THE EXPRESSION OF YOEB AND PEZT BACTERIAL TOXIN GENES IN MICROALGA *Chlorella vulgaris* AND THE EFFECTS ON CELL VIABILITY

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ABSTRACT

Chlorella vulgaris is an oleaginous microalgae which is a potential candidate for the harvesting of valuable cellular contents, particularly for the production of biofuels. However, the microalgae's rigid cell wall proved to be a challenge faced during microalgae transformation as well as the downstream harvesting of cellular contents. Toxin-antitoxin (TA) systems are genetic entities that are almost ubiquitous in prokaryotic genomes and have been implicated in programmed bacterial cell death and to date, no TA homologs are found in eukaryotic cells. A TA system is usually made up of two genes, an antitoxin gene that encodes a labile antitoxin, which can either be an untranslated RNA or a protein, and the toxin gene which encodes for the more stable toxin protein. The expression of several TA toxins were reported to be functionally lethal in several eukaryotic organisms such as zebrafish, *Arabidopsis thaliana,* yeast as well as human cell lines. In this study, the utility of a two-component chemical-inducible expression system which was originally developed for the *Arabidopsis* plant system was investigated in the green microalgae, *C. vulgaris* UMT-M1 for the expression of the YoeB$_{Spn}$ and PezT toxin from the Gram-positive bacterium, *Streptococcus pneumoniae.* Both the activator vector, pMDC150 that harbored the constitutive CaMV 35S promoter together with responder vector (pMDC221) cloned with translational fusions of either *yoeB$_{Spn}$-GFP* or *pezT*-GFP, respectively, were co-transformed into *C. vulgaris* UMT-M1 via *Agrobacterium tumefaciens*-mediated transformation. The XVE transcription activator encoded on the pMDC150 vector was constitutively expressed under the control of CaMV 35S promoter. In the presence of 17-β-estradiol as the inducer, an *XVE*-responsive promoter (OlexTATA) readily initiates transcription of the *yoeB$_{Spn}$-GFP* and *pezT-GFP* fusion genes that were cloned separately into the pMDC221 responder vector. Following *Agrobacterium*-mediated transformation, PCR analysis confirmed that the transgenes were present in the transformed *C. vulgaris* lines. Upon 17-β-estradiol

treatment to express the *yoeB_{Spn}-GFP* and *pezT-GFP* fusions, GFP signals were observed in transgenic *C. vulgaris* cells which showed signs of cellular damage and lysis. Expression of the YoeB_{Spn} and PezT toxins greatly affected the cell viability of the transgenic *C. vulgaris* cells. This is the first report demonstrating the simultaneous transformation of two vectors into *C. vulgaris* as well as the functionality of the XVE-based two-component expression system in *C. vulgaris.* This is also the first demonstration of the lethality of bacterial TA toxins in eukaryotic microalgae as evidenced by the morphological changes and cell lysis of transgenic *Chlorella vulgaris* subsequent to the activation of YoeB and PezT toxins. The conditional expression of the bacterial toxin in microalgae can be used to develop novel means to efficiently harvest microalgal cellular contents through the lysis of transgenic microalgal cells triggered by toxin activation upon induction with the appropriate signal.

ABSTRAK

Chlorella vulgaris ialah sejenis mikoralga penghasil-minyak yang merupakan calon potensi untuk pengumpulan kandungan selular yang berharga, terutamanya untuk penghasilan biofuel. Walaubagaimanapun, dinding sel yang tegar terbukti adalah halangan yang dihadapi ketika transformasi mikroalga beserta dengan pemperosesan hiliran untuk mendapatkan kandungan selular mikroalga. Sistem toksin-antitoksin (TA) merupakan entiti genetik yang hampir selalu wujud di dalam genom prokariot dan dikaitkan dengan kematian sel yang telah diprogramkan dan sehingga kini, tiada TA homolog pernah dijumpai di dalam sel-sel eukariot. Sistem TA biasanya terdiri daripada dua gen, satu gen antitoksin yang mengekod antitoksin yang labil, yang boleh menjadi samada RNA tidak diterjemahkan atau protein, dan gen toxin yang mengekod toksin protein yang lebih stabil. Expresi beberapa TA toxin telah dilaporkan berfungsi dalam membawa kemautan kepada beberapa organisma eukariot seperti ikan "zebra", *Arabidopsis thaliana,* yis dan juga titisan sel manusia. Dalam kajian ini, utiliti sistem ekspresi dua komponen yang berdasarkan induksi kimia yang pada mulanya direka untuk sistem tumbuhan *Arabidopsis* telah diuji di mikroalga hijau, *C. vulgaris* UMT-M1 untuk ekspresi YoeB$_{Spn}$ dan PezT toxin dari bakteria Gram-positif, *Streptococcus pneumoniae.* Kedua-dua vektor pengaktif, pMDC150 yang memuatkan pendorong CaMV 35S bersama-sama dengan vektor pembalas (pMDC221) klon dengan gabungan translasi samada *yoeB$_{Spn}$-GFP* or *pezT*-GFP, masing-masing, telah ditransformasikan bersama-sama ke dalam *C. vulgaris* UMT-M1 melalui transformasi *Agrobacterium tumefaciens.* Transkripsi pengaktif XVE yang dikodkan pada vektor pMDC150 itu diekspresikan secara berlanjutan di bawah kawalan pendorong CaMV 35S. Dalam kehadiran 17-*β*-estradiol sebagai induksi, pendorong-responsif XVE (OlexTATA) memulakan transkripsi daripada gen-gen gabungan yoeB$_{Spn}$-GFP dan pezT-GFP yang diklon secara berasingan ke dalam pMDC221 vektor pembalas. Berikutan transformasi *Agrobacterium*,

analisis PCR mengesahkan bahawa transgen hadir dalam sel transgenik *C. vulgaris*. Setelah rawatan 17-β-estradiol untuk mengekspresikan gabungan gen *yoeB$_{Spn}$-GFP* dan *pezT-GFP*, isyarat GFP diperhatikan dalam sel-sel transgenik *C. vulgaris* yang menunjukkan tanda-tanda kerosakan selular dan sel lisis. Ekspresi YoeB$_{Spn}$ dan PezT toksin mejejaskan daya hidup sel-sel transgenik *C. vulgaris*. Ini adalah laporan pertama yang menunjukkan transformasi serentak untuk dua vektor ke dalam *C. vulgaris* serta fungsi sistem ekspresi dua komponen yang berdasarkan XVE dalam *C. vulgaris*. Ini juga demonstrasi pertama kemautan yang disebabkan oleh bakteria toksin TA dalam mikroalga eukariot berdasarkan kepada perubahan morforlogi dan lisis sel-sel transgenik *C. vulgaris* selepas ekspresi YoeB$_{Spn}$ dan PezT toksin . Ekspresi bersyarat toksin bakteria dalam mikroalga boleh digunakan untuk membangunkan kaedah novel yang efisien untuk pengumpulan kandungan selular microalgal melalui lisis sel transgenik microalgal oleh pengaktifan toksin apabila isyarat induksi yang sesuai diaplikasikan.

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LIST OF SYMBOLS AND ABBREVIATIONS

AmpR	Ampicillin resistance gene
AS	Acetosyringone
BBM	Bold's Basal Medium
bp	base pairs
$CaCl_2$	Calcium chloride
CaMV 35S	Promoter of the cauliflower mosaic virus gene
$^\circ$C	Degrees Celsius
CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
$\times g$	G-force
GFP	Green fluorescent protein
GFP	Green fluorescent protein gene
gus	*β*-glucuronidase gene
hpt	Hygromycin phosphotransferase gene
IM	Induction Media
Kan^R	Kanamycin resistance gene
kb	Kilo base pairs
kV	kilo volts
LB	Luria-Bertani Medium
µg	Microgram
µL	Microliter
mg	Milligram
mL	Milliliter
mM	Milimolar

ng	Nanogram
nos	nopaline synthase gene
*O*lexA TATA	XVE-responsive promoter
OD$_{260}$	Optical density at 260 nm
OD$_{280}$	Optical density at 280 nm
OD$_{600}$	Optical density at 600 nm
%	Percentage
PCR	Polymerase chain reaction
PPF	Photosynthetic photon flux
rpm	Revolutions per minute
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase-polymerase chain reaction
TA	Toxin-antitoxin
TAs	Toxin-antitoxin system
TAE	Tris-Acetate-EDTA
T-DNA	Transferred DNA
w/v	weight/volume
V	Volt

CHAPTER 1:

INTRODUCTION

Chlorella vulgaris is a unicellular eukaryotic microalga that shares many similar characteristics with plants such as capturing sunlight for photosynthesis and aiding in the capture of global carbon dioxide (Mallick *et al.*, 2012). Under optimal condition, *C. vulgaris* undergoes rapid cell division, doubling in 24 hours (Safi *et al.*, 2014). Owing to their ubiquitous nature, this microalga can easily thrive in natural waters such as ponds or lakes and even in wastewater ponds.

Since *C. vulgaris* is extensively favored for its potential for the production of several forms of bioenergy, many studies have focused on garnering the optimal cellular contents for downstream processing as reviewed in Jones & Mayfield (2012). Nonetheless, issues such as, expensive set–ups and equipment as well as the prerequisite to degrade the cell wall that encompasses the microalgal cellular content (Gerken *et al.*, 2013) need to be overcome for the venture to be financially viable. If the cell content release can be induced at a molecular level, the downstream processing can then completed in a shorter time with reduced effort. The expression of bacterial toxin that belong to toxin-antitoxin system were reported to attack cellular target and cause cell lysis in various types of organism, thus leading to death. The ability of bacterial toxin to induce cell lysis can be utilized in eukaryotic microalgae to develop a method that can boost the rapid release of cell contents into the surrounding environment without the requirements of enzymatic pre-treatment or expensive equipment.

Toxin-antitoxin systems are small genetic entities found in almost all prokaryotes that function as stress regulators and are associated with the regulation of bacterial cell death. Prokaryotes are generally exposed to various stress triggers in their environment. Harboring TA systems confers a survival advantage to the bacterial populations by forming biofilms and persister cells (Yamaguchi & Inouye, 2011). The mechanism of TA

relies heavily on the differential stability of the two components, namely the stable toxin and the labile cognate antitoxin (Yamaguchi *et al.*, 2011). In general, the antitoxin is produced abundantly to bind and neutralize the toxin under normal growth conditions. However, when bacterial cells are under stress, endogenous proteases that degrades the antitoxin proteins are expressed causing the liberation of its cognate toxin from the complex. The toxin will act on its specific cellular targets, often occasioning in cell lysis and death. TA systems were originally found encoded in plasmids and were elucidated to be involved in the maintenance of plasmid prevalence (Yarmolinsky, 1995). The remaining stable toxins, following cell division will destroy the daughter cells that did not inherit the TA genes. This phenomenon is also known as post segregational killing and ensures that the plasmid is maintained among the bacterial populations (Yarmolinsky, 1995). TA systems were also designated "addiction modules" as their presence enabled the bacterial host to become addicted to the plasmid which contained them (Engelberg-Kulka & Glaser, 1999). With the discovery of chromosomally-encoded TA systems, their diversity greatly expanded and along with their increasing diversity, various cellular functions have been ascribed to them. TA systems are currently classified into six different types (types I – VI) depending on the type and mode of action of the antitoxin for example, in the type II TAs, where both antitoxins and their toxin partners are proteins, are predominant. Antitoxins of type II TAs generally function to neutralize the lethality of their cognate toxins by tight binding, occluding the toxin's active site and forming an inactive toxin-antitoxin complex (Chan *et al.*, 2016). Type II toxins target various essential components of the cellular machinery, with majority of type II toxins discovered so far functioning as endoribonucleases that target either mRNA or tRNA, thus disrupting the cellular translational apparatus (Yamaguchi *et al.*, 2011). Other toxin targets include DNA gyrase, elongation factor EF-Tu, and synthesis of the cell wall peptidoglycan layer (Mutschler *et al.*, 2011). Chromosomally-encoded TA systems have been linked with

programmed bacterial cell death where under stress conditions such as nutrient depletion, a portion of cells in a bacterial population will trigger activation of the toxins, leading to an altruistic cell death, killing and releasing nutrients that enable the rest of the population to survive (Engelberg-Kulka & Glaser, 1999). TA systems have also been implicated in the cell stress response, a form of cellular stasis state known as persistence, formation of biofilms, maintenance of various mobile genetic elements in the bacterial genomes, as an anti-phage infection system, and in the virulence of several pathogenic bacteria (Hayes & Van Melderen, 2011)

To date, no eukaryotic homologue of bacterial TA systems has been reported but some bacterial TA toxins have been shown to be functional in eukaryotic cells. For instance, the expression of the Kid toxin from the Kis-Kid TA module was found to cause cell death in zebrafish, frog embryo cells and human cells (de la Cueva-Mendez *et al.*, 2003). The induction of Kid toxin in zebrafish generates a genetically modified zebrafish lines that aids in differentiating the wildtype among the transgenic populations. The co-expression of the Kis-Kid TA genes and the desired transgene offered a positive selection of transgenic mammalian cells (Nehlsen *et al.*, 2010). The *Escherichia coli*-coded RelE toxin was found to be exceedingly toxic to the yeast *Saccharomyces cerevisiae* and the TA gene was engaged as part of containment study to avoid accidental release of the transgenic yeasts into environment (Andreev *et al.*, 2008). The functional expression of these bacterial TA systems in eukaryotic cells has led to various applications such as a potential containment system for genetically modified yeast (Andreev *et al.*, 2008), novel specific cell ablation system (Slanchev *et al.*, 2005) as well as potential antiviral and anticancer gene therapy as reviewed in Chan *et al.* (2012).

It was recently demonstrated that expression of the YoeB$_{Spn}$ toxin was lethal in the model plant *Arabidopsis thaliana* (Abu Bakar *et al.*, 2015). A two-component XVE-based expression system comprising of an activator vector and a responder vector (Brand *et al.*,

2006) was utilized with the inducer 17-*β*-estradiol to express the cloned *yoeB_{Spn}* transgene (Abu Bakar *et al.*, 2015). Until now, there has yet to be any report on the heterologous expression of bacterial TA toxins in eukaryotic microalgae. In this study, the same two-component XVE-based expression system (Abu Bakar *et al.*, 2015) was used to investigate the functionality of the YoeB_{Spn} as well as PezT toxin from *S. pneumoniae* in the green eukaryotic microalga, *C. vulgaris* UMT-M1. The lethality of the bacterial YoeB_{Spn} and PezT toxins in *C. vulgaris* would ultimately contribute to a novel method of cellular content-harvesting for transgenic microalgae. To date, the rigidity of microalga cell wall has been reported to impede the harvesting of cell content due to the pre-requisition for cell wall degradation (Mussgnug *et al.*, 2010). However, the use of bacterial YoeB_{Spn} as well as PezT toxins are predicted to induce a rapid cell lysis in microalgal cells which will in turn release the valuable cell content. The toxin-induced lysis can minimise the harvesting efforts in terms of cost and also time. Accidental escape of transgenic microoganism to the environment is a major concern. The use of XVE-based expression system can induce a controlled expression of the bacterial toxin whenever necessecary to lyse the transgenic microorganism thus preventing them from being released to the surrounding environment. The XVE-based expression system allows flexibility and control of the transgene expression and has potential to be developed into a gene containment system for transgenic organisms. There are many applications that can be developed using this system and this leads to the aims of this research as follows:

1. To produce stable transgenic *C. vulgaris* by co-transforming activator vector encoding the XVE chimeric transcription activator and responder vector harboring the bacterial *yoeB_{Spn}* and *pezT* toxin genes using *Agrobacterium tumefaciens*-mediated transformation.

2. To investigate the lethality of the YoeB_{Spn} and PezT bacterial toxins in *C. vulgaris* using the XVE inducible system.

CHAPTER 2:

LITERATURE REVIEW

2.1 Microalgae

2.1.1 Introduction

Microalgae are an extremely diverse group that comprises many thousands of known species. The origins of microalgae can be outlined back to the first establishment of a culture of *Chlorella* by Beijerinck in 1890 (Tomaselli, 2003). A classification system proposed by Lee (1989) separated the current algal division into four specific groups (Tomaselli, 2003; Khan *et al.*, 2009). The first group includes the prokaryotic algae, Cyanobacteria and Prochlorophyta while the following three groups are classified according to the characteristics of their chloroplast. The second group includes Glaucophyta, Rhodophyta and Chlorophyta which have chloroplasts surrounded by two chloroplast membranes. On the other hand, the members of the third and fourth groups have chloroplasts surrounded by one (Dinophyta and Euglenophyta) or two extra endoplasmic reticulum membranes (Cryptophyta, Chrysophyta, Prymnesiophyta, Bacillariophyta, Xanthophyta, Eustigmatophyta, Raphidophyta and Phaeophyta), respectively.

2.1.2 Significance of microalgae as a sustainable fuel source

With the ever-expanding population and increasing economic activities, there is an inevitable advance in the utilization of fossil fuels. Petroleum, which is partially derived from ancient deposits, is a limited supply that will ultimately come to an end (Kerr, 2011). The challenge now is to develop renewable energy that is sustainable and to supply the demands of the growing population. Over the years, oils from terrestrial plants, such as palm and soy, have been utilised to produce biodiesel (Tomei & Upham, 2009; Johari *et al.*, 2015). This strategy is operative on a small scale but they are not maintainable when

usage is increased, as extra agricultural land will be required to revenue significant amounts of biodiesel (Laurance *et al.*, 2014).

Microalgae-based fuels are now considered as a prospective alternative fuel source. Many microalgae species possess novel metabolic features that can be exploited for the production of biofuels. Microalgae are ubiquitous as they can propagate in a wide range of environments, from freshwater to saline water (Mata *et al.*, 2010). The very promising trait of microalga-based biofuel is the high growth rate in which microalgae biomass can be rapidly generated, some species doubling in as little as 3.5 hours during exponential growth (Taher *et al.*, 2011).

Microalgae have the ability to naturally store high oil levels by weight of dry biomass (Rodolfi *et al.*, 2009). *Botryococcus braunii* was cultivated as a renewable source of liquid fuel, owning to the high hydrocarbon content that is up to 50% of its dry biomass (Eroglu *et al.*, 2011). Besides that, under nitrogen starvation, *C. vulgaris* was demonstrated to accumulate high lipid content that resulted in a favorable fatty acid profile for biodiesel production (Yeh & Chang, 2011). Owing to high biomass productivity, rapid lipid accumulation and high carbohydrate storage capacity, microalgae are considered as highly attractive candidates for the production of biofuel.

Microalgae are sunlight-driven and efficiently consume CO_2 during photosynthesis which can significantly help in the CO_2 fixation (Sydney *et al.*, 2010). Besides that, microalgae duplicate by cell division, hence, have additional advantage over terrestrial plants, as the duration of microalgae replication only take hours compared to weeks and up to years in higher plants. Microalgae have also been regarded as bioremediator of wastewater and used agricultural land (Pittman *et al.*, 2011; Gressler *et al.*, 2014) as they are more effective, more cost-efficient and provide a safer approach than physical or chemical remediation methods (Christenson & Sims, 2012; Fathi *et al.*, 2013). The production of bio-energy from microalgae is of major significance as this can in the long

run replace the use of agricultural terrestrial plants and production can be done at a large scale (Laurance *et al.*, 2014). Relative to terrestrial plants, microalgae also have higher biomass productivity to land area ratio (Fathi *et al.*, 2013). In addition, there has also been significant advances in the genetic engineering of microalgae resulting in the introduction of specific traits to produce valuable transgenic microalgae strains as likely candidates to produce biofuels (Radakovits *et al.*, 2010).

To make microalgae an economically feasible platform to counterbalance petroleum, several hurdles still need to be overcome. Nutrient requirement for microalgae growth is not an issue of concern since they are very competent in sequestering these nutrients when present in the natural environment (Marchetti *et al.*, 2009). However, like terrestrial plants, microalgae require essential nutrients such as nitrate, phosphorus and sulfate, which are the main constituents of fertilizers. It is a worry that massive microalgae cultures that are necessary for large-scale commercial biofuel production would necessitate the need for financially untenable supplies of such nutrients. In addition, one of the other impediments for using microalga-based fuel is that the downstream processes for oil extraction are relatively expensive (Krichnavaruk *et al.*, 2008). Moreover, microalgae cultures are also at risk of invasion by pests and pathogens and hence, crop protection is vital for microalgae culture sustainability. On the plus side, some microalgae exhibit chemical defenses such as secretion of antimicrobials (Desbois & Smith, 2010) or toxic exopolysaccharides (Mandal *et al.*, 2011) into the surrounding environment that help mitigate the risk of pathogen infection.

2.1.3 Production of microalga-based fuel

The organic materials produced by microalgae namely carbohydrates, lipids and proteins, could positively impact the economics of bioenergy in future (Figure 2.1). The most common molecules are the fatty acids that are naturally produced in microalgae and have potential as biofuel feedstock. Microalgal fatty acids chains range between 12-22

hydrocarbons and include a combination of saturated and non-saturated fatty acids. A few microalgae species accumulate longer hydrocarbon chains in their fatty acid molecules (Niehaus *et al.*, 2011). Microalgae yield as high as 50-60% of lipid storage which upon trans esterification, can be a sustainable source of biodiesel (Griffiths & Harrison, 2009).

Sugar crops such as sugarcane and sugar beet and the by-product molasses contribute to 61% of bioethanol production through fermentation (Halim *et al.*, 2013). However, there are setbacks to using these as feedstock as these crops are grown on restricted lands and require high amount of fertilizers which can lead to soil erosion. Microalgae have been recommended as prospective feedstock for bioethanol production. The high composition of carbohydrates in microalgae can be converted to bioethanol via anaerobic yeast fermentation (Hannon *et al.*, 2010). The equipment set-up for microalga-based bioethanol is simpler and can effectively reduce the cost. The faster replication time of microalgae enabled microalgal biomass to be harvested in a shorter time frame and hence, could supply the bioethanol demand better when compared to using plant feedstock (Halim *et al.*, 2013). Moreover, the by-products or residual microalgal biomass can be utilized as fertilizer, thus minimalizing waste products (Harun *et al.*, 2009). Since microalgae are not lignified, there is no need to allocate the cost for degradation of the rigid hemicellulose layer (Choi *et al.*, 2010), unlike in the case of using lignocellulosic feedstock that are also currently explored for bioethanol production.

While harvesting endogenous lipids is the most promising avenue from microalgae biofuel production, the production of microalgae-based hydrogen has also been widely discussed (Rumpel *et al.*, 2014). The production of biohydrogen using *C. vulgaris* was deemed attractive as it requires only solar energy and can be used as gas fuel for electricity generation (Bala Amutha & Murugesan, 2011). Biohydrogen is considered attractive to be used as gas fuel for electricity generation because of its high conversion efficiency, non-polluting nature and high energy content (Balat, 2008). Photo-autotrophic organisms

such as green microalgae or cyanobacteria generate hydrogen through biophotolysis in which water serves as the electron donor (Srirangan *et al.*, 2011). Biophotolysis requires only the freely available solar energy and CO_2 and is anticipated to be a practical approach for hydrogen production. *C. vulgaris* MSU 01 strain was demonstrated to be involved in hydrogen production by utilizing its anaerobic fermentation process (Bala Amutha & Murugesan, 2011). The study applied different source of nutrients in the growth media to establish an optimal condition for *Chlorella* hydrogen production. Based on the gas chromatography results, the highest hydrogen production was obtained from the *Chlorella* culture grown on the growth media supplemented with optimal corn stalk concentration (Bala Amutha & Murugesan, 2011).

Biogas is the by-product of anaerobic digestion of organic matter and with methane (CH_4) constituting 55-75% of biogas. Biogas has been regarded with growing interest as a renewable energy source to generate heat and electricity. There are two options in obtaining biogas production from microalgae. First, microalgae will be used for biofuel production and the microalgae residues will then be used as substrate for anaerobic digestion that will justify the high nitrogen and phosphorus requirement for biofuel-producing microalgae growth. Biogas has been produced as renewable energy from *Botrycus braunii* with 30% lipid content (Neumann *et al.*, 2011). The second option involved using the whole microalgae for biogas production. The organic matter of microalgae such as carbohydrates, lipids and proteins, will be digested and transformed into methane and carbon dioxide (Zamalloa *et al.*, 2011). Mussgnug *et al.* (2010) concluded that methane gas from *Chlamydomonas reinhardtii* can be 7-13% higher compared to biogas from crop plants like maize. To date, biogas production using microalgae biomass still has limitations that need to be addressed for example, the space required for the set-up for the equipment (Collet *et al.*, 2011) and the breakdown of the thick microalgae cell wall (Mussgnug *et al.*, 2010). Besides that, the elimination of CO_2

to remove impurities in order to obtain a higher calorific value of biogas is also an issue. Meier *et al.* (2015) suggested using photosynthetic microalgae as a feasible alternative of biogas upgrading which can also directly reduce CO_2 emissions into the environment.
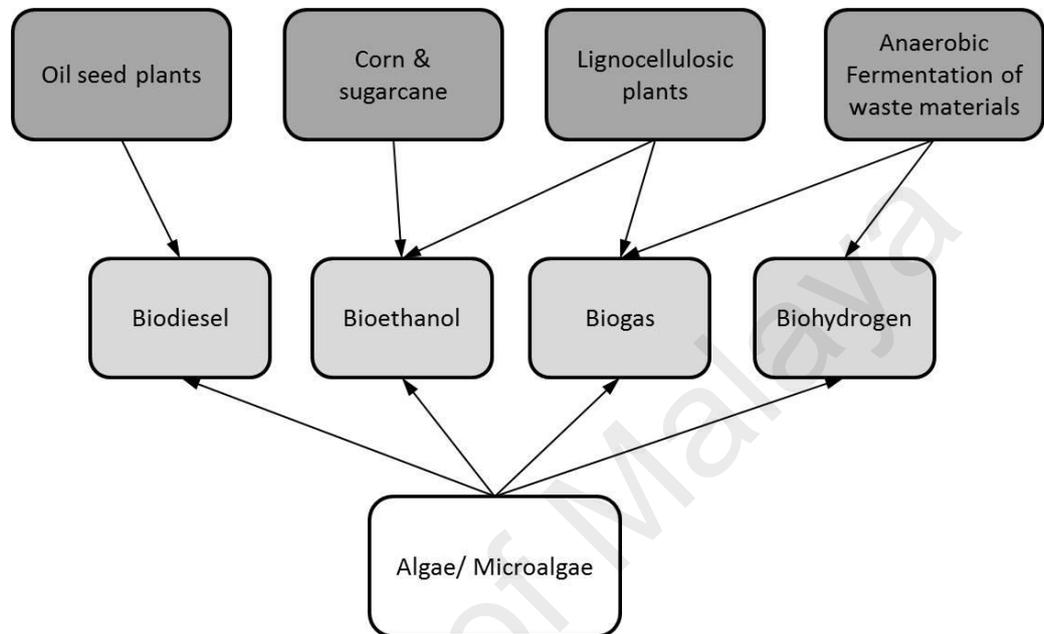
Figure 2.1: The bio-energies that can be harvested from the organic matters of microalgae (Pittman *et al.*, 2011).

2.1.4 Genetic engineering of microalgae

Several microalgae species have been established as model systems for studying fundamental cellular processes such as photosynthesis, flagella function, photoreception and nutrient accumulation (Harris, 2001). Microalgae have also been suggested for the expression of heterologous proteins (Franklin & Mayfield, 2004; Walker *et al.*, 2005). Successful nuclear transformation of microalgae species has been established in three eukaryotic microalgae groups, including chlorophytes (Bogaert *et al.*, 2004; Cha *et al.*, 2012; Neupert *et al.*, 2012), diatoms (Zhang & Hu, 2014) and dinoflagellates (ten Lohuis & Miller, 1998).

The foundation of microalgae transformation is to induce temporary permeabilization of the cell membrane which will enable foreign DNA molecules to enter the host cell. A successful transformation involves the targeted cells surviving the temporary destruction of the cell membrane and DNA insertion, followed by the perpetuation of cell division. An assortment of transformation methods have been applied for different microalgal species. Each of the transformation approaches has advantages and restrictions. The choice of transformation method should be carefully evaluated in terms of species, parameter optimization and equipment availability.

2.1.4.1 *Agrobacterium tumefaciens*-mediated transformation of microalgae

The phytopathogenic soil bacterium *A. tumefaciens* transforms plants by integrating T-DNA from the *Ti* (tumor inducing) plasmid into the nuclear genome of plant cells (Tzfira & Citovsky, 2006). This bacterium is capable of transforming a wide spectrum of plants and also eukaryotic species from the fungi *Blastocladiella emersonii* (Vieira & Camilo, 2011) to even human cells (Tzfira *et al.*, 2006). *Agrobacterium*-mediated transformation was initially applied in dicotyledonous plants which are the natural host for this pathogen, but soon was employed among monocotyledonous plants as well (Zhang *et al.*, 2013). The gene copies introduced via *Agrobacterium*-mediated

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transformation is usually low and therefore reduces the occurrence of gene silencing (Qin *et al.*, 2012).

The first successful *Agrobacterium*-transformation of green algae was reported by Kumar *et al.* (2004). In comparison to a glass bead method of transformation, a 50-fold increase of transformation frequency was observed when *Agrobacterium*-mediated transformation was used to transfer T-DNA harboring *gfp, uidA* and *hpt* transgenes into the *Chlamydomonas* genome. Following that, protocols to transform the model microalgae *Chlamydomonas reinhardtii* were improved and the *hpt* and GUS gene were sucessfully amplified from the transgenic microalgae cells (Pratheesh *et al.,* 2014).

Dunaliella bardawil which was previously resistant to this method of transformation due to its halophilic nature, has also been successfully transformed using *A. tumefaciens* (Anila *et al.*, 2011). High salt in the media culture of *D. bardawil* can considerably diminish the antibiotics efficacy to inhibit the growth of these microalgae, hence, Anila *et al*. (2011) optimized the salt concentration which permitted the growth of both *D. bardawil* and *A. tumefaciens* during co-cultivation. During the selection of resistant transformants, the salt concentration was gradually increased to 1.0 M. Southern hybridization results showed that the transgene were stably integrated in *D. bardawil* even up to 18 months after transformation (Anila *et al.*, 2011).

Transgenic *Schizochytrium* colonies have also been positively generated using *Agrobacterium*-mediated transformation. The diatom *Schizochytrium* sp. is known to produce large amounts of oil in which 45% of the fatty acid is composed of docosahexaenoic acid (DHA) (Cheng *et al.*, 2011). The sucessful genetic transformation of *Schizochytrium* allowing exploration of biomass and lipid production of this diatom.

Cha *et al.* (2012) reported the optimization of factors affecting *Agrobacterium*-mediated transformation of the green algae *Chlorella vulgaris.* Six critical parameters (pre-culture duration, co-cultivation duration, co-cultivation temperature, co-cultivation

medium pH and the use of acetosyringone during co-cultivation) influencing the transfer of *A. tumefaciens* T-DNA were tested and optimized in this study. The successful establishment of *Chlorella* transformants was verified by the detection of GUS and GFP signals along with the PCR molecular characterization of the integrated selectable marker gene. Interestingly, the use of optimal acetosyringone concentration during co-cultivation enhanced gene expression while *Chlorella* cells cultivated on acetosyringone-free medium exhibited no GUS expression (Cha *et al.*, 2011). Plant phenolic compounds such as acetosyringone are essential for stimulating bacterial attachment as the *vir* gene function will be boosted during *Agrobacterium*-mediated transformation (Gelvin, 2000).

