gene, and promoter strength study [68]. The tdTomato gene expressed orange fluorescent protein with an excitation/emission of 554/581 nm. The orange fluorescent protein was the brightest of all expressed fluorescent protein in *C. reinhardtii* CC1690 but the fluorescent protein forms a dimer which can be difficult to express in oxidizing environment [68]. The mCherry (Ds) and mCherry (Cr) gene expressed red fluorescent protein and green fluorescent protein respectively with an excitation/emission of 587/610 nm and 587/610 nm respectively. The red and green fluorescent proteins are monomer and well suited for expression in *C. reinhardtii* and other microalgae for study [68].

The sGFP gene transformed in *C. reinhardtii* CC-125 expressed superfolder green fluorescent protein which has an excitation/emission of 500/530 nm [69]. The pHRed gene transformed in *C. reinhardtii* expressed red fluorescent protein which has an excitation/emission of 600/620 nm [70]. The red fluorescent protein is pH sensitive and can be used to study the pH effects in intracellular components of cell [71].

The GUS gene transformed in *T. chuii* expressed β-glucuronidases enzyme, which has an excitation/emission of 365/455 nm [44]. The β-glucuronidases enzyme hydrolyzes 4-MUG to 4-MU, which can be quantified. The uidA gene transformed in marine

microalgae *Nannochloropsis* sp. expressed β-glucuronidases enzyme which has an excitation/emission of 365/455 nm [72]. The β-glucuronidases enzyme hydrolyzes 4-MUG to 4-MU which can be quantified.

The Gluc gene transformed in *C. reinhardtii* expressed the firefly luciferase enzyme [73]. Coelenterazine is a substrate which reacts with luciferase enzyme to produces light and the expression level can be quantified by reading the emission at 560 nm. The crluc gene transformed in *C. reinhardtii* strain JL173 expressed the renilla luciferase enzyme [74]. Coelenterazine reacts with renilla luciferase enzyme to produces light that is quantified by reading the emission at 480 nm.

Table 2.5 - Fluorescent proteins in microalgae

Fluorescent gene	Fluorescent protein expressed	Strain	Place of transformation	Detection wavelength	Comments	Reference
shCP	Purple chromophore protein	*Nannochloropsis oculata*	Either nuclear or organellar	574 nm	Can detect visually the dark brown color for selection of transformed cells, no need of UV- visible spectrophotometer for detection	[66]
VFP	vivid Verde Fluorescent Protein	*Chlamydomonas reinhardtii* TN72	Chloroplast	Excitation /Emission - 491/503 nm	Fluorescence level was very low but detectable by Western blot	[65]
VFP	vivid Verde Fluorescent Protein	*Chlamydomonas reinhardtii* Bst	Chloroplast	Excitation /Emission – 491/503 nm	Showed very low levels of VFP hence detection is difficult	[65]
mTagBFP	Blue fluorescent protein	*Chlamydomonas reinhardtii* CC1690	Nuclear	Excitation /Emission - 402/457 nm	Has high auto-fluorescence, and excellent candidate for protein–protein interaction studies	[68]
mCerulean	Cerulean			Excitation	Has high auto-	

				/Emission - 433/475 nm	fluorescence, and excellent candidate for protein–protein interaction studies	
Venus	Yellow fluores cent protein			Excitation /Emission - 515/528 nm	Has good signal to noise ratio, good for protein tagging, reporter gene, and promoter strength study	
tdTomato	Orange fluorescent protein			Excitation /Emission - 554/581 nm	This was the brightest of all expressed fluorescent protein but is a dimer	
mCherry (Ds)	Red fluorescent protein			Excitation /Emission - 587/610 nm	Second brightest fluorescent protein and is a monomer. Well suited for *C. reinhardtii* studies	
mCherry (Cr)	Green fluorescent protein			Excitation /Emission - 587/610 nm	Well suited for *C. reinhardtii* studies	
sGFP	Superfolder green fluorescent protein	*Chlamydomonas reinhardtii* CC-125	plastid	Excitation /Emission – 500/530 nm	Expression of 2-lysophosphatidic acid acyltransferase led to > 20 % increase in oil content under nitrogen	[69]

					deficient conditions	
pHRed	Red fluorescent protein	*Chlamydomonas reinhardtii*	chloroplast	Excitation /Emission - 600/620 nm	pH sensitive protein, useful tool to study pH effects in intracellular cell	[70] and [71]
GUS	β-glucuronidases	*Tetraselmis chuii*	Nuclear	Excitation /Emission – 365/455 nm	4-MUG is hydrolyzed to 4-MU	[44]
uidA	β-glucuronidase	*Nannochloropsis* sp.	Nuclear	Excitation /Emission – 365/455 nm	4-MUG is hydrolyzed to 4-MU	[72]
Gluc	Firefly luciferase	*Chlamydomonas reinhardtii*	Chloroplasts	Emission – 560 nm	Coelenterazine reacts with luciferase which is converted to light (produces greenish yellow light)	[73]
crluc	Renilla luciferase	*Chlamydomonas reinhardtii* strain JL173 mutant	Nuclear	Emission - 480 nm	Coelenterazine reacts with luciferase which is converted to light (produces blue light)	[74]

2.1.6　Promoters and terminators

Promoters are an important component in the development of transgenic microalgae system because they drive the expression of foreign gene expression. In development of transgenic microalgae, two genes, two promoters, and two terminators are used universally. The first section of gene cassette is promoter, terminator, and selection marker for isolation of transgenic microalgae. The second section of gene cassette is promoter, terminator, and gene of interest for expression of gene of interest protein. The promoter is located upstream of the selection marker gene/gene of interest and the terminator is located downstream of the selection marker gene/gene of interest. **Table 2.6** list some of the promoters and terminators that have been used over the last decade for the development of transgenic microalgae.

Scranton et al. [75] developed twenty-five synthetic algal promoters by using POWRS software for *C. reinhardtii*. They carried out a comparison study of their synthetic algal promoters with the current best endogenous promoter in *C. reinhardtii* (hsp70/rbs2 promoter) and found that synthetic algal promoter 11 (containing an rbcs2 intron) performed better expression of recombinant protein, and a new DNA motif essential for promoter function was discovered [75]. In *C. reinhardtii* TN72, vivid Verde Fluorescent gene was expressed under the atpA promoter/5' UTR, and 3' UTR rbcL terminator. Under heterotropic conditions 3.3 mg of VFP /g dry biomass was expressed [65]. In the marine microalgae *D. salina*, an RBCS2 promoter and a 35 S terminator were used to express the β-carotene hydroxylase gene (isolated from *C. reinhardtii*). They found a 3-fold increase in violaxanthin during the growth phase and 2-fold increase in zeaxanthin but no change in cartenoid [4].

In another marine microalgae *D. tertiolecta*, the psbD/5′ UTR promoter and psbA/3′ UTR terminator were used to successfully express five hydrolysis enzymes [55]. In the diatom *Phaeodactylum tricornutum* Bohlin, a novel diatom-infecting viruses (DIVs) promoter was used to express enhanced green fluorescent protein and terminated by fucoxanthin chlorophyll a/c-binding protein terminator (Tpfcp). The novel DIV promoter was shown to have higher transcription and translation compared to the endogenous promoter fucoxanthin chlorophyll a/c-binding protein (FCP) gene [76]. Park et al. [77] have expressed erythropoietin in *Chlorella ellipsoidea* KMCC C-20 using a 33kDa promoter and 3^1 UTR terminator. They compared the expression of the 33kDa promoter with the CaMV 35S and found the expression level to be similar [77].

In the marine microalgae *D. salina* (UTEX-1644), a white spot syndrome virus (WSSV) subunit VP28 gene was expressed successfully using a maize ubiquitin promoter (Ubi1-Ω) and Nopaline synthase gene terminator (Nos). They expressed 3 ng/mg of white spot syndrome virus subunit vaccine by using the Ubi1-Ω promoter [78]. Kilian et al. [79] isolated a new bidirectional promoter, Violaxanthin/chlorophyll a-binding protein (VCP2-Promter bidirectional) and its terminator, VCP2 3^1 UTR. The promoter was shown to work in either direction and allow protein expression. The promoter can be used for expression of a selection marker and a recombinant protein. It was also, shown that a transformation efficiency of up to 80 % could be achieved using the VCP2 bidirectional promoter.

 Renilla luciferase was expressed in *C. reinhardtii* cw15 under the control of a strong PsaD constitutive promoter and PsaD terminator. The Renilla luciferase was expressed efficiently in *C. reinhardtii* [80]. *Symbiodinium microadriaticum* and *Amphidinium* were

transformed with *Agrobacterium* p1′2′ bidirectional promoter and 35 S terminator. They expressed the selection marker hygromycin B phosphotransferase gene (hpt) and β-glucoronidase (GUS) reporter gene [81]. Using this bidirectional promoter gene of interest and selectable marker can be expressed and this can be achieved with a single transformation.

Díaz-Santos et al. [82] carried out a comparison study of three heterologous promoters expression in *C. reinhardtii* 704. The promoters tested were cauliflower mosaic virus 35S RNA promoter (CaMV 35S), nopaline synthase promoter (NOS), and HSP70A/RBCS2 promoter. The CaMV 35S and NOS promoters had a NOS terminator; whereas, HSP70A/RBCS2 promoter had RBCS2 terminator. All three promoters expressed aminoglycoside 3′-phosphotransferase encoding gene (APHVIII). The mRNA transcript showed that HSP70A/RBCS2 promoter > NOS promoter > CaMV 35S. Sakaue et al. [83] expressed β-glucoronidase (GUS) enzyme (uidA gene) under three heterologous virus promoters in diatom *Phaeodactylum tricornutum Bohlin* (UTEX 642). The promoters tested were cytomegalovirus (PCMV - used in mammalian), rous sarcoma virus long terminal repeat (PRSV-LTR - used in avian) and cauliflower mosaic virus 35s (PCaMV35s - used in plant). All promoters showed variable β-glucoronidase activity. The highest activity was from PRSV-LTR promoter and lowest activity was from the PCMV promoter.

Table 2.6 - Promoters and terminators in microalgae

Strain	Promoter	Terminator	Total soluble protein	Place of transfor-mation	Remark	Reference
Chlamydomonas reinhardtii	Synthetic algal promoter 11 + rbcs2 5′	rbcs2 5′ and 3′ UTRs	N/A	Nuclear	A new DNA motif essential for promoter function is discovered	[75]
Chlamydomonas reinhardtii TN72	atpA promoter/5'UTR element	3' UTR *rbcL*	Under heterotrophic condi-tion 3.3 mg VFP /g dry biomass	Chloroplast	Fluorescence level of VFP was very low but detectable by Western blot	[65]
Dunaliella salina	RBCS2 (rubisco promoter along with transit peptide)	35 S	3-fold increase in violaxanthin during growth phase and 2-fold increase in zeaxanthin	Nuclear	No difference in total carotenoid and chlorophyll with transformed and untransformed	[4]
Dunaliella tertiolecta	*psbD* and 5′ UTR	*psbA* and 3′ UTR	Xylanase - 57.8 ±10 U/mg, α-Galactosidase - 480 ± 340 mU/mg, α-Galactosidase - 980 ± 200 mU/mg,	Chloroplast	Measurable quantities of all enzymes were produced, and transgenes were codon optimized	[51]

			Phytase - 180 ± 10 mU/mg, and Phosphatase - 2.1 ± 0.2 mU/mg			
Phaeodactylum tricornutum Bohlin	DIV promoter	Tpfcp	The egfp and sh*ble* mRNA was 1200/rps in ClP1	Nuclear	High levels of expression were reported in cells in the stationary phase compared to the exponential phase of growth	[76]
Chlorella ellipsoidea KMCC C-20	33kDa promoter	3^1 UTR	0.37 OD$_{450}$ nm of Erythropoietin protein by ELISA analysis	Nuclear	*Chlorella* viral promoter drive protein expression, and the expression level is equivalent to CaMV 35S	[77]
Dunaliella salina (UTEX-1644)	Ubi1-Ω	Nopaline synthase gene terminator (Nos)	3 ng/mg of white spot syndrome virus subunit vaccine	Nuclear	Bar gene is used for selection and the concentration used is 3 µg/ml phosphinothricin	[78]
Nannochloropsis sp. W2J3B	Violaxanthin/ chlorophyll a-binding protein (VCP2-Promter bidirectional)	*VCP2* 3′ UTR	80 % transformation efficiency was achieved with VCP2 bidirectional promoter whereas no transformation was seen in cell	Nuclear	Can be used to express 2 different proteins at a time	[79]

			lacking promoter			
Chlamydomonas reinhardtii cw15	*PsaD* promoter	*PsaD* terminator	Renilla luciferase	Nuclear	Efficient promoter for *Chlamydomonas reinhardtii*	[80]
Symbiodinium microadriaticum	p1′2′ bidirectional promoter	35 S	366 pmol MUG /min/mg of protein	Nuclear	Can be used to express 2 different proteins at a time	[81]
Amphidinium sp.	p1′2′ bidirectional promoter	35 S	783 pmol MUG /min/mg of protein	Nuclear	Can be used to express 2 different proteins at a time	
Chlamydomonas reinhardtii 704	Cauliflower mosaic virus 35S RNA promoter (CaMV 35S)	*NOS* ter	Relative mRNA transcript = 0.05	Nuclear	The mRNA transcript level is low	[82]
	Nopaline synthase promoter (*NOS*)	*NOS* ter	Relative mRNA transcript = 0.25	Nuclear	NOS promoter works in *C. reinhardtii* and it is better than *CaMV 35S* by 5-fold	
	HSP70A/RBCS2	*RBCS2* ter	Relative mRNA transcript = 0.50	Nuclear	This is the best promoter system for *C. reinhardtii*	
Phaeodactylum tricornutum Bohlin	Fucoxanthin chlorophylla/ c-binding	n/a	Average GUS activity with air and high CO_2 ~ 12	Nuclear	The fcpA promoter works in diatom	[83]

(UTEX 642)	protein A gene (fcpA)		and 15 nmol pnp/mg protein/min		
	Cytomegalo virus promoter (mPCMV)		Average GUS activity with air and high CO_2 ~ 4 and 4 nmol pnp/mg protein/min	Nuclear	When Pptca::mPCMV fused gene are used GUS activity with air is ~ 175 nmol pnp/mg protein/min, and mammalian promoter work in diatoms
	Rous sarcoma virus long terminal repeat promoter (PRSV-LTR)		Average GUS activity with air and high CO_2 ~35 and 20 nmol pnp/mg protein/min	Nuclear	Mammalian and avian promoter system work in diatoms
	Cauliflower mosaicvirus 35s promoter (PCaMV35s)		Average GUS activity with air and high CO_2 ~15 and 20 nmol pnp/mg protein/min	Nuclear	Plant promoter system works in diatoms

2.1.7   Inducible systems

Inducible systems allow conditional expression of genes of interest. Here a two-component based inducible systems is discussed. The first component is called activator/driver and it is typically a chimeric transcription factor which binds to tightly controlled promoter. The second component is called effector/reporter and it contains the binding sites for activator/driver and it controls the expression of gene of interest. In inducible systems expression of gene of interest is not turned on or turned off until the inducer is added to the cells. The inducer can bind to a chimeric transcription factor hence the expression of gene of interest can occur. Inducible systems are still at their nascent stage in microalgae. They have wide application in metabolic engineering of microalgae, expression of toxic proteins, and expression of cell lysis enzyme (easy recovery of lipids). **Table 2.7** shows some of the inducible system that have been developed in microalgae over last decade.

Iwai et al. [84] used a sulphoquinovosyldiacyl glycerol synthase 2 (SQD2) promoter in *C. reinhardtii* CC-408 which is upregulated during phosphorous starvation for expression of diacylglycerol acyl-CoA acyltransferase (CrDGTT4) gene. They found the TAG increased by 1.5 to 2.5-fold by using SQD2 promoter when compared to wildtype [84]. However, the promoter is activated during phosphorous starvation and would affect the physiology of the cell. Iwai et al [85] used the same SQD2 promoter to express CrDGTT4 gene in marine microalgae *Nannochloropsis* strain NIES-2145 and found that TAG increased by 1.3 to 1.7-fold. The promoter is also activated during phosphorous starvation. They have shown the inducible system can be used in oleaginous marine microalgae.

Inducible systems have also been developed in the diatom *P. tricornutum*. The alkaline phosphatase promoter (pPhAP1) is induced by phosphorous starvation. Enhanced green fluorescent protein (egfp) gene was expressed under high phosphorous concentration in the culture media the green fluorescent protein was not expressed whereas, when the phosphorous concentration was 3.6 μM or lower the enhanced green fluorescent protein was expressed. The high concentration of phosphorous was found to have no effect on cell growth [86]. In another strain of *P. tricornutum* 646, a nitrogen starvation inducible (DGAT1) promoter was used to express *Haematococcus* oil globule protein (HOGP) and enhanced green fluorescent protein. The transgenic diatoms showed 25 to 30 % higher TFA [87].

In *C. reinhardtii* JL173, a light-inducible protein promoter gene (LIP) was used to express the Renilla luciferase gene. The -400 bp, -800 bp, and -1700 bp LIP promoters showed over 1000-fold increase in luciferase activity. Also, the LIP promoter isolated from *Dunaliella* sp. is functional in *C. reinhardtii* [74]. In *C. reinhardtii* CC-849, the β-carotene ketolase inducible promoter (bkt1) was used to express the bleomycin resistance protein (Ble). The bkt1 gene was induced by high light and high sodium acetate. The mRNA transcript of the bleomycin resistance protein gene was analyzed. Wang et al. [88] found that the bkt1-RBCS2 fusion promoter had 12-fold more ble mRNA transcript compared to wild type.

C. reinhardtii CC849 was transformed with a heat inducible promoter gene (HSP70A-RBCS2). Artificial miRNA of oxygen evolving enhancer (OEE2) gene was expressed under the HSP70A-RBCS2 inducible promoter. Li et al. [89] found a 2-fold increase in H_2 production by heat induction. The artificial miRNA OEE2 with HSP70A-RBCS2

inducible system can be used in further application in microalgae. The CYC6 inducible promoter system induced by nickel ions and repressed by copper was developed in *C. reinhardtii* cw15. Renilla luciferase gene was used to study the inducibility. For induction 10 µM of copper chelator was sufficient whereas, up to 50 µM of nickel is necessary for induction [80].

β-glucuronidase (gusA) gene was expressed under the control of light inducible AcRbcS promoter in *A. convolutes*. A 24-hour study under light and dark condition was carried out. It was found that the gusA mRNA transcript was high when grown in presence of light and low when grown in dark [90]. Tahri et al. [91] developed a new High affinity phosphate transporter (HAPT) inducible system. The system is induced by less than 50 µM phosphate and suppressed by 50 – 100 µM of phosphate. The HAPT was transformed into *O. tauri* 0TTH95 and used luciferase to monitor the inducible system. They found that with HAPT, 40-fold inducibility (40-fold increase in luciferase activity when < 50 µM phosphate was used compared to control where no induction at 50 – 100 µM phosphate) could be achieved.

Marine microalgae *N. oceanica* CCMP1779 was transformed with Lipid droplet surface protein (LDSP) promoter. The LDSP gene was responsive to nitrogen starvation. Δ12-desaturase (NoD12) gene was expressed under the control of the LDSP promoter. The transgenic microalgae under nitrogen starvation showed 10 to 12-fold higher NoD12 expression compared to wild type [92].

Shemesh et al. [87] developed a novel nitrogen starvation inducible system (DGAT1 gene promoter) for *P. tricornutum* 646. To study the inducibility, N-terminus of

Haematococcus oil globule protein (HOGP) fused with EGFP was expressed under the control of DGAT1 promoter gene. They found a slight increase in TFA and TGA accumulation compared to wild type [87]. Ng et al. [93] has developed a novel 17-β-estradiol inducible system in *C. vulgaris* UMT-M1. 17-β-estradiol inducible system is a two-component system (activator and reporter) derived from the *Arabidopsis thaliana* plant system. They expressed yoeBSpn and pezT toxin genes fused with green fluorescent protein gene. The system was found to be a non-leaky inducible system and demonstrated that toxic proteins can be expressed using this system [93].

Niu et al. [94] used a nitrate reductase inducible promoter to drive the expression of chloramphenicol acetyltransferase (CAT) in *C. vulgaris*. The nitrate reductase inducible promoter was derived from *P. tricornutum*. Expression of CAT was induced in transgenic *C. vulgaris* by nitrate whereas there was no CAT expressed in presence of ammonium. Also, the heterologous gene from a diatom was shown to be active in *C. vulgaris* [94].

Table 2.7 - Inducible systems in microalgae. In the below table, all the gene transformation are carried out are nuclear transformation

Strain	Source	Inducible system gene	Activator	Gene expressed	Remark	Reference
C. reinhardtii CC-408	*C. reinhardtii*	Sulphoquinovos-yldiacyl glycerol synthase 2 (SQD2) promoter	Phosphorous starvation	Diacylglycerol acyl-CoA acyltransferase (CrDGTT4)	TAG increased between 1.5 to 2.5-fold	[84]
C. reinhardtii JL173	*Dunaliella* sp.	Light-inducible protein promoter (LIP)	Light	Renilla luciferase	The -400 bp, -800 bp, and -1700 bp LIP promoters showed over 1000-fold increase in luciferase activity	[74]
C. reinhardtii CC-849	*H. pluvialis*	β-carotene ketolase promoter gene (bkt1)	High light and high sodium acetate concentrati-on	Ble gene (Bleomycin resistance protein)	bkt1-RBCS2 fusion promoter had increased ble mRNA transcript by 4.11- and 9.0-fold at 12 and 24-hour post induction	[88]
C. reinhardtii	*C. reinhardtii*	HSP70A-RBCS2	Heat	amiRNA OEE2	By heat induction,	[89]

CC849			inducible		amiRNA OEE2 gene was expressed which led to 2 -fold increase in H$_2$ production	
C. reinhardtii cw15	*C. reinhardtii*	CYC6 promoter	Repressed by copper and induced by nickel ions	Renilla luciferase	For induction 10 μM of copper chelator is sufficient whereas, up to 50 μM of nickel is necessary for induction	[80]
Nannochloropsis NIES-2145	*C. reinhardtii*	Sulfoquinovosyldiac-yl glycerol synthase 2 (SQD2) promoter	Phosphorous starvation	Diacylglycerol acyl-CoA acyltransferase (CrDGTT4)	mRNA transcript increased between 7 to 112-fold and TAG increased between 1.3 to 1.7-fold	[85]
P. tricornutum	*P. tricornutum*	Alkaline phosphatase promoter (pPhAP1)	Phosphorous starvation	Enhanced green fluorescent protein	Tight inducible system	[86]
P. tricornutum 646	*P. tricornutum*	Nitrogen starvation inducible (DGAT1) promoter	Nitrogen starvation	*Haematococcus* oil globule protein (HOGP) and Enhanced green	Transgenic cells showed 25–30% higher TFA content	[87]

				fluorescent protein		
P. tricornutum 646	*C. reinhardtii*	DGAT1 promoter gene	Nitrogen starvation	*Haematococcus* oil globule protein (HOGP) was fused with EGFP (HOGP::EGFP)	Slight increase in accumulation of TFA and TGA	[95]
A. convolutus	*A. convolutus*	*AcRbcS* promoter	Light	β-glucuronidase (*gusA*)	The *gus*A mRNA transcript was high when grown in presence of light whereas, *gus*A mRNA transcript was low when grown in dark	[90]
O. tauri 0TTH95	*O. tauri*	High Affinity Phosphate Transporter (HAPT) gene	Phosphate overdose	Luciferase	50–100 μM of β-glycerol phosphate or NaH$_2$PO$_4$ is needed for suppression of luciferase expression, 40-fold inducibility was achieved, and the induction system may have some	[91]

					physiological changes on cells	
N. oceanica CCMP1779	*N. oceanica* CCMP1779	Lipid droplet surface protein (LDSP) promoter	Nitrogen starvation	Δ12-desaturase (NoD12)	Under nitrogen starvation transgenic microalgae showed 11 to 12-fold higher NoD12 expression compared to wild type	[92]
C. vulgaris UMT-M1	*A. thaliana*	OlexA-TATA	17-β-estradiol	*yoeB*Spn and *pezT* toxin genes	Non-leaky inducible system, and can be used for expressing toxic proteins	[93]
C. vulgaris	*P. tricornutum*	Nitrate reductase promoter	Nitrate starvation	Chloramphenicol acetyltransferase (CAT)	CAT was induced in transgenic *C. vulgaris* by nitrate whereas there was no CAT expressed in presence of ammonium	[94]

2.1.8   Affinity tags

Affinity tags are small peptide sequences that are genetically attached to the gene of interest either upstream or downstream. The main reason for using affinity an tag in recombinant protein production is for separation of recombinant protein from other contaminants. Affinity purification is a simple method of separation of recombinant protein. Affinity tags are also used for Western blotting, immunoprecipitation and immunofluorescence assays. **Table 2.8** lists some of the affinity tags that have been expressed in microalgae.

Hemagglutinin (HA) epitope tag is derived from human influenza hemagglutinin. It is a nine amino acid long peptide. The tag is inserted either at the N terminal or C terminal of the protein and it is found to not interact with recombinant proteins that are expressed. *C. reinhardtii* TN72, *C. reinhardtii* Bst, and *C. reinhardtii* were transformed with HA-tag containing recombinant protein. The recombinant protein size were analyzed by using the HA-tag Western blot [70] and [60].

FLAG-tag is an eight amino acid peptide which was first used in 1984 by Munro and Pelham. The tag has been used on the N and C terminal ends of recombinant protein. The tag has been used in microalgae for immunoblot assay. The 3x FLAG-tag was expressed in *C. reinhardtii* CC1010 and used immunoblot for analysis [96]. Jones et al. [97] expressed a serum amyloid A protein (SAA) tag in *C. reinhardtii* 137c and used Western blotting, and ELISA for analysis.

Table 2.8 - Affinity tags in microalgae. In the below table, all the gene transformation are carried out are chloroplast transformation

Strain	Affinity tag	Transformation technique	Type of assay	Type of affinity purification	Reference
C. reinhardtii TN72	Hemagglutinin (HA) epitope tag	Glass bead agitation	Western blot	Anti HA agarose	[60]
C. reinhardtii Bst	Hemagglutinin (HA) epitope tag	Glass bead agitation	Western blot	Anti HA agarose	[60]
C. reinhardtii	HA-tag	Glass bead agitation	Western blot	Anti HA agarose	[70]
C. reinhardtii CC1010	3x FLAG-tag	Electroporation	Immunoblot	Anti-FLAG agaorse	[96]
C. reinhardtii 137c	Serum amyloid A protein (SAA)	Particle bombardment	Western blotting, ELISA	n/a	[97]

2.1.9   Export signal

In both eukaryotic and prokaryotic cells, proteins are transported within the cell, to the cell membrane, surface of cell membrane, and secreted to the culture medium. This is achieved by signal peptides. The signal peptide is usually made of 11-27 residue. They comprise of three domains. The first region is comprised of positively charged n-region made up 1 to 5 residue long. The second region is comprised of a central hydrophobic h-region made up of 7 to 15 residue long. The third region is comprised of a polar c-region made up of 3 to 7 residue long. **Table 2.9** shows some of the export signal that has been developed over the past decade.

Proteins in the chloroplast are transferred to the thylakoid membrane by the Sec or Tat pathways. Zedler et al. [70] has developed a TorA signal peptide (Tat pathway) in *C. reinhardtii*. The TorA signal peptide introduced at the N-terminal of the recombinant proteins allowed the protein to be transferred from the chloroplast to the thylakoid lumen. They expressed a red fluorescent protein and scFv antibody fragment sucessfully. This would be a useful tool to express recombinant proteins that require an oxidizing environment or for low pH stable proteins.

León et al. [98] used a rubisco transit peptide and ferredoxin transit peptide in *C. reinhardtii* 704 to express β-carotene ketolase. The rubisco transit peptide and ferredoxin transit peptide were used to transport β-carotene ketolase to the chloroplast. The analysis revealed 4-keto-lutein/ketozeaxanthin expression.

Lauersen et al. [99] carried out a nuclear transformation of *C. reinhardtii* UVM4. They used the Carbonic anhydrase 1 (cCA) secretion signal of at the N-terminal of the

recombinant protein gaussia luciferase and lolium perenne ice binding protein (LpIBP). 10 mg/l of recombinant gaussia luciferase and 12 mg/l of lolium perenne ice binding protein was secreted into culture medium, respectively. The cCA can be used for secretion of recombinant proteins from *C. reinhardtii*. Rasala et al. [100] carried out nuclear transformation of *C. reinhardtii* cc3395 (cell wall deficient strain). They used Ars1 secretion signal at the N-terminal of recombinant protein xylanase. The secretion signal Ars1 was effectively expressed. The secreted xylanase activity was higher in culture media compared to intracellular activity. Rubisco transit peptide was expressed in marine microalgae *D. salina* for transport of β-carotene hydroxylase to chloroplast [4].

