Generating and evaluating salinity and temperature resilient cyanobacteria for tropical outdoor cultivation in Australia

Thesis Submitted by

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Statement of Contribution by Authors

This thesis includes collaborative work with my current supervisors, Associate Professor Kirsten Heimann, Senior Lecturer Lynne van Herwerden and JCU/CSIRO research fellow Dr. Diane Jarvis, my previous supervisor Associate Professor Patrick Schaeffer, and scientist Dr. Cherie Moti and Diane Brinkman from the Australian Institute of Marine Science (AIMS). Gobalakrishnan Subashchandrbose conducted the identification of the microalgal industry requirements and developed the research with multiple aims and objectives to generate and evaluate salinity and temperature resilient cyanobacteria. The experimental design, data collection and interpretation were performed by myself, Gobalakrishnan Subashchandrabose by seeking advice from many of my supervisors. The research conducted in this thesis complies with all bio-security ethical measures established by James Cook University and outlined by the Bio-Safety Ethics Committee of James Cook University. This research thesis is divided into six chapters and the author's contribution are listed in Table SCO.1.

Table SC0.1. Summary of author's contribution to the research presented in the chapters of this thesis

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2	GS,KH,LVH	GS	GS,KH,LVH	GS,KH,LVH	GS,KH,LVH
3	GS,KH,PS	GS	GS,KH,PS,LVH	GS,KH,PS,LVH	GS,KH,LVH
4	GS,KH, PS, CM, DB	GS,KH,CM, DB	GS,KH,CM, LVH	GS,KH,CM,LVH	GS,KH,CM,LVH
5	GS,KH,LVH,DJ	GS	GS,KH,LVH,DJ	GS,KH,LVH,DJ	GS,KH,LVH,DJ
6	GS,KH,LVH,DJ	GS	GS,KH,LVH,DJ	GS,KH,LVH,DJ	GS,KH,LVH,DJ

GS=Gobalakrishnan Subashchandrabose, **KH**=Kirsten Heimann, **LvH**= Lynne van Herwerden, **PS**= Patrick Schaeffer, **CM**=Cherie Moti, **DJ**=Diane Jarvis, **DB**=Diane Brinkman.

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Abstract

Global population levels, anticipated to increase to >9 billion by 2050, present serious worldwide challenges, such as energy-, food- and freshwater security. In addition, rising greenhouse gas (GHG) emissions lead to climatic instability, reduce the availability of freshwater and challenge agricultural productivity, which is exacerbated by decreasing arable land availability. Hence, the carbon- and freshwater-constrained economy demands industries to limit freshwater usage and carbon emissions. In this context, photosynthetic microalgae or cyanobacteria offer great promise for remediating carbon-dioxide emissions and high-nutient wastewaters, which can be coupled with renewable resource production to cater for large-volume low-value markets, such as animal feed, bio-fertiliser, and energy-production. The required scale of production for these markets, however, has to date not been realised, as outdoor cultivation presents severe challenges, including access to sufficient non-arable land in close proximity to water, nutrients (inorganic fertilisers: nitrogen and phosphate) and carbon-dioxide sources. In addition, high temperature and variable salinities are major limitations to cost-effective commercial microalgal production, as these factors are challenging to control.

Ectoine, a valuable osmolyte, is produced by extremophile microbes in response to variable salinities and high temperature stress. Ectoine synthesis is mediated by an *ectABC* gene cassette. Given this, my research aimed at engineering a *de novo* biosynthesis pathway for ectoine production into the freshwater cyanobacterium *Synechococcus elongatus* PCC 7942 - to examine: a) its effect on temperature and/or salinity tolerance and b) potential downstream effects of ectoine on fertilisation requirements and biochemical profiles of this cyanobacterium, as the latter affects bio-product potential. *Synechococcus elongatus* PCC7942 was chosen as a model cyanobacterium, as its genome is small and fully sequenced, commercial vectors for transformation are available and it is exempt from restriction of laboratory transformation experiments by the Office of Gene Technology Regulator (OGTR).

For *ectABC* transformation of *S. elongatus* PCC7942, a codon-optimised *ectABC_pSyn_6* plasmid was constructed, based on the *ectABC* gene nucleotide sequence from the temperature- and salinity-tolerant bacterium *Halomonas elongata* DSM4043. *ectABC*-

transformed, untransformed *pSyn_6* vector (lacking ectABC insert) controls and wild-type (no vector, WT) *S. elongatus* PCC 7942 were subjected to a three temperature (35, 40, and 45°C), three salinity (0, 18, 36 ppt) factorial design experimental challenge without acclimation. Our data confirmed that *ectABC*-transformed *S. elongatus* PCC7942 had improved temperature tolerance up to 45°C and salinity tolerance up to 18 ppt at 35°C, compared to WT and *pSyn_6* empty vector controls. Limited growth was observed at 36 ppt salinity in WT, *pSyn-6* and *ectABC* transformants, irrespective of temperature. *ectABC*-transformant population growth rates were highest at 35°C. High pressure liquid chromatography analysis of these *ectABC* transformants confirmed ectoine production, albeit minimal. Further studies are necessary at the molecular level to resolve impediments associated with the low level of ectoine expression, should ectoine be chosen as a high-value co-product for the cosmetics industry.

In terms of commercial production, it is vital to assess *ectABC*-transformed *S. elongatus* PCC 7942 fertilisation requirements. Results showed that nitrogen-requirements of *ectABC*-transformants were higher than that of WT and *pSyn_6* empty vector controls at an

elevated salinity of 18 ppt, but lower at 45°C temperature stress. Phosphate uptake was lowest in *ectABC*-transformants at temperature and salinity stress of 45°C and 18 ppt, respectively. Fertilisation costs require serious consideration for commercial-scale cultivation of large-volume, low-value bio-products markets. Thus, the stress-induced increased nitrogen fertilisation requirements of *ectABC*-transformants suggest that co-location with nitrogen-rich wastewater streams would be beneficial, thereby also reducing nutrient run-off into the local river systems.

Regarding the biochemical profile of hydrocarbon-based biofuel production, *ectABC* transformants had increased lipid and fatty acid production under both temperature (45°C) and salinity (18 ppt) stress. Thus, this research addresses an area of importance for transitioning to a bio-economy as a whole and for implementing environmentally and economically sustainable production of renewable biofuels, animal feed, bio-fertilisers, which are perhaps best achieved through co-production of some high-value bio-products, such as ectoine or the high-value pigment - c-phycocyanin.

To investigate this potential, a modelling approach using multi-criteria analysis and geographical information system analysis was adopted. ArcGIS was used to evaluate potential sites suitable for co-locating microalgal and sugarcane production in the Great Barrier Reef (GBR) catchment region in Queensland, Australia – whilst taking into account climatic, land-use and economic factors that consider energy balances for each facility. Critical resource inputs such as land, water, CO_2, energy and climatic factors such as temperature and rainfall were considered when estimating the available resources at sugar mills in the Wet Tropics region, adjacent to the GBR. Our economic analysis revealed that co-locating microalgal biomass production with such an industry is economically feasible in the Wet Tropics, by achieving significant cost-reductions and improved economic performance. As such, this research produces valuable information for investors, policy makers, government and industry to make informed decisions about the location potential for microalgal production sites that focus on salinity- and temperature-resilient microalgal cultivation for high-value compounds (e.g. the osmolyte ectoine) or low-value animal feed as their principal commodity, whilst reducing CO_2 emissions and nutrient runoff to the GBR, both of which attract tradeable credits which offer

additional economic returns over and above the returns from the production and sales process.

List of Abbreviations

AFDW	Ash-free dry weight
AIMS	Australian Institute of Marine Science
ANOVA	Analysis of variance
ATP	Adenosine tri-phosphate
A. thaliana	*Arabidopsis thaliana*
AUD	Australian dollar
BADH	Betaine aldehyde dehydrogenase
BLAST	Basic local alignment search tool
BVCMO	Choline monooxygenase from *Beta vulgaris*
BG11	Blue-green algae medium-11
bp	Base pair
CAI	Codon adaptation index
Cas9	CRISPR-associated protein 9
CDH	Choline dehydrogenase
CO_2	Carbon di-oxide
C. reinhardtii	*Chlamydomonas reinhardtii*
CRISPR	Clustered regularly interspaced short palindromic repeats
CSG	Coal seam gas

CSIRO	Commonwealth Scientific & Industrial Research Organisation
DNA	Deoxyribo nucleic acid
DNTP's	Deoxynucleotide triphosphates
D. salina	*Dunaliella sailna*
EctABC	Ectoine gene cassette
ectA	L-2, 4-Diaminobutyric acid acetyltransferase
ectB	L-2, 4-diaminobutyric acid transaminase
ectC	L-Ectoine synthase
E. coli	*Escherichia coli*
Ectoine	1, 4, 5, 6-Tetrahydro-2-methyl-4-pyrimidine-carboxylic acid
ENC	Effective number of codons
ESI-MS	Electrospray ionization-Mass spectrometry
FAME	Fatty acid methyl ester
GBR	Great Barrier Reef
G + C	Guanidine + Cytosine
GFP	Green fluroscent protein
GHG	Greenhouse gas

GMO	Genetically modified organism
GM crops	Genetically modified crops
GUS	β-Glucuronidase
HPLC	High-pressure liquid chromatography
IRA	Individual Research Account
IPCC	Intergovernmental Panel on Climate Change
JCU	James Cook Univerity
JCUPRS	James Cook University Post Graduate Research Scholarship
JCAT	Java Codon Adaptation Tool
KanR	Kanamycin resistance
LC-MS	Liquid chromatography-Mass spectrometry
MBD	Mid-west Biodiesel
mb	Mega bases
mRNA	Messenger RNA

NQAIF	North Queenslad Algal Identification/ Culturing Facility
NS1	Neutral site 1
NS2	Neutral site 2
NO_X	Nitrous oxide
OD	Optical density
OGTR	Office of Gene Technology Regulator
PCR	Polymerase chain reaction
ppt	Parts per thousand
ppm	Particle per million
pSyn	Plasmid of *Synechococcus elongatus* PCC 7942
PUFA	Poly-unsaturated fatty acid
P. tricornutum	*Phaeodactylum tricornutum*
RBS	Ribosome binding site
RNA	Ribonucleic acid

ROS	Reactive oxygen species
rpm	Round per minute
RSCU	Relative Synonymous Codon Usage
R_t	Retention time
SARDI	South Australian Research and Development Institute
SCOA	Statement contribution of authors
S. elongatus PCC 7942	*Synechococcus elongatus* PCC 7942
SO_X	Sulphur oxides
TAI	Translation adaptation index
TCA cycle	Tri-carboxylic acid cycle
TEV recognition site	Tobacco Etch Virus recognition site
T. pesudonana	*Thalassiosira pseudonana*
UDP-Glucose	Uridine diphosphate glucose
UNESCO	United Nations Educational, Scientific and Cultural Organization

USD	United States of American dollars
UV spectroscopy	Ultra-violet spectroscopy
WT	Wild-type

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1 CHAPTER ONE

GENERAL INTRODUCTION

1.1 Background

The world population is expected to rise to more than 9 billion by 2050, which is associated with increasing demands for energy, food and freshwater - all of which are required for stable economic growth and social stability Lau (2017). Many current methods of energy production are considered to be unsustainable as fossil fuel reserves are finite, as are global phosphate reserves. Additionally, climate change-induced unstable weather patterns- and water scarcity are current global challenges (Heimann, 2016; Lewandowski et al., 2018; Ummenhofer & Meehl, 2017). These challenges are interlinked with increasing greenhouse gas (GHG) emissions, exacerbated by a rapidly growing human population with increased energy, food and fuel demands (IPCC, 2014). Clean freshwater sources and arable land are already scarce in some developing and developed nations. The impact of climate-induced changes on global rainfall patterns has further reduced freshwater availability, and increased temperatures and sea levels (Rasul, 2016). These changes are predicted to add pressures and may worsen the availability of these scarce resources along with mobilising millions of displaced refugees (Fischer et al., 2017; Paul, 2017). As a result, countries with densely populated areas and little

arable land availability are more challenged to expand food production in a carbon- and freshwater-constrained future (Lewandowski et al., 2018). This causes serious problems in maintaining high living standards, while high GHG emissions further drive the imbalance in economic growth between countries (IPCC, 2014).

In addition to the socio-economic impact, climate change is expected to adversely affect food quality and human health, resulting in increased disease, exacerbated by freshwater/water quality reductions (Bank, 2016). Considering the aforementioned problems, there is an urgent need to identify and develop alternative renewable energy resources to replace fossil fuel energy, and support agriculture production through mitigating GHG emissions and wastewater remediation concurrently (ABS, 2001; Byrnes et al., 2013).

Unlike plants, microalgae or cyanobacteria, are often hailed as the ‘miracle cure’ for the world’s ailments, as microalgal biomass can be generated on non–arable land and in water sources not suitable for human consumption (nutrient-rich waste water, seawater) and such microalgal biomass is suitable to derive many bio-products including food, feed, fertiliser and fuel (Heimann, 2016). Algae are capable of abating GHG

emissions and their production represents a renewable resource, whilst preserving scarce potable freshwater resources, as they require less water than conventional crops and can (in part) contribute to additional food production (Heimann et al., 2015b). Based on photosynthetic performance, algae can be used to mitigate CO_2-rich industrial waste gases and remediate nutrient– and metal-rich waste water streams from agriculture and other industries (Heimann, 2016; von Alvensleben et al., 2016).

Despite these exciting possibilities, industrial-scale outdoor cultivation of freshwater microalgae or cyanobacteria faces severe economic and energy bottlenecks (Li et al., 2012). This is mainly due to large volumes of water and land area required for cultivation (Li et al., 2012). For example, Australia is the largest producer of the commercial production of marine microalgae for a high-value low-volume pigment (carotene) market (Borowitzka et al., 1984; Li et al., 2012). However, freshwater microalgal production for low-value high-volume bio-products such as food, feed, fuel and fertiliser production is not sustainable, as this microalgal production would compete with agriculture production for land and water resources in coastal areas (Borowitzka & Moheimani, 2013b). Therefore, identifying suitable land with sufficient water is essential for sustainable, successful

microalgal cultivation. Examining the land requirements for industrial-scale production of large-volume microalgal products (bio-fuel, bio-fertilisers) in Australia leads to alternative solutions: 1) Inland cultivation, where land is more accessible. However, this is associated with highly variable ground water salinity and extreme temperatures (Kolkovski, 2011), which negatively impact growth/biomass performance of microalgae or cyanobacteria and potentially alter the biochemical profile thereby reducing the suitability of the biomass for the desired end product (Li et al., 2012). 2) Cultivation that is co-located with other coastal area industries, such as sugarcane, aquaculture or solar farms, where ample sunlight, water and nutrients (in the case of sugarcane and aquaculture co-location) are available, offers a viable alternative. The latter option may also provide additional cost benefits by either providing bioremediation of nutrient-rich wastewater or by accessing low-cost solar-powered energy for microalgae cultivation, depending on the industry with which the microalgae production is co-located.

In addition, temperature and salinity management are two cultivation parameters that remain challenging for environmentally and economically sustainable production of microalgal biomass for high-volume, low-value

bio-products such as fuel, feed and fertiliser (Adenan et al., 2013; Heimann et al., 2015b). These factors are not readily controllable, because they require high energy inputs for cooling and freshwater to maintain suitable salinities (von Alvensleben et al., 2016). Whilst co-locating microalgal biomass production with other industrial facilities to source CO_2 may offer a solution, this is an expensive approach, as extraction and solubilisation of sufficient amount of CO_2 is difficult (Williams & Laurens, 2010). In addition, for CO_2 extraction from high temperature flue gas stream and cultivation of microalgae on a nutrient-rich saline wastewater require extremo-halophilic microalgae that tolerate high temperature and salinity (Varshney et al., 2015). In order to overcome this hurdle, strain selection for high temperature and salinity tolerance is possible, but is challenging in the context of maintaining growth and biochemical performance criteria for the desired end product (Xu, 2015). It appears that developing salinity- and temperature-tolerant cultivars is the only feasible solution to deliver on the microalgal promise in Australia (von Alvensleben, 2015). In order to develop salinity- and temperature-tolerant microalgae, genetically engineered microalgal strains are required to yield industrially suitable microalgae with improved resilience to extreme temperature and salinity

(Guihéneuf et al., 2016), whilst achieving the desired growth/biochemical profile performance characteristics for such large volume aquaculture enterprises. This strategy has already been implemented in several higher plants, bacteria and cyanobacteria to improve stress resilience (Moghaieb et al., 2011; Ning et al., 2016; Ruffing et al., 2013). Despite these possibilities, the use of genetically modified crops on limited arable land likely poses environmental risks and economic loss (Blakeney, 2017). In Australia, cultivating GM crops on arable land is fairly limited due to cross pollination and contamination by native strains (Blakeney, 2017). Furthermore, GM crop farms in Australia require approval from both federal and state governments (Blakeney, 2017). At present, the only genetically modified crops produced on limited arable lands in Australia are cotton and canola, the latter being approved for oil production after extensive consultation satisfied the risk assessment and management plan requirements with both federal and state governments (Blakeney, 2017).

Several organisms including bacteria, archaea and higher plants are well studied with regards to temperature and salinity stress (Chaudhuri et al., 2017). Such studies suggest that high salinity and temperature stress could be associated with the ability to synthesize osmolytes such as proline,

ectoine, and trehalose. (Chaudhuri et al., 2017). Biofuel production from the halo-tolerant marine *Dunaliella salina,* which responds to salinity changes through accumulating osmolytes (e.g. glycerol), is typically not considered, as other high-value products (e.g. β-carotene) are commercial favoured (Borowitzka & Vonshak, 2017). Osmolytes synthesized by extremo-halophilic bacteria have many applications in biotechnology and pharmaceutical industries for their protective effects and therefore have high commercial values (Cavicchioli et al., 2011). Accumulation of these osmolytes has been recognised to infer tolerance to high temperatures, drought- and salinity-stress by acting as compatible solutes that protect cell membrane fluidity, hydration and dehydration status (Chaudhuri et al., 2017). Although several osmolytes found in many organisms provide tolerance to salinity and temperature stress, very few are well known for their broad applications in agricultural and biotechnological industries (Cavicchioli et al., 2011).

The osmolyte ectoine is synthesized by an extremo-halophilic bacterium *Halomonas elongata* in response to extreme salinity and temperature conditions (Waditee-Sirisattha et al., 2016). Such extremophiles are much more tolerant to high temperatures and salinities, as they are adapted to

survive in the ocean depths at up to ~3,800 m and in extreme heat and salinity (Giddings & Newman, 2015). Ectoine biosynthesis is originally linked to the main energy pathway of the TCA cycle, which exists in all aerobic organisms. Under extreme stress conditions, ectoine synthesis is mediated by a three-step enzymatic reaction (catalysed by *ectA*, *ectB* and *ectC* gene products) which converts the aspartic acid precursor, an intermediate amino acid into ectoine during metabolism (see **Figure 2.1** for ectoine synthesis pathway) (Saum & Müller, 2008). Ectoine is a hydrophilic amino acid and its production has been genetically engineered in agricultural crops and bacteria due to the salinity resilience it induces. For instance, agriculture crops such as tobacco were genetically engineered for salinity- and temperature-stress resilience by adapting the metabolic pathways from halo-tolerant extremophilic organisms (Moghaieb et al., 2011; Nakayama et al., 2000).

Thus far, these technologies have not been extrapolated to microalgae to improve salinity- and temperature-stress resilience. Recent studies reported that individual transfection with either *ectA* (Lunde, 2012), *ect B* (Afzal, 2014) or *ectC* (Holck, 2014) genes into the green freshwater microalga *Chlamydomonas reinhardtii* did not lead to ectoine accumulation.

This is due to the fact that all three genes are required in one cassette to synthesize ectoine. Thus, a hypothesis was formulated that ectoine gene cassette transformation may generate microalgae that are temperature- and salinity-resilient, like the extremophile bacterium (*Halomonas elongata)* that produces ectoine naturally. Amongst algae, cyanobacteria are often favoured over eukaryotic algae for genetic manipulation because genetic manipulation in cyanobacteria is easier than in eukaryotic microalgae and the tools for gene transformation are readily available and applied (Knoot et al., 2017).

In this study, a unicellular (non- filamentous) freshwater cyanobacterium, *Synechococcus elongatus* PCC7942, was chosen for transfection with the *ectABC* gene cassette from *Halomonas elongata*, in order to improve salinity- and temperature-resilience. It is the first cyanobacterium proven to be effective for metabolic engineering (Holtman et al., 2005). It possesses a small, fully sequenced genome and molecular tools are commercially available for easy genetic manipulation. *Synechococcus elongatus* PCC7942 is a widely used model species to study- nutrient regulation under environmental stress (Holtman et al., 2005). The above context reveals that *Synechococcus elongatus* PCC7942 is well suited to be

genetically modified with the *ectABC* gene cassette to: 1) induce temperature- and salinity-resilience; 2) evaluate *ectABC* transformation efficiency; and 3) assess the growth, nutritional requirements, biochemical profile and ectoine expression of *ectABC*-transformed vs WT and empty vector-transformed *Synechococcus elongatus* PCC7942 controls in response to temperature- and salinity-stress.

Accordingly, my thesis contains the following five chapters:

Chapter 1 introduces the reader to the study as a whole, detailing the purpose of the research and the thesis structure.

Chapter 2 mainly focuses on reviewing the literature and investigates the need to develop cyanobacterial GMOs and probable sources of osmolytes from extremo-halophilic bacteria that could be engineered into cyanobacteria to produce salt- and temperature-tolerant.

Chapter 3 details the *ectABC* gene cassette design, codon optimization, plasmid construction, and transformation of *ectABC* into S. *elongatus* PCC7942, recombinant selection, confirmation of *ectABC* integration and a pilot study to assess temperature- and salinity-tolerance of selected *S. elongatus* PCC7942 transformants vs WT and empty vector controls.

Chapter 4 mainly focuses on two key areas: 1) the evaluation of cyanobacterial biomass and production levels of the bio-products of the *ectABC* transformed *versus* empty vector and WT controls of *S. elongatus* PCC7942; and 2) characterizing the nutritional requirements and biochemical profile (lipids, fatty acids and ectoine) of *ectABC*-transformed versus empty vector and WT controls of *S. elongatus* PCC7942.

Chapter 5 proposes a framework for an integrated bio-economic model whereby a facility for developing low-value, high-volume bio-products such as feed, fertiliser and biofuel from *ectABC*-transformed *S. elongatus* PCC7942 could be co-located with a sugar mill facility within the Great Barrier Reef catchment region of Far North Queensland, Australia. An indicative model was developed to estimate the financial viability of such a co-located facility, finding that such a development can indeed be financially viable, subject to a number of specified underpinning assumptions, whilst also offering environmental benefits to the region.

Chapter 6 provides a general discussion, limitations of this PhD research, future research direction and concluding remarks

CHAPTER TWO

AN OVERVIEW OF LITERATURE

2 Factors affecting microalgal biomass production and economic feasibility with an emphasis on tolerance to the stressors: temperature and salinity

2.1 Introduction

Microalgae and cyanobacteria are aquatic organisms capable of converting waste resources into valuable bio products (Collotta et al., 2018). Although they are a promising resource for renewable bio-energy production, the nascent microalgal industry is still facing challenges for sustainable biomass production (Heimann et al., 2015a). They can be cultivated in open ponds which are ideally suited for low-energy cultivation in remote communities, because the biomass produced fits into established and low-cost production (Heimann et al., 2015a). In open pond systems, algae are grown in shallow water and a moving paddle wheel produces sufficient flow to keep the organisms suspended and well mixed (Dalgleish, 2017). When compared to open pond systems, closed bioreactor systems are placed either indoors or outdoors, with the first option enabling cultivation under controlled environmental conditions (Dalgleish, 2017). The closed bioreactor system design is expensive and photosynthetic activity of the culture

leads to high oxygen saturation of the water in the solar compartment, leading to inhibition of photosynthesis, thereby limiting biomass productivity (Sousa, 2013). Therefore, open pond systems appear to be the only feasible option for industrialists or researchers who wish to take advantage of Australia for microalgal biomass production (Li et al., 2012). However, open pond systems are characterised by large water surface area and cultivation space requirements (Chanakya et al., 2012; Heimann et al., 2015a). The main identified challenges for such cultivation are: climate-induced temperature changes, contamination susceptibility (Heimann et al., 2015a), water use inefficiency (Płaczek et al., 2017) and salinity (primarily through evaporative water loss) (Heimann et al., 2015a). These hurdles need to be overcome along with site-selection and species-selection for low-value and high-volume bio-products such as biofuels, bio-fertilisers and animal feed for economically feasible development.