2.1.4.2 Glass bead transformation of microalgae

Glass bead transformation is a simple technique to introduce DNA into the targeted organism. Using a vortex mixer, cells of the targeted organism are agitated with glass beads together with DNA to induce a state of competency in the cell wall to uptake the foreign DNA. The glass bead method, which was originally developed for yeasts (Costanzo & Fox, 1988), had been reportedly used to successfully transform microalgae cells with a high transformation efficiency (Kindle, 1990). One of the advantages of the glass bead method is that there is no prerequisite for expensive apparatus, such as for electroporation. The transformation efficiency of a vector carrying the *GUS* gene in *Dunaliella salina* using this method was related to the rotation speeds, agitation periods, concentrations of PEG and plasmid DNA concentration (Feng *et al.*, 2009). However, the cells have to be enzymatically treated before glass bead transformation to ensure a successful DNA uptake by the cell. (Kindle, 1990).

2.1.4.3 Electroporation-mediated transformation of microalgae

Electroporation involves the formation of transient holes in the cell membrane for DNA to pass through when electrical pulses are applied. This method has been broadly

tested on animal, plant, microalgae and bacterial cells. As with glass bead transformation methods, the cell wall has to be degraded using enzymatic treatment or zinc-containing metallo-protease gametolysin to achieve high transformation efficiency in microalgae (Kubo *et al.*, 2001). However, cells with no cell wall are mostly fragile and the degradation process can be time-consuming. Yamano *et al.* (2013) transformed *C. reinhardtii* via electroporation without the need to remove the cell wall. Using an electroporator, the microalgae cells were transformed with three-step multiple electroporation pulse and higher transformation efficiency with alleviated cell damage were achieved.

Electroporation in the diatom *Phaeodactylum tricornutum* produced the maximum number of transformants using a linearized vector with salmon sperm DNA integrated as carrier DNA and the electric field strength was optimized at 0.5kV (Zhang & Hu, 2014). Four different parameters (plasmid concentration, osmolarity, pulse duration and voltage) were optimized in the electroporation transformation of *Scenedesmus obliquus* to obtain a high number of transformants (Zhang & Hu, 2014). External factors such as temperature, osmolarity, electric conditions, field strength, and pulse time of discharge and DNA concentrations have to be well-controlled for a successful electroporation. The drawback of electroporation is that it typically results in extremely inconsistent transgene copy numbers and low copy transformants.

2.1.4.4 Micro particle bombardment

Micro-particle bombardment, or biolistics, is an approach whereby small gold or tungsten particles are coated with DNA, then literally shot into the host cells by helium driven gun (Coll, 2006). This method has been established for the transformation of several microalgae species which were recalcitrant to other transformation methods (Johnson *et al.*, 2007; Leon & Fernandez, 2007). However, particle bombardment is not widely preferred due to the difficulty in generating transformants and the expensive

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laboratory equipment required. The biolistics approach often leads to transformation of large number of gene copies into the microalgae genome that eventually results in gene silencing (Muskens *et al.*, 2000). Ideally, the amount of cassette DNA to be integrated should be precise to achieve single copy number integration (Lowe *et al.*, 2009). Microalgae with intact cell-walls that have been effectively transformed using biolistics include *C. reinhardtii* (Kindle *et al.*, 1989) and diatoms specifically, *P. tricornutum* (Apt *et al.*, 1996)*, Cyctocella cryptica* (Dunahay *et al.*, 1995) and *Navicula saprophila* (Dunahay *et al.*, 1995). More recently, the volvocine green algae *Eudorina elegans* was successfully co-transformed using biolistic with two vectors carrying the gene of interest (aminoglycoside 3′-phosphotransferase VIII (*aph*VIII) gene of *Streptomyces rimosus*) and a selectable marker (luciferase (*gluc*) gene). Both transgenes were reported to be stably incorporated in the *E. elegans* genome with efficient heterologous genes expression (Lerche & Hallmann, 2013).

2.1.4.5 Difficulties with the production of genetically modified microalgae

Promoter selection is essential in genetic transformation to guarantee efficient expression in microalgae expression systems. Generally, strong constitutive or inducible, and preferably, endogenous promoters are obligatory for efficient expression of transgenes (Rosa *et al.*, 2004). The cauliflower mosaic virus (CaMV 35S) and Simian virus 40 (SV 40) promoters are commonly used although they do not contain any endogenous genetic material of transformed hosts (Wang *et al.*, 2010; Anila *et al.*, 2011). For any microalgae expression system, endogenous promoters are still considered to be the most suitable and efficient promoters (Leon & Fernandez, 2007). For instance, the endogenous promoter of the plastid-targeted protein Rubisco SSU (*rbcS*) from *Lotharella amoebiformis* was isolated and effectively functional in transgenic studies (Hirakawa *et al.*, 2008). Stable transformation of *D. salina* was also achieved using a duplicated carbonic anhydrase 1 (*DCA1)* endogenous promoter (Li *et al.*, 2010).

At times, protein production in microalgae was unsuccessful at times due to codon differences in the transformed host or because the genes encoding these proteins may have inadequate expression regulatory sequences that cannot be recognized in the host organism. This is problematic when transferring exogenous DNA into marine microalgae which will cause a significant change in the translational and expression rates. Potvin & Zhang (2010) mentioned that codon usage optimization can increase transgene expression and diminish the rates of gene silencing. Codon optimization of marine microalgae before genetic transformation is important and is gradually becoming a common practice especially when dealing with *de novo* DNA synthesis (McArthur & Fong, 2010).

The selectable marker is an important tool employed in heterologous gene expression to discriminate the transformed cells from a population that are non-transformed. Genes that confer antibiotic resistance in cells are the usual choice for the selection of microalgae transformants. However, there are escalating concerns of antibiotic safety when these transgenic organisms are released into the environment thus, encouraging the advancement of marker-free selection systems (Manimaran *et al.*, 2011). Marker-free selection systems in microalgae are however, not favorable when the microalgae will be mass-cultivated in open ponds or open seas as the large transgenic population will be difficult to control if they are accidentally released to surrounding environment and there is no marker-free system to distinguish the transgenic population within the non-transgenic ones (Miki & McHugh, 2004). As microalgae proliferate rapidly, a marker-free system will need to be cautiously evaluated to gain public acceptance.

Reporter genes such as *β*-Glucuronidase (*GUS*) and Green Fluorescent Protein (*GFP*) are used to track protein expression and subcellular localization when expressed with target proteins. The green fluorescent protein of the jellyfish *Aequorea victoria* has been widely used to identify protein subcellular localization (Hirakawa *et al.*, 2008; Wang *et al.*, 2010; Cha *et al.*, 2012). However, one of the restriction of using GFP in marine

microalgae is that microalgae can display strong endogenous pigments which at times disguise GFP expression. Hence, strong promoters that can emit strong GFP signals are mandatory in such instances (Falciatore *et al.*, 1999; Fuhrmann *et al.*, 1999; Poulsen & Kroger, 2005). Several alternate fluorescent proteins with different emission wavelengths have recently been developed such as enhanced GFP (EGFP), yellow fluorescent protein (YFP) blue fluorescent protein (BFP) and red fluorescent protein (RFP). The *GUS* gene that encodes for *E. coli β*-glucuronidase has been successfully used in several algae species such as *D. salina* (Tan *et al.*, 2005), *C. vulgaris* (Cha *et al*., 2012), *Amphidinium* sp. and *Symbiodinium microadriaticum* (ten Lohuis & Miller, 1998). When examining the reporter gene expression, controls must be included so that weak background noise of the reporter genes can be omitted to obtain accurate results.

When it comes to generating a transgenic microalgae, there is always a possibility that the transgene is not expressing as anticipated, even though all regulatory elements necessary for transcription and translational are fully integrated. This may result from a positional effect as foreign DNA is likely to integrate randomly in eukaryotic nuclear genomes by non-homologous recombination. Positional effects associated with the control of development and to the response of the cell to viruses, transposable elements, or other unnaturally placed foreign DNA can also mainly lead to gene silencing (Wu-Scharf *et al.*, 2000). The lack of homologous recombination events has prevented the intensive study of algal genomics and biology in *Chlamydomonas* (Zorin *et al.*, 2009). Transgene expression may also be affected by post-transcriptional gene silencing , an event that researchers commonly report with transgenic higher plants. The weak transgene expression level is often correlated with high copies of the transgene (Marenkova & Deineko, 2010; Potvin & Zhang, 2010). By using low concentrations of DNA (100 ng or less), Gonzalez-Ballester *et al.* (2005) demonstrated single DNA copy-integration in *Chlamydomonas*, thus considerably lowering the risk of gene silencing.

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2.2 Prokaryotic toxin-antitoxin systems

Free-living prokaryotes strive in an ever-changing environment that is constantly exposed to a myriad of stress conditions. To survive, bacteria often acquire beneficial genes from other prokaryotic genomes via horizontal transfer. Toxin-antitoxin (TA) systems were first discovered on bacterial plasmids but have since been found in almost all bacteria as well as archaea (Gerdes *et al*., 2005). TA systems have been associated with mobile genetic elements including plasmids, transposons and genomic islands and they play various roles in bacterial, contributing to their survival in contrast to cells that do not possess these systems (Yamaguchi *et al*., 2011; Chan *et al*., 2012).

2.2.1 Classification of the types of TA systems

A TA system is customarily composed of two genes – the toxin gene encoding a stable proteic toxin and an antitoxin gene encoding a relatively labile antitoxin (Van Melderen & Saavedra De Bast, 2009). The antitoxin gene normally precedes the toxin gene; both genes often have overlapping stop and start codons, indicative of coupled translation. In some cases, the gene order is reversed (Chan *et al.*, 2012). Each TA system is dependent on the antitoxin to tightly regulate toxin expression or to keep toxin levels low unless required. The antitoxin functions as the repressor of the operon while the toxin, usually acts as co-repressor. Antitoxins are synthesized at a more rapid rate but also degraded faster, comparative to the cognate toxin (Yamaguchi *et al.*, 2011). Under normal circumstances, antitoxins are expressed in abundance, forming complexes with toxins and thus, nullifying the toxin effects. However, in the presence of stress, ATP-dependent proteases degrade the unstable antitoxins thus bringing about the release of toxin proteins from the complexes. The liberated toxins cause dire effects to the organisms depending on the mechanisms of the toxins (Yamaguchi *et al.*, 2011). Bacterial TA systems are currently characterized into six types, Types I, II, III, IV, V and VI according to the composition of the TA system.

2.2.1.1 Type I TA systems

In Type I TA systems, the antitoxin is an antisense RNA whereas the toxin is a protein (Figure 2.2a). The toxin and antitoxin genes are positioned on opposite strands in a reverse orientation (Fozo *et al.*, 2008). The antisense RNA antitoxin base-pairs with the toxin mRNA, forming a complex that stimulates the degradation of the toxin mRNAs thereby preventing translation of the toxin (Gerdes & Wagner, 2007). Type I TA systems have been discovered in both Gram-positive and Gram-negative bacteria and are represented by the Hok-Sok (Chukwudi & Good, 2015), SymR-SymE (Kawano *et al.*, 2007) and IstR-TisB (Unoson & Wagner, 2008) TA systems. All type I TA toxins are predicted to be localized in the inner membrane and prevent ATP synthesis by forming pores to interrupt the membrane potential (Unoson & Wagner, 2008).

One of the well-characterized type I systems is the *hok-sok* locus of plasmid R1 in *E. coli.* Expression of the Hok toxin is controlled by the *sok* antisense RNA. The *hok* mRNA sequence is complementary to *sok* antisense RNA, but the complementarity does not comprise the *hok* Shine-Dalgarno sequence. Fascinatingly, a third protein encoded by the *mok* gene exists (Fineran *et al.*, 2009) to assist the Hok protein translation as the open reading frame overlaps with that of the Hok toxin. Thus, if antitoxin RNA binds to the *mok* transcript, the toxin translation will be suppressed (Fozo *et al.*, 2010). Killing of cells that do not carry the TA-encoding plasmid is accomplished through differential stability of the *hok-sok* pair. The rapid decay of the antitoxin in the plasmid-free cell causes the accumulation of the *hok* toxin mRNA which is then translated into Hok toxin proteins. This toxin exerts its lethal effect by causing cell membrane depolarization that affects the membrane integrity leading to inhibition of DNA replication and translation (Gerdes & Wagner, 2007).

2.2.1.2 Type II TA systems

The type II TA system is the most well-studied among the various TA systems and can be exemplified by MazE-MazF (Park *et al.*, 2012), RelE-RelB (Li *et al.*, 2009), YefM-YoeB (Zhang & Inouye, 2009) and MqsR-MqsA (Brown *et al.*, 2009; Christensen-Dalsgaard *et al.*, 2010) systems. The mutual feature shared among Type II TA systems is the formation of a complex between a labile antitoxin protein with the stable toxin protein, leading to inhibition of toxin activity (Singletary *et al.*, 2009). Typically, the type II TA system is encoded by an operon and its expression is regulated at the level of expression by the TA complex. The type II antitoxin usually functions as a weak transcriptional repressor whereas its repression activity is enhanced when the antitoxin is in complex with the toxin (Chan *et al.*, 2016). In most Type II TA systems, the antitoxin genes are situated upstream of toxin genes, so that antitoxins have a lead for their production over their cognate toxins (Figure 2.2b), although there are several exceptions.

The toxins liberated are adept at disrupting essential cellular processes in the prokaryotic hosts that harbor the TA system. The CcdB toxin disrupts DNA replication by targeting DNA gyrase. The toxin first binds to the dimerization domain of GyrA and then inhibits the formation of a functional conformation of GyrA (Loris *et al.*, 1999). CcdB can also impair supercoiling which is disastrous for *E. coli* cells (Dao-Thi *et al.*, 2005*)*. The RI plasmid of *E. coli* expresses a Type II TA complex, Kis-Kid. The Kid toxin specifically acts on DnaB which functions as a DNA helicase during replication (Kamphuis *et al.*, 2006). Messenger RNA (mRNA) is most commonly targeted by type II bacterial toxins. Toxins that are recognized as mRNA interferases can be further classified into two types, ribosome-independent and ribosome-dependent (Yamaguchi *et al.*, 2011). The former is typified by the MazF toxin that cleaves mRNA at ACA sequences to effectively prevent protein translation (Zhang *et al.*, 2003). Ribosome-dependent mRNA interferases exhibit little or no endoribonuclease activity on their own

(Zhang & Inouye, 2009). The RelE toxin of the RelB-RelE TA system binds to 30S ribosomes and links with the ribosome A-site, leading to cleavage of mRNA specifically between the nucleotides of the termination codon (Pedersen *et al.*, 2003). YoeB blocks translation initiation by binding to 50S ribosomes leading to the cleavage of mRNAs downstream of the initiation codon (Zhang & Inouye, 2009). Interestingly, although RelE and YoeB share similar roles, the former binds to 30S to hinder translation termination while the latter interacts with 50S to inhibit translation elongation (Yamaguchi *et al.*, 2011).

2.2.1.3 Type III TA systems

This class of TA system was established with the characterization of the ToxI-ToxN TA system from plasmid pECA1039 of the Gram-negative pathogen, *Pectobacterium atrosepticum* (Blower *et al.*, 2012). This type of TA system was first known as abortive infection systems that guards bacteria from bacteriophage attacks (Fineran *et al.*, 2009). Type III TA loci contain an array of nucleotide repeats of antitoxin RNA that precedes the toxin gene. The ToxN toxin activity was neutralized by direct binding of the *toxI* antitoxin-RNA, forming an inactive protein-RNA complex (Figure 2.2c). ToxN was established to be an endoribonuclease as the structure is highly similar to that of Kid and MazF toxins (Blower *et al.*, 2011).
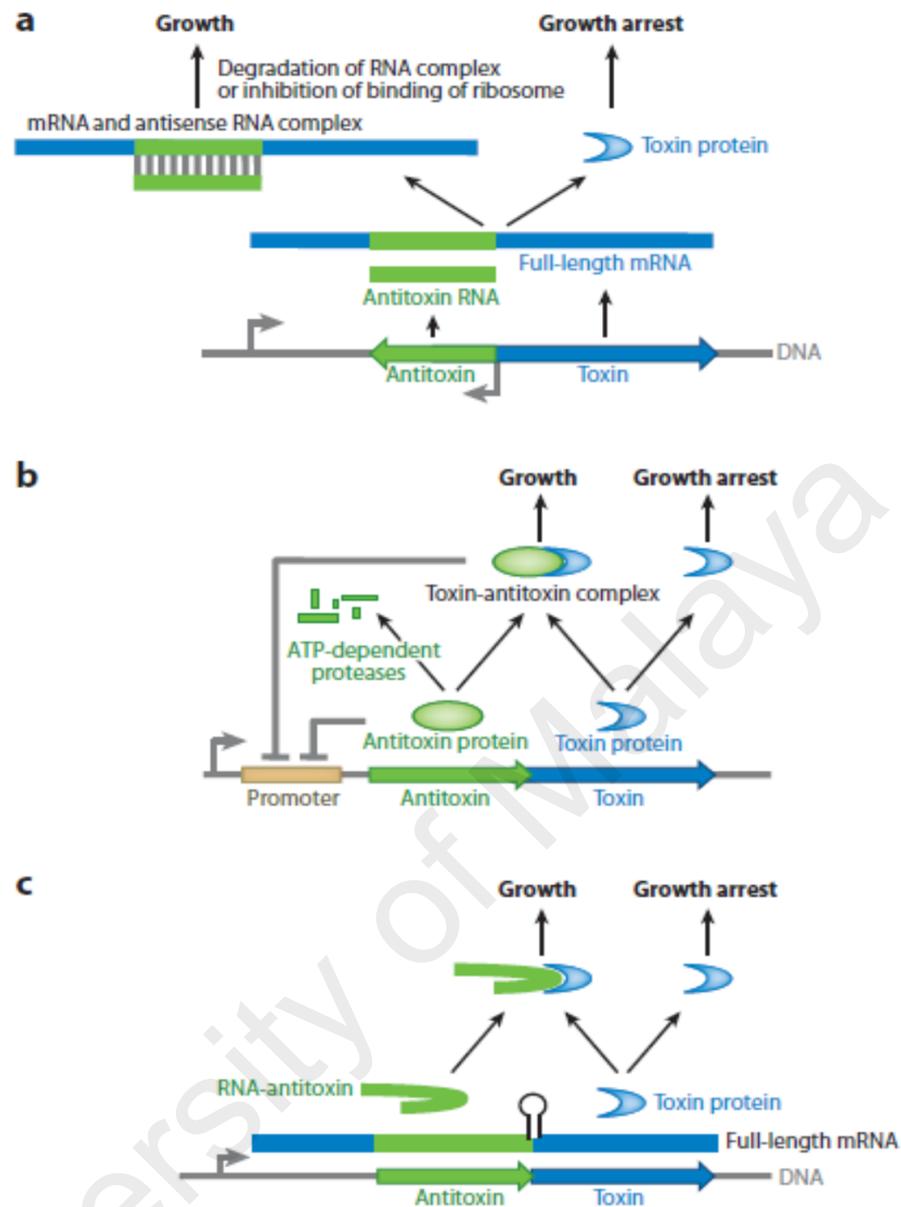
Figure 2.2: Three types of TA systems. (a) Type I TA systems. Toxin protein and antitoxin antisense-RNA are transcribed separately. RNA-antitoxin binds to mRNA-toxin forming a duplex that leads to degradation of the toxin mRNA; (b) Type II TA systems. Toxin and antitoxin mRNAs are transcribed from the same promoter and are then translated into proteins. The formation of TA complex blocks toxin effect. Under stress conditions, the more labile antitoxin will be digested by endogenous ATP-dependent cellular proteases. The liberated toxin will exert its lethality by attacking specific cellular target(s) causing growth arrest and cell death. (c) Type III TA systems. The lethality of the toxin protein is suppressed by direct binding with antitoxin-RNA. Image taken from Yamaguchi *et al.* (2011).

2.2.1.4   Type IV TA systems

The YeeV (CbtA) protein is the foremost toxin to date that was described to stop bacterial growth by binding to the cytoskeletal proteins, MreB and FtsZ (Tan *et al*., 2011). YeeV interacts with FtsZ and blocks self-interaction among FtsZ that is essential for GTP activity and polymerization. In addition to that, YeeV also binds to MreB, a prokaryotic actin-like protein that is important for cell division and in turn, obstructs MreB function. Upon YeeV expression, the morphology of *E. coli* was altered from the normal rod-shaped to contorted lemon-shaped cells (Masuda *et al*., 2012). YeeU is a novel type of Type IV antitoxin that does not form a complex with its cognate YeeV toxin but functions to antagonize the lethal effect of the toxin by promoting polymerization of MreB and FtsZ. Hence, in type IV TA systems, there is no direct contact between the toxin and antitoxin, but rather each binds to the target proteins and exerts opposite effect to each other.

2.2.1.5   Type V TA systems

A different type of TA system, categorized as a Type V TA system, was recently discovered and is represented by the GhoST TA system (Wang *et al*., 2012). Instead of the toxin functioning as an endoribonuclease, the GhoS is the first antitoxin to act as a ribonuclease that precisely cuts GhoT toxin mRNA in sites that are rich in U and A. The GhoT toxin is described as a membrane lytic peptide that induced ghost cell formation (lysed cells with intact membrane), leading to cell death. Besides that, the expression of the GhoT toxin can cause loss of membrane integrity which in turn rendered the cells with increased tolerance towards ampicillin and induced the formation of persister cells (Wang *et al.*, 2012).

2.2.1.6   Type VI TA systems

Similar to Type II and IV TA system, the antitoxin and toxin of Type VI TA systems exist as proteins. However, the toxins of type VI TA systems are labile when compared

to their cognate antitoxins, in contrast to type II TA toxins. The type VI TA system was typified by the SocAB TA system that was discovered in a Gram-negative bacterium, *Caulobacter crescentus* that is usually found in aquatic environments. The SocAB TA system is comprised of the SocA antitoxin and SocB toxin (Aakre *et al.*, 2013). The SocB toxin is labile and susceptible to degradation by protease ClpXlp. Instead of directly neutralizing SocB toxin, the SocA antitoxin functions as a ClpXlp protease adaptor necessary for the toxin degradation. The toxin, SocB acts to inhibit replication elongation by causing the collapse of DNA replication fork (Aakre *et al.*, 2013).

2.2.2 Plasmid-encoded TA systems

TA systems were originally found in low copy number plasmids where they function to ensure stable plasmid maintenance by deploying a killing strategy known as Post-Segregational Killing (PSK). PSK increases the plasmid prevalence (number of plasmid-containing cells/total number of cells) in a bacterial population by the elimination of daughter cells that did not inherit a plasmid copy during cell division, due perhaps to a replication defect or mis-segregation of the replicated plasmids (Figure 2.3). PSK depends on the differential stability of the toxin and antitoxin. In general circumstances and for type II TA systems, the stable toxin binds to its cognate antitoxin to form an inactive complex. During vertical transmission, the daughter cell that inherits the plasmid-containing TA continues to survive. In contrast, in a plasmid-free daughter cell, the antitoxin concentration is not replenished occasioning in the liberation of toxin from the complex to exert its toxicity. Plasmid-encoded TA systems were consequently also known as "addiction modules" as the bacterial host becomes reliant (or "addicted") on the continual existence of the TA system, which is accountable for the lethal consequences of plasmid withdrawal from the bacterial host (Van Melderen & Saavedra De Bast, 2009).
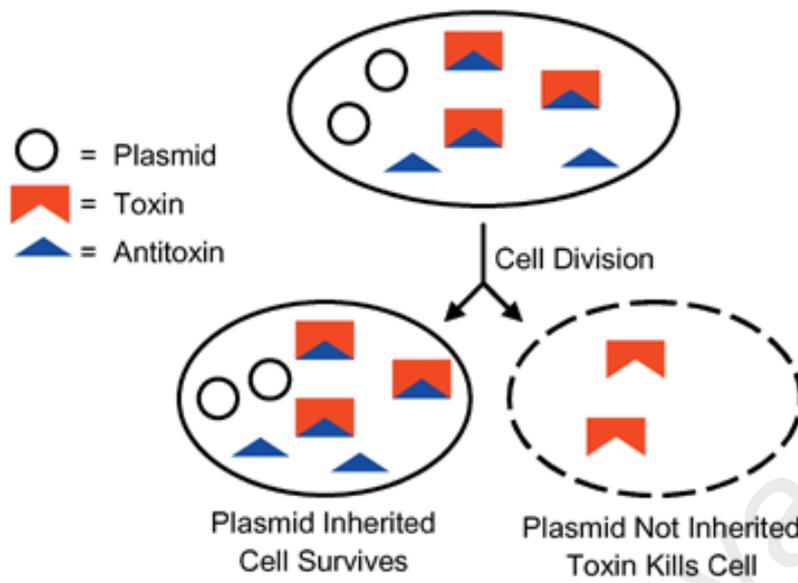
Figure 2.3: Plasmid maintenance via post-segregational killing. The presence of a TA system increases plasmid prevalence by selecting cells that inherit plasmids. If resulting daughter cells do not inherit a plasmid copy, endogenous ATP-dependent proteases degrade remaining antitoxin proteins, thus liberating the remaining toxins that will lead to selective killing of the plasmid-free cells Image taken from DeNap & Hergenrother (2005).

2.2.3 Chromosomally-encoded TA systems

Bacterial chromosomes also harbor TA cassettes but the role of these chromosomally-encoded TA systems are indistinct and this was a matter of intense intellectual debate over the past decade. Programmed Cell Death (PCD) is a common physiological process that occurs during development in eukaryotic organism. As unicellular organisms, bacteria were believed not to undergo PCD, yet, the opposing theory that bacteria may possess PCD was presented as a result of observations with the MazEF chromosomal TA system of *E. coli* that was triggered under nutritional stress (Engelberg-Kulka & Glaser, 1999; Mutschler *et al.*, 2011). PCD in bacteria was hypothesized to enable surviving cells to forage nutrients from the dead cells under nutrient starvation conditions and was touted as an example of the multicellular behavior of bacteria (Engelberg-Kulka & Glaser, 1999).

The chromosomally-encoded toxins can also act as cell cycle arrest factors causing cells to enter a dormant state as a defense mechanism when faced with external stress. This occurrence is known as persistence and described the ability of some cells to persevere in the presence of antibiotics although not inheriting genetic resistance. Persistence has been correlated with elevated expression levels of chromosomal-encoded TA systems (Shah *et al.*, 2006). For example, the elevated activity of the TisB toxin caused an increase in the manifestation of ciprofloxacin-induced persister *E. coli* cells (Dorr *et al.*, 2010). TisB is a small hydrophobic protein that binds to the cell membrane and interrupts the proton motive force that brought to a decline in ATP production (Unoson & Wagner, 2008). Cells that expressed TisB stopped growing and the declined ATP levels shut down ciprofloxacin targets and induced cells to enter into dormancy (Dorr *et al.*, 2010).

The chromosomal *mazEF* TA system was first identified in *E. coli* and elucidated to be involved in stress management (Engelberg-Kulka & Glaser, 1999). Upon activation of stress and a cellular death factor, *mazEF* expression is repressed leading to the

degradation of the unstable MazE antitoxin by ATP-dependent protease and liberation of the MazF toxin from the protein complex (Engelberg-Kulka & Glaser, 1999), as shown in Figure 2.4 The *E. coli*-encoded MazF is an endoribonuclease that cleaves mRNA at 5'-ACA-3' sites as well as 16S rRNA within the 30S ribosomal subunit (Zhang *et al.*, 2003; Vesper *et al.*, 2011). On the other hand, the MazF-mt6 toxin that is encoded by *Mycobacterium tuberculosis* digests 23S rRNA in the ribosome active center to block protein synthesis leading to cell growth arrest and eventually, cell death (Schifano *et al.*, 2013).
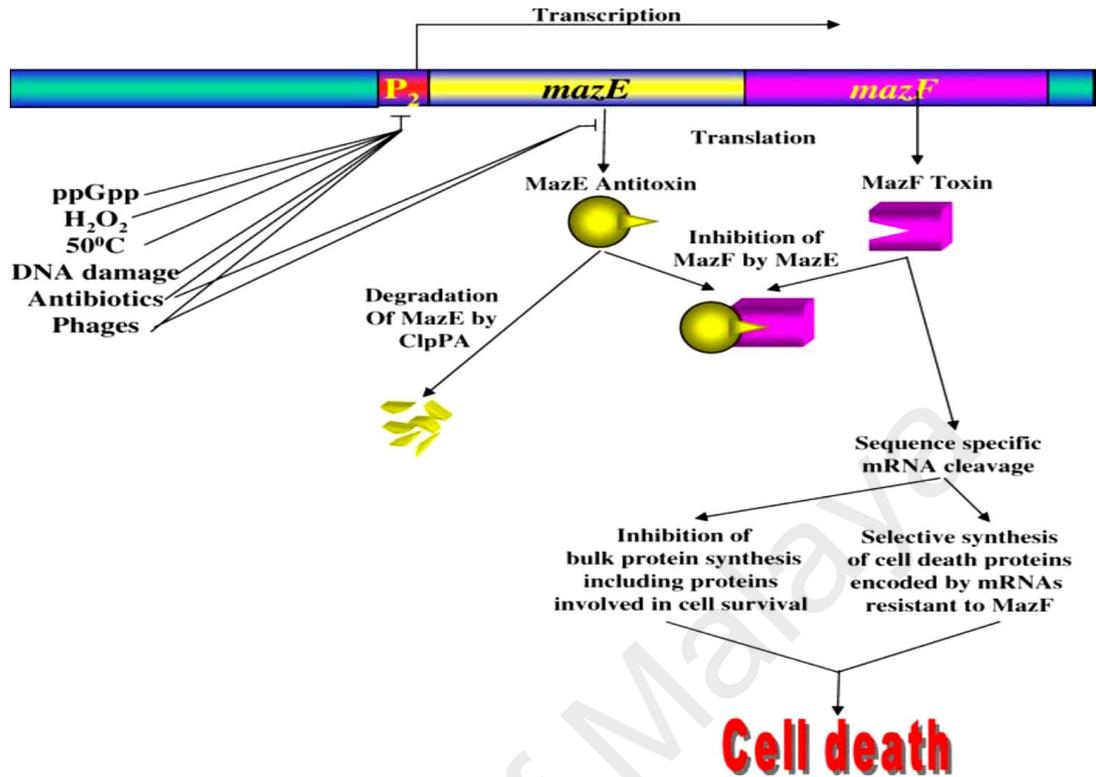
Figure 2.4: Schematic presentation of the *mazEF* TA system of *E. coli* postulated to be involved in bacterial PCD. Stress elements trigger the degradation of MazE antitoxins and liberates MazF toxin. MazF cleaves cell mRNA and blocks translation that leads to cell growth arrest and eventually, cell death. Image taken from Engelberg-Kulka *et al.* (2006).

However, in a different line of research, amino acid starvation was shown to induce the *relBE* TA system in *E. coli* but expression of this TA system did not result in cell death but rather, the dormancy of the host cells until a more favorable growth condition resumed. The RelE toxin hinders translation and brings about a reversible bacteriostatic response when production of RelB antitoxin resumes (Cataudella *et al.*, 2012). In this matter, both *mazEF* and *relBE* were proposed to be modulators of physiological stress response to poor nutritional conditions rather than bona fide PCD (Pedersen *et al.*, 2002). The altruistic death of 95% of the bacterial population upon *mazEF* induction was speculated to allow the surviving cells to gain more nutrients. In short, chromosomal TA system act to alter the physiology of the cell to external cues, by inducing PCD (Engelberg-Kulka *et al.*, 2006) to confer fitness advantage to host cells.

Saavedra De Bast *et al.* (2008) proposed the possibility that chromosomally-encoded TA systems may also act as an anti-addiction molecule. TA systems that undergo horizontal gene transfer are integrated into the new host chromosome and these systems may interfere with plasmid-encoded TA homologs. The chromosomally-encoded *ccd* (*c*ontrol of *c*ell *d*eath) (*ccd_{Ech}*) in *Erwinia chrysanthemi* functioned as anti-addiction molecules to protect the cells against PSK by the F-plasmid *ccd* (*ccd*_F) homolog.