Table 2.9 - Export signal in microalgae

Strain	Type of signal	Place of transport	Place of transformation	Recombinant protein expressed	Remark	Reference
C. reinhardtii	TorA signal peptide	Thylakoid lumen	Chloroplast	Red fluorescent protein and scFv antibody fragment	Useful tool for expressing toxic and low pH stable proteins in microalgae	[72]
C. reinhardtii 704	Rubisco transit peptide	Chloroplast	Nuclear	β-carotene ketolase	4-keto-lutein or ketozeaxanthin was expressed	[100]
C. reinhardtii 704	Ferredoxin transit peptide	Chloroplast	Nuclear	β-carotene ketolase	4-keto-lutein or ketozeaxanthin was expressed	[100]
C. reinhardtii UVM4	Secretion signal of Carbonic anhydrase 1 (cCA)	Culture medium	Nuclear	Gaussia luciferase	10 mg/l of recombinant protein is produced, and cCA can secrete 84% more recombinant protein than luciferase secretion signal	[101]
C. reinhardtii cc3395	Ars1 secretion	Culture medium	Nuclear	Xylanase	The secreted xylanase activity	[102]

	signal sequence				was higher in culture media compared to intracellular activity	
C. reinhardtii UVM4	Secretion signal of Carbonic anhydrase 1 (cCA)	Culture medium	Nuclear	Lolium perenne ice binding protein (LpIBP)	12 mg/l of recombinant protein is produced by photomixotrophically in a 10-liter wave-bag bioreactor	[103]
D. salina	Rubisco transit peptide	Chloroplast	Nuclear	β-carotene hydroxylase	3-fold increase in violaxanthin during growth phase and 2-fold increase in zeaxanthin	[4]

2.2   Conclusions

Microalgae are a diverse group of photosynthetic microbes which grow in a wide array of environmental conditions. Most of the microalgae have thick cell wall and their characteristics vary widely from species to species. Microalgae have wide biotechnological application in recombinant protein production, biofuel, oils, fatty acids, polysaccharides and bioactive compounds. This can further be enhanced by understanding the characteristics, genomics, proteomics and developing new genetic engineering tools. This review gives an outline of the new developments over the past decade in genome sequencing of new microalgae, methods of gene transformation, selection markers for marine and fresh water microalgae, marker free selection system for mutant strains, fluorescent proteins, promoters and terminators, inducible systems, affinity tags, and export signals.

With the advent of microarrays, the gene sequencing cost has come down drastically. There are several microalgae that have been identified but not yet sequenced. Genome sequencing can lead to unlocking unknown bioactive compounds and pathways which have industrial and biotechnological application. Since the transformation of the first microalgae *C. reinhardtii* in 1989 by Kindle et al. [102]. Gene transformation of microalgae has come a long way and vastly improved. To date, the most effective transformation in microalgae has been electroporation. However, the voltage needed for transformation varies from microalgae to microalgae due to the cell wall thickness and the few other characteristics differences between strains. Recently, Bae et al. [35] developed a new high throughput transformation system which is ~ 10-fold better than electroporation in *C. reinhardtii*. Furthermore, a non-invasive method developed by Kim

et al. [31] in *C. reinhardtii* can be extended to other microalgae since the method does not require any expensive instruments, and is simple, efficient, safe and reproducible.

Selection markers plays an important role in isolation of transformed clones from non-transformed clones. To date, microalgae have been selected using appropriate resistance genes for antibiotics, herbicides, photoautotrophy, and nutrient deficiency. Herbicide resistance markers are found to be effective for marine microalgae and antibiotic markers are found to be effective for fresh water microalgae. Several new selection markers like TETX gene resistance to tetracycline, RPL44 (P56Q) gene resistance to cycloheximide, *pds*-L540R gene resistance to norflurazon, and protox rs-3 gene resistance to oxadiazon/oxyfluorfen have been developed and the concentration of antibiotics and herbicide needed is also very low. In nutrient-based selection marker systems like the *ptxD* gene system where phosphite is oxidized to phosphate, can be used to develop transgenic microalgae for outdoor cultivation. When arginine minus mutants is transformed with the *ARG9* gene, the mutants which have taken up the gene can be isolated. These selection markers could be expressed in different microalgae.

Reporter genes are used to confirm transformed clones, estimate the transformation efficiency, check the strength of promoters, and check the expression of protein. Some of the new reporters that have been reported recently are vivid Verde fluorescent protein, superfolder green fluorescent protein and purple chromophore protein.

The gene expression level depends on the strength of promoters. In microalgae, several microalgae and plant based virus promoters are shown to be active like the *33kDa* promoter, the CaMV 35S promoter, and the NOS promoter. Also, two bidirectional

promoters, p1′2′, and VCP2 have expressed active protein. This could be used to express two different proteins in microalgae at the same time. To date the best promoter in microalgae is HSP70A/RBCS2 and further research on promoters needs to be carried out.

Inducible systems in microalgae have not been well developed to date when compared to plant, microbial and mammalian cell lines. The inducible systems would be helpful in expression of toxic proteins, or to restrict the expression during mid log phase. Some of the inducible systems that have been developed in microalgae are : the OlexA-TATA promoter which is induced by 17-β-estradiol and is considered a non-leaky system; alkaline phosphatase promoter induced by phosphorous starvation; and the *AcRbcS* promoter which is induced by light. However, these inducible systems may have some physiological effect on microalgae. Further work on exploring other inducible system is necessary.

Affinity tags are used in biotechnology for simple separation of recombinant protein from cell lysate, and can be used for Western blotting, immunoprecipitation and immunofluorescence assays. Some of the affinity tags that have expressed till date in microalgae are the HA-tag, Flag tag, SAA tag, and His tag.

Export signals are used for transporting the proteins within the cell to different organelles, for secretion of proteins, for expression on the cell surface, or to localize the protein in the membrane. In microalgae, expression of some complex proteins that need proper folding and glycosylation are carried out in the chloroplast. Some the chloroplast transporting signals include: the TorA signal peptide, rubisco, and the ferredoxin transit

peptide. Secretion signals that are expressed in microalgae include carbonic anhydrase 1, and Ars1 secretion signal.

Over the past decade there have been several advances in genetic engineering of microalgae. Several tools needed have been improved from gene transformation to protein expression. To realize the full potential of microalgae more genetic engineering work should be focused on recombinant protein expression level, development of more synthetic promoters that drive high expression, and development of inducible systems. Continuous innovation and the huge market potential of microalgae will aid further advancement in transgenic microalgae.

2.3　References

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Chapter 3

3 General materials and methods

Materials and methods

This chapter describes all the common materials and methodology used in this thesis. Commonly used analytical techniques are also presented. Subsequent chapters hereafter describe relevant materials and methods pertinent for that chapter only.

3.1 Strains, growth, and transformation

3.1.1 *Escherichia coli*

Escherichia coli (*E. coli*) strain NEB 5-alpha competent strain, purchased from New England Biolabs (Whitby, ON, Canada) was used for cloning work. The cells were stored in – 80 ^{0}C until use. Cells were cultured in Luria-Bertani (LB) medium at 37 ^{0}C and 200 RPM. Appropriate antibiotics were added for the vector transformed.

The cells were transformed by heat shock method. For heat shock transformation, 20 µl of NEB 5-alpha competent cells were kept on ice for 10 minutes. After thawing the cells, 3 µl of 50 ng of plasmid DNA was mixed with NEB 5-alpha competent cells. The mixture was flicked 4-5 times to mix cells with DNA. The mixture was then placed on ice for 30 minutes. The heat shock was carried out by placing the mixture in 42 ^{0}C water bath for 30 seconds. Then the mixture was immediately placed on ice for 5 minutes. 980 µl of SOC media was added to the mixture and incubated at 37 ^{0}C for 60 minutes at 200 rpm. The transformed cells were diluted 10 times and 100 µl was spread on a selection

plate. The transformed cells were kept at 37 ^{0}C. The cells were visible after overnight incubation.

3.1.2   *Agrobacterium tumefaciens* LBA 4404

Agrobacterium tumefaciens LBA 4404 was kindly provided by Dr. Abdelali Hannoufa (Agriculture and Agri-Food Canada, London, ON CANADA). Cells were cultured in Yeast Extract Beef (YEB) medium containing streptomycin 100 µg/ml, and rifampicin 20 µg/ml at 25 ^{0}C and 200 RPM or on agar plate. Appropriate antibiotics were added for the vector transformed.

Preparation of Electrocompetent Cells

Single colony of *Agrobacterium tumefaciens* LBA 4404 was inoculated in 15 ml of YEB media containing streptomycin 100 µg/ml, and rifampicin 20 µg/ml at 30 ^{0}C and 200 RPM. The 15 ml of overnight culture was inoculated into 1.5 liter of YEB media containing streptomycin 100 µg/ml, and rifampicin 20 µg/ml at 30 ^{0}C and 200 RPM. The cells were grown overnight to a density of 5–10 x 10^7 cells/ml. The cells were then centrifuged at 3000 x g for 10 min at 4 °C. The supernatant was discarded and cell pellet was kept on ice. The pellet was washed with 10 % of ice cold glycerol and centrifuge the cells were centrifuged at 3000 x g for 10 min at 4 °C. The supernatant was discarded and keep the cell pellet was kept on ice. The cells were washed twice with 10 % of ice cold glycerol. The cells were suspended in ice cold 1 M sorbitol and 20 µl aliquots of cell suspension were added to 1.5 ml eppendorf tubes and stored at -70 °C.

Electroporation of *Agrobacterium tumefaciens* LBA 4404

50 μg of 5 μl of plasmid was pipetted into a 1.5 ml eppendorf tube and kept on ice. 20μl of electro competent *A. tumefaciens* LBA 4404 cells were kept on ice for 10 minutes to thaw. Sterile 0.1 cm electroporation cuvette was kept on ice. 20 μl of electro competent *A. tumefaciens* LBA 4404 cells and 5 μl of plasmid were pipetted into an electroporation cuvette and tapped gently and kept on ice. The micropulser was set to "Agr". The plasmid cell mixture cuvette was placed in the chamber slide and the slide was pushed into chamber until the cuvette was seated in the base of the chamber. The cuvette was pulsed once. Immediately 1 ml of YM broth was put into the cuvette and suspension was transferred into 17 x 100 mm tube. The cells were incubated for 3 hrs at 30 °C and 250 rpm. The cells were diluted 10-fold and suspended on YEB agar with appropriate antibiotics in the dark.

3.1.3   *Chlorella vulgaris* (UTEX 2714)

Chlorella vulgaris (UTEX 2714) was purchased from UTEX Culture Collection of Algae (Austin, TX, USA). The cells were cultured in Tris-Acetate-Phosphate (TAP) medium at 25 ^{0}C, 150 RPM, 90 % humid atmosphere, and under 140 μmol m^{-2} s^{-1} white light illumination of 16 h on/8 h off.

Co-transformation

A. tumefaciens LBA 4404 was grown on YEB agar plate with appropriate antibiotics. A single colony *Agrobacterium* from the plate was inoculated in 10 ml of YEB medium containing 20 μg/ml rifampicin, 50 μg/ml streptomycin, and 50 μg/ml kanamycin at 28 ^{0}C, 200 RPM overnight. 1 OD$_{600}$ unit of *A. tumefaciens* LBA 4404 was added into

induction medium (100 mM acetosyringone and 1 mM glycine betaine in TAP liquid medium and pH was adjusted to 5.2) and incubated at 25 ^{0}C and 100 RPM for 4 hours.

A single colony of *C. vulgaris* was grown in liquid TAP medium until log phase. 1 x 10^6 cells/ml were taken and pelleted at 5,000 rpm for 5 minutes. The pellet was re-suspended in TAP induction medium containing *Agrobacterium* culture and incubated at 25 ^{0}C for 30 minutes with mild agitation for infection. Post-infection, the cells were pelleted at 5,000 rpm for 5 minutes. The pellet was re-suspended in 250 µl of TAP medium and spread plated onto TAP agar plate. The plate was kept in the dark for 2 days at 25 ^{0}C for co-cultivation. After co-cultivation, the cells were washed 3 times in 500 µg/ml cefotaxomine. The cells were centrifuged at 200 rpm to separate the *Agrobacterium* from *C. vulgaris.* The *Agrobacterium* would be suspended while *C. vulgaris* would pellet (when centrifuged at 200 rpm). The suspended *Agrobacterium* was discarded and the *Chlorella vulgaris* pellet was spread onto TAP agar plate containing 500 µg/ml cefotaxomine and 30 µg/ml G418. The transformed cells were visible after 4 weeks of incubation at 25 ^{0}C, 150 RPM, 90 % humid atmosphere, and under 140 µmol m^{-2} s^{-1} white light illumination of 16 h on/8 h off.

3.2   Polymerase chain reaction

The primers were designed by using Snap Gene software (GSL Biotech LLC, Chicago, IL, USA). The primers were ordered from UWO oligo factory (UWO, London, Ontario, CANADA). For carrying out PCR, Q5 high fidelity polymerase was purchased from New England Biolabs (Whitby, ON, Canada). The annealing temperature (Tm) of the primers was calculated by NEB calculator (http://tmcalculator.neb.com/#!/). The reaction mixture

was prepared on ice and in PCR tubes. The reaction was set as shown in **Table 3.1** and **Table 3.2**.

Table 3.1 - PCR composition

Components	50 µl reaction
Q5 High-Fidelity 2X Master Mix	25 µl
10 µM Forward Primer	2.5 µl
10 µM Reverse Primer	2.5 µl
Template DNA	2.0 µl (0.5 ng/µl)
Nuclease-Free Water	18 µl

Table 3.2 – Thermocycler cycler setting for PCR

Step	Temperature	Time
Initial Denaturation	98 ^{0}C	30 seconds
	98 ^{0}C	10 seconds
30 cycles	50 - 66 ^{0}C	30 seconds
	72 ^{0}C	30 seconds/kb
Final extension	72 ^{0}C	2 minutes
Hold	4 ^{0}C	

3.2.1   Colony PCR

Colony PCR was carried out to isolate the proper construct. The *E. coli* colonies were grown on agar plate containing appropriate antibiotics. 20 to 30 colonies were streaked on agar plate (and numbered) containing appropriate antibiotics. The agar plate was kept

overnight at 37 ^{0}C in an incubator. Small amounts of colony were put in 50 µl of water in a PCR tube and suspended. The PCR tubes were kept in the PCR machine at 95 ^{0}C for 5 minutes to break the cells. 2 µl of the lysed cell was used as template for colony PCR. The reaction mixture was prepared on ice in PCR tubes. The reaction was set as shown in **Table 3.3** and **Table 3.4**.

Table 3.3 – Colony PCR composition

Components	50 µl reaction
OneTaq Master Mix	25 µl
10 µM Forward Primer	2.5 µl
10 µM Reverse Primer	2.5 µl
Template DNA	2.0 µl
Nuclease-Free Water	18 µl

Table 3.4 – Colony PCR thermocycler setting

Step	Temperature	Time
Initial Denaturation	95 ^{0}C	120 seconds
30 cycles	95 ^{0}C	20 seconds
	45 - 65 ^{0}C	30 seconds
	68 ^{0}C	60 seconds/kb
Final extension	68 ^{0}C	10 minutes
Hold	4 ^{0}C	

- 4-6 µl of each PCR reaction was mixed with loading dye and directly loaded onto an agarose gel, alongside an appropriate DNA ladder.

3.3   Agarose gel preparation and gel electrophoresis

1 gm of agarose was taken and added to 90 ml of water. The agarose was dissolved by heating the mixture on a hot plate with constant stirring. The mixture was cooled down to 60 ^{0}C and 10X TAE buffer was added (containing BlueView). The molten agarose was poured into the gel casting apparatus. 50 ml of 10X TAE buffer (containing BlueView) was added to 450 ml of DI water. 10 µl of DNA was mixed with 2 µl of loading dye and loaded to the gel well. The gel was run at 50 V for 2 hours.

3.4   Glycerol stock

100 µl of glycerol was added to in 1.5 ml eppendorf tubes. The eppendorf tubes were autoclaved at 121 ^{0}C for 20 minutes. The autoclaved glycerol was left to cool overnight. Single colony of *E. coli* was grown overnight in LB media with appropriate antibiotics at 37 ^{0}C and 200 RPM. 900 µl of log phase microbe was pipetted into 100 µl of autoclaved glycerol in 1.5 ml eppendorf tube. The mixture was vortexed and kept in - 80 ^{0}C for long term storage.

3.5   Composition of Lysogeny broth

The lysogeny broth (LB), yeast extract broth (YEB), Tris-Acetate-Phosphate (TAP) and super optimal broth with catabolite repression (SOC) media composition is as given in **Table 3.5, Table 3.6, Table 3.7, and Table 3.8**. The mixture was autoclaved at 121 ^{0}C for 20 minutes and used after overnight cooling.

Table 3.5 - Composition of Lysogeny broth

Components	Weight (g/liter)
Tryptone	10
NaCl	10
Yeast extract	5
Water	Make up the volume to 1000 ml

Table 3.6 - Composition of YEB medium

Components	Weight (gm/liter)
Beef extract	5.00
Yeast extract	1.00
Peptone	5.00
Sucrose	5.00
$MgCl_2$	0.50
Water	Make up the volume to 1000 ml

Table 3.7 - Composition of TAP medium

Components	Concentration in final medium (1000 ml)
Tris base	2×10^{-2} M
TAP salts	
NH_4Cl	7×10^{-3} M
$MgSO_4 . 7H_2O$	8.3×10^{-4} M
$CaCl_2 . 2H_2O$	4.5×10^{-4} M

Phosphate solution	
K_2HPO_4	1.65×10^{-3} M
KH_2PO_4	1.05×10^{-3} M
Trace elements solution	
$Na_2EDTA . 2H_2O$	1.34×10^{-4} M
$ZnSO_4 . 7H_2O$	1.36×10^{-4} M
H_3BO_3	1.84×10^{-4} M
$MnCl_2 . 4H_2O$	4.00×10^{-5} M
$FeSO_4 . 7H_2O$	3.29×10^{-5} M
$CoCl_2 . 6H_2O$	1.23×10^{-5} M
$CuSO_4 . 5H_2O$	1.00×10^{-5} M
$(NH_4)_6MoO_3$	4.44×10^{-6} M
Glacial acetic acid (CH3COOH)	1 ml

Table 3.8 - SOC media

Components	Weight (gm/liter)
Tryptone	20
Yeast extract	5
5 M NaCl	2 ml
1 M KCl	2.5 ml
1 M $MgCl_2$	10 ml
1 M $MgSO_4$	10 ml
1 M Glucose	20 ml

3.6   Agar plate preparation

15 grams of agar was added to 1 liter of medium. The mixture was autoclaved at 121 ^{0}C and 20 minutes. The medium was cooled to 50 – 60 ^{0}C and appropriate antibiotics were added. 10 ml of agar medium was added to the plate. The plates were kept for cooling for 2 hours. The cooled plates were flipped and kept in 4 ^{0}C until used.

3.7   Plasmid preparation and storage

Single colony of *E. coli* containing appropriate plasmid was grown overnight in LB media with appropriate antibiotics at 37 ^{0}C and 200 RPM. The plasmids were isolated according to manufacturer's protocol (plasmid extraction kit). The concentration of plasmid was measured by Nano quant in microplate reader (Tecan M 200). The plasmids were stored in 4 ^{0}C for short term storage and – 20 ^{0}C for long term storage.

Chapter 4

4 Expression of exo-inulinase gene from *Aspergillus niger* 12 in *E. coli* strain Rosetta-gami B (DE3) and its characterization

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Abstract

Inulin is a linear carbohydrate polymer of fructose subunits (2-60) with terminal glucose units, produced as carbon storage in selected plants. It cannot directly be taken up by most microorganisms due to its large size, unless prior hydrolysis through inulinase enzymes occurs. The hydrolyzed inulin can be taken up by microbes and/or recovered and used industrially for the production of high fructose syrup, inulo-oligosaccharides, biofuel and nutraceuticals. Cell-free enzymatic hydrolysis would be desirable for industrial applications, hence the recombinant expression, purification and characterization of an *Aspergillus niger* derived exo-inulinase was investigated in this study. The eukaryotic exo-inulinase of *Aspergillus niger* 12 has been expressed, for the first time, in an *E. coli* strain (Rosetta-gami B (DE3)). The molecular weight of recombinant exo-inulinase was estimated to be approximately 81 kDa. The values of K_m

and V_{max} of the recombinant exo-inulinase toward inulin were 5.3 ± 1.1 mM and 402.1 ± 53.1 µmol min^{-1} mg^{-1} protein, respectively. Towards sucrose, the corresponding values were 12.20 ± 1.6 mM and 902.8 ± 40.2 µmol min^{-1} mg^{-1} protein, respectively. The S/I ratio was 2.24 ± 0.7, which is in the range of native inulinase. The optimum temperature and pH of the recombinant exo-inulinase towards inulin was 55 ^{0}C and 5.0, while they were 50 ^{0}C and 5.5 towards sucrose. The recombinant exo-inulinase activity towards inulin was enhanced by Cu^{2+} and reduced by Fe^{2+}, while its activity towards sucrose was enhanced by Co^{2+} and reduced by Zn^{2+}.

Keywords – Exo-inulinase, Aspergillus niger 12, E. coli, Inulin and Enzyme Activity

4.1   Introduction

Inulin is a linear carbohydrate polymer composed of fructose units joined together by β-(2, 1)-D-fructosyl-fructose bonds and a terminal glucose unit joined by α-(2,1)-D-glucose bond. The inulin solubility in aqueous solution depends on the degree of polymerization [1]. Inulin is produced by plants as an energy storage in their roots. Some of the plants with high inulin content are Jerusalem artichoke, chicory, stevia and onions. Recently, inulin has found application in pharmaceuticals and food additives [1]. Hydrolyzed inulin has been used for the production of bio-fuels, ultra-high fructose syrup and oligosaccharides [2]. The hydrolysis of inulin can be carried out thermally or via acid, base and enzymatic catalysts to obtain oligosaccharides, fructose and glucose molecules [1].

Based on the respective mode of action, inulinase enzymes are mainly classified into endo-inulinase and exo-inulinase. Endo-inulinase (2,1-β-D-fructan fructohydrolase; EC

3.2.1.7) hydrolyzes inulin internal linkages to give inulo-oligosaccharides and sucrose molecules while exo-inulinase (β-D-fructan fructohydrolase; EC 3.2.1.80) hydrolyzes inulin from the fructose terminal end to give fructose and glucose molecules [3].

Common sources of inulinases are yeasts, bacteria and molds [4]. Yeasts and molds are known to produce both exo-inulinase and endo-inulinase. However, after hydrolysing inulin these microbes utilize fructose and fructose oligosaccharides for growth, which is an undesirable characteristic from an industrial perspective. Some of the applications targeting inulin as a feedstock aim to produce ethanol [5],[6], and [7], single cell oil [8] and [9], citric acid [10], lactic acid [11], 2,3-butanediol [12] and [13] and butanol [14]. Purification of naturally occurring enzymes would take multiple steps and the final yield is low. Hence, several studies on over expression of inulinase has been carried out in *Yarrowia lipolytica* [15]*, Saccharomyces cerevisiae* [16]*, Pichia pastoris* [17]*, Klyuveromyces lactis* [18] and *Escherichia coli* [19].

To the best of our knowledge, no study on the expression of an exo-inulinase (EX-INU) gene from *A. niger* 12 in *E. coli* has been reported until now. In this study, exo-inulinase enzyme from *Aspergillus niger* 12 was expressed in *E. coli* strain Rosetta-gami B (DE3). The PCR product of exo-inulinase cDNA gene was expressed in *E. coli* and purified by Ni^{2+}-NTA column chromatography. The activity of purified exo-inulinase was measured using inulin and sucrose as substrates.

4.2 Materials and methods

4.2.1 Strains, plasmids and reagents

Aspergillus niger 12 (ATCC 10579) purchased from Cedarlane (Burlington, ON, Canada) was cultured in our laboratory. *E. coli* strain DH5α purchased from New England Biolabs (Whitby, ON, Canada) was used for cloning and Rosetta-gami B (DE3) purchased from Novagen (Etobicoke, ON, Canada) was used for protein expression. The expression vector pET-32a(+) was purchased from Novagen (Etobicoke, ON, Canada). Q5 high fidelity polymerase was purchased from New England Biolabs (Whitby, ON, Canada). Fast cloning pack, Fast digest restriction enzymes and PCR clean up kit was purchased from Fermentas (Ottawa, ON, Canada). Reverse transcriptase kit (catalog # 205110), Plasmid purification kit (catalog # 27104) and RNA extraction kit (catalog # 74903) were purchased from Qiagen (Toronto, ON, Canada). Inulin from Dahlia tubers and all other materials were purchased from Sigma-Aldrich (Oakville, ON, Canada) and were of analytical grade.

4.2.2 RNA extraction, cloning and gene sequencing

Aspergillus niger 12 was grown for 3 days based on a protocol by Moriyama *et al*[20]. RNA was isolated from RNeasy plant mini kit (Qiagen, Toronto, ON Canada) as suggested by the manufacturer. The reverse transcription polymerase chain reaction (RT-PCR) was carried out using the primer 1 (**Table 4.1**) and manufacturer protocol was followed for cDNA synthesis. The pET-32a(+) plasmid was double digested with NotI and BamHI and purified by running on 0.7 % deoxyribonucleic acid (DNA) agarose gel electrophoresis. The entire exo-inulinase DNA was amplified using cDNA as template

and primer 2 and 3 (**Table 4.1**) for amplification of the DNA. A BamHI was introduced

at the 5^1 (**Table 4.1**, primer 2) and a NotI site (**Table 4.1**, primer 3) was introduced at the

3^1 by PCR. The PCR product was double digested with BamHI and NotI. A GeneJET

PCR purification kit (Ottawa, ON, Canada) was used to purify the double digested PCR

product. Ligation of linearized pET-32a(+) and PCR product was carried out with

Fermentas fast cloning pack (Ottawa, ON, Canada) according to manufacturer's

instructions. 2.6 fmol of double digested pET-32a(+), 7.8 fmol of double digested PCR

product, 4 µl of 5X rapid ligation buffer and 1 µl of T4 DNA ligase (5 u/µl) was mixed

together and kept at 22 ^{0}C for 5 minutes for ligation. Purity and length of DNA constructs

were characterized with Lonza FlashGelTM DNA System (Rockland, ME, USA)

according to the manufacturer's instructions. 20 ng of double digested PCR product,

double digested pET-32a(+), double digested pET-32a+EX-INU and quick load 1 kb

DNA ladder from New England Biolabs (Whitby, ON, Canada) were loaded in flash gel

and run at 50 V for 45 minutes. The pET-32a+EX-INU (**Figure 4.1**) plasmid constructed

was sequenced at Robarts Research Institute (London, ON, Canada). The primers 4, 5

and 6 were used for gene sequencing (**Table 4.1**).

Table 4.1 - List of primers used for reverse transcription, construction of exo-inulinase gene in pET-32a+ and gene sequencing of pET-32a+EX-INU.

Primer description	Primer	Primers sequence
RT-PCR PRIMER (entire length of Exo-inulinase)	1	5^1-TTAATTCCACGTCGAAGTAAT-3^1
Exo-inulinase	2	FP - 5^1-ATA**GGATCC**GCTCGTCTTTTGAAGGC-3^1
primers	3	RP-5^1-TA**GCGGCCGC**TTAATTCCACGTGAAGTAAT-3^1

	4	FP - 5[1]- TTTGTTAGCCGGATCTCAGTGGTG-3[1]
Gene sequencing primers	5	FP - 5[1]-GAATGTGGTTCCGTCGAACTCGCCCAC-3[1]
	6	RP - 5[1]-GACGACAAGGCCATGGCTGATATCGGA-3[1]
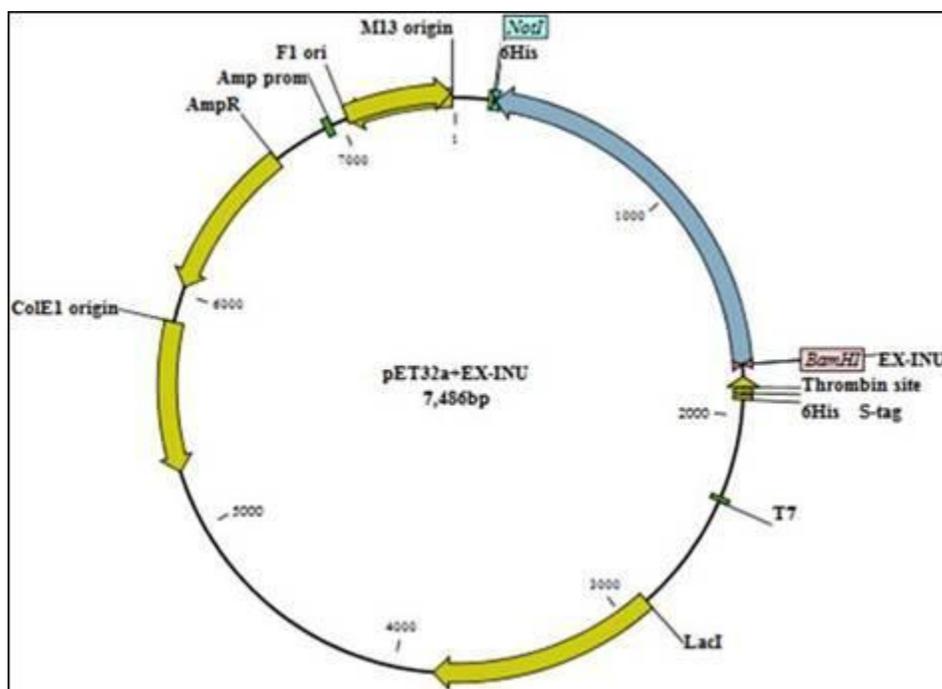
Figure 4.1 - pET-32a+EX-INU expression plasmid construct

4.2.3   Cultivation and cell lysis

The pET-32a+EX-INU plasmid containing the inserts were transformed into Rosetta-gami B(DE3) *E. coli* strain via the heat shock method. The transformed cells were grown on agar plates. Single colonies were isolated and grown overnight on LB+A_p+K_n+T_c+C_m (Lysogeny broth[21], A_p- 50µg/mL, K_n-15 µg/mL, T_c -12.5 µg/mL and C_m-34 µg/mL) at 37 ^{0}C. An appropriate volume of inoculum was added to 95 mL fresh media to achieve an initial OD_{600} value of 0.1. The fresh media contained LB+A_p+K_n+T_c+C_m+1% glucose solution. The cells were grown at 30 ^{0}C and 150 revolutions per minute (RPM) until the

cell culture reached between 0.5 - 0.6 OD_{600} units. The cultures were kept on ice for 5 minutes and 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce protein expression. The culture was grown at 16 ^{0}C and 150 RPM until early stationary phase. The cells were harvested and centrifuged at 3,500 RPM for 20 minutes at 4 ^{0}C and washed thrice with phosphate buffered saline (PBS) buffer pH 7. The cells were suspended in Ni-NTA binding buffer (300 mM NaCl, 50 mM sodium phosphate buffer, 10 mM imidazole and pH 8) (1 gm wet weight of cells in 10 mL of buffer) which contained 30 KU of lysozyme and 10 µl of protease inhibitor. The suspension was kept in a shaking incubator at 30 ^{0}C and 50 RPM for 30 minutes and later placed in ice for 10 minutes. Cell lysis was carried out in Misonic XL-2000 series and 1/8"tip at 9 W power for 15 cycles (1 cycle – 30 seconds on and 60 seconds off). The lysed cells were centrifuged at 10,000 x g for 20 minutes at 4 ^{0}C. The clear supernatant and insoluble lysate were separated. The clear supernatant was purified to obtain recombinant exo-inulinase.