2.2 Critical factors affecting large-scale microalgal cultivation

Cheap cultivation systems, availability of freshwater, saline or wastewater close to production facilities and species that grow across a

wide range of temperatures and salinities are all required criteria for microalgal mass cultivation - the latter is crucial to limit freshwater use (Li et al., 2012). In Australia, commercial production of high-value pigments such as β-carotene from the halo-tolerant marine microalga *Dunaliella salina* is the only sustainable microalgal production (Li et al., 2012). In contrast, freshwater algae suitable for production of low-value, high-volume bio products such as feed, fuel and fertiliser are not sustainable (Borowitzka et al., 2012). This is mainly due to strain-tolerance limitations to elevated salinity - and temperature stress. To date, only two studies (Borowitzka, 2018; Fogg, 2001) reported on microalgal stress factors, the rest focused on system design and improvement to achieve cost-effective microalgal biomass production. This clearly identifies existing knowledge gaps, which need to be filled by investigating routes that infer both temperature- and salinity-tolerance for freshwater microalgal production, in order to realise the renewable energy promise. Knowledge of the details of stress-related gene expression and regulation, adopting genetic transformations to improve salinity- and temperature-resilience, evaluating biomass production and identifying potential sites for cultivating microalgal GMOs for feasible

commercial production will strongly advance implementing microalgae farming of low-value, high-volume bio-products that use organisms designed and optimised for this purpose. This literature review discusses the challenges and opportunities of co-locating microalgal production with other industrial facilities, species characteristics and genetic transformation strategies to improve salinity- and temperature-resilience with an emphasis on the requirement for public acceptance of microalgal GMOs.

2.3 History of microalgal mass cultivation and development in Australia.

Success in mass microalgae cultivation was first achieved in 1951 in Massachusetts (Borowitzka & Moheimani, 2013b). Since that time, the main biological and technical challenges for industrial cultivation of microalgae - particularly for high-volume, low-value product markets - are still largely unresolved and/or unimplemented at industrial scales. Microalgal mass cultivation techniques have remained essentially unchanged over the last 68 years, open pond cultivation being the only commercial cultivation technique presently implemented at industrial scales (Borowitzka & Moheimani, 2013a; Chen et al., 2010;

Raeisossadati et al., 2019). Currently, there are two microalgae pilot plants in Australia: (1) Cognis, a leading producer in the algal industry, cultivates the marine microalga *Dunaliella salina* for β-carotene production at Hutt lagoon in Western Australia and Whyalla in South Australia (Li et al., 2012). (2) Muradel Pty Ltd, a microalgal company established in 2010 as a joint venture between Murdoch University and Adelaide University, close to the Western Australian Rio Power station, for cultivating marine microalgal biofuel production in open ponds (Borowitzka & Moheimani, 2013a; Li et al., 2012). In order to encourage further development and to expand the nascent microalgal industries, microalgal research is being carried out by several Australian universities, government and private industries. The focus is on achieving multiple objectives, including i) to identify potential sites to co-locate microalgal biomass production with other industrial facilities (Borowitzka et al., 2012; Sedghamiz, 2017), ii) system design improvement (Berner et al., 2015; Heimann, 2016; Palma et al., 2017) and iii) harvesting and extracting the biomass to develop bio-products (Estime et al., 2017; Koley et al., 2017; Soomro et al., 2016). In reality,

climate extremes and resource bottlenecks are major constraints in large-scale biomass production.

2.4 Major resource bottlenecks in large-scale microalgal cultivation

2.4.1 Site selection and land availability

Site selection and land availability are prime factors for large-scale cultivation to ensure sustainable microalgal production (Olofsson, 2015). Appropriate topography, climate, proximity to water (whether freshwater, inland saline water, marine water, or wastewater) and nutrient supplies would have to be matched carefully to ensure successful and sustainable algal production, with the need to avoid/reduce the costs and energy consumption expended transporting those resources to cultivation facilities (NRC USA, 2013).The majority of Australia's land is considered arid or semi-arid. Australia is a land of extremes with temperatures ranging from highs of > 40°C in the central desert region and exceeding 30°C in densely populated areas (ABS, 2012). Although suitable microalgal cultivation sites can be found near densely populated urban or suburban centres or coastal recreation areas, production would only support a small-scale high-value microalgal industry, as the price of

land and land availability would be prohibitive for large-scale low-value production (Geoff L. Allan, 2008). It has been shown that solar energy derived from coastal areas could contribute 20% of Australia's total energy needs for the next 10 years (James, 2010). Theoretically, cultivation of microalgae in marine environments could hold promise for future generations of bioenergy (Olofsson, 2015) and low-value products particularly for aquaculture feeds, but various concerns need to be taken into account. Setting up such an industry in the ocean is logistically extremely difficult and would require anchorage and construction of the production platforms required for the harvesting / dewatering of large-scale systems. These structures would need to be weatherproofed (e.g. harnessed against damage by approaching cyclones etc.) and even if weatherproofed, serious damage to coral reefs and other aquatic ecosystems is a likely impact of such structures. In addition, shading of vast areas by these structures and subsequent interference with primary production in the ocean (along with the ecological flow-on effects via depletion of oxygen levels, disruption of food chains and therefore organism livelihoods at higher trophic levels) is at present unpredictable (Kachel, 2008). In addition, transportation needs and fuel consumption

during set up, maintenance and transport of the biomass produced is likely to be large - probably drawing a positive carbon balance that counteracts the intended sustainable and renewable (carbon-neutral) bio-product production goal (Klein-Marcuschamer et al., 2013). Realistically, considering land requirements for industrial-scale production of low-value, high-volume algal products (e.g. bio-fuel, bio-fertiliser, animal feed) in Australia leads to one solution: co-locating production facilities with other related industrial facilities to minimise resource utilisation and to share infrastructure facilities. In Australia, coal/gas-fired power stations are the largest industry sector and generate more than 70% of electricity, but they heavily contribute to greenhouse gas emissions (188 million tonnes in 2015) (Senate, 2016). Greenhouse gas abatement by microalgae has been heralded as the potential solution to the world's energy security, due to their photosynthetic activity (CO_2 assimilation) (Laurens et al., 2017). Hence, the establishment of large-scale microalgal production systems adjacent to a coal-fired power station offers advantages to remediate industry-released greenhouse gases in a carbon-constrained future. However, Australia's target to meet 26-28% reduction of total GHG emissions by

2030 based on 2005 levels requires further development of policies and robust modelling of abatement opportunities. (Greg Bourne & Brailsford., 2018). Aiming to meet the 2030 emissions reduction target, some of the Australian state and territory governments have taken a range of actions, implementing renewable energy production at large-scale through solar- and wind farms, hydro-plants and biomass-burning stations (e.g. sugar mills) (QREE, 2016) (**Table 2.1**).

Table 2.1. Renewable energy target plan by Australian states

Australian states	Target plan
Australian Capital Territory	100 % by 2020
South Australia	50% by 2025
Victoria	25 % and 40 % by 2020 and 2025
New South Wales	20 % by 2020
Queensland	50 % by 2030
Northern Territory	50% by 2030

Adapted from Queensland Renewable Energy Expert Panel Draft Report (QREE, 2016)

The Queensland Government has set a goal of achieving a 50 % renewable target by 2030 due to its prime goal to develop a strong renewable industry. In Queensland, renewable energy is currently mainly produced from biomass and hydro-power stations. While hydro-

power produces zero emissions, renewable energy production from biomass-burning does not help Australia to achieve the target plan, as it contributes to carbon emissions during the biomass-burning process, but they can also provide additional options to coal- or gas-fired power stations for microalgal biomass production in Queensland. However, opportunity to co-locate microalgal production with biomass-burning stations has not been well studied in Queensland, as the primary focus was microalgal biomass production at the then prevalent coal- and gas-fired power stations and aquaculture industries (**Table 2.2**). In Australia, Pacific Biotechnologies Ltd (formerly MBD) is a medium-scale algae-based company whose aim was to co-locate with coal-fired power plants in New South Wales, Victoria and Queensland (**Table 2.2**), using waste CO_2 as a cheap carbon source for large-scale microalgal biomass production (**Table 2.2**). However, most of these proposed ideas were not taken into further developments (see **Table 2.2**) after abolishment of the carbon tax by the Australian government in 2014. Pacific Biotechnologies Ltd is currently involved in recirculating aquaculture wastewater using settlement ponds from prawn farming for microalgae production for aquatic animal feed and astaxanthin at Ayr in North

Queensland (**Table 2.2**). In addition, Cubic QED Ltd conducted a feasibility study to construct a 100 ha algae-based wastewater treatment plant in New South Wales (**Table 2.2**). Algae Tec Ltd had a plan to use waste CO_2 released from an ethanol refinery in New South Wales (**Table 2.2**). Muradel constructed and operated a 1 ha algal pilot plant for two years adjacent to the Rio Tinto Yurralyi Maya power station in Karatha Western Australia, however the faciltity was shut down due to inefficient economic performance and moved to Whyalla, South Australia in 2014, where seawater for microalgal production is readily accessible. Recently, Muradel focused on the development of a technology called 'Green2Black' platform focused on biocrude oil production from solid wastes such as car tyres (**Table 2.2**). Another feasibility study by SARDI, CSIRO and Flinders University was undertaken to develop an algal plant adjacent to nearby Port Rivers and a coal-fired power station, to use nutrients and waste CO_2, respectively (**Table 2.2**). Many of these proposed ideas and some successful developments (**Table 2.2**) set examples to integrate or co-locate microalgal production facilities with other industries, providing opportunity to utilise waste CO_2, and nutrients, and whilst avoiding competition with agricultural land and

freshwater requirements. Yet, there are problems associated with open pond cultivation, particularly: water and nutrient availability in close proximity; microalgal strain-tolerance to increased salinities of wastewater; climate-induced and seasonal temperature changes – all of which are major obstacles to achieving economic performance and sustainable production.

Table 2.2. Past and current remarkable initiatives on integration and co-location of microalgal biomass production in Australia

Region	Algae Industry	Co-location	Significance of co-location	Outcomes	Reference
New South Wales	Pacific Biotechnologies Ltd (formerly MBD)	Eraring power plant	CO_2 sequestration	Proposed but not developed	(Borowitzka et al., 2012; Khan et al., 2017)
	Algae Tec Ltd	Manildra ethanol refinery	CO_2 sequestration	Scaled up sucessfully	(Li et al., 2012)
	Cubic QED Ltd	Mineral spa industry	Wastewater remediation	Proposed but not developed	(Li et al., 2012)
Victoria	Pacific Biotechnologies Ltd (formerly MBD)	Loy Yang power plant	CO_2 sequestration	Proposed but not developed	(Borowitzka et al., 2012; Khan et al., 2017)
	EnergyTransformed Flagship (CSIRO)	Melbourne water treatment plants	Waste water remediation	Scaled up successfuly for biofuel development	(Li et al., 2012)
Queensland	Pacific Biotechnologies Ltd (formerly MBD)	Stanwell power plant	CO_2 sequestration	Pilot plant constructed and demonstrated successfully	(Borowitzka et al., 2012; Khan et al., 2017)
	Pacific Biotechnologies Ltd (formerly MBD)	Pacific Reef Fisheries (currently known as Pacific Biotechnologies Ltd)	Recirculation of aquaculture waste water and nutrient remediation for clean water	Industry partners united their business under Pacific Biotechnologies Ltd and operation is ongoing	(Austrade, 2015)

Western Australia	Muradel	Rio Tinto Yurralyi Maya power station	CO_2 sequestration	Pilot plant operated for two years and was shut down due to not being profitable	(Borowitzka et al., 2012; Stockwell, 2013)
South Australia	Muradel	Seawater	Sea water from salt water storage plant	Industry changed their focus from algae to solid wastes for biocrude oil production	(Castello et al., 2018; Energy & Australia, 2015)
	Research collaboration SARDI, CSIRO and Flinders University	Torrens Island Port River and adjacent power plants	Nutrients in saline water and CO_2 sequestration	Funding was terminated by DRET due to delays in securing industry partner	(Li et al., 2012; Nayar, 2013)

2.4.2 Water availability and salinity

Water availability is an important criterion for successful, sustainable long-term, large-scale microalgae cultivation (Borowitzka & Moheimani, 2013b; von Alvensleben, 2015). Scarcity of freshwater resources requires that industries recirculate their wastewater for sustainable water supplies. In Australia, 20-30% (approximately 300,000 ML) of wastewater is recycled every year to meet freshwater demand (Ishika et al., 2017). In open pond microalgal biomass cultivation for bio-products such as biofuels, bio-fertilisers and even animal feed, enormous volumes of water are required in every aspect of their production; this is identified as one of the main challenges as even if water is partially recycled this adds significantly to production costs (Shilton et al., 2012). Consequently, industrial wastewater derived from sugar mills (Lohrey & Kochergin, 2012; Sedghamiz, 2017; Shashirekha et al., 2016) or aquaculture fish or prawn farming industreis (Nogueira et al., 2018; Tossavainen et al., 2018; Venkatesan et al., 2006) may provide wastewater for microalgae aquaculture production. In addition, ground water is abundant in Australia, with salinity of less than 5 ppt (von Alvensleben et al., 2015). There are also several major highly saline

groundwater areas in Australia with salinity ranging from 20 to 40 ppt, including the Murray River basin (Kolkovski, 2011). In addition, the use of saline wastewater streams for bioenergy production should ideally be mandatory to avoid competition for scarce freshwater resources (Ishika et al., 2017; Sing et al., 2014). This is of particular concern in temperate regions where evaporative water loss is higher than in tropical areas (Prosser, 2011; Sunbather, 2016). According to the Bureau of Meterology 2006, evaporation rates of 5% day^{-1} are common in Southeast Queensland during the summer season in December. Accordingly, von Alvensleben et al. (2015) cited that open pond cultivation containing 100,000 L of water, with a starting salinity of 5 ppt would increase to 7.5 and to 10 ppt in 10 and 20 days, respectively. Even when using sea water for cultivation, evaporation requires freshwater make-up water to maintain permissible salinity levels and to maximise algal biomass production (Ishika et al., 2017). Wastewater produced at different coal-fired power stations often range from freshwater (2 ppt) to seawater salinity (36 ppt) (von Alvensleben, 2015). In Queensland, coal seam gas (CSG) water production has, to date, averaged about 300 GL litres per annum (Underschultz et al., 2018).

Water produced from CSG has generally been underground for a long time, with very little freshwater penetration. As a result, CSG wastewater often contains salt, mainly sodium chloride and bicarbonate plus trace metals required for algal growth (Underschultz et al., 2018). For instance, a CSG water-based algal culture medium was developed for cultivating *Dunaliella tertiolecta* for lipid production (Aravinthan & Harrington, 2014). Co-location of algal farms coupled with sugarcane-processing industries could also provide an added advantage to improve water quality of wastewater generated during sugarcane-processing (Sedghamiz, 2017). For example, processing one tonne of sugarcane requires 1,500-2,000 L of freshwater and generates 1,000 L of wastewater (Sahu, 2018). The wastewater generated contains suspended solids, nitrogen, phosphates, calcium, chlorides, magnesium, oil and grease and can be pre-treated and filtered through settlement ponds, before being recirculated for microalgal cultivation (Lohrey & Kochergin, 2012; Sahu, 2018). Although co-location of microalgal production in saline wastewater offers significant advantages, water requirements for microalgal production are quite often species-specific. Most marine algae require salinities of greater than 18 ppt, while

freshwater microalgae often require less than 11 ppt (Heimann et al., 2015b). While it is important to make a wise decision on selecting species, the selection of species that can tolerate a wide range of salinities for industrial-scale cultivation is challenging. Therefore, it is essential to identify sites with sustainable water supplies, choose strains that have high biomass and bio-product productivities without large fertilisation requirements, and investigate routes to improve salinity- and temperature-tolerance of species for higher biomass production to broaden the suitable species range. Salinity- and temperature-tolerant engineered microalgae have been shown to have higher biomass productivities (Radakovits et al. 2010), providing an ideal opportunity for micoalgal production using saline groundwater resources and/or mine/coal seam gas waste waters in remote locations, overcoming two site-specific concerns (land availability and water resources). Several industrially important microalgal species and their salinity-tolerance are given in **Table 2.3**. An alternate, utilising waste CO_2 from high temperature gas streams from sugar mills, is reviewed in a broader context in the following section.

2.4.3 CO2 supply and temperature-tolerance

The concentration of atmospheric CO_2 is 0.04% in air (Smith et al., 2015). Large-scale cultivation of microalgae in open ponds aerated with atmospheric CO_2 has limited biomass productivity of ~3 g $m^{-2}d^{-1}$, which is less profitable (Benemann, 2013). As a result, most microalgal industries require external supply of pure CO_2 ($800 ton^{-1}) (Schenk, 2016) to achieve profitable biomass productivities >20 g $m^{-2}d^{-1}$ (Benemann, 2013; Fischetti, 2010; Heimann et al., 2013; Schenk et al., 2008). This leads to increasing costs for operation and production and thus often industry failure (Fischetti, 2010). In order to reduce the costs associated with large-scale microalgal biomass production, many microalgal industries aim to co-locate with CO_2 emitting industries (Borowitzka et al., 2012; Fischetti, 2010). Microalgae fix CO_2, i.e one tonne of algal biomass is formed from the assimilation of 1.83 tonnes of CO_2 (Guo et al., 2019; Heimann et al., 2015b). Electricity generation from coal/ gas power stations and biomass-burning sugar mills release waste flue gas, containing 12-15 % CO_2, which is ideal for algal cultivation (Ben-Amotz, 2011; Sedghamiz, 2017). Waste flue gas release has a temperature of ~120°C (Szulc & Tietze, 2017) and further cooling

brings it down to 40°C (Feron, 2016). This high temperature prevents use with a wide range of microalgal species, which possess a narrow optimal temperature range between 16-27°C (Joseph & Ajith Kumar, 2015). Therefore, cooling is necessary to reduce the coal/gas power station flue gas temperature to that suitable for reuse by microalgae. After initial cooling, this can be achieved by choosing locations further away from the flue gas producer (natural cooling, as was done at the Stanwell pilot plant, Queensland) or cooling through mixing with additional freshwater input, which could be managed by recycling power station generated wastewater. Similarly, if microalgae cultivation were co-located with sugar mills, the final flue gas temperature of the boiler is ~75°C and further cooling to 40°C requires sugar mill-generated wastewater, which is mostly fresh. In addition, bagasse burned at 800°C (Clark et al., 2017) generates steam, which is converted to electricity, which could be used to provide power for microalgal biomass production (Lohrey & Kochergin, 2012). While these scenarios avoid the costs of electricity and CO_2 purchase from a commercial supplier, algae are rarely tolerant to increased temperature (Barghbani et al., 2012; Gupta et al., 2017). Whilst biomass growth, yields and composition are

species-specific, growth rates and biochemical profiles are also strongly influenced by CO_2 and temperature (García et al., 2012; Huerlimann et al., 2010; Islam et al., 2013; Rai & Rajashekhar, 2014). Species selection for co-location of microalgal biomass production for CO_2 utilisation with sugar mills needs to be considered both from an industrial and economic perspective. Very few microalgae can grow in high CO_2 concentrations and tolerate temperatures above 40°C. An exception is the thermophilic eukaryotic microalga *Cyanidium caldarium* which can tolerate temperatures of >50°C and survive under 100% pure CO_2 concentrations. The thermophilic cyanobacterium *S. elongtaus* survives in hot springs, tolerating temperatures of up to 60°C and can grow under 60 % high CO_2 concentrations (Onai et al., 2004). Another mesophilic WT *S. elongatus* PCC 7942 survives in freshwater with an optimal temperature 35°C and can tolerate 60% CO_2 concentration (**Table 2.3**). Although the marine diatom *Phaeodactylum tricornutum* can tolerate 10-15% CO_2 (**Table 2.3**), carbon uptake by *P. tricornutum* was reported to be less efficient and therefore not suitable for mass cultivation co-located with waste CO_2 industrial facilities (Usher et al., 2014). A summary of the CO_2 and cultivation temperatures of industrially

important microalgal species, and their co-location potential identified based on their biomass-derived product outcome, is presented in **Table 2.3**.

2.4.4 Species Selection

In the context of co-locating microalgal biomass production with wastewater or related aquaculture industrial facilities, species selection becomes an important issue not only from an economic point of view, but also, and, perhaps equally importantly, from a product perspective. The biochemical profile of the produced biomass must match the product requirements (e.g. for nutritional value, fuel characteristics or carbon, nitrogen, phosphate content for bio-fertilisers). In general, cultivation of a single species has more challenges in open pond systems, mainly due to temperature and to contamination by invasive species such as protozoa, bacteria, and fungi that potentially reduce the product biomass (Ben-Amotz, 2008). The latter can be avoided if pre-treated or filtered saline wastewater is used. To ensure long-term sustainable industrial-scale production, it is important to choose strains or species that are resource-wise, which requires a complex series of selection criteria (Heimann et al., 2015a). For example, commercial

production of the marine microalga *Dunaliella salina* is a reality for the high-value, low-volume pigment market, e.g. β-carotene, which responds to changes in salinity through accumulation of the osmolyte glycerol (Borowitzka et al., 1984). High salinity- and temperature-tolerance in parallel for industrial co-location cultivation is low (marine algae do not withstand low salinities of ground water typically around 5-10 ppt), i.e. *D. salina* cultivation requires hyper-saline waters (30-300 ppt), conditions which also limit occurrence of contamination. Another very versatile organism is the euryhaline green microalga *Picochlorum atomus*, which tolerates salinities from 0 to >45 ppt and prefers higher temperatures, with high biomass productivity (**Table 2.3**) (von Alvensleben et al., 2013). The biochemical profile of *P. atomus* restricts its use to animal feed supplements or for use as a bio-fertiliser (von Alvensleben et al., 2013). The latter requires more investigation, as sustainability would depend on its own fertilisation requirements to attain high biomass productivity. While selecting microalgal species for industrial co-location, it is important to understand the nutritional and light requirements of target species (Heimann et al., 2015b). Nutritional requirements for microalgal production are often species-specific (Heimann et al., 2015b).

Provision of organic carbon in the form of acetate is typically restricted to the cultivation of freshwater species as it negatively affects growth of marine species (Heimann et al., 2015b). Although most microalgae are photosynthetic, three different nutritional strategies are represented amongst different strains such as autotrophy, heterotrophy and mixotrophy (Heimann et al., 2015b). Heterotrophic and mixotrophic cultures can be grown in saline wastewater as organic carbon is required (i.e. heterotrophic species are not photosynthetic and require acetate, glycerol or sugar addition for carbon assimilation while mixotrophic species are photosynthetic require low light but will utilise organic carbon to supplement requirements) (Perez-Garcia & Bashan, 2015). This has consequences for cultivation systems, where the absence of light will assist in cultivating heterotrophic species and low light conditions will favour mixotrophic species (Heimann et al., 2015a; Perez-Garcia & Bashan, 2015). Mixotrophic cultivation is very rarely considered for outdoor large-scale industrial environments (Lutzu, 2012). In contrast to this, autotrophic species require sufficient light and inorganic carbon supplementation (CO_2). Accordingly, huge differences in biomass productivity and biochemical content are evident amongst

different microalgal species. For example, lipid productivity of *Chlorella vulgaris* varies from 4 mg L^{-1} d^{-1} in autotrophic conditions whereas productivity was 54 mg L^{-1} d^{-1} in mixotrophic and 27 to 35 mg L^{-1} d^{-1} in heterotrophic conditions (Liang et al., 2009). Similarly, *Scenedesmus* sp., yielded higher lipid productivity in mixotrophic and heterotrophic conditions (Shen et al., 2018). However, supplying the nutrients in the form of either acetate or glucose will add additional cost in large-scale production. Therefore, it is worthwhile to consider co-location of photoautotrophic microalgal production with other industrial facilities to capture waste CO_2 as a cheap carbon source (Lutzu, 2012). Several microalgal species suitable for industrial co-location and their salinity, cultivation temperature and CO_2 tolerance, as well as product potential/realisation are given in **Table 2.3.** Although microalgal production can be co-located with coal/ gas-fired power stations or sugar mills for utilising the resources such as CO_2, wastewater and nutrients, it is equally important to locate end biomass using industries to co-locate with. This has implications in biomass distribution for several end use industries, and would reduce energy costs associated with transportation.