2.2.4   TA systems in *Streptococcus pneumoniae*

Streptococcus pneumoniae is a Gram-positive pathogen accountable for pneumococcal pneumonia that ranges from asymptomatic to acute infections in humans (Kadioglu *et al.*, 2008). Pneumococcal infections cause high mortality rates with estimations that pneumonia kills approximately 1.4 million children under the age of 5 years annually, more than AIDS, malaria and tuberculosis combined (O'Brien *et al.*, 2009). The bacterium can be found naturally in the upper respiratory tracts of 5 to 70% of healthy individuals, and opportunistic infection resulting in pneumonia happens when the immune system of the individual is compromised (Kadioglu *et al.*, 2008).

Chan *et al.* (2012) performed bioinformatics searches with expanded data (Makarova *et al.*, 2009) for TA systems in the sequenced *S. pneumoniae* genomes that were available in the NCBI databases. Only HicAB, Phd-Doc, RelBE, HigBA, YefM-YoeB, PezAT, Xre-COG2856, and Xre-Bro TA systems were found and were postulated to exist in the genomes of *S. pneumoniae.* However to date, only four of these chromosomally-encoded pneumococcal TA pairs have been established to be functional, namely, *relBE2* (Nieto *et al.*, 2006), *pezAT (*Khoo *et al.*, 2007), *yefM-yoeB* (Chan *et al.*, 2011) and *phd-doc* (Chan *et al.*, 2014).

2.2.4.1 RelBE-1 and RelBE-2

Nieto *et al.* (2010) described the existence of two putative pneumococcal TA pairs, namely *relBE1* and *relBE2*, which are homologous to the *E. coli*-encoded *relBE* TA genes. The *relBE* family is present in three of the first sequenced *S. pneumococcus* strains (TIGR4, D39 and R6). The pneumococcal *relBE2* genes were confirmed to be functional (Nieto *et al.*, 2007), while *relBE1* was shown to be non-functional as the *relE1* toxin was unable to cut mRNA in *E. coli* (Christensen & Gerdes, 2003). The pneumococcal *relB2* antitoxin precedes its cognate *relE2* toxin gene, with the former gene overlapping the atter by eight nucleotides (Pandey & Gerdes, 2005) (Figure 2.5). The pneumococcal RelE2 toxin cleaves ribosomal messenger RNA at the A site, showing that the toxin expression causes cell growth arrest in *E. coli* and *S. pneumoniae* (Nieto *et al*., 2006). The lethal effect of RelE2 toxin can be reversed by the introduction of its cognate *relB2* antitoxin gene; however, prolonged exposure to the RelE2 toxin ultimately led to *E. coli* cells inability to form colonies, indicative that the antitoxin is only able to rescue RelE2 toxin-affected cells within a certain time frame (Nieto *et al.*, 2006).

2.2.4.2 YefM-YoeB$_{Spn}$

The pneumococcal *yefM-yoeB* genes were originally identified on the basis of homology to the Axe-Txe TA system in the pRUM multidrug resistant plasmid of *Enterococcus faecium* as well as to the *E. coli* counterpart (Nieto *et al.*, 2007). The gene arrangement in the *yefM-yoeB$_{Spn}$* operon consists of two stop codons of the *yefM$_{Spn}$* antitoxin gene located upstream of the ATG start codon of *yoeB$_{Spn}$* toxin (Chan *et al.*, 2012) (Figure 2.5). An interesting feature of the pneumococcal *yefM-yoeB$_{Spn}$* operon is the presence of a BOX element immediately upstream. BOX elements are short repeat sequences which are involved in the regulation of gene expression and appeared to be unique to pneumococci (Martin *et al.*, 1992) The presence of a BOX elements that was inserted upstream of the original *yefM-yoeB$_{Spn}$* promoter is likely to be beneficial to *S. pneumoniae* as it was proposed to increase the expression of the *yefM-yoeB$_{Spn}$* locus through the formation of an additional constitutive promoter (Chan *et al.*, 2013). Inhibition of cell growth and reduction in the number of colony-producing *E. coli* cells was observed when the pneumococcal YoeB$_{Spn}$ toxin was overproduced. However, the co-expression of both *yefM-yoeB$_{Spn}$* genes was non-toxic to cells as the later transcription of *yefM$_{Spn}$* offers a window period for cell recovery (Nieto *et al.*, 2007).

The cellular target of pneumococcal YoeB$_{Spn}$ has not yet been determined, but *E. coli*-encoded YoeB was elucidated to prevent translation initiation by cleaving mRNA near the initiation codons (Zhang & Inouye, 2009). *E. coli*-encoded YoeB alone does not have RNase activity and only displays mRNA cleavage when associated with the 50S ribosomal subunit in 70S ribosomes (Zhang & Inouye, 2009).

2.2.4.3 PezT-PezA

The pneumococcal *pezAT* genes were termed after their homologue, the *epsilon-zeta* TAS, which was identified in plasmid pSM19035 of *S. pyogenes* (Khoo *et al.*, 2007). In the *pezAT* TA operon, the antitoxin *pezA* gene precedes the toxin *pezT* gene in which the

TAA stop codon of the antitoxin gene overlaps the ATG initiation codon of the toxin gene (Khoo *et al.*, 2007). Both *pezAT* genes are co-transcribed from a single promoter. PezA functions as a repressor by binding to a palindrome sequence upstream of the *pezA* gene whereas PezT functions as a co-repressor in the TA complex to further block transcription from the *pezAT* promoter (Khoo *et al.*, 2007). The PezT toxin functions to inhibit bacterial cell wall synthesis by phosphorylating UDP-*N*-acetylglucosamine (UNAG) to UDP-*N*-acetylglucosamine-3'-phosphate (UNAG-3G) which in turn, blocks MurA, the catalytic enzyme in the initial stage of peptidoglycan biosynthesis and finally, cell autolysis (Mutschler *et al.*, 2011). The lethality of this toxin is abolished when *pezA* gene was co-expressed (Khoo *et al.*, 2007).
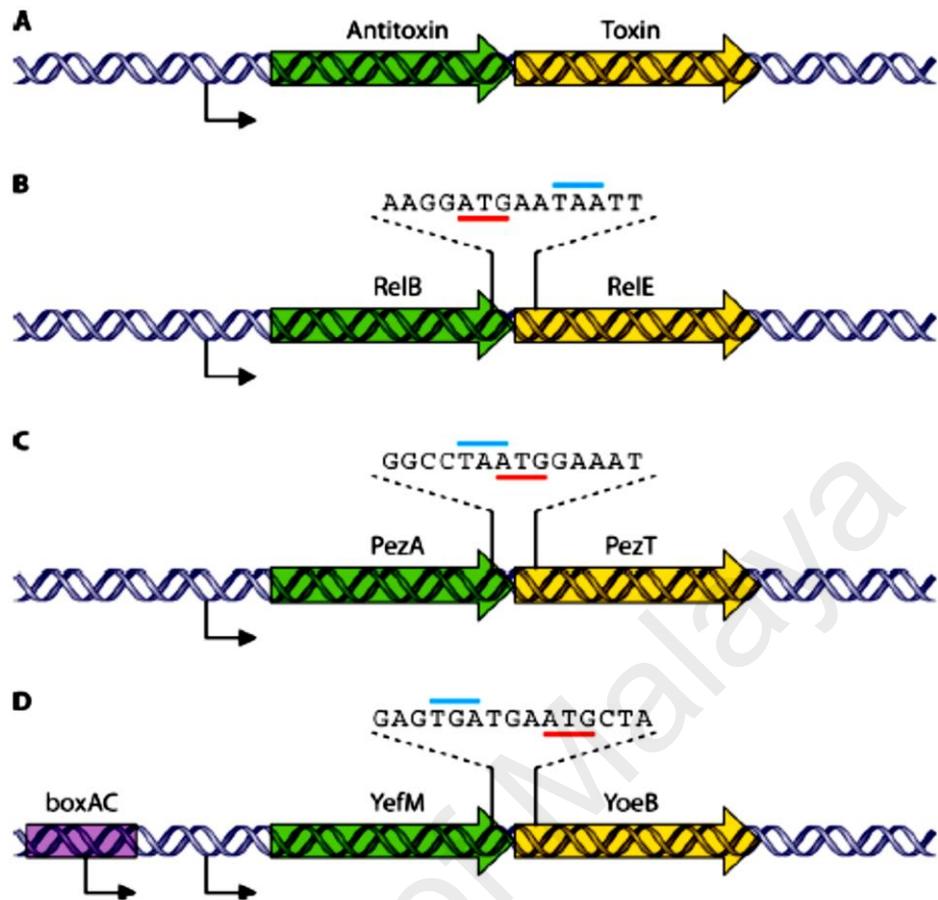
Figure 2.5: Genetic organization of pneumococcal Type II TA systems. (**A**) A typical organization of TA complex consists of the antitoxin gene that precedes the toxin gene. (**B** to **D**) The organization of the three well-studied pneumococcal TA systems in which the TA genes of are overlapped by 8 nt (*relBE2*) (**B**) or by 1 nt (*pezAT*); or separated by 3 nt (*yefM-yoeB_{Spn}*). The antitoxin and toxin genes are shown in green and yellow, respectively. The lines indicate the start (Red) and stop (blue) codons. Image taken from Chan *et al.* (2012).

2.2.4.4 Phd-Doc

The fourth functional TA system in *S. pneumoniae* was recently discovered and designated Phd-Doc after its *E. coli* counterpart (Chan *et al.*, 2014). This TA system shared the conventional sense of Type II TA characteristic in which the toxin protein is liberated under stress triggers and the toxin lethality can be nullified by the translated antitoxin protein. Expression of the Doc toxin was found to be lethal in *S. pneumoniae* whereas the expression of is cognate Phd antitoxin was able to neutralize the lethality of the Doc toxin. The target of the *E. coli*-encoded Doc toxin is the conserved theorine (Thr382) of the translation elongation factor EF-Tu, which is phosphorylated leading to the inability to bind to aminoacylated tRNAs, thereby inhibiting translation (Castro-Roa *et al.*, 2013).

2.2.5 Expression of bacterial TA systems in eukaryotic cells

Several TA toxins exhibit activity when expressed heterologously in yeast and mammalian cells and they may be valuable for various applications in eukaryotic systems. The Kis-Kid TA system was employed for ablating the germ line in transgenic zebra fish enabling their fertility to be regulated by induction of Kid toxicity (Slanchev *et al.*, 2005). This system can help to distinguish wild type fish populations from the transgenic fishes, which represents a major concern in fish aquaculture.

Gene silencing in eukaryotic cells due to epigenetic effects is a major shortcoming in the generation of stably high-expressing cell lines. A new technique by means of bacterial TA systems was established to overcome this challenge (Nehlsen *et al.*, 2010). In mammalian cells, the desired transgene is tightly co-expressed with the Kis antitoxin in cells which also expresses the Kid toxin that functions as mRNA ribonuclease. This system offers a positive selection for transgenic cells that co-express the transgene and the antitoxin at high levels, as the antitoxins are able to block Kid toxicity, thus avoiding

the demise of transgenic cells. Over time, enrichment of cells with toxin genes in a given type of cells and antitoxin expression were observed (Nehlsen *et al.*, 2010).

Andreev *et al.* (2008) demonstrated that RelE can efficiently and specifically cut mRNA in the A site of the eukaryotic ribosome. The *E. coli*-encoded *relE* toxin gene was expressed in the yeast *Saccharomyces cerevisiae* and proved to be toxic. The *relE-relB* toxin-antitoxin gene pair was therefore projected as part of a gene containment system for genetically modified yeasts to prevent the survival of these transgenic yeasts that are accidentally released into the environment (Kristoffersen *et al.*, 2000).

2.3 Genetic ablation in plants

Cell-specific ablation approaches can be applied to analyze the origin and fate of various cell types, to study the interaction and communication between different cell types and to investigate cellular function. Physical ablation has been done by using methods like micro-dissection or laser ablation. These methods were effectively employed in *Drosophila* (Broughton *et al.*, 2005) and *Caenorhabditis elegans* (Qi *et al.*, 2012), but there are challenges for these ablation methods to be applied in complex eukaryotic organisms that are less receptive to physical and genetic manipulations.

Genetic ablation has been demonstrated to overcome impediments encountered by conventional cell ablation techniques. It is achieved by the expression of a cytotoxic gene from a tissue-specific promoter that will cause cell death. Genetic ablation has been successfully employed in a diverse group of tissues in mammals (Saito *et al.*, 2001) and plants (Roque *et al.*, 2007) as well as *Drosophila* (Sweeney *et al.*, 2012). Genetic ablation permits the destruction of many cells particularly those that are inaccessible using physical ablation, such as primordial cells. A slight drawback of genetic ablation is the limited number of promoters currently available for the expression of toxin genes in a given types of cells.

The production of male-sterile plants by genetic ablation in plant anther was achieved by Roque *et al.* (2007). This study involved the manipulation of *PsEND1*, an anther-specific gene that displays very early expression in the anther primordium cells. The *PsEND1* promoter was fused to the bacterial *barnase* toxin gene and the expression caused ablation in the cell layers. Since bacterial barnase toxin functions as a ribonuclease, the co-expression of *PsEND1* and *barnase* gene in two Solanaceae (*Nicotiana tabacum* and *Solanum lycopersicon*) and two Brassicaceae (*Arabidopsis thaliana* and *Brassica napus*) species have been successful in obtaining male-sterile plants. In a related study, the effectiveness of *Nicotiana tabacum* lines expressing gene responsible for male-(*p108: barnase*) and female-(*sp41: barnase*) sterility were observed (Gardner *et al.*, 2009). Both gene constructs were fused with the bacterial *barnase* toxin gene and exhibited no pollen production, stipulating sterility (Roque *et al.*, 2007).

Diphtheria toxin is a naturally occurring toxin of *Corynebacterium diphtheria* that is synthesized into a polypeptide and subsequently cleaved into A and B chains (Collier, 1975). The B chain is responsible for the recognition of most eukaryotic cells via a specific membrane receptor and for the import of a chain into the cell. Upon entry into plant cells, the A chain hinders protein translation. Diphteria toxin protein is an enzyme that is fatal at low concentration (Yamaizumi *et al.*, 1978). The tightly controlled expression of toxin A subunit under the control of *Arabidopsis HAP2* (*GCS1*) promoter blocked sperm development before the final cell mitosis thus, resulting in pollen tube with one single sperm, rather than two that are required for male plants flowering (Frank & Johnson, 2009).

The YoeB$_{Spn}$ toxin that belongs to the YefM-YoeB TA module of *S. pneumoniae* was demonstrated to act as a potent mRNA interferase that blocks translation initiation (Chan *et al.*, 2011). Expression of bacterial YoeB$_{Spn}$ toxin was reported to be lethal in the model plant *Arabidopsis thaliana* (Abu Bakar *et al.*, 2015). The YoeB$_{Spn}$ toxin was expressed

using a two component, XVE-based gene inducible system and resulted in the distortion of the transgenic plant leaves with lesion formation 3 days after 17-β-estradiol treatment. All transgenic plants that were induced for YoeB$_{Spn}$ expression were dead by the eight day post-induction sample time (Abu Bakar *et al.*, 2015). The development of this cell ablation technique using a bacterial toxin gene is potentially beneficial as a bio-containment strategy to control the spread of the transgenic plant when necessary.

2.4 Heterologous protein expression systems in higher plants and microalgae

Higher plants and microalgae are promising expression systems for the production of heterologous proteins (Specht *et al.*, 2010; Dugdale *et al.*, 2013). They are prospective substitutes to conventional expression host as plants and microalgae expression systems offer more advantages in terms of low cost productions and lower capital (Specht *et al.*, 2010). While the study of microalgae expression system is still limited, the higher plant expression system are established and were successfully used for the production of heterologous protein. For example, Guerrero-Andrade *et al.* (2006) reported the expression of fusion (F) gene of Newcastle Disease Virus (NDV) in *Zea Mays L* maize plants. NDV causes infection in birds and F protein mediates penetration into host-cells and causes virus-induced hemolysis (Morrison, 2003). Chickens that fed on the transgenic F-containing maize developed antibodies against the virus, thus showing potential as vaccines (Guerrero-Andrade *et al.*, 2006). The chemically-inducible gene expression systems discussed below are focused on higher plants as there has yet to be any report on microalgae inducible expression systems.

2.4.1 Chemically-inducible gene expression systems

Constitutive promoters have been used over the years to transcribe the gene of interest, but a major limitation of these is the over- or under-expression of the heterologous genes which at times leads to deleterious effects on the tested organisms (Zuo & Chua, 2000).

Chemically-inducible gene expression systems offers a general solution as such systems are inactive in the absence of the inducer and gene expression is switched on by the presence of a specific signal or inducer. Based on the malleable nature of this system, the gene expression can be hypothetically controlled at specific stages of plant development or for a specific duration of time. An ideal expression system should have the following features: firstly, the basal expression should be insignificant in the absence of inducer and there should be rapid response upon induction or withdrawal of inducing agent. Secondly, the inducer should not exert toxicity on the plants nor to the environment. Thirdly, the system should be highly responsive to a broad range of inducer concentrations and efficient even at low concentrations (Zuo & Chua, 2000).

A number of chemical inducible systems have been established to regulate gene expression in plants, some of which have been successfully implemented for use in microalgae. The systems discussed in the following paragraphs are the recent and more promising studies and include: (1) Nitrate-reductase (NR) based, nitrate-inducible system; (2) Alcohol-regulated promoter (AlcR) based, ethanol-inducible system; (3) glucorticoid receptor (GR) based, steroid-inducible system and (4) XVE-based, steroid-inducible system.

Niu *et al.* (2011) showed that the nitrate reductase (NR) promoter from the microalga *Phaeodactylum tricornutum* in green microalga *C. vulgaris* can drive the inducible expression of a chloramphenicol acetyltransferase (*CAT*) gene in the presence of nitrate and is switched off in the presence of ammonium. Since nitrate is a source of nitrogen of *Chlorella* growth condition, the use of an endogenous and inducible NR promoter can be applied to control the expression of heterologous genes in *Chlorella* (Zhang & Hu, 2014).

The *Aspergillus nidulans alcA* promoter is one of the most generally used promoters in a wide range of plants (Caddick *et al.*, 1998; Garoosi *et al.*, 2005). The *alcR* gene encodes a protein that regulates the transcriptional activator, namely the *alc*-inducible

regulon that is responsible for the oxidation of ethanol. The activator AlcR binds to specific sequences on the target promoter to activate the transcription of alcohol dehydrogenase, in the presence of ethanol. Filichkin *et al.* (2006) investigated the effectiveness of controlled expression of the *β*-glucuronidase (*GUS*) gene in transgenic *Populus* plant using this alcohol-inducible gene expression system. GUS expression can be detected in the aerial and root parts in the presence of as low as 0.5% ethanol concentration. Moreover, the team found that ethanol was the most effective inducer and did not confer phytotoxicity to the plant when used at concentrations of 2% or below.

Aoyama & Chua (1997) constructed a chimeric transcriptional activator GVG that contains the DBD domain of the yeast GAL4 transcription factor (G), the activating sequence of VP16 (V), and the regulatory region of the rat GR (G). A study conducted by Fuoco *et al.* (2013) showed that in the tobacco plant *Nicotiana langsdorffii*, the alteration of hormonal profile up-regulated the activation of secondary metabolites involved in the response to stress. When exposed to the phytohormone inducer, the gene encoding for the rat GR in the transgenic plants induced a lower uptake of heavy metals (cadmium and chromium), relative to the wild-type plants that did not have the GR. Moreover, the stress-responsive hormones such as S-abscisic acid (S-ABA), 3-indole acetic acid (IAA), and salicylic acids were recorded to be raised upon induction. This showed that GR and phytohormones together are responsible for the physiological changes in the transgenic *N. langsdorffii* (Fuoco *et al.*, 2013).

LEAFY COTYLEDON 2 (LEC2) is a key gene in embryo development and accountable for the morphology, maturation progression, identity specification and suppression of premature germination of *Arabidopsis thaliana* seeds (Stone *et al.*, 2001). A functional inducible LEC2: GR under the control of constitutive CaMV 35S promoter was developed to study the effects of LEC2 expression in *Arabidopsis* plant. Upon steroid dexamethasone induction, the lipid composition in the leaves was modified and led to the

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accumulation of seed-specific mRNAs coding for oleosin which is a triacylgyceride. This study suggested that the leaf metabolism may have reverted to storage type upon DEX induction (Mendoza *et al.*, 2005).

The chimeric transcription activator XVE is a fusion protein of three constituents: the bacterial repressor LexA (X), the acidic trans-activating domain of herpes simplex virus, VP16 (V) and the regulatory region of human estrogen receptor (E) (Zuo & Chua, 2000). The XVE activator can be readily activated by the steroid hormone 17-β-estradiol as the inducer and in the absence of the 17-β-estradiol, background activities were not detected (Zuo *et al.,* 2000). The XVE-responsive promoter contains eight copies of the LexA-binding sites fused to the -46 35S minimal promoter (designated as *O*lexA TATA) that controls transcription of target genes. The XVE system has been successfully applied to various types of dicotyledonous plants such as *Arabidopsis* (Brand *et al.*, 2006; Abu Bakar *et al.*, 2015), tomato (Zhang *et al.*, 2006) and rice (Okuzaki *et al.*, 2011). Brand *et al.* (2006) constructed a series of Gateway-compatible, two-component XVE-based expression vectors for the inducible expression of heterologous genes in plants. The pMDC150 activator vector contained the XVE-encoded gene while the pMDC221 or pMDC160 responder vectors contained the XVE-responsive promoter (*O*lexA TATA) for the expression of the heterologous transgene of interest. The utility of these vectors was demonstrated when a pMDC150:35S activator construct was co-transformed with a pMDC160-GUS responder construct into *Arabidopsis thaliana*. Transgenic *Arabidopsis thaliana* plants that developed clearly showed GUS (β-glucuronidase) activity in the presence of 17-β-estradiol (Brand *et al.*, 2006). Strong GUS expression was also observed in seedlings germinated on 17-β-estradiol-containing plates while wild-type seedlings did not show any expression. This experiment proved that the XVE system is suitable for the study of conditional gene expression as the gene of interest can be activated only during induction and at the desired parts of plants (Brand *et al.*, 2006). The XVE system was

employed recently (Abu Bakar *et al.*, 2015) to express the pneumococcal YoeB$_{Spn}$ toxin in *Arabidopsis thaliana*. Induction of the *yoeB$_{Spn}$* toxin gene with 17-*β*-estradiol was lethal in the tested transgenic *Arabidopsis*.

Zhang *et al*. (2006) successfully introduced a synthetic *Bacillus thuringiensis* endotoxin gene into tomato (*Solanum lycopercisum*) using Cre/loxP site-specific DNA excision system, thus creating insect-resistant transgenic tomatoes that are free of selectable marker genes. The Cre/loxP system involves Cre recombinase that specifically recognizes and excises selectable marker DNA flanked between two loxP recognition sites *(Dale & Ow, 1991)*. In this study, both the selectable marker (kanamycin resistance gene) and the Cre recombinase gene were co-transformed on the same T-DNA region. Induction using 17-*β*-estradiol activated the expression of the recombinase protein which then excised its own coding sequence and removed the selectable marker gene. This inducible auto-excision approach aided by the XVE system produced tomato plants free of either selectable marker gene or the recombinase gene (Zhang *et al.*, 2006).

The efficacy of 17-*β*-estradiol levels induced by the XVE system to activate GFP expression in *Oryza sativa L.* (Okuzaki *et al.*, 2011). This study focused on the GFP expression patterns and the amount of inducer required to drive gene expression in specific parts of the plants. The XVE system rapidly induced GFP expression in the rice roots and calli with 5 µM 17-*β*-estradiol but lower expression was observed in leaves even with 50 µM 17-*β*-estradiol treatment. GFP signals in rice calli were still detected up to 10 days after 17-*β*-estradiol induction indicating that the XVE system was still functioning as long as the 17-*β*-estradiol is still present in the media used (Okuzaki *et al.*, 2011). The results obtained indicated the utility of the XVE-based expression system for controlled target gene expression in rice and other monocotyledonous plants.
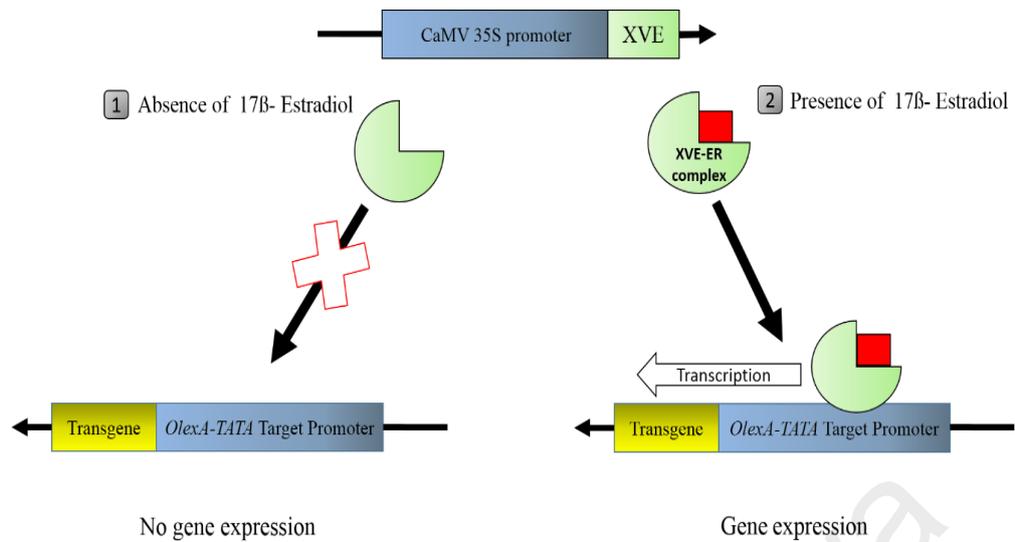
Figure 2.6: The XVE-based inducible expression system. In the absence of the inducer (17-β-estradiol), the expression of the transgene is inhibited as the XVE activator could not bind to the target promoter. In the presence of inducer, the XVE activator binds to 17-β-estradiol, resulting in a conformational change which enables it to bind to the target promoter to initiate expression of the transgene. Figure adapted and modified from Gatz & Lenk (1998).

<div align="center">

CHAPTER 3:

MATERIALS AND METHODOLOGY

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3.1 Materials

3.1.1 Bacterial, microalgae and plasmids used in this study

3.1.1.1 Recombinant *Agrobacterium tumefaciens* strains

A two-component 17-β-estradiol-inducible expression system comprising the pMDC150 activator vector and the pMDC221 responder vector (Brand *et al.*, 2006) was used for the expression of the two prokaryotic toxins genes in this study. Isolates of the recombinant *A. tumefaciens* (LBA 4404) strains used in this study were constructed and provided by Fauziah Abu Bakar from Faculty of Science, University of Malaya (Abu Bakar *et al.*, 2015). The three recombinant *A. tumefaciens* strains carried the (1) pMDC150_35S activator vector, (2) the pMDC221_yoeB$_{Spn}$GFP; and (3) pMDC221_pezTGFP responder vectors (Abu Bakar *et al.*, 2015) as shown in Figure 3.1 In pMDC221_yoeB$_{Spn}$GFP, the *yoeB*$_{Spn}$ gene was cloned as a translational fusion with the *GFP* gene (Abu Bakar et al., 2015) whereas in pMDC221_pezTGFP, the *pezT* gene was cloned as a translational fusion with the *GFP* gene (Ng et al., 2016). The pMDC221_GFP is the *GFP*-expressing recombinant responder vector (Abu Bakar *et al.*, 2015) and was included as a positive control in this study.
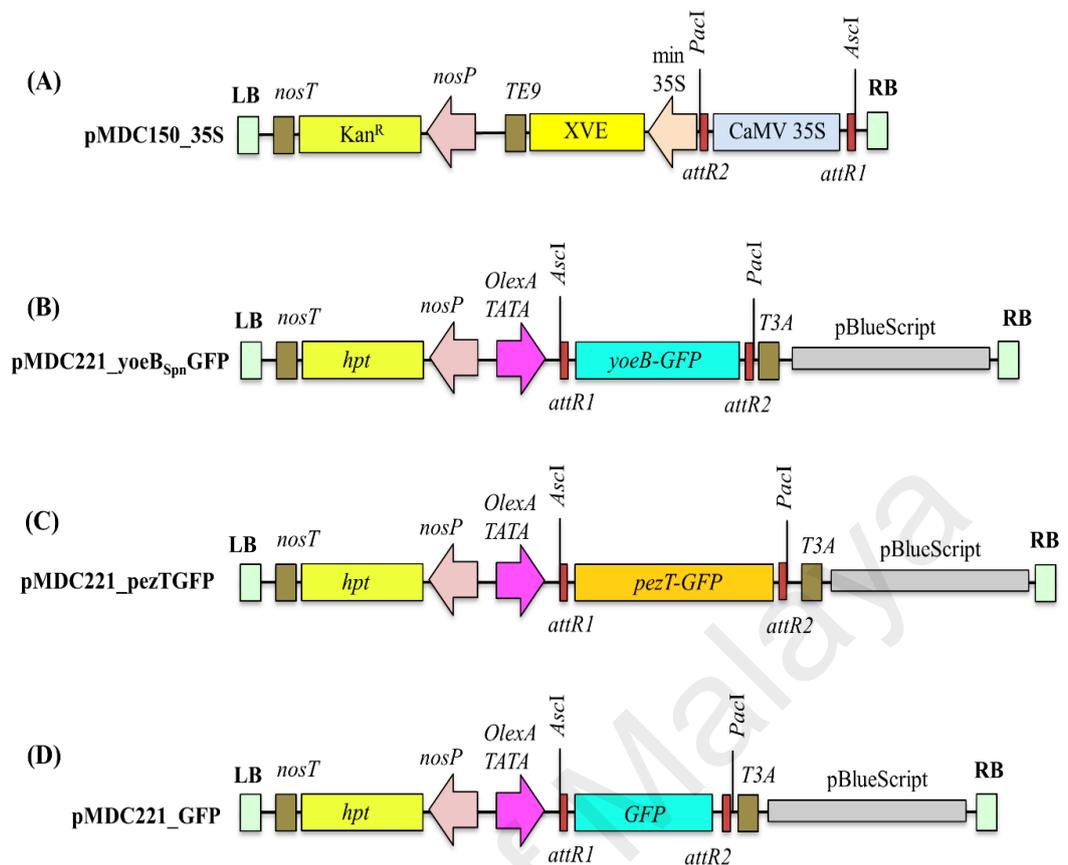
Figure 3.1: Schematic illustration of the T-DNA of the vector constructs used in this study as adapted from Brand *et al*. (2006). The activator vector (**A**) pMDC150-35S contained the XVE transcriptional activator gene driven by the constitutive CaMV 35S promoter. The responder vectors are (**B**) pMDC221_yoeB$_{Spn}$GFP; (**C**) pMDC221_pezTGFP and (**D**) pMDC221_GFP, in which the transgenes of interest were expressed from the XVE-responsive promoter (designated *O*lexA TATA). The recombinant pMDC221_GFP vector (with the GFP gene driven by the XVE-responsive promoter) is used as a positive control in this study. Constructions and details of the recombinant vectors which were kindly provided by F. Abu Bakar (Abu Bakar *et al.*, 2015; Ng *et al.*, 2016). LB, RB are the left and right borders of the T-DNA, respectively; Kan^R is the kanamycin resistance gene; *hpt* is the hygromycin resistance gene, used as selectable marker for transgene selection; *TE9*, *TE3* and *nosT* are terminator sequences; *attR1* and *attR2* are recombination sites used for Gateway cloning and pBlueScript refers to the *E. coli* cloning vector pBlueScript backbone, which is included in the T-DNA to enable plasmid rescue in *E. coli* (Brand *et al.,* 2006).

3.1.1.2 *Chlorella vulgaris* strain

The *C. vulgaris* strain (UMT-M1) was isolated from the coastal area near Universiti Malaysia Terengganu, Terengganu (Cha *et al.*, 2011) and was provided by Dr Cha Thye San from the Institute Marine of Biology, University Malaysia Terengganu.