4.2.4   Recombinant exo-inulinase purification

The exo-inulinase was purified by Ni^{2+} affinity chromatography from Amicon pro purification system (catalog # ACS505012) (Etobicoke, ON, Canada). The purification was carried out according to manufacturer's recommendation with minor modifications. The crude extract in binding buffer (300 mM NaCl, 50 mM sodium phosphate buffer and pH - 8) was applied to the Ni^{2+}-NTA column. The protein binding step was carried out for 3 hours at 22 ºC in a shaker at 10 RPM. Contaminants and unbound proteins were removed by centrifugation (1000 x g, 1 minute). The column was washed with 1.5 mL wash buffer (300 mM NaCl, 50 mM sodium phosphate buffer, 20 mM imidazole and pH

- 8) to further remove contaminants and unbound proteins from the column. The exo-inulinase was eluted from the column with elution buffer (300 mM NaCl, 50 mM sodium phosphate buffer, 250 mM imidazole and pH - 8). The buffer exchange was carried out in an Amicon ultra 0.5 device with nominal molecular weight limit (NMWL) of 50 K. The buffer exchange step was carried out with 50 mM acetate buffer, pH - 5 and centrifugation was carried out at 3,000 x g for 30 minutes.

4.2.5   Protein quantification

The protein quantification was carried out by Pierce BCA protein assay kit (Rockford IL, USA). The assay was carried out according to manufactures protocol in a 96 well plate. The experiment was carried out in duplicate.

4.2.6   Western blot and SDS-PAGE of recombinant exo-inulinase

Bio-Rad laboratories precast 4-20 % mini-protean TGX gels (Mississauga ON, Canada) were used. 0.5 µg of protein was prepared in Laemmli sample buffer. The gel was run at 100 V for 90 min. The gel was stained with Invitrogen InVision His-tag In-gel stain (Carlsbad CA, USA) according to manufacturer's recommendation. The image was captured using Bio-Rad ChemiDoc XRS+ imager. Next, the same gel was washed with water and stained in Pierce GelCode Blue Stain (Rockford IL, USA) reagent according to the manufacturer's recommendations.

4.2.7   Measurement of expressed exo-inulinase activity

Exo-inulinase activity was quantified via dinitrosalicylic acid (DNS) method in 2 mL micro-centrifuge tubes. The DNS reagent was prepared according to Miller *et al.*[22]. Enzyme activity assays were carried out in 50 mM acetate buffer, pH – 5.0 and 40 ^{0}C in a

water bath. For the exo-inulinase assay, inulin concentrations from 1-50 mg/mL were prepared in 50 mM acetate buffer, pH 5.0. For invertase assay, sucrose concentrations ranging from 1-50 mg/mL were prepared in 50 mM acetate buffer, pH 5.0. Substrate and enzyme were pre-incubated in a water bath at 40 ^{0}C for 5 minutes prior to mixing, where 450 µl of substrate and 50 µl of appropriately diluted enzyme were kept for 10 minutes. The enzyme activity was stopped by adding 500 µl of DNS reagent. The mixture was kept in a boiling water bath for 10 minutes and subsequently cooled to room temperature. The samples were diluted 5 times in milli Q water. Exactly 200 µl of the sample was transferred to each well of a 96 well clear flat bottom plates and the samples absorbance was measured at 540 nm in a plate reader (Tecan, Männedorf, Switzerland). Fructose was prepared in 50 mM acetate buffer, pH 5.0 and used as standard. The initial reaction rate was plotted versus initial substrate concentration to determine whether the enzyme followed Michaelis-Menten kinetics, and V_{max} and K_m were determined by non-linear regression of the Michaelis-Menten equation $\{v=V_{max}[s]/K_m+[s]\}$. The S/I (Inulinase/Invertase activity) ratio was also determined. One unit of enzyme activity was defined as the quantity of enzyme required to liberate 1 µmol of fructose equivalent per minute at 40^{0}C, and specific activity was defined as units per mg protein. The inulin concentration was based on a molecular mass of 5000 g mol^{-1} and average chain length of 30. The experiments were carried out in duplicate.

4.2.8   pH Optimum and Stability studies

The effect of pH on exo-inulinase and invertase activity was investigated at 50 ^{0}C, pH 3.0 − 7.0 and 50 mM citrate-phosphate buffer. For invertase activity, sucrose solutions at 50 mg/ml, and for exo-inulinase activity, inulin solutions at 50 mg/ml, in appropriately

buffered pH were prepared. The relative activities at different pH values were calculated by assigning the highest activity as 100 %. For inulinase, relative activity at pH 5.0 was used as 100 %, and for invertase, relative activity at pH 5.5 was used as 100 %. The pH stability was determined by measuring the residual enzyme activities after incubating purified enzyme without substrate at various pH at 50 ^{0}C for 24 hours. Purified enzyme, not subjected to incubation, was used as a control. After 24 hours of incubation the residual activity was estimated via equation 1.

$$Residual\ activity = \left(\frac{Activity\ of\ 24\ hour\ incubated\ enzyme}{Activity\ of\ 0\ hour\ incubated\ enzyme}\right) \times 100\ \% \tag{1}$$

4.2.9   Temperature optimum and thermostability

The effect of temperature on exo-inulinase and invertase activity was evaluated at different temperatures (30 ^{0}C – 70 ^{0}C), pH 5.0, 50 mM citrate-phosphate buffer and a substrate concentration of 50 mg/ml was used. The relative activities at different temperatures were calculated by assigning the highest measured activity as 100 %. For inulinase, relative activity at 50 ^{0}C was used as 100 % and invertase relative activity at 55 ^{0}C was used as 100 %. The effect of temperature on enzyme activity was determined by incubating the purified enzyme for 24 hours at different temperatures. Purified enzyme, not subjected to incubation at different temperatures, was used as a control. After 24 hours of incubation the residual activity was calculated via equation 1.

4.2.10   Effect of metal ion on recombinant exo-inulinase and invertase activity

The effect of metal ions on enzyme activity was analyzed by pre-incubating the enzyme in a buffered solution containing 5 mM metal ions for 1 hour at 50 ^{0}C, pH 5.0, 50 mM

citrate-phosphate buffer and substrate concentration of 50 mg/ml. Purified enzyme, not containing the metal ions but otherwise incubated under the same condition as above, was used as a control.

Statistical analysis

Correlation coefficient (r) between duplicates were found for optimum pH, optimum temperature, pH stability and temperature stability study. Two tailed student's t-test was carried out for effect of metal ion on exo-inulinase and invertase study. DeWinter has shown that t-test could be carried out with n=2 when the effect size is high [23]. Goodness of fit test was carried out for enzyme kinetics study. All statistical analysis were carried out in GraphPad Prism 5.

4.3   Results

4.3.1   Expression in Rosetta-gami B(DE3)

Several eukaryote proteins are not well expressed in prokaryotes due to a combination of factors like codon bias, protein folding, phosphorylation and glycosylation [24]. However, this can be overcome by using a unique *E. coli* strain Rosetta-gami B(DE3). The Rosetta-gami B(DE3) strain harbors the pRARE plasmid which reduces the codon bias, by expressing a tRNA pools which is not naturally produced by *E. coli* [25].

The Rare Codon Calculator (RaCC) (http://nihserver.mbi.ucla.edu/RACC/) was used to check the exo-inulinase codons that are not optimally expressed in *E. coli*. The rare codons were found to be arginine (AGG (3), CGA (3)), leucine (CTA (6)), isoleucine (ATA (2)) and proline (CCC (15)). Thus in the exo-inulinase gene used for expression of recombinant exo-inulinase, 29 codons were found from a total of 538 codons (i.e. 5.4 % of total codons) which are not optimal for expression. The number of sub-optimal codons

are high in our study. Also, the Rosetta-gami B(DE3) strain has thioredoxin reductase and glutathione reductase mutation which makes the cytoplasm environment oxidizing and helps in stable disulfide bond formation in the protein molecule[26]. The exo-inulinase of *Aspergillus niger* 12 has a disulfide bond[20], hence this expression system was chosen. As a precautionary measure we used the Rosetta-gami B(DE3) strain, low temperature and low IPTG concentration for recombinant exo-inulinase induction since our primary objective was the expression of active and soluble recombinant exo-inulinase. The specific growth was 0.48 h^{-1} (based on OD_{600} measurements; data not shown).

4.3.2   Purification of over expressed recombinant exo-inulinase

The histidine tag on the N-terminal of the recombinant exo-inulinase allowed for affinity purification of the fusion protein by Ni^{2+}-NTA column chromatography in a single step. The purity and molecular weight of exo-inulinase was analyzed by SDS-PAGE and Western blot. As shown in **Figure 4.2** (Lane 1), the purified protein showed a single band and the contaminants from column flow-through in **Figure 4.2** (Lane 2). The expected protein size of 81 kDa[20] was seen on the gel **Figure 4.2** (Lane 1). Also, a single band was seen in the Western blot at 81 kDa (**Figure 4.3**, Lane 1) and no bands were visible in the flow through (**Figure 4.3**, Lane 2). The exo-inulinase was of the expected size[20].
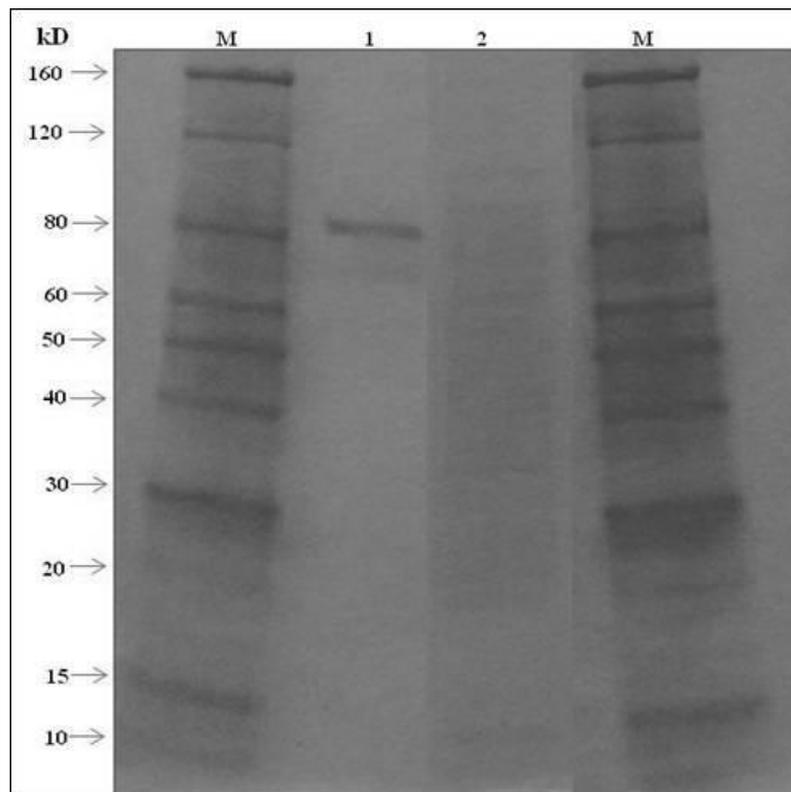
Figure 4.2 - SDS-PAGE analysis of exo-inulinase enzyme expressed in Rosetta-gami B(DE3). M- Marker, Lane 1 – 0.5 µg of purified Histidine tagged exo-inulinase protein; Lane 2 – 0.5 µg flow through fraction from Ni2+-NTA purification. The gel was run at 100 V for 90 minutes. The gel was stained in GelCode Blue Stain reagent according to manufactures recommendation.
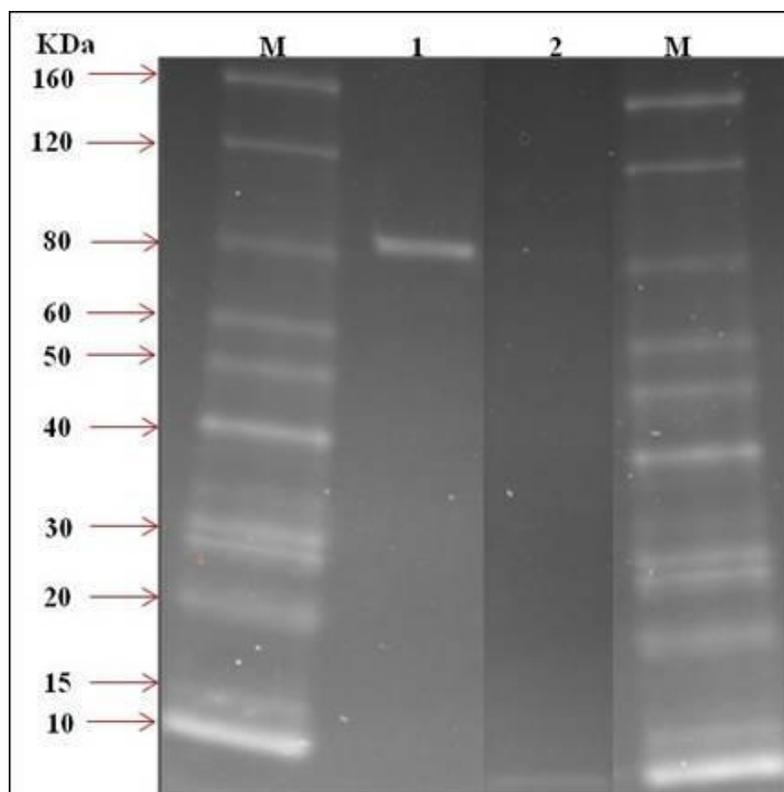
Figure 4.3 - Western blot analysis of Histidine tag exo-inulinase expressed in Rosetta-gami B(DE3). M – Marker; Lane 1 – 0.5 µg of purified Histidine tagged exo-inulinase protein; Lane 2 – 0.5 µg flow through fraction from Ni2+-NTA purification. The gel was run at 100 V for 90 minutes. The gel was stained with InVision His-tag In-gel stain according to manufactures recommendation.

Recombinant exo-inulinase assay

The initial rate of recombinant exo-inulinase for inulin and sucrose were determined at 40 ^{0}C and pH 5.0. The initial substrate and initial rate were curve fitted by non-linear regression. Goodness of fit test carried out on enzyme kinetics showed coefficients R^2 – 0.98 for inulin and R^2 – 0.96 for invertase indicating the experimental data fitted well by the Michaelis–Menten equation. Increasing concentration of inulin showed increase in formation of fructose. However, from the Michaelis–Menten graph it can be seen that

(**Figure 4.4**), the enzyme was not saturated by inulin as the graph did not plateau for inulin concentrations upto 50 mg ml^{-1}. Beyond a concentration of 50 mg ml^{-1}, inulin is less soluble making accurate determination of inulinase kinetics difficult. For invertase, enzyme activity increased with increased sucrose concentration and it followed a typical Michaelis–Menten pattern (**Figure 4.5**). The enzyme could be further saturated up to 100 mg ml^{-1} sucrose to enable more accurate enzyme kinetics.
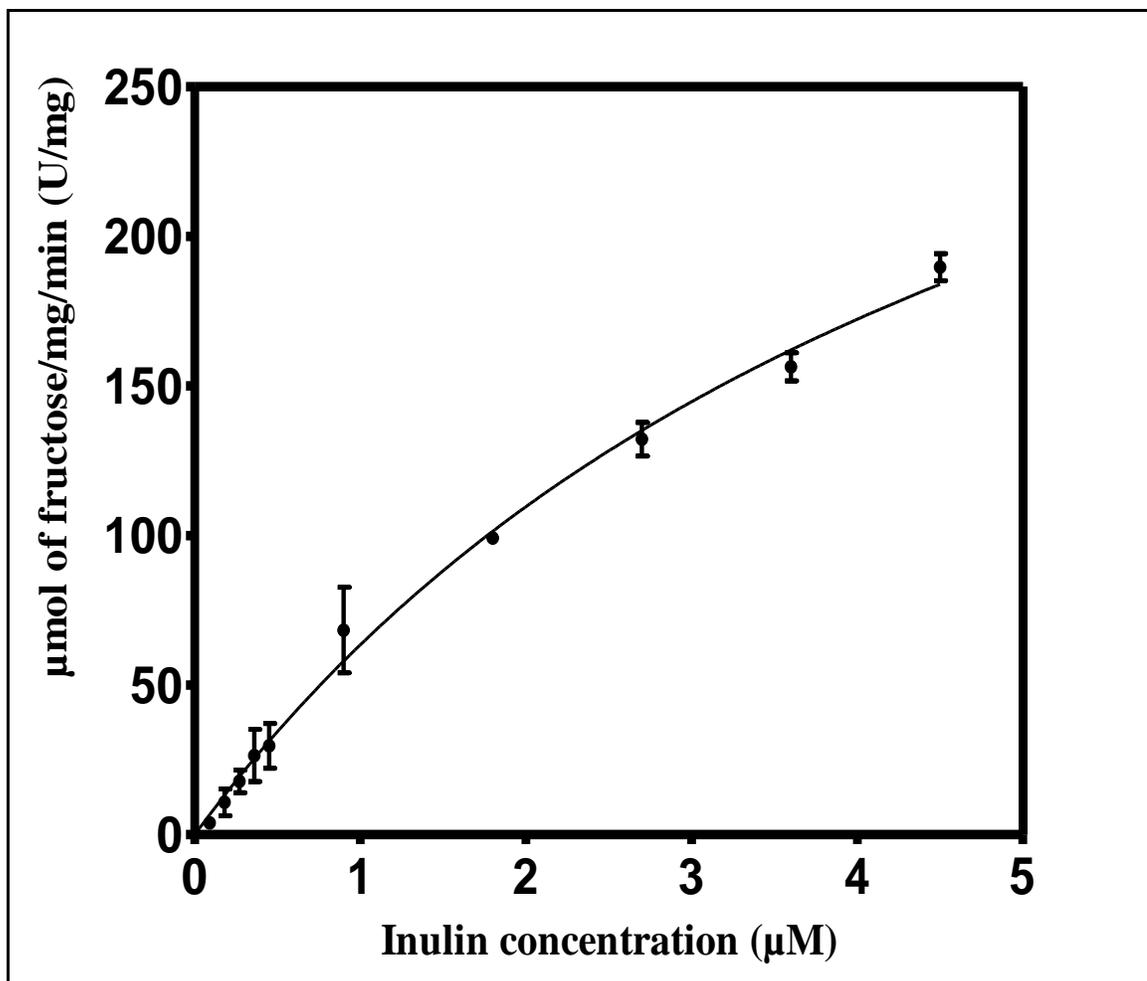
Figure 4.4 - Michaelis–Menten kinetics plot for recombinant exo-inulinase activity. The inulinase activity was carried out at 40 0C, pH 5.0 and 50 mM acetate buffer. The inulin concentration ranging from 1-50 mg/mL was used for assay. Micro moles of fructose liberated are shown by Michaelis–Menten kinetics. n=2, average ± SE and R^2 – 0.98.
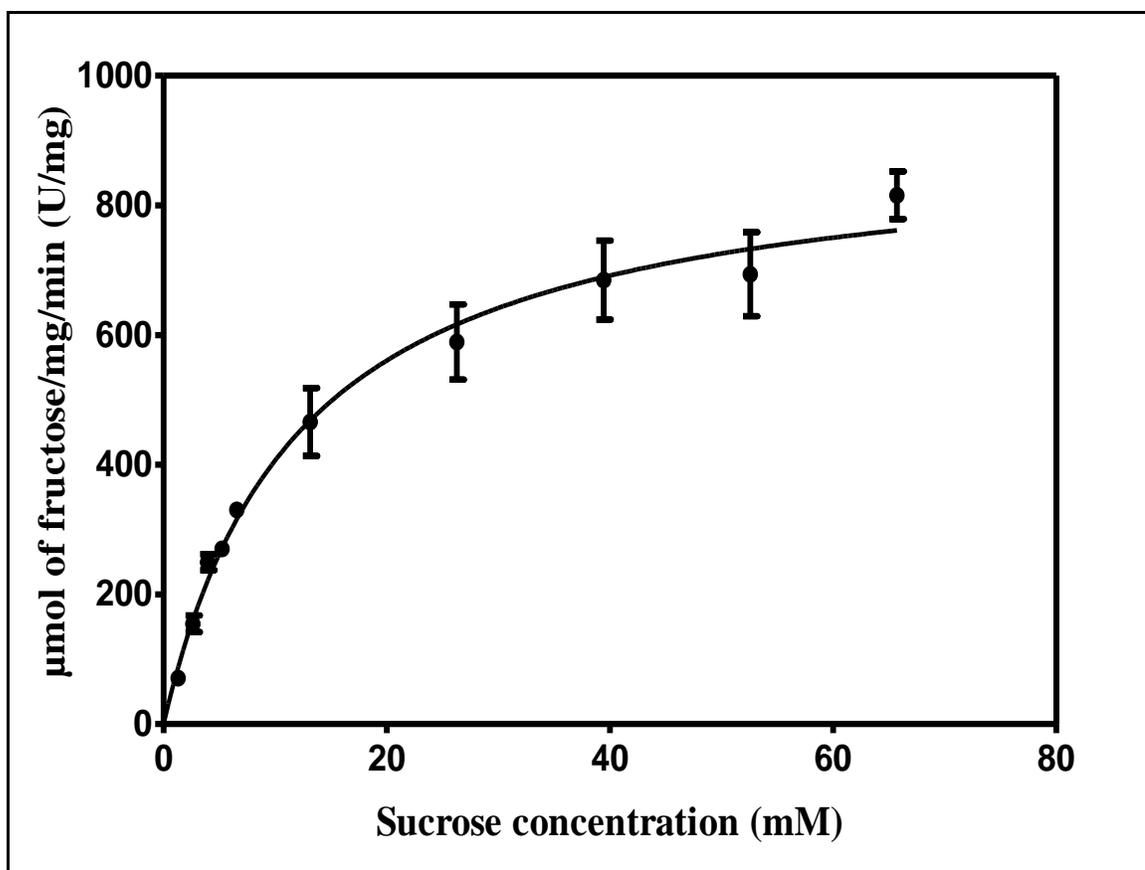
Figure 4.5 - Michaelis–Menten kinetics plot for recombinant invertase activity of recombinant exo-inulinase. The invertase activity was carried out at 40 0C, pH 5.0 and 50 mM acetate buffer. The inulin concentration ranging from 1-50 mg/mL was used for assay. Micro moles of fructose liberated are shown by Michaelis–Menten kinetics. n=2, average ± SE and R2 – 0.96.

The values of kinetic constants K_m and V_{max} for inulin were found to be 5.3 ± 1.1 mM and

402.1 ± 53.1 µmol min^{-1} mg^{-1} protein respectively (**Figure 4.4**). The values of the kinetic

constants K_m and V_{max} for sucrose were 12.20 ± 1.6 mM and 902.8 ± 40.2 µmol min^{-1} mg^{-1} protein (**Figure 4.5**). Inulinase activity (I) is generally compared with invertase activity

(S) of the same enzyme preparation. The S/I ratio is used to characterize inulinase (I)[27].

The S/I ratio for the recombinant exo-inulinase was 2.24 ± 0.7 which appears to be 1.9

fold lower than for purified non-recombinant exo-inulinase from *Aspergillus niger* 12[20]

(**Table 4.2**). A comparison with a few exo-inulinases expressed by non-recombinant and

recombinant microbes from literature is shown in **Table 4.2** and discussed below.

Table 4.2 - Comparison of exo-inulinase and invertase activity from different micro-organism and recombinant exo-inulinase and invertase activity from current study. (*) E. coli from current study; n=2 and average ± SE.

	Exo-inulinase				Invertase				S/I	Relative S/I fold change compared to current study
	V (U/mg)	K_m (mM)	K_{cat} (10^3 min^{-1})	1/ K_{cat} (10^{-3} sec)	V (U/mg)	K_m (mM)	K_{cat} (10^3 min^{-1})	1/ K_{cat} (10^{-3} sec)		
A. niger 12[20]	52.8	-	4.27	14	227	-	18.38	3.3	4.3	+ 1.91
E. coli [*]	402.1 ± 53.1	5.3 ± 1.1	32.5 ± 4.2	1.8	902.8 ± 40.2	12.20 ± 1.6	73.4 ± 3.2	0.8	2.24 ± 0.7	-
P. pastoris[20]	55.5	-	4.77	12.6	293	-	25.19	2.4	5.27	+ 2.35
E. coli [19]	2500	0.7	43.2	1.38	6000	42	102	0.58	2.4	+ 1.07
Streptomyces sp.[28]	450.0 ± 5.6	1.63 ± 0.03	20.45	2.9	260.0 ± 2.5	66.66 ± 0.87	11.81	5.07	0.57	- 3.9
E. coli [29]	1000.0 ± 0.1	7.1 ± 0.2	63	0.95	12,037.0 ± 801.9	347.6 ± 25.9	761.8	7.87	12.03	+ 5.37
E. coli [40]	21.69	1.72	0.12	494	78.7	41	0.442	135.7	3.62	+ 1.61
E. coli [41]	294.1	10.2	16.42	3.65	1666.7	6.5	93.1	0.64	5.66	+ 2.52

Kwon *et al.* [19] reported both K_m and V_{max} values for inulin and sucrose hydrolysis for recombinant exo-inulinase from *Bacillus polymyxa* expressed in *E. coli.* These values were 0.7 mM and 2500 µmol min^{-1} mg^{-1} protein for inulin and 42 mM and 6000 µmol min^{-1} mg^{-1} protein for sucrose respectively. For the above study, the S/I ratio was 2.4 and

this was 1.07 fold higher than our current study. A similar investigation conducted by Sharma and Gill *et al*. [28] found K_m and V_{max} for inulin and sucrose hydrolysis for an exo-inulinase (purified from *Streptomyces sp)* to be 1.63 mM and 450 µmol min^{-1} mg^{-1} of protein for inulin and 66.6 mM and 260 µmol min^{-1} mg^{-1} of protein for sucrose. The S/I ratio in this study was 3.9-fold lower than our current study. Other studies as in **Table 4.2** [29] also indicate that the S/I ratios found in our study are within the range of values obtained by other investigators. The recombinant exo-inulinase in our study displays typical inulinase characteristics since its S/I ratio falls in the range of 0.5-18.5, whereas true invertases display S/I ratios of several thousand [27].

4.3.3   Characterization of exo-inulinase

The optimum pH, temperature and stability were evaluated for purified recombinant exo-inulinase. The optimum pH of the enzyme with inulin and sucrose as substrates was found to be pH 5.0 and 5.5 respectively (**Figure 4.6A**). For the optimum pH study, the correlation coefficient (r) for inulin and sucrose was found to be 0.98 for both. The pH optimum for native *A. niger* 12 exo-inulinase and invertase has not been evaluated here, however Moriyama *et al*. have used pH 5.0 to find enzyme kinetics in their study[20]. Typical pH values for inulinases expressed from yeast, fungi and their recombinant systems are in the range of 4.0 – 5.5, such as a recombinant exo-inulinase (pH 4.0) and invertase (pH 5.0)[29], *Aspergillus niger* (pH 4.4)[30], *Aspergillus ficuum* JNSP5-06 exo-inulinase and invertase (pH 4.5)[31], marine yeast *Crytococcus aureus* G7a (pH 5.0) and *Kluyveromyces marianus* YS-1 (pH 5.5)[32].