Table 2.3. Cultivation conditions of suitable microalgal species for industrial co-location

Character	Salinity tolerance [ppt]	Temperature tolerance [°C]	CO_2 tolerance [%]	Possible industries for co-location	Major industrial product or potential*	Reference
Haematococcus pluvialis	<10	25-27	16-34	Ash-dam wastewater	Astaxanthin	(Borowitzka et al., 1991; Solovchenko & Khozin-Goldberg, 2013; von Alvensleben et al., 2016)
Dunaliella salina	30-300	0-45	10-15	Seawater or other saline water	ß-carotene	*(Hosseini Tafreshi & Shariati, 2009; Preetha et al., 2012; Singh & Ahluwalia, 2013)*
Botryococcus braunii	-	20-30	10	Coal/ gasfired power station	Biofuel*	(Ansari et al., 2017; Kashyap et al., 2018; Li et al., 2012; Singh & Singh, 2015)
Chaetoceros sp.	7-35	10-30	30	Aquaculture	Aquatic feed	(Borowitzka et al., 2016; Lucas & Southgate, 2019)
Chlamydomonas	0-5	25	15	Brewery, sugarmill	Biofuel and feed additives	(Carr et al., 2018; Kong et al., 2010; Mata et al., 2014; Ono & Cuello, 2003; Scranton et al., 2015)
Chlorella	0-5	45	40	Aquaculture and Piggeries	Feed	(Ansari et al., 2017; Salih, 2011; Sergejevová & Masojídek, 2012)

Chlorococcum littorale	35	25	60	Dairy effluent or power station and sugarmills	Ethanol*	(Ansari et al., 2017; Solovchenko & Khozin-Goldberg, 2013; Ueno et al., 1998)
Cyanidium caldarium	-	57	Pure CO_2 (100%)	Coal-fired power station	High CO_2 absorber	(Ono & Cuello, 2003)
Thalassiosira pseudonana	-	10-20	-	Aquaculture	Aquatic feed	(Lucas & Southgate, 2019)
Thalassiosira weissflogii	0-35	-	10-20	Aquaculture	Feed	(Daniel, 2017; Ishida, 2000)
Skeletonema costatum	14-35	10-20	-	Aquaculture	Aquatic feed	(Lucas & Southgate, 2019)
Isochrysis sp.	7-35	15-30	-	Aquaculture	Aquatic feed	(Lucas & Southgate, 2019)
Pavlova salina	21-35	15-30	-	Aquaculture	Aquatic feed	(Lucas & Southgate, 2019)
Pavlova lutheri	7-35	10-25	-	Aquaculture	Aquatic feed	(Lucas & Southgate, 2019)
Tetraselmis sp.	7-35	10-30	14	Aquaculture	Aquatic feed	(Lucas & Southgate, 2019; Ono & Cuello, 2003)
Nannochloropsis sp.	7-35	10-30	15	Aquaculture	Aquatic feed	(Borowitzka et al., 2016; Lucas & Southgate, 2019; Ono & Cuello, 2003)
Scenedesmus strain K34		40	100	Aquaculture	High CO_2 absorber	(Varshney et al., 2015)

Scenedesmus obliquus		50	18	Aquaculture	Biodiesel*	(Mandal & Mallick, 2012; Varshney et al., 2015)
Synechococcus elongatus	0-5	30-34	60	Freshwater waste resources	Biodiesel*	(Almeida et al., 2017; Solovchenko & Khozin-Goldberg, 2013; Voshol, 2015)
Thermophilic *S. elongatus*	-	60	60	Coal-fired power station	High CO_2 absorber	(Miyairi, 1995; Ono & Cuello, 2003)
Phaeodactylum tricornutum	10-40	30	15	Saline wastewater resources	Aquaculture	(Borowitzka et al., 2016; Branco-Vieira et al., 2017; Okauchi & Tokuda, 2003)
Picochlorum atomus	36	24-35	-	Seawater or saline wastewater	Animal feed	(von Alvensleben et al., 2013)

2.5 The potential for cultivating better adapted microalgae

Almost all organisms have their own protective mechanism to cope with a range of salinity and temperature stresses. Undeniably, green algae are closely related to higher plants (Heimann et al., 2015b) and have similar responses to temperature and salinity stress (Mendez-Alvarez et al., 1999; Yoshida et al., 2003). However, while these stress mechanisms are well studied in higher plants, they are poorly understood or not clearly explained in relation to the biology of algae (Borowitzka, 2018). To date, only two studies have attempted to define and explain stress concepts in algae. The first attempt to examine stress in algae was undertaken by Fogg (Fogg, 2001) and the second study was published recently by Borowitzka (Borowitzka, 2018). Different organisms have different mechanisms to maintain their water balance under extreme salinity- and temperature-conditions. For example, halophilic bacteria (halophiles) belonging to the *Halobacteriaceae* family are well known for their extreme salinity- and temperature-tolerances (Saum & Müller, 2008). These halophilic bacteria follow two different strategies to maintain cell volume. The first strategy involves accumulation of K^+ and other inorganic ions to cope with salinity- and

temperature-stress. This is known as the ‘salt in strategy’ (Ma et al., 2010). The second strategy involves synthesis of osmolytes to reduce the osmotic gradient between the outside environment and the cellular interior (Ma et al., 2010). These osmolytes are compatible solutes that are not harmful to the organism, even if accumulated in high concentration and do not interfere with cellular metabolism (Slama et al., 2015). All other organisms, including algae, cyanobacteria and most higher plants follow a compatible solute strategy, which requires 100-times more energy than the “salt in” strategy of some halophiles (Rai & Gaur, 2012). Although osmolytes are widely distributed among all organisms, this does not guarantee salinity- or temperature-tolerance. In addition, accumulation of osmolytes by bacteria, algae and higher plants in freshwater environments has a lesser effect than for species living in halophilic environments (Brown, 1978). For example, glycerol as an osmolyte was found in microalgae inhabiting both freshwater (*Chlamydomonas reinhardtii*) and marine (*Dunaliella* species) environments (Brown, 1978). However, freshwater *Chlamydomonas* species are less salinity tolerant than the marine *Dunaliella* species (30-300 ppt) (Keerthi et al., 2015). Therefore, many freshwater organisms,

including bacteria, archaea and higher plants, are genetically engineered to improve their stress tolerance by importing osmolyte synthesis pathways from halophiles (Erdmann & Hagemann, 2001). This provides a strategy to breed microalgae or cyanobacteria for salinity- and temperature-resilience (Hanin et al., 2016).

2.5.1 Compatible solutes

Compatible solutes are very small organic molecules dissolved in water (Chaudhuri et al., 2017). Compatible solutes accumulate in high concentrations, which raises the internal osmolalities of cells, thereby maintaining cellular turgor pressure and volume. Compatible solutes play a vital role in maintaining biomolecules of the cell (Chaudhuri et al., 2017). They act as molecular chaperones to prevent protein misfolding and protein aggregation, maintain enzyme and cell structure integrities, protect photosynthetic rates against heat stress, all of which leads to increased biomass productivities, over production of recombinant proteins (in GMOs only) and maintenance of membrane fluidity, thereby avoiding dehydration of cells (Chaudhuri et al., 2017). These compatible solutes are classified into five major categories based on their chemical

nature: amino acids, sugars, sugar alcohols, methylamines and quaternary amines.

Many organisms (including bacteria and higher plants) accumulate proline as an osmolyte. Proline biosynthesis regulates general protein synthesis in higher plants and was first identified in *E. coli* 45 years ago (Hayat et al., 2012). Several reports suggest that proline accumulation is a protective mechanism against salinity-, drought- and cold-stress in higher plants but is not activated by heat stress (Fogg, 2001). However, proline accumulation in *Arabidopsis thaliana* was often found too low to play a vital role in osmoprotection (Ghars et al., 2008; Liu & Zhu, 1997; Slama et al., 2015) . Accordingly, proline biosynthesis gene was engineered into *Arabidopsis* and tobacco to reduce salinity-stress and improve drought-resistance, respectively (Erdmann & Hagemann, 2001; Székely et al., 2008). Similarly, in *Dunaliella*, over-expression of proline increased tolerance against cold-stress (Fogg, 2001; Rai & Gaur, 2012)

Ectoine (1,4,5,6,-tetrahydro-2-methyl-4-pyrimidine carboxylic acid) is an osmolyte produced by the extremophilic halo-tolerant bacterium *Halomonas elongata* (**Figure 2.1**), confering tolerance to high temperatures and salinities by acting as a compatible solute (Saum &

Müller, 2008). Ectoine also protects proteins, DNA and RNA through providing sufficient hydration water (Chaudhuri et al., 2017).

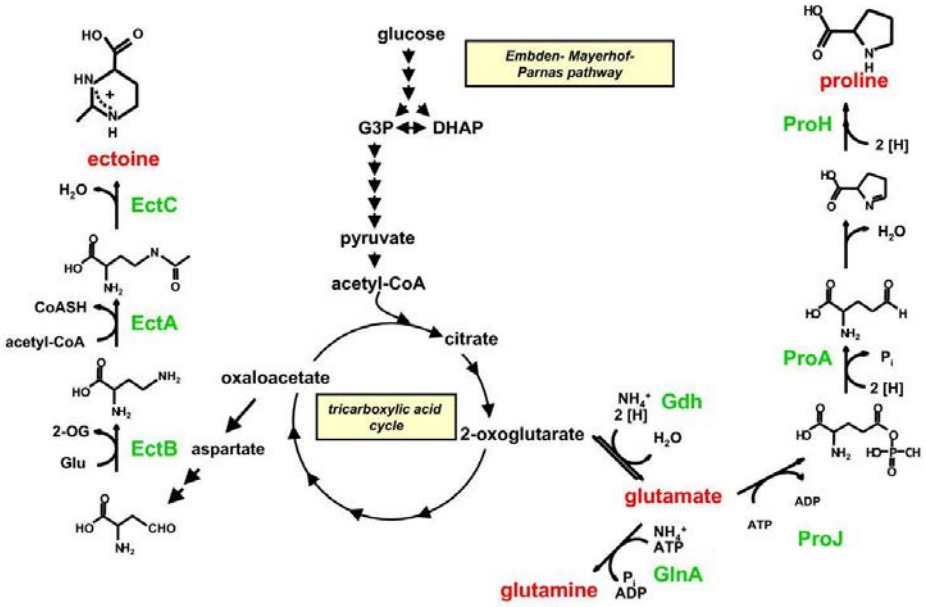


Figure 2.1. *ectA*, *ectB* and *ectC* mediated ectoine biosynthesis in halophilic bacteria (Saum & Müller, 2008)

Another osmolyte, betaine, was reported as abundant in sugar beet in 1860 (Sadler, 2014). It is also present in higher plants, including most crop plants and accumulates under soil salinity and water deprivation (Herrmann & Obeid, 2011). Betaine is also widely distributed in animals and microorganisms, generally acting as an osmolyte. It is used as a dietary supplement advocated to enhance general protein synthesis and improve human body composition by regulating homocysteine metabolism (Cholewa et al., 2013; Olthof et

al., 2005). Betaine was also reported to increase animal resilience to heat stress (Cronje, 2005). It is highly soluble in water and stable at high temperatures (e.g. when oven-baked) (Chaudhuri et al., 2017).

In general, biosynthesis of betaine is a two-step oxidation reaction beginning with choline in the cytoplasm, where it is oxidized into betaine aldehyde, which is further oxidized to yield glycine betaine (Chaudhuri et al., 2017). It is found in the chloroplasts of higher plants and accumulation of glycine betaine increases stress-tolerance against various abiotic stresses in plants, such as drought and salinity (Hussain Wani et al., 2013). It is known to protect proteins and enzyme activities under water-deficient conditions. In microalgae and cyanobacteria glycine betaine increases salinity-tolerance (Chaudhuri et al., 2017).

Another osmolyte, trehalose is a non-reducing sugar found in many bacteria, yeast, fungi and some heat-tolerant plants (resurrection plants) (Fernandez et al., 2010). Trehalose is a disaccharide that has a protective role under abiotic stress (Fernandez et al., 2010). Biosynthesis of trehalose has been recognized in yeast for protection against heat stress (Gibney et al., 2015). Apart from osmoprotection, trehalose has many applications in medicine and science (Jain & Roy,

2009). Moreover, it serves as a carbon source and is a compatible solute in many halophiles and cyanobacteria (Welsh, 2000). Trehalose also plays a vital role in fungal virulence in *Candida albicans*, *Fusarium graminearum*, *Cryptococcus neoformans* and is a main component of insect hemolymphs (Chaudhuri et al., 2017). Trehalose overproduction enhances photosynthetic activity and increases carbohydrate levels which may be advantageous for large-scale microalgal cultivation (Chaudhuri, 2017 #27; Thammahong, 2017 #365). Polyols also function as osmoprotectants, due to the presence of a hydroxyl group which allows them to form hydration shells around macromolecules and thus prevent inactivation under low osmotic potential (Williamson et al., 2002). For example, the well-known osmolyte glycerol was found in a commercially important marine microalga, *Dunaliella salina* in response to salinity changes (30-300 ppt) (Keerthi et al., 2015). Another interesting osmolyte and major photosynthetic product, mannitol, is widely distributed in higher plants and algae (Patel & Williamson, 2016; Ymele-Leki et al., 2013). Sorbitol, also an osmolyte, is synthesized from glucose and mainly found in higher plants (McQueen & Minchin, 2005). Sucrose, also a sugar osmolyte, is mainly found in the cyanobacteria

Synechosystis sp. and *Synechococcus* species (Kirsch et al., 2018; Lunn, 2002). Sucrose accumulation in cyanobacteria is a great starting material for bioethanol production (Möllers et al., 2014). **Table 2.4** summarises the biological significance of naturally synthesized compatible solutes and osmolytes in different organisms.

Table 2.4 Naturally synthesised osmolytes and their biological significance

Classification	Osmolytes	Biological significance	Source of organism	Reference
Amino acids	Proline	Salt-, drought- and cold-resistance	*Escherichia coli* and *Arabidopsis thaliana*	(Liang et al., 2013)
	Glycine	Maintains osmoregulation at shallow depths	Mollusks and crustaceans	(Chaudhuri et al., 2017)
	Ectoine	High temperature- and salinity-tolerance, increases productivity, Can serve as a nitrogen source under nitrogen limitation	*Halomonas elongata* DSM 2581T	(Schwibbert et al., 2011)
	Hypotaurine	Sulfide detoxification in animals present in hydrothermal vents	Invertebrates	(Chaudhuri et al., 2017)
	Glutamate		*Salmonella typhimurium* and *E. coli*	(Chaudhuri et al., 2017)
Methylamines	Betaine	Protecting subcellular structure, transcription and translational machineries, refolding of molecular chaperones	Higher plants, animals and bacteria	(Hussain Wani et al., 2013)
	Choline oxidase	Salt-stress	*Arthrobacter globiformis*	(Hussain Wani et al., 2013)
Quaternary Amines	Glycine betaine	Maintains membrane integrity, salt-, cold-, heat- and freezing-resilient	Higher plants and *E. coli*	(Sakamoto & Murata, 2002)
	Proline betaine	Salt-stress	Alfalfa plants, *Sinorhizobium meliloti*	(Alloing et al., 2006; Trinchant et al., 2004)

	Alanine betaine	Facilitating oxygen use during hypoxia conditions	Plants in salt marshes	(Chaudhuri et al., 2017)
	Glutamate betaine	Salt-stress	Bacteria and cyanobacteria (*Calothrix* spp.)	(Javor, 2012; Oren, 1998; Viña, 2002)
Polyols	Glycerol	Salt-resistance	*Saccharomyces cerevisae; Dunaliella* sp.,	(Chaudhuri et al., 2017; Oren, 2017)
	Sorbitol	Protection from hypertonicity, freezing	Renal cells and plants (Plantaginaceae family)	(Chaudhuri et al., 2017)
	Mannitol	Free radicals scavengers	Yeast and fungi	(Chaudhuri et al., 2017)
Sugars	Glucose	Protection from heavy metal-stress, freeze-tolerant	Plants and amphibians	(Gupta et al., 2015; Storey & Storey, 2017)
	Sucrose	Protection from salt- and heavy metal-stress	Tomato and other plants	(Gupta et al., 2015; Singh & Dar, 2017)
	Trehalose	Protection from hypoxia and carbon source in high osmolarity medium	*Drosophila* and *E. coli*	(Chaudhuri et al., 2017; Singh & Dar, 2017)
	Mannose	Salt-tolerance	Mangroves and celery plants	(Chaudhuri et al., 2017; Kim, 2016)
	Raffinose	Membrane protection against several stresses.	Higher plants	(Jenks & Hasegawa, 2008)
	Xylose	Heat-stress	Higher plants	(Chang, 2003)

2.5.1 Compatible solute selection for genetic engineering

Although osmolytes are widely distributed and accumulate under stress conditions in all living organisms, this does not guarantee salinity- or temperature-tolerance (Chaudhuri et al., 2017). Therefore, many freshwater organisms including bacteria, archaea and higher plants are genetically engineered to improve their stress-tolerance by imparting an osmolyte synthesis pathway into an organism (**Table 2.5**). Recent progress in genetic engineering enhanced the level of stress-tolerance in several transgenic organisms (Fogg, 2001). For example, the *p5CS* gene, responsible for biosynthesis of proline, was engineered into tobacco and *Arabidopsis* for salinity- and drought-resistance, respectively (Erdmann & Hagemann, 2001). Likewise, choline dehydrogenase, choline oxidase and betaine aldehyde dehydrogenase were engineered into tobacco and canola for glycine betaine production (**Table 2.5**)**.** Similarly, other osmolytes such as mannitol, sorbitol and polyamines were engineered for improved stress-tolerance (**Table 2.5**). Selection of an osmolyte for metabolic engineering of microalgae or cyanobacteria for improved stress-tolerance have not been extensively studied before, except Fogg (2001), and thus critical review is required.

Not all osmolytes are considered for genetic manipulation. Several studies reported that proline concentration increases in response to increased salinity in bacteria, cyanobacteria, protozoa, marine invertebrates, algae and higher plants (Chaudhuri et al., 2017; Fogg, 2001; Hayat et al., 2012; Saum & Müller, 2008). However, proline is considered a weak osmolyte in stabilizing protein folding during salinity-stress, but its accumulation is often reported as a consequence of damage due to salinity stress rather than as playing a vital role in improving salinity stress-tolerance (Atkinson, 2005). Therefore, proline cannot be considered for genetic engineering of microalgae or cyanobacteria, aiming to improve their salinity- and temperature-tolerance. Another osmolyte, glycerol, occurs in the industrially important marine halophilic microalga, *Dunaliella salina* (Borowitzka et al., 1984; Borowitzka, 2018). Glycerol may be an osmolyte suitable for genetic engineering of eukaryotic microalgae but not for cyanobacteria as glycerol usage was mainly restricted to the domain Eukarya (Fogg, 2001; Oren, 1999). Very few green microalgae and some yeasts are known for glycerol synthesis (Borowitzka et al., 1984; Brown, 1978; Klein et al., 2017). Although glycerol synthesis is the least energy

demanding osmolyte (requiring 30 ATP per glycerol molecule), glycerol accumulation may not be possible for bacteria, archaea or other prokaryotes, as their cell membranes are highly permeable whereas halophilic marine microalgae like *Dunaliella* posess specially adapted membrane structures, characterised by lower permeability, retaining glycerol intracellularly and thus improving salinity-tolerance (Fogg, 2001; Oren, 1999; Rai & Gaur, 2012).

In addition to glycerol, other polyhydric alcohols, such as sorbitol and mannitol, are also compatible solutes present in algae, higher plants and fungi, but not in cyanobacteria and bacteria, and their synthesis requires intermediate energy (57 ATP equivalents per molecule) (Fogg, 2001; Rai & Gaur, 2012). Accumulation of sugar molecules requires the most energy (109 ATP equivalents per molecule) and confers the lowest tolerance to salinity (Fogg, 2001; Rai & Gaur, 2012). Therefore, over-production of sugar molecules cannot be exploited for improving salinity- and temperature-resilience. The cheapest energy demanding osmolyte, ectoine (requiring 50 ATP equivalents per molecule) can be engineered into microalgae or cyanobacteria, as it is naturally found in salinity- and temperature-extremophilic organisms (Fogg, 2001; Rai &

Gaur, 2012). Several osmolytes listed in **Table 2.5** were transfected into higher plants or other microorganisms, resulting in increased tolerance to high salinity and temperature, but this has not been exploited in algae or cyanobacteria. Glycine betaine naturally occurs in *Synechococcus elongatus* PCC7942 and can be introduced into other cyanobacteria (Erdmann & Hagemann, 2001). Choline oxidase also increased salinity-tolerance in *Synechoccous* sp. (Deshnium et al., 1995). While plants and some algae appear quite similar on first sight, technology used for terrestrial crops and related species for increased temperature- and salinity-resistance has not been extrapolated to algae (Erdmann & Hagemann, 2001). Ectoine, an osmolyte in the extremophilic bacterium *Halomonas elongata* DSM2581T, was successfully introduced into tobacco and tomato and can be applied to other microalgae and cyanobacteria (Moghaieb et al., 2006; Moghaieb et al., 2011).

Table 2.5. Osmolyte pathways genetically engineered into organisms for enhanced temperature- and salinity-resilience

Osmolytes	Gene	Target organism	Stress improvement	Reference
Proline	*p5CS*	Tobacco Tobacco Petunia	Salt	(Erdmann & Hagemann, 2001)
	p5CS1	*Arabidopsis*	Salt	(Székely et al., 2008)
	Pyrroline-5-carboxy late synthetase	*Oryza sativa*	Drought	(Erdmann & Hagemann, 2001)
	Atprodh Antisense-proline DH	*Arabidopsis thaliana*	Cold	(Erdmann & Hagemann, 2001)
Ectoine	*ectABC*	Tomato Tobacco *Chlamydomonas reinhardtii*	Salinity	(Moghaieb et al., 2006; Moghaieb et al., 2011; Mwansa, 2017)
	ectA	*Chlamydomonas reinhardtii*	Salt	(Lunde, 2012)
	ectB	*Chlamydomonas reinhardtii*	salt	(Afzal, 2014)
	ectC	*Chlamydomonas reinhardtii*	salt	(Holck, 2014)
Glycine betaine	*CDH+ BADH*	Tobacco	salt	(Holmström et al., 2000)
	CodA	*Arabidopsis*, Rice	Salt	(Erdmann & Hagemann, 2001)
	COX	Canola, tobacco	salt	(Huang, 2016)
	GSMT+DMT	Jatropha	salt	(Tsuchimoto et al., 2012)
	BvCMO	Tobacco	salt	(Zhang et al., 2008)
	BADH	Tobacco	Salt	(Zhou et al., 2008)
Mannitol	*mtlD*	*Populus tomentosa*,	Salt	(Hu et al., 2005; Karakas et al.,

		Tobacco *Arabidopsis*		1997; Thomas et al., 1995)
Sorbitol	*stpdI*	Tobacco	salt	(Salie, 1998)
Trehalose	*tpsI, otsA (+ otsB)*	Tobacco	salt	(Tamminen et al., 2002)
	Tre-P synthase (+ Tre-P phosphatase)	Potato	ROS	(Erdmann & Hagemann, 2001)

2.6 Problems with genetic engineering of microalgae

Recent advances in molecular cell biology have enabled researchers to produce genetically modified plants, animals and human embryos for abiotic stress resilience, drug resistance and to cure congenital disease, respectively (Hasanuzzaman et al., 2019; Teti & Teitelbaum, 2019). Quite a few studies have demonstrated the genetic modification microalgae. For example, stable nuclear transformation of *Phaeodactylum tricornutum* (Apt et al., 1996; Niu et al., 2012; Zaslavskaia et al., 2000; Zhang & Hu, 2014) and *Chaetoceros gracilis* (Ifuku et al., 2015). Qin et al. (2012) detailed gene selection methods for vector construction and transformation of marine microalgae. Other recent studies (Doron et al., 2016; Huang & Daboussi, 2017) provided information on appropriate genetic engineering tools and selectable markers for foreign gene expression in microalgae. Yet, genetic engineering of algae and cyanobacteria still lags in knowledge and know-how compared to mammalian, plant and yeast genetic enginieering tools, because of the chemical diversity of their rigid cell walls, making them difficult to penetrate using a single transformation approach (Chen et al., 2019). This hurdle has been overcome in higher

plants, but the problem remains unsolved in microalgal transformations (Jeon et al., 2017). This is mainly due to morphological, structural and physiological differences between species (Jeon et al., 2017). For instance, the eukaryotic microalga *Thalassiosira pseudonana* possesses a cell wall composed of silica (known as frustule), which is not the case in higher plants and cyanobacteria (Popper & Tuohy, 2010). The frustule also contains many pores offering an advantage for inserting DNA into the host cell, but it requires a multistep, time-consuming protocol (Poulsen et al., 2006; Poulsen & Kröger, 2005) .

Limitations yet to be overcome include availability of complete genome sequence information; molecular tools such as species-specific primers; bias in codon usage; suitable vectors to carry the gene of interest; protocols for transformation; selectable markers and appropriate promoter genes for the chosen organism (Hallmann, 2007; Young & Purton, 2016). Further problems include long delays for existing policy reviews; developing risk management plans for obtaining approval from federal governments; negotiating with local bodies and adjacent landowners to release GMOs into the environment if successful and being approved in the laboratory environment. These

serious algal genetic engineering hindrances are reviewed in the following sections.

2.6.1 Variable interspecific characteristics in microalgal genetic engineering

2.6.1.1 Codon usage bias

In the early 1980's, there was a trend that synonymous codons should not appear equally or randomly in the sequence of any particular protein (Salim & Cavalcanti, 2008). However, some codons are repeatedly used in preference to others in some organisms, termed codon bias (Behura & Severson, 2013). Bias in codon usage is important criterion to consider in heterologous transformation, because expression of a gene construct may not be compromised, despite possessing all regulatory elements required for transcription and translation (Doron et al., 2016; Hallmann, 2007). This is mainly due to variability of codon frequencies in source and target organisms, with rare codons in the target organism displaying low translation rates compared to the source organism that has an abundance of the rare codons (Wang et al., 2012). To avoid this problem, careful selection of desired genes and target organisms should aim for a similar codon

usage to that of source organisms. Another way to overcome this issue is to optimize codons of the desired gene sequence, effectively replacing uncommon codons in the target organism without changing the translation of the specific amino acids originally encoded by the source organism's gene sequence. (Ng et al., 2017). Among the 20 amino acids encoded, most are coded by multiple codons, except the amino acids methionine (AUG-start codon) and tryptophan (TGG) (Pathak et al., 2017).