3.1.1.3 *Escherichia coli* strain

The chemically competent *E. coli* TOP 10 strain (Invitrogen, USA) was used in this study as the host for pGEM-T-derived cloning vectors (Promega, USA) that were used to clone the PCR amplicons obtained for sequencing to validate the amplified products.

3.2 Methods

3.2.1 Culture Conditions for transgenic *A. tumefaciens* strains

Recombinant *A. tumefaciens* LBA4404 cells were cultured in Luria-Bertani (LB) broth and on LB agar solidified with 1.2 % (w/v) bacto-agar, supplemented with the appropriate antibiotics. For *A. tumefaciens* strains harboring the recombinant pMDC150-derived activator vector, the antibiotics that used were rifampicin (50 µg mL^{-1}) and spectinomycin (50 µg mL^{-1}); whereas for *A. tumefaciens* harboring recombinant pMDC221-derived responder vector, the two antibiotics used were rifampicin (50 µg mL^{-1}) and ampicillin (100 µg mL^{-1}) (Brand *et al.*, 2006). Bacterial culture on agar was incubated at 27 °C while those in broth was incubated at 27 °C with shaking at 220 rpm. Both agar and broth cultures of *A. tumefaciens* were incubated in the dark at all times as rifampicin is a light-sensitive antibiotic.

3.2.2 Culture Conditions for *C. vulgaris*

C. vulgaris UMT-M1 (Cha *et al.*, 2012) was cultured in Bold's Basal Medium (BBM) broth and on BBM agar solidified with 1.2% w/v bacto-agar. Microalgae cultures on agar were incubated at 27 °C while those in broth were incubated at 27 °C with shaking at 220

rpm. Both agar and broth cultures of *C. vulgaris* UMT-M1 were exposed to a continuous photosynthetic photon flux (PPF) of 40 µmol m^{-2} s^{-1}. For selection, the transformed *C. vulgaris* UMT-M1 was cultured on selective BBM supplemented with both kanamycin (50 µg mL^{-1}) and hygromycin (20 µg mL^{-1}). The agar and broth cultures of transformed *C. vulgaris* UMT-M1 were incubated in the dark for the first 24 h before exposure to light as hygromycin is a light-sensitive antibiotic.

3.2.3 Co-transformation of two-component inducible expression vectors into *C. vulgaris* UMT-M1

A. tumefaciens-mediated transformation of *C. vulgaris* UMT-M1 was carried out according to Cha *et al.* (2012) with modifications. Approximately 5×10^7 *C. vulgaris* UMT-M1 cells from a log-phase culture (OD$_{600}$ = 0.5-1.0) were pre-cultured for 5 days in BBM broth and the cells were then harvested. Prior to co-cultivation, the *C. vulgaris* UMT-M1 cells were treated with 500 mg mL^{-1} lysozyme and 500 mg mL^{-1} cellulase for 18 h at 28°C. The treated microalgae cells were washed with induction medium (IM) broth (BBM + 100 µM acetosyringone, pH 5.6) before being subjected to *Agrobacterium*-mediated transformation. The recombinant *A. tumefaciens* cells were cultured in LB broth supplemented with the appropriate antibiotics for 18 hours until the cells reached OD$_{600}$=0.4. For co-cultivation, each *C. vulgaris* UMT-M1 culture (~100 µL) was incubated with two *A. tumefaciens* LBA4404 strains (~200 µL), one carrying the activator vector pMDC150_35S and the other carrying the respective recombinant responder vector, i.e., either pMDC221_GFP (as the positive control), pMDC221_yoeBGFP, or pMDC221_pezTGFP and plated on IM media solidified with 1.2% (w/v) bacto-agar for 3 days incubation.

Following co-cultivation, the mixed culture was harvested and inoculated into 25 mL BBM broth supplemented with cefotaxime (500 µg mL^{-1}) and incubated at 27 °C in the dark for 2 days to eliminate remaining *A. tumefaciens* cells. The cell culture was then

centrifuged at 5,000 ×*g* for 5 min at 25 °C and the supernatant was discarded. The cell pellet was resuspended with 50 µL of BBM broth before plating on BBM agar. The transformed cells were incubated for 7 days at 27 °C for recovery in the dark. The transformed microalgae cells were then transferred to selective BBM agar supplemented with kanamycin (50 µg mL^{-1}) and hygromycin (20 µg mL^{-1}) and incubated at 27 °C in the dark for 2 days, before exposure to light. The plates were kept in the dark for 2 days before being exposed to light as the antibiotics that were used for the selection of transformants were light sensitive. Transformed colonies were cultured on selective BBM agar. The positive transformants were visible within 3 weeks and were sub-cultured on non-selective BBM after 4 weeks, for further experiments.

3.2.4 Selection and maintenance of transformed *C. vulgaris* lines

The selected transformed *C. vulgaris* lines were cultured alternately on selective media (BBM containing kanamycin and hygromycin) and then, non-selective media (BBM with no supplemented antibiotic) with three-month's duration for each sub-culture (culture conditions as described in Section 3.2.2). After one year of culture maintenance, the transformed *C. vulgaris* lines were then subjected to further experiments.

3.2.4.1 Genomic DNA extraction

Genomic DNA extraction of transformed *C. vulgaris* UMT-M1 was carried out using the Wizard® Genomic DNA Purification Kits (Promega, USA). A 1 mL aliquot of transformed *C. vulgaris* UMT-M1 culture was transferred into a clean 1.5 mL microcentrifuge tube and the cells were harvested by centrifugation at 5,000 ×*g* for 5 min at room temperature. The cell pellet was resuspended in 600 µL nuclei lysis solution and transferred to a clean mortar and pestle. The cell suspension was subjected to grinding for 5 min and then transferred to a clean 1.5 mL microcentrifuge tube followed by incubation at 65 °C for 15 min. A total of 3 µL RNase solution was added into the sample and

incubated at 37 °C for 15 min, and allowed to cool at room temperature for 5 min. A total of 200 µL of protein precipitation solution was added to the sample followed by vortexing and centrifugation at 14, 5000 $\times g$ for 3 min. The resulting clear supernatant was then transferred into a sterile tube containing 600 µL isopropanol to precipitate the DNA. The sample was inverted 6 times and further subjected to centrifugation at 14,500 $\times g$ for 1 min. The resulting supernatant was decanted and 600 µL of 70% ethanol were added to the DNA pellet. The sample was again centrifuged at 14,500 $\times g$ for 1 min and the supernatant was discarded. Any residual ethanol was aspirated and the pellet was left to air-dry. To rehydrate the DNA pellet, 100 µL DNA rehydration solution were added and incubated at 65 °C for 1 hour.

3.2.4.2 DNA quantitation and visualization

A 2 µL aliquot of the extracted DNA was diluted with 98 µL sterile distilled water to prepare each 50 $\times$ diluted sample. Using UV spectrophotometry (Eppendorf® BioPhotometer, Germany), the DNA concentration was then measured at the optical density of 260 nm (in which 1 OD_{260} unit is equal to 50 μg mL^{-1} for double stranded DNA) and the purity of the DNA sample was indicated by calculating the OD_{260}/OD_{280} ratio values with pure samples having a ratio of 1.8 (Barbas *et al.*, 2007).

3.2.4.3 Separation of DNA using gel electrophoresis

An aliquot of 3 µL of the extracted genomic DNA was mixed together with a 1 µL of 6 $\times$ bromophenol blue loading dye. The DNA-dye mixture was loaded into the lane and electrophoretically separated on a 1.0% w/v agarose gel stained with ethidium bromide (0.5 μg mL^{-1}). The extracted genomic DNA were subjected to electrophoresis along with an appropriate DNA marker (Vivantis Technologies, USA) in 1$\times$ TAE buffer with a voltage of 100 V for 40 min. DNA was then visualized by exposing the gel to UV light

in a gel imager (Quantum ST4- 3026, Vilber Lourmat, Australia) using the Quantum-Capt (Vilber Lourmat, Australia) software.

3.2.5 Screening of positive microalgae transformants

3.2.5.1 Primer design and synthesis

The gene-specific primers used in this study are listed in Table 3.1. The locations of the gene-specific primers in the recombinant vectors are shown in Figure 3.2. The primers marked with asterisk (*) were previously described (Abu Bakar *et al.*, 2015) whereas the remaining primers were designed with reference to the DNA sequences of the recombinant vectors pMDC150_35S, pMDC221_yoeB_{Spn}GFP and pMDC221_pezTGFP. Designed primers were then checked for melting temperature estimation, presence of secondary structures and hairpin formation using Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/). The optimal primers were chosen for each set and these primers were commercially synthesized by Integrated DNA Technologies, USA.

The primers designed for PCR amplification were also used for the screening of positive transformants and RT-PCR. The primer pairs were designed to be 20-30 nucleotides in length and with similar melting temperature (± 3 ℃). The GC content was fixed between 40-60% with a uniform distribution of G and C nucleotides throughout the primer. Each primer was designed to avoid self-complementarity or complementarity with the other primer in the reaction mixture to avoid thermostable secondary structure and hairpins, primer-dimers and homodimers under the PCR annealing conditions.

Table 3.1: Gene-specific primers used in PCR amplification of desired transgenes

Primer name	Primer sequence (5'-3')
yoeB_F2	GCTACTCAAGTTTACAGAAG
yoeB_R2	CACGCTATCTCCATCCATC
pezT_F*	CACCATGGAAATCCAAGAT
GFP_R*	TTATAATCCCAGCAGCTGTT
pezT_F2	GCAAGGAATCTTCGTTCACTG
pezT_R2	TCCTTCTCTACCTGACTCCAC
GFP_F*	GGATCCATGGTAGATCTGA
GFP_scR	CAGCTGTTACAAACTCAAGAAG
XVE_F1	CAAGAGGTGTTTGATCTCATC
XVE_R1	TGTCCAAGAGCAAGTTAG
18S_F	CCTGCGGCTTAATTTGACTCAACACG
18S_R	TAGCAGGCT GAGGTCACGTTCG

Note: Asterisk (*) indicates the primers were obtained from Abu Bakar *et al.* (2015); other

primers were designed in this study.
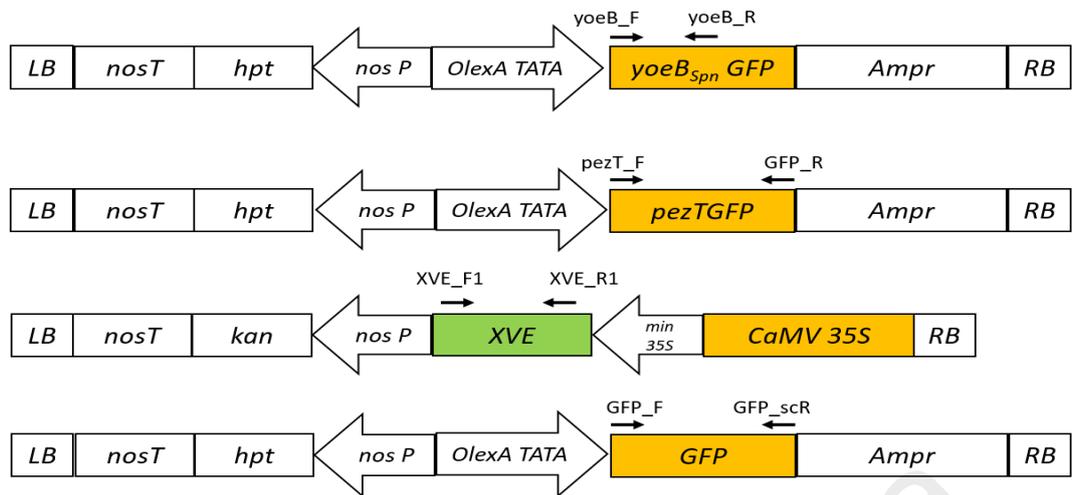
Figure 3.2: A schematic illustration of the locations of the gene specific primers used in this study for transgene PCR amplification. **(A)** Recombinant T-DNA of pMDC221_yoeB$_{Spn}$GFP; **(B)** T-DNA of pMDC221_pezTGFP; **(C)** T-DNA of pMDC150_35S; **(D)** T-DNA of pMDC221-GFP. LB is the left border; RB is the right border of the T-DNA; *nosT*, *nos* terminator are the terminator sequences; *hpt* is the hygromycin resistance gene for plant selection; *O*lexA TATA is the *XVE* responsive promoter and *Ampr* is the ampicillin resistance gene for bacterial selection.

3.2.5.2 PCR analysis

Each PCR reaction consisted of 100 ng DNA template, 1× MyFi reaction buffer (5 mM dNTPs, 15 mM MgCl2, stabilizers and enhancers) (Bioline, USA), 0.4 µM each of the reverse and forward primers, 0.4 U of MyFi DNA polymerase (Bioline, USA) and sterile deionized distilled water in a final volume of 25 µL. PCR was carried out in a T100™ thermal cycler (Eppendorf, Germany) with the following conditions: initial denaturation of DNA template at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 15 s, primer annealing at 57 °C for 15 s, extension at 72 °C for 45 s and final extension at 72 °C for 5 min. The PCR products were subjected to agarose gel electrophoresis as described in Section 3.2.4.3.

3.2.5.3 Purification of PCR products from agarose gels

PCR products from agarose gel electrophoresis were purified using Wizard® SV Gel and PCR Clean-Up System (Promega, USA). The band of interest observed following agarose gel electrophoresis was excised using a clean sterile blade and the gel slice (weighing approximately 200 mg) placed in a 1.5 mL microcentrifuge tube. A 10 µl of membrane binding solution was added for every 10 mg of the gel slice followed by incubation at 65 °C until the gel slice was fully dissolved. The dissolved gel mixture was transferred into a SV minicolumn which was placed in a collection tube, left for 1 min at room temperature and subsequently subjected to centrifugation at 14,500 ×g for 1 min using a bench top microcentrifuge MiniSpin® plus (Eppendorf, Germany). The flow-through was discarded and the minicolumn was re-inserted into the collection tube. The washing step was initiated by adding 700 µL wash solution followed by centrifugation at 14,500 ×g for 1 min. The flow-through was discarded and the minicolumn was re-inserted into collection tube. The washing step was repeated with 500 µL wash solution and centrifugation at 14,500 ×g for 5 min. The collection tube was emptied and subjected to another round of centrifugation at 14,500 ×g for 1 min to discard any residual ethanol

within the minicolumn. The minicolumn was then transferred to a clean 1.5 ml microcentrifuge tube and 30 µL of sterile distilled water was added and incubated at room temperature for 1 min before subjected to centrifugation at 14,500 $\times g$ for 1 min. The minicolumn was discarded and an aliquot (3 µL) of the eluted DNA was subjected to agarose gel electrophoresis for validation before being kept for storage at -20 °C until use. The PCR products were subsequently cloned into pGEM®-T Easy Vector System (Promega, USA) using protocols as described in Section 3.2.5.4.

3.2.5.4 Ligation

The cloning of the PCR products into pGEM®T Vector System (Promega, USA) utilizes the ligation of A overhangs at the 5' ends of PCR products and the T overhangs found at the 3' ends of the pGEM-T vector. The ligation reaction was set up as follows: $1 \times$ Ligation Buffer [60 mM Tris-HCl (pH 7.8), 20mM $MgCl_2$, 20 mM dithiothreitol, 2mM ATP and 10% polyethylene glycol], T4 DNA Ligase (Promega, USA), 5 ng of pGEM®-T vector , 0.2 µg of PCR product, 0.3 Weiss units/ µL of T4 DNA ligase and sterile distilled water added to a final reaction volume of 10 µL. The ligation mixture was subjected to incubation at 4 °C overnight.

3.2.5.5 Preparation of chemically-induced *E. coli* competent cells

The *E. coli* TOP 10 competent cell kept as glycerol stock was cultured on a LB plate overnight at 37 °C. A single *E. coli* colony was picked and inoculated overnight in 10 mL LB broth at 37 °C with shaking at 220 rpm. Following that, a total of 1 mL overnight *E. coli* culture was inoculated into 40 mL LB broth and incubated for 1.5 h until the cells reached log phase (OD_{600}= 0.4). The flask containing the bacterial culture was then placed on ice and 20 mL of the culture was transferred to pre-chilled centrifuge tubes and cells collected by centrifugation at 6,000 $\times g$ at 4 °C for 20 min. The supernatant was discarded and the cell pellet was resuspended using 10 mL of chilled 0.1 M $CaCl_2$. The mixture was

then left on ice for 30 min. Following that, the cells were again centrifuged at 6,000 $\times g$ at 4 °C for 20 min and the supernatant discarded. The cell pellet was resuspended with 2 mL CaCl$_2$ solution (85 mM CaCl$_2$ and 15% glycerol). Finally, 200 µL aliquot of the bacterial culture was placed into each 1.5 mL centrifuge tubes, flash-frozen in liquid nitrogen and stored in a -80 °C freezer overnight or until used.

3.2.5.6 Transformation of chemically-induced *E. coli* competent cells

Frozen *E. coli* TOP 10 competent cells were retrieved from the -80 °C freezer and left to thaw on ice for 15 min. A total of 6 µL DNA ligation reaction mixture (as described in Section 3.2.5.4) was added into the thawed competent cells and the tube incubated on ice for a further 30 min. The cells were then subjected to heat shock at 42 °C for precisely 1 min and immediately placed back in ice for 2 min. Then, 700 µL of LB broth was aliquoted into the competent cells and incubated at 37 °C with shaking at 220 rpm for 90 min. The transformed bacterial cells were plated on LB agar plate supplemented with ampicillin (100 µg mL^{-1}) and incubated overnight at 37 °C.

3.2.5.7 Colony PCR

Selected bacterial colonies that harbored the recombinant pGEM®T vectors were picked using sterile toothpicks and subcultured on LB agar supplemented with ampicillin (100 µg mL^{-1}) and incubated overnight at 37 °C. The same toothpick was then used to touch the base of 0.2 mL microcentrifuge tube. PCR mastermix was prepared accordingly to Section 3.2.5.2 using the gene specific primers (Table 3.1) for the *yoeB$_{Spn}$, pezT* and *XVE* transgenes. PCR was carried out as described in Section 3.2.5.2.

3.2.5.8 Plasmid DNA extraction

Plasmid DNA was extracted using GeneJET™ Plasmid Miniprep Kit (Fermentas, USA). The overnight bacterial culture was centrifuged at 14,500 $\times g$ and the resulting

supernatant was discarded. The cell pellet was re-suspended with 250 µL resuspension solution and vortexed followed by the addition of 250 µL of lysis solution and the tube was inverted 4-6 times to achieve cell lysis. A total of 350 µL neutralization solution was then added and the tube was again inverted. The mixture was centrifuged at 14,500 ×g for 5 min in a benchtop MiniSpin® plus microcentrifuge (Eppendorf, Germany). The clear supernatant was carefully transferred to a spin column placed in a collection tube and subjected to centrifugation at 14,500 ×g for 1 min. The flow-through was discarded and 500 µL of 70% ethanol was added and centrifuged at 14,500 ×g for 1 min to wash the spin column. This step was repeated twice. The flow-through was discarded and the empty column was subjected to further centrifugation at 14,500 ×g for 1 min to allow the evaporation of any residual ethanol. The spin column was then transferred to a clean 1.5 mL microcentrifuge tube and 30 µL of sterile distilled water was added and incubated for 2 min at room temperature to enable elution of the plasmid DNA. The tube was subjected to final centrifugation at 14,500 ×g for 2 min. The concentration and the purity of the eluted plasmid DNA was measured spectrophotometrically as described in Section 3.2.4.2. Plasmid DNA samples were sequenced by a commercial vendor (1st Base) and the sequencing results were analyzed using BioEdit version 7.2.5. and BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome).

3.3 Cell lysis assay

3.3.1 Enumeration of transgenic *C. vulgaris* UMT-M1 cells using modified Miles and Misra method

To determine the effect of bacterial toxin expression on the viability of transgenic *C. vulgaris* UMT-M1 cells, a modified Miles and Misra method (Tran *et al.*, 2013) was carried out independently for UMT-M1 (GFP), UMT-M1 (yoeB$_{Spn}$-GFP) and UMT-M1 (pezT-GFP) cell lines. A total of 1 mL of transformed *C. vulgaris* UMT-M1 cells were

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inoculated in 20 mL of BBM broth at 27 °C for 5 days with shaking at 220 rpm until log phase (OD_{600}=0.5-1.0). Transformed *C. vulgaris* UMT-M1 cell numbers were standardized to 5×10^6 using haemocytometer counts and appropriate dilutions. Prior to 17-*β*-estradiol treatment, the transformed *C. vulgaris* UMT-M1 cells were treated with lysozyme (500 µg mL^{-1}) and cellulase (500 µg mL^{-1}) for 16 h to degrade the cell wall and incubated overnight at 27 °C with shaking at 220 rpm in the dark. The enzyme-treated microalgae cells were centrifuged at 8,000 $\times g$ for 10 min and then washed with 1 mL BBM broth. The supernatant was discarded and the cell pellet was resuspended with 1 mL BBM broth. A total of 1 mL of transformed *C. vulgaris* UMT-M1 cells in 1.5 mL microcentrifuge tube were treated with 100 µM 17-*β*-estradiol and agitated at 220 rpm for 24 h. The 17-*β*-estradiol-treated cells were kept in the dark at 27 °C for the entire duration of treatment as 17-*β*-estradiol is light-sensitive and prone to degradation. An overview of the modified Miles and Misra dilution method used in this study is illustrated in Figure 3.3. At each time point (0.5, 6, 12 and 24 h), 11 µL of the17-*β*-estradiol-treated cells were collected and inoculated into 99 µL BBM broth to make up a total of 110 µL cell suspension. A 10-fold serial dilution up to 10^{-8} from the 110 µL of the cell suspension was prepared. A total of five replicates (five BBM plates) of each sample were prepared for each time point. Each plate was divided into 8 sectors and labelled with dilutions. A total of 20 µL of the appropriate dilution was dropped from a height of 2 cm and the drop was allowed to spread naturally. The plates were left upright on the bench to dry before they were inverted and incubated at 27 °C for 7 days. Once the transformed *C. vulgaris* UMT-M1 cells had grown to full size discrete colonies, colony counts were performed using the following equation:

(CFU)/mL= Average number of colonies for a dilution × 20 (volume of cell suspension dropped onto BBM media) × dilution factor.
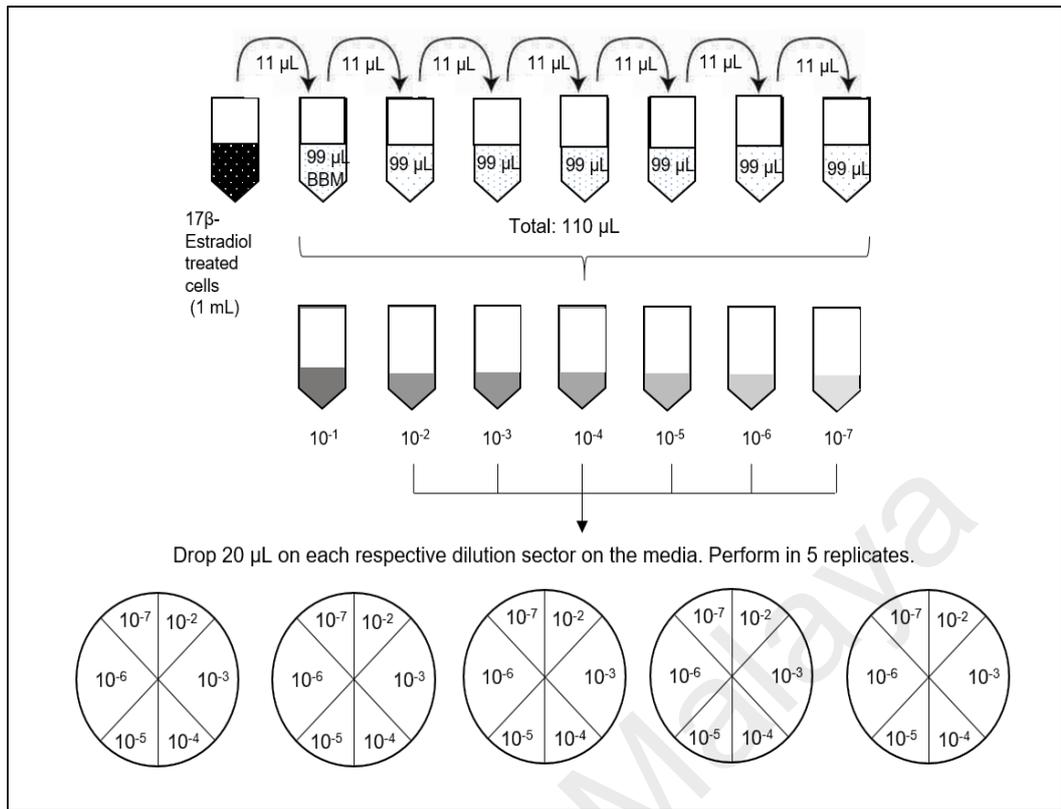
Figure 3.3: An overview of the Miles and Misra method used to enumerate viable transgenic *C. vulgaris* following 17-*β*-estradiol-treatment for the expression of the bacterial toxin.

3.3.1.1 Statistical analysis

All the data were expressed as the mean ±SD. The statistical significance was set at $p < 0.05$ that was considered as significant. The t-test was used to statistically analyze the samples' differences using Microsoft Excel® 2013.

3.3.2 Detection of GFP fluorescence and cell lysis activity in transgenic *C. vulgaris* UMT-M1 using fluorescence and bright field microscopy

The effects of 17-β-estradiol treatment on the cell morphology of transformed *C. vulgaris* UMT-M1 cells were determined by observing for GFP fluorescence under fluorescence microscopy and for the cellular appearance under bright field microscopy. From each time point following 17-β-estradiol treatment (i.e., 0.5, 6, 12 and 24 h), a 5 µL aliquot of the 17-β-estradiol-treated cells of transformed UMT-M1 (GFP), UMT-M1 (yoeB$_{Spn}$-GFP) and UMT-M1 (pezT-GFP) were placed onto glass slides. Each sample was viewed under bright field and fluorescence microscope at 1000× magnification. The fluorescent imaging was carried out using Leica DM LB2 (Leica, Germany). GFP fluorescence was visualized with an I3 filter (470-nm excitation filter/525-nm barrier filter). Concurrently, the transformed *C. vulgaris* UMT-M1 cells were also observed under Bright Field microscope at 1000× magnification to determine their morphology and observe for signs of cell lysis.

3.4 Determination of transcript levels using reverse transcription-PCR

To validate that the *yoeB$_{Spn}$* and *pezT* transgenes were expressed in the 17-β-estradiol-treated transgenic *C. vulgaris* cells, reverse transcription-PCR (RT-PCR) was performed on total RNA extracted from the transgenic cell lines.

3.4.1 Total RNA extraction

RNA extraction of microalgae cells were carried out using the GF-1 Total RNA extraction kit (Vivantis Technologies, USA). A total of 30 mL of each transgenic *C.*

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vulgaris UMT-M1 line was treated with 100 µM 17-*β*-estradiol and agitated at 220 rpm for a duration of 24 h. A total of 5 mL of estradiol-treated cells were collected at different time points (0.5, 6, 12 and 24 h) and the cells pelleted by centrifugation at 5,000 ×*g* for 5 min at room temperature. The cell pellet was flash-frozen with liquid nitrogen, ground into fine powder using a pre-chilled mortar and pestle, and the resulting powder transferred into a cold RNase-free microcentrifuge tube. A total of 400 µL of Buffer TR containing 0.143 M *β*-mercaptoethanol was added to the cell powder and mixed thoroughly by vigorous vortexing. The suspension was then subjected to centrifugation at 14,500 ×*g* for 3 min in a benchtop MiniSpin® plus microcentrifuge (Eppendorf, Germany) and the supernatant containing the cell lysate was transferred into a homogenization column that was placed in a collection tube. The column containing the cell lysate was centrifuged at 14,500 ×*g* for 2 min followed by the addition of 350 µL 80% ethanol to the flow-through.

The sample was mixed thoroughly by pipetting and then transferred into a RNA binding column that was assembled in a collection tube. The sample was subjected to centrifugation at 10,000 ×*g* for 1 min and the flow-though was discarded. A total of 500 µL wash buffer was added to the sample and centrifuged at 14,500 ×*g* for 1 min. The flow-through was again discarded and 70 µL of a DNase I Digestion mix was pipetted directly onto the membrane of the RNA binding column and incubated at room temperature for 15 min. Following that, 500 µL of inhibitor removal buffer was added to the sample and centrifuged at 14,500 ×*g* for 1 min, and the resulting flow-through was discarded. The column was again washed with 500 µL wash buffer and centrifuged at 10,000 ×*g* for 1 min, discarding the flow-through, and repeating this step once. The empty column was centrifuged at 10,000 ×*g* for 1 min again to remove any traces of buffer. The column was placed into a new microcentrifuge tube and 40 µL RNase-free water was aliquoted directly onto the membrane and allowed to stand for 1 min before subjected to

centrifugation at 10, 000 ×*g* for 1 min. The 2 µL of extracted RNA was diluted with 98 µL sterile distilled water to prepare a 50 × dilution. Using UV spectrophotometry (Eppendorf® BioPhotometer, Germany), the diluted RNA concentration was then measured at the optical density of 260 nm (OD_{260}) and the level of protein contamination in the RNA sample was determined by the OD_{260}/OD_{280} ratio values (Barbas *et al.*, 2007). An OD_{260}/OD_{280} ratio value of 2.0 is considered good quality for RNA samples (Barbas *et al.*, 2007)

3.4.2 cDNA synthesis

M-MuLV reverse transcriptase (Thermo Scientific, USA) was used to reverse-transcribe RNA into complementary DNA (cDNA). The components to generate the first strand of cDNA were all briefly centrifuged and kept on ice. Total RNA template (1 µg) and 1 µL of 10 µM gene specific primer (GFP_scR) (Table 3.1) were added into a nuclease-free tube, followed by the addition of DEPC-treated water to make up a volume of 11.5 µL. To remove any RNA secondary structure, this mixture was mixed gently, centrifuged briefly, incubated at 65 °C for 15 min and placed back on ice. The components for the first strand cDNA synthesis were as follows: 0.5 µg of total RNA, 0.4 µmol of GFP_scR gene specific primer, 1× MyFi reaction buffer, 20 U of RiboLock RNase Inhibitor (Thermo Scientific), 0.5 mM of dNTP mix, 200 U of RevertAid Reverse Transcriptase (Thermo Scientific) and sterile distilled deionized water added to a final volume of 20 µL. The mixture was mixed gently and briefly centrifuged, followed by incubation at 37 °C for 60 min. The reverse transcription reaction was then terminated by incubating the mixture at 70 °C for 10 min. The final product was kept at -20 °C, if it was not immediately utilized for PCR reaction.

3.4.3 Reverse Transcriptase PCR

The first strand cDNA was used as DNA template for RT- PCR. The RT-PCR settings and conditions were as described in Section 3.2.5.2. The gene-specific primers used to

amplify the *yoeB* transcript were yoeB_F2 and yoeB_R2 whereas for the *pezT* transcript, the pezT_R2 and pezT_R2 gene-specific primers were used (Table 3-1). The 18S rRNA gene was used as the housekeeping control and amplified using 18S rRNA-specific primers (Table 3.1).

3.5 Transgene stability assay

A single line each from the transgenic line UMT-M1 (yoeB$_{Spn}$-GFP) and UMT-M1 (pezT-GFP) transgenic line was selected from the selection plate that was maintained for about a year as described in Section 3.1.1.2 Following that, the transgenic cells were subjected to a series of five consecutive subcultures (~ 75 days) of alternating presence and absence of antibiotic-selection pressure (i.e., growth in the presence of kanamycin and hygromycin, followed by growth in antibiotic-free media) (Figure 3.4). After the alternate sub-culturing, the cells from the final subculture were randomly selected for DNA extraction and PCR analysis. The presence of gene transcripts was determined by RT-PCR amplification (as described in Section 3.2.5.2) using gene-specific primers (Table 3.1).