Complete hydrolysis of inulin at relevant substrate and enzyme loadings requires at least 24 h[14] hence this period was chosen for incubation. For the pH stability study, the

correlation coefficient (r) for inulin and sucrose was found to be 0.72 and 0.94. In this study the residual activity of exo-inulinase was lowest at pH 4.0 where it retained only 14.5% of the activity of the control (**Figure 4.6B**). Between pH 5.5 – 7.0 the enzyme retained 26 – 30 % activity. The highest residual activity was retained at pH 6.5 and was 31.9 %. The enzyme had higher invertase residual activity than the exo-inulinase activity. The highest invertase residual activity was found at pH 5.0 where the enzyme retained 72 % activity.

Figure 4.6 - Effect of pH on recombinant exo-inulinase and invertase activity and their stability. (A) Effect of pH on recombinant exo-inulinase and invertase activity; (B) Effect of pH on recombinant exo-inulinase and invertase stability. I – Inulin; S – Sucrose. n=2, average ± SE. Correlation coefficient for effect of pH on exo-inulinase – 0.98 and invertase – 0.98. Correlation coefficient for effect of pH stability on exo-inulinase – 0.72 and invertase – 0.94.

The optimum temperature studies were carried out at pH 5.0, 50 mM citrate phosphate buffer at appropriate temperature (30 ^{0}C – 70 ^{0}C) for 24 hours. The optimum temperatures with inulin and sucrose as substrates were found to be 55 ^{0}C and 50 ^{0}C (**Figure 4.7A**), within the typical range of 50 ^{0}C – 60 ^{0}C. For optimum temperature study, the correlation coefficient (r) for inulin and sucrose was found to be 0.86 and 0.85.

The thermal stability study was also studied for 24 hours at up to 50 ^{0}C without substrate. For the thermal stability study, the correlation coefficient (r) for inulin and sucrose was found to be 0.96 and 0.98. For exo-inulinase activity, interestingly, at lower temperature (30 ^{0}C and 35 ^{0}C) the residual enzyme activity was lower (78 % and 84 %) (**Figure 4.7B**) whereas the enzyme retained more than 90 % activity between 40 ^{0}C – 55 ^{0}C and at 60 ^{0}C it retained only 75 % activity. The enzyme activity was completely lost at 65 ^{0}C and 70 ^{0}C implying, that thermal denaturation occurs above 60 ^{0}C. For invertase activity, the residual enzyme activity was more than 94 % for 30 ^{0}C, 35 ^{0}C, 40 ^{0}C, 55 ^{0}C and 60 ^{0}C. Interestingly, the residual activity was lower at 45 ^{0}C and 50 ^{0}C (83.8 % and 87.4 %) compared to 55 ^{0}C and 60 ^{0}C. The enzyme activity was completely lost at 65 ^{0}C and 70 ^{0}C. Most of the thermal stability studies found in the literature have been carried out for 1 – 3 hours [29] and [33] whereas this study carried out stability studies for 24 h since high temperatures are used in many industrial processes and this study would hence help in understanding the long term stability of enzyme. It is interesting to see that *K. marxianus* enzymes, when incubated with inulin at 50 ^{0}C, lose activity completely after 2.5 hr[34]. On the contrary, in this study the enzyme is relatively stable for 24 hr when kept at 50 ^{0}C without substrate.
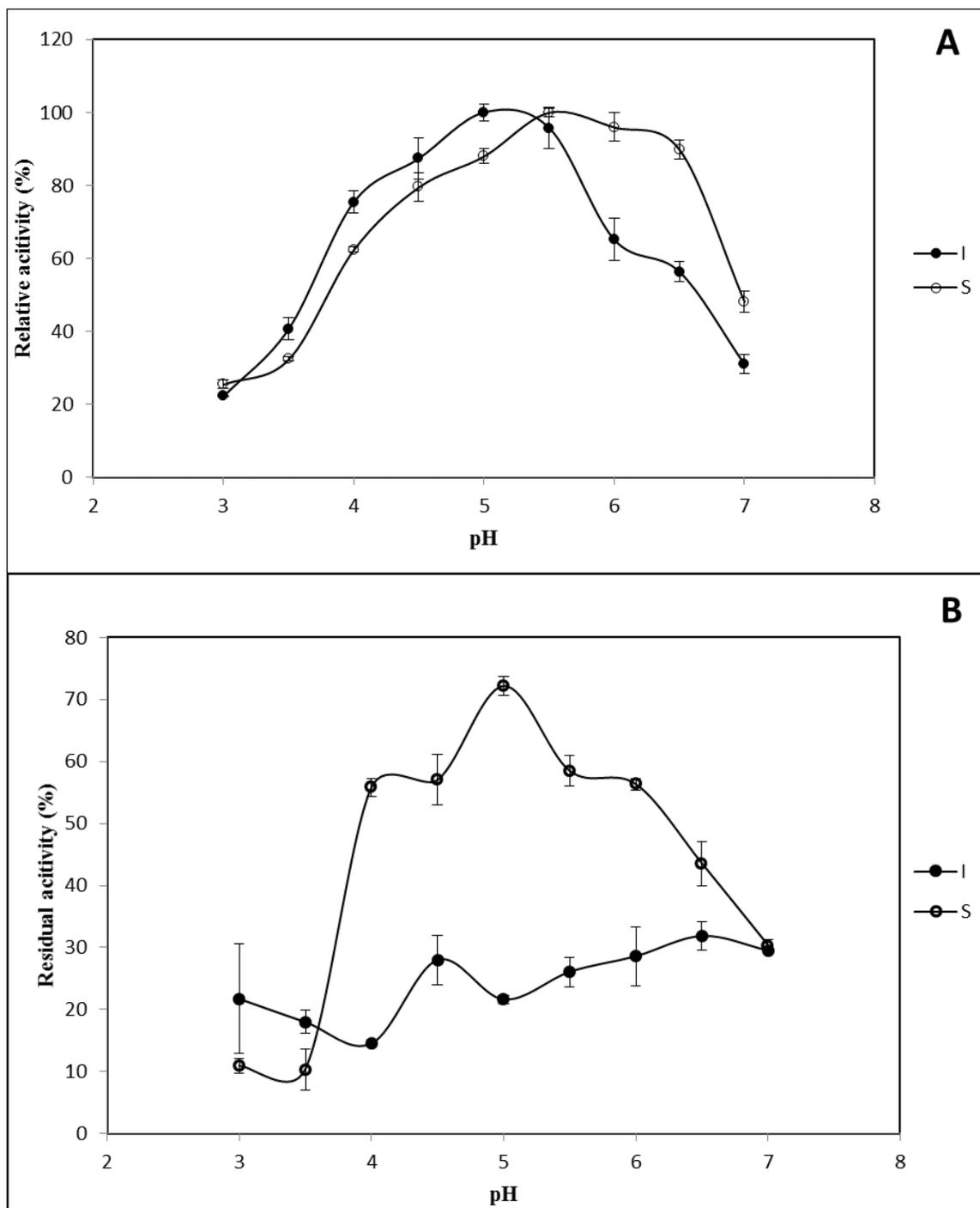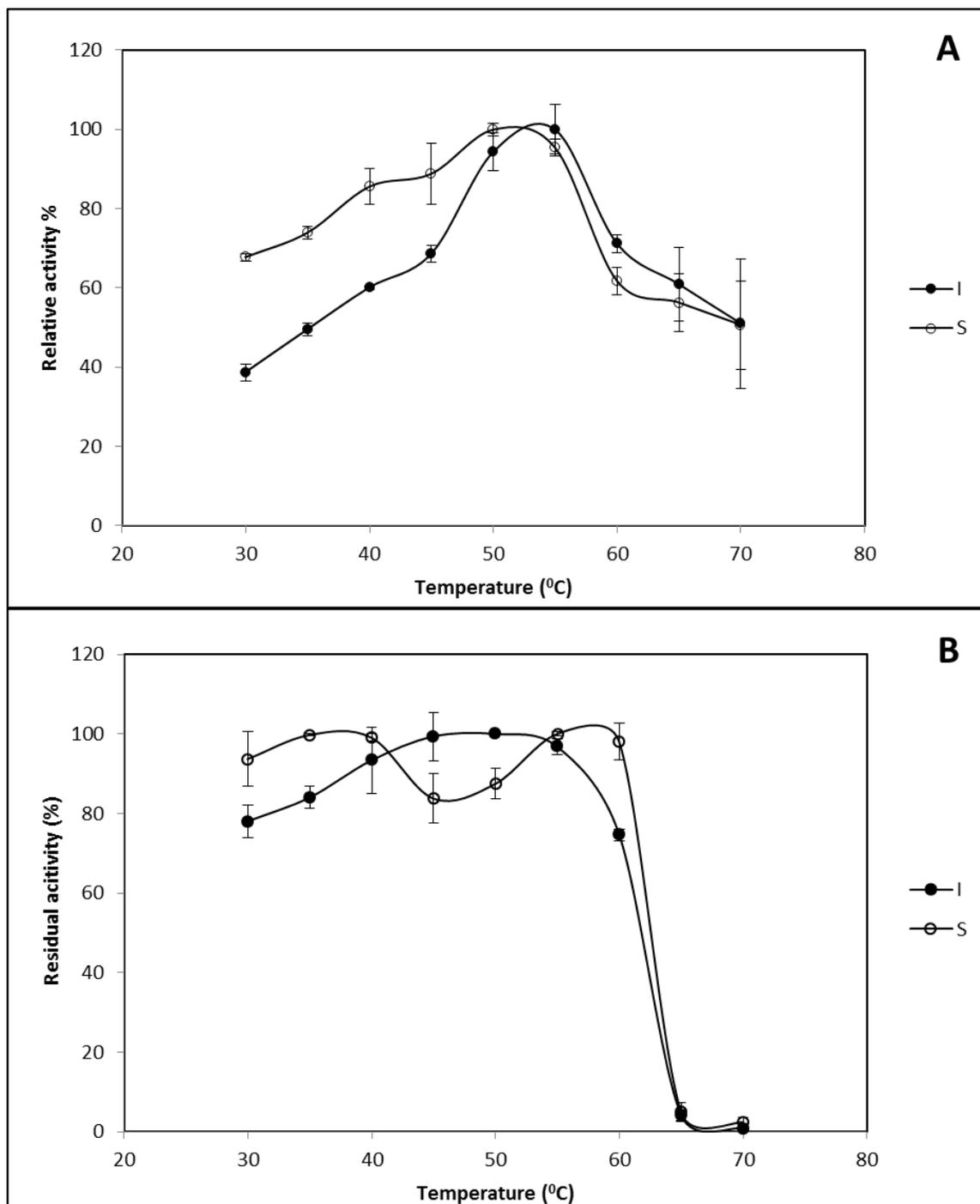
Figure 4.7 - Effect of temperature on recombinant exo-inulinase and invertase activity and their stability. (A) Effect of temperature on recombinant exo-inulinase and invertase activity; (B) Effect of temperature on recombinant exo-inulinase and invertase stability. I – Inulin; S – Sucrose. n=2 and average ± SE. Correlation

coefficient for effect of temperature on exo-inulinase – 0.86 and invertase – 0.85. Correlation coefficient for effect of temperature stability on exo-inulinase – 0.96 and invertase – 0.98.

The effect of metal ions on enzyme stability was also investigated. For exo-inulinase activity (**Table 4.3**) Fe^{2+}, Ca^{2+}, Mg^{2+} and Ba^{2+} was found to decrease the enzyme activity and are in agreement with *A. ficuum* JNSP5-06 and its recombinant inulinase[31] and [29]. Na^+, Mn^{2+}, Fe^{3+} and EDTA did not have any significant effect on enzyme activity. Cu^{2+}, Co^{2+}, Zn^{2+} and K^+ increased the enzyme activity, in agreement with findings on yeast inulinase[33] but not in agreement with fungul inulinase[29] [31]. Fe^{2+} had the greatest inhibitory effect (62 %) and Cu^{2+} had greatest enhancer effect (144 %). Whereas, Cu^{2+} was previously found to be a complete inhibitor on recombinant fungal inulinase[29]. For two tailed student's t-test on exo-inulinase, it was statistically significant when compared with the control ($P < 0.5$) for Fe^{2+}, Cu^{2+}, Co^{2+}, K^+ and Ba^{2+} and statistically insignificant when compared with the control ($P > 0.5$) for Na^+, Zn^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{3+} and EDTA.

For invertase activity Zn^{2+} was found to decrease the enzyme activity (**Table 4.3**). Fe^{2+}, Na^+, Cu^{2+}, K^+, Ca^{2+}, Mg^{2+}, Ba^{2+}, Fe^{3+} and EDTA did not have any significant effect on enzyme activity and the effect of most metal ions are in general agreement with what has been reported for fungal and bacterial inulinase [29], [31], [35]. Zn^{2+} had the greatest inhibitory effect (89.7 %) in agreement with fungal inulinase [31], [29], while Co^{2+} and Mn^{2+} had the greatest enhancer effect (3.7 and 1.4 fold increase). For two tailed student's t-test on invertase, it was statistically significant when compared with control ($P < 0.5$) for Co^{2+} and Mn^{2+} and statistically insignificant when compared with control ($P > 0.5$) for Fe^{2+}, Na^+, Cu^{2+}, Zn^{2+}, K^+, Ca^{2+}, Mg^{2+}, Ba^{2+}, Fe^{3+} and EDTA.

Table 4.3 - Effect of metal ions on recombinant exo-inulinase and invertase activity.
The purified enzyme was pre-incubated for 1 hr with 5 mM of metal ion in pH 5, 50 mM Citrate-Phosphate buffer at 50 0C. The control was used to calculate the relative activity. n=2 and average ± SE. Two tailed student's t-test was carried.

Metal ions	Relative activity (%)	
	Inulin	Sucrose
Control	100 ± 5.0	100 ± 5.2
Fe^{2+}	62.0 ± 0.2^a	111.7 ± 4.5^b
Na^+	94.0 ± 1.9^b	109.5 ± 0.9^b
Cu^{2+}	143.8 ± 7.7^a	104.9 ± 4.3^b
Co^{2+}	137.9 ± 0.1^a	371.1 ± 1.2^a
Zn^{2+}	103.3 ± 0.4^b	89.7 ± 2.5^b
K^+	115.1 ± 7.1^b	99.8 ± 0.2^b
Ca^{2+}	85.9 ± 1.2^b	104.8 ± 4.2^b
Mg^{2+}	90.7 ± 1.4^b	104.5 ± 2.1^b
Mn^{2+}	97.7 ± 3.3^b	142.8 ± 0.2^a

Ba^{2+}	78.5 ± 1.8^a	97.1 ± 3.3^b
Fe^{3+}	97.9 ± 7.3^b	97.04 ± 5.0^b
EDTA	96.9 ± 3.2^b	96.2 ± 1.1^b

(a) Statistically significant when compared with control ($P < 0.5$) and (b) Statistically insignificant when compared with control ($P > 0.5$).

4.4   Discussion

These combined results indicate that some biochemical properties of the recombinant exo-inulinase and invertase are different from those of the fungi, yeast and other bacterial sources.

The purity of the enzyme also affects the enzyme specific activity[36] and an accurate determination of K_m was difficult for inulin because, hydrolysis of inulin from terminal end leads to smaller chain length molecules also functioning as substrates to exo-inulinase enzyme hence affects the rate of hydrolysis[27] and requiring a more complex mathematical model.

The His tag, S Tag and thrombin site at the N-terminal of the recombinant exo-inulinase may influence the post translation modification and possibly increase the catalytic efficiency by allowing for easier access of inulin and sucrose to the active site of the recombinant enzyme. Yeon *et al* [37] found the recombinant 3-hydroxybutyrate dehydrogenase enzyme to be 1,200 fold more active with an N-terminal His-tag when compared to a C-terminal His tag. Also, glycosylation may play a role in the post

translation modification of the protein. Glycosylation occurs in *A. niger* 12 and *P. pastoris* but not in *E. coli.*

The DNS method carried out by Moriyama *et al.* [20] has some limitations. The absorbance used in Millers *et al.*[38] technique is 575 nm. In our method 540 nm wavelength was used and all the samples in our method were diluted 5 times to make sure they fell in the detection limit of 0.1 to 1 absorbance[39]. Also, commercial inulinase enzyme activity was carried out as the vendor suggested and found a good correlation with their number (data not shown).

The degree of polymerization (DP) of inulin is also shown to have an effect on the exo-inulinase activity[30]. Even though Moriyama *et al*[20] and this study used dahlia inulin, the difference in activity might be due to difference in degree of polymerization.

4.5   Conclusions

In this study, exo-innulinase was successfully expressed in *E. coli* strain Rosetta-gami B (DE3), by taking advantage of the strain's expression of rare codons and its oxidizing cytoplasm environment which aids in disulphide bond formation. Further characterization was done to obtain K_m and V_{max} values as well as an S/I ratio of the recombinant exo-inulinase. The results were found to fall within the expected range of native enzymes. The optimum temperature and long term stability indicate the potential to use the enzyme in large-scale inulin hydrolysis.

4.6   References

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Chapter 5

5 High throughput screening of β-glucuronidase (GUS) reporter in transgenic microalgae transformed by *Agrobacterium tumefaciens*

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Abstract

GUS (β-glucuronidase) is a chemiluminescent reporter gene that has been used in *E. coli*, fungi, yeast, mammalian cells, plant cells and microalgae. Currently, the GUS gene fusion system used for detection of GUS enzyme activity is carried out by cell lysis and requires 500 μl cell lysate. Hence, GUS activity cannot be assessed by high throughput screening (HTS) method. This study addresses this by using a HTS technique to quickly isolate transgenic strains (shown using *Chlorella* vulgaris) expressing high GUS activity in a 96 well microplate format. In this technique, quantitative results were obtained without carrying out cell lysis and all the experiments were carried out in a 96 well black microplate. The method developed is cost effective, less labor intensive and can be carried out in a timely manner.

For this, a new GUS reporter vector pBIN+TetR+TetO was developed, followed by transformation (*Agrobacterium tumefaciens*), screening and characterization of the

transgenic *Chlorella vulgaris*. In the screening study, strain number 18 the showed highest fluorescence intensity (16,988 ± 1,168). The GUS enzyme was found to be stable for more than 8 hours for intact cell and lysed cell studies. The optimum concentration of Triton X-100 to release the product (4-Methylumbelliferone) into buffer was 0.1 % and the fluorescence intensity was 28,397± 787. The values of K_m and V_{max} of the recombinant GUS for lysed cells were 0.1304 ± 0.0101 mM and 0.35 ± 0.004 pmol 4-MU/min/ml of crude cell lysate respectively.

Keywords - β-glucuronidase, *Agrobacterium tumefaciens*, High throughput screening, 4-MUG, 4-MU, and *Chlorella vulgaris*.

5.1   Introduction

Microalgae are a diverse group of oxygenic, photosynthetic eukaryotic and prokaryotic (cyanobacteria) microorganisms. They thrive in a wide range of environments, growing on simple nutrients, and carbon dioxide [1]. Potential autotrophic growth, frequently high levels of intracellular lipids and novel pathways and enzymes make microalgae interesting candidates for biotechnological and industrial applications [2]. However, for industrial application of microalgae, the ability to use genetic engineering is advantageous [3]. The number of genetically engineered microalgae are very few when compared to yeast, bacteria, plants, and mammalian system. This shows the development of new molecular tools and techniques are necessary to realize the full potential of microalgae [4].

In the development of transgenic microalgae, the gene of interest is typically inserted into the microalgae genome by homologous and non-homologous integration. To date, most

of the transgenic microalgae developed are by non-homologous integration [5]. The non-homologous integration has been carried out using several transformation techniques like electroporation, particle bombardment, glass bead method, polyethylene glycol, conjugation, and the *Agrobacterium* system [4]. Some of the drawbacks of current molecular techniques in transgenic microalgae are gene silencing, random integration, codon usage, and low transgene expression level [6], [7].

The selection of proper high recombinant protein expressing microalgae is a challenge due to variation in transgene expression level due to random integration into the genome. The foremost technique currently used to isolate high expression transgenic microalgae is by RT-PCR. But the mRNA used in RT-PCR is prone to degradation, contamination, and the cells lysis is needed to isolate it. This method requires 30 to 50 transgenic microalgae are used in screening [8], [9]. Some of the drawbacks of this technique are that it is expensive, and that the success of the experiment depends on the primers and their design. The screening is further labor intensive, and involves several experimental steps and research expertise [10].

High-throughput screening (HTS) is an approach used mainly in drug discovery and has gained widespread acceptance over the last decade. HTS is a process of carrying out large numbers of assays with small sample, substrate, and buffer volumes. HTS in biotechnological application are used in drug development [11], isolating transgenic microbes expressing high level of recombinant protein [12], to carryout enzyme kinetics and characterization studies [13], to study the effects of different chemicals and bio-chemicals on individual cells [14], [15], and to quantify the expression of reporter and marker proteins in transgenic strains [16].

The β-glucuronidase reporter protein hydrolyzes 4-MUG (4-Methylumbelliferyl β-D-glucuronide) to 4-MU (4-Methylumbelliferone). The 4-MU is a fluorescence molecule which emits light at 460 nm when excited at 365 nm. This can be exploited for calculating the GUS activity, and protein expression levels. Several transgenic strains have been studied with β-glucuronidase reporter protein expression. Some of the microbes are *Escherichia coli* [17], *Mycobacterium tuberculosis* [18], *yeast,* and *filamentous fungi* [19]. The system has also been used in mammalian cells [20], extensively in plant cells [21] and for the past decade in microalgae [22].

The *Agrobacterium tumefaciens* (*A. tumefaciens*) transformation technique has been successfully applied in *Chlorella vulgaris* [23], *Chlamydomonas reinhardtii* [24], *Dunaliella salina* [25], *Haematococcus pluvialis* [26], *Schizochytrium* [26], *Isochrysis galbana*, and *Isochrysis* sp. However, the *Agrobacterium* mediated transformation has some drawbacks such as low transformation, low transgene expression level, low copy number of transfer DNA (T-DNA) integrated into the genome of microalgae, integration of T-DNA far from transcriptional activating elements or enhancers, integration of T-DNA into transcriptionally silent regions of the microalgae genome [27].

To overcome the above listed constraints, several screening steps are used such as growing the transgenic strain on appropriate concentration of antibiotics, PCR of genomic DNA for T-DNA integration, mRNA transcript analysis for expression of transgenic transcript by RT-PCR, GUS histochemical assay with X-gluc for preliminary screening of transgenic strain, GUS activity for quantitative analysis of β-glucuronidase [25]. Currently, the conventional method used for isolating the proper construct is by GUS histochemical analysis. The method is tedious and prone to biased interpretation of

the result since the observations are made under the microscope. Also, to quantify GUS activity cell lysis is necessary and requires a cell lysate volume of ~ 500 µl. Hence, the GUS activity cannot be carried out by HTS method.

In order to quantify the florescence intensity in intact cell study, it is necessary to study the effect of Triton X- 100 on the permeability of 4-MUG through the cell membrane and release of 4-MU into the assay buffer. Hence, we have tried different concentration of Trion X-100 to determine its effect on 4-MU release into the assay buffer.

Here a GUS expression vector was constructed and transformed into *C. vulgaris* via *A. tumefaciens*. Also, a new HTS method for intact cell screening of the transgenic *C. vulgaris* expressing β-glucuronidase was developed in a 96 well plate format. The new technique will enable GUS expressing microalgae screening to be carried out in cost effective, less labor intensive and time effective manner.

5.2   Materials and methods

Oligonucleotides were purchased from UWO Oligo Factory (London, ON, Canada). Q5 high fidelity polymerase was purchased from New England Biolabs (Whitby, ON, Canada). Fast cloning pack, Fast digest restriction enzymes and PCR clean up kit were purchased from Fermentas (Ottawa, ON, Canada). Plasmid purification kits (catalog # 27104) were purchased from Qiagen (Toronto, ON, Canada). Antibiotics, X-gluc, 4-MUG, 4-MU and all other materials were purchased from Sigma-Aldrich (Oakville, ON, Canada) and were of analytical grade.

5.2.1 Strains, plasmids, and culture condition

pUC 19 plasmid was purchased from New England Biolabs (Whitby, ON, Canada) and was used for cloning work. Vectors pTET1/pBin Tet R and pBin HygTx–GUS-INT were kindly provided by Prof. Dr. Christiane Gartz (University of *Göttingen, Göttingen*, Germany). *Escherichia coli* strain DH5α was purchased from New England Biolabs (Whitby, ON, Canada) and was used for cloning work. Cells were cultured in Luria-Bertani (LB) medium containing 50 µg/ml of ampicillin or 50 µg/ml of kanamycin at 37 ^{0}C and 200 RPM. *A. tumefaciens* (LBA 4404) was kindly provided by Dr. Abdelali Hannoufa (Agriculture and Agri-Food Canada, London, ON CANADA). Cells were cultured in Yeast Extract Beef (YEB) medium containing streptomycin 100 µg/ml, rifampicin 20 µg/ml, and kanamycin 50 µg/ml at 25 ^{0}C and 200 RPM. *C. vulgaris* (UTEX 2714) was purchased from UTEX Culture Collection of Algae (Austin, TX, USA). The cells were cultured in Tris-Acetate-Phosphate (TAP) medium at 25 ^{0}C, 150 RPM, 90 % humid atmosphere, and under 140 µmol m^{-2} s^{-1} white light illumination of 16 h on/8 h off.

5.2.2 Construction of expression vector

Vector pUC19+Tx-GUS-INT was generated by double digestion of pUC19 and pBin HygTx–GUS-INT with EcoRI and HindIII. The digested vectors were gel purified. The double digested pUC19 was used as a backbone vector to ligate the HygTx–GUS-INT gene from pBin HygTx–GUS-INT. They were gel purified and ligated with fast ligase enzyme. The vector was transformed into *E. coli* by heat shock method and blue white screening was done to isolate the proper construct.

The final expression vector pBin19+TetR-TetO was generated by PCR amplifying CaMV 35S –GUS-INT– 35 S 3^1 poly tail cassette from pUC19+Tx-GUS-INT to introduce HindIII and ClaI restriction site at 5^1 and 3^1 termini, using primers F_HindIII for (5^1 –AAA AAA AAG CTT CGT CTA AGA AAC CAT TAT TAT CAT GAC ATT AAC C – 3^1) and R_ClaI (5^1 –AAA AAA ATC GAT CTT GCA TGC CTG CAG GTC AC– 3^1). The PCR fragment and pBin Tet R were double digested with HindIII and ClaI. They were gel purified and ligated with fast ligase enzyme to get the final vector pBin19+TetR-TetO. The vector was transformed into *E. coli* by heat shock method. The precise construct was isolated by carrying out colony PCR and restriction digest analysis.

5.2.3 Electroporation of *A. tumefaciens* and transformation of *C. vulgaris*

Electrocompetent *A. tumefaciens* LBA 4404 cells were transformed with pBin19+TetR-TetO vector in a Bio-Rad MicroPulser (Hercules, CA). The transformation was carried according to the MicroPulser manual [28]. For transformation of *C. vulgaris,* the Pratheesh et al. protocol was followed [24]. For selection of transformed *C. vulgaris*, the cells were plated on TAP agar plate containing 500 µg/ml cefotaxomine and 30 µg/ml G418 Sulfate.

Isolation and growth of single colony

After 1 month of growth on the selection plate, colonies where visible. Single colonies were suspended in 5 ml of TAP media containing 30 µg/ml G418 Sulfate for 2 weeks.

GUS Histochemical analysis

The G418 sulfate -resistant colonies were analyzed for GUS activity by staining with X-gluc (5-bromo-4-chloro-3-indoyl b-D-glucuronide). Transgenic *C. vulgaris* culture were pelleted and re-suspended in X-gluc solution and incubated at 37 °C overnight. After incubation, cells were pelleted, bleached with ethanol and analyzed under the microscope.

5.2.4   Quantitative Measurement of GUS Activity

GUS activity was measured by monitoring cleavage of 4-MUG to 4-MU by the β-glucuronidase enzyme [29][21]. The formation of 4-MU was measured by a Tecan M-1000 multimode plate reader (Männedorf, Switzerland) with an excitation of 365 nm and emission at 455 nm. The assay was adapted so that large numbers of samples could be assayed and measured in a 96-well plate format (Greiner 96 Flat Bottom Black Polystyrol). Microalgae cultures were mixed for 30 second in the plate reader before taking readings. For all the fluorescence intensity measurement the gain was set manual and the Z-position to 20,000 µm. All results are typically the mean (±SD) of at least three replicates unless stated otherwise.

5.2.5   Screening for GUS activity in recombinant culture

Single colonies from the selection plate were grown in 5 ml of TAP media containing 30 µg/ml G418 Sulfate. Untransformed *C. vulgaris* was used as control. For this study, 150 µl of transgenic cells were pelleted and washed twice with 100 mM, pH 7.4 PBS buffer. The supernatant was discarded and the cells was suspended in 150 µl of assay buffer and transferred to a 96-well microplate. The microplate was sealed with microplate sealing film during the duration of the assay. The assay buffer was composed of 50 mM sodium phosphate, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, and 1 mM 4-MUG. The samples

were incubated at 37 °C for 2 hours. At the end of the incubation period, 50 µL of 1 M Na$_2$CO$_3$ (stop solution) was added to each well and 4-MU fluorescence intensity was measured.