Foreign gene expression in microalgae was greatly affected by codon usage of an organism (Urtubia et al., 2016). For example, expression of the green fluorescent protein (GFP) is greatly affected by codon bias in *Chlamydomonas reinhardtii (Urtubia et al., 2016)*. Consequently, codon optimization was for the luciferase gene for genetic engineering into the green alga *Gonium pectoral*, yielding improved gene expression (Lerche & Hallmann, 2009). Similarly, transformation of the red seaweed *Porphyra yezoensis* with a codon-optimized β-glucuronidase gene improved gene expression (Royer, 2017; Uji et al., 2014). In cyanobacteria, codon usage is well studied in *Synechcocystis* 6803 for heterologous gene transfer of the *kudzu IspS*

gene (Lindberg et al., 2010). This study revealed that transfection with the codon-optimized *kudzu IsPs* gene resulted in 10-fold higher expression compared to the native gene under same promoter (Lindberg et al., 2010; Wang et al., 2012). In addition, it was reported that codon usage was greatly influenced by the composition of G+C content in genomes or genes. Variation of GC content ranges from 25 to 75 % in total genomes and 7 to 95 % in genes (Pathak et al., 2017). It is concluded that GC content is the driving force for codon usage and the connection between GC content, amino acid composition and codon usage can be modified by mutation and natural selection (Li et al., 2015). Several studies concluded that most of the extremo-halophilic organisms have increased GC contents (60-70%) in their genome (Siddiqui & Thomas, 2008), which appears to be correlated with extreme temperature tolerance (Siddiqui & Thomas, 2008). Interestingly, another major finding concluded that GC variation-induced changes in amino acid composition (codon redundancy) is mainly due to the third nucleotide position of the codons, whereas the first and second nucleotides have a lesser effect on codon usage variation (Li et al., 2015). Therefore, the importance of codon

optimization in algal genetic engineering is undeniable. A codon usage database (http://www.kazusa.or.jp/codon/) constructed by Kazusa DNA Research Institute, Japan, contains codon usage information for 35,799 organisms. Recently, a team of researchers from The George Washington University also constructed and published a codon usage database (hive.biochemistry.gwu.edu/review/codon) organized to contain Genbank sequence information for 665,044 species (Athey et al., 2017). Widely used measures that quantitate codon bias are CAI (codon adaptation index), ENC (effective number of codons), relative synonymous codon usage (RSCU) and adaptation index (Helliot & Mortain-Bertrand) (Athey et al., 2017). The CAI measures range from 0 to 1, where a CAI value of 0 reveals that all codons are used equally and indicates an absence of codon bias. In contrast, the largest CAI value (1) suggests complete codon usage bias in the source organism compared to the target organism (Sharp & Li, 1987). ENC values range from 61 to 20, where 20 indicates high codon bias and lower codon usage bias for 61 (Uddin; Wright, 1990). Several online software tools are freely available to optimize the codon usage of a target gene (**Table 2.6**).

Table 2.6. Codon optimization tools to evaluate and optimize DNA sequences of genes for transfection of algae and cyanobacteria

Optimization tool	Advantages	Disadvantages	Reference
Codon optimization online (Chin et al.) http://cool.syncti.org/	Minimise or maximise of CAI and GC, multiple optimization criteria can be selected and Pareto plot (with colour).	Licensing required for commercial users	(Chin et al., 2014)
JCAT http://www.jcat.de	CAI, GC maximize only, Open source software	Single criteria optimization output	(Chin et al., 2014; Grote et al., 2005)
Synthetic gene designer http://userpages.umbc.edu/~wug1/codon/sgd/	CAI, GC maximize only	No GC content measurement and Pareto plot containing codon frequency	(Chin et al., 2014; Wu et al., 2006)
Optimizer http://genomes.urv.es/OPTIMIZER/	Maximise or minimize CAI only	Not suitable for multi objective optimization	(Chin et al., 2014; Puigbo et al., 2007)
Eugene http://bioinformatics.ua.pt/eugene/	Minimise or maximise of CAI and GC, multiple optimization criteria can be selected	Pareto plot (without colour)	(Chin et al., 2014; Gaspar et al., 2012)
Gene designer http://www.dna20.com/resources/genedesigner	Open source software, multi-objective orientation, maximizing individual codon usage	No measurement for CAI and GC	(Chin et al., 2014; Villalobos et al., 2006)
Genscript rare codon analysis tool https://www.genscript.com/tools/rare-codon-analysis	Open source software	Optimization restricted to a limited range of organisms	https://molbiol-tools.ca/Translation.htm

2.6.1.2 Limitations of plasmid vectors

Plasmid vectors are an important tool for transfer of the foreign DNA into the targeted organism (Urtubia et al., 2016). They can be of two types: i) integrative vectors which alter the genome of the targeted organisms by recombination or transposition; and ii) Shuttle vectors, which are self-replicating plasmids capable of expressing genes independent of the host genome (Urtubia et al., 2016). In microalgae and cyanobacteria, few cloning vectors are available. Constructing microalgal species-specific cloning vectors is often laborious and time-consuming (Bañuelos-Hernández et al., 2017). In addition, eukaryotic microalgal vector construction often encounters problems with complex post-translational modifications and recombinant protein secretion (Bañuelos-Hernández et al., 2017). Very recently, significant efforts have been made to develop a customized microalgal species-specific vector for *Thalassiosira pseudonana* and *Phaeodactylum tricornutum* using *CRISPR/Cas9* genome editing (Hopes et al., 2016; Stukenberg et al., 2018). Alternately, a recent study recommended viral-based vectors for microalgal genetic engineering (Bañuelos-Hernández et al., 2017). In cyanobacteria, another recent study established an online

bioinformatics tool to assemble cyanobacterial plasmids (Taton et al., 2014). An alternative approach, which we would recommend, are to use commercially available vectors for algal or cyanobacterial genetic engineering. To date, each group (microalgae and cyanobacteria) has only one choice of vector available in the market. For eukaryotic microalgae, Chlamy_4 for chloroplast-targeted transformation in *C. reinharditii* and the *pSyn* family vectors specific for the cyanobacterium *Synechococcus elongatus* PCC7942.

2.6.1.3 Physiological constraints and transformation protocols

The structure of the cell wall of organisms is a major hurdle for algal genetic engineering. For example, diatoms like *Phaeodactylum tricornutum* and *Thalassiosira pseudonana* possess silicified cell walls, which are harder to break during transformation (Hallmann, 2007). However, natural transformation has been recently described for the diatoms *Phaeodactylum tricornutum* and *Thalassiosira pseudonana*, in which, episomal vectors were inserted by conjugation with *E. coli* (Karas et al., 2015). Genetic transformation of filamentous cyanobacteria is more complicated and often the desired gene can integrate with the filaments, but not with the cells (Urtubia et al., 2016).

In addition, screening and selection of recombinants that consist of mixed wild-type and mutant filaments, results in the inability to establish and report what the transformation efficiency of colony forming units are (Urtubia et al., 2016). Other problems like cell division and differentiation mechanisms may differ between filaments within the organisms (Urtubia et al., 2016). Some strains of cyanobacteria have very few copy numbers of their chromosomes, which challenges recombinant screening and strain selection (Urtubia et al., 2016). To date, only three different procedures - natural transformation, electroporation, and conjugation - are available for introducing foreign DNA into cyanobacteria (Urtubia et al., 2016). Success in cyanobacterial transformation is fully dependent on species and their physiological and biochemical barriers (Urtubia et al., 2016). For integrating foreign DNA into the genome of *Synechococcus elongatus* PCC7942, two neutral sites were advantageous for homologous recombination (Almeida et al., 2017).

The eukaryotic microalga *Chlamydomonas reinhardtii* is a widely used model organism for genetic transformation. However, nuclear transformation was inefficient and chloroplast transformation was an easier approach to deliver foreign DNA into *C. reinhardtii* cells (Muñoz

et al., 2018). The lack of transformation protocols for microalgal genetic engineering is another issue, which needs to be addressed by understanding the biochemistry of cellular function and by further investigating cell differentiation and compartmentalised cell functions in filamentous cyanobacteria (Flores & Herrero, 2010; Rehnstam-Holm & Godhe, 2003). To date, Biolistics has been a widely used approach that was very efficient for transforming eukaryotic microalgae and electroporation was used for cyanobacteria (Urtubia et al., 2016). Some microalgae were successfully transformed using *Agrobacterium tumefaciens*, conjugation, electroporation and methods involving glass beads and carbon whiskers (Muñoz et al., 2018). In the past few decades, several studies focused on a metabolic engineering approach restricted to *Chlamydomonas reinhardtii* (Scaife et al., 2015). Despite being well characterized and having transformation protocols that were well developed, progress made for *C. reinhardtii* has not resulted in commercial product development, as the species does not have high potential for production (Muñoz et al., 2018). Conversely, industrially important oleaginous microalgae such as *Chlorella vulgaris*, *Nannochloropsis* sp., *Neochloris oleoabundans* and *Acutodesmus obliquus* offer great potential for the production of high- and low-value

products, but these promising species still lack appropriate vector tools for cloning, and protocols for transformation are yet to be developed (Muñoz et al., 2018). Recent studies Muñoz et al. (2018) aimed to optimize the transformation feasibility for *Chlorella vulgaris*, *Nannochloropsis* sp., *Neochloris oleoabundans* and *Acutodesmus obliquus* but successful transformation was only achieved for *Acutodesmus obliquus* and further optimization and protocol validation are required for other microalgae.

2.6.1.4 Selectable markers and reporters

For any type of transformation, selectable markers are required to identify transgenic microalgae or cyanobacteria. Most notable selectable markers are antibiotic-resistance genes, which are commonly used in genetic engineering (OGTR, 2017a) (**Table 2.7)**. Another class of selectable markers are herbicide-resistance genes and some specific metabolic markers are important components to establish herbicide- and metabolic-resistant transgenic organisms (**Table 2.7)**. Without incorporating such markers into target organisms, transformed cells cannot be distinguished from untransformed cells. For example, the *aphA6* gene derived from *Actinobacter baumanni*

confers antibiotic-resistance to the antibiotics kanamycin or amikacin during chloroplast transformation of *C. reinhardtii*. Similarly, the *ble* gene from S*treptoalloteichus hindustanus* confers antibiotic-resistance to zeomycin and phleomycin (used for nuclear transformation of *C. reinhardtii)*. Even an *E. coli* gene was effectively used as a selectable marker for *C. reinhardtii*, *D. salina*, *H. pluvialis* and *Nannochloropsis* species transformation (Esland et al., 2018). The *psbA* gene, which encodes photosystem II protein D1, was used as a metabolic marker for the photoautrophic model organism *C. reinhardtii (*Ahmad et al., *2016).* Several examples of antibiotic- and herbicide- and metabolic-markers used in microalgal or cyanobacterial genetic engineering studies are listed in **Table 2.7.**

Table 2.7. Selectable markers used for microalgae and cyanobacteria genetic modification.

Selectable markers	Source	Target organism	Compartment	Selective agent
Antibiotic resistance genes				
aph8	*Streptomyces rimosus*	*Chlamydomonas reinhardtii* *Pandorina morum*	Nuclear	Paramomycin
aphA7	*C. reinhardtii*	*C. reinhardtii*	Chloroplast	Kanamycin and Amikacin
aphA6	*Actinobacter baumannii*	*Chalmydomonas reinhardtii* *Chlorella ellipsoidea* *Phaeodactylum tricornutum*	Chloroplast	Kanamycin and Amikacin
aadA	*E. coli*	*Chlamydomonas reinhardtii*	Nuclear and chloroplast	Spectinomycin and streptomycin
Ble	*Streptoalloteichus hindustanus*	*Chlamydomonas reinhardtii* *Phaeodactylum tricornutum* *Chlorella ellipsoidea* *Nannochloropsis sp,*	Nuclear	Zeomycin and Phloemycin
Cat	*E. coli*	*Phaeodactylum tricornutum* *Arthrospira (*formerly *Spirulina platensis)* *Chlorella vulgaris* *Scenedesmus obliquus*	Nuclear	Chloramphenicol
hpt	*E. coli*	*Chlamydomonas reinhardtii* *Dunaliella salina* *Haematococcus pluvialis* *Chlorella vulgaris*	Nuclear	Hygromycin-B
phtR		*Tetraselmis chuii*	Nuclear	Phloemycin
rrns	*C. reinhardtii*	*Chlamydomonas reinhardtii*	Chloroplast	Spectinomycin and streptomycin
rrnl	*E. coli*	*Chlamydomonas reinhardtii*	Chloroplast	Erythromycin

Herbicide resistance genes				
AHAS	Higher plants	*Porphyridium* species	Chloroplast	Sulfometuron methyl
Bar/pat	*Streptomyces hygroscopicus*	*Dunaliella salina*	Nuclear	Gulfosinate, Basta and Phosphinothricin
GAT	*Bacillus licheniformis*	*Chlamydomonas reinhardtii CC3491*	Nuclear	EPSPS inhibitor and Glyphosphate
pds	*Haematococcus pluvialis*	*H. pluvialis, Isochrycis* sp., *Tisochrysis* (formerly *Isochrysis*) *galbana Chlamydomonas reinhardtii*	Nuclear	Norflurazon, Fluridone and Flurochloridone
Protox, PPO	*C. reinhardtii*	*Chlamydomonas reinhardtii CC3491*	Nuclear	Oxadiazon and oxyflurofen
psbA mutant	*C. reinhardtii*	*Chlamydomonas reinhardtii*	Chloroplast	Resistance to dichlophenyl dimethyl urea and metribuzin
Metabolic marker genes				
atpB	*C. reinhardtii*	*Chlamydomonas reinhardtii*	Chloroplast	Photoautotrophy
arg9	*C. reinhardtii*	*Chlamydomonas reinhardtii*	Chloroplast	Ariginine
nifH	*C. reinhardtii*	*Chlamydomonas reinhardtii*	Chloroplast	-
petB	*C. reinhardtii*	*Chlamydomonas reinhardtii*	Chloroplast	Photoautotrophy
psbA	*C. reinhardtii*	*Chlamydomonas reinhardtii*	*Chloroplast*	Photoautotrophy
tscA	*C. reinhardtii*	*Chlamydomonas reinhardtii*	Chloroplast	Photoautotrophy

Source: (Day & Goldschmidt-Clermont, 2011; Doron et al., 2016; Esland et al., 2018; Rathod et al., 2017; Urtubia et al., 2016; Yan et al., 2016)

2.6.1.5 Fully sequenced microalgae and cyanobacteria as model organisms for genetic engineering.

Genetic engineering approaches require model organisms which are representative of particular taxonomic groups (Yan & Fong, 2017). The advances in sequencing technology enabled to determine the genome sequences of several microalgae and cyanobacteria over the last two decades (Urtubia et al., 2016). For instance, *C. reinhardtii* has a 121 Mb genome size, which was published in 2006 (Merchant et al., 2007). Recently, the unicellular non-flagellated marine microalga *Osterococcus tauri* - considered a model organism to study iron metabolism in green phytoplankton (Lelandais et al., 2016) - genome was published (Derelle et al., 2006). It has a very small 12 Mb genome for a eukaryotic microalga. *Dunaliella salina* had its whole genome sequenced in August 2017. *Dunaliella salina* has a large genome of approximately 343.7 Mb and is used as a model organism to understand salt adaptation strategies in oceanic environments (Polle et al., 2017; Wang et al., 2019). Similarly, *Nannochloropsis* sp., is known for biofuel production attempts, and is becoming a prominent model organism to study lipid production (Ajjawi et al., 2017; Sirakov et al., 2015). The genome

particulars for six different *Nannochloropsis* species range from 25 – 32 Mb (Wang et al., 2014). Interestingly, a recent report from Texas A&M University, College of Agriculture Science reported the sequences of the whole genome of *Botryococcus braunii* (Showa), a biofuel-producing strain with a genome size of approximately 166.2±2.2 Mb (Browne et al., 2017). As a result, *Botryococcus sp.,* has recently received much attention for renewable biofuel production (Banerjee et al., 2002). Another major group of microalgae, the diatoms, which possess completely or partially silicified cell walls, which contribute ~ 40% of oceanic primary production, are of great interest (Rocha et al., 2018). *Thalassiosira pesudonana* and *Phaeodactylum tricornutum* are popular species for genetic research and the latter also in aquaculture (Huang & Daboussi, 2017). Both have been fully sequenced and are used as models to study larger diatom communities (Siaut et al., 2007). The first fully sequenced marine diatom was *T. pseudonana*, which was published in 2004, followed by *P. tricornutum* in 2008 (Bowler et al., 2008; Hadi, 2004). The *P. tricornutum* genome is slightly smaller than that of *T. pseudonana* (Bowler et al., 2008). They have been well studied to understand physiological features like silica cell wall morphogenesis,

nutrient storage and salinity-mediated metabolism in ocean environments (Obata et al., 2013; Poulsen & Kröger, 2004). Compared to the above eukaryotic microalgae, cyanobacteria (blue green algae) are widely used as a valuable models to understand the genes involved in evolutionary mechanisms of endosymbiosis (origin of chloroplasts), nitrogen fixation and carbon fixation through oxygenic photosynthesis (Archibald, 2015; Kulasooriya & Magana-Arachchi, 2016; Zhang et al., 2017). These cyanobacteria are known for their impressive ability and flexibility to manipulate gene expression of foreign genes (Al-Haj et al. 2016).

In the cyanobacteria, more than 300 species genomes were completely sequenced (Fujisawa et al., 2016). *Synechocystis* sp. was the first completely sequenced cyanobacterial genome, followed by *Synechococcus elongatus* PCC7942 in 1996, which are largely used due to their small genome size and publicly available genetic information (Triana et al., 2014). The advantages and disadvantages of several fully sequenced microalgae and cyanobacteria are listed in **Table 2.8.** In Australia, *S. elongatus* PCC7942 is an exempted species under “dealing with genetically modified organisms” (GMOs) regulations in laboratory

environments. Therefore, the research in my thesis investigates ectoine (*ectABC*) gene transformation of *Synechococcus elongatus* PCC7942 in order to improve both salinity- and temperature-resilience.

Table 2.8. Advantages and disadvantages of fully sequenced model algae and cyanobacteria for genetic engineering

Microalgae / Cyanobacteria	Genome size	Advantages	Drawback	Reference
Botryococcus braunii	166 Mb	Biofuel production	Slow growth	(He et al., 2018)
Chlamydomonas reinhardtii	120 Mb	Cloning vector for chloroplast transformation commercially available	60% of the function of genes unknown	(Bai et al., 2012)
Chlorella	46.2 Mb	Wide spread and adaptable; and feed application	Lack of genetic tools-	(Benemann, 2013; Eckardt, 2010; Kim et al., 2018)
Dunaliella salina	343.7 Mb	ß-carotene production and high salinity stress-tolerance	Lack of transformation protocols	(Borowitzka et al., 1984; Borowitzka et al., 2016; Polle et al., 2017)
Nannochloropsis sp.,	25.38 to 32.07Mb	Smaller genome than *C. reinhardtii* High coding potential	Lack of transformation	(Wang et al., 2014)
Ostreococcus tauri	12.56 Mb	Smallest free living eukaryote, non-flagellated and fast growing	Highly complex genome structure	(Derelle et al., 2006)
Phaeodactylum tricornutum	27Mb	Genetic tools developed	Silicified cell wall	(Poulsen et al., 2006; Siaut et al., 2007)
Thalassiosira pseudonana	34Mb	Genetic tools developed	Silicified cell wall	(Poulsen et al., 2006)
Synechosystis sp.,	3.6 Mb	Small size genome and highly amenable for genetic	Slow productivity	(Yu et al., 2013)

		manipulation.		
Synechococcus elongatus PCC7942	2.7 Mb	Small genome, species-specific molecular tools such as vector, selectable markers are commercially available commercially for transformation; c-phycocyanin market potential	Some disadvantages of low lipid content and –productivity and no PUFA content	(Delaye et al., 2011; Santos-Merino et al., 2018)
Anabaena sp.,	5.5 Mb	Nitrogen fixation	Highly active restriction sites degrade foreign DNA	(Pfeffer & Brown, 2016; Sani & Rathinam, 2018)

2.7 Justification on species and target gene selection for improving salinity- and temperature-resilience

Although *Chlamydomonas reinhardtii* has been well characterized, and plasmid vectors and tools for genetic engineering are commercially available, our research identified that this microalga is unlikely to be suitable for large-scale cultivation in open ponds, due to slow growth, low oil content and sensitivity to high solar radiation (Scranton et al., 2015). Metabolic engineering of several other eukaryotic algae such as *Thalassiosira pseudonana* (Poulsen & Kröger, 2004) and *Phaeodactylum tricornutum* (Eilers et al., 2016) have been explored for the production of high-value products. Compared to eukaryotic microalgae, cyanobacteria are known for their impressive ability and flexibility for gene manipulation and expression of foreign genes (Al-Haj et al. 2016). Of the cyanobacteria, our research identified *Synechococcus elongatus* PCC7942 as a species of choice, due to its small genome size and publicly available genetic information. Moreover, in Australia, *Synechococcus elongatus* PCC7942 is exempted from restrictions of dealing with genetic modification in laboratory environments (OGTR, 2017a; OGTR, 2017b). Therefore, this research will investigate gene transformation in *Synechococcus*

elongatus PCC7942 with the osmolyte ectoine (*ectABC*) to improve salinity- and temperature-resilience.

2.8 Objectives and approach

Given the importance of salinity- and temperature-resilient cyanobacteria for culture under non-ideal conditions, the molecular aspect of the proposed research aims to transfer an *ectABC* gene cassette into *Synechococcus elongatus* PCC7942 in order to generate salinity- and temperature-tolerant cyanobacteria for high-volume, low-value products and ectoine production. With this in mind, this research aims to:

1. Transform the ectoine gene cassette (*ectABC*) into *Synechococcus elongatus* PCC7942 for improvement of salinity- and temperature-tolerance (chapter three).

2. Assess the capacity of *ectABC*-transfected *Synechococcus elongatus* PCC7942 to withstand salinity and temperature challenges and investigate the impact on biomass, nutrient consumption and biochemical profile (lipids, fatty acid contents and ectoine) (chapter four).

3. Evaluate the potential real-term opportunities of *ectABC*-transformed *Synechococcus elongatus* PCC7942 for tropical outdoor industrial cultivation (Chapter 5).

4. Synthesize the outcome of all of the above and discuss limitations of this research (Chapter 6).

CHAPTER 3

3 Designing a *Halomonas elongata* DSM3043 e*ctABC* gene cassette sequence variant for efficient transformation into *Synechococcus elongatus* PCC7942: Codon optimization, recombinant plasmid construction, genetic transformation and temperature resilience study

3.1 Introduction

Microalgae or cyanobacteria can be considered as a renewable resource requiring less water than conventional crops (Kumar et al., 2019). Microalgae can be cultivated on non-arable land using water sources not suitable for human consumption (Heimann et al., 2015a). Mass cultivation of microalgae provides the benefit of acting as a carbon sink to mitigate CO_2 emissions and the harvested biomass can be processed into food, feed, fuel and fertilizer (Heimann, 2016; Santos et al., 2017). Thus, microalgae can make a partial contribution to expanding food production. Efficient recycling of waste CO_2 and water from coal-fired gas power stations and sugar mills requires co-location of algae production facilities to maximise the use of waste resources through integrated resource management practices (Khan et al., 2017; Sedghamiz, 2017). However, microalgal cultivation co-location with other industrial facilities particularly in temperate regions where temperature is

high during summer, requires algae that can tolerate high temperature and salinity (Mondal et al., 2017). Regardless of co-location, climate-induced changes requires associated temperature and salinity-management, which remain additional challenges for environmentally and economically sustainable production of microalgal biomass for high-volume, low-value bio-products, such as bio-fertilisers (Ebrahimi & Salarzadeh, 2016). These factors are not readily controllable, because they require high-energy inputs for cooling and freshwater for maintaining appropriate salinity levels (Tredici et al., 2015). The only solution to this remaining bottleneck is strain selection for high temperature and salinity tolerance, which is challenging in the context of maintaining growth and biochemical performance criteria for the desired product (Heimann et al., 2015b). Examining the body of literature **(Please see Chapter 2)** clearly identifies that selection for salinity- and temperature-tolerant cultivars is the only option to bring the promise of a large volume microalgal industry to fruition. Given this, it is important to investigate routes that confer both temperature- and salinity- tolerance to microalgae that also have a suitable biochemical profile and biomass productivity, which is required by the industry.

Ectoine is an osmolyte produced by extremophilic-halo-tolerant bacteria and infers tolerance to high temperatures and salinities. Ectoine Ectoine biosynthesis is mediated by a three step enzymatic reaction requiring *ectABC* genes in one cassette **(Please see Chapter 2 for detailed summary of biosynthesis pathway and properties of ectoine)**. Ectoine- transformed tomato (Moghaieb et al., 2011) and tobacco plants (Moghaieb et al., 2006) showed increased salinity resilience, while transfection of the eukaryotic green freshwater microalga *Chlamydomonas reinhardtii* with either the *ectA (Lunde, 2012)*, *ectB* (Afzal, 2014) or *ectC* (Holck, 2014) genes yielded no positive outcomes, as all three *ectABC* genes are required to carry out ectoine synthesis (Lunde, 2012). Having not succeeded with the eukaryotic model organism *Chlamydomonas reinhardtii*, this study focused on the freshwater cyanobacterium *Synechococcus elongatus* PCC7942, which was selected for *ectABC* gene cassette transformation in order to investigate whether temperature- and salinity- tolerance could be imparted successfully by enabling ectoine expression. This cyanobacterium is an ideal candidate organism for this study, as it fulfills the Office of Gene Technology Regulations (OGTR) accreditation requirements to deal with genetic modification (OGTR, 2017b) and

avoids further constraints due to: a) the availability of the complete genome sequence; b) access to existing species-specific commercial vectors; and c) transformation protocols within Australia.