Culture maintenance for one year

1

2   S

3   NS

} Repeat 5 times

4

5

1. After a year or culture maintenance, a single transformed microalgal cell line was selected and cultured on a new non-selective BBM
2. A single colony was randomly selected and then cultured on selective BBM using spread plate method
3. The microalgal cells were harvested and transferred to non-selective BBM using spread plate method
4. Steps 3 and 4 were repeated for 5 times
5. A single loop of cell was then transferred to a non-selective BBM
6. Single colonies were sub-cultured on grid plates

* For each steps, the culture were incubated at optimal conditions (as described in Section 3.2.2) until visible colonies appeared before proceeding to the subsequent steps.
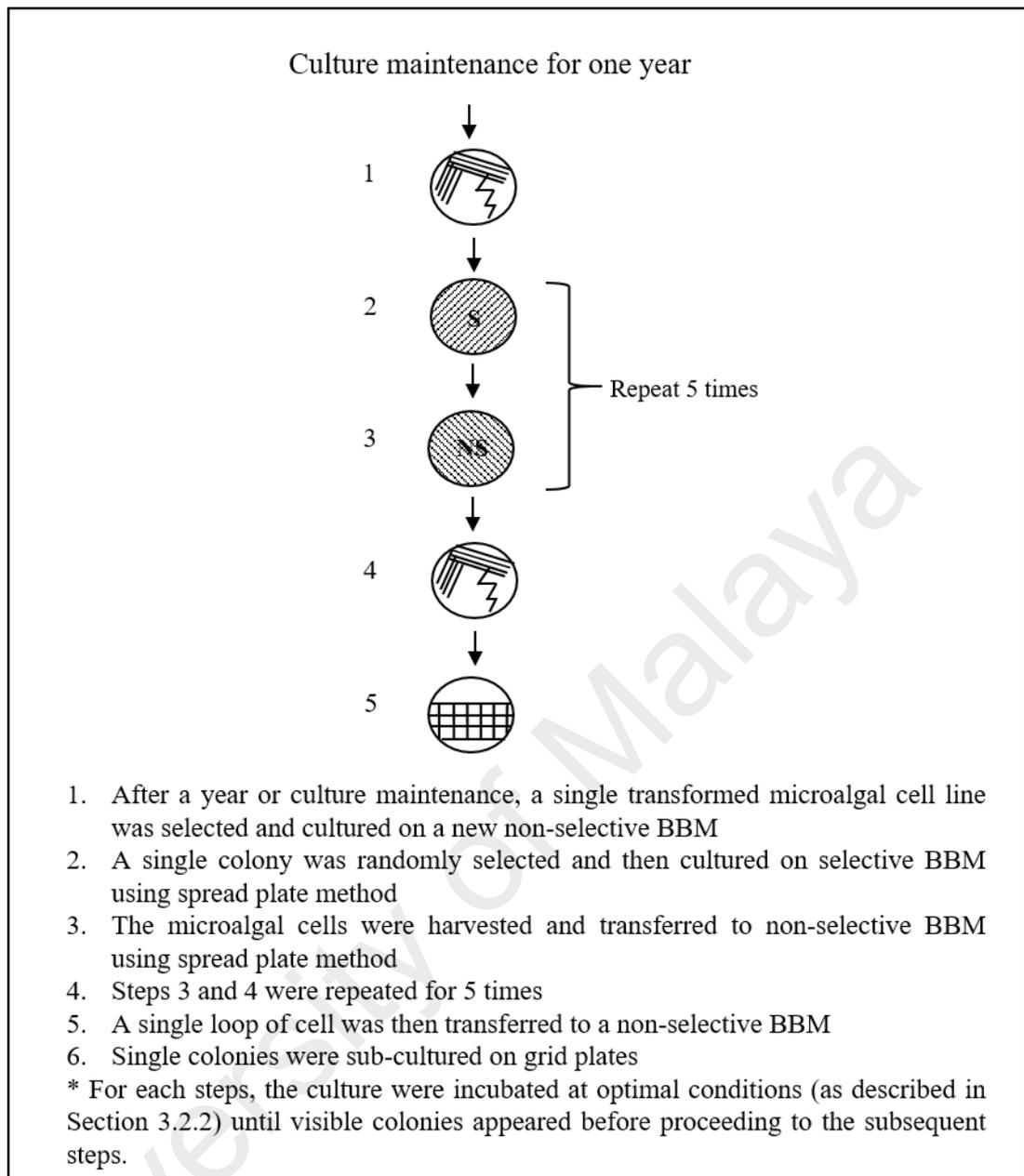
Figure 3.4: An overview of the protocol for determining stable transgenes integrations in transgenic *C. vulgaris* UMT-M1 lines.

CHAPTER 4:

RESULTS

4.1 Co-transformation of the two-component expression vectors

The two component XVE-based expression system, which comprised of the responder vector (pMDC150) and an activator vector (pMDC221) was co-transformed into *Chlorella vulgaris* UMT-M1 microalgae using *Agrobacterium*-mediated transformation. The *C. vulgaris* cell lines that were transformed with (1) pMDC221_yoeBGFP together with pMDC150_35S and (2) pMDC221_pezTGFP together with pMDC150_35S were designated as UMT-M1 (yoeB$_{Spn}$-GFP) and UMT-M1 (pezT-GFP), respectively. The *C. vulgaris* cell line transformed with pMDC221-GFP and pMDC150-35S was designated as UMT-M1 (GFP) and was included in the experiments as a control with only GFP expression.

4.1.1 Screening of transformed UMT-M1 (yoeB$_{Spn}$-GFP) lines

The putative transformant colonies of UMT-M1 (yoeB$_{Spn}$-GFP) grew on selective BBM supplemented with hygromycin (20 µg mL^{-1}) and kanamycin (50 µg mL^{-1}) four weeks after co-transformation. Six transformants colonies were randomly selected from BBM (Figure 4.1) for genomic DNA extraction and PCR analysis. Using the yoeB_F2 and yoeB_R2 primers (T-able 3.1), the *yoeB$_{Spn}$* toxin gene from the pMDC221_yoeB$_{Spn}$GFP vector was amplified with the expected band size of 223 bp (Figure 4.2). The *XVE* gene from the activator vector pMDC150_35S was also amplified with the expected band size of 873 bp (Figure 4.3) using the XVE_F1 and XVE_R1 primers (Table 3.1). The *E. coli*-recombinant purified plasmids (i.e., pMDC221_yoeB$_{Spn}$GFP and pMDC150_35S) that were used as positive controls also showed the amplification of the *yoeB$_{Spn}$* and *XVE* genes whereas the genes were not present in the DNA of the wild type *C. vulgaris* UMT-M1 that was used as negative

control. Thus, the PCR data reveal that the $yoeB_{Spn}$ and XVE genes were present in all six transformant lines four weeks after co-transformation with the responder and activator plasmid constructs.

Figure 4.1: Screening for transformant colonies of UMT-M1 (yoeB$_{Spn}$-GFP) cultured on BBM.Green colonies (1 – 25) indicate clones with resistance to hygromycin and kanamycin.

Figure 4.2: PCR detection of *yoeB$_{Spn}$* gene in transformed UMT-M1(yoeB$_{Spn}$-GFP). PCR amplification of the 223 bp *yoeB$_{Spn}$* gene from the transformed colonies in Lane 1-6 with plasmid pMDC221_yoeBGFP as the positive control (lane marked "+ve"). The negative control (lane "-ve") used is the genomic DNA of wild-type *C. vulgaris* UMT-M1. Lane M is the 100 bp DNA marker (Vivantis).

Figure 4.3: PCR detection of the *XVE* gene in transformed UMT-M1(yoeB$_{Spn}$-GFP). PCR amplification led to the presence of the expected 873 bp amplified product from the transformed colonies in Lanes 1-6 with plasmid pMDC150_35S as the positive control (lane marked "+ve"). The negative control (lane "-ve") used is the genomic DNA of wild-type *C. vulgaris* UMT-M1. Lane M is the 100 bp DNA marker (Vivantis).

4.1.2 Screening of the transformed UMT-M1 (pezT-GFP) lines

After *Agrobacterium*-mediated co-transformation with pMDC221_pezTGFP and pMDC150_35S, the UMT-M1 (pezT-GFP) cells were then cultured on selective BBM supplemented with hygromycin (20 µg mL^{-1}) and kanamycin (50 µg mL^{-1}). Four putative transformants were then randomly selected from the selective BBM plates (Figure 4.4) for PCR amplification. The *pezT-GFP* transgene amplified using *pezT-GFP* primers (pezT_F and GFP_R) (Table 3.1) yielded an expected band of 1494 bp (Figure 4.5). The *XVE* gene from the activator vector pMDC150_35S with the expected band size of 873 bp was also amplified from the selected transformants (Figure 4.6) using the XVE_F1 and XVE_R1 primers (Table 3.1). The *E. coli*-purified plasmids (pMDC221_pezTGFP and pMDC150_35S) that were used as positive control also showed amplification of the *pezT* and *XVE* genes. The genes were absent in the DNA of the wild type *C. vulgaris* UMT-M1 that was used as negative control. Thus, the PCR data reveal that the *pezT* and *XVE* genes were present in all four transformant lines four weeks after co-transformation with the responder and activator plasmid constructs.

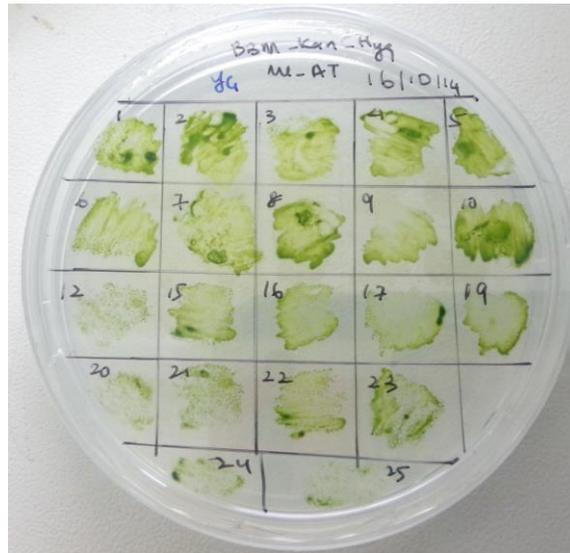**Figure 4.4:** Screening for transformant colonies of UMT-M1 (pezT-GFP) cultured on BBM. Green colonies (1 – 28) indicate clones with resistance to hygromycin and kanamycin.

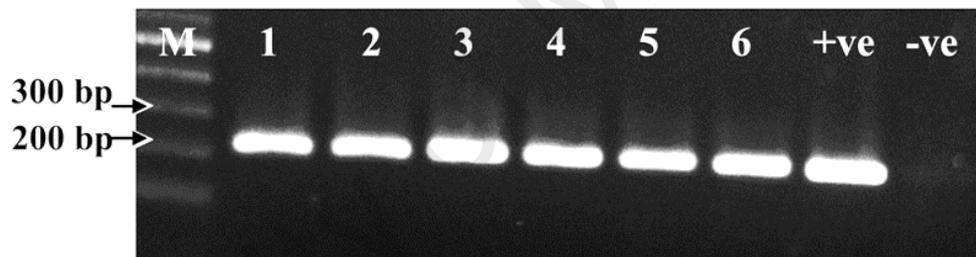**Figure 4.5:** PCR detection of *pezT-GFP* gene in transformed UMT-M1 (pezT-GFP) lines. PCR amplification yielded the expected 1494 bp *pezT-GFP* amplicon from the transformed colonies in Lanes 1-4 with plasmid pMDC221_pezTGFP as the positive control (lane marked "+ve"). The negative control (lane "-ve") used is the genomic DNA of wild-type *C. vulgaris* UMT-M1. Lane M is the 100 bp DNA marker (Vivantis).
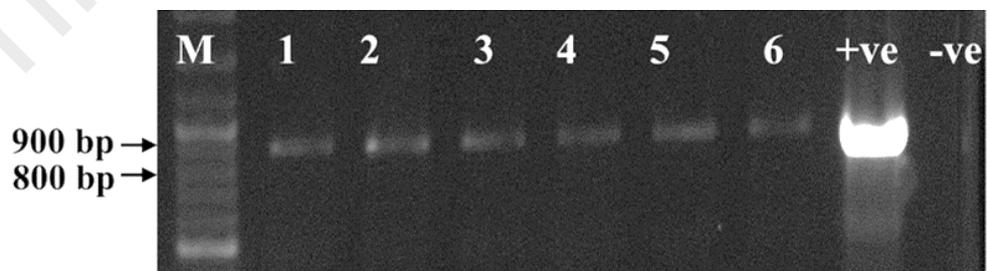

Figure 4.6: PCR detection of the *XVE* gene in transformed UMT-M1 (pezT-GFP) lines. PCR amplification yielded the expected band size of 873 bp from the transformed colonies in lanes 1-4 with plasmid pMDC150_35S as the positive control (lane marked "+ve"). The negative control (lane "-ve") used is the genomic DNA of wild-type *C. vulgaris* UMT-M1. Lane M is the 100 bp DNA marker (Vivantis).

4.1.3   Screening of transformed UMT-M1 (GFP)

The UMT-M1 (GFP) that carried the vector with GFP without the toxin was included as a control in the toxin expression studies. The putative transformant colonies of UMT-M1 (GFP) grew on selective BBM supplemented with hygromycin (20 µg mL^{-1}) and kanamycin (50 µg mL^{-1}) 4 weeks after co-transformation. Four of the putative transformant colonies of *C. vulgaris* UMT-M1 (GFP) were randomly selected from BBM (Figure 4.7) and screened by PCR amplification for the presence of the *GFP* and *XVE* transgenes. The *GFP* transgene was successfully amplified using gene specific primers GFP_F and GFP_scR (Table 3.1) with the expected band size of 691 bp (Figure 4.8). The *XVE* transgene from pMDC150_35S was also amplified from the UMT-M1 (GFP) transformants with the expected band size of 873 bp (Figure 4.9). Thus, the PCR data confirm that the *GFP* and *XVE* genes were present in all four transformant lines four weeks after co-transformation with the responder and activator plasmid constructs.
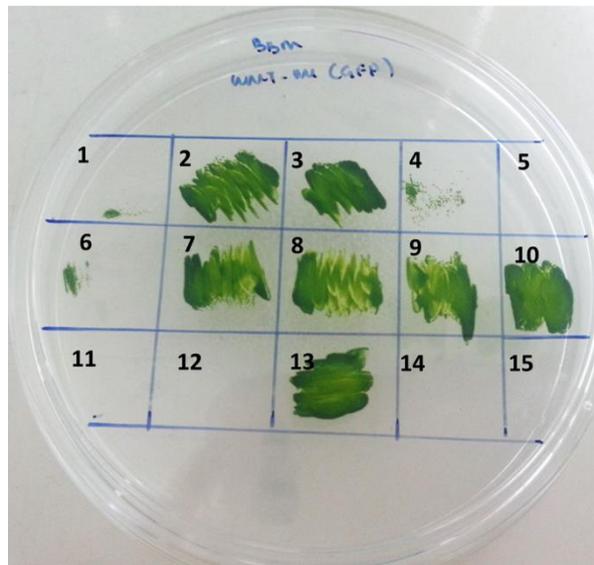
Figure 4.7: Screening for transformant colonies of UMT-M1 (GFP) cultured on BBM.Green colonies (1–4, 6-10 and 13) indicate clones with resistance to hygromycin and kanamycin.
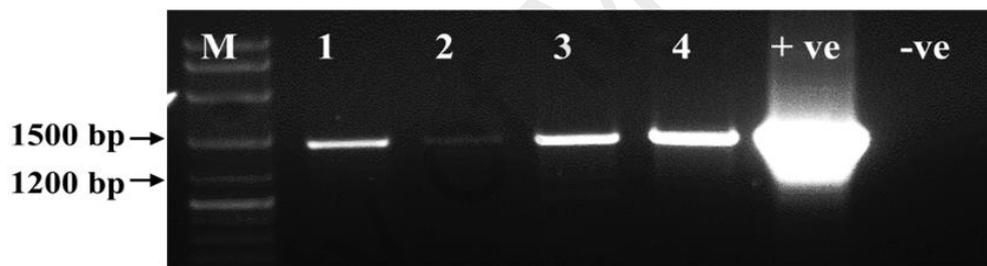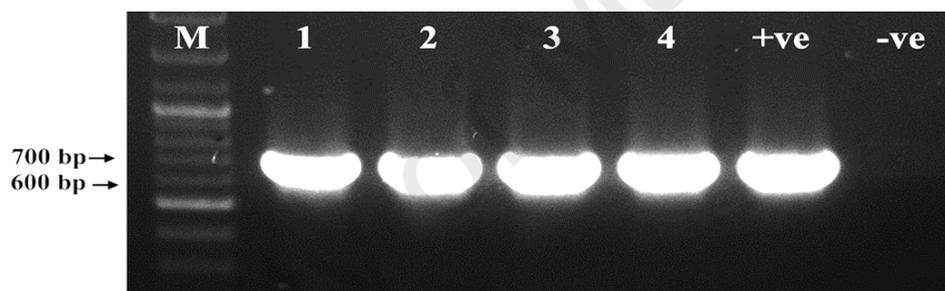

Figure 4.8: PCR detection of *GFP* gene in transformed UMT-M1 (GFP) lines PCR amplification yielded the expected band size of 873 bp from the transformed colonies in Lanes 1-4 with plasmid pMDC221_GFP as the positive control (lane marked "+ve"). The negative control (lane "-ve") used is the genomic DNA of wild-type *C. vulgaris* UMT-M1. Lane M is the 100 bp DNA marker (Vivantis).
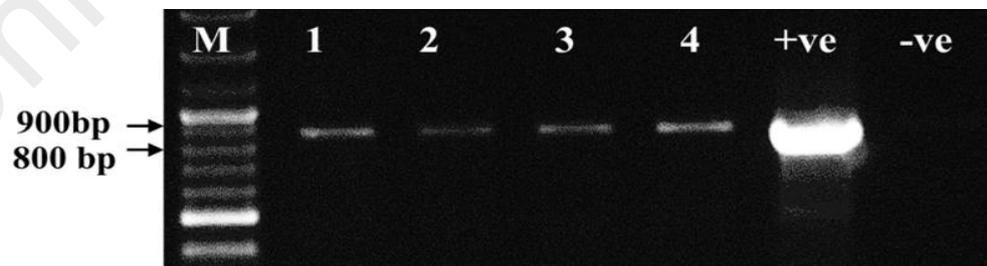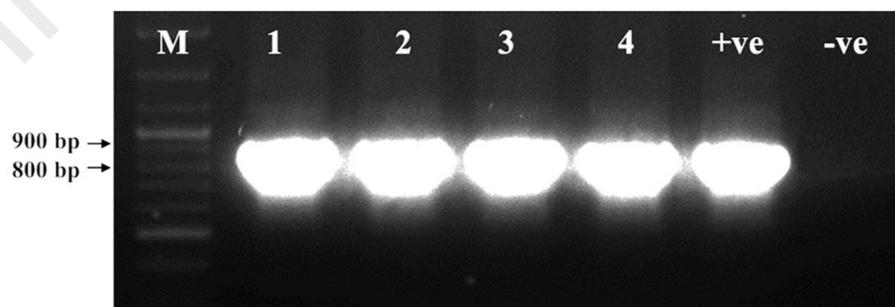

Figure 4.9: PCR detection of the *XVE* gene in transformed UMT-M1 (GFP) lines. PCR amplification yielded the expected band size of 691 bp from the transformed colonies in lanes 1-4 with plasmid pMDC150_35S as the positive control (lane marked "+ve"). The negative control (lane "-ve") used is the genomic DNA of wild-type *C. vulgaris* UMT-M1. Lane M is the 100 bp DNA marker (Vivantis).

4.2 Stability of transgenes in *C. vulgaris* UMT-M1

After maintaining the transgenic *C. vulgaris* UMT-M1 cell lines [six for UMT-M1 (yoeB$_{Spn}$-GFP) and four for UMT-M1 (pezT-GFP)] for about one year, a single line each from UMT-M1 (yoeB$_{Spn}$-GFP) and UMT-M1 (pezT-GFP) were subjected to a series of five consecutive subcultures (~ 75 days) of alternating presence and absence of antibiotic-selection pressure (i.e., with kanamycin and hygromycin, followed by antibiotic-free media). The *yoeB$_{Spn}$* transgene was amplified using gene specific primers (yoeB_F2 and yoeB_R2) resulting in an expected band size of 223 bp, while the *pezT* transgene was amplified by the pezT_F2 and GFP_R gene specific primers and produced the expected band size of 1494 bp. Thus, the result demonstrates that the *yoeB$_{Spn}$* and *pezT* transgenes were present in the transgenic UMT-M1 (yoeB$_{Spn}$-GFP) and UMT-M1 (pezT-GFP) cell lines, respectively (Figures 4.10 and 4.11), following the five consecutive subcultures.
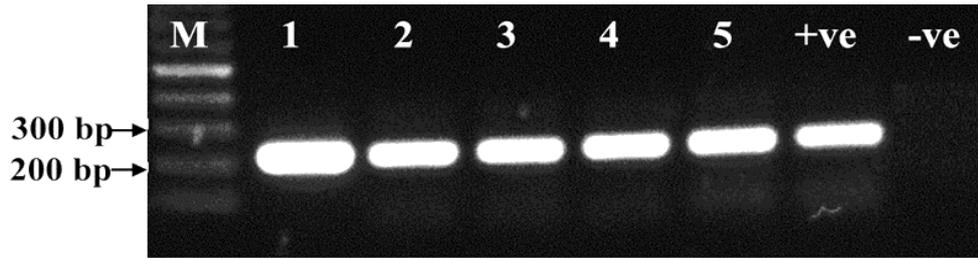
Figure 4.10: Transgene stability of *yoeB_{Spn}GFP* in subcultured transgenic UMT-M1 (yoeB_{Spn}-GFP) after multiple passages of alternating antibiotic selection pressure.PCR amplification of *yoeB_{Spn}* transgene (223 bp) from UMT-M1 (yoeB_{Spn}-GFP) transgenic lines (lanes 1-5) with plasmid pMDC221_yoeBGFP as the positive control (lane marked "+ve"). The negative control (lane "-ve") used is the genomic DNA of wild-type *C. vulgaris* UMT-M1. Lane M in is a 100 bp DNA marker (Vivantis).
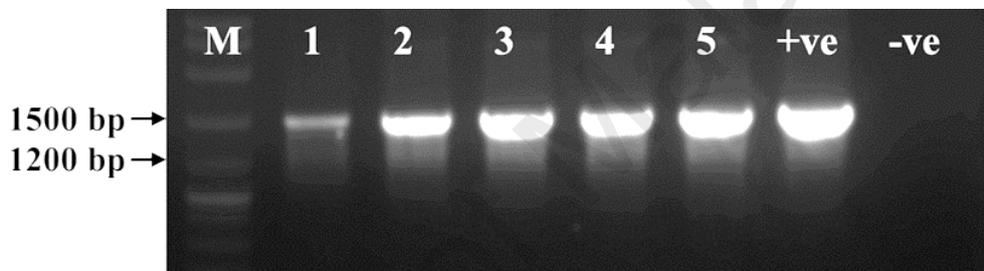
Figure 4.11: Transgene stability of *pezT-GFP* transgene in subcultured transgenic colonies UMT-M1 (pezT-GFP) after multiple passages of alternating antibiotic selection pressure. Detection of the *pezT-GFP* fusion transgene (1494 bp) from UMT-M1 (pezT-GFP) transgenic lines (lanes 1 – 5) with plasmid pMDC221_pezTGFP as the positive control (lane marked "+ve"). The negative control (lane "-ve") used is the genomic DNA of wild-type *C. vulgaris* UMT-M1. Lane M is a 100 bp DNA marker (Vivantis).

4.3 Functionality of the XVE-based two-component expression system in transgenic *C. vulgaris* UMT-M1 (GFP)

To assess the functionality of the XVE-based two-component expression system that was developed for terrestrial plants (Brand *et al.*, 2006) in the *C. vulgaris* microalgae, the *C. vulgaris* UMT-M1 (GFP) transgenic cells were subjected to 17-*β*-estradiol treatment and observed for GFP fluorescence. Fluorescence was observed in the randomly-selected UMT-M1 (GFP) cell line at 0.5 h until 48 h after 17-*β*-estradiol treatment (Figure 4.12A) but not in cells that were not treated with 17-*β*-estradiol (Figure 4.12B). This indicated that the XVE-based two-component expression system is functional and enabled the inducible expression of *GFP* in *C. vulgaris* UMT-M1. No morphological differences were observed in the induced transgenic *C. vulgaris* UMT-M1 (GFP) cells when compared with the wild-type (Figure 4.12C).
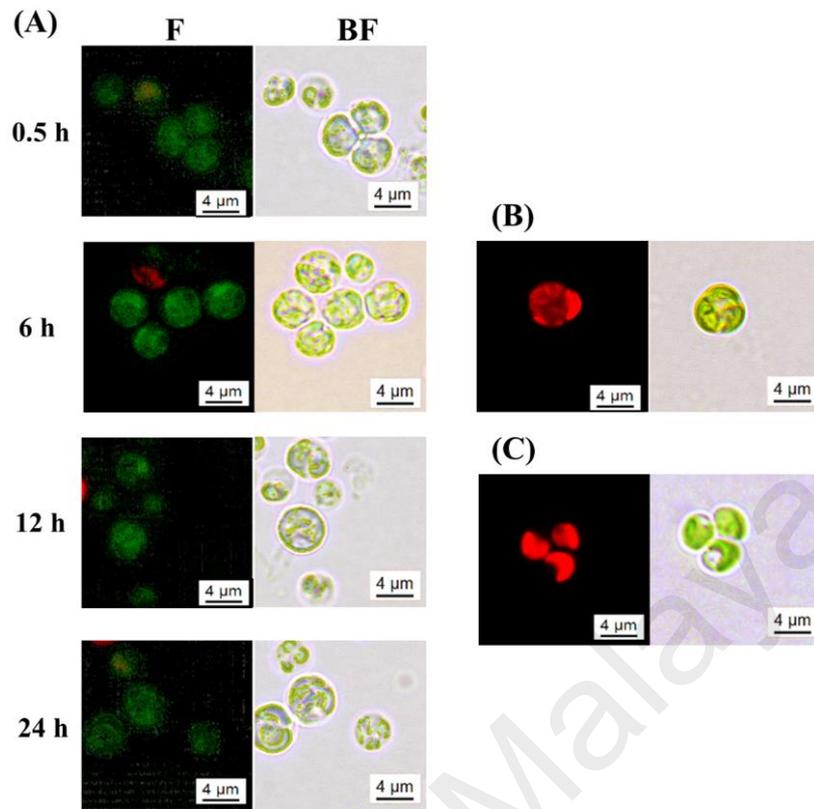
Figure 4.12: GFP fluorescence in transformed UMT-M1 (GFP) line. **(A)** UMT-M1 (GFP) cells emitted GFP signals at 0.5 to 24 h after 17-*β*-estradiol treatment. Absence of GFP fluorescence in **(B)** UMT-M1 (GFP) cells that were not treated with 17-*β*-estradiol and in **(C)** 17-*β*-estradiol-treated wild-type *C. vulgaris* UMT-M1. F, image obtained under fluorescence microscopy; BF, image obtained under bright field microscopy. Magnification: 1000×. Each 10 µL aliquot examined contained an estimated 5000 cells.

4.4 Altered cell morphologies in transgenic *C. vulgaris* UMT-M1 expressing the bacterial toxins

Two transformed lines each from the PCR-positive UMT-M1 colonies for the *yoeB_Spn* [designated UMT-M1 (yoeB_Spn-GFP)] and *pezT* [designated UMT-M1 (pezT-GFP)] transgenes that had been maintained for a year were used for this experiment. The original untransformed wild-type (WT) *C. vulgaris* UMT-M1 served as a negative control. The transformed cells were treated with 17-β-estradiol and then assessed for GFP activity and toxin-induced cell lysis through fluorescence and bright field microscopy. Upon 17-β-estradiol-treatment, GFP fluorescence was detected in both the transformed UMT-M1 (yoeB_Spn-GFP) and UMT-M1 (pezT-GFP) lines. The expression of the bacterial YoeB_Spn and PezT toxins in transformed UMT-M1 (yoeB_Spn-GFP) and UMT-M1 (pezT-GFP) lines produced altered phenotypes in the transgenic cells, as described below.

4.4.1 Effect of YoeB_Spn toxin on the cell morphologies of UMT-M1 (yoeB_Spn-GFP)

Two one-year old transgenic UMT-M1 (yoeB_Spn-GFP) lines were randomly selected and designated as YG_1 and YG_2. Both YG_1 and YG_2 transgenic lines displayed strong emission of GFP fluorescence at all sampled time-points (0.5 –48 h) following 17-β-estradiol treatment (Figure 4.13) with the GFP intensity increasing gradually YG_1 and YG_2. At the later time points (6 h and beyond), the contents of damaged cells were scattered in the media surrounding the cells and were associated with GFP fluorescence. Besides that, the GFP-fluorescing cells also showed abnormal cell morphologies indicative of cell lysis and damage (Figure 4.13). The cell lysis activity was prominent in the transgenic lines YG_1 and YG_2 from the time points 0.5 h to 48 h. When viewed under bright field microscopy, the signs of cell damage included the loss of chlorophyll, clumping of the damaged cells, bursting of cells and the scattering of the cellular contents into surrounding environment (Figure 4.13). A careful observation showed that the transgenic YG_1 cells were intact from 0.5 h to 4 h post-treatment and started to display

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severe cell damage from 8 h post-treatment onwards. In contrast, the transgenic YG_2 cells were already showing signs of cell lysis even after 30 min of 17-*β*-estradiol-treatment (Figure 4.13). In contrast, the wild-type UMT-M1 cells treated with 17-*β*-estradiol showed normal cellular morphologies and exhibited only red chlorophyll auto-fluorescence throughout the entire experimental time frame.
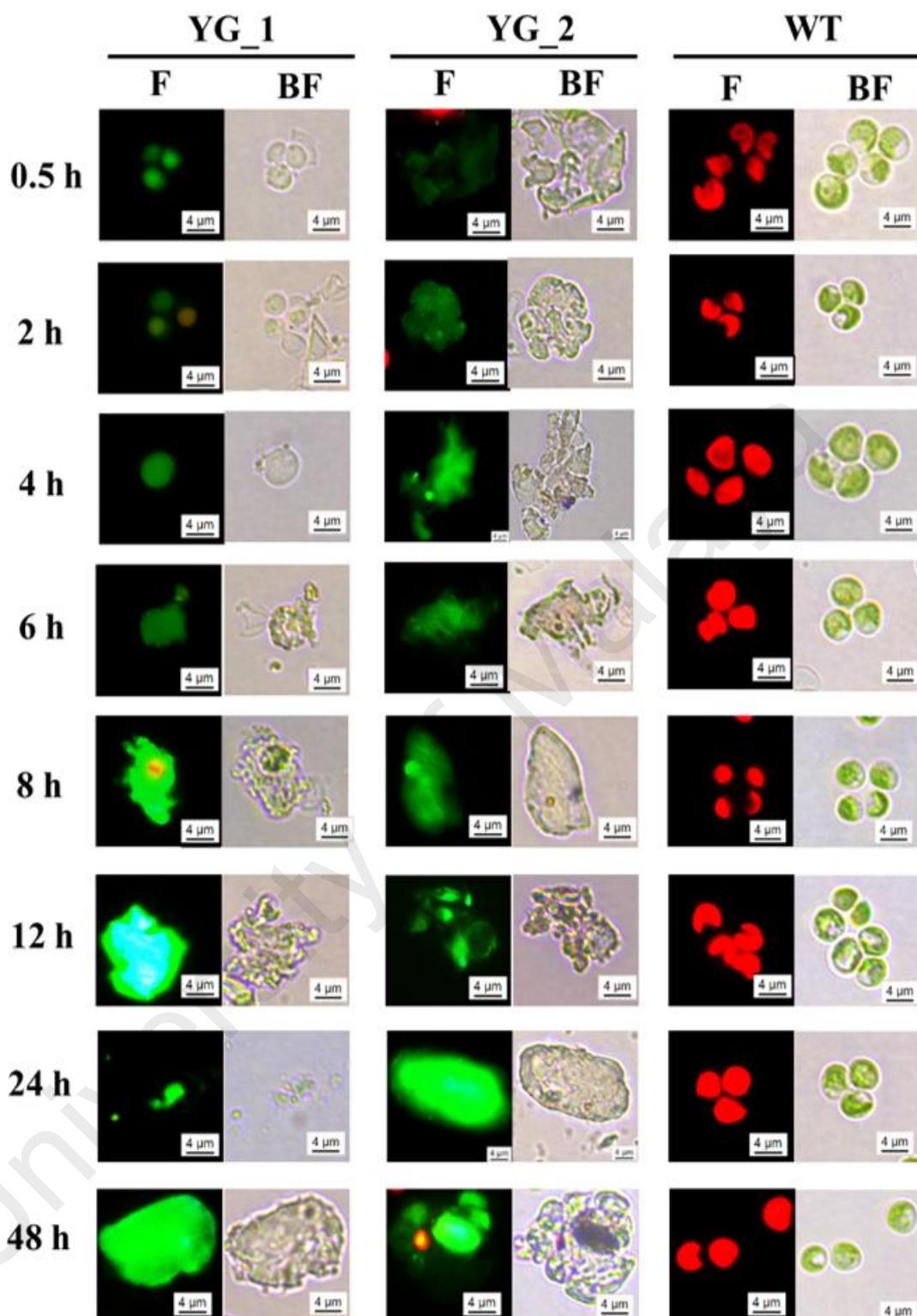
Figure 4.13: Effects of the expression of the *yoeB_{Spn}-GFP* transgene in transgenic UMT-M1 (yoeB_{Spn}-GFP) and wild-type *C. vulgaris* UMT-M1 as viewed under fluorescent (F) and bright-field (BF) microscope at 1000× magnification. GFP fluorescence and cell damage were detected in transgenic UMT-M1 (yoeB_{Spn}-GFP) cell lines YG_1 and YG_2. The wild-type *C. vulgaris* UMT-M1 (WT) *yoeB_{Spn}-GFP* transgene the negative control. Magnification: 1000×. Each 10 µL aliquot examined contained an estimated 5000 cells.