5.2.6   GUS activity in culture supernatant

GUS activity in the culture supernatant was determined by measuring the fluorescence intensity. For this, 200 µl of 1 OD$_{600}$ unit of cell culture was taken. The cells were centrifuged at 13,000 rpm for 5 minutes. The supernatant was used for carrying out the experiments. The supernatant (75 µl) was mixed with GUS assay buffer (75 µL) in a 96-well microplate. The GUS assay buffer composed of 2 mM 4-MUG, 50 mM Na-phosphate pH 7.0, 1 mM EDTA, 0.1 % Triton X-100, 10 mM β-mercaptoethanol. The microplate was sealed with microplate sealing film during the duration of the assay. The reaction was carried out at 37 ^{0}C for 3 hours. The reaction was stopped with 50 µl of 1 M Na$_2$CO$_3$. The 4-MU fluorescence intensity was measured.

5.2.7   Quantification of GUS activity in intact cell

150 µl of 1 OD$_{600}$ unit of 7 days old transgenic cells were pelleted and washed twice with 100 mM, pH 7.4 PBS buffer. The cells were suspended in 150 µl of assay buffer and transferred to 96-well microplate. The microplate was sealed with microplate sealing film during the duration of the assay. The samples were incubated at 37 °C. At the end of the incubation period, 50 µL of 1 M Na$_2$CO$_3$ (stop solution) was added to each well and 4-MU fluorescence intensity was measured. Activity was expressed as molarity of 4-MU from calibration curve of the standards. The standards were prepared for low range (150 µL of 500 – 30 pM 4-MU in assay buffer, plus 50 µL of 1 M Na$_2$CO$_3$) and for high range (150 µL of 65.5 – 10 µM 4-MU in assay buffer, plus 50 µL of 1 M Na$_2$CO$_3$).

5.2.8   Quantification of GUS activity in lysed cell

The recombinant cell pellet was washed twice with 100 mM, pH 7.4 PBS buffer. The cells were suspended in extraction buffer (100 mM potassium phosphate, 1 mM DTT, pH 7.5). The suspended cells were lysed by sonicator (UP400S, Hielscher, Teltow, Germany). The sonicator was set to 5 min on/ 5 min off for 30 minutes. The cells were kept on ice during sonication. The lysed cells were centrifuged for 1 hour at 3,500 rpm. The clear cell lysate was used to carry out the experiments. The clear cell lysate (75 µL) was mixed with GUS assay buffer (75 µL) in a 96-well microplate. The experiments were carried out at 37 °C and covered with microplate sealing film for the duration of the assay. At the end of the incubation period, 50 µL of 1 M Na_2CO_3 (stop solution) was added to each well and 4-MU fluorescence intensity was determined. Activity was expressed as molarity of 4-MU based on a calibration curve prepared with appropriate standards.

Protein quantification

The protein quantification was carried out by Pierce BCA protein assay kit (Rockford IL USA). The assay was carried out according to manufacturer's protocol in a 96-well plate (Costar 96 Flat Bottom Transparent Polystyrol). The experiment was carried out in duplicate.

5.2.9   Effect of different concentration of Triton X-100 on GUS activity

The effect of Triton X-100 on GUS activity of intact cell was studied. For this study, 150 µl of 1 OD_{600} unit of 7 days old transgenic cells were pelleted and washed twice with 100

mM, pH 7.4 PBS buffer. The cells were suspended in 150 µl of assay buffer and transferred into 96-well microplate. The microplate was covered with microplate sealing film during the duration of the assay. The assay buffer was prepared with different concentration of Triton X-100 (0%, 0.01 %, 0.1 % and 1 %). The samples were incubated at 37 °C for different time intervals. At the end of the incubation period, 50 µL of 1 M Na$_2$CO$_3$ (stop solution) was added to each well and 4-MU fluorescence intensity was measured.

After measuring the fluorescence of intact cell, the cells were separated by centrifugation at 13,000 rpm for 10 minutes. The supernatant fluorescence intensity without cells (200 µl) was measured.

5.2.10   GUS enzyme kinetics

For carrying out GUS activity, the recombinant cells were lysed as mentioned earlier. GUS activity assays were carried out at 37 °C in a water bath for 10 minutes. The experiment was carried out in 0.3 ml in a 96 well PCR plate. The clear supernatant (75 µL) was pre-incubated in a water bath for 5 minutes and mixed with GUS assay buffer (75 µL) containing different concentration of 4-MUG (0.06 mM to 5.0 mM). The reaction was carried out for 10 minutes and stopped by adding 50 µL of 1 M Na$_2$CO$_3$. The sample was transferred into a 96-well microplate (Greiner 96 Flat Bottom Black Polystyrol). The amount of product formed (4-MU fluorescence) was directly determined using a microplate reader. The initial reaction rate was plotted versus initial substrate concentration to determine whether the enzyme followed Michaelis-Menten kinetics. The V_{max} and K_m were determined by non-linear regression of Michaelis-Menten equation.

5.3   Results

5.3.1   Construction of Expression Vector

Prior to the construction of the expression vector an intermediate vector was constructed.

The binary vector pBIN-HYG-TX-GUS-INT contains the cassette for the expression of

the GUS enzyme. The cassette was PCR amplified and ligated into pUC19 plasmid which

was digested with the same restriction enzyme. The vector was named pUC19+TX-GUS-

INT. The proper construct (in DH5α) was isolated by blue white screening (data not

shown). The vector pUC19+Tx-GUS-INT was double digested with EcoRI and HindIII

and the expected gene size of 2,600 bp and 2,900 bp was visualized on DNA Flash gel
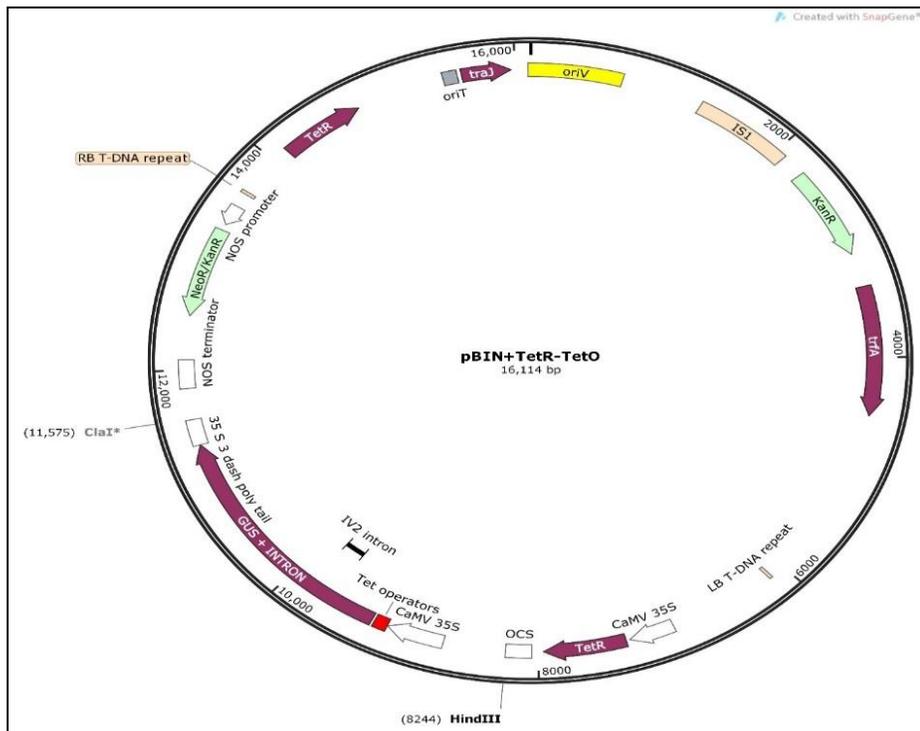
(**Figure 5.2**).

Figure 5.1 - Schematic representation of expression vector pBIN+TetR-TetO constructed.

For the construction of the expression vector, the cassette in pUC19+Tx-GUS-INT was PCR amplified and ligated into pBIN-Tet R vector which was digested with the same restriction enzymes. The new construct was named as pBin+TetR-TetO (**Figure 5.1**). The pBin+TetR-TetO was double digested with HindIII and BspTI and the expected gene size of ~ 12,299 bp and ~ 3,815 bp was visualized on DNA Flash gel (**Figure 5.2**).
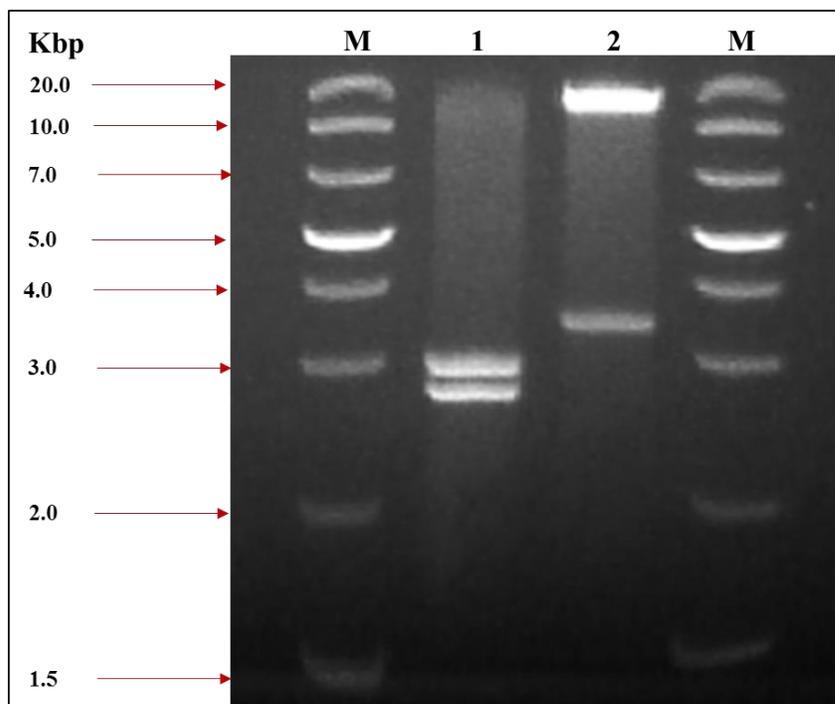

Figure 5.2– DNA Flash gel run at 50 V. M – Generuler 1 kb Plus DNA Ladder, 1 – pUC19+TX-GUS-INT double digest with EcoRI and HindIII (expected gene size of 2.6Kb and 2.9 Kb). 2 – pBin+TetR+TetO double digested with HindIII and BspTI (expected gene size of 12,299 bp and 3815 bp)

5.3.2 Antibiotic resistance study of *C. vulgaris*

Log phase *C. vulgaris* cells were inoculated in 5 ml of TAP medium with 1 µg/ml, 5 µg/ml, 10 µg/ml, 20 µg/ml, 30 µg/ml, 40 µg/ml and 50 µg/ml of G418 sulphate

antibiotics. The cells grew in 1 µg/ml, 5 µg/ml, and 10 µg/ml whereas the cell did not grow in 20 µg/ml, 30 µg/ml, 40 µg/ml and 50 µg/ml (data not shown).

5.3.3 Transformation and reporter gene expression

Log phase *C. vulgaris* and log phase transformed *A. tumefaciens* were co-cultivated for 3 days. 10^6 cells of co-cultivated *C. vulgaris* was plated on TAP agar plate containing cefotaxime (500 µg/ml) and G418 sulphate (30 µg/ml). Transformed colonies were visible after 1 month (data not shown).

Single colonies of transformed colonies were grown in TAP liquid medium containing G418 sulphate (30 µg/ml) for 2 weeks. The cells were analyzed by GUS histochemical assay. 40 samples were analyzed and only 1 clone showed the integration of DNA into the microalgae (data not shown).

5.3.4 Direct screening of intact recombinant *C. vulgaris*

The recombinant cells grown in TAP liquid media were used for carrying out the screening. 32 samples of cells were screened with sample number 1 being the control. 150 µl of cells were pelleted and washed with PBS buffer. The pelleted cells were suspended in assay buffer and incubated for 2 hours. From the study, it was found that only sample number 18 had shown GUS activity. The fluorescence intensity was 16,988 ± 1,168. While the activity with rest of the samples were negligible (**Figure 5.3**).

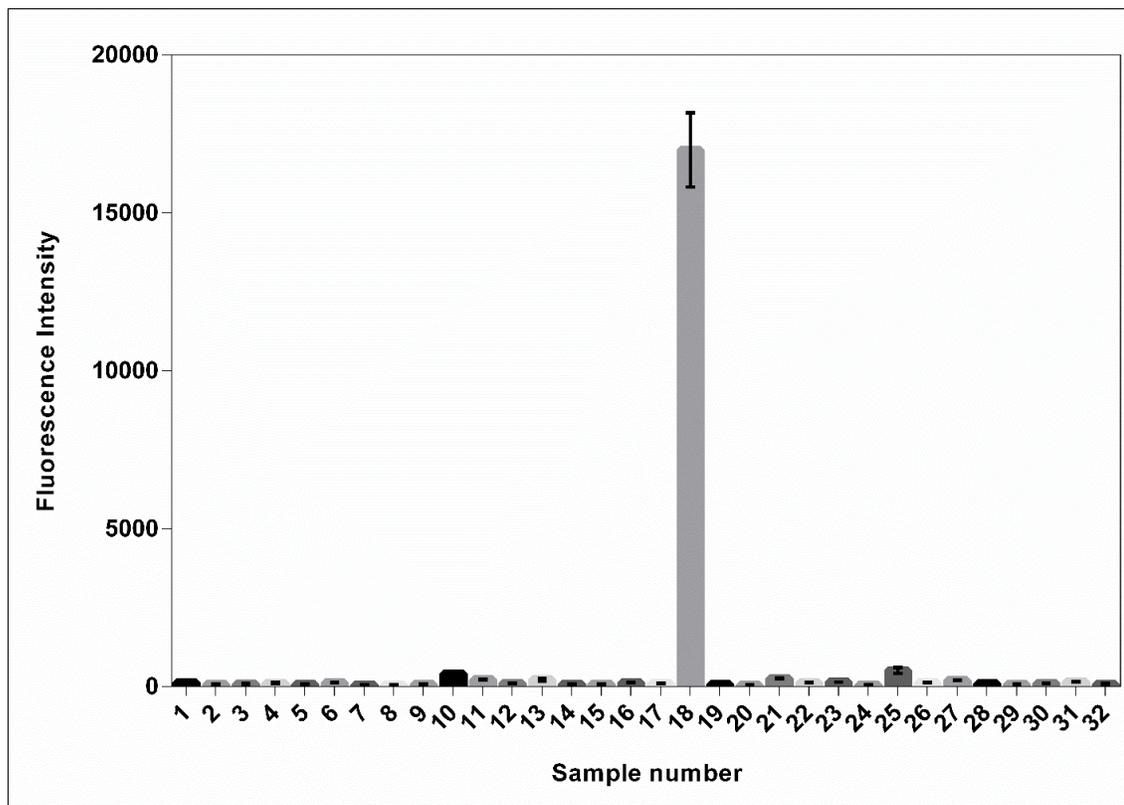**Figure 5.3- Direct screening of intact recombinant C. vulgaris. 31 transgenic c. vulgaris and 1 control sample were screened in 96 well microplate. Sample number 1 being control. Sample number 18 showed highest GUS activity. 150 μl of cells were pelleted and washed with PBS buffer. The pelleted cells were suspended in assay buffer and incubated for 2 hours and the fluorescence intensity was measured by excitation/emission wavelength of 365/455 nm.**

5.3.5　GUS activity in culture supernatant

GUS activity in the culture supernatant was determined by measuring the fluorescence intensity. The fluorescence intensity was 1,107 ± 4 for transgenic *C. vulgaris* (**Figure 5.4**). Whereas, the fluorescence intensity was 376 ± 5 for untransformed *C. vulgaris.*
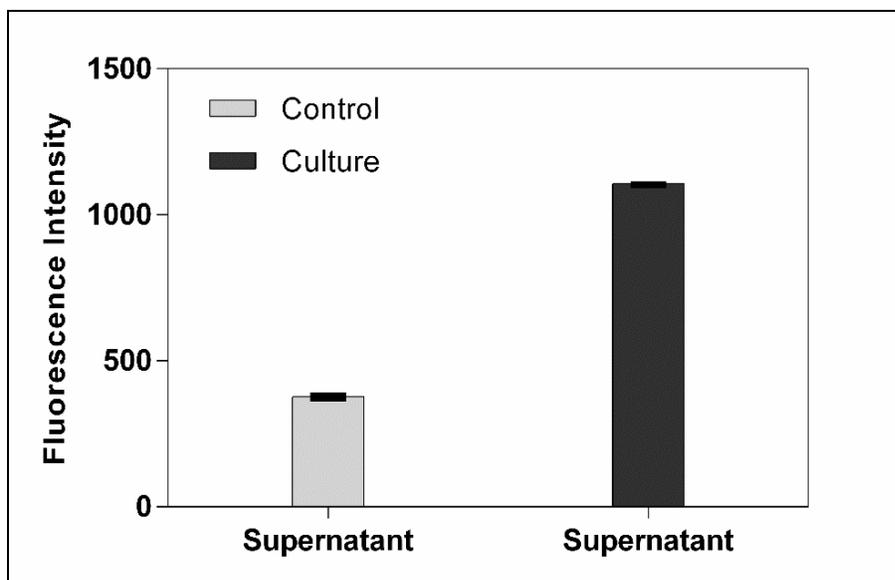

Figure 5.4 – GUS activity in transgenic cell culture supernatant. 75 µl of transgenic cell culture supernatant was mixed with 75 µl of assay buffer in 96 well microplate and incubated at 37 ⁰C for 3 hours. The reaction was stopped by adding 50 µl of 1 M Na2CO3. At the end of 3 hours, the fluorescence intensity recorded was 1,107 ± 4.

5.3.6　Detection of GUS activity in intact recombinant *C. vulgaris*

Recombinant *C. vulgaris* carrying the GUS reporter gene were grown in TAP media containing 30 µg/ml of G418 sulphate. 150 µl of culture was pelleted by centrifugation, the culture supernatant was discarded and the cell pallet were incubated directly in GUS assay buffer, which contained Triton X-100 (0.1%) to enhance the ability to penetrate the cell wall for both the substrate 4-MUG and the product 4-MU throughout the cell.

The release of the product 4-MU was monitored by fluorescence which occurred with a delay of 10 minutes. The fluorescence increased greatly over time up to 4 hours for intact cell and slight increase between 4 to 7 hours. The highest 4-MU recorded was 172 ± 8 µM/ OD_{600} at 7 hours (**Figure 5.5**).
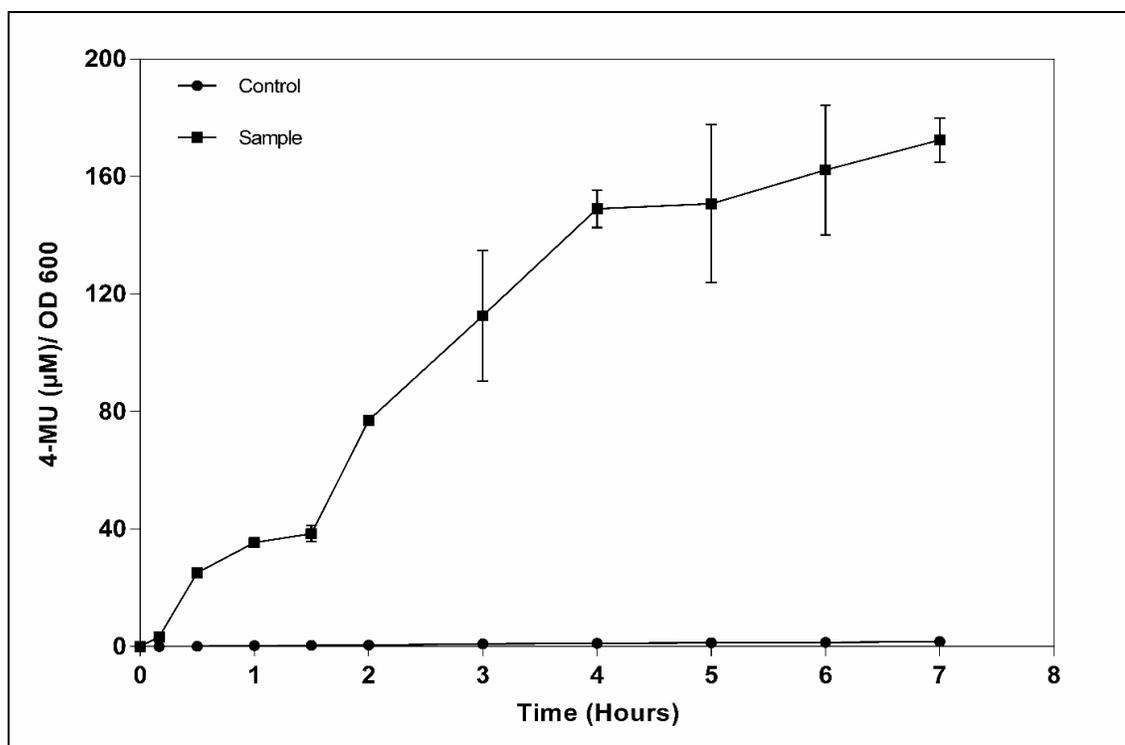

Figure 5.5 – Cell lysate GUS activity with mixing. 150 µl of pelleted cells was suspended in assay buffer in 96 well microplate and incubated at 37 ^{0}C and 150 rpm in shaker. The reaction was stopped by adding 50 µl of 1 M Na_2CO_3. At the end of 7 hours, the 4-MU formed was 172 ± 8 µM/ OD_{600}.

5.3.7   Detection of GUS activity in lysed recombinant *C. vulgaris*

The current technique to quantify GUS activity in lysed cell microalgae is carried out by using ~ 500 µl of cell lysate per time point. Here, we have reduced the amount of cell lysate needed to 75 µl and the experiment was carried out in a microplate.

GUS activity was determined in lysed recombinant *C. vulgaris*. In this study the cell lysate was subjected to the same treatment as the intact cell before. However, 2 mM 4-MUG was used instead of 1 mM 4-MUG. Also, 75 µl of cell lysate was mixed with 75 µl substrate. The fluorescence increased with time linearly. There was no decrease in fluorescence even up to 8 hours and the GUS enzyme was stable. The highest 4-MU was seen at 8 hour, the calculated fluorescence reading was $4,303 \pm 55$ µM/mg of crude cell lysate. It was interesting to note that there was increase fluorescence reading in the non-GUS containing control with increase in time. The highest reading of the control was 96 ± 1 µM/mg of crude cell lysate (**Figure 5.6**). Unlike intact cell there was no delay in fluorescence during the first 30 minutes in sample assay.
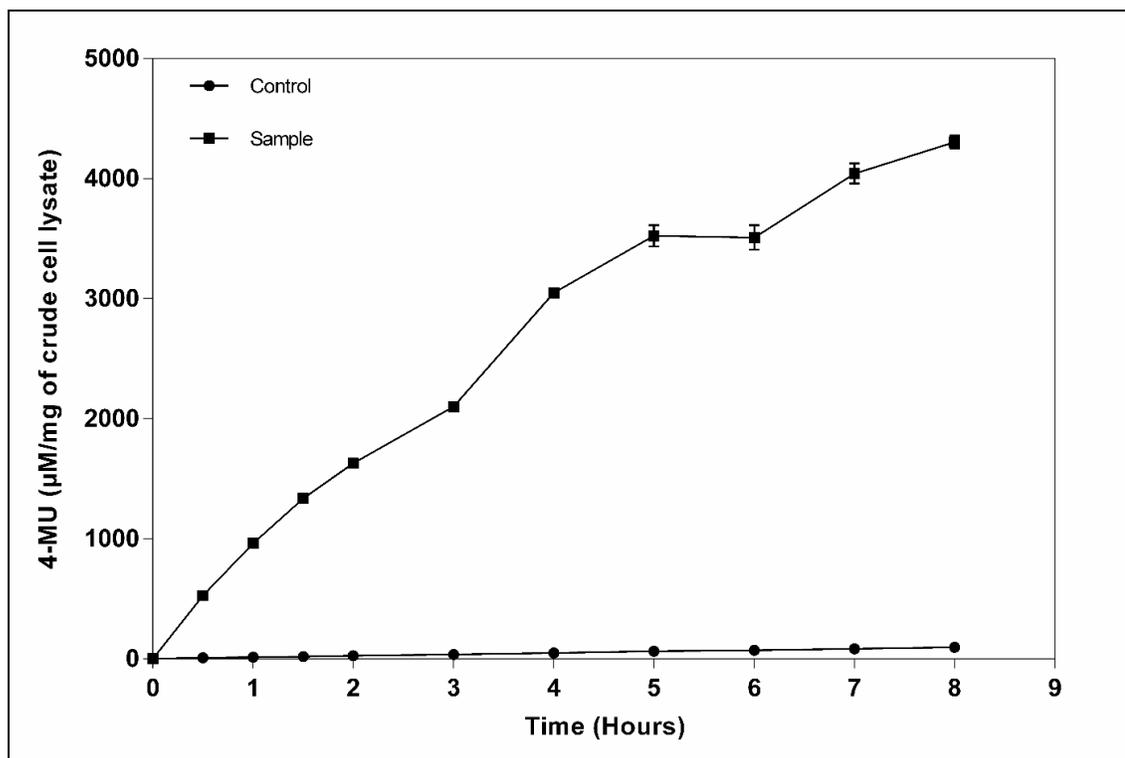

Figure 5.6 – Intact cell GUS activity with mixing. 75 µl of cell lysate was mixed with 75 µl of assay buffer in 96 well microplate and incubated at 37 ^{0}C and 150 rpm in

shaker. The reaction was stopped by adding 50 µl of 1 M Na$_2$CO$_3$. At the end of 8 hours, the 4-MU recorded was 4,303 ± 55 µM/mg of crude cell lysate.

5.3.8 Effect of Triton X-100 on GUS activity in intact cell and its supernatant

In this experiment, the effect Triton X-100 on cell permeability to substrate (4-MUG) and product (4-MU) was studied. Triton X-100 is known to provide permeabilization access to intracellular components of the cell [30]. Triton X-100 is a non-ionic detergent made up of uncharged hydrophilic head groups that consist of polyoxyethylene moieties. It is better suited for breaking lipid-lipid and lipid protein interactions than protein protein interactions. They solubilize the membrane protein and the critical micelle concentration is relatively unaffected by the ionic strength of the buffer [30].

Four different concentration of Triton X-100 (0%, 0.01%, 0.1% and 1 %) were studied to see which would lead to enhanced permeability of 4-MUG into the cell and release of 4-MU into the supernatant. The fluorescence intensity was measured with intact cells in the assay buffer and the culture supernatant (for this step the intact cells were centrifuged to separate cells from assay buffer; and the assay buffer fluorescence was measured) in 96 well microplate. The fluorescence intensity was measured over a 4-hour period. In the intact cell study, there was an increase in fluorescence with time. The data for TritonX addition is shown in **Figure 5.7**. Overall, for 0%, 0.01%, 0.1% and 1% there was an increase in fluorescence intensity of 7.4-fold, 7.7 fold, 8.3 fold and 6.8 fold from 0th hour to 4th hour. The highest change in fluorescence intensity was seen in 0.1% Triton X-100 while lowest change in fluorescence intensity was seen in 1% Triton X-100 over a 4 hour period.
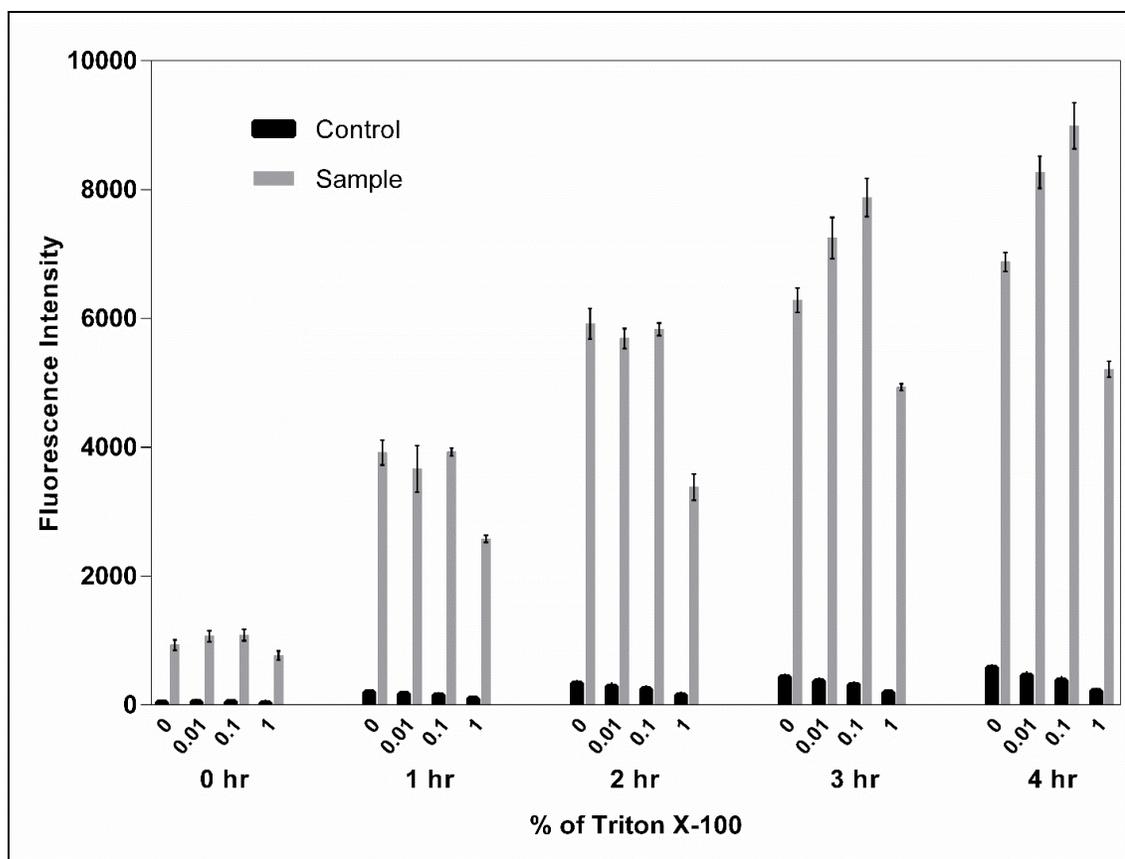
Figure 5.7 – Effect of Triton X-100 on intact cell GUS activity. 150 µl of pelleted cells was suspended in assay buffer containing different concentration of Triton X-100 (0 %, 0.01 %, 0.1 % and 1 %) in 96 well microplate and incubated at 37 ^{0}C. The reaction was stopped by adding 50 µl of 1 M Na$_2$CO$_3$. At the end of 4 hours, the highest fluorescence intensity recorded was 8,994 ± 357 for 0.1 % Triton X-100.