3.2 Materials and Methods

3.2.1 Cyanobacterial strains and Plasmids

The cyanobacterial strain *Synechococcus elongatus* PCC7942 and the *pSyn-6* vector were obtained from Invitrogen (Life Technologies) for *ectABC* gene cassette transformation. *Synechococcus elongatus* PCC7942 cells were grown in BG 11 medium at 34ºC ± 1ºC with light intensities of 50–100 µE m^{-2} s^{-1} on a shaker set to 100 rpm. Cultures were maintained in a temperature-controlled cabinet with continuous aeration as per the supplier's manual.

3.2.2 *ectABC* cassette design and *pSyn-6* vector

The *ectABC* gene cassette is typically organized as a single operon containing 2869 bp (**Figure 3.1**), taken from *Halomonas elongata* DSM3043 (Cánovas et al., 1998). The coding regions of *ectABC* genes were determined using a BLAST search and interleaved non-coding regions were removed.

The *pSyn_6* vector (**Figure. 3.2**) is a publically and commercially available tool for introducing foreign genes into *Synechococcus elongatus* PCC7942 (https://www.thermofisher.com/order/catalog/product/A24230). The *pSyn_6* vector contains several elements such as an origin of replication (pUC), a promoter (*psbA*), a selectable marker (Spectinomycin resistance gene), a ribosome binding site (RBS) and a termination region (*rrnb*) **(Table 3.1)** all of which are required for *ectABC* gene expression and transformant selection (**Figure 3.3**). It contains a strong constitutive *psbA* promoter from *Synechococcus elongatus* PCC7942 for high-level expression of desired genes. The ribosome binding site initiates translation of the desired ectoine gene and the origin of replication maintains high plasmid copy numbers within transformed cells. Multiple restriction sites are present to assist in cloning the desired gene and facilitate including histidine tags into the vector. The latter allows N- and C-terminal poly histidine antibodies to be applied to visually confirm gene expression of the cloned genes by the *ectABC* transformed *S. elongatus* PCC7942. Additionally, the *pSyn-6* vector contains neutral sites (*NS1 & NS2*) which facilitate homologous recombination for integrating the vector into the

Synechococcus elongatus PCC7942 genome. The inclusion of a spectinomycin-resistance gene in the vector allows only recombinants to be selected from non- transformed cells, by adding spectinomycin to growth media during the culture of transformed microalgae. Finally, including a termination site (*rrnb)* ensures that ectoine transcription is terminated. The 2869 bp *ectABC* gene sequence inserted into the *pSyn-6* vector was obtained from *Halomonas elongata* DSM3043 (Genbank accession no: AJ011103) (**Figure 3.1**).

Figure 3.1. *ectABC* gene sequence from *Halomonas elongata* DSM3043 (2869 bp)

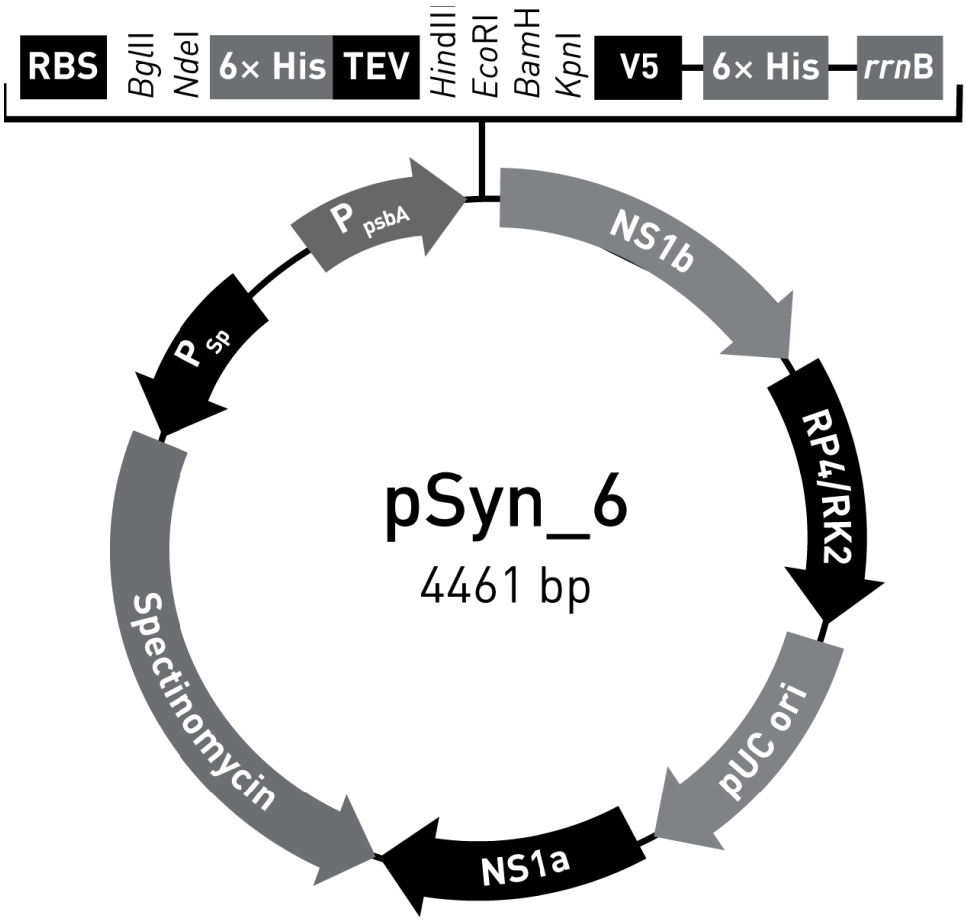


Figure 3.2. Circular structure of *pSyn_6* vector (4461 bp). The inset shows the composition of the cloning site into which the *ectABC* casette will be inserted during the construction of the plasmid for transforming *S. elongatus* PCC7942using this vector, as shown in Figure 3.3

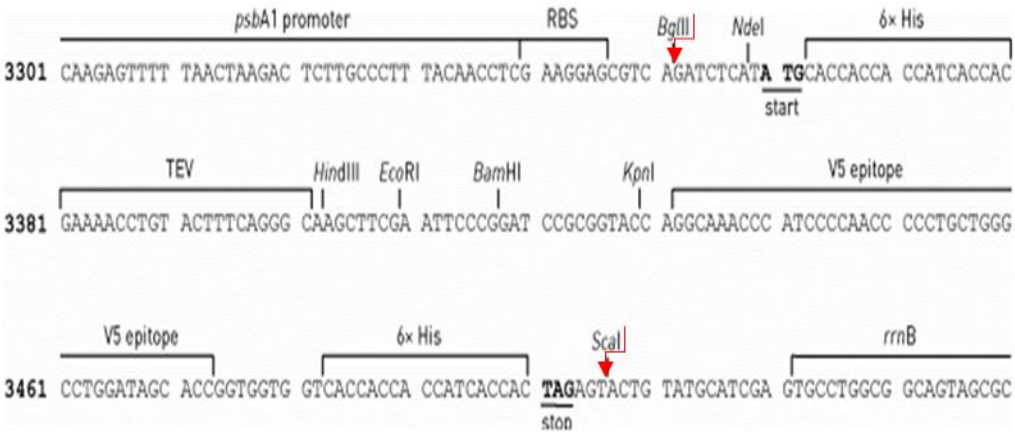


Figure 3.3. Focus on linearised *pSyn_6* vector cloning region for insertting the *ectABC* gene cassette.

As the *pSyn_6* vector contains a single ribosome-binding site, it could only initiate translation of one ectoine gene component, *ectA,* as presented in **Figure 3.4**. In order to incorporate the *ectABC* cassette into the *pSyn_6* vector, a range of restriction enzyme sites (*BglII*, *NdeI*, *HindIII*, *EcoRI*, *BamHI*, *KpnI* and *ScaI*) were employed. Of interest are *BglII* and *ScaI,* between which the *ectABC* gene cassette was inserted. To do this, we calculated the optimal aligned spacing between the ribosome-binding site (RBS) and the transcription initiation codon (*NdeI* site) in the *pSyn_6* vector and inserted another RBS region sequence (13-nt spacing between RBS to start codon) upstream of the *ectB* and *ectC* genes **(Figure 3.4)**.

Table 3.1. Cloning elements of *pSyn_6* vector

Cloning elements for integration		Nucleotides location and size (bp)
A. *ectABC* gene cassette (2,869 bp)		
	EctA (Diaminobutricacid amino transferase)	594
	ectB (Diaminobutricacid acetyltransferase)	1272
	ectC (Ectoine Synthase)	393
B. *pSyn_6* vector (4461 bp)		
	RP4/RK2 bom site	1-304
	pUC origin	362-977
	*NS1*a (neutral site 1a)	1049-1847
	Spectinomycin resistance gene	1970-2980
	Spectinomycin promoter	2981-3114
	psbA promoter (P_{psbA})	3117-3339
	RBS (ribosome binding site)	3340-3346
	6x His tag	3363-3380
	TEV recognition site	3381-3401
	V5 epitope	3432-3473
	rrnB transcriptional termination region	3522-3679
	NS1 b (neutral site 1b)	3680-4457

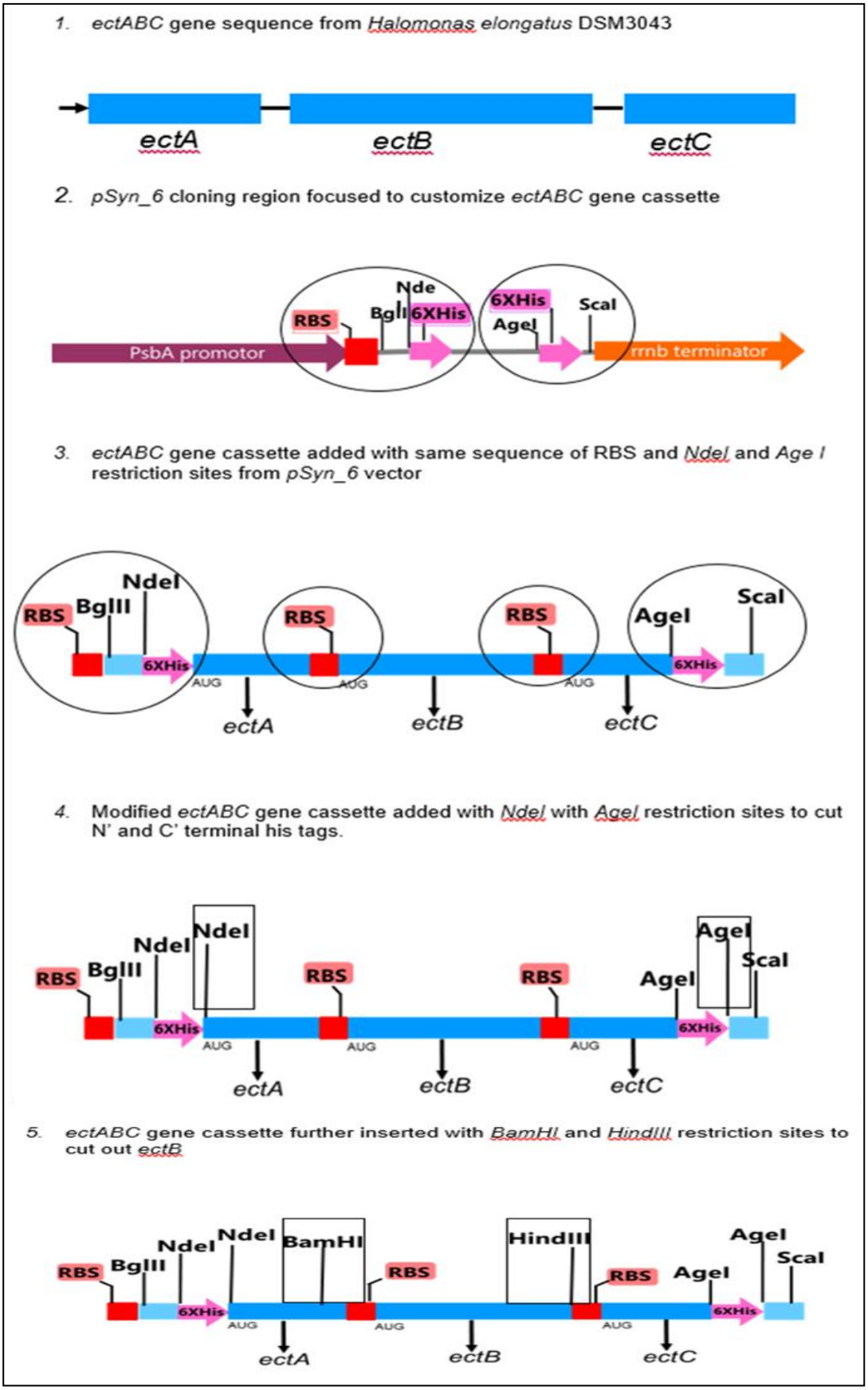


Figure 3.4. Schematic representation of *ectABC* customization to clone into *pSyn_6* vector

Two poly-histidine sequences (identified as 6xHis) on the *pSyn_6* vector diagram were incorporated at the C' and N' terminals of the *ectABC* gene cassette to evaluate *ectA* and *ectC* gene expression, if required. Additionally, a *BamHI* (*GGATCC*) site was also included upstream of *ectB* and a *HindIII* (AAGCTT) restriction site was included upstream of *ectC* **(Figure 3.4)**. This was necessary to be able to perform various restriction digests, as required, to remove *ectB* gene expression if required for further evaluation. Further, *BglII* and *ScaI* sites were replaced with different bases to ensure no other *BglII* or *ScaI* restriction sites were present elsewhere in the *ectABC* gene cassette.

3.2.3 Codon optimization

Codon optimization of the *ectABC* gene cassette sequence to be added to *Synechococcus elongatus* PCC7942 was performed by Gene optimizer software (Thermo Fisher Scientific). Codon optimization was performed and codon adaptation indices were calculated based on previous studies (Sharp & Li, 1987). The *Synechococcus elongatus* PCC7942 genome contains 3,000 codons and the entire genome has a high G+C content (~55.5% G+C; 1st letter G+C ~64%, 2nd letter G+C, ~44%, and 3rd letter G+C ~60%) **(Table 3.2)**. The expression levels of recombinant genes are significantly improved, often to the level of gene

expression of the original host organism, if the gene of interest is optimised to match the codon bias of the new host's (*Synechococcus elongatus*) codon usage (**Table 3.2**).

Table 3.2. Codon usage preference for *S. elongatus* PCC 7942

***Synechococcus elongatus* PCC 7942** [gbbct]: 3000 CDS's (911886 codons)
Fields : [triplet] [frequency : **per thousand**] ([number])

UUU 19.2 (17496)	UCU 6.6 (5975)	UAU 10.7 (9763)	UGU 4.5 (4100)
UUC 15.5 (14123)	UCC 7.6 (6962)	UAC 13.7 (12526)	UGC 6.3 (5731)
UUA 8.7 (7908)	UCA 5.6 (5131)	UAA 1.1 (985)	UGA 0.9 (830)
UUG 25.2 (22961)	UCG 11.7 (10713)	UAG 1.3 (1185)	UGG 17.5 (15955)
CUU 9.3 (8463)	CCU 10.3 (9368)	CAU 8.3 (7535)	CGU 10.3 (9425)
CUC 26.7 (24371)	CCC 18.3 (16731)	CAC 8.9 (8111)	CGC 30.5 (27818)
CUA 11.2 (10224)	CCA 9.5 (8620)	CAA 30.1 (27443)	CGA 10.2 (9266)
CUG 41.6 (37974)	CCG 16.5 (15063)	CAG 30.8 (28115)	CGG 14.1 (12824)
AUU 26.1 (23843)	ACU 10.1 (9244)	AAU 13.4 (12255)	AGU 11.7 (10706)
AUC 27.3 (24888)	ACC 20.1 (18339)	AAC 14.1 (12824)	AGC 16.8 (15338)
AUA 1.0 (879)	ACA 7.2 (6544)	AAA 13.8 (12542)	AGA 1.6 (1419)
AUG 15.2 (13852)	ACG 14.6 (13280)	AAG 12.4 (11274)	AGG 1.0 (880)
GUU 15.9 (14510)	GCU 25.5 (23271)	GAU 33.4 (30419)	GGU 18.4 (16771)
GUC 22.8 (20784)	GCC 33.4 (30421)	GAC 17.8 (16266)	GGC 31.7 (28871)
GUA 4.6 (4198)	GCA 18.8 (17145)	GAA 32.4 (29535)	GGA 8.8 (8018)
GUG 23.2 (21116)	GCG 27.4 (24963)	GAG 24.3 (22192)	GGG 12.7 (11604)

Coding GC 55.96% 1st letter GC 63.76 % 2nd letter GC 44.01% 3rd letter GC 60.10%

(https://www.kazusa.or.jp/codon/cgibin/showcodon.cgi?species=1140)

3.2.4 Plasmid vector construction

Plasmid *v*ector construction is an important step for any gene transformation and is often a time-consuming process (Bok and Keller 2012). Cloning the *ectABC* gene cassette into the *pSyn_6* vector was carried out by Gene Art Technologies, Germany. The muti-step

protocol of recombinant plasmid construction of *ectABC_pSyn_6* is illustrated in **Figure 3.5**. Firstly, *ectABC* genes were subcloned into a pMK-RQ (kanR) vector using the restriction enzyme *SfiI* and *SfiI* cloning sites for creating a single linear DNA molecule (**Figure 3.5)**. Later, this modified *ectABC* fragment was transformed into *E. coli* K12 strain (**Figure 3.5)**. Secondly, the constructed plasmid DNA was purified from *E. coli* K12 and concentration was determined to be optimal (by UV spectroscopy). Subsequently, the *ectABC*-containing plasmid was cloned into the *pSyn_6* expression vector for transformation of *Synechococcus elongatus* PCC7942 using this engineered vector (**Figure 3.5)**. The final construct was verified by sequencing (Thermo Fisher Scientific).

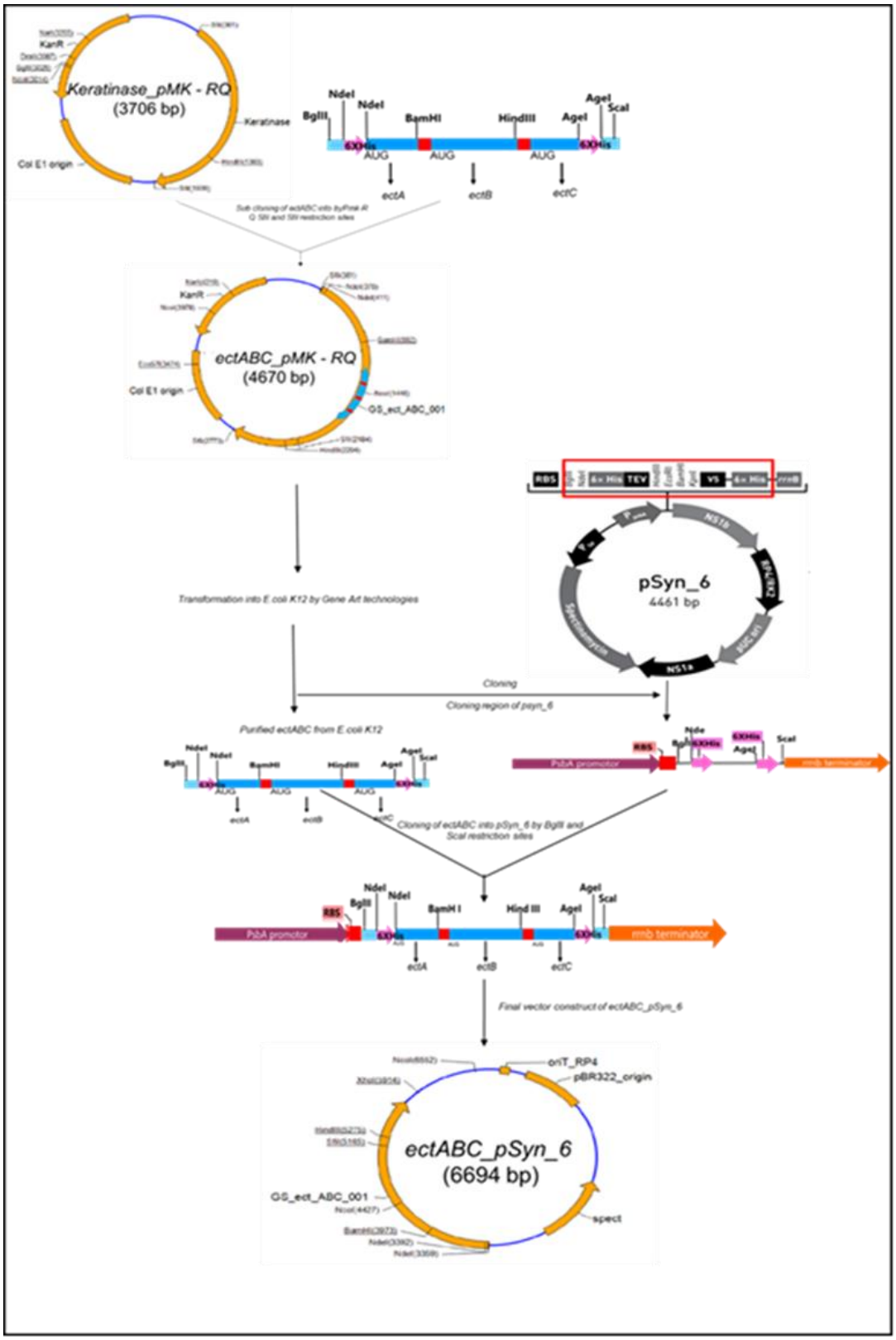


Figure 3.5. Schematic representation of *ectABC* cloning and plasmid construction for *S. elongatus* PCC7942 transformation

3.2.5 Transformation of *S. elongatus* PCC 7942 using the *pSyn_6_ectABC* construct

Synechococcus elongatus PCC7942 cells were grown to an optical density (OD_{750nm}) of 1, containing more than $1.15x10^6$ cells mL^{-1}, which were harvested by centrifugation at 14,000 rpm for 3 min at room temperature. The supernatant was removed and the cells were resuspended and washed in 1 mL of BG11 medium and re-centrifuged. The pellets were resuspended in 100 µL of BG11 medium and 100 ng of supercoiled plasmid DNA (i.e., *pSyn_6* construct containing *ectABC* gene cassette) was added to the resuspended cells. A negative control was also prepared by adding 100 ng of supercoiled plasmid DNA of the *pSyn_6* vector that did not contain the *ectABC* construct (*pSyn_6* empty vector control). The DNA cell suspensions were mixed gently by flicking tubes and the cell-DNA mixture(s) were incubated at 34°C in a water bath with a dark lid for 4 h. The tubes were removed from the water bath and wiped with 70% ethanol after incubation. Transformation mixtures of 80 µL of cells transformed with *ectABC_pSyn_6* (actual plasmid target) and of cells transformed with *pSyn_6* (empty vector control) and non-*ectABC_pSyn_6* (WT *S. elongatus* PCC7942) vector were plated onto

separate BG11 agar plates pre-warmed to room temperature, containing 10 µg/mL of spectinomycin (**Figure 3.6)**.

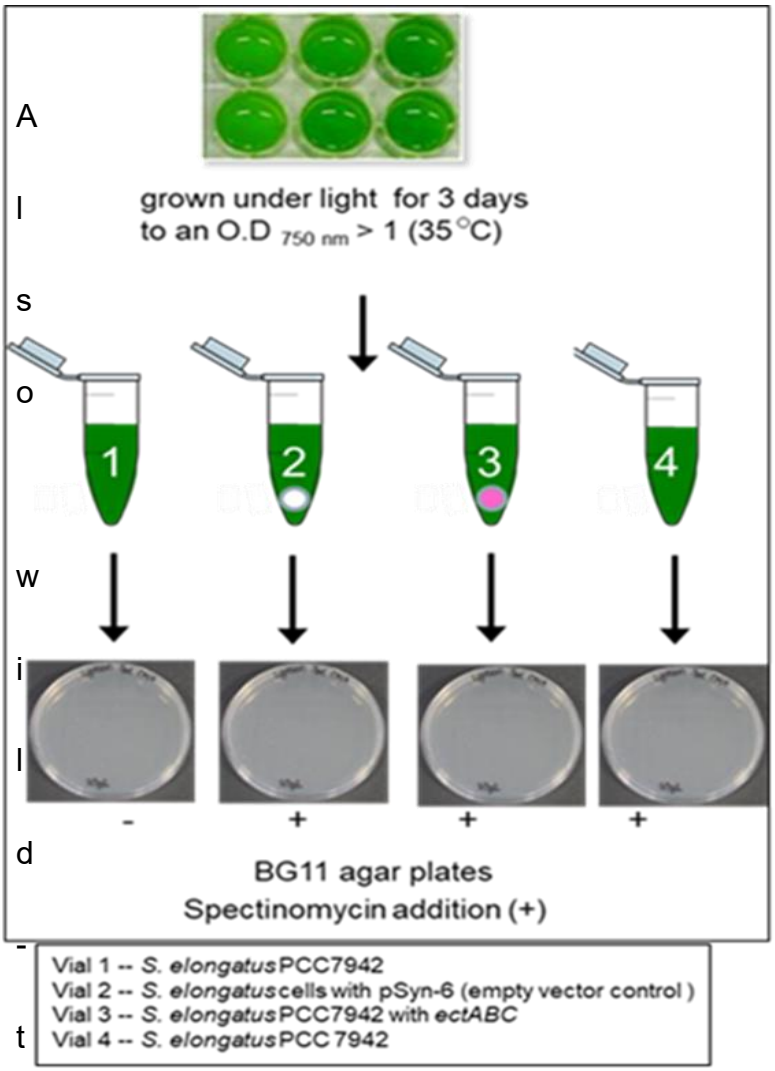


Figure 3.6. Transformation and spectinomycin resistance of *ectABC_pSyn_6* transformants and empty vector control (*pSyn_6*) versus WT *S.elontgatus PCC 7942.*

Also WT (untransformed) *Synechococcus elongatus* PCC7942 were taken in separate aliquots and plated on BG11 agar plates with and without spectinomycin to ensure WT cells were not spectinomycin-resistant and would not grow on BG11 agar plates in the presence of

Spectinomycin, but *ectABC* transformed *S. elongatus* PCC7942 would grow. All plates were placed on illuminated shelves, agar side down at room temperature (25–30°C) for 3 days of incubation.