4.4.2 Effect of PezT toxin on the cell morphology of UMT-M1 (pezT-GFP)

Two one-year old transgenic lines of UMT-M1 (pezT-GFP) were randomly selected and designated as PG_1 and PG_2. Following 17-*β*-estradiol-treatment, the two transgenic lines exhibited GFP fluorescence at all sampled time points (0.5 h to 48 h) (Figure 4.14). The emitted GFP signals were detected in the transgenic cells at 0.5 h and was observed to be more intense from 2 h onwards. The wild-type UMT-M1 cells treated with 17-*β*-estradiol exhibited only red chlorophyll auto-fluorescence. Besides GFP activity in the transgenic lines PG_1 and PG_2, the transgenic cells showed obvious cellular damage and lysis, particularly at 2 h post-treatment (Figure 4.14). The damage displayed in transgenic cells PG_1 and PG_2 was similar to that in the transgenic UMT-M1 (yoeB$_{Spn}$-GFP) cells (Section 4.4.1), i.e., chlorophyll removal, clumping of damaged cells, bursting of cells with spillage of the cellular contents into the surrounding environment. The cell lysis activity was most prominent in transgenic cells at 24 h and 48 h after 17-*β*-estradiol-treatment in which the cell contents were observed to be distributed into the surrounding environment. Although the transgenic PG_1 and PG_2 cells showed serious cellular damage, strong GFP signals in the mass of clumped cells and released cell contents were still observed (Figure 4.14).
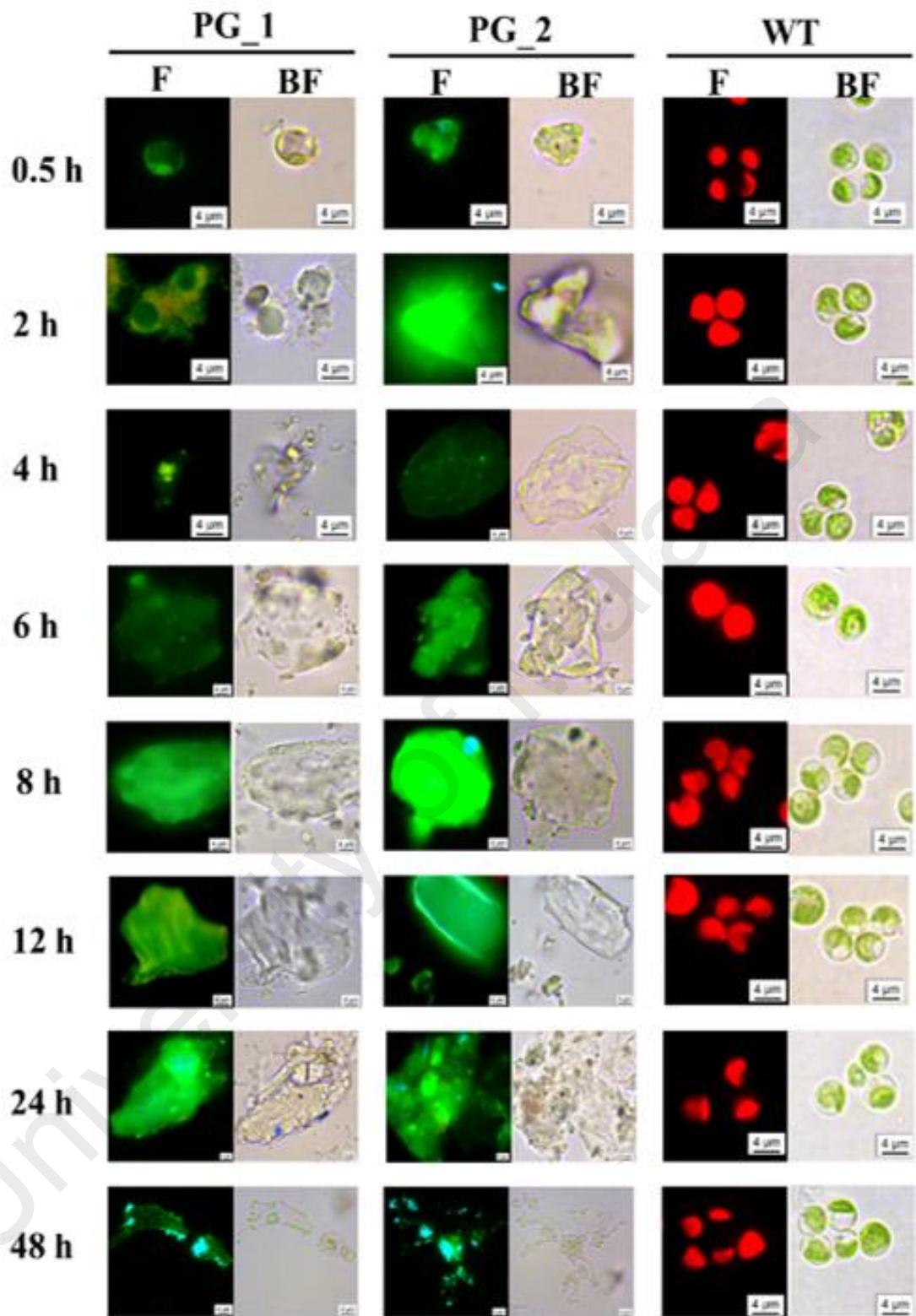
Figure 4.14: Effects of the expression of the *pezT-GFP* transgene in transgenic UMT-M1 (pezT-GFP) and wild-type *C. vulgaris* UMT-M1 as viewed under fluorescent (F) and bright-field (BF) microscope at 1000× magnification. GFP fluorescence and cell damage were detected in transgenic UMT-M1 (pezT -GFP) cell lines PG_1 and PG_2. Wild-type *C. vulgaris* UMT-M1 (WT) the *pezT-GFP* transgene in the negative control. Magnification: 1000×. Each 10 µL aliquot examined contained an estimated 5000 cells.

4.5 The expression of the YoeB$_{Spn}$ and PezT toxins in *C. vulgaris* UMT-M1 affected cell viability

To determine if the expression of the YoeB$_{Spn}$ and PezT toxins in *C. vulgaris* UMT-M1 affected cell viability, the modified Miles and Misra method (as described in Section 3.3) was used to determine the CFU/mL values for the transgenic cells following 17-β-estradiol treatment. Wild-type *C. vulgaris* UMT-M1 (designated WT) and transgenic UMT-M1 (GFP) cells treated with 17-β-estradiol were used as controls and CFU/mL values were plotted relative to the WT values which was set as 1.

4.5.1 Effect of YoeB$_{Spn}$ toxin on the cell viability

There is a significant difference between the CFU/mL values of the UMT-M1 (yoeB$_{Spn}$-GFP) cell lines (i.e., YG_1 and YG_2) and the controls used which were UMT-M1 (GFP) control as well as WT (Figure 4.15). Relative to WT, the UMT-M1 (yoeB$_{Spn}$-GFP) cell lines YG_1 and YG_2 treated by 17-β-estradiol displayed reduced cell viability (Figure 4.16). Although the YG_1 cells displayed an initial increased CFU/mL value at 0.5 h post-treatment, the CFU/mL values then decreased gradually from 6 h post-treatment (reduction of 57.8% relative to the WT) and beyond (reduction of 72.7% relative to the WT at 24 h post-induction) (Table 4.1; Figure 4.16). In contrast, the reduction of cell viability in YG_2 was greater with a 91.9% reduction (relative to the WT) observed as early as 0.5 h post-17-β-estradiol-treatment. By the 24 h post-treatment time point, cell viability had dropped to 99.9 % relative to the WT (Table 4.1; Figure 4.16). The CFU/mL values for the positive control UMT-M1 (GFP) cells (that expresses only GFP) displayed reduced CFU/mL values relative to WT from 0.5 h to 12 h but a significant reduction noted was only at 6 h post-treatment (Figure 4.15). The overall CFU/mL values of UMT-M1 (GFP) after 17-β-estradiol treatment increased gradually at all time points, from 0.5 h to 24 h (Figures 4.16).
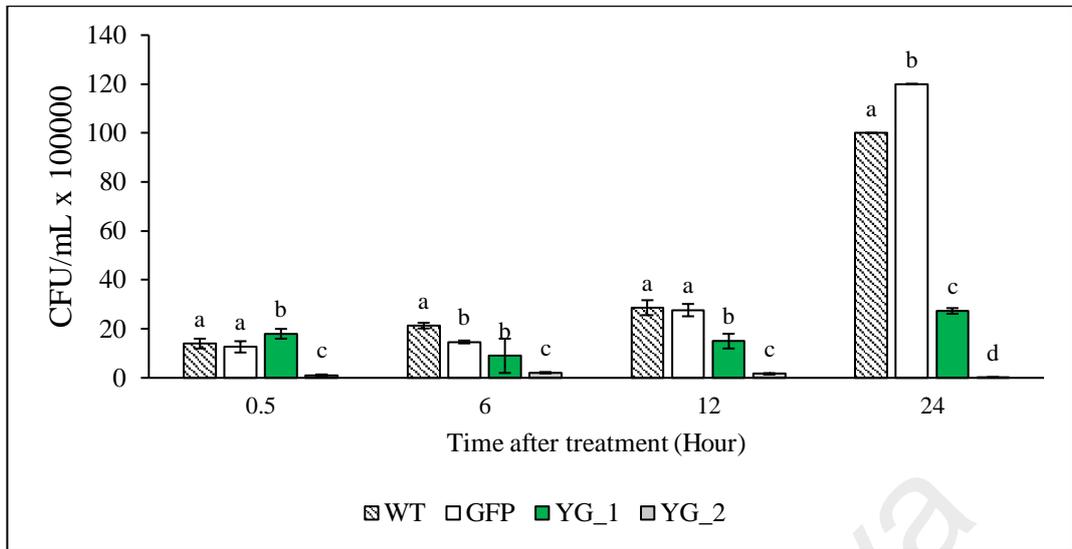
Figure 4.15: The effects of YoeB toxin in UMT-M1 (yoeB$_{Spn}$-GFP) viability presented in CFU/mL values. The randomly selected transgenic lines for UMT-M1 (yoeB$_{Spn}$-GFP) were YG_1 and YG_2. GFP: The positive control UMT-M1 (GFP); WT: Wild type *C. vulgaris* UMT-M1. Each bar represents the mean and standard error while the different letters indicate values that are significantly different ($p<0.05$).

Figure 4.16: The viability of transgenic UMT-M1 (yoeB$_{Spn}$-GFP) cell lines after 17-β-estradiol treatment. The UMT-M1 (yoeB$_{Spn}$-GFP) transgenic lines used were YG_1 and YG_2. The transgenic *C. vulgaris* UMT-M1 (GFP) that only expressed GFP was included as a control. All the CFU/mL obtained were shown relative to the wild type (WT), which was set to 1. Each bar represents the mean and standard error while the different letters indicate values are significantly different ($p<0.05$).

Table 4.1: Reduction rate of CFU/mL values in transgenic UMT-M1 (yoeB$_{Spn}$-GFP) YG_1 and YG_2 cell lines. The reduction rates of CFU/mL in the tested transgenic lines were determined relative to WT which was set at 1. The values in red indicate reduction while values in black indicate increase of CFU/mL relative to WT.

Time / Samples	0.5 h	6 h	12 h	24 h
YG_1	28.6%	-57.8%	-47.7%	-72.7%
YG_2	-91.9%	-90.0%	-94.9%	-99.9%
GFP	-9.5%	-31.3%	-3.5%	20.0%

4.5.2 Effect of PezT toxin on the cell viability of UMT-M1 (pezT-GFP)

Both transgenic UMT-M1 (pezT-GFP) lines (i.e., PG_1 and PG_2) showed significant reductions in CFU/mL values compared to WT and UMT_M1 (GFP) (Figure 4.17). As shown in Figure 4.18, both transgenic lines PG_1 and PG_2 also displayed a large decrease in the relative CFU/mL values to the WT control following 17-β-estradiol treatment. The CFU/ml values of UMT-M1 (GFP) plotted relative to the wild-type showed increasing cell viability (i.e., CFU/mL values following 17-β-estradiol treatment) (Figure 4.18). Within the first 0.5 h after 17-β-estradiol treatment, the PG_1 transgenic line showed a reduction of 99.7% while the PG_2 line displayed a 96.3% reduction in viable cells. The reduction in cell viability of 99.9% was sustained in both transgenic lines up to 24 h post-treatment (Figure 4.18; Table 4.2). There were no obvious differences in the patterns of reduced cell viability observed between the PG_1 and PG_2 lines up to and including the 24-h post-treatment time point.
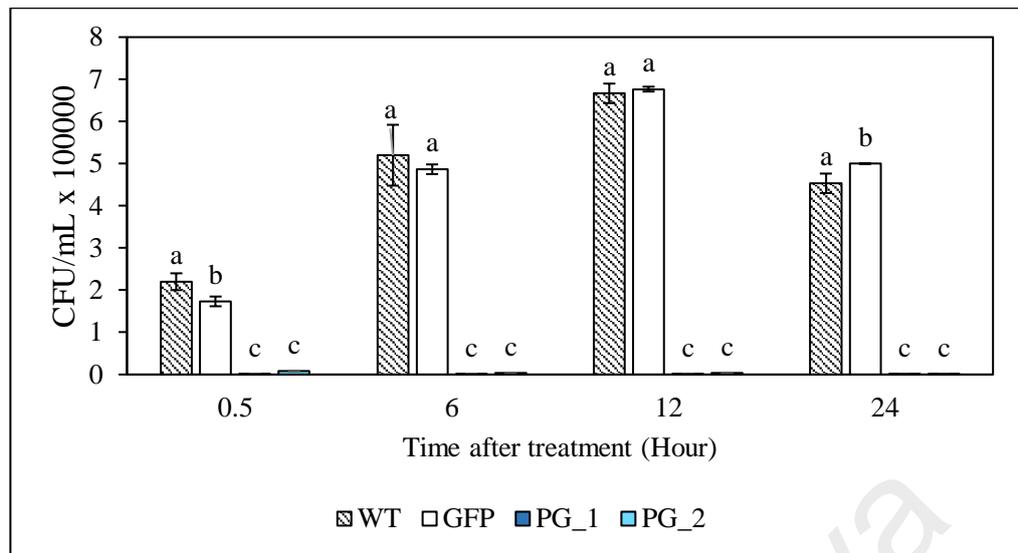
Figure 4.17: The effects of PezT toxin on UMT-M1 (pezT-GFP) viability based on CFU/mL values. The selected transgenic lines for UMT-M1 (pezT-GFP) were PG_1 and PG_2. GFP: The positive control UMT-M1 (GFP); WT: Wild type *C. vulgaris* UMT-M1. Each bar represents the mean and standard error while the different letters indicate values that are significantly different (*p*<0.05).
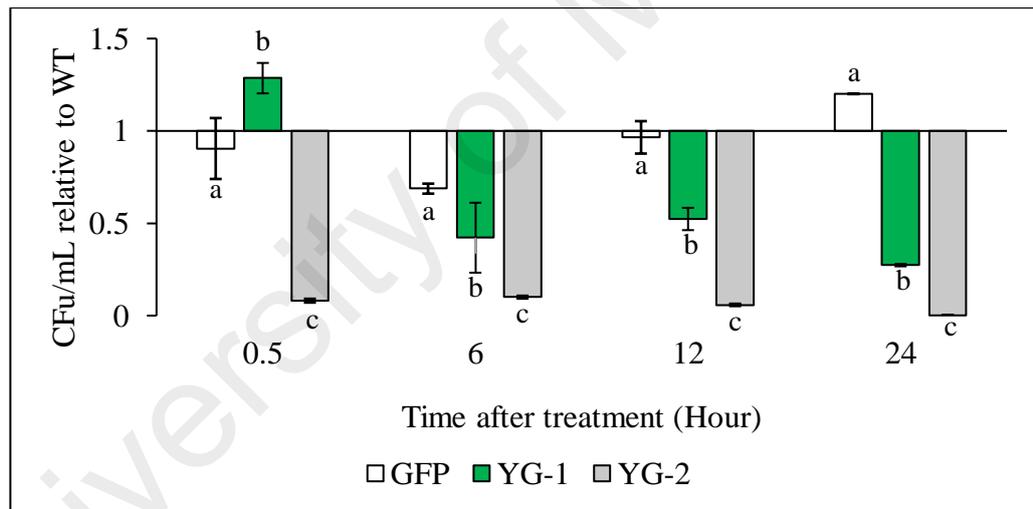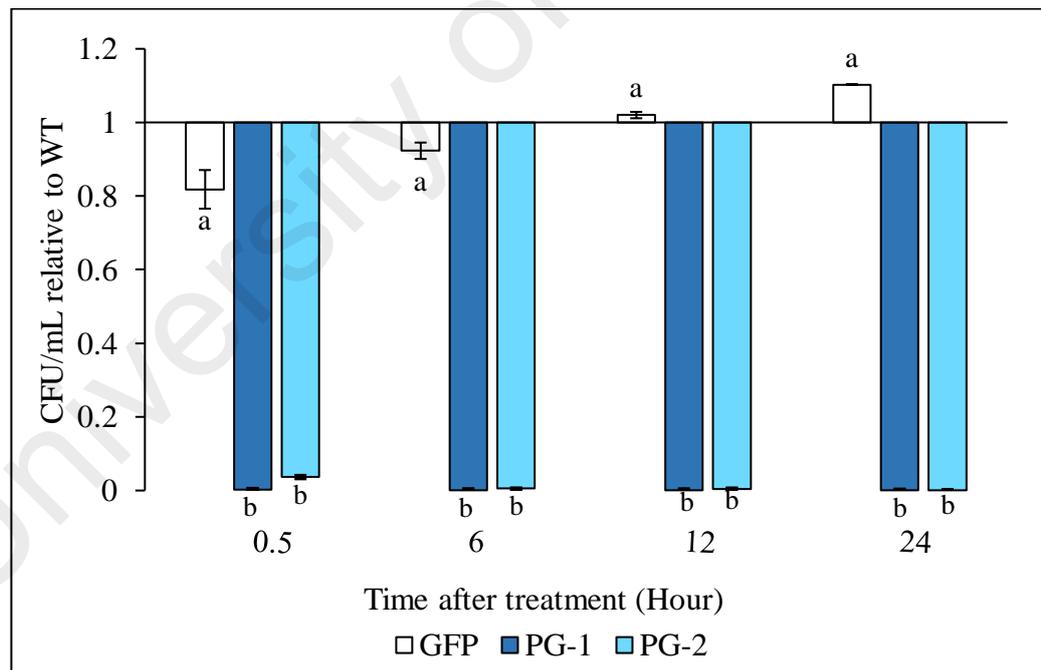

Figure 4.18: The viability of transgenic UMT-M1 (pezT-GFP) cell lines after 17-*β*-estradiol treatment. The effects of PezT toxin in the PG_1 and PG_2 lines of UMT-M1 (pezT-GFP) cells. The transgenic *C. vulgaris* UMT-M1 (GFP) was included as a control to show that GFP expression did not have any adverse effects on transgenic *C. vulgaris*. All the CFU/mL obtained were shown relative to the wild type (WT), which was set to 1. Each bar represents the mean and standard error while the different letters indicate values that are significantly different (*p*<0.05).

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Table 4.2: Reduction rate of CFU/mL values in transgenic UMT-M1 (pezT-GFP) PG-1 and PG-2 cell lines. The reduction rates of CFU/mL in the tested transgenic lines were calculated in relative to WT which was set at 1. The values in red indicate reduction while values in black indicate increase of CFU/mL relative to WT.

Time Samples	0.5 h	6 h	12 h	24 h
YG-1	-99.7%	-99.9%	-99.9%	-99.9%
YG-2	-96.3%	-99.5%	-99.5%	-99.9%
GFP	-18.2%	-7.7%	2.0%	10.3%

4.6 Detection of toxin gene transcripts in transgenic *C. vulgaris* UMT-M1

Total RNA was extracted from the UMT-M1 (yoeB_Spn-GFP) and UMT-M1 (pezT-GFP) cell lines 0.5 h, 6 h, 12 h and 24 h after treatment with 17-β-estradiol. The RNA concentration was measured spectrophotometrically and the RNA integrity checked using agarose gel electrophoresis. The presence of the *yoeB_Spn* and *pezT* transcripts in the total RNA extracted was then determined by reverse-transcriptase PCR.

4.6.1 Detection of the *yoeB_Spn* transcript

Using RT-PCR, the *yoeB_Spn* transcript was PCR-amplified using the yoeB_F2 and yoeB_R2 primers (Table 3.1), producing an expected band size of 223 bp in the 17-β-estradiol-treated transgenic lines (YG_1 and YG_2) of UMT-M1 (yoeB_Spn-GFP) (Figure 4.19). The *yoeB_Spn* transcript was successfully amplified from 17-β-estradiol-treated transgenic cells at 0.5 h, 6 h and 12 h post-treatment time points. RT-PCR was only carried out up to 12 h post-treatment for the 17-β-estradiol-treated-transgenic cells due to insufficient RNA that could be extracted from the 24 h sample (the concentration of total RNA obtained was less than 20 ng/µL and the minimum RNA concentration required for RT-PCR was 100 ng/µL (according to manufacturer's protocol) whereas the total RNA obtained for the other time points were > 100 ng/µL). The *yoeB_Spn* transcript was not present in the untransformed UMT-M1 that was designated as wild-type (WT) (Figure 4.19). The 18S rRNA was used as the housekeeping control for RT-PCR and amplified using 18S mRNA-specific primers (18S_F and 18S_R) (Table 3.1), producing the expected amplified 18S mRNA band from the transgenic lines (YG_1 and YG_2) and WT at 0.5 h to 12 h (Figure 4.19). As semi-quantitative PCR is not highly accurate and only one sample was used for each time point, no assumptions were made about expression levels based on this data other than absence or presence of transcripts.

Figure 4.19: RT-PCR analysis of the 17-β-estradiol-treated transgenic UMT-M1 (yoeB$_{Spn}$-GFP). Detection of the *yoeB$_{Spn}$* toxin gene mRNA transcript in the transgenic *C. vulgaris* UMT-M1 (yoeB$_{Spn}$-GFP) lines (YG_1 and YG_2). The 18S mRNA was included as housekeeping control. The 0.5, 6 and 12 indicates the number of hours after which the RNA was extracted.

4.6.2 Detection of the *pezT* transcript

The *pezT* transcript was successfully amplified by RT-PCR from the 17-*β*-estradiol-treated UMT-M1 (pezT-GFP) transgenic line (PG_1). Using gene specific primers (pezT_F2 and pezT_R2) (Table 3.1), the *pezT* transcript was detected at all the sampled time-points, from 0.5 h to 24 h (Figure 4.20). RT-PCR analysis was only carried out for the PG_1 transgenic line as there was insufficient total RNA recovered from the PG_2. The concentrations of total RNA extracted at all time points from PG_2 line were lower than 50 ng/µL (Figure 4.20). The *pezT* transcript was not detected in the untransformed wild-type UMT-M1 (Figure 4.20). The 18S rRNA that served as the housekeeping control was amplified from the transgenic line PG_1 and WT using 18S rRNA-specific primers (18S_F and 18S_R) (Table 3.1) and was detected at all the time points (0.5 h to 24 h) (Figure 4.20). As semi-quantitative PCR is not highly accurate and only one sample was used for each time point, no assumptions were made about expression levels based on this data other than the absence or presence of transcripts.

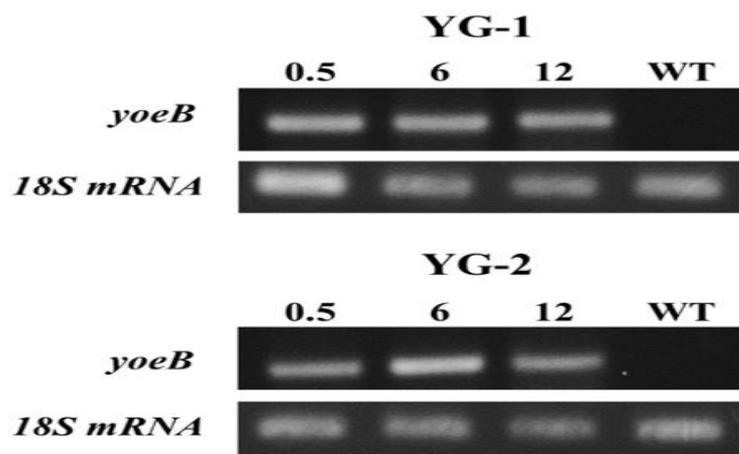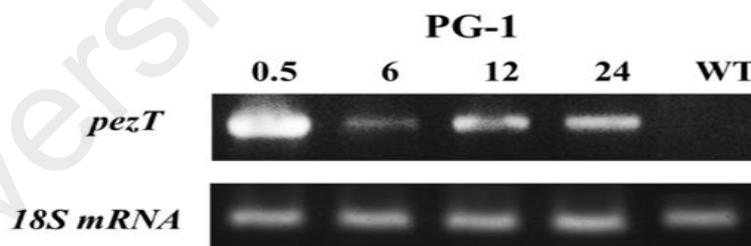**Figure 4.20**: RT-PCR analysis of the 17-*β*-estradiol-treated transgenic UMT-M1 (pezT-GFP). Detection of the *pezT* toxin gene mRNA transcript in transgenic *C. vulgaris* UMT-M1 (PG_1). The 18S rRNA transcript was used as the housekeeping control for the RT-PCR reactions. The 0.5, 6 and 12 indicates the number of hours after which the RNA was extracted.

CHAPTER 5:

DISCUSSION

In this study, a two-component inducible plant expression system that was initially developed for the terrestrial model plant, *Arabidopsis thaliana*, was used to investigate the lethality of the *Streptococcus pneumoniae*-encoded YoeB$_{Spn}$ and PezT toxins in the green eukaryotic marine microalga, *Chlorella vulgaris*. There have been several reports of *Agrobacterium tumefaciens*-mediated transformation that had been established in various types of microalgae such as *Chlamydomonas reinhardtii* (Kumar et al., 2004)*, C. vulgaris* (Cha *et al.*, 2012) and *Nannochloropsis salina* (Beacham & Ali, 2016). However, such transformation experiments have been limited to introducing only a single vector at a time into microalgae species, for example, the introduction and subsequent expression of the chloramphenicol acetyltransferase (*CAT*) (Niu *et al.*, 2011) and heat shock protein 90 (HSP 90) transgenes (Liu *et al.*, 2014) that were reported for *C. vulgaris* which utilized the pPTt-ApCAT and pMD-18T vectors, respectively. The stacking of multiple transgenes such as the desired insert and antibiotic selectable markers within a single cassette may face limitations due to the vector size and difficulty to deliver a long T-DNA strand into plants or microalgae (Dafny-Yelin & Tzfira, 2007). A large construct carried on a single vector may also face issues for genetic stability in plant or microalgae cells (Dafny-Yelin & Tzfira, 2007).

Here, the XVE-based expression system that was comprised of two vectors (i.e., activator vector pMDC150 and responder vector pMDC221) seemed to be tightly regulated in transgenic *C. vulgaris* as GFP fluorescence was only detected in 17-*β*-estradiol-treated UMT-M1 (GFP) but not in 17-*β*-estradiol-treated wild-type (WT) and untreated UMT-M1 (GFP) cells (Figure 4.12). This tight regulation of expression was also observed when the same vectors were introduced into *A. thaliana* (Brand *et al.*, 2006; Abu Bakar *et al.*, 2015). The detection of GFP fluorescence in 17-*β*-estradiol-treated

UMT-M1 (GFP) indicated successful co-transformation of the responder and activator vectors into *C. vulgaris* UMT-M1 using *Agrobacterium*-mediated transformation, which is the first time such a procedure has been reported.

Co-cultivation of *C. vulgaris* UMT-M1 with (1) recombinant *Agrobacterium tumefaciens* containing pMDC221_yoeBGFP together with another *Agrobacterium* harboring pMDC150_35S and; (2) *Agrobacterium* harboring pMDC221_pezTGFP together with the recombinant *Agrobacterium* containing pMDC150_35S yielded positive *C. vulgaris* transformants on the selection plates (Figures 4.1 and 4.4) that were subsequently validated by PCR (Figures 4.2, 4.3, 4.5 and 4.6). The PCR amplification data showed that the transgenes (*yoeB_{Spn}*, *XVE* and *pezT*) were present in all the selected putative transformant colonies, respectively, indicative of efficient *Agrobacterium*-transformation. However, it is important to note here that transformation efficiency was not determined as the focus of this study is to obtain stable transgenic *C. vulgaris* lines instead of optimizing *Agrobacterium*-transformation protocol in microalgae. In this study, an enzymatic degradation step using lysozyme with the addition of cellulase prior to co-cultivation with *Agrobacterium* was included to degrade the *C. vulgaris* UMT-M1 rigid cell wall as was also previously tested in other *C. vulgaris* cells (Gerken *et al.*, 2013) in order to simultaneously transform the two different vector cassettes into *C. vulgaris* UMT-M1.

GFP is a stable and non-invasive reporter protein used for the detection of gene localization and the screening of a large number of transgenic lines (Sun *et al.*, 2009; Ding *et al.*, 2011). As the pneumococcal YoeB_{Spn} and PezT toxins were cloned as translational fusions with GFP in this study, the one-year old transgenic *C. vulgaris* UMT-M1 cells expressing the YoeB_{Spn}-GFP and PezT-GFP fusions exhibited GFP fluorescence and cellular damage with lysis upon 17-*β*-estradiol treatment, (Figures 4.13 and 4.14). In contrast, wild-type *C. vulgaris* UMT-M1 cells fluoresced red due to chlorophyll auto-

fluorescence in the absence of GFP green fluorescence. The detection of GFP signals and alteration of cell morphologies in transgenic *C. vulgaris* UMT-M1 cells are indicative of the functional expression of the YoeB$_{Spn}$-GFP and PezT-GFP fusion proteins following 17-β-estradiol treatment. However, the percentage of cell damage was not measured due to the different extents of cell damage induced by bacterial toxin and the functional gene expression was further validated by RT-PCR to detect the relevant gene transcripts in the 17-β-estradiol treated transgenic cell lines (Figures 4.19 and 4.20).