In the second stage, the cells were separated by centrifugation and the fluorescence was measured with the released 4-MU into assay buffer without cells (supernatant). The data is shown in **Figure 5.8**. Overall, for 0%, 0.01%, 0.1% and 1% there was increase in fluorescence intensity of 9.3-fold, 4.9-fold, 6.6-fold and 3.7 fold from 0th hour to 4th hour. The highest change in fluorescence intensity was seen in 0.1% Triton X-100 while the lowest change in fluorescence intensity was seen in 1% Triton X-100 over the 4 hour period.

There was ~ 3-fold increase in fluorescence intensity without cells when compared to with cells. This may be due to absorption of fluorescence by the cells in the sample. Vivek *et al.* has carried out similar work in *Arabidopsis thaliana*. The GUS activity was measured with *Arabidopsis thaliana* seedling present in the lysis medium and the GUS activity was measured with *Arabidopsis thaliana* seedling removed from the lysis medium. They found > 95 % of the 4-MU was released into the medium [31].
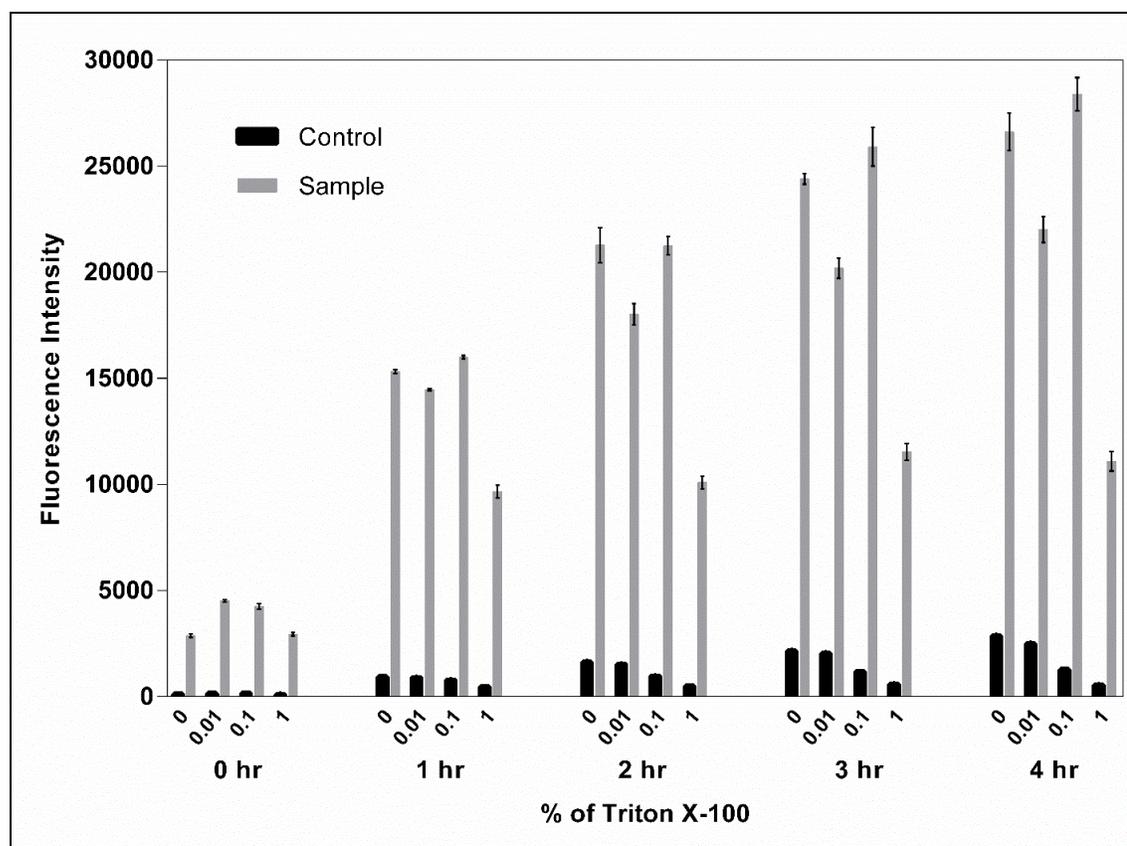

Figure 5.8 – Effect of Triton X-100 on intact cell supernatant GUS activity. 150 μl of pelleted cells was suspended in assay buffer containing different concentration of Triton X-100 (0 %, 0.01 %, 0.1 % and 1 %) in 96 well microplate and incubated at 37 ^{0}C. The reaction was stopped by adding 50 μl of 1M Na_2CO_3. At the end of 4 hours, the highest fluorescence intensity recorded was 28,397 ± 787 for 0.1 % Triton X-100.

5.3.9   GUS enzyme activity

The GUS activity was characterized for cell lysates. The activity was found by adding

different concentration of 4-MUG substrate to the crude cell lysate for 10 minutes at 37

^{0}C in a water bath. The reaction was stopped by adding stop solution. The GUS activity

experiments were carried out in a 96 well PCR plate. The values of kinetic

constants K_m and V_{max} for GUS were found to be 0.1304 ± 0.0101 mM and 0.35 ± 0.004

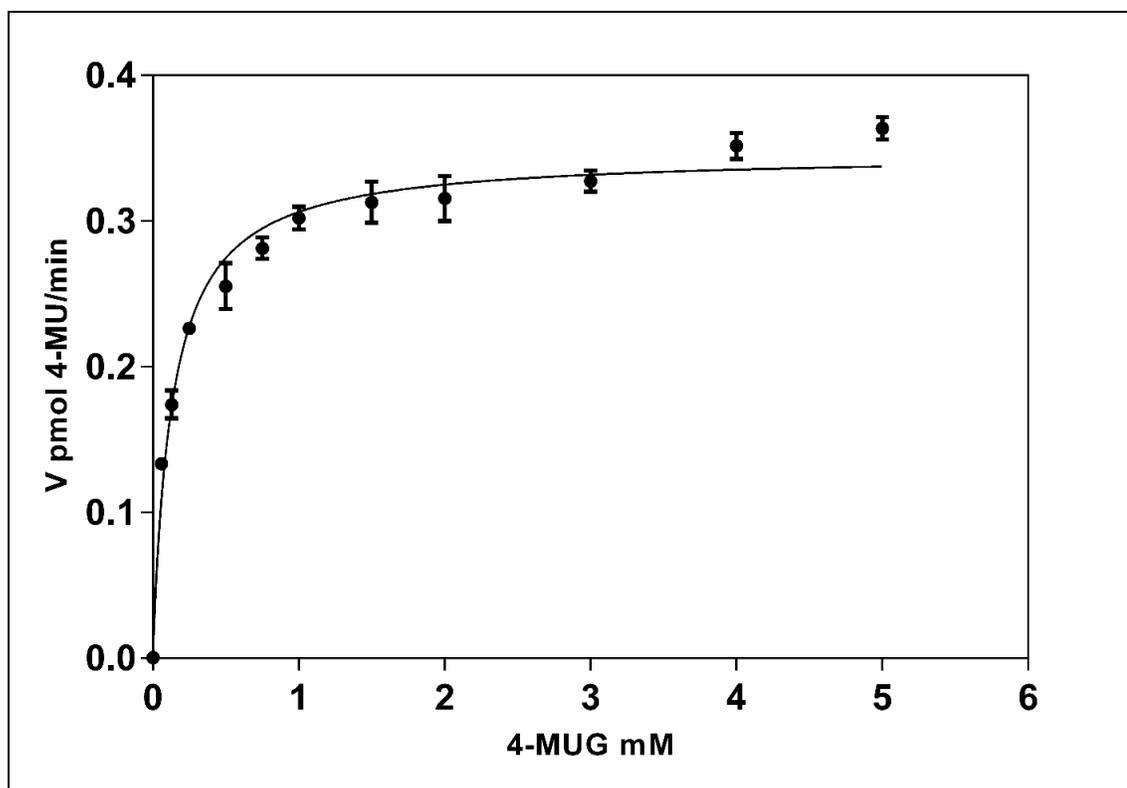pmol 4-MU/min/ml of crude cell lysate respectively (**Figure 5.9**).

Figure 5.9 – GUS enzyme Michaelis–Menten kinetics plot. 75 µl of cell lysate was mixed with 75 µl of assay buffer containing different concentration of 4-MUG (0.06 mM to 5.0 mM) in 96 well PCR microplate and incubated at 37 ^{0}C in water bath for 10 minutes. The reaction was stopped by adding 50 µl of 1 M Na_2CO_3. The values of kinetic constants K_m and V_{max} for GUS were found to be 0.1304 ± 0.0101 mM and 0.35 ± 0.004 pmol 4-MU/min/ml of crude cell lysate respectively.

5.4   Discussion

By using the newly developed High throughput screening technique it was possible to screen 32 samples rapidly in a 96 well microplate. The isolated strain number 18 had ~ 16,000 fold higher fluorescence intensity when compared to the other strains. Strain number 10 and strain number 25 were found to have minuscule fluorescence intensity. However, the fluorescence intensity of strain number 10 and strain number 25 was negligible when compared to strain number 18.

The GUS enzyme was found to be secreted by the microalgae into the culture medium. The culture supernatant also showed GUS activity. The activity was found to be ~ 3 fold higher compared to the control sample (untransformed cell supernatant). The fluorescence intensity observed in transgenic *C. vulgaris* supernatant maybe due the GUS enzyme secreted out into the culture or lysed cell may have released GUS enzyme into the culture. However, the GUS enzyme appears stable in the culture supernatant for long duration. The observed fluorescence intensity in control supernatant may be due to thermal hydrolysis of 4-MUG to 4-MU during 3 hours of incubation at 37 ^{0}C.

For intact and lysed cells, it was found that the GUS enzyme is stable at 37 ^{0}C for more than 8 hours since the product formation increased with time. Also, there was no substrate or product inhibition of GUS enzyme over the studied duration.

The lysed cell GUS activity was found to be ~ 26-fold higher than the intact cell study. This may due to interference of the cells to emission/excitation wavelength which may absorb and emit different wavelength. The substrate 4-MUG used in lysed cell study was

2 mM while in intact cell it was 1 mM. An increase of 2-fold substrate concentration led to higher product formation.

In a cell lysate study, Cheng *et al.* carried out GUS activity in marine microalgae *Schizochytrium* found the highest GUS activity was ~ 3000 florescence intensity [33]. Úbeda-Mínguez *et al.* transformed *Tetraselmis chuii* with GUS gene found the GUS activity to ~ 2800 pmol min/mg of protein [34]. Dautor *et al.* expressed GUS gene in *Scenedesmus almeriensis* and found the strain expressing highest GUS activity was only ~ 20 pmol min/mg of protein [35]. Whereas in our study, the enzyme activity was found to be 0.35 ± 0.004 pmol 4-MU/min/ml of crude cell lysate which is very low.

The study on Triton X-100 concentration showed some interesting observation on florescence intensity, and product (4-MU) release into the assay buffer.

The fluorescence intensity increase was in the order of Triton X-100 concentration 1%, 0 %, 0.01 % and 0.1 % and the order of fluorescence intensity magnitude change was same with respect to time. In 1 % Triton X-100 concentration there was decrease in fluorescence intensity this may be due to interference of Triton X-100 in emission/excitation wavelength or an unknown effect. However, it was initially expected that higher concentration of Triton X-100 would aid in removing the cell membrane more effectively and hence access of substrate (4-MUG) into the cell for converting into product (4-MU).

Whereas, it was found that the fluorescence intensity increase in the cell pellet supernatant was in the order of Triton X-100 concentration 1%, 0.01 %, 0 % and 0.1 % and the order of fluorescence intensity magnitude change was same with respect to time.

The fluorescence intensity was higher in 0.01 % and 0 % whereas in the intact cell study, the fluorescence intensity was higher in 0 % and 0.01 %. This shows that more product (4-MU) is released into the liquid medium (assay buffer/supernatant) with Triton X-100 concentration of 0 %. Also, the experiment can be carried out without Triton X-100 in the assay buffer with slight reduction in fluorescence intensity.

There was increase in fluorescence intensity with increase in time in control samples also. This may be due to partial hydrolysis of substrate (4-MUG) to product (4-MU) at 37 ^{0}C with longer duration of incubation.

The V_{max} and K_m of the crude cell lysate were found out by Michaelis-Menten kinetic plots and the specific activity of crude cell lysate was found using a BCA protein assay. The V_{max} was 0.3463 ± 0.004 pmol 4-MU/min/ml of cell lysate, the K_m was 0.1304 ± 0.0101 mM and specific activity was 0.979 ± 0.011 pmol 4-MU/mg of crude protein.

The β-Glucuronidase assay carried out by GE Lifesciences using β-glucuronidase (GUS, liquid suspension from *E. coli*, Boehringer Mannheim 127 680) in DyNA Quant 200 Fluorometer found out that the V_{max} was 14.7 pmol/min and K_m was 0.57 mM. The V_{max} was ∼ 42 fold and K_m was ∼ 4 fold lower in our study when compared to GE Lifesciences study [32]. The low enzyme activity in the present study maybe due to very low expression of β-glucuronidase in transgenic *C. vulgaris*, also protease might have been present in the lysate negatively affecting the β-glucuronidase activity.

5.5　　Conclusion

This study developed a new high throughput screening method for isolating recombinant microalgae carrying a GUS reporter. The direct application in a 96 well microplate

format for screening requires minimum amounts of sample handling and sample volume while directly providing quantitative data. This assay is superior when compared to the GUS histochemical assay. The GUS histochemical assay involves staining, overnight incubation and strains are screened based on visual appearance under a microscope. The visual evaluation is prone to biased hit selection. Also, the volume of sample, buffer, and substrate is reduced by ~ 6.6-fold when compared to the standard GUS assay using a HTS fluorometry method. The present setup allows to quantify 1 control and 31 samples in triplicates in a single 96 well microplate cutting down the time requirement. The GUS activity carried out with intact cell, with cells removed after incubation with substrate, and lysed cells were quantifiable. Hence, the methodology described here is accurate, reliable, and robust and could be used in high throughput screening of recombinant microalgae carrying the GUS gene.

5.6   References

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Chapter 6

6 Application of high throughput screening technique for transgenic *Chlamydomonas reinhardtii* colonies on agar plates

Abstract

GUS (β-glucuronidase) is a reporter gene that is used in several microbial systems to investigate several factors which affects expression levels. GUS hydrolyses 4-MUG to 4-MU. The 4-MU formed can be quantified by using a fluorescence reader since the 4-MU has excitation/emission wavelength of 365/455 nm. Currently for screening, cell lysate is used. Here, we have applied high throughput screening (HTS) technique that was developed for *Chlorella vulgaris* (**Chapter 5**) to *Chlamydomonas reinhardtii.* This study was carried out to show the usefulness of the HTS technique to quickly isolate intact transgenic *Chlamydomonas reinhardtii* cell expressing high GUS activity in a 96 well microplate format. The method used is less labour intensive, cost effective, and can be carried out in a timely manner.

For this we transformed GUS reporter expression vector pBIN-Hyg-Tx–GUS-INT into *Chlamydomonas reinhardtii* by co-cultivation with *Agrobacterium tumefaciens.* The transformed cells which grew on TAP agar plate containing Hygromycin B antibiotic were screened for intact cell GUS activity in 96 well black microplates. 124 transgenic *Chlamydomonas reinhardtii* were screened and strain number 50 showed the highest

fluorescence intensity of 1,113. Strain number 103 was found to have 5.6-fold lower fluorescence intensity compared to strain number 50. The technique can be used to screen transformed colonies that have GUS activity directly from agar plate.

Keywords - β-glucuronidase, *Agrobacterium tumefaciens*, High throughput screening, 4-MUG, 4-MU, and *Chlamydomonas reinhardtii*.

6.1   Introduction

Chlamydomonas reinhardtii has been a model organism for studying microalga [1]. It is one of the most well studied microalgae to date. The complete genome sequence is available for mitochondria [2], chloroplast [3], and nuclear genomes [4]. These advantages make it an ideal platform to test new techniques. Several transformation techniques have been developed [5], [6], [7].

However, nuclear transformation is still wildly used and one of the shortcomings is that the transgene integrates at random locations in the nuclear genome [8]. This leads to variation in transgene expression level from one transgenic strain to another. Here we address this issue by using HTS method for GUS expressing transgenic *C. reinhardtii.*

We applied the previously developed HTS in *C. vulgaris* to *C. reinhardtii* with few modifications. In the previous work, transgenic *C. vulgaris* grown in liquid culture was used for HTS. For the present work, transgenic colonies from agar plates were directly used to carry out the HTS method. This saves time of growing colonies from agar plate in liquid culture as it would ideally take 1-2 weeks to obtain.

Here we have transformed GUS expression vector pBIN-Hyg-Tx–GUS-INT into *C. reinhardtii* via *A. tumefaciens.* The transgenic *C. reinhardtii* expressing β-glucuronidase was screened by the HTS method for intact cell screening in a 96 well plate format. The cells scraped from agar were directly screened. The new technique developed will enable GUS expressing microalgae screening to be carried out in a cost effective, less labor intensive and timely manner.

6.2   Materials and methods

Unless stated all the techniques used here are similar to Chapter 5.

6.2.1   Antibiotic test

C. reinhardtii sensitivity was tested for different concentration of Hygromycin B. Log phase cells were inoculated in 5 ml of TAP media containing 0 µg/ml, 2.5 µg/ml, 5 µg/ml, 10 µg/ml, 20 µg/ml, and 30 µg/ml. The OD_{600} was measured every 24 hours for 8 days.

6.2.2   Expression vector

The GUS expression vector pBIN-Hyg-Tx–GUS-INT was used in this work. Transformation of *A. tumefaciens* and co-cultivation of *C. reinhardtii* was the same as in Chapter 5.

6.2.3   Isolation and growth of single colony

After 1 month of growth on the selection plate, colonies were visible. A single colony was re-streaked on TAP agar plate containing 10 µg/ml Hygromycin B. The plate was

incubated for 2 weeks at 25 ^{0}C, 90 % humid atmosphere, and under 150 µmol m^{-2} s^{-1} white light illumination with 16 h on/8 h off.

6.2.4 Screening for GUS activity in transgenic *Chlamydomonas reinhardtii*

The transgenic cells were scraped off the agar plate and suspended in 150 µl of assay buffer in a 96-well black microplate. The assay buffer composed of 50 mM sodium phosphate, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, and 1 mM 4-MUG (4-methylumbelliferyl β-D-glucuronide). The microplate was incubated at 37 °C for 2 hours. The microplate was sealed with microplate sealing film during the duration of the incubation. At the end of the incubation period, 50 µL of 1 M Na$_2$CO$_3$ (stop solution) was added to each well and 4-MU fluorescence intensity was measured.

6.3   Results

6.3.1   Transformation of *E. coli*

Analogous to pBIN+TetR-TetO, the expression vector pBIN-Hyg-Tx–GUS-INT (**Figure 6.1**) was developed for tetracycline inducible system in *Arabidopsis thaliana*. However, the vector has an origin of replication from *E. coli*. The difference to the previously used vector is that there is no Tetracycline repression gene (TetR) in the vector.
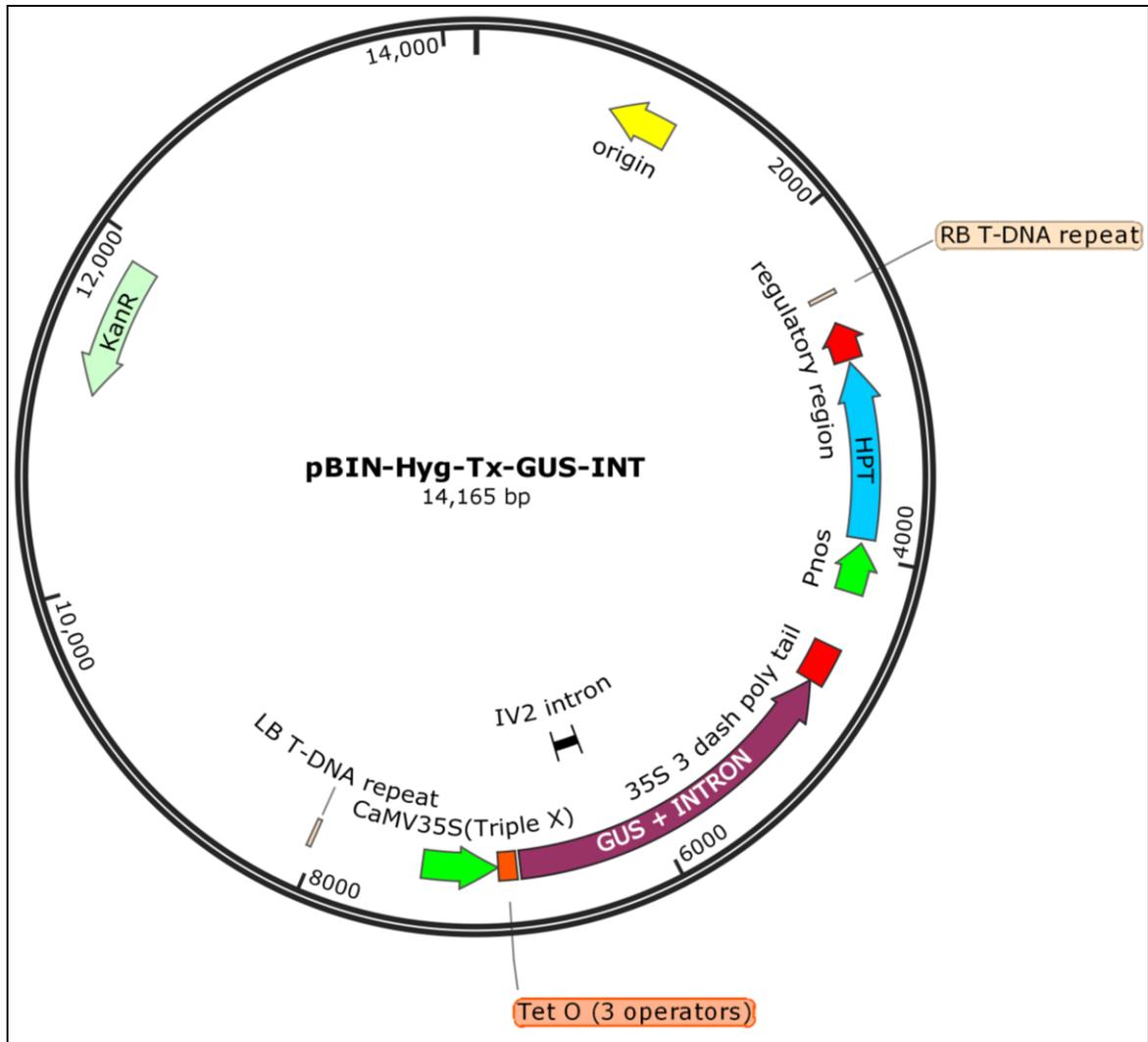
Figure 6.1 – GUS expression vector pBIN-Hyg-Tx-GUS-INT used for transformation of Chlamydomonas reinhardtii

The expression vector was used to propagate for restriction digest analysis and transformation steps. The vector was transformed into *E. coli* strain DH5α by heat shock method (**Figure 6.2**).
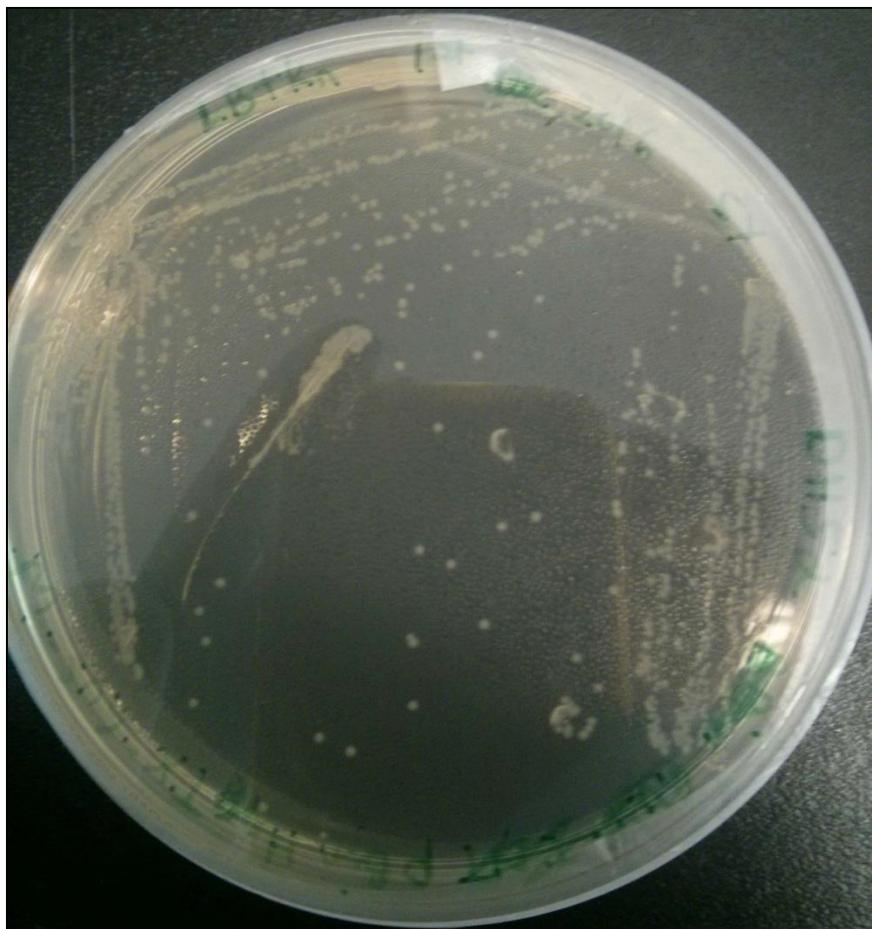
Figure 6.2- Heat shock method of transformation - The vector pBIN-Hyg-Tx-GUS-INT was transformed into *E. coli* strain DH5α. 50 µl of cells and 1 µl of 100 ng of vector were mixed and kept on ice for 30 minutes. The cells were transformed by keeping in water bath (42 ^{0}C) for 30 seconds and 950 µl of SOC was added and grew the cells by keeping at 37 ^{0}C for 60 minutes. 50 µl of cells were streaked on the Lb agar plate containing K$_n$ antibiotic. The recombinant cells were visible after 24 hr incubation at 37 ^{0}C.