3.2.6 Primer design for the *ectABC* gene and PCR amplification

To confirm that transformation was successful, colony PCR amplification of the *ectABC* gene was carried out on the negative control, the "empty" *pSyn_6* vector (lacking the *ectABC* cassette) and the *ectABC*-modified *pSyn_6* vector. The *ectABC* gene cassette was targeted for amplification using the three alternative transformants as templates for amplification and two sets of primer pairs to amplify. The first primer pair (Forward 5' ACG CCT ACA ACC GAG AAC TTC '3; Reverse 5' CGT TCG AGA ATC TGG GTC'3) was designed to amplify the *ectA*&*B* regions only. The second primer pair (Forward 5' GTA ACC TGG AAG AAT GCC GCA '3; Reverse 5' AGT ACT CTA GTG GTG ATG GTG '3) was designed to amplify the *ectC* region only. PCR was conducted using the *ectA&B*- and *ectC*-specific primer pairs along with the PCR Super Mix High Fidelity premix (Thermo Fisher Scientific product catalogue no: 12532016). The first PCR reaction conducted to amplify *ectA&B* was performed as follows: an initial denaturation at 95ºC was performed for 5 min, this was followed by 34 amplification cycles at 94°C, denaturation for 1 min, 55°C

annealing of primers for 1min, 72°C extension for 1 min and a final extension step at 72°C for 7 min. The second PCR reaction to amplify only *ectC* using primer set II was as follows: an initial denaturation occurred at 94ºC for 2 min, followed by 34 cycles at 94°C for 1 min, 52.5°C for 1min, 72°C for 1 min and a final extension at 72°C for 7 min.

3.2.7 Temperature pilot study

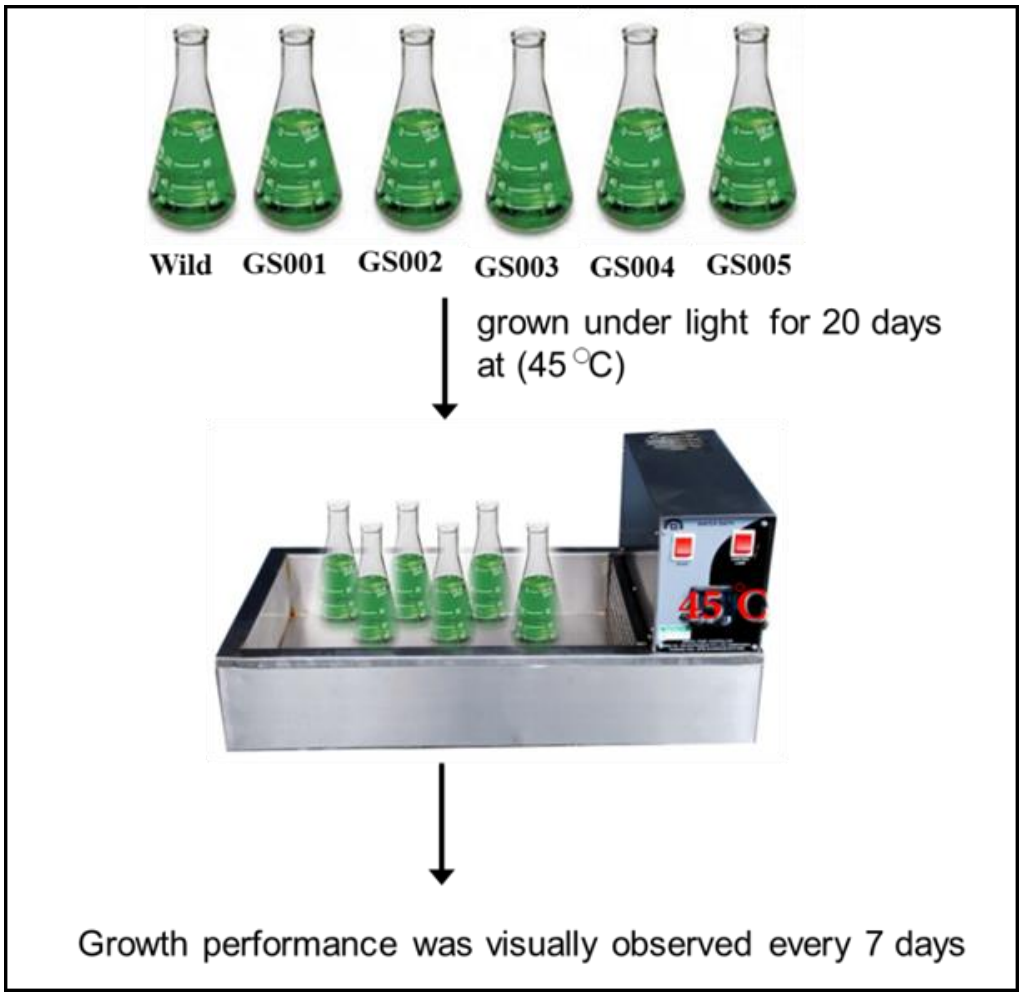


Figure 3.7. *ectABC* transfromants temperature challenge experiment at 45ºC

In total, five different *ectABC*-transformed *Synechococcus elongatus* PCC7942 clones (*Synechococcus elongatus* PCC7942_*ectABC* GS001, GS002….GS005) along with a WT *S. elongatus* PCC7942. (Untransformed strain) and "empty" vector control-transformed *S. elongatus* PCC7942, lacking the *ectABC* insert (5+1+1=7) were selected for a range-finder temperature pilot study. The selected strains were grown in 90 mL BG11 medium at an inoculation density of 10 mL at temperatures of 45ºC for twenty-one days in a water bath **(Figure 3.7)**. Culture growth was observed and recorded every seventh day for 21 days.

3.3 Results

3.3.1 Codon optimization

Gene Art Technologies using Gene Optimizer Software evaluated codon optimization of *ectABC* expression within the host organism *Synechococcus elongatus* PCC7942. The *ectABC* gene cassette optimization for *S. elongatus* PCC7942 was estimated using codon adaptation index values. Gene optimizer software results revealed that CAI values between non-optimized and optimized *ectABC* were different. Non- optimized *ectA*, *ectB* and *ectC* CAI values were 0.82,

0.84 and 0.8, respectively whereas optimized CAI values for *ectA*, *ectB* and *ectC* were 0.95, 0.92 and 0.95, respectively (**Figure 3.8**)

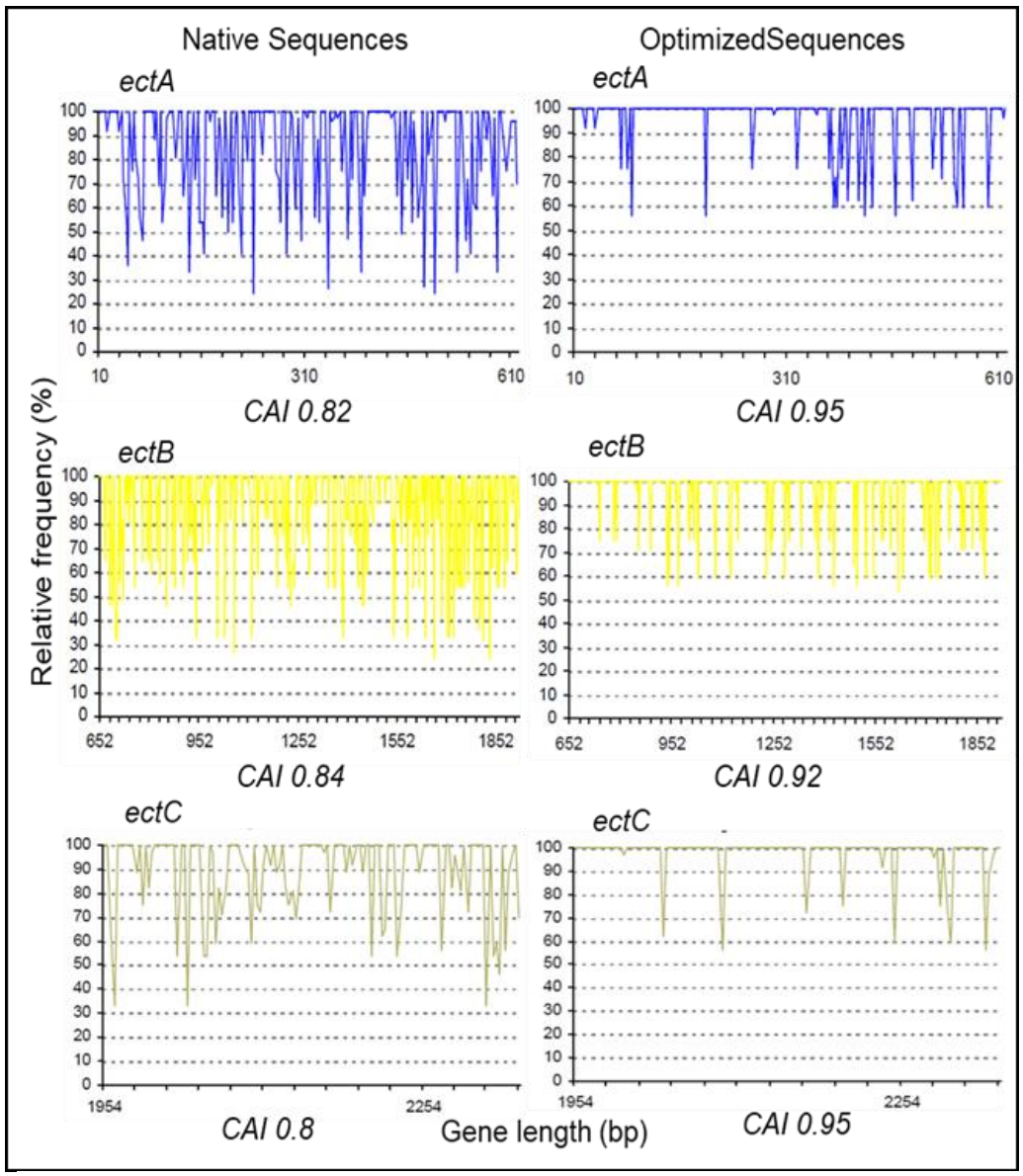


Figure 3.8. Codon adaptation index for optimized and non-optimized *ectABC*

The frequency of optimal codons parameter, of 100 is set for the codon with the highest usage frequency for a given amino acid in the host organism *Synechococcus elongatus* PCC 7942. In this study, codon

frequency of the optimized *ect A*, *ect* B and *ect* C genes have shown 90, 88 and 92 percent codon frequency in the optimized sequences, respectively (**Figure 3.9**).

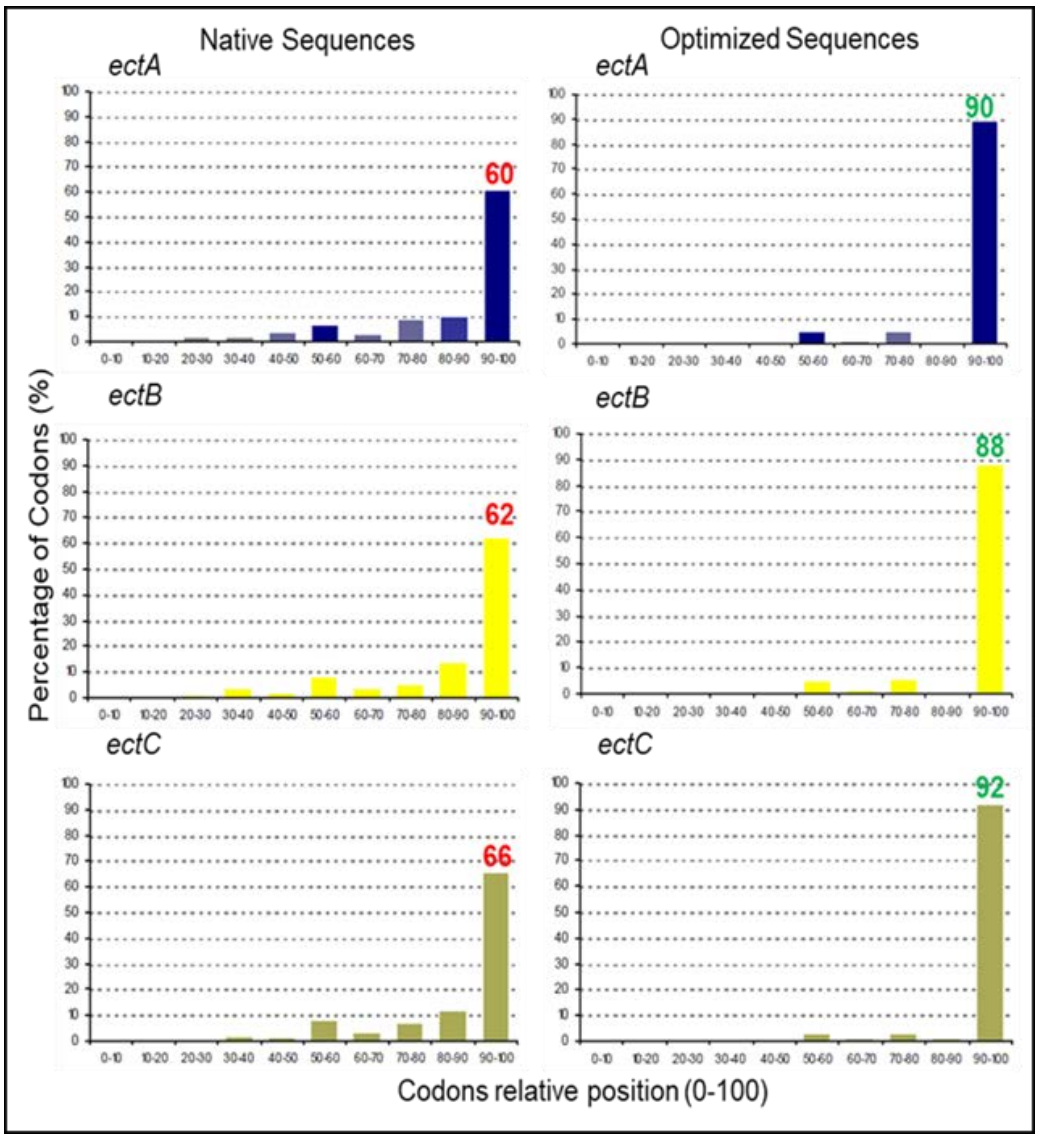


Figure 3.9 Codon translation frequency optimization in non-optimized (native) and optimized (*ectABC)* sequences.

In addition, the *ectABC* genes GC content optimization was determined to be 56 % (**Figure 3.10)**, as this was indicated in *S. elongatus* PCC7942 preferred codon usage (**Table 3.2).**

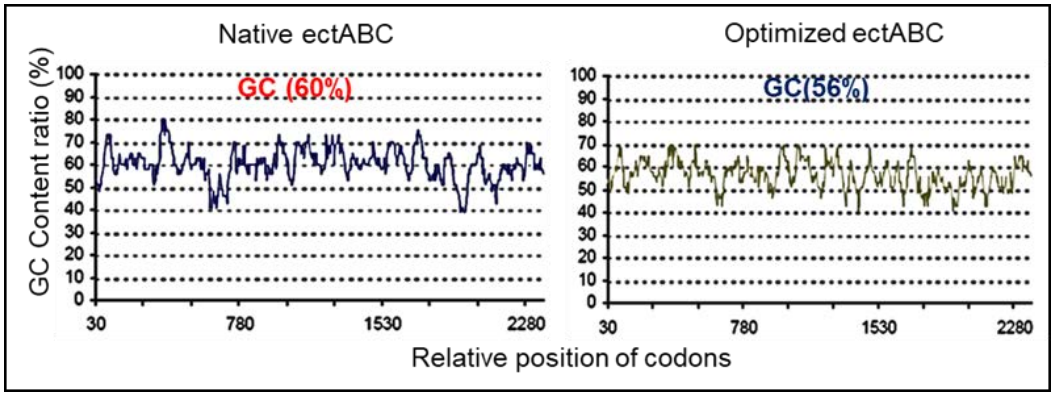


Figure 3.10. GC content ratio of native *ectABC* vs optimized *ectABC* genes

3.3.2 *pSyn_6* with *ectABC* plasmid construction and transformation into *S.elongatus* PCC 7942.

After successful optimization, the cloning of *ectABC* into *pSyn_6* was carried out following *BglII* and *ScaI* restriction digestion (as shown in **Figure 3.5**). Sequencing of constructed plasmid was verified by sequencing Thermo Fisher Scientific to confirm the cloning of *ectABC* gene cassette into *pSyn_6*. The transformation of S. elongatus PCC7942 relies on homologous recombination between cell's chromosome and exogenous DNA that is not automatically replicating.and contains sequences homologous to the chromosome. (http://tools.thermofisher.com/content/sfs/manuals/geneart_synechococc

us_engineering_man.pdf). Thus facilitating recombination of the ectopic sequence into the chromosomal DNA of the target organism When transformed with vectors containing an antibiotic resistance cassette and neutral site sequences, a double homologous recombination event occurs between the neutral site vector and the *S. elongatus* chromosome. The selective marker (spectinomycin resistance) and the gene of interest driven by a promoter are inserted into the neutral site together, whilst the vector backbone (pUC) is lost, allowing the recombinant genes in *S. elongatus* PCC 7942. PCC 7942 to be expressed (https://www.thermofisher.com/order/catalog/product/A24230).

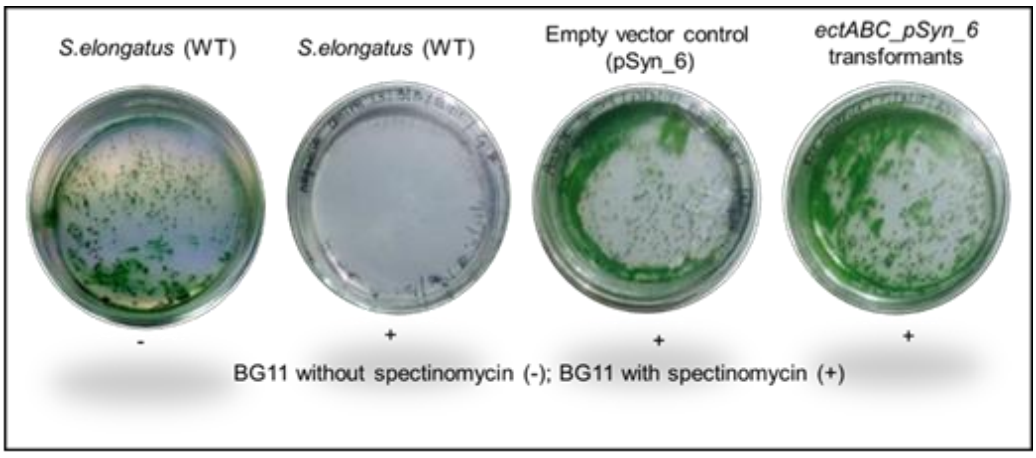


Figure 3.11. Growth of spectinomycin-resistant clones of *ectABC_pSyn_6 S. elongatus* PCC7942 and *pSyn_6* empty vector controls versus WT strain

The spectinomycin containing BG11 agar plates (**Figure 3.11**). The *pSyn_6* empty vector and the *ectABC _ pSyn_6* plasmid transformation into *S. elongatus* PCC7942 was determined by measuring *S. elongatus* PCC7942 growth on The WT *S. elongatus* PCC7942 failed to grow on spectinomycin-containing BG11 agar plates, confirming that the WT strain is not spectinomycin resistant. Colonies were observed on agar plates containing *pSyn_6* constructs with the *ectABC* gene cassette and the *pSyn_6* vector control, respectively. Colonies were re-streaked onto fresh BG-11 agar plates containing spectinomycin (**as shown in supplementary material S.3**). Colonies were randomly chosen from spectinomycin containing plates for culture and further analysis.

3.3.3 PCR amplification of the *ectABC* gene cassette

In total, 14 colonies of transformed *ectABC* gene containing cells were screened by colony PCR to confirm that the *ectABC* gene sequence was integrated into the *Synechococcus elongatus* PCC 7942 genome. Colony PCR was performed using two primer sets (I and II) to amplify target regions of *ectA&B* and *ectC,* respectively. Primer set I targeted the *ectA*&*B* region (670 bp) and primer set II targeted *ectC* (429bp). The *ectA&B* and *ectC*-specific primers yielded no amplification products for

the empty vector control *pSyn_6* (data not shown), demonstrating that the genes for ectoine synthesis are absent in *Synechococcus elongatus* PCC7942. The colony PCR using *ectABC* gene-specific primers on randomly chosen 14 transformants and successful *ectABC* integration was tested and confirmed in by PCR amplification of *ectA&B* and *ectC*, (**Figure 3.12**). Among the above *ectA&B* (670 bp) (**Figure 3.12 A**) and *ectC* (429 bp) (**Figure 3.12 B**) expressing 14 transformants, only five transformants were chosen for secondary screening based on the *ectA&B* amplified band intensity (**Figure 3.12 A**). The same transformants *ectC* integration are highlighted in **Figure 3.12 B.**

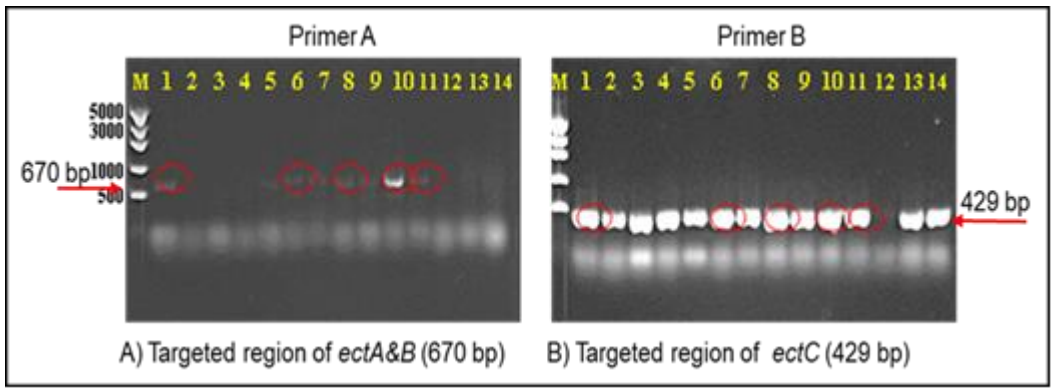


Figure 3.12. Primary screening of *ectABC* transformed *S. elongatus* PCC7942 for *ectA&B* and *ectC* inserts by colony PCR

Among 14 transformants, only five were selected for further confirmation of *ectABC* integration into *S. elongatus* PCC7942. The selected five transfromants were re-streaked (**as shown in supplementary material S.3**) on spectinomycin containing BG-11 agar plates and incubated for

three to four days. The colonies of the five transformants containing *ectA&B* and *ectC* were again amplified using the designed primers. This secondary screening further confirmed the successful integration of *ectABC* by *ectA&B* and *ectC* targeted region amplification using designed primers. **(Figure.3.13).**

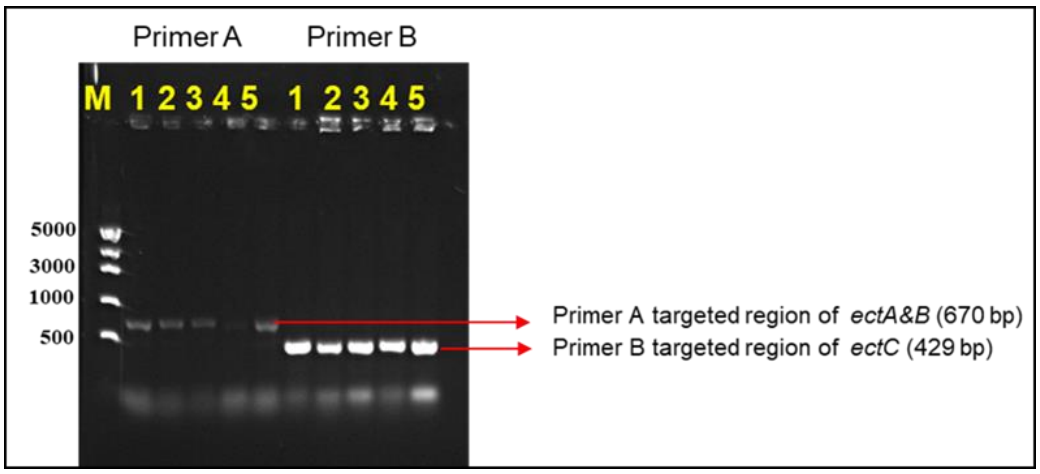


Figure 3.13. Secondary screening of *ectABC* transformants for the presence of *ectA&B* and *ectC* inserts

3.3.4 Temperature resilient pilot sudy

Temperature resilient pilot study revealed the dark blue-green coclour of GS001-GS004 on days 7, 14 and 20 were indicative of temperature-tolerance of *ectABC*-transformed *S. elongatus* PCC7942 (**Figure. 3.13**). The light green colour of transformant GS005 and the WT revealed sensitivity to temperature stress (**Figure. 3.14**). Wild-type *S. elongatus* PCC7942 and GS005 cultures were pale green/ yellowish on days 14

and 20, indicating a negative impact of temperature stress on those cultures. The *ectABC transformed S. elongatus* PCC7942 strain GS003 was chosen for further studies, as it appeared most resilient to 45ºC temperature stress (**Figure.3.14**).