The cell viability of the UMT-M1 (YoeB$_{Spn}$-GFP) and UMT-M1 (pezT-GFP) transgenic lines were significantly reduced upon 17-β-estradiol treatment as opposed to the WT and UMT-M1 (GFP) cells that were only expressing GFP (Figures 4.15 and 4.17). The transgenic lines for UMT-M1 (YoeB$_{Spn}$-GFP) (ie., YG_1 and YG_20 showed declining patterns of CFU/mL values from 0.5 to 24 hours after 17-β-estradiol treatment (Figure 4.15). A smilar declining trend was also demonstrated by both the UMT-M1 (pezT-GFP) lines (ie., PG_1 and PG_20) albeit the reduction of CFU/mL values in UMT_M1 (pezT-GFP) lines were more pronounced (Figure 4.17). This decrease in CFU/mL values in UMT-M1 (YoeB$_{Spn}$-GFP) and UMT-M1 (PezT-GFP) is most likely caused by the expression of their respective YoeB$_{Spn}$ and PezT toxins and not caused by GFP. This is supported by the findings of a previous study, where the endoribonuclease activity of the YoeB$_{Spn}$ toxin was reported to cleave the mRNA of *E. coli* cells thus inhibiting translation and resulting in cell death and the inability to form viable colonies (Nieto *et al.*, 2007). The expression of YoeB$_{Spn}$-GFP in *A. thaliana* also caused cell death of the plant that was associated with apoptosis (Abu Bakar *et al.*, 2015). It is likely that in this study, the expression of YoeB$_{Spn}$-GFP also led to the reduction of cell viability in YG_1 and YG_2 transgenic lines due to the endoribonucleolytic activity of YoeB$_{Spn}$. It is interesting to note that even though the YG_1 and YG_2 transgenic lines displayed significantly reduced cell viabilities relative to wild-type *C. vulgaris* UMT-M1, YG_1

responded more slowly, with relatively higher cell counts at 0.5 h time point compared to YG_2 (Figure 4.16), which suggested that the expression level of the *yoeB~Spn~-GFP* transgene could perhaps be lower and only induced bacteriostatic effect in the YG_1 line. In addition, the different YoeB~Spn~ expression levels in the two cell lines were also suggested by the different amount of cellular damage and GFP fluorescence observed in both YG_1 and YG_2 lines whereby YG_1 cells only showed signs of cellular damage 6 h after 17-β-estradiol treatment as compared to YG_2 cells which showed damage even at 0.5 h after treatment (Figure 4.16). The different levels of gene expression may be attributed to the integration of *yoeB~Spn~-GFP* transgene at different loci in the genomes of the YG_1 and YG_2 transgenic lines as similarly reported from the findings of Day *et al.* (2000) in which the integration sites of the *gus* gene at different target loci of tobacco protoplast led to low expression that could be associated with gene silencing. In addition, the integration of transgenes within the transcriptionally-active chromosomal euchromatin regions can promote transgene expression whereas integration within the inert heterochromatin regions can result in suppression of gene transcription instead (Richards & Elgin, 2002). It is possible that in YG_1 transgenic lines, the chromatin structure was very compact or the *yoeB~Spn~-GFP* transgene may have integrated into the heterochromatin regions; either event could have led to reduced transcription of the transgenes. For example, the *lacZ* reporter gene was inserted into different loci of the *Saccharomyces cerevisiae* genome and the gene expression levels were inferred from the β-galactosidase activity measured in the various transgenic yeast strains. There was an 8.7-fold difference between the transgenic yeast strains with the highest and lowest β-galactosidase activity (Flagfeldt *et al.*, 2009).

In contrast to the UMT-M1 (YoeB~Spn~-GFP) transgenic lines, both the PG_1 and PG_2 transgenic lines of UMT-M1 (PezT-GFP) displayed reduction of cell viability after 17-β-estradiol treatment but with no significant difference between the two transgenic lines

(Figure 4.17). The rapid and strong expression of the PezT toxin was apparent 30 min post-treatment as evidenced by the abrupt drop in the CFU/mL values of both PG_1 and PG_2 lines (Figure 4.17). The cell viability of both PG_1 and PG_2 lines was affected even 24 h after 17-β-estradiol treatment, indicating that the expression of PezT toxin was lethal in transgenic UMT-M1 (PezT-GFP). Mutschler *et al.* (2011) reported that overproduction of PezT toxin caused inhibition of bacterial peptidoglycan synthesis that was lethal in its native host, *S. pneumoniae* as well as in *E. coli* cells. PezT toxin is a kinase that targets UDP-N-acetylglucosamine (UNAG), the key intermediate in the synthesis of bacterial peptidoglycan that makes up the bacterial cell wall, leading to the formation of UDP-N-acetylglucosamine-3'-phosphate (UNAG-3P). Since UNAG-3P is a metabolite that is rendered unusable for peptidoglycan biosynthesis and is also a competitive inhibitor of MurA, the enzyme that catalyzes the first step in peptidoglycan synthesis, the synthesis of bacterial peptidoglycan is thus inhibited by expression of the PezT toxin (Mutschler *et al.*, 2011). In a related study, the expression of the *S. pyogenes*-encoded ζ toxin, a homolog of PezT toxin, was reported to also elicit cell lysis in yeast cells (Zielenkiewicz *et al.*, 2009) and this likely occurred through depletion of the UNAG component that is required for chitin synthesis in yeast cells (Mutschler & Meinhart, 2011). The rigid cell wall of *Chlorella* sp. is composed of *N*-acetylglucosamine chains that is present as chitin-like glycan (Kapaun & Reisser, 1995) thus sharing a similar cell wall composition with bacteria and yeast cell wall. Therefore, it is likely the expression of the PezT toxin in transgenic *C. vulgaris* UMT-M1 (PezT-GFP) also caused an inhibition of microalgal cell wall biosynthesis leading to cell death.

The cellular damage and subsequent lysis in transgenic *C. vulgaris* UMT-M1 indicated the functionally lethal expression of the YoeB$_{Spn}$ and PezT toxins. Both YoeB$_{Spn}$ and PezT toxins caused cell lysis in transgenic cells and reduction of cell viability up to 24 h after 17-β-estradiol treatment, which indicated that the transgenic cells have completely lysed

or were too weak to be revived thereafter (Figures 4.16 and 4.18). The cell lysis in UMT-M1 (PezT-GFP) cells was extreme and very noticeable in comparison to those in UMT-M1 (YoeB$_{Spn}$-GFP), (Figure 4.16 and 4.18), indicating that the toxicity of YoeB$_{Spn}$ in transgenic *C. vulgaris* UMT-M1 was less potent than that of the PezT toxin. This can be attributed to the different modes of action employed by YoeB$_{Spn}$ and PezT toxins in transgenic UMT-M1 (YoeB$_{Spn}$-GFP) and UMT-M1 (PezT-GFP), respectively. YoeB toxin targets the mRNA within the cells (Nieto *et al.*, 2007) and it is now known that the ectopic expression of endoribonuclease toxins such as YoeB, RelE and MazF in bacteria is not necessarily lethal but in certain cases causes the host cells to enter a state of dormancy known as persistence as a means of coping with environmental stresses (Lewis, 2010). The PezT toxin targets the cell wall which is more vulnerable as it is the outermost layer of a cell and expression of PezT or ζ was proposed to be bactericidal (Mutschler *et al.*, 2011) although under certain conditions, expression of the ζ toxin was reported to facilitate the entry of *Bacillus subtilis* cells into a dormant state (Lioy *et al.*, 2012).

After more than a year of maintenance and repeated alternate subcultures of the transformed *C. vulgaris* lines, the *yoeB$_{Spn}$* and *pezT* toxin transgenes in transgenic UMT-M1 (YoeB$_{Spn}$-GFP) and UMT-M1 (PezT-GFP) cell lines were still present (Figures 4.10 and 4.11). Besides that, the YoeB and PezT toxins still showed activity in the one-year old transgenic *C. vulgaris* lines as demonstrated by the cellular damage observed (Figures 4.13 and 4.14), indicating that co-transformation with two-component XVE-based expression system can generate stable transgenic *C. vulgaris* cells. Due to the rapid life cycle in many species of microalgae, the elimination of the foreign DNA after several rounds of cell division can cause the loss of desirable traits and also impede further experiments requiring transgenic cells (Neupert *et al.*, 2009; Barampuram & Zhang, 2011). The events that can lead to the loss of transgene could be caused by transient expression where the transgene was expressed for a short window of time in the

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transformed cells but the transgene was not integrated into the host genome and thus no replication could occur and the transgene would be lost following subsequent cellular replication cycles (Doron *et al.*, 2016). Therefore, the stability of transgenes introduced into eukaryotic microalgae is an important consideration for feasibility of applications that require long-term maintenance of cultures. Previous studies have shown promising results including stable expression of GFP in *C. vulgaris* even after 16 consecutive subcultures (about 8 months) with alternating antibiotic pressure (Yang *et al.*, 2015) and stable GUS activity in *Chlorella ellipsoidea* transformants that had been maintained under alternating antibiotic selection pressure for about 10 months (Liu *et al.*, 2013).

CHAPTER 6:

CONCLUSION

This study is the first to demonstrate the functional lethality of the bacterial TA toxins YoeB$_{Spn}$ and PezT from pathogenic *Streptococcus pneumoniae* in the eukaryotic microalgae *Chlorella vulgaris* UMT-M1. The co-transformation and functionality of the XVE-based two-component inducible gene expression system that was initially developed for the model plant *Arabidopsis thaliana* (Brand *et al.*, 2006) was applied for the first time in the eukaryotic microalgae *C. vulgaris* and was shown to be a functional expression system in the organism. The expression of the YoeB$_{Spn}$ and PezT toxins was detrimental to transgenic *C. vulgaris* UMT-M1 causing cell damage and lysis upon 17-β-estradiol treatment. It was noted that different expression levels were displayed for YG_1 and YG_2 lines of transgenic UMT-M1 (YoeB$_{Spn}$-GFP) which suggested that the transgene integration sites may vary in the genomes of these different transgenic lines. The sites of transgene integration were not further investigated in this study, however they could be further investigated in the future by use of sequencing methods to determine the exact locus of transgene integration and by quantifying the transcript levels of YoeB$_{Spn}$-GFP using quantitative real-time PCR. Transgene stability is an important aspect that needs to be considered when developing transgenic microalgae for biotechnological purposes to ensure the sucess of an experimental procedure. In our study, the stability of the transgenes in *C. vulgaris* was demonstrated following alternate sub-culturing on selective and non selective media over a period of more than a year in which PCR-amplification demonstrated that the transgenes are still present and functional in the transgenic lines of UMT-M1 (YoeB$_{Spn}$-GFP) and UMT-M1 (PezT-GFP). Subsequent to the 17-β-estradiol treatment, the YoeB$_{Spn}$ and PezT toxins were also shown to cause cell lysis in the one-year old transgenic microalgae cells indicating that transgene stability and functionality in *C. vulgaris.* In future studies, quantitative-PCR could be used to measure

the levels of gene expression more accurately and for a greater numbers of biological replicates since insertion of transgene in different loci might give even better results.

The demonstrated functionality of the bacterial TA toxins using the XVE-based two-component expression system in *C. vulgaris* UMT-M1 could pave the way for several interesting biotechnological applications. The YefM-YoeB and PezAT TA systems can be potentially developed as gene containment systems for transgenic microalgae in future studies. For example, the toxin gene could be placed under the control of a nutrient-responsive promoter and in a favorable environment (such as the nutrient-rich conditions in a bioreactor), the expression of the toxin gene would be repressed. However, if the transgenic cells were to accidentally escape into the environment, the nutrient-poor conditions outside of a bioreactor would trigger the activation of the toxin causing the transgenic cells to die. Compared to the need to quarantine affected areas and major-cleanup of the transgenic organisms that have escaped into the environment, having an in-built kill switch utilizing a TA toxin as gene containment system would be more time- and cost-effective as the PezT as well as $YoeB_{Spn}$ toxins do act in a more rapid manner in *C. vulgaris*. Beside their potential use for biological containment, TA systems could also be developed into a promising alternative to harvest valuable microalgal cell contents. As the $YoeB_{Spn}$ and PezT toxins were demonstrated to induce cell lysis in transgenic *C. vulgaris* UMT-M1, the YefM-$YoeB_{Spn}$ and PezAT TA systems can be further investigated to assess their utility in efficiently harvesting the cellular contents of microalgae, particularly lipids that can be used for bio-energy. The $YoeB_{Spn}$ or PezT toxins could be induced to express in mass cultures of transgenic oleaginous microalgae when the cellular lipid accumulation is at a maximum causing cellular lysis of the microalgal cells, thus leading to the release of their valuable oil content. The released oil content can then be collected, extracted and purified in the downstream processing of biofuel. The conditional expression of the bacterial toxins in these transgenic microalgae

would target a particular time frame whereby there is maximum microalgal lipid accumulation. This would promise the highest biomass yield and save in the production cost as repetitions of oil-harvesting procedures can be eliminated by targeting only a specific time frame to optimally harvest oil. The findings presented here could also be applied to other eukaryotic microalgae besides *C. vulgaris*. The functional lethality of the bacterial YoeB$_{Spn}$ and PezT toxins in *C. vulgaris* demonstrated in this study thus provides the algal biotechnologist with additional molecular tools that could be utilized for the engineering of transgenic microalgae for the production of biofuels.

REFERENCES

Aakre, C. D., Phung, T. N., Huang, D. & Laub, M. T. (2013). A bacterial toxin inhibits DNA replication elongation through a direct interaction with the beta sliding clamp. *Molecular Cell, 52*(5): 617-628.

Abu Bakar, F., Yeo, C. C. & Harikrishna, J. A. (2015). Expression of the *Streptococcus pneumoniae yoeB* chromosomal toxin gene causes cell death in the model plant *Arabidopsis thaliana*. *BMC Biotechnology, 15*: 26-35.

Andreev, D., Hauryliuk, V., Terenin, I., Dmitriev, S., Ehrenberg, M. & Shatsky, I. (2008). The bacterial toxin RelE induces specific mRNA cleavage in the A site of the eukaryote ribosome. *RNA, 14*(2): 233-239.

Anila, N., Chandrashekar, A., Ravishankar, G. A. & Sarada, R. (2011). Establishment of *Agrobacterium tumefaciens* mediated genetic transformation in *Dunaliella bardawil*. *European Journal of Phycology, 46*(1): 36-44.

Aoyama, T. & Chua, N. H. (1997). A glucocorticoid-mediated transcriptional induction system in transgenic plants. *The Plant Journal, 11*(3): 605-612.

Apt, K. E., Kroth-Pancic, P. G. & Grossman, A. R. (1996). Stable nuclear transformation of the diatom *Phaeodactylum tricornutum*. *Molecular Genetics and Genomics, 252*(5): 572-579.

Bala Amutha, K. & Murugesan, A. G. (2011). Biological hydrogen production by the algal biomass *Chlorella vulgaris* MSU 01 strain isolated from pond sediment. *Bioresource Technology, 102*(1): 194-199.

Balat, M. (2008). Potential importance of hydrogen as a future solution to environmental and transportation problems. *International Journal of Hydrogen Energy, 33*(15): 4013-4029.

Barampuram, S. & Zhang, Z. J. (2011). Recent advances in plant transformation. *Methods in Molecular Biology, 701*: 1-35.

Barbas, C. F., Burton, D. R., Scott, J. K. & Silverman, G. J. (2007). Quantitation of DNA and RNA. *Cold Spring Harbour Protocols, 2007*: 47.

Beacham, T. A. & Ali, S. T. (2016). Growth dependent silencing and resetting of *DGA1* transgene in *Nannochloropsis salina*. *Algal Research, 14*: 65-71.

Blower, T. R., Pei, X. Y., Short, F. L., Fineran, P. C., Humphreys, D. P., Luisi, B. F. & Salmond, G. P. (2011). A processed noncoding RNA regulates an altruistic bacterial antiviral system. *Nature Structural and Molecular Biology, 18*(2): 185-190.

Blower, T. R., Short, F. L., Rao, F., Mizuguchi, K., Pei, X. Y., Fineran, P. C., Luisi, B. F. & Salmond, G. P. (2012). Identification and classification of bacterial Type III toxin-antitoxin systems encoded in chromosomal and plasmid genomes. *Nucleic Acids Research, 40*(13): 6158-6173.

Bogaert, D., van Belkum, A., Sluijter, M., Luijendijk, A., de Groot, R. & Rumke, H. C. (2004). Colonisation by *Streptococcus pneumoniae* and *Staphylococcus aureus* in healthy children. *Lancet, 363*: 1871-1872.

Brand, L., Horler, M., Nuesch, E., Vassalli, S., Barrell, P., Yang, W., Jefferson, R. A., Grossniklaus, U. & Curtis, M. D. (2006). A versatile and reliable two-component system for tissue-specific gene induction in *Arabidopsis*. *Plant Physiology, 141*(4): 1194-1204.

Broughton, S. J., Piper, M. D., Ikeya, T., Bass, T. M., Jacobson, J., Driege, Y., Martinez, P., Hafen, E., Withers, D. J., Leevers, S. J. & Partridge, L. (2005). Longer lifespan, altered metabolism, and stress resistance in *Drosophila* from ablation of cells making insulin-like ligands. *Proceedings of the National Academy of Sciences of the United States of America, 102*(8): 3105-3110.

Brown, B. L., Grigoriu, S., Kim, Y., Arruda, J. M., Davenport, A., Wood, T. K., Peti, W. & Page, R. (2009). Three dimensional structure of the MqsR:MqsA complex: a novel TA pair comprised of a toxin homologous to RelE and an antitoxin with unique properties. *PLOS Pathogens, 5*(12): e1000706.

Caddick, M. X., Greenland, A. J., Jepson, I., Krause, K. P., Qu, N., Riddell, K. V., Salter, M. G., Schuch, W., Sonnewald, U. & Tomsett, A. B. (1998). An ethanol inducible gene switch for plants used to manipulate carbon metabolism. *Nature Biotechnology, 16*(2): 177-180.

Castro-Roa, D., Garcia-Pino, A., De Gieter, S., van Nuland, N. A., Loris, R. & Zenkin, N. (2013). The Fic protein Doc uses an inverted substrate to phosphorylate and inactivate EF-Tu. *Nature Chemical Biology, 9*(12): 811-817.

Cataudella, I., Trusina, A., Sneppen, K., Gerdes, K. & Mitarai, N. (2012). Conditional cooperativity in toxin-antitoxin regulation prevents random toxin activation and promotes fast translational recovery. *Nucleic Acids Research, 40*(14): 6424-6434.

Cha, T. S., Chen, J. W., Goh, E. G., Aziz, A. & Loh, S. H. (2011). Differential regulation of fatty acid biosynthesis in two Chlorella species in response to nitrate treatments

and the potential of binary blending microalgae oils for biodiesel application. *Bioresource Technology, 102*(22): 10633-10640.

Cha, T. S., Yee, W. & Aziz, A. (2012). Assessment of factors affecting *Agrobacterium*-mediated genetic transformation of the unicellular green alga, *Chlorella vulgaris*. *World Journal of Microbiology and Biotechnology, 28*(4): 1771-1779.

Chan, W. T., Espinosa, M. & Yeo, C. C. (2016). Keeping the wolves at bay: antitoxins of prokaryotic type II toxin-antitoxin systems. *Frontiers in Molecular Biosciences, 3*: 9-28.

Chan, W. T., Moreno-Cordoba, I., Yeo, C. C. & Espinosa, M. (2012). Toxin-antitoxin genes of the Gram-positive pathogen *Streptococcus pneumoniae*: so few and yet so many. *Microbiology and Molecular Biology Reviews, 76*(4): 773-791.

Chan, W. T., Moreno-Córdoba, I., Yeo, C. C. & Espinosa, M. (2013). Toxin-antitoxin loci in *Streptococcus pneumoniae*. In K. Gerdes (Ed.), *Prokaryotic Toxin-Antitoxins* (pp. 315-339). Berlin, Germany: Springer Berlin Heidelberg

Chan, W. T., Nieto, C., Harikrishna, J. A., Khoo, S. K., Othman, R. Y., Espinosa, M. & Yeo, C. C. (2011). Genetic regulation of the yefM-yoeB toxin-antitoxin locus of *Streptococcus pneumoniae*. *Journal of Bacteriology, 193*(18): 4612-4625.

Chan, W. T., Yeo, C. C., Sadowy, E. & Espinosa, M. (2014). Functional validation of putative toxin-antitoxin genes from the Gram-positive pathogen *Streptococcus pneumoniae*: phd-doc is the fourth bona-fide operon. *Frontiers in Microbiology, 5*: 677-689.

Cheng, R. B., Lin, X. Z., Wang, Z. K., Yang, S. J., Rong, H. & Y., M. (2011). Establishment of a transgene expression system for the marine microalga *Schizochytrium* by 18S rDNA-targeted homologous recombination. *Journal of Microbiology and Biotechnology, 7*: 737-741.

Choi, S. P., Nguyen, M. T. & Sim, S. J. (2010). Enzymatic pretreatment of *Chlamydomonas reinhardtii* biomass for ethanol production. *Bioresource Technology, 101*(14): 5330-5336.

Christensen-Dalsgaard, M., Jorgensen, M. G. & Gerdes, K. (2010). Three new RelE-homologous mRNA interferases of *Escherichia coli* differentially induced by environmental stresses. *Molecular Microbiology, 75*(2): 333-348.

Christensen, S. K. & Gerdes, K. (2003). RelE toxins from bacteria and Archaea cleave mRNAs on translating ribosomes, which are rescued by tmRNA. *Molecular Microbiology 48*(5): 1389-1400.

Christenson, L. B. & Sims, R. C. (2012). Rotating algal biofilm reactor and spool harvester for wastewater treatment with biofuels by-products. *Biotechnology and Bioengineering, 109*(7): 1674-1684.

Chukwudi, C. U. & Good, L. (2015). The role of the hok/sok locus in bacterial response to stressful growth conditions. *Microbial Pathogenesis, 79*: 70-79.

Coll, J. M. (2006). Review: Methodologies for transferring DNA into eukaryotic microalgae. *Spanish Journal of Agricultural Research, 4*(4): 316-330.

Collet, P., Helias, A., Lardon, L., Ras, M., Goy, R. A. & Steyer, J. P. (2011). Life-cycle assessment of microalgae culture coupled to biogas production. *Bioresource Technology, 102*(1): 207-214.

Collier, R. J. (1975). Diphtheria toxin: mode of action and structure. *Bacteriological Reviews, 39*(1): 54-85.

Costanzo, M. C. & Fox, T. D. (1988). Transformation of yeast by agitation with glass beads. *Genetics, 120*(3): 667-670.

Dafny-Yelin, M. & Tzfira, T. (2007). Delivery of multiple transgenes to plant cells. *Plant Physiology, 145*(4): 1118-1128.

Dale, E. C. & Ow, D. W. (1991). Gene transfer with subsequent removal of the selection gene from the host genome. *Proceedings of the National Academy of Sciences of the United States of America, 88*(23): 10558-10562.

Dao-Thi, M. H., Van Melderen, L., De Genst, E., Afif, H., Buts, L., Wyns, L. & Loris, R. (2005). Molecular basis of gyrase poisoning by the addiction toxin CcdB. *Journal of Molecular Biology, 348*(5): 1091-1102.

Day, C. D., Lee, E., Kobayashi, J., Holappa, L. D., Albert, H. & Ow, D. W. (2000). Transgene integration into the same chromosome location can produce alleles that express at a predictable level, or alleles that are differentially silenced. *Genes & Development 14*: 2869-2880.

de la Cueva-Mendez, G., Mills, A. D., Clay-Farrace, L., Diaz-Orejas, R. & Laskey, R. A. (2003). Regulatable killing of eukaryotic cells by the prokaryotic proteins Kid and Kis. *The EMBO Journal, 22*(2): 246-251.

DeNap, J. C. & Hergenrother, P. J. (2005). Bacterial death comes full circle: targeting plasmid replication in drug-resistant bacteria. *Organic & Biomolecular Chemistry, 3*(6): 959-966.

Desbois, A. P. & Smith, V. J. (2010). Antibacterial free fatty acids: activities, mechanisms of action and biotechnological potential. *Applied Microbiology and Biotechnology, 85*(6): 1629-1642.

Ding, Y., Liang, S., Lei, J., Chen, L., Kothe, E. & Ma, A. (2011). *Agrobacterium tumefaciens* mediated fused *egfp-hph* gene expression under the control of gpd promoter in *Pleurotus ostreatus*. *Microbiological Research, 166*(4): 314-322.

Doron, L., Segal, N. & Shapira, M. (2016). Transgene Expression in Microalgae-From Tools to Applications. *Frontiers in Plant Science, 7*: 505.

Dorr, T., Vulic, M. & Lewis, K. (2010). Ciprofloxacin causes persister formation by inducing the TisB toxin in *Escherichia coli*. *PLOS Biology, 8*(2): e1000317.

Dugdale, B., Mortimer, C. L., Kato, M., James, T. A., Harding, R. M. & Dale, J. L. (2013). In plant activation: an inducible, hyperexpression platform for recombinant protein production in plants. *The Plant Cell, 25*(7): 2429-2443.

Dunahay, T. G., Jarvis, E. E. & Roessler, P. G. (1995). Genetic transformation of the diatoms *Cyclotella cryptica* and *Navicula saprophila*. . *Journal of Phycology, 31*: 1004-1012.

Engelberg-Kulka, H., Amitai, S., Kolodkin-Gal, I. & Hazan, R. (2006). Bacterial programmed cell death and multicellular behavior in bacteria. *PLOS Genetics, 2*(10): 1528-1536.

Engelberg-Kulka, H. & Glaser, G. (1999). Addiction modules and programmed cell death and antideath in bacterial cultures. *Annual Review of Microbiology, 53*: 43-70.

Eroglu, E., Okada, S. & Melis, A. (2011). Hydrocarbon productivities in different *Botryococcus* strains: comparative methods in product quantification. *Journal of Applied Phycology, 23*(4): 763-775.

Falciatore, A., Casotti, R., Leblanc, C., Abrescia, C. & Bowler, C. (1999). Transformation of Nonselectable Reporter Genes in Marine Diatoms. *Marine Biotechnology, 1*(3): 239-251.

Fathi, A. A., Azooz, M. M. & Al-Fredan, M. A. (2013). Phycoremediation and the potential of sustainable algal biofuel production using watewater. *American Journal of Applied Sciences, 10*(2): 189-194.

Feng, S., Xue, L., Liu, H. & Lu, P. (2009). Improvement of efficiency of genetic transformation for *Dunaliella salina* by glass beads method. *Molecular Biology Reports, 36*(6): 1433-1439.

Filichkin, S. A., Meilan, R., Busov, V. B., Ma, C., Brunner, A. M. & Strauss, S. H. (2006). Alcohol-inducible gene expression in transgenic *Populus*. *Plant Cell Reports* *25*(7): 660-667.

Fineran, P. C., Blower, T. R., Foulds, I. J., Humphreys, D. P., Lilley, K. S. & Salmond, G. P. (2009). The phage abortive infection system, ToxIN, functions as a protein-RNA toxin-antitoxin pair. *Proceedings of the National Academy of Sciences of the United States of America, 106*(3): 894-899.

Flagfeldt, D. B., Siewers, V., Huang, L. & Nielsen, J. (2009). Characterization of chromosomal integration sites for heterologous gene expression in *Saccharomyces cerevisiae*. *Yeast, 26*(10): 545-551.

Fozo, E. M., Hemm, M. R. & Storz, G. (2008). Small toxic proteins and the antisense RNAs that repress them. *Microbiology and Molecular Biology Reviews, 72*(4): 579-589.

Fozo, E. M., Makarova, K. S., Shabalina, S. A., Yutin, N., Koonin, E. V. & Storz, G. (2010). Abundance of type I toxin-antitoxin systems in bacteria: searches for new candidates and discovery of novel families. *Nucleic Acids Research, 38*(11): 3743-3759.

Frank, A. C. & Johnson, M. A. (2009). Expressing the diphtheria toxin A subunit from the HAP2(GCS1) promoter blocks sperm maturation and produces single sperm-like cells capable of fertilization. *Plant Physiology, 151*(3): 1390-1400.

Franklin, S. E. & Mayfield, S. P. (2004). Prospects for molecular farming in the green alga *Chlamydomonas*. *Current Opinion in Plant Biology, 7*(2): 159-165.

Fuhrmann, M., Oertel, W. & Hegemann, P. (1999). A synthetic gene coding for the green fluorescent protein (GFP) is a versatile reporter in *Chlamydomonas reinhardtii*. *The Plant Journal 19*(3): 353-361.

Fuoco, R., Bogani, P., Capodaglio, G., Del Bubba, M., Abollino, O., Giannarelli, S., Spiriti, M. M., Muscatello, B., Doumett, S., Turetta, C., Zangrando, R., Zelano, V. & Buiatti, M. (2013). Response to metal stress of *Nicotiana langsdorffii* plants wild-type and transgenic for the rat glucocorticoid receptor gene. *Journal of Plant Physiology 170*(7): 668-675.

Gardner, N., Felsheim, R. & Smith, A. G. (2009). Production of male- and female-sterile plants through reproductive tissue ablation. *Journal of Plant Physiology 166*(8): 871-881.

Garoosi, G. A., Salter, M. G., Caddick, M. X. & Tomsett, A. B. (2005). Characterization of the ethanol-inducible *alc* gene expression system in tomato. *Journal of Experimental Botany, 56*(416): 1635-1642.

Gatz, C. & Lenk, I. (1998). Promoters that respond to chemical inducers *Trends in Plant Science, 3*(9): 352-358.

Gelvin, S. B. (2000). Agrobacterium and Plant Genes Involved in T-DNA Transfer and Integration. *Annual Review in Plant Physiology and Plant Molecular Biology, 51*: 223-256.

Gerdes, K. & Wagner, E. G. (2007). RNA antitoxins. *Current Opinion in Microbiology, 10*(2): 117-124.

Gerken, H. G., Donohoe, B. & Knoshaug, E. P. (2013). Enzymatic cell wall degradation of *Chlorella vulgaris* and other microalgae for biofuels production. *Planta, 237*(1): 239-253.

Gonzalez-Ballester, D., de Montaigu, A., Higuera, J. J., Galvan, A. & Fernandez, E. (2005). Functional genomics of the regulation of the nitrate assimilation pathway in *Chlamydomonas*. *Plant Physiology, 137*(2): 522-533.

Gressler, P. D., Bjerk, T. R., Schneider Rde, C., Souza, M. P., Lobo, E. A., Zappe, A. L., Corbellini, V. A. & Moraes, M. S. (2014). Cultivation of *Desmodesmus subspicatus* in a tubular photobioreactor for bioremediation and microalgae oil production. *Environmental Technology, 35*(1-4): 209-219.

Griffiths, M. J. & Harrison, S. T. L. (2009). Lipid productivity as a key characteristic for choosing algal species for biodiesel. *Journal of Applied Phycology, 21*(5): 493-507.

Guerrero-Andrade, O., Loza-Rubio, E., Olivera-Flores, T., Fehervari-Bone, T. & Gomez-Lim, M. A. (2006). Expression of the Newcastle disease virus fusion protein in transgenic maize and immunological studies. *Transgenic Research, 15*(4): 455-463.