6.3.2   Restriction digest analysis of expression vector

The expression vector was isolated from *E. coli* strain DH5α carrying pBIN-Hyg-Tx-

GUS-INT using a Qiagen mini prep kit. To check the integrity of expression vector

pBIN-Hyg-Tx-GUS-INT, the expression vector was double digested with EcoRI and

HindIII and analyzed in Flashgel. The double digest was of expected size 2858 bp and 11986 bp (**Figure 6.3**).
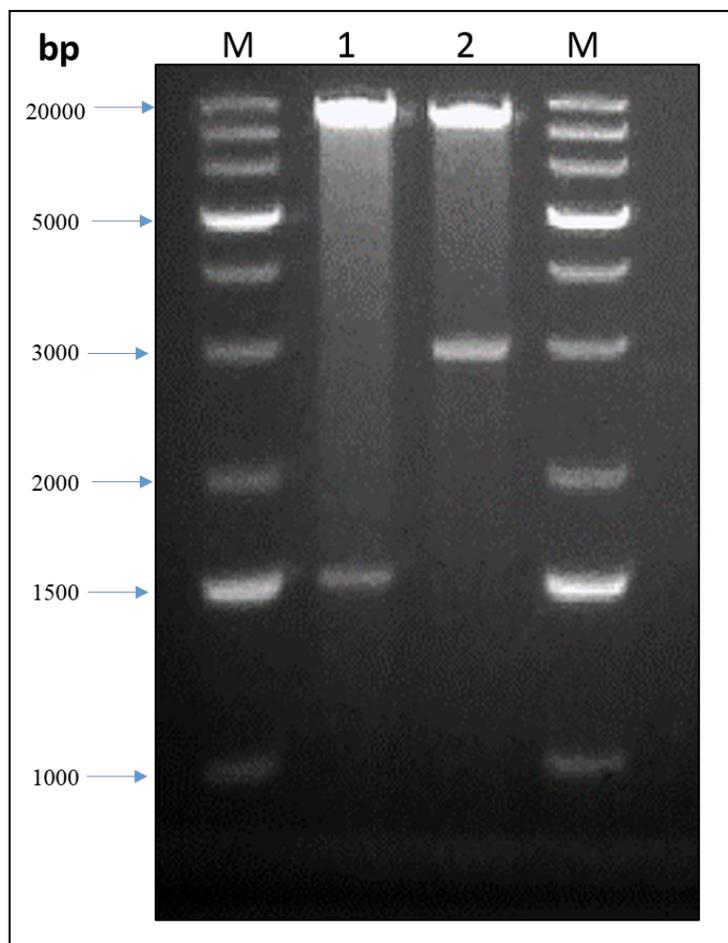

Figure 6.3- Agarose gel electrophoresis of vector restriction digestion. M: 1 kb Plus DNA Ladder; Lane 2: Double digest of pBIN-HygTX-GUS-INT with EcoRI and HindIII (2858 bp and 11986 bp). The flash gel was run at 50 V for 90 minutes.

6.3.3 Gene transformation of *Agrobacterium tumefaciens*

The expression vector grown in *E. coli* strain DH5α was purified using a Qiagen plasmid purification kit in order to transform the vector into *A. tumefaciens*. Cell growth of *A. tumefaciens* after transformation was visible after two days of incubation (**Figure 6.4**).
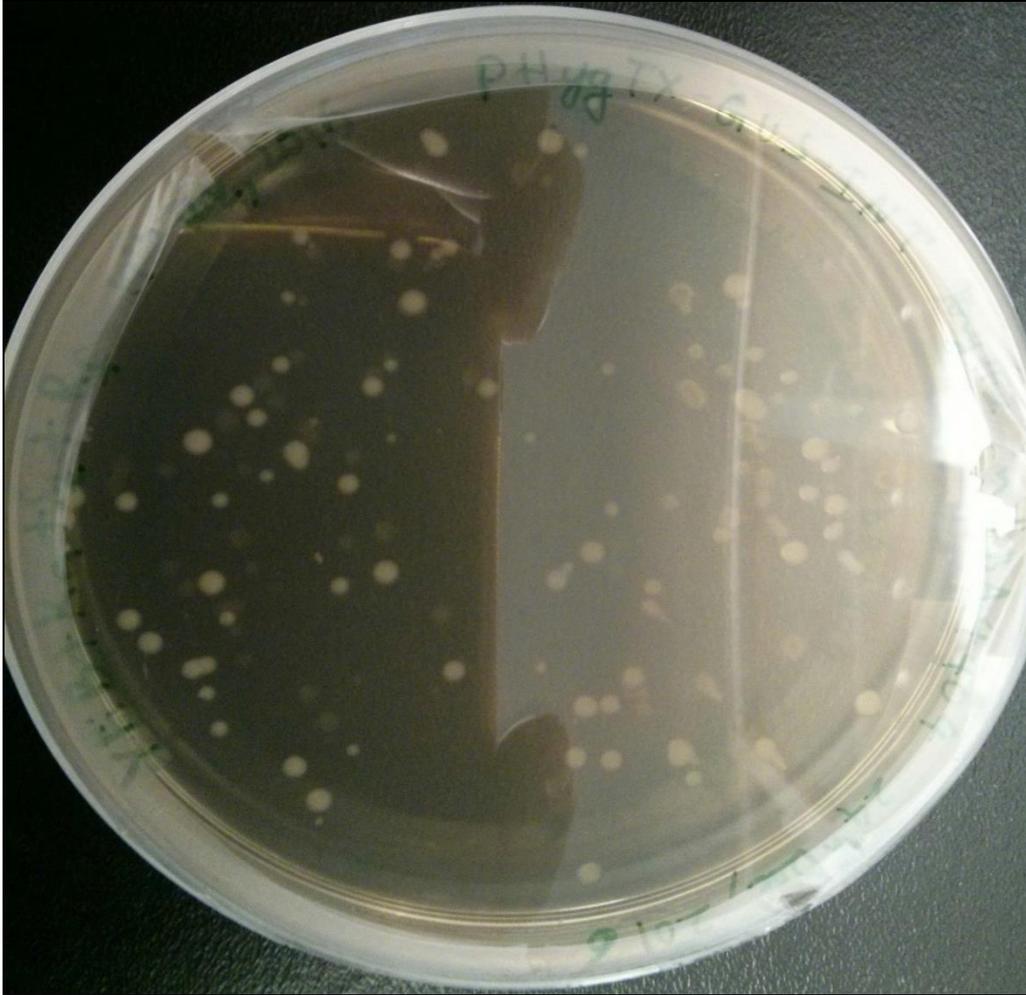
Figure 6.4 - Electroporation transformation of *Agrobacterium tumefaciens*. The vector pBIN-HygTX-GUS-INT was transformed into *Agrobacterium tumefaciens* strain LBA4404. 20 μl of electrocompetent cells and 2 μl of total 100 ng of vector were mixed and kept on ice. The mixture was transferred into a chilled 0.1 cm cuvette and electroporated. The electroporated cells were grown in 1 ml of SOC media at 30 ^{0}C for 60 minutes. 50 μl of cells were streaked on the YEB agar plate containing K_n + S_t + R_f antibiotics. The recombinant cells were visible after 48 hr incubation at 30 ^{0}C.

6.3.4   Culturing and antibiotic resistance study of *C. reinhardtii*

For antibiotic tests, cells from log phase were inoculated in 5 ml of TAP medium with 0 μg/ml, 2.5 μg/ml, 5 μg/ml, 10 μg/ml, 20 μg/ml, and 30 μg/ml of Hygromycin B antibiotics. Cell growth was monitored over a period of 8 days by measuring OD_{600}.

The cells grew well in 0 µg/ml and with reduced growth in 2.5 µg/ml, whereas the cells did not grow in 5 µg/ml, 10 µg/ml, 20 µg/ml, and 30 µg/ml (**Figure 6.5**).
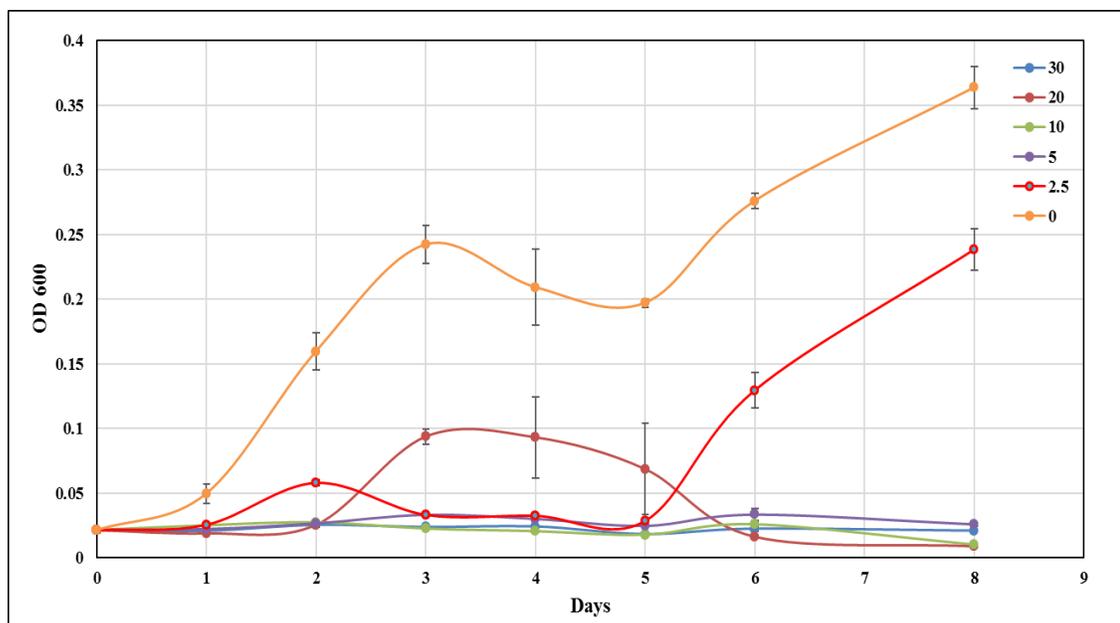

Figure 6.5 - Hygromycin B resistance of *C. reinhardtii*. The *C. reinhardtii* cells were inoculated in 5 ml of TAP media and different concentrations of Hygromycin B were added to the media. The cell growth was monitored by OD$_{600}$ absorbance. The cells were unable to grow between 5 µg/ml - 30 µg/ml of antibiotic.

6.3.5 Co-cultivation of *A. tumefaciens* and *C. reinhardtii*

The binary vector pBIN-HygTX-GUS-INT, able to express GUS reporter gene was transferred into electrocompetent *A. tumefaciens* LBA 4404 by electroporation. Transformed cells were grown on YEB agar medium containing kanamycin and streptomycin antibiotics. Log phase *C. reinhardtii* and log phase transformed *A. tumefaciens* were co-cultivated on TAP agar plate for 3 days in the dark. 10^6 cells of co-cultivated *C. reinhardtii* was spread on a TAP agar plate containing cefotaxime (500 µg/ml) and hygromycin B (10 µg/ml) and kept in dark for 2 days at 25 ^{0}C. The plate was

transferred into 25 °C, 90 % humid atmosphere and under 140 µmol m^{-2} s^{-1} white light illumination of 16 h on/8 h off. Transformed colonies were visible after 1 month (**Figure 6.6**).

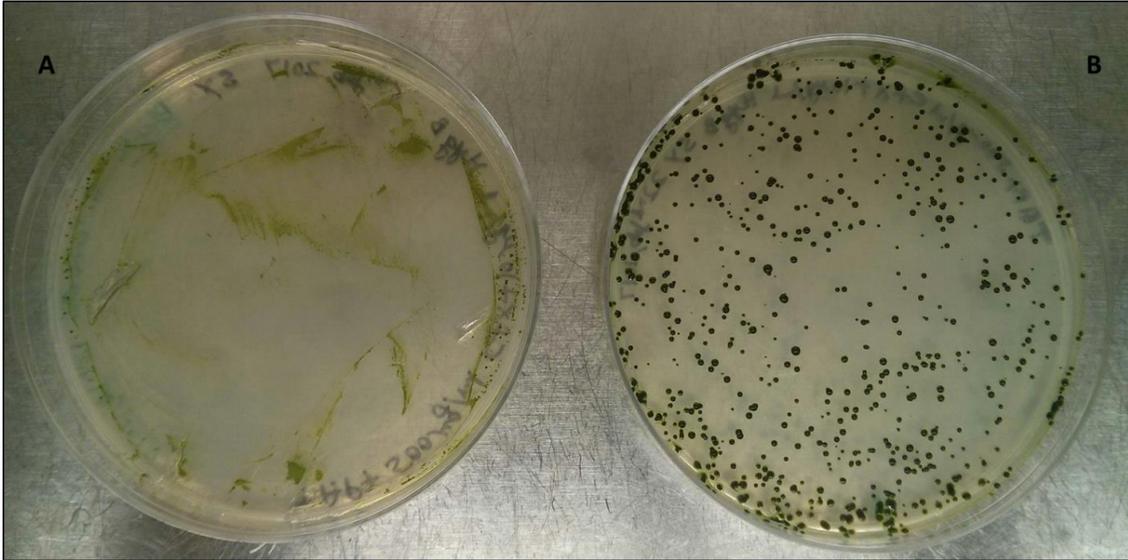**Figure 6.6 - Transformed *C. reinhardtii* – (A) Untransformed *C. reinhardtii* (control) grown on TAP agar plate containing 10 µg/ml of Hygromycin B. (B) *C. reinhardtii* transformed with binary vector pBIN-HygTX-GUS-INT grown on TAP agar plate containing 10 µg/ml of Hygromycin B. The untransformed cells did not grow and transformed cells grew as expected.**

6.3.6   Re - streaking of transformed colonies

Single transformed colonies were re-streaked on a TAP agar plate containing 10 µg/ml of Hygromycin B to keep the antibiotic pressure on the transgenic cells. Each agar plate was divided into small squares and single colonies were streaked. 126 single colonies were streaked. The cells were visible after 2 weeks of growth on agar plate (**Figure 6.7**).
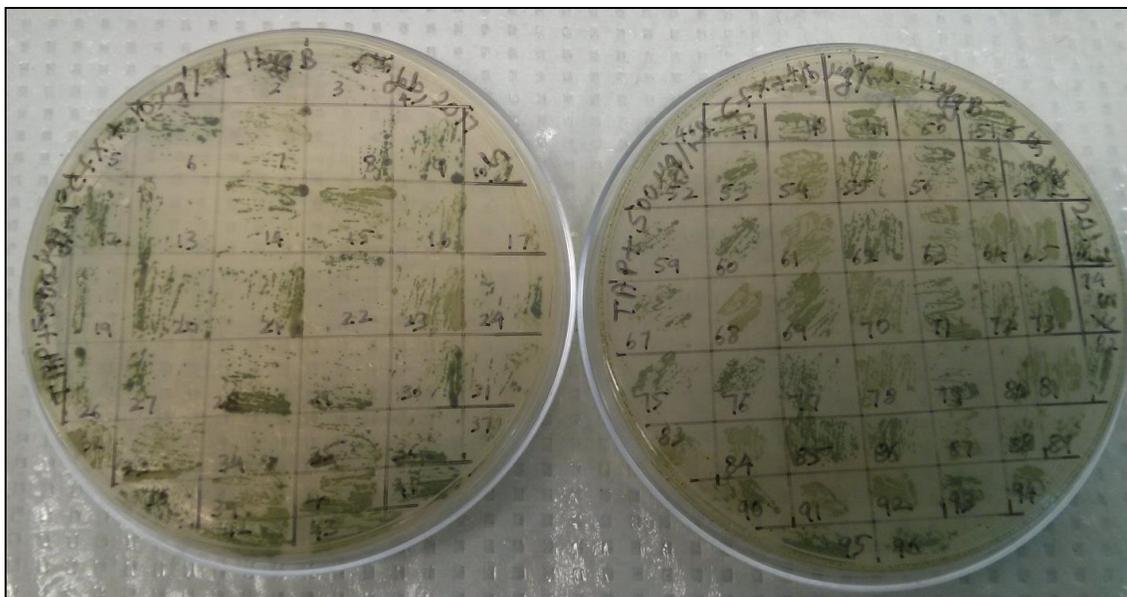
Figure 6.7 – Re - streaking of transformed colonies. Single colonies were streaked in a single box and grew at 25 °C, 150 RPM, 90 % humid atmosphere and under 140 µmol m^{-2} s^{-1} white light illumination of 16 h on/8 h off. The streaked cells were visible after 2 weeks of growth.

6.3.7   Direct screening of intact recombinant *C. reinhardtii*

No quantitative data regarding expression level can be obtained from the plates as shown in **Figure 6.7**. The transgenic cells grown on TAP agar were therefore used for a novel screening assay based on the previously developed HTS system (**Chapter 5**). 126 samples of cells were screened with sample number 1 being control. A fraction of each colony was physically removed and suspended in assay buffer and incubated for 2 hours before the reaction was stopped by adding stop solution. From the study, it was found that only sample number 50 had GUS activity. The fluorescence intensity was 1,113. While the activity with rest of the samples and control were ~ 5 to 10-fold lower than strain number 50 (**Figure 6.8**).
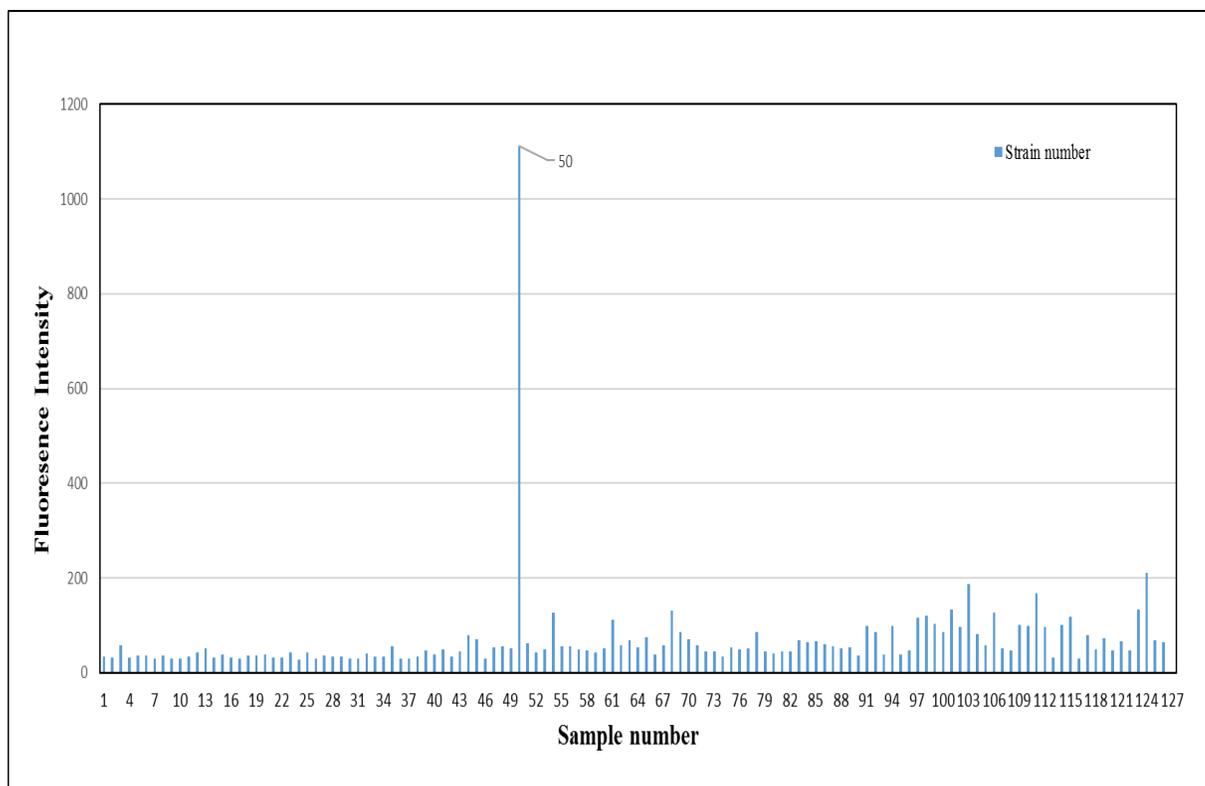
Figure 6.8 - Direct screening of intact recombinant *C. reinhardtii*. 126 transgenic *C. reinhardtii* and 1 control sample were screened in 96 well microplates. Sample number 1 being control. Sample number 50 showed highest GUS activity. The scraped cells from the TAP agar plate were suspended in 150 µl of assay buffer and incubated for 2 hours and reaction was stopped by adding 50 µl of stop solution and the fluorescence intensity was measured by excitation/emission wavelength of 365/455 nm. At the end of 2 hours, the fluorescence intensity recorded was 1,113.

6.4   Discussion

In this technique, a high GUS expressing strain was isolated. The highest GUS activity strain was, strain number 50. The florescence intensity was 1,113-fold. A few other strains were also found to have some GUS activity.

By using the newly developed High throughput screening technique it was possible to screen 126 samples rapidly in 96 well black microplates. The isolated strain number 50

had ~ 32 fold higher fluorescence intensity when compared to control (strain number 1). Strain number 124 and strain number 103 were found to have ~ 5.6-fold lower fluorescence intensity compared to strain number 50. The fluorescence intensity of strain number 124 and strain number 103 was lower when compared to strain number 50.

Based on the literature of this field an increase of fluorescence intensity of 4-MU can be considered a success [9], [10], [11], the selected strain can therefore be considered transgenic. The success rate of ~1 in 50 is comparable with what is found in the literature [12], [13], [14], hence the developed assay can be considered a valuable contribution in this field.

6.5   Conclusions

Here we have shown the application of HTS developed earlier (**Chapter 5**) for *C. vulgaris* in *C. reinhardtii*. This work shows that, the HTS method can be applied for other microalgae system. We further reduced the time needed to carry out the HTS, by directly using transgenic *C. reinhardtii* colonies from the agar plate containing appropriate antibiotics. Carrying out GUS activity for colonies from agar plate is advantageous for slow growing microalgae's since, growing a single colony in liquid culture can 2 to 4 weeks.

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Chapter 7

7   Conclusions and Recommendations

This chapter outlines the conclusions of the study. In addition, some recommendations for future work are proposed.

7.1   Conclusions

The work presented in this thesis investigated the development of molecular tools, transformation and expression of recombinant enzymes in *E. coli*, *Chlorella vulgaris*, and *Chlamydomonas reinhardtii* and carried out the characterization work of the recombinant enzymes expressed.

A review of the current literature shows that, though microalgae are creating substantial interest as hosts for biotechnology applications, the availability of genetic tools is at the date of writing this thesis, still very limited. Genetic engineering of bacteria, yeast, various mammalian cell cultures and plants has led to major scientific and economic breakthroughs. Until now, such an event has not occurred for micro-algae based applications and products, largely due to the lack of generally applicable tools catered for microalgae. A key contribution in this thesis was the development of a new HTS-assay to quantify gene expression in recombinant microalgae. The advantage of this technique the is large number of transgenic strains that can be screened at a relatively low cost when compared to RT-PCR. The, HTS is carried out at translation step (GUS enzyme) information about mRNA expression level will not be available. The technique can also

help in understanding the effects of enhancers and inhibitors on recombinant GUS activity in microalgae system.

In chapter 4, we have successfully constructed an expression vector and expressed the exo-inulinase gene isolated from eukaryotic microbe *Aspergillus niger* in prokaryotic microbe *E. coli.* The expression of eukaryotic protein in *E. coli* can be overcome by using a genetically modified *E. coli* strain, Rosetta-gami B (DE3) which can overcome codon bias and provide an oxidizing environment for stable disulfide bond formation. The molecular weight of recombinant exo-inulinase was of expected size. The exo-inulinase and invertase activity, optimum pH, temperature, stability and effect of metal ions were studied on substrates inulin and sucrose. These results would help the researchers to carry out hydrolysis of inulin and sucrose to apply for biofuel and high value compounds production from non-photosynthetic microbes.

In chapter 5, we developed an expression vector and expressed GUS reporter gene in *Chlorella vulgaris.* Also, a new high throughput screening technique was developed. The high throughput screening technique was found to reduce the amount of substrate, buffer, sample, and time needed to carry out the experiment. Also, the time and labor work needed is reduced drastically compared to RT-PCR. In RT-PCR, the transcription (mRNA) is quantified. However, this does not give information about the expression of recombinant protein and its activity. The technique developed here can directly tell whether the enzyme expressed is active and soluble inside the cell. This can be used by researchers for high throughput screening of transgenic microalgae strains grown in liquid culture containing the GUS gene.

In chapter 6, application of the HTS method developed in chapter 5 was applied to *C. reinhardtii.* Compared to chapter 5, where liquid culture was used for screening. In this work, transgenic cells from an agar plate were used to carry out HTS for GUS activity. The technique further reduced the time and labor needed drastically. This can be used by researcher for high throughput screening of transgenic microalgae strains grown in agar plate containing GUS gene.

Overall, during our research work we have developed new expression vectors for expression of exo-inulinase and GUS reporter gene in *E. coli* and fresh water microalgae. To our knowledge, this is the first reported work for expression of *A. niger* 12 exo-inulinase enzyme in *E. coli.* Also, this is the first report on high throughput screening method developed for GUS reporter enzyme in microalgae in both liquid culture and colonies from agar plate.

7.2   Recommendations

Exo-inulinase can be expressed on the surface of cell membrane by using ice nucleation protein or secreted into culture medium by using bacteriocin release protein. This would aid in hydrolysis of inulin to fructose which can be utilized by the cells for its cell growth and production of bio-chemicals. Also, simultaneous expression of exo-inulinase and endo-inulinase on cell surface or secreation into culture medium can be explored.

The GUS gene HTS technique can be used to develop and study the transformation technique in new microalgae species which have not been genetically transformed till date. Similar high throughput screening technique can be developed for expression of Firefly Luciferase in microalgae. Different promoter strength can be analyzed by using

the GUS activity by high throughput screening technique. Effect of inhibitors and inducers on recombinant GUS activity can be studied.

The expression vector pHyg-TX-GUS-INT has three tetracycline operator genes between CaMV 35 promoter and GUS reporter. The transgenic *C. reinhardtii* carrying pBIN-HygTX-GUS-INT gene can be transformed with another vector pBin-TetR that expresses the tetracycline repressor protein, which binds to the tetracycline operator gene to block the expression of GUS enzyme. This can be used developed an inducible system to express GUS gene and recombinant proteins especially proteins that are toxic to the microalgae.

Appendices

## Appendix A: Specification of the UV-Star 96 well microplate

Specification	
Description	UV-Star® Microplate, 96 well, clear film F-bottom (flat), alphanumeric well coding, chimney well
Plate Length	Plate: length: 127,76 mm (± 0,2 mm) width: 85,48 mm (± 0,2 mm) curvature: 200 μm Foil: 135 μm (± 10 μm)
Volume	Total volume: 392 μl (mathematically calculated) Working volume: 25 - 340 μl
Material/ Resin	Plate and foil :Cycloolefine, free of heavy metal
Color	658801 : Clear
Sterilization	No
Quality Control	Raw Material-Control: physical testing Product-Control: testing of attributive and variable characteristics in accordance with the valid specification
Usage	For single use only
Basic Features	Free of detectable DNase/RNase, human DNA and pyrogens
Temperature Range	-20°C to +40°C

|	
Autoclavability	No
Centrifugation, max. RCF	4800 x g: swinging-bucket rotor
Chemical Resistance	See homepage: www.gbo.com/bioscience ®Products ®Literature ®Technical Information ®Chemical Resistance of Resins
Shelf Life	Not applicable

Appendix B: Schematic representation of intermediate vector pUC19+Tx-GUS-INT
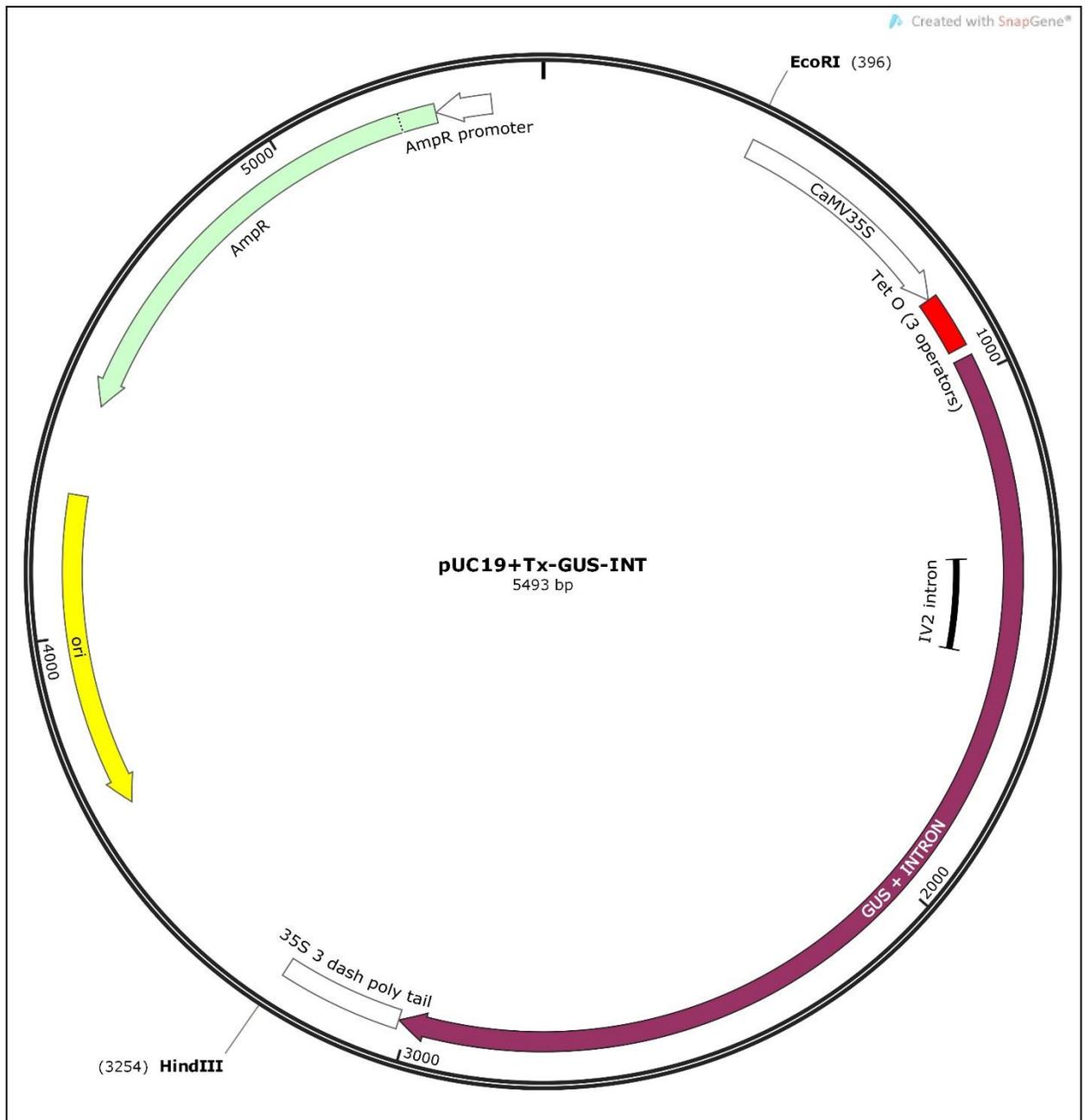
Schematic representation of intermediate vector pUC19+Tx-GUS-INT.