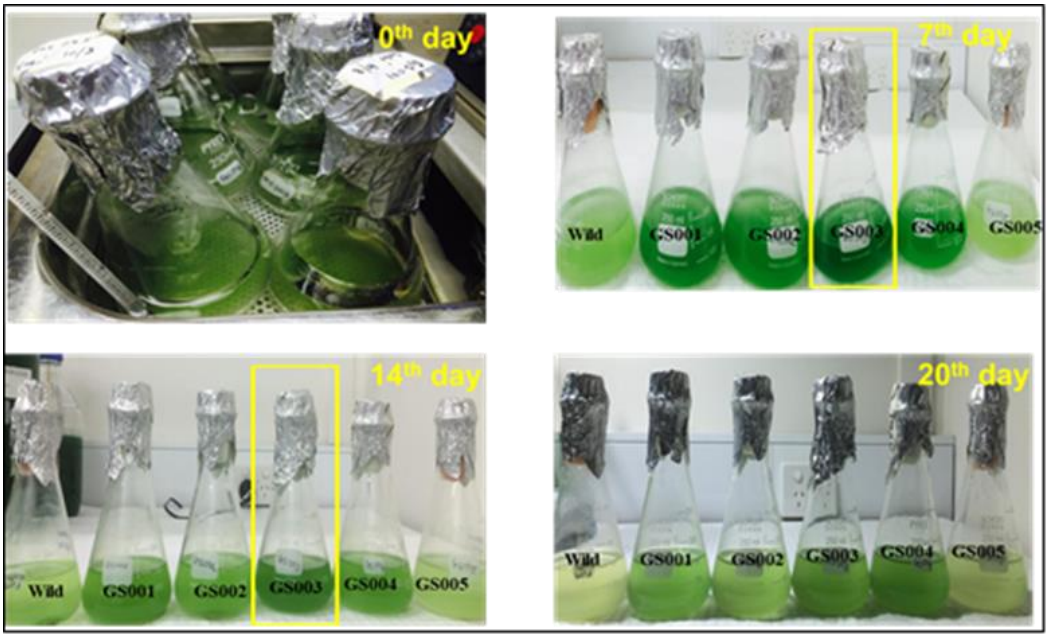


Figure 3.14. 20-day time course of growth performance of *ectABC*-transformed and WT *S. elongatus* PCC7942 at 45ºC

3.4 Discussion

Although microalgae and cyanobacteria have potential for genetic transformation, very few have been targeted for genetic manipulation to produce valuable products (Nesamma et al., 2015). Thus far, genetic manipulation of microalgae and cyanobacteria for abiotic stress

(temperature and salinity) resilience is limited due to the non-availability of a transformation protocol and the lack of publicly available genome information (León-Bañares et al., 2004). Moreover, ectoine synthesis is a complex mechanism, which involves three different genes organised as a single operon and both the codon optimization and cloning of this complex *ectABC* gene cassette into a vector that is suitable for further transformation into a host organism is complicated. In this study, optimization of appropriate codon usage for *S. elongatus* PCC7942 was used, so that the formation of secondary mRNA structures and it's folding in the ribosome binding site (RBS) and translation initiation region was avoided. In this case, the native gene contained tandem rare codons that could reduce the translation efficiency or even disengage the translational machinery. Codon optimization was used to overcome the codon usage bias in *S. elongatus* PCC7942 by upgrading the CAI from 0.82 to 0.95. G+C content and unfavorable peaks were optimized to prolong the mRNA halfilife of transformed microalgal cultures. The stem-Loop structures, which impact ribosomal binding and mRNA stability were removed during codon optimization. Our optimization process included screening and successfully modifying any negative cis-acting signals (listed in the results section).

After successful optimization, recombinant plasmid construction involved sub-cloning the *ectABC* cassette into an expression vector pMK –RQ and transformation into the *E. coli* K12 strain. The level of sequence identity was ensured to be 100 % in each step. Later, the transformation of the *ectABC_pSyn_6* vector into *S. elongatus* PCC7942 involved screening and selection of recombinants to confirm that the *ectABC* cassette integration was successful, thereby confirming the integration was successful. Random selection of spectinomycin resistant transformants grown on BG11 agar plates further confirmed the successful integration of *ectABC* by colony PCR using specific primers to amplify fragments of known size to identify both *ectA&B* and *ectC* in *ectABC* transformants. The intensity of the *ectA&B* band was weaker than for *ectC*. Several factors, such as low DNA concentration, differences in nucleotide composition, sequence length, G+C content ratio and melting temperature(Tm) or differing gel eclectrophoretic conditions for the amplicons (Corthell, 2014; Pandey et al., 2016). This may be the reason for primer set amplifying *ectA&B* being less efficient than primers designed to amplify *ectC* fragment.

Although previous studies of *ectABC* gene cassette transformation in several bacteria (primarily in *E. coli* and land based agricultural crops

such as tobacco and tomato) have been successful, this was not the case for microalgae (**Table 3.2**). Ectoine genes transformation into tomato and tobacco have been shown to increase salinity tolerance whereas the freshwater green microalga, *Chlamydomonas reinh*ardtii targeted choloroplast transformation was not successful. (**Table 3.2**). In addition, transfection of green freshwater microalgae using the *ectA* gene yielded no positive outcomes, as it targeted the chloroplast, relying on an *ectB* equivalent enzyme to carry out the first step for ectoine synthesis (**Table 3.2**).

Table 3.3. Model organisms transformed with etoine genes for ectoine accumulation

Species	Genes trasformed	Ectoine synthesis	Reference
E.coli DH5∞	*ectABC*	Positive	(Schubert et al., 2007a)
E.coli	*ectABC*	Positive	(Rajan et al., 2008)
E.coli K-12	*ectABC*	Positive	(He et al., 2015)
E.coli	*ectABC*	Positive	(Ning et al., 2016)
Tobacco	*ectABC*	Positivet	(Moghaieb et al., 2006)
Tomato	*ectABC*	Positive	(Moghaieb et al., 2011)
C.reinhardtii (Chloroplast transformation)	*ectA*	Negative	(Lunde, 2012)
C.reinhardtii (Chloroplast transformation)	*ectA and ectB*	Negative	(Afzal, 2014)
C.reinhardtii (Chloroplast transformation)	*ectA and ectC*	Negative	(Holck, 2014)
C.reinhrdtii (Chloroplast transformation)	*ectABC*	N.d	(Mwansa, 2017)

*N.d; Not detected

In this first of its kind study, the *ectABC* gene cassette was designed, optimized, cloned into the *pSyn_6* vector and then the *ectABC_pSyn_6* plasmid construct was successfully transformed into the cyanobacterium *S. elongatus* PCC7942 in a single transformation step. Although the demonstrated pilot study revealed that 4 out of 5 transformants were resilient to high temperature at 45°C, at 20 days post exposure, further studies are required to evaluate concurrent salinity and temperature stress resilience. Additionally, the promising outcomes with regards to temperature-resilience of *ectABC*-transformed *S. elongatus* PCC7942 could enable algal industries targeting c-phycocyanin (Jiang et al., 2017) as a high-value product to adopt such transformation procedures and to develop in areas with typicallty unsuitably high temperatures.

CHAPTER 4

4 Effect of temperature and salinity on growth performance and nutrient uptake of *ectABC*-transformed *S. elongatus* PCC7942 vs WT and *pSyn_6* empty vector controls

4.1 Introduction

Salinity and temperature affect outdoor industrial-scale microalgal or cyanobacterial cultivation (Linares et al., 2017; Mallick et al., 2016). Integration of microalgal cultivation with coal/ gas-fired power stations and biomass-burning sugar mills require salinity- and temperature-tolerant microalgae (Lohrey & Kochergin, 2012; Ono & Cuello, 2003). In general, most microalgae have narrow temperature optima, from 18 to 28°C (Heimann et al., 2015b). In open pond cultivation, water- and energy-wise microalgal biomass production is difficult to achieve, due to increase in salinity through evaporative water loss and seasonal temperature fluctuations (Berner et al., 2015; Heimann et al., 2015b). Investigating routes that infer both temperature- and salinity-tolerance to microalgae with a suitable biochemical profile and biomass productivity is of critical importance to the industry (Ono & Cuello, 2003). Ectoine is an osmolyte produced by extremophilic halo-tolerant bacteria and has been shown to infer tolerance to high salinity- and temperature-stress by acting as a compatible solute (Pastor et al., 2010). Ectoine-transformed

tomato (Moghaieb et al., 2006) and tobacco plants showed increased salinity-tolerance (Nakayama et al., 2000). Transfection of the green freshwater microalga *Chlamydomonas reinhardtii* with the *ectA* (Lunde, 2012) and *ectC* genes (Holck, 2014) yielded no positive outcomes, as it targeted the chloroplast, relying on an *ectB*-equivalent enzyme to carry out the first step for ectoine synthesis. This study concluded that ectoine synthesis might rely on all three three genes (*ectABC*) and insertion as an organised single cassette may also improve outcomes. Therefore, our previous study (chapter 3*)* ensured successfull integration of the *ectABC* gene cassette from *Halomonas elongata* DSM3043 into the freshwater cyanobacterium *S. elongatus* PCC7942. In this research, to ensure sustainable long-term microalgal production, we investigated, whether transformation with the *ectABC* gene cassette could impart salinity- and temperature-resilience in *Synechococcus elongatus* PCC7942. We also assessed the effect of *ectABC*-transformation on nutrient requirements and biochemical profiles by comparing *ectABC*-transformed *versus* WT and empty vector controls of *S. elongatus* PCC7942.

4.2 Materials and Methods

4.2.1 Culture growth condition

Wild-type *Synechococcus elongatus*, *S. elongatus* PCC7942_*ectABC* (GS003) and *pSyn_6* empty vector-transformants were chosen based on results presented in chapter 3 (*ectA&B* and *ectC* amplification). Wild-type *S. elongatus* PCC7942 and the *pSyn_6* (empty vector control) transformed *S. elongatus* PCC7942 were used to ensure that observed temperature- and salinity-tolerance of the *ectABC*-transformants was truly the consequence of *ectABC*-expression. Cultures were grown in 250 mL Erlenmeyer flasks containing freshly sterilized BG11 liquid medium at 34ºC ± 1ºC with a photoperiod of 12:12 h and an irradiation of 50–100 µE m^{-2} s^{-1} as instructed by supplier manual[1] at the North Queensland Algal Identification / Culturing Facility (NQAIF) (James Cook University, Townsville).

4.2.2 Experimental set up for factorial temperature- and salinity-tolerance experiment

Synechococcus elongatus PCC7942-*ectABC* GS003, *S. elongatus* PCC7942_*pSyn-6* and WT *S. elongatus* PCC7942 were grown in 1 L aerated batch cultures in BG11 medium and acclimated to three

[1] (http://tools.thermofisher.com/content/sfs/manuals/geneart_synechococcus_engineering_man.pdf)

different salinities (0, 18 and 36 ppt) and temperatures (35, 40 and 45°C) for 20 days. For culture medium salinity adjustments, BG11 medium was prepared in de-ionized water for 0 ppt, while for sea water-based BG11, the seawater was filtered through 0.45 µm (Whatman GF/C, Durapore, and Millipore). For a salinity of 18 ppt, the sea water was diluted with deionized water (50:50 v/v), while the sea water was used undiluted for a salinity of 36 ppt. Salinity- and temperature-acclimatized *S. elongatus* PCC7942_*ectABC*, *S. elongatus* PCC7942_*pSyn_6* and WT *S. elongatus* PCC7942 were cultivated at the nominated salinities and temperatures for 7 days using the above lighting and photo-period specifications.

4.2.3 Dry weight and ash-free dry weight determination

Dry weight and ash-free dry weight analyses followed standard procedures. 20 mL of cultures samples were harvested and centrifuged (Eppendorf, 5810R) at 21952 RCF for 20 min at room temperature. The supernatant was preserved for nutrient consumption analyses, while the pellets were resuspended in 5 mL of BG-11 medium and transferred to pre-ashed and pre-weighed 5 mL beakers before drying for 48 h at 100ºC. After cooling to room temperature in a dessicator, dry weights were calculated by subtracting the pre-ashed beaker weight

from the dried biomass in the beakers. Dried samples were then subjected to ashing at 500ºC for 24 h. After cooling to room temperature in a dessicator. The ash content was calculated by subtracting the pre-ashed beaker weight from the ash-beaker weight. Ash-free dry weight was determined by subtracting the ash content of the biomass from the dry weights obtained previously.

4.2.4 Growth rate

Specific growth rates and population growth rates were determined following von Alvensleben et al. (2015). Growth rate was calculated by using the formula,

$$\text{Growth rate } (\mu) = \ln (DW_2 - DW_1) / (t_2 - t_1) \quad \text{eq. 2.1}$$

Where, DW_2 and DW_1 = final and initial ash-free dryweight; t_1 and t_2= initial and final timepoints [days], respectively.

4.2.5 Nutrient uptake

Nutrient uptake was measured to identify the fertilisation requirements of *S. elongatus* PCC7942_*ectABC* GS003, *S. elongatus* PCC7942_*pSyn-6* and WT *S. elongatus* PCC7942 for a period of 7 days. To quantify nutrient uptake, 5 mL culture samples were centrifuged at 3000 × g at 20°C for 20 min at room temperature

(Eppendorf, 5810R), followed by 0.45 µm filtration (Durapore; Millipore Kilsyth) of the supernatant, to remove any suspended material. Supernatants were stored at -80°C for later use. Medium nitrate, nitrite and phosphate concentrations were measured following von Alvensleben et al. (2015) .

4.2.6 Total Lipids

Total lipids were measured gravimetrically following von Alvensleben et al. (2015). Percentage of total lipids were calculated by using solvent (hexane) extracts in a pre-weighed galss vial dried under nitrogen gas and weighed to determine total lipids.

4.2.7 Fatty acid content

Fatty acids extraction and analysis were carried out at the Australian Institute of Marine Science, Townsville following von Alvensleben et al. (2015). Identification and quantification of fatty acids was carried out with an Agilent 7890 GC equipped with a flame ionization detector (FID) and connected to an Agilent 5975C Electron Ionisation (EI) Turbo Mass Spectrometer (Agilent Technologies Australia Pty Ltd). Quantification of FAMEs determined by comparison of peak areas of authentic external standards (Sigma Aldrich), and was corrected for

recovery of internal standard (C19:0). Total fatty acid content was determined as the sum of all FAMEs.

4.2.8 Quantification of ectoine

Quantification of secreted and intracellular ectoine was carried out at the Australian Institute of Marine Science (AIMS) in Townsville. WT, empty vector controls (*pSyn_6*), and temperature- or salinity-tolerant *ectABC*-transformed *S. elongatus* PCC7942 were analysed for intracellular and extracellular ectoine using standards from Sigma following Bergmann et al. (2013) and Ning et al. (2016). Firstly, one mL of liquid culture was centrifuged at 13,000 rpm for 2 min. The supernatant was then transferred to another Eppendorf tube and diluted for extracellular ectoine detection. The remaining pellet was resuspended with water, centrifuged for 10 min at 14,000 g at room temperature. For cell disruption, 120 mg of polished glass beads (diameter 0.1 mm) and 500 µL of a methanol/chloroform/water solution (volume ratio 10:5:4) was added to the pellet and treated in a bead beater for 4 min at 4.0 m s^{-1}. Subsequently, 130 µl chloroform and 130 µl H_2O were added, followed again by 1 min homogenization at 4.0 m s^{-1}. The suspension was centrifuged for 10 min at 14,000 g at room temperature to achieve two phase separation. The aqueous phase was

collected for HPLC analysis and the pellet was resuspended in water equal to the removed supernatant, and then treated with ultrasound to break up cells that were not disrupted by bead beating to release remaining intracellular ectoine. Samples for intracellular ectoine resulting from the bead beating and ultrasound treatments were combined for the analysis of intracellular ectoine. All samples were diluted to make the concentration of ectoine between 0.01~0.1 g/L and filtered through a 0.22 μm microporous filter before injection into the HPLC (Thermo Scientific UltiMate 3000) fitted with a TSK-GEL C18 column (TOSOH, Japan). Ectoine concentration of the samples was quantified by isocratic HPLC with an acetonitrile/water mixture (2:98 v/v) at a flow rate of 1 mL min^{-1} as the mobile phase. Ectoine was monitored by a UV detector at a wavelength of 210 nm. The sample injection volume was 20 μL with column temperature was 30°C. To confirm ectoine expression, intracellular fractions collected from HPLC were further analysed by mass spectrophotometry analysis.

4.2.9 Mass spectrometry analysis

Fractions containing intracellular ectoine, collected at R_t = 3.8 min from the HPLC, were used for mass spectrometry analysis. Low-resolution mass spectral data were measured on a Bruker Daltonics Esquire

3000plus quadrupole ion trap electrospray ionisation mass spectrometer (ESI-MS) with an Apollo source operating at 40 eV. Direct infusion of the sample (~0.1 mg mL^{-1}) was carried out using a Cole Palmer 74900 syringe pump at a flow rate of 150 µL h^{-1}. All MS data were collected using Bruker Daltonics Esquire Control v5.3 and Hystar v3.1 software operating on Windows XP Professional.

4.2.10 Statistical analysis

To ascertain significance of ectoine transformation on growth (population growth rate r), nutrient uptake, total fatty content and profiles and ectoine production, statistical analyses were preformed using ANOVA in Statistica 13.2 (Statsoft). Alpha was set to 0.05 and data were log-transformed when assumptions of homogeneity of variance and normality were not met. Homogeneity of variance was analysed using the Cochran C or Levene's tests (as indicated in the statistics tables in the Appendix to this chapter), while normality was assessed using P-P plots, Shapiro Wilk tests and histograms, as indicated in the statistical figures and tables in the Appendix to this chaper. A Tukey's post hoc analysis was performed to determine drivers of significance. Significance of the effect of salinity on intra- and extracellular, as well as total ectoine concentrations of *ectABC*-transformed *S. elongatus* PCC7942 was

determined via t-test (Statistica 13.2, Softstat) and variance was determined using Box and Whisker plots of the salinity groups of 0 and 18 ppt.

4.3 Results

4.3.1 Growth performance

A 7-day growth time-course experiment showed that ectoine transformation of *S. elongatus* PCC7942 resulted in improved temperature-tolerance up to 45°C and salinity-tolerance up to 18 ppt at permissible temperature of 35°C, compared to WT and empty vector controls (**Figure 4.1**). Very low growth, resulting from the first 2 days of cultivation only, was observed for WT, *pSyn_6* and *ectABC*-transformants at a salinity of 36 ppt, irrespective of temperature or at 18 ppt at elevated temperatures.

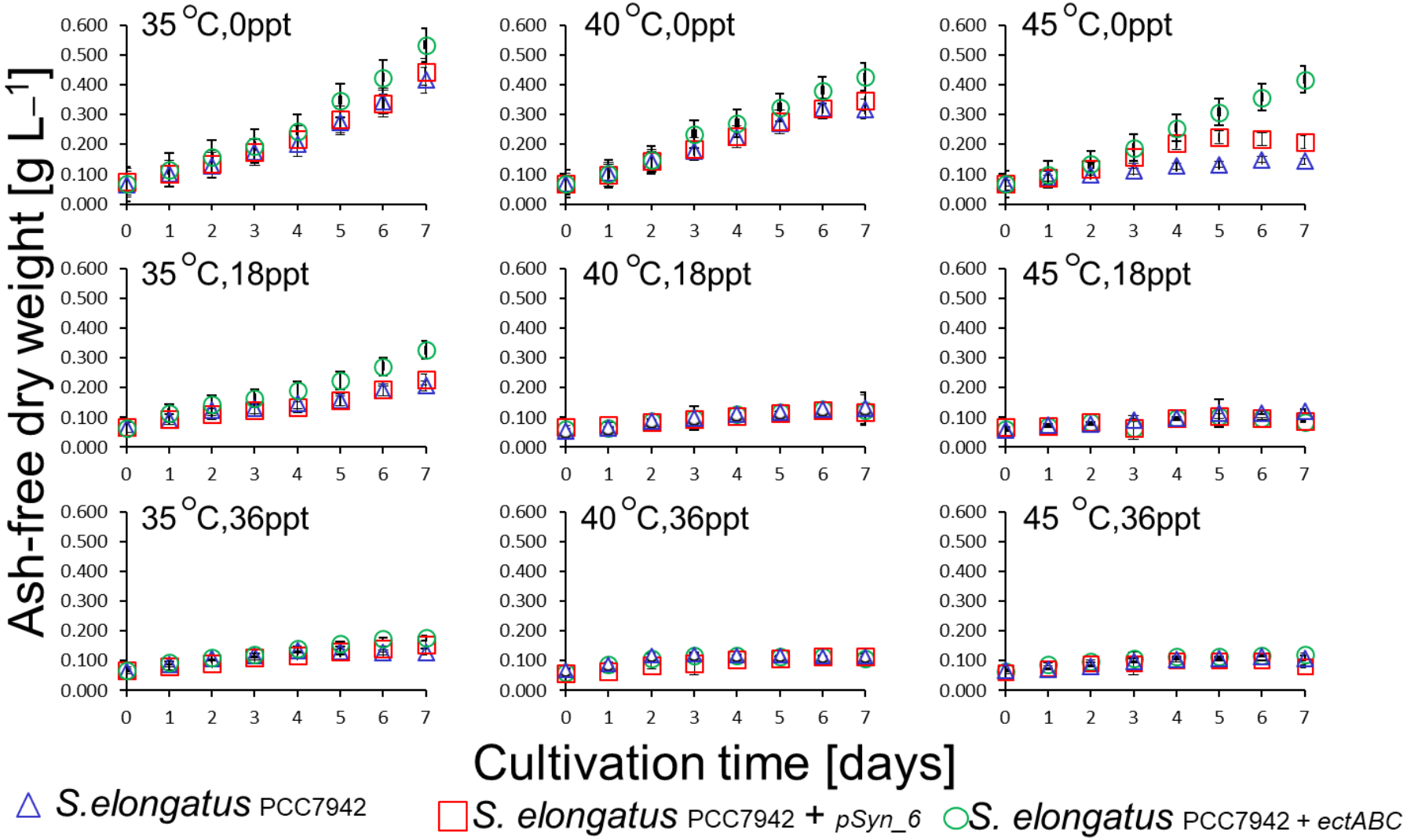


Figure 4.1 Effect of temperature and salinity on growth performance (ash-free dry weight) of wild-type, empty vector control (*pSyn_6*) and ectoine-transformed (*ectABC*)-*Synechococcus elongatus* PCC7942

4.3.2 Population Growth rate

Population growth rates (*r*) were highest at permissible temperature for the *ectABC*-transformants and *r* was comparable to the growth of WT and *pSyn_6* controls (**Table 4.1**). Population growth rates remained high at 40 and 45°C for the *ectABC*-transformants, comparable to performance at the permissible temperature, but were approximately halved for WT and *pSyn_6* controls at the challenging temperature of 45°C (**Table 4.1**). Combined stress of elevated temperature and increased salinity affected growth performance of the *ectABC*-transformants to a similar degree as WT and empty vector (*pSyn_6)* controls, except for the *ectABC*-transformants under moderate salinity stress and permissible temperature. Population growth rates at 35°C and 18 ppt salinity of the *ectABC*-transformant were comparable to those of the WT and *pSyn_6* cultures at 40°C without salinity stress (**Table 4.1**).Population growth rate was normally distributed for groups ($p = 0.077$; **Appendix Table A4.1**) (**Appendix Fig. A4.1**). Likewise, variances were homogenous for all groups (strain, salinity and temperature) (**Appendix Table A4.1**). A factorial ANOVA on the significance of strain, salinity and temperature determined that these variables had a significant effect on *r* ($F_{(2,54)} = 87.147$; $p = < 0.0001$;

$F_{(2,54)}$ = 655.230; p < 0.0001; $F_{(2,54)}$ = 241.872; p < 0.0001) and there was significant interaction of “strain*salinity” ($F_{(4,54)}$ = 11.357; p = 0.000001), “strain*temperature” ($F_{(4,54)}$ = 6.509; p = 0.000238, “salinity*temperature” ($F_{(4,54)}$ = 19.151; p < 0.0001), “strain*salinity*temperature” ($F_{(8,54)}$ = 10.579; p < 0.0001) (**Appendix Table A4.2**). A Tukey’s post hoc analysis confirmed that significance was driven by the higher population growth rate of the *ectABC*-transformed *S. elongatus* PCC7942 at the highest temperature and a salinity of 18 ppt, whilst population growth rates of *pSyn-6* controls and WT were largely not significantly different to each other (**Appendix Table A4.3**).