Halim, R., Harun, R., Webley, P. A. & Danquah, M. K. (2013). Bioprocess Engineering Aspects of Biodiesel and Bioethanol Production from Microalgae. *Advanced Biofuels and Bioproducts*: 601-628.

Hannon, M., Gimpel, J., Tran, M., Rasala, B. & Mayfield, S. (2010). Biofuels from algae: challenges and potential. *Biofuels, 1*(5): 763-784.

Harris, E. H. (2001). *Chlamydomonas* as a Model Organism. *Annual Review of Plant Physiology and Plant Molecular Biology, 52*: 363-406.

Harun, R., Singh, M. & Forde, G. M. (2009). Bioprocess engineering of microalgae to produce a variety of consumer products. *Renewable & Sustainable Energy Reviews, 14*: 1037-1047.

Hayes, F. & Van Melderen, L. (2011). Toxins-antitoxins: diversity, evolution and function. *Crit Rev Biochem Mol Biol, 46*(5): 386-408.

Hirakawa, Y., Kofuiji, R. & Ishida, K. (2008). Transient transformation of a chlorarachniophyte alga, *Lotharella amoebiformis* (chlorarachniophyceae), with uidA and egfp reporter genes. *Journal of Phycology, 44*: 814-820.

Johari, A., Nyakuma, B. B., Mohd Nor, S. H., Mat, R., Hashimi, H., Ahmad, A., Zakaria, Z. Y. & Tuan Abdullah, T. A. (2015). The challenges and prospects of palm oil based biodiesel in Malaysia. *Energy, 81*: 255-261.

Johnson, E. A., Rosenberg, J. & McCarty, R. E. (2007). Expression by Chlamydomonas reinhardtii of a chloroplast ATP synthase with polyhistidine-tagged beta subunits. *Biochimica et Biophysica Acta, 1767*(5): 374-380.

Jones, C. S. & Mayfield, S. P. (2012). Algae biofuels: versatility for the future of bioenergy. *Current Opinion in Biotechnology, 23*(3): 346-351.

Kadioglu, A., Weiser, J. N., Paton, J. C. & Andrew, P. W. (2008). The role of *Streptococcus pneumoniae* virulence factors in host respiratory colonization and disease. *Nature Reviews Microbiology, 6*(4): 288-301.

Kamphuis, M. B., Bonvin, A. M., Monti, M. C., Lemonnier, M., Munoz-Gomez, A., van den Heuvel, R. H., Diaz-Orejas, R. & Boelens, R. (2006). Model for RNA binding and the catalytic site of the RNase Kid of the bacterial parD toxin-antitoxin system. *Journal of Molecular Biology, 357*(1): 115-126.

Kapaun, E. & Reisser, W. (1995). A chitin-like glycan in the cell wall of *Chlorella* sp. (Chlorococcales, Chlorophyceae). *Planta, 197*(4): 577-582.

Kawano, M., Aravind, L. & Storz, G. (2007). An antisense RNA controls synthesis of an SOS-induced toxin evolved from an antitoxin. *Molecular Microbiology, 64*(3): 738-754.

Kerr, R. A. (2011). Peak oil production may already be here. *Science, 331*(6024): 1510-1511.

Khan, S. A., Rashmi, H., M.Z., Prasad, S. & Banerjee, U. C. (2009). Prospects of biodiesel production from microalgae in India. *Renewable & Sustainable Energy Reviews, 13*(9): 2361-2372.

Khoo, S. K., Loll, B., Chan, W. T., Shoeman, R. L., Ngoo, L., Yeo, C. C. & Meinhart, A. (2007). Molecular and structural characterization of the PezAT chromosomal toxin-antitoxin system of the human pathogen *Streptococcus pneumoniae*. *The Journal of Biological Chemistry, 282*(27): 19606-19618.

Kindle, K. L. (1990). High-frequency nuclear transformation of *Chlamydomonas reinhardtii*. *Proceedings of the National Academy of Sciences of the United States of America, 87*(3): 1228-1232.

Kindle, K. L., Schnell, R. A., Fernandez, E. & Lefebvre, P. A. (1989). Stable nuclear transformation of *Chlamydomonas* using the *Chlamydomonas* gene for nitrate reductase. *The Journal of Cell Biology, 109*(6 Pt 1): 2589-2601.

Krichnavaruk, S., Shotipruk, A., Goto, M. & Pavasant, P. (2008). Supercritical carbon dioxide extraction of astaxanthin from *Haematococcus pluvialis* with vegetable oils as co-solvent. *Bioresource Technology, 99*(13): 5556-5560.

Kristoffersen, P., Jensen, G. B., Gerdes, K. & Piskur, J. (2000). Bacterial toxin-antitoxin gene system as containment control in yeast cells. *Applied and Environmental Microbiology, 66*(12): 5524-5526.

Kubo, T., Saito, T., Fukuzawa, H. & Matsuda, Y. (2001). Two tandemly-located matrix metalloprotease genes with different expression patterns in the *Chlamydomonas* sexual cell cycle. *Current Genetics, 40*(2): 136-143.

Kumar, S. C., Misqitta, R. W. & Reddy, V. S. (2004). Genetic transformation of the green alga *Chlamydomonas reinhardtii* by *Agrobacterium tumefaciens*. *Plant Science, 166*: 731-738.

Laurance, W. F., Sayer, J. & Cassman, K. G. (2014). Agricultural expansion and its impacts on tropical nature. *Trends in Ecology & Evolution, 29*(2): 107-116.

Lee, R. E. (1989). *Phycology* (2nd ed.). New York: Cambridge University Press.

Leon, R. & Fernandez, E. (2007). Nuclear transformation of eukaryotic microalgae: historical overview, achievements and problems. *Advances in Experimental Medicine and Biology, 616*: 1-11.

Lerche, K. & Hallmann, A. (2013). Stable nuclear transformation of *Eudorina elegans*. *BMC Biotechnology,* *13*: 11.

Lewis, K. (2010). Persister cells. *Annual Review of Microbiology,* *64*: 357-372.

Li, G. Y., Zhang, Y., Inouye, M. & Ikura, M. (2009). Inhibitory mechanism of *Escherichia coli* RelE-RelB toxin-antitoxin module involves a helix displacement near an mRNA interferase active site. *The Journal of Biological Chemistry,* *284*(21): 14628-14636.

Li, J., Lu, Y., Xue, L. & Xie, H. (2010). A structurally novel salt-regulated promoter of duplicated carbonic anhydrase gene 1 from *Dunaliella salina*. *Molecular Biology Reports,* *37*(2): 1143-1154.

Lioy, V. S., Machon, C., Tabone, M., Gonzalez-Pastor, J. E., Daugelavicius, R., Ayora, S. & Alonso, J. C. (2012). The zeta toxin induces a set of protective responses and dormancy. *PLOS One,* *7*(1): e30282.

Liu, L., Wang, Y., Zhang, Y., Chen, X., Zhang, P. & Ma, S. (2013). Development of a new method for genetic transformation of the green alga *Chlorella ellipsoidea*. *Molecular Biotechnology,* *54*(2): 211-219.

Liu, Z., Zhang, L., Pu, Y., Liu, Z., Li, Z., Zhao, Y. & Qin, S. (2014). Cloning and expression of a cytosolic HSP90 gene in *Chlorella vulgaris*. *BioMed Research International,* *2014*: 1-11.

Loris, R., Dao-Thi, M. H., Bahassi, E. M., Van Melderen, L., Poortmans, F., Liddington, R., Couturier, M. & Wyns, L. (1999). Crystal structure of CcdB, a topoisomerase poison from *E. coli*. *Journal of Molecular Biology,* *285*(4): 1667-1677.

Lowe, B. A., Prakash, N. S. W., M., Mann, M. T., Spencer, T. M. & Boddupalli, R. S. (2009). Enhanced single copy integration events in corn via particle bombardment using. *Transgenic Research 18*: 831-840.

Makarova, K. S., Wolf, Y. I. & Koonin, E. V. (2009). Comprehensive comparative-genomic analysis of type 2 toxin-antitoxin systems and related mobile stress response systems in prokaryotes. *Biology Direct,* *4*: 19.

Mallick, N., Mandal, S., Singh, A., Bishal, M. & Dash, A. (2012). Green microalga *Chlorella vulgaris* as a potential feedstock for biodiesel. *Journal of Chemical Technology and Biotechnology 87*(1): 137-145.

Mandal, S. K., Singh, R. P. & Patel, V. (2011). Isolation and characterization of exopolysaccharide secreted by a toxic dinoflagellate, *Amphidinium carterae*

Hulburt 1957 and its probable role in harmful algal blooms (HABs). *Microbial Ecology, 62*(3): 518-527.

Manimaran, P., Ramkumar, G., Sakthivel, K., Sundaram, R. M., Madhav, M. S. & Balachandran, S. M. (2011). Suitability of non-lethal marker and marker-free systems for development of transgenic crop plants: present status and future prospects. *Biotechnology Advances 29*(6): 703-714.

Marchetti, A., Parker, M. S., Moccia, L. P., Lin, E. O., Arrieta, A. L., Ribalet, F., Murphy, M. E., Maldonado, M. T. & Armbrust, E. V. (2009). Ferritin is used for iron storage in bloom-forming marine pennate diatoms. *Nature, 457*(7228): 467-470.

Marenkova, T. V. & Deineko, E. V. (2010). Transcriptional gene silencing in plants. *Genetika, 46*(5): 581-592.

Martin, B., Humbert, O., Camara, M., Guenzi, E., Walker, J., Mitchell, T., Andrew, P., Prudhomme, M., Alloing, G. & Hakenbeck, R. (1992). A highly conserved repeated DNA element located in the chromosome of *Streptococcus pneumoniae*. *Nucleic Acids Research, 20*(13): 3479-3483.

Masuda, H., Tan, Q., Awano, N., Yamaguchi, Y. & Inouye, M. (2012). A novel membrane-bound toxin for cell division, CptA (YgfX), inhibits polymerization of cytoskeleton proteins, FtsZ and MreB, in *Escherichia coli*. *FEMS Microbiology Letters, 328*(2): 174-181.

Mata, T. M., Martins, A. A. & Caetano, N. S. (2010). Microalgae for biodiesel production and other application: A review. *Renewable & Sustainable Energy Reviews, 14*(1): 217-232.

McArthur, G. H. t. & Fong, S. S. (2010). Toward engineering synthetic microbial metabolism. *Journal of Biomedicine and Biotechnology 2010*: 459760.

Meier, L., Perez, R., Azocar, L., Rivas, M. & Jeison, D. (2015). Photosynthetic CO2 uptake by microalgae: An attractive tool for biogas upgrading. *Biomass & Bioenergy, 73*: 102-109.

Mendoza, M. S., Dubreucq, B., Miguel, M., Caboche, M. & Lepiniec, L. (2005). LEAFY COTYLEDON 2 activation is sufficient to trigger the accumulation of oil and seed specific mRNAs in *Arabidopsis* leaves. *FEBS Letters, 579*(21): 4666-4670.

Miki, B. & McHugh, S. (2004). Selectable marker genes in transgenic plants: applications, alternatives and biosafety. *Journal of Biotechnology 107*(3): 193-232.

Morrison, T. G. (2003). Structure and function of a paramyxovirus fusion protein. *Biochimica et Biophysica Acta, 1614*(1): 73-84.

Muskens, M. W., Vissers, A. P., Mol, J. N. & Kooter, J. M. (2000). Role of inverted DNA repeats in transcriptional and post-transcriptional gene silencing. *Plant Molecular Biology, 43*(2-3): 243-260.

Mussgnug, J. H., Klassen, V., Schluter, A. & Kruse, O. (2010). Microalgae as substrates for fermentative biogas production in a combined biorefinery concept. *Journal of Biotechnology, 150*(1): 51-56.

Mutschler, H., Gebhardt, M., Shoeman, R. L. & Meinhart, A. (2011). A novel mechanism of programmed cell death in bacteria by toxin-antitoxin systems corrupts peptidoglycan synthesis. *PLOS Biology, 9*(3): e1001033.

Mutschler, H. & Meinhart, A. (2011). epsilon/zeta systems: their role in resistance, virulence, and their potential for antibiotic development. *Journal of Molecular Medicine, 89*(12): 1183-1194.

Nehlsen, K., Herrmann, S., Zauers, J., Hauser, H. & Wirth, D. (2010). Toxin-antitoxin based transgene expression in mammalian cells. *Nucleic Acids Research, 38*(5): 32-39.

Neumann, P., Torres, A., Azocar, L., Meier, L., Vergace, C. & Jeison, D. (2011). Biogas production as a tool for increasing sustainability of biodiesel production from microalgae *Botryococcus braunii, X Latinamerican and Symposium on Anaerobic Digestion*. Ouro Preto, Brazil.

Neupert, J., Karcher, D. & Bock, R. (2009). Generation of *Chlamydomonas* strains that efficiently express nuclear transgenes. *The Plant Journal, 57*(6): 1140-1150.

Neupert, J., Shao, N., Lu, Y. & Bock, R. (2012). Genetic transformation of the model green alga *Chlamydomonas reinhardtii*. *Methods in Molecular Biology, 847*: 35-47.

Ng, S. L., Harikrishna, J. A., Abu Bakar, F., Yeo, C. C. & Cha, T. S. (2016). Heterologous expression of the *Streptococcus pneumoniae yoeB* and *pezT* toxin genes is lethal in *Chlorella vulgaris*. *Algal Research, 19*: 21-29.

Niehaus, T. D., Okada, S., Devarenne, T. P., Watt, D. S., Scivipa, V. & Chapell, J. (2011). Identification of unique mechanisms for triterpene biosynthesis in *Botryococcus braunii*. *PNAS, 108*(30): 12260-12265.

Nieto, C., Cherny, I., Khoo, S. K., de Lacoba, M. G., Chan, W. T., Yeo, C. C., Gazit, E. & Espinosa, M. (2007). The yefM-yoeB toxin-antitoxin systems of *Escherichia coli* and *Streptococcus pneumoniae*: functional and structural correlation. *Journal of Bacteriology, 189*(4): 1266-1278.

Nieto, C., Pellicer, T., Balsa, D., Christensen, S. K., Gerdes, K. & Espinosa, M. (2006). The chromosomal relBE2 toxin-antitoxin locus of *Streptococcus pneumoniae:* characterization and use of a bioluminescence resonance energy transfer assay to detect toxin-antitoxin interaction. *Molecular Microbiology, 59*(4): 1280-1296.

Nieto, C., Sadowy, E., de la Campa, A. G., Hryniewicz, W. & Espinosa, M. (2010). The relBE2Spn toxin-antitoxin system of *Streptococcus pneumoniae:* role in antibiotic tolerance and functional conservation in clinical isolates. *PLOS One, 5*(6): e11289.

Niu, Y. F., Zhang, M. H., Xie, W. H., Li, J. N., Gao, Y. F., Yang, W. D., Liu, J. S. & Li, H. Y. (2011). A new inducible expression system in a transformed green alga, *Chlorella vulgaris. Genetics and Molecular Research, 10*(4): 3427-3434.

O'Brien, K. L., Wolfson, L. J., Watt, J. P., Henkle, E., Deloria-Knoll, M., McCall, N., Lee, E., Muhollan, K., Levine, O. S. & Cherian, T. (2009). Burden of disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global estimates. *Lancet, 374*(9693): 893-902.

Okuzaki, A., Konagaya, K., Nanasato, Y., Tsuda, M. & Tabei, Y. (2011). Estrogen-inducible GFP expression patterns in rice (*Oryza sativa* L.). *Plant Cell Reports, 30*(4): 529-538.

Pandey, D. P. & Gerdes, K. (2005). Toxin-antitoxin loci are highly abundant in free-living but lost from host-associated prokaryotes. *Nucleic Acids Research, 33*(3): 966-976.

Park, J. H., Yamaguchi, Y. & Inouye, M. (2012). Intramolecular regulation of the sequence-specific mRNA interferase activity of MazF fused to a MazE fragment with a linker cleavable by specific proteases. *Applied and Environmental Microbiology, 78*(11): 3794-3799.

Pedersen, K., Christensen, S. K. & Gerdes, K. (2002). Rapid induction and reversal of a bacteriostatic condition by controlled expression of toxins and antitoxins. *Molecular Microbiology, 45*(2): 501-510.

Pedersen, K., Zavialov, A. V., Pavlov, M. Y., Elf, J., Gerdes, K. & Ehrenberg, M. (2003). The bacterial toxin RelE displays codon-specific cleavage of mRNAs in the ribosomal A site. *Cell, 112*(1): 131-140.

Pittman, J. K., Dean, A. P. & Osundeko, O. (2011). The potential of sustainable algal biofuel production using wastewater resources. *Bioresource Technology, 102*(1): 17-25.

Potvin, G. & Zhang, Z. (2010). Strategies for high-level recombinant protein expression in transgenic microalgae: a review. *Biotechnology Advances, 28*(6): 910-918.

Poulsen, N. & Kroger, N. (2005). A new molecular tool for transgenic diatoms: control of mRNA and protein biosynthesis by an inducible promoter-terminator cassette. *The FEBS Journal, 272*(13): 3413-3423.

Pratheesh, P. T., Vineetha, M. & Kurup, G. M. (2014). An efficient protocol for the Agrobacterium-mediated genetic transformation of microalga Chlamydomonas reinhardtii. *Mol Biotechnol, 56*(6): 507-515.

Qi, Y. B., Garren, E. J., Shu, X., Tsien, R. Y. & Jin, Y. (2012). Photo-inducible cell ablation in *Caenorhabditis elegans* using the genetically encoded singlet oxygen generating protein miniSOG. *Proceedings of the National Academy of Sciences of the United States of America, 109*(19): 7499-7504.

Qin, S., Lin, H. & Jiang, P. (2012). Advances in genetic engineering of marine algae. *Biotechnology Advances, 30*(6): 1602-1613.

Radakovits, R., Jinkerson, R. E., Darzins, A. & Posewitz, M. C. (2010). Genetic engineering of algae for enhanced biofuel production. *Eukaryotic Cell, 9*(4): 486-501.

Richards, E. J. & Elgin, S. C. (2002). Epigenetic codes for heterochromatin formation and silencing: rounding up the usual suspects. *Cell, 108*(4): 489-500.

Rodolfi, L., Chini Zittelli, G., Bassi, N., Padovani, G., Biondi, N., Bonini, G. & Tredici, M. R. (2009). Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. *Biotechnology and Bioengineering, 102*(1): 100-112.

Roque, E., Gomez, M. D., Ellul, P., Wallbraun, M., Madueno, F., Beltran, J. P. & Canas, L. A. (2007). The PsEND1 promoter: a novel tool to produce genetically engineered male-sterile plants by early anther ablation. *Plant Cell Reports, 26*(3): 313-325.

Rosa, L., David, G., Aurora, G. & Emilio, F. (2004). Transgenic microalgae as green-cell factories. *Trends in Biotechnology, 22*(1): 45-52.

Rumpel, S., Siebel, J. F., Fares, C., Duan, J., Reijerse, E., Happe, T., Lubitz, W. & Winkler, M. (2014). Enhancing hydrogen production of microalgae by redirecting electrons from photosystem I to hydrogenase. *Energy & Environment Science, 7*: 3296-3301.

Saavedra De Bast, M., Mine, N. & Van Melderen, L. (2008). Chromosomal toxin-antitoxin systems may act as antiaddiction modules. *Journal of Bacteriology, 190*(13): 4603-4609.

Safi, C., Xebib, B., Merah, O., Pontalier, P.-Y. & Vaca-Garcia, C. (2014). Morphology, composition, production, processing and applications of *Chlorella vulgaris:* A review. *Renewable & Sustainable Energy Reviews, 35*: 265-278.

Saito, M., Iwawaki, T., Taya, C., Yonekawa, H., Noda, M., Inui, Y., Mekada, E., Kimata, Y., Tsuru, A. & Kohno, K. (2001). Diphtheria toxin receptor-mediated conditional and targeted cell ablation in transgenic mice. *Nature Biotechnology, 19*(8): 746-750.

Schifano, J. M., Edifor, R., Sharp, J. D., Ouyang, M., Konkimalla, A., Husson, R. N. & Woychik, N. A. (2013). Mycobacterial toxin MazF-mt6 inhibits translation through cleavage of 23S rRNA at the ribosomal A site. *Proceedings of the National Academy of Sciences of the United States of America, 110*(21): 8501-8506.

Shah, D., Zhang, Z., Khodursky, A., Kaldalu, N., Kurg, K. & Lewis, K. (2006). Persisters: a distinct physiological state of *E. coli. BMC Microbiology, 6*: 53-61.

Singletary, L. A., Gibson, J. L., Tanner, E. J., McKenzie, G. J., Lee, P. L., Gonzalez, C. & Rosenberg, S. M. (2009). An SOS-regulated type 2 toxin-antitoxin system. *Journal of Bacteriology, 191*(24): 7456-7465.

Slanchev, K., Stebler, J., de la Cueva-Mendez, G. & Raz, E. (2005). Development without germ cells: the role of the germ line in zebrafish sex differentiation. *Proceedings of the National Academy of Sciences of the United States of America, 102*(11): 4074-4079.

Specht, E., Miyake-Stoner, S. & Mayfield, S. (2010). Micro-algae come of age as a platform for recombinant protein production. *Biotechnology Letters, 32*(10): 1373-1383.

Srirangan, K., Pyne, M. E. & Perry Chou, C. (2011). Biochemical and genetic engineering strategies to enhance hydrogen production in photosynthetic algae and cyanobacteria. *Bioresource Technology, 102*(18): 8589-8604.

Stone, S. L., Kwong, L. W., Yee, K. M., Pelletier, J., Lepiniec, L., Fischer, R. L., Goldberg, R. B. & Harada, J. J. (2001). LEAFY COTYLEDON2 encodes a B3 domain transcription factor that induces embryo development. *Proceedings of the National Academy of Sciences of the United States of America, 98*(20): 11806-11811.

Sun, N., Lee, A. & Wu, J. C. (2009). Long term non-invasive imaging of embryonic stem cells using reporter genes. *Nature Protocols, 4*(8): 1192-1201.

Sweeney, S. T., Hidalgo, A., de Belle, J. S. & Keshishian, H. (2012). Genetic systems for functional cell ablation in *Drosophila*. *Cold Spring Harbour Protocols, 2012*(9): 950-956.

Sydney, E. B., Sturm, W., de Carvalho, J. C., Thomaz-Soccol, V., Larroche, C., Pandey, A. & Soccol, C. R. (2010). Potential carbon dioxide fixation by industrially important microalgae. *Bioresource Technology, 101*(15): 5892-5896.

Taher, H., Al-Zuhair, S., Al-Marzouqi, A. H., Haik, Y. & Farid, M. M. (2011). A review of enzymatic transesterification of microalgal oil-based biodiesel using supercritical technology. *Enzyme Research, 2011*: 4061-4085.

Tan, C., Qin, S., Zhang, Q., Jiang, P. & Zhao, F. (2005). Establishment of a micro-particle bombardment transformation system for *Dunaliella salina*. *Journal of Microbiology, 43*(4): 361-365.

Tan, Q., Awano, N. & Inoyue, M. (2011). YeeV is an *Escherichia coli* toxin that inhibits cell division by targeting the cytoskeleton proteins, FtsZ and MreB. *Molecular Microbiology, 79*: 109-118.

ten Lohuis, M. R. & Miller, D. J. (1998). Genetic transformation of dinoflagellates (*Amphidinium* and *Symbiodinium*): Expression of GUS in microalgae using heterologous promoter constructs. *The Plant Journal, 13*: 427-435.

Tomaselli, L. (2003). The Microalgal Cell. In A. Richmond (Ed.), *in Handbook of Microalgal Culture: Biotechnology and Applied Phycology* (pp. 3-19). Oxford, UK: Blackwell Publishing Ltd

Tomei, J. & Upham, P. (2009). Argentinean soy-based biodiesel: An introduction to production and impacts. *Energy, 37*(10): 3890-3898.

Tran, D. T., Chen, C. L. & Chang, J. S. (2013). Effect of solvents and oil content on direct transesterification of wet oil-bearing microalgal biomass of *Chlorella vulgaris* ESP-31 for biodiesel synthesis using immobilized lipase as the biocatalyst. *Bioresource Technology, 135*: 213-221.

Tzfira, T. & Citovsky, V. (2006). Agrobacterium-mediated genetic transformation of plants: biology and biotechnology. *Current Opinion in Biotechnology, 17*(2): 147-154.

Tzfira, T., Kunik, T., Gafni, Y. & Citovsky, V. (2006). Mammalian cells. *Methods in Molecular Biology, 344*: 435-451.

Unoson, C. & Wagner, E. G. (2008). A small SOS-induced toxin is targeted against the inner membrane in *Escherichia coli*. *Molecular Microbiology, 70*(1): 258-270.

Van Melderen, L. & Saavedra De Bast, M. (2009). Bacterial toxin-antitoxin systems: more than selfish entities? *PLOS Genetics, 5*(3): e1000437.

Vesper, O., Amitai, S., Belitsky, M., Brygasov, K., Kaberdina, A. C. & Engelberg-Kulka, H. (2011). Selective translation of leaderless mRNAs by specialized ribosomes generated by MazF in *Escherichia coli*. *Cell, 147*: 147-157.

Vieira, A. L. & Camilo, C. M. (2011). *Agrobacterium tumefasciens*-mediated transformation of the aquatic fungus *Blastocladiella emersonii*. *Fungal Genetics and Biology, 48*(8): 806-811.

Walker, T. L., Purton, S., Becker, D. K. & Collet, C. (2005). Microalgae as bioreactors. *Plant Cell Reports, 24*(11): 629-641.

Wang, J. F., Jiang, P., Cui, Y. L., Guan , X. Y. & Qin, S. (2010). Gene transfer into conchospores of *Porphyra haitanensis* (Bangiales, Rhodophyta) by glass bead agitation. *Phycologia, 49*: 355-360.

Wang, X., Lord, D. M., Cheng, H. Y., Osbourne, D. O., Hong, S. H., Sanchez-Torres, V., Quiroga, C., Zheng, K., Herrmann, T., Peti, W., Benedik, M. J., Page, R. & Wood, T. K. (2012). A new type V toxin-antitoxin system where mRNA for toxin GhoT is cleaved by antitoxin GhoS. *Nature Chemical Biology, 8*(10): 855-861.

Wu-Scharf, D., Jeong, B., Zhang, C. & Cerutti, H. (2000). Transgene and transposon silencing in *Chlamydomonas reinhardtii* by a DEAH-box RNA helicase. *Science, 290*(5494): 1159-1162.

Yamaguchi, Y. & Inouye, M. (2011). Regulation of growth and death in *Escherichia coli* by toxin-antitoxin systems. *Nature Reviews Microbiology, 9*(11): 779-790.

Yamaguchi, Y., Park, J. H. & Inouye, M. (2011). Toxin-antitoxin systems in bacteria and archaea. *Annual Review of Genetics, 45*: 61-79.

Yamaizumi, M., Mekada, E., Uchida, T. & Okada, Y. (1978). One molecule of diphtheria toxin fragment A introduced into a cell can kill the cell. *Cell, 15*(1): 245-250.

Yamano, T., Iguchi, H. & Fukuzawa, H. (2013). Rapid transformation of *Chlamydomonas reinhardtii* without cell-wall removal. *Journal of Bioscience and Bioengineering, 115*(6): 691-694.

Yang, B., Liu, J., Liu, B., Sun, P., Ma, X., Jiang, Y., Wei, D. & Chen, F. (2015). Development of a stable genetic system for *Chlorella vulgaris*- A promising green alga for CO2 biomitigation. *Algal Research, 12*: 134-141.

Yarmolinsky, M. B. (1995). Programmed Cell-Death in Bacterial-Populations. *Science, 267*(5199): 836-837.

Yeh, K. L. & Chang, J. S. (2011). Nitrogen starvation strategies and photobioreactor design for enhancing lipid content and lipid production of a newly isolated microalga *Chlorella vulgaris* ESP-31: implications for biofuels. *Biotechnology Journal, 6*(11): 1358-1366.

Zamalloa, C., Vulsteke, E., Albrecht, J. & Verstraete, W. (2011). The techno-economic potential of renewable energy through the anaerobic digestion of microalgae. *Bioresource Technology, 102*(2): 1149-1158.

Zhang, C. & Hu, H. (2014). High-efficiency nuclear transformation of the diatom *Phaeodactylum tricornutum* by electroporation. *Marine Genomics, 16*: 63-66.

Zhang, W. J., Dewey, R. E., Boss, W., Phillippy, B. Q. & Qu, R. (2013). Enhanced *Agrobacterium*-mediated transformation efficiencies in monocot cells is associated with attenuated defense responses. *Plant Molecular Biology, 81*(3): 273-286.

Zhang, Y. & Inouye, M. (2009). The inhibitory mechanism of protein synthesis by YoeB, an *Escherichia coli* toxin. *The Journal of Biological Chemistry, 284*(11): 6627-6638.

Zhang, Y., Li, H., Ouyang, B., Lu, Y. & Ye, Z. (2006). Chemical-induced autoexcision of selectable markers in elite tomato plants transformed with a gene conferring resistance to lepidopteran insects. *Biotechnology Letters, 28*(16): 1247-1253.

Zhang, Y., Zhang, J., Hoeflich, K. P., Ikura, M., Qing, G. & Inouye, M. (2003). MazF cleaves cellular mRNAs specifically at ACA to block protein synthesis in *Escherichia coli*. *Molecular Cell, 12*(4): 913-923.

Zielenkiewicz, U., Kowalewska, M., Kaczor, C. & Ceglowski, P. (2009). In Vivo Interactions between Toxin-Antitoxin Proteins Epsilon and Zeta of Streptococcal Plasmid pSM19035 in *Saccharomyces cerevisiae*. *Journal of Bacteriology, 191*(11): 3677- 3684.

Zorin, B., Y., L., Sizova, I. & Hegemann, P. (2009). Nuclear gene targeting in *Chlamydomonas* as exemplified by disruption of the PHOT gene. *Gene 432*(1-2): 91-96.

Zuo, J. & Chua, N. H. (2000). Chemical-inducible systems for regulated expression of plant genes. *Current Opinion in Biotechnology, 11*(2): 146-151.

Zuo, J., Niu, Q. W. & Chua, N. H. (2000). Technical advance: An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. *The Plant Journal, 24*(2): 265-273.

LIST OF PUBLICATIONS AND PAPERS PRESENTED

Publications

1. Ng, S. L., Harikrishna, J. A., Abu Bakar, F., Yeo, C. C. and Cha, T. S. (2016). Heterologous expression of the *Streptococcus pneumoniae yoeB* and *pezT* toxin genes is lethal in *Chlorella vulgaris*. *Algal Research 19*: 21-29.

Seminar

1. Ng, S. L., Harikrishna, J. A., Cha, T. S., Yeo, C. C. (2015). The Expression of Bacterial *yoeB* and *pezT* Toxin Genes in *Chlorella vulgaris* and its Toxicity Effect on Cell Viability. Institute Biological Sciences, Postgraduate Seminar. 13th May, 2015. Institute Biological Science, Faculty of Science, University of Malaya, Kuala Lumpur.

Conferences

1. Ng, S. L., Harikrishna, J. A., Cha, T. S., Yeo, C. C. (2014). Assessment of an Inducible Gene Expression System for the Expression of the Bacterial YoeB Toxin in the Microalgae *Chlorella vulgaris*. 21st Malaysian Society for Molecular Biology & Biotechnology (MSMBB). 1-3 October, 2014, Kuala Lumpur, Malaysia.

    This poster presentation was awarded **3rd place (Genetics & Omics).**

2. Ng, S. L., Harikrishna, J. A., Cha, T. S., Yeo, C. C. Utilizing a Two-component Inducible Gene Expression System to Demonstrate the Lethality of Expressing the Bacterial YoeB toxin in the Microalgae *Chlorella vulgaris*. 20th Biological Sciences Graduate Congress (BSGC). 9-11th December, 2015, Chulalongkorn University, Bangkok. Thailand.

    This poster presentation was awarded **2nd place (Applied Science and Biotechnology).**