Appendix C: Blue white screening
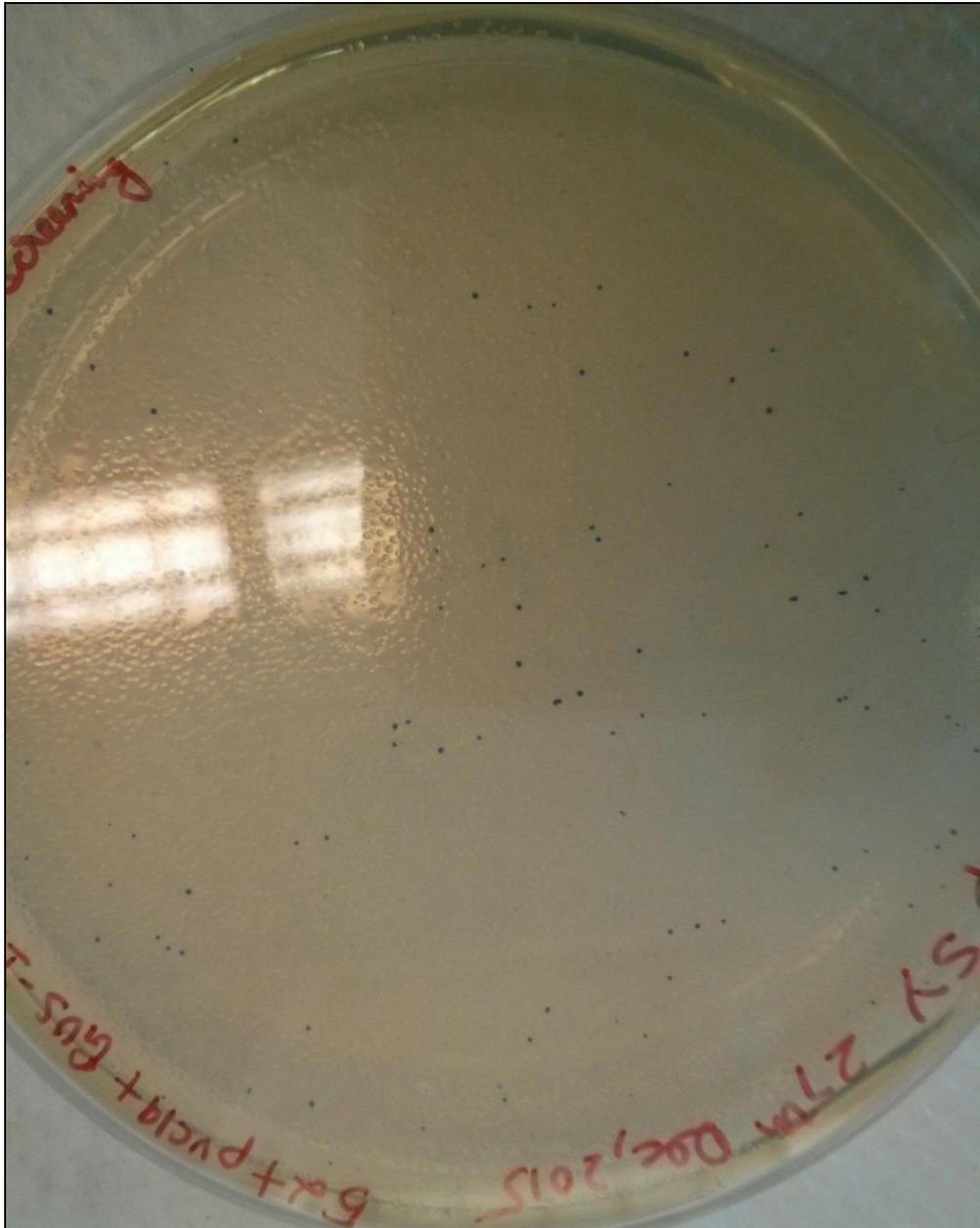
Blue white screening of intermediate vector pUC19+Tx-GUS-INT in DH5α.

**Appendix D:** *A. tumefaciens* **LBA 4404**
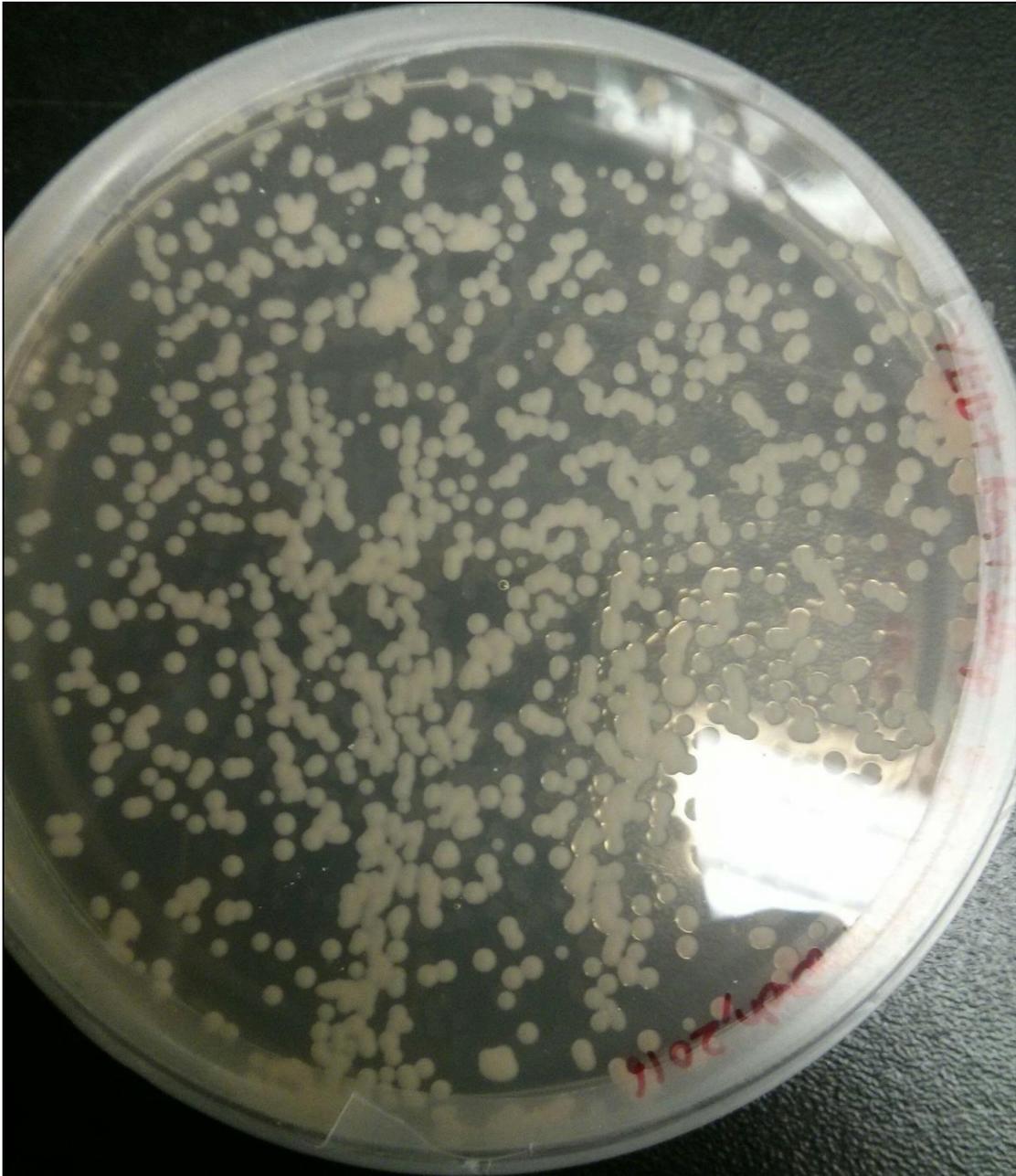
Expression vector pBIN+TetR-TetO transformed into *A. tumefaciens* LBA 4404 by electroporation. The electroplated cells were diluted 10-fold and grown at 30 ^{0}C for 48 hours on YEB agar plate containing streptomycin 100 μg/ml, rifampicin 20 μg/ml, and kanamycin 50 μg/ml

Appendix E: Transformed *C. vulgaris*
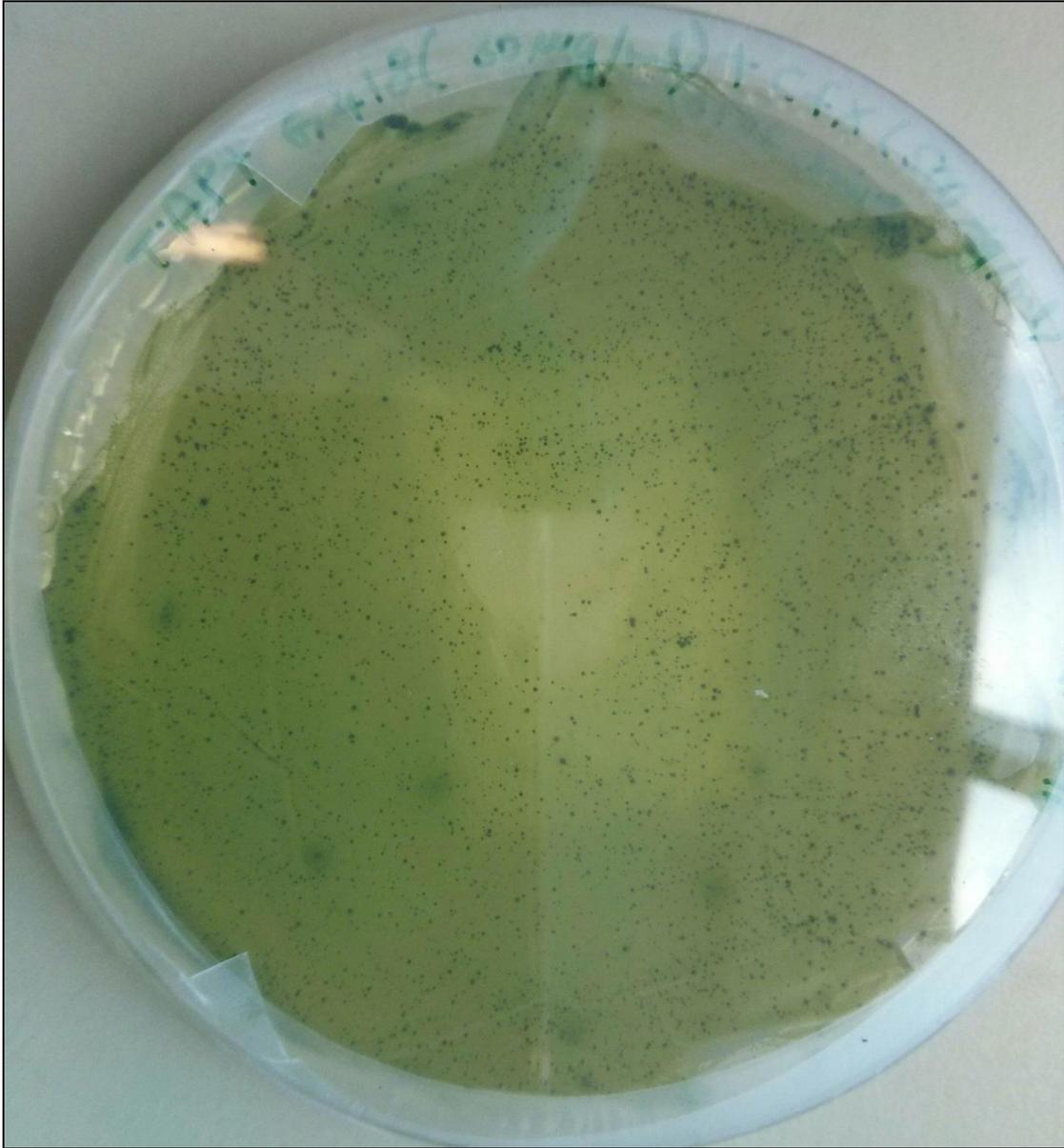
Transformed *Chlorella vulgaris* visible after growing them for 1 month on agar plate containing 500 μg/ml cefotaxomine and 30 μg/ml G418 sulfate. The plate was incubated at 25 ^{0}C, 90 % humid atmosphere, and under 150 μmol m^{-2} s^{-1} white light illumination with 16 h on/8 h off.
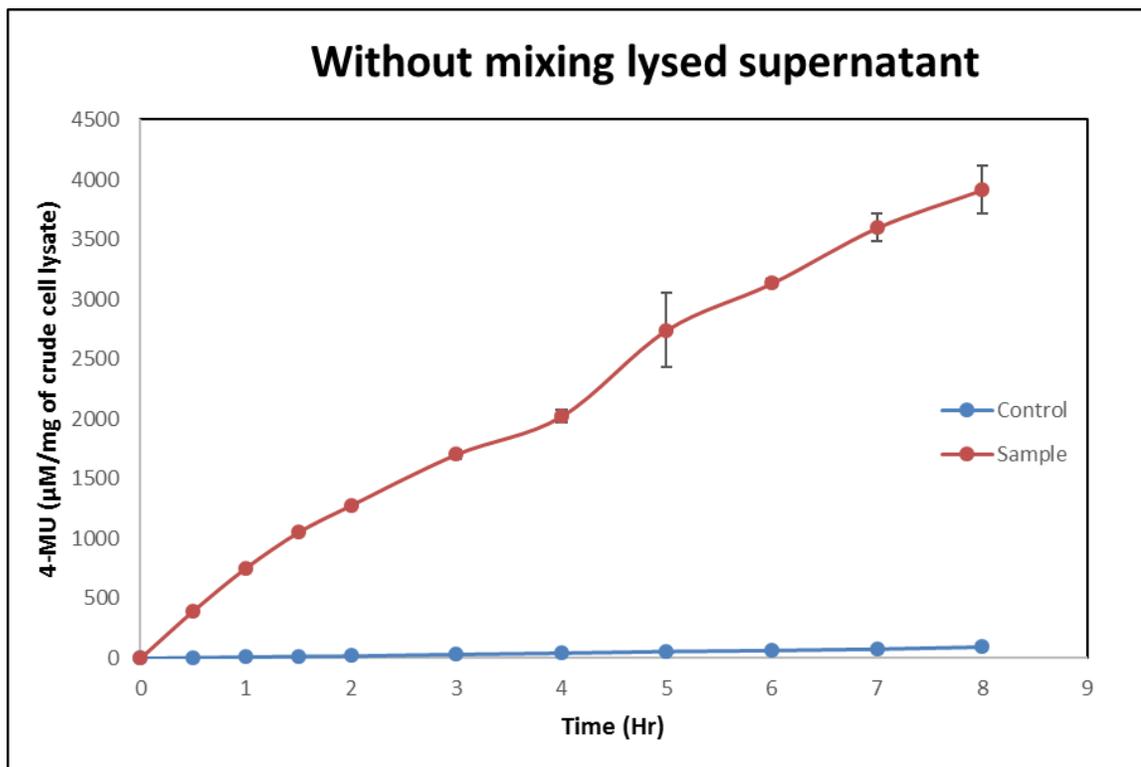
Appendix F: GUS histochemical analysis

GUS histochemical analysis – Control and transgenic *C. vulgaris* culture were incubated in X-gluc solution overnight at 37 ^{0}C and washed with ethanol.

**Appendix G: Intact cell GUS activity without mixing**
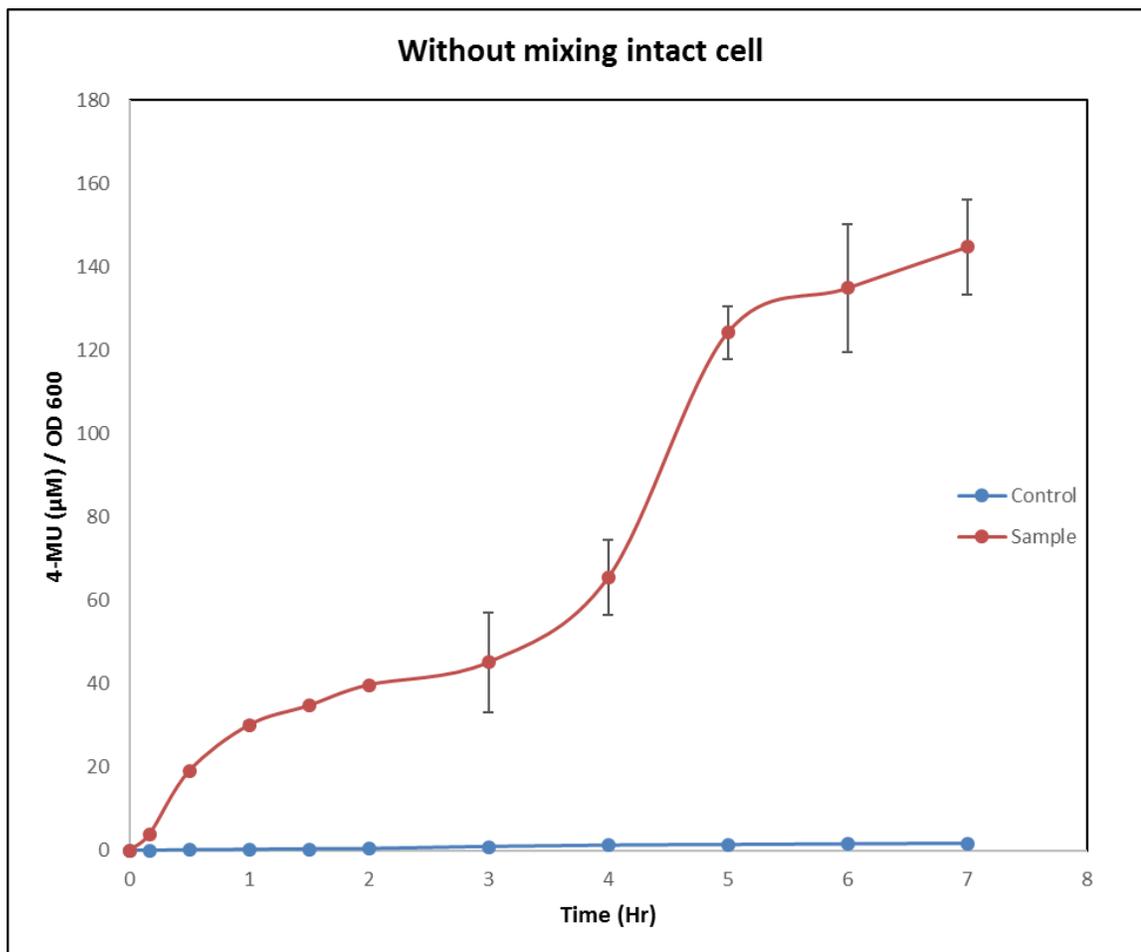
Intact GUS activity without mixing – 150 µl of pelleted cells was suspended in assay buffer in 96 well microplate and incubated at 37 ^{0}C. The reaction was stopped by adding 50 µl Na_2CO_3. At the end of 7 hours, the 4-MU recorded was 144.79 ± 11.45 µM/ OD_{600}
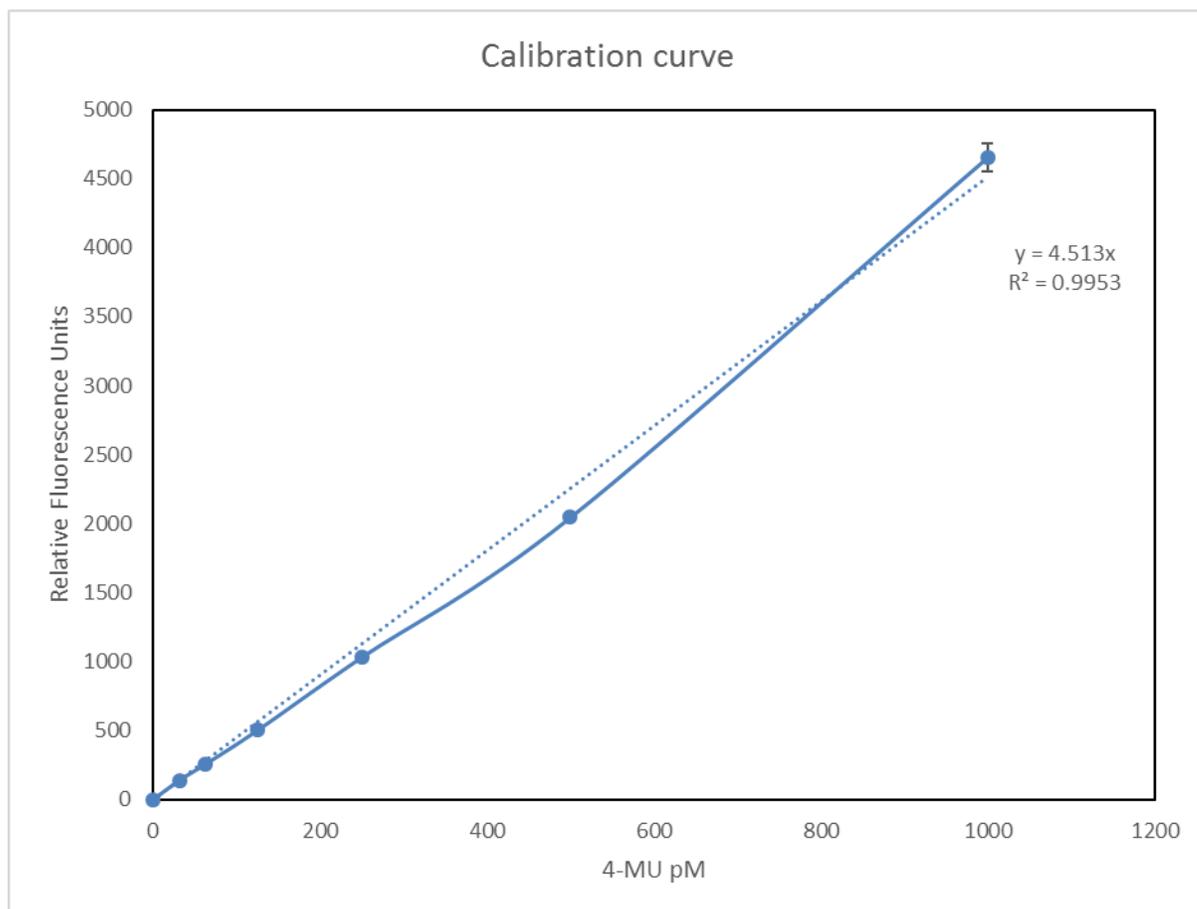
Appendix H: Cell lysate GUS activity without mixing

Cell lysate GUS activity without mixing – 75 µl of cell lysate was mixed with 75 µl of assay buffer in 96 well microplate and incubated at 37 ^{0}C. The reaction was stopped by adding 50 µl Na_2CO_3. At the end of 8 hours, the 4-MU recorded was 3919.69 ±197.07 µM/mg of crude cell lysate.

Appendix I: GUS histochemical analysis
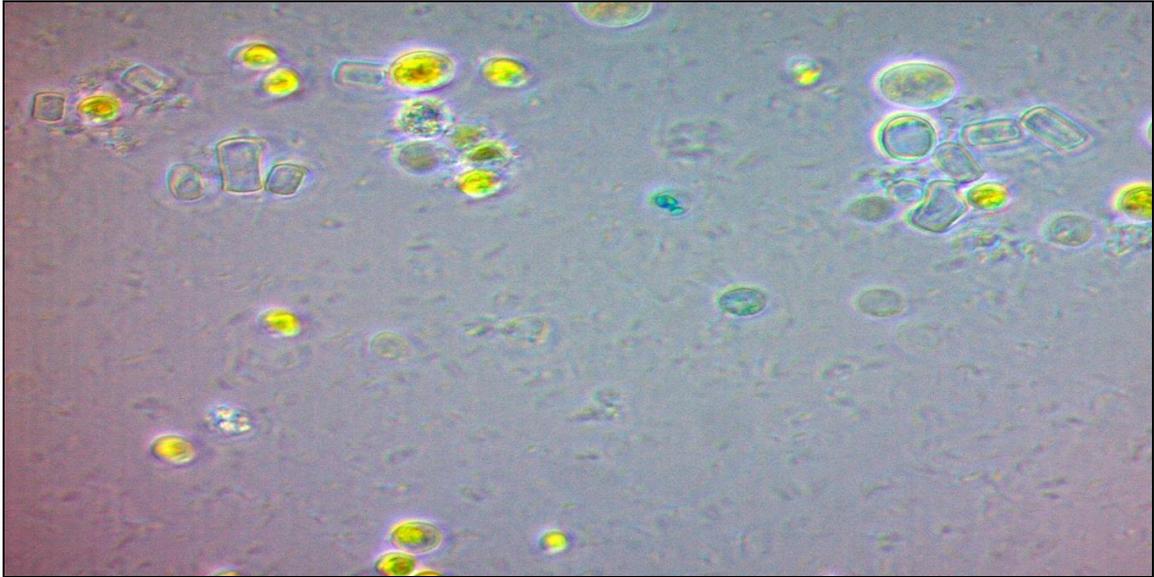
GUS histochemical analysis – The transgenic *C. vulgaris* culture were incubated in X-gluc solution overnight at 37 ^{0}C and washed with ethanol. The cells were observed under the microscope.

Appendix J: Protein calibration curve

Protein calibration curve

y = 0.0016x
$R^2 = 0.9973$

Absorbance 562 nm (y-axis)
Protein concentration (µg/ml) (x-axis)
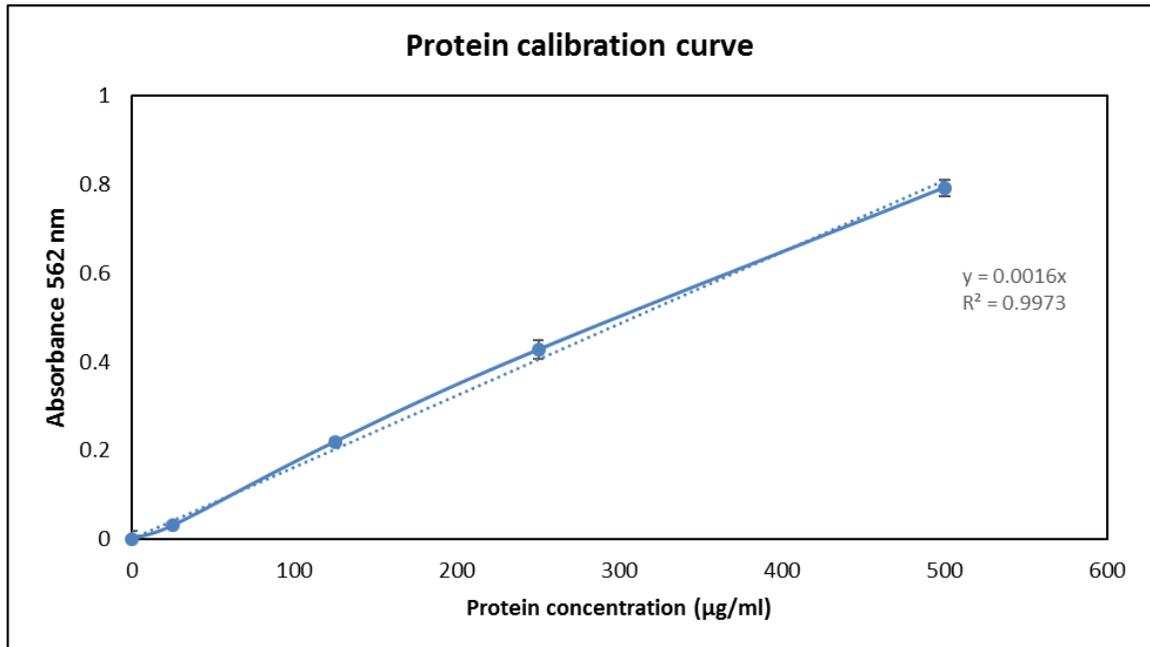
Appendix K: 4-MU calibration curve

Calibration curve

$y = 4.513x$
$R^2 = 0.9953$

Relative Fluorescence Units

4-MU pM

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6th International Conference on Algal Biomass, Biofuels and Bioproducts
26-29 June, 2016 – San Diego, USA

Book chapter:

Mengyue Gong, Yulin Hu, **Shreyas Yedahalli**, and Amarjeet Bassi, Oil extraction processes in microalgae. Accepted for the e-book *Microalgae as a Source of Bioenergy: Products, Processes and Economics. 2017 Bentham eBooks, Bentham Science Publishers*