4.3.3 Nutrient uptake

The effect of *ectABC* transformation on nutrient uptake -nitrite-corrected nitrate (**Figure 4.2**) and phosphate uptake (**Figure 4.3**) was investigated in WT, empty vector control (*pSyn_6*) and *ectABC*-transformants of *S. elongatus* PCC7942 at salinities and temperatures for which growth was observed. Measurements of media nitrite-corrected nitrate levels and phosphate levels showed that neither medium nitrate nor medium phosphate concentrations limited uptake

(**Appendix Figure. A4.2**), thereby excluding that observed nutrient utilisation patterns were determined by nutrient availability.

A 7-day time course experiment on nitrite-corrected nitrate uptake standardised for biomass increase (mg g^{-1} AFDW) showed that elevated salinity (18 ppt) led to 4-5 times higher nitrogen uptake (**Figure 4.2**). Especially *ectABC*-transformants of *S. elongatus* PCC7942 showed a significantly higher nitrite-corrected nitrate uptake during the first 5 days compared to empty vector (*pSyn_6*) and WT controls (**Figure 4.2**). In contrast, at a salinity of 0 ppt, lowest uptake was observed at 40°C and no difference in uptake was observed for WT, *pSyn_6* and *ectABC*-transformants at 35 and 40°C, while at 45°C uptake was highest in WT and lowest in *ectABC*-transformants on day 7 (**Figure 4.2**).

Total biomass-increase standardized nitrite-corrected nitrate uptake was almost 2 to 3-fold higher in WT *S. elongatus* PCC7942 at 45°C at 0 ppt and 35°C and 18 ppt salinity, respectively, compared to uptake under control temperatures and salinity (**Table 4.1**). Likewise, total nitrogen uptake was also 2 to 3-times higher for *pSyn_6* controls and *ectABC*-transformants when challenged with salinity stress of 18 ppt, but were only moderately increased by temperature stress (45°C) for

pSyn_6 controls and no increase was observed for *ectABC*-transformants (**Table 4.1**).

The effect of temperature and salinity on nitrite-corrected biomass-standardised uptake was analysed in two separate factorial ANOVA analyses, as uptake was only evaluated for actively growing cultures. Based on the observed nitrite-corrected biomass-standardised uptake patterns, data for temperature effect on total nitrite-corrected nitrate uptake were not expected to be homogeneously distributed, but homogeneity of variance was met for 'strain*temperature" (**Appendix Table A4.3**). Likewise, while total nitrite-corrected nitrate uptake was not normally distributed (Shapiro-Wilks test: W = 0.90684' p = 0.019; **Appendix Figure. A4.2**), the histogram showed a sufficiently normal distribution for ANOVA analysis.

The ANOVA determined that strain ($F_{(2,17)}$ = 29.36; $p < 0.0001$) and temperature ($F_{(2, 17)}$ = 10.07; p = 0.0013) significantly affected total nitrite-corrected nitrate uptake and 'strain*temperature' showed significant interaction ($F_{(4,17)}$ = 6.93; p = 0.0017) (**Appendix Table A4.5**). A Tukey's post hoc analysis determined that significance was driven by WT *S. elongatus* PCC7942 showing significantly different nitrite-corrected nitrate uptake at 45°C, compared to *pSyn_6* and

ectABC-transformants (**Appendix Table A4.6**), confirming the 7-day nitrite-corrected nitrate uptake behaviour (**Figure 4.2**).

A Cochran C test showed that total nitrite-corrected nitrate uptake was homogeneously distributed for 'strain' and 'salinity*strain', but, as expected, not for salinity (**Appendix Table A4.7**) and data were not normally distributed (Shapiro-Wilk test: W = 0.80671; p = 0.00189; **Appendix Figure A4.3**). The ANOVA determined that strain ($F_{(2,12)}$ = 10.68; p =0.002) and salinity ($F_{(1,12)}$ = 646.4; p < 0.0001) had a significant effect and the interaction 'salinity*strain' ($F_{(2,12)}$ = 6.31; p = 0.013) was also significant (**Appendix Table A4.8**). A Tukey's post hoc analysis determined that total nitrite-corrected nitrate uptake of WT *S. elongatus* PCC7942 at 0 ppt salinity was significantly different *to pSyn_6* controls and *ectABC*-transformants, while *pSyn_6* was not significantly different to *ectABC*-transformants at the individual salinities (**Appendix Table A4.9**), confirming the 7-day time course uptake data (**Figure 4.2**).

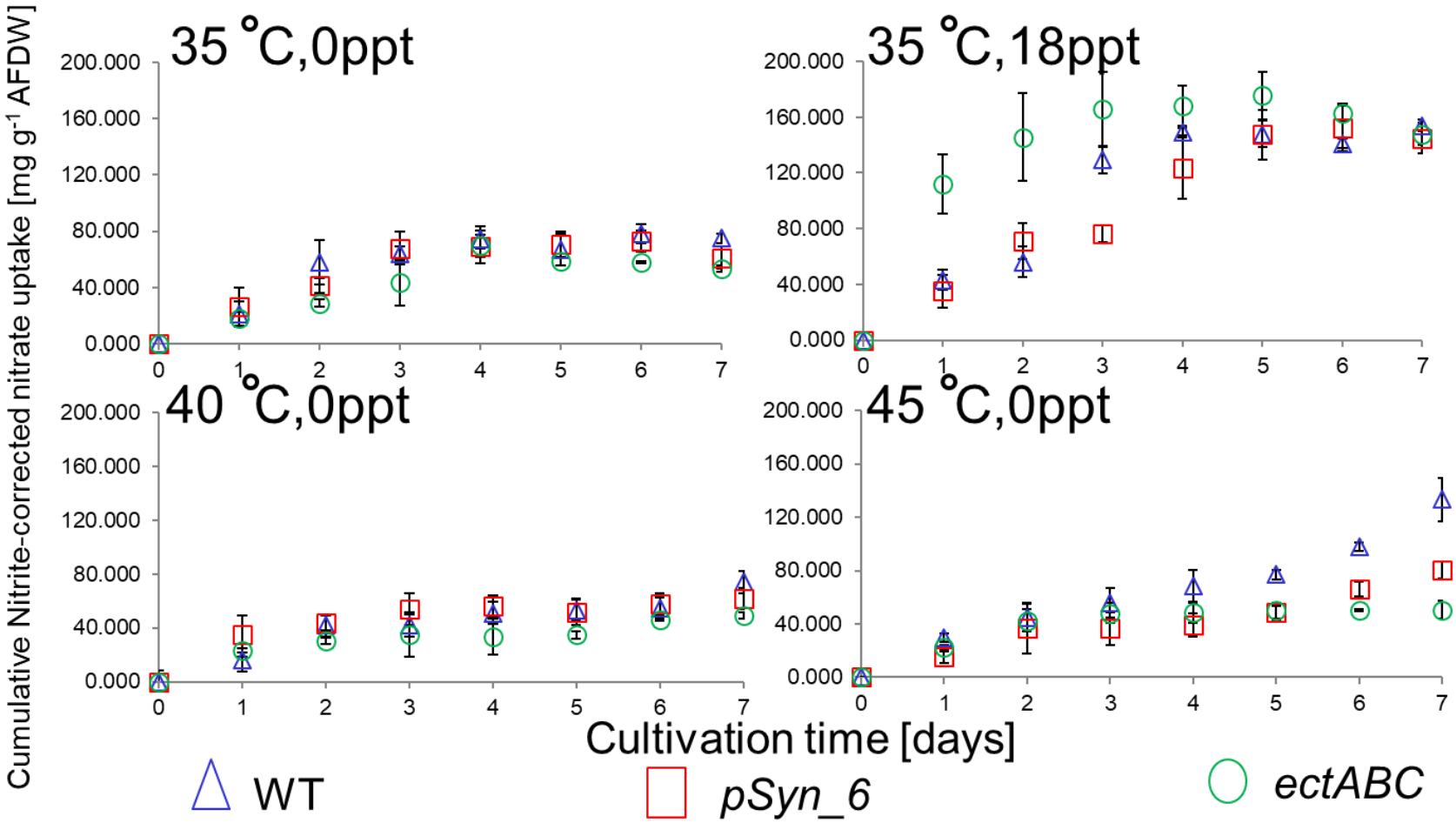


Figure 4.2. Effect of temperature and salinity on biomass-standardised cumulative nitrite-corrected nitrate uptake of wild-type (WT), empty vector control (*pSyn_6*) and ectoine-transformed (*ectABC*) *Synechococcus elongatus* PCC7942

Similar to nitrite-corrected nitrate uptake, ash-free dry weight-standardised phosphate uptake was significantly higher under elevated salinity stress (18 ppt) at permissible temperature, but highest uptake was observed in WT and lowest in *ectABC*-transformants on day 7 (**Figure 4.3**). Like for nitrogen uptake, at a salinity of 0 ppt, temperature stress (45°C) also induced higher phosphate uptake in WT and the empty vector (*pSyn_6*) control, whilst phosphate uptake of the *ectABC*-transformant was not different to that observed at 35 and 40°C, with no difference in phosphate uptake was observed for WT, *pSyn_6* and *ectABC*-transformants for the latter two temperatures (**Figure 4.3**). Total phosphate uptake was 2 to >3-fold higher in WT *S. elongatus* PCC7942 challenged with high temperature and salinity stress at permissible temperature, respectively (**Table 4.1**). Salinity and temperature stress also elevated total phosphate uptake of *pSyn_6* controls and *ectABC*-transformants, but to a lesser degree, with lowest total phosphate uptake observed for the *ectABC*-transformants under these stress conditions (**Table 4.1**).

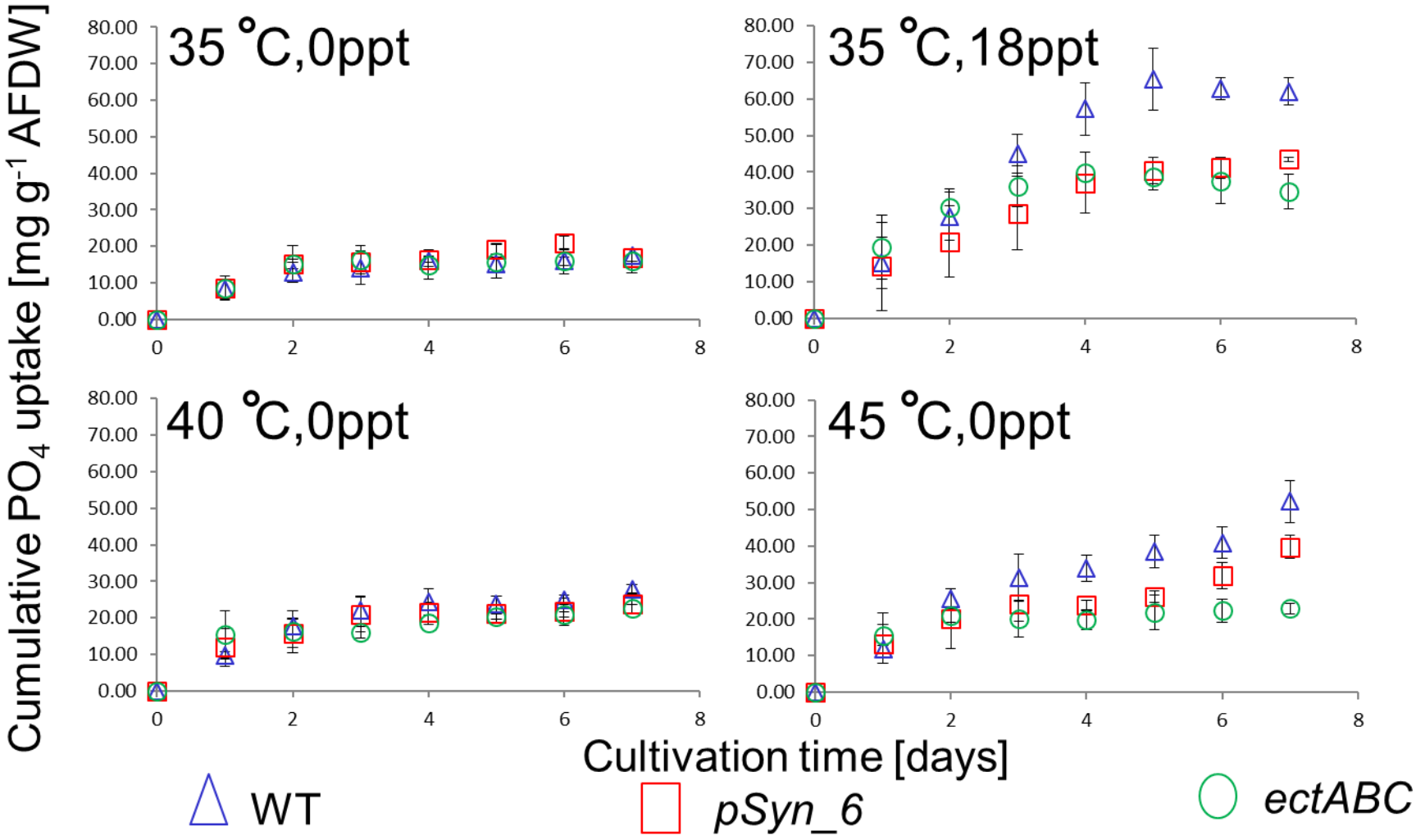


Figure 4.3 Effect of temperature and salinity on biomass-standardised cumulative phosphate uptake of wild-type (WT), empty vector control (*pSyn_6*) and ectoine-transformed (*ectABC*) *Synechococcus elongatus* PCC7942

The significance of temperature and salinity on phosphate uptake was analysed in two separate factorial ANOVAs, as uptake data were limited to actively growing cultures. As expected based on the 7-day time course uptake studies, total phosphate uptake showed homogeneous variances for 'strain' and 'temperature' (**Appendix Table A4.3**) and a normal distribution (Shapiro-Wilk test: W = 0.94094; p = 0.12852, **Appendix Fig. A4.3**). A factorial ANOVA determined significance of 'strain' ($F_{(2,17)}$ = 4.037; p = 0.037), 'temperature' ($F_{(2,17)}$ = 16.862; p < 0.0001) and a significant interaction of 'strain*temperature' ($F_{(4,17)}$ = 3.55; p = 0.028) (**Appendix Table A4.10**). A Tukey's post hoc analysis determined that significance on total phosphate uptake was driven by WT *S. elongatus* PCC7942 at 45°C, which was, however, not significantly different to 40°C for itself or the *pSyn_6* empty vector control, but significantly different to *ectABC*-transformants. Phosphate uptake by WT at 40°C was not significantly different to either the *pSyn_6* controls or the *ectABC*-transformant for any temperature (**Appendix Table A4.13**).

A Cochran C test determined that variances were homogeneously distributed for total phosphate uptake at the two salinities (0 and 18 ppt) (**Appendix Table A4.7**), but marginally failed the Shapiro-Wilk test for

normality (W = 0.89173; p = 0.04132; **Appendix Fig. A4.3**). A factorial ANOVA determined a significant effect of 'strain' ($F_{(1,12)}$ = 11.41; p = 0.0016) and 'salinity' ($F_{(2,12)}$ = 294.65; p < 0.0001) and 'strain' showed significant interaction with 'salinity' ($F_{(2,12)}$ = 6.18; p = 0.014) (**Appendix Table A12**). A Tukey's post hoc analysis determined that total phosphate uptake was not significantly different for the different strains at 0 ppt salinity, but total phosphate uptake of WT *S. elongatus* PCC7942 differed significantly from uptake by *pSyn_6* empty vector controls and *ectABC*-transformants at 18 ppt, while total phosphate uptake of *ectABC*-transformants was not significantly different to *pSyn_6* controls at that salinity (**Appendix Table A4.13**).

Table 4.1 Effect of temperature and salinity on population growth rate (*r*) and total uptake of biomass- standardised nitrite – corrected nitrate and phosphate in WT, *pSyn_6* and *ectABC* – transformed *Synechoccous elongatus* PCC7942.

Salinity [ppt]		**0**			**18**			**36**		
Temperature [°C]		**35**	**40**	**45**	**35**	**40**	**45**	**35**	**40**	**45**
Population growth rate (r)[day^{-1}]										
Strains	WT	0.263 ± 0.008	0.221 ± 0.002	0.105 ± 0.015	0.143 ± 0.036	0.112 ± 0.018	0.045 ± 0.014	0.096 ± 0.051	0.078 ± 0.032	0.086 ± 0.008
	pSyn_6	0.256 ± 0.013	0.233 ± 0.002	0.158 ± 0.015	0.172 ± 0.007	0.079 ± 0.035	0.047 ± 0.017	0.117 ± 0.024	0.097 ± 0.004	0.037 ± 0.011
	ectABC	0.289 ± 0.013	0.271 ± 0.007	0.269 ± 0.010	0.228 ± 0.006	0.133 ± 0.011	0.097 ± 0.006	0.141 ± 0.064	0.08 ± 0.021	0.082 ± 0.016
Total nitrite – corrected nitrate uptake [mg g^{-1} AFDW]										
Strains	WT	74.8 ± 3.2	74.2 ± 3.9	133.9 ± 16.1	153.8 ± 4.2	0	0	0	0	0
	pSyn_6	61.2 ± 7.9	62.0 ± 6.6	80.3 ± 6.0	144.8 ± 11.1	0	0	0	0	0
	ectABC	53.5 ± 2.4	49.3 ± 11.1	50.6 ± 6.5	147.9 ± 7.5	0	0	0	0	0
Total phosphate uptake [mg g^{-1} AFDW]										
Strains	WT	17.4 ± 1.6	27.8 ± 1.1	52.1 ± 5.6	61.9 ± 3.7	0	0	0	0	0
	pSyn_6	16.9 ± 2.3	23.8 ± 2.8	39.7 ± 3.1	43.4 ± 0.6	0	0	0	0	0
	ectABC	16.07 ± 3.3	22.5 ± 1.12	22.8 ± 1.3	34.6 ± 4.6	0	0	0	0	0

*Total nitrate and phosphate uptake was not measured for strains that showed significantly lower growth rates under combined stress of high salinity and temperature (18 ppt at 40°C and 45°C, and 36 ppt at 35°C, 40°C and 45°C).

4.3.4 Total lipid

Lipid content was highest in *ectABC*-transformants of *S. elongatus* PCC7942 under salinity stress at permissible temperature, followed by the empty vector control *pSyn_6* for the same cultivation conditions, whilst contents were lowest and comparable in both transformants without salinity stress at the permissible temperature (**Fig. 4.4**). Temperature stress (40 and 45°C) increased total lipid content to a similar extent for both transformants (**Fig. 4.5**).

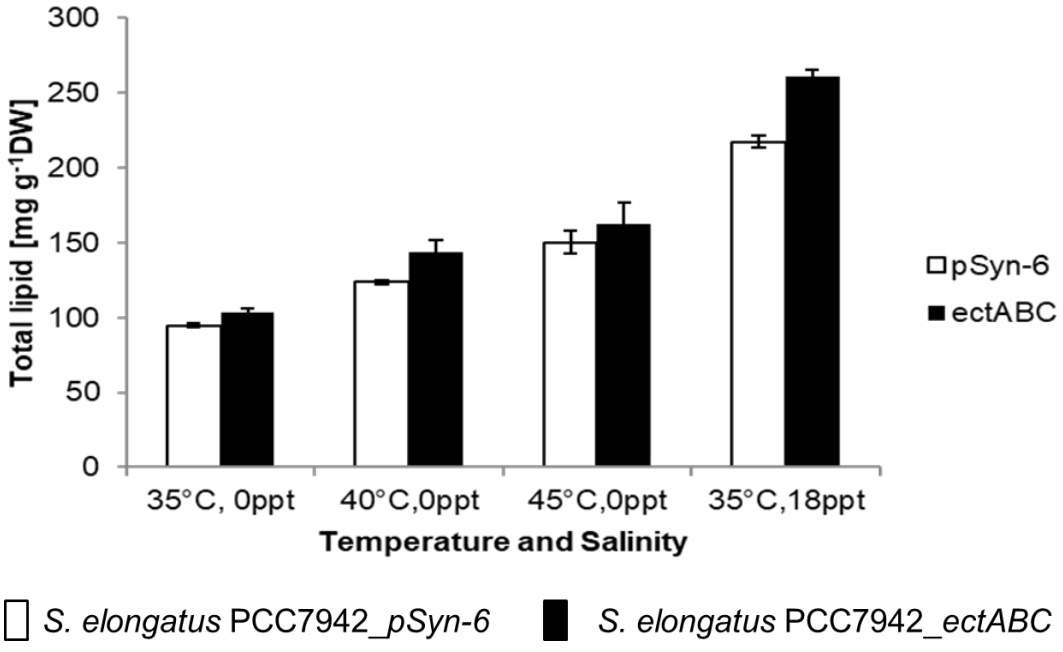


Figure 4.4 Effect of salinity and temperature on total lipid content of *ectABC*-transformed *S. elongatus* PCC7942 and the empty vector control *pSyn_6.*

Data for total lipids were normally distributed (**Shapiro-Wilks test, Appendix Table A4.14**) and variances were homogeneous for strain but not for temperature ($F_{(2,15)}$ = 5.52; p = 0.016) (**Leven's test, Appendix Table A.15)**. A factorial ANOVA showed that temperature and strain significantly affected total lipids ($F_{(2,12)}$ = 36.04; p <0.0001 and $F_{(1,12)}$ = 13.91; p = 0.003, respectively) (**Appendix Table A4.16)**. A Tukey's post hoc confirmed that total lipid contents of *ectABC*-transformants of *S. elongatus* PCC7942 were not significantly different to each other or the empty vector control at 40 and 45°C, but that WT was significantly different to both transformants at 35°C and to *pSyn-6* at 40°C (**Appendix Table A4.17**). Similarly, while data were normally distributed for the effect of salinity (**Appendix Table A4.18**), data on total lipid content were not homogeneously distributed for the effects of 'strain' and 'salinity' ($F_{(1,10)}$ = 6.14; p = 0.032 and $F_{(1,10)}$ = 7; p = 0.025, respectively) (**Levene's test, Appendix Table A4.19**). A factorial ANOVA showed that 'strain' ($F_{(1,8)}$ = 34.7, p 0.0004) and 'salinity' ($F_{(1,8)}$ = 1539.1; p < 0.0001) had a significant effect on total lipid content, but the interaction of 'strain*salinity' was not significant (**Appendix Table A4.20**). A Tukey's post hoc confirmed that total lipids content were significantly different at a salinity of 18 ppt for *ectABC*-transformants and *pSyn_6* empty vector

controls, and between both strains, while total lipid content of the *ectABC* –transformant was not significantly different to the empty vector control at a salinity of 0 ppt (**Appendix Table A4.21**).

The effect of temperature and salinity on fatty acid content and profile was also investigated (**Tables 4.2. and 4.3**). Total fatty acid (Σtot FA), the sum of saturated fatty acids (ΣSFA) and of mono-unsaturated fatty acid (ΣMUFA) contents [mg g^{-1} AFDW] were highest in *ectABC*-transformed *S. elongatus* under 18 ppt salinity stress (**Table 4.3**), while it did not affect the percent contribution to ΣSFA (**Fig. 4.5A, Appendix Table 4.22**). Temperature stress of 45°C also elevated levels (**Table 4.2**), with highest percent contribution to Σtot FA achieved at 40°C for ΣSFA (61.2%) and ΣMUFA at 45°C (49%) (**Figure 4.5A, Appendix Table 4.22**). Contents were lowest in empty vector *pSyn_6* controls at 45°C, but represented the highest contribution to Σtot FA at 45 and 40°C, representing 72% and 46% for ΣSFA and ΣMUFA, respectively (**Figure 4.5A, Appendix Table 4.22**). Percent contribution and amounts were comparable at 35°C without salinity stress for *ectABC*-transformants and *pSyn_6* controls **(Fig.4.5 A and B and Appendix Table 4.22, and Table 4.22**). Total fatty acid, ΣSFA and ΣMUFA contents were similar in the empty vector *pSyn_6* control at 35°C and

both salinities and comparable to contents of the *ectABC*-transformant at a salinity of 0 ppt (**Table 4.3**). These data suggest that temperature and salinity affect fatty acid profiles in the *ectABC*-transformed *S. elongatus* PCC7942. To determine significance, univariate ANOVAs were carried out for the effect of temperature and salinity. A Shapiro Wilk test and the Levene's test showed that log-transformed total fatty acid contents (Σtot FA), the sums of saturated fatty acids (ΣSFA) and monounsaturated fatty acids (ΣMUFA) were normally distributed with homogeneous variances for *pSyn-6* empty vector controls and *ectABC*-transformed *S. elongatus* PCC7942 at the different growth temperatures of 35 (permissible temperature), 40 and 45°C (**Appendix Tables A4.14 and 4.15)** for the effect of temperature. Likewise, data were normally distributed and variances were homogenous for the effect of salinity for *ectABC*-transformants and *pSyn_6* empty vector controls of *S. elongatus* PCC7942 (**Appendix Table A4.18 and 4.19**). The factorial ANOVAs showed that neither temperature nor salinity had a significant effect (data not shown).

Irrespective of temperature or salinity, Palmitic acid (C16:0) and Palmitoleic acid (C16:1) were by far the most dominant fatty acids of the fatty acid profile of *S. elongatus* PCC7942 either transfected with

ectABC or the *pSyn_6* empty vector, whilst Heptadecanoic acid (C17:0) was present in minute (barely detectable levels) and poly-unsaturated fatty acids were not detected (**Tables 4.2 and 4.3**). Amounts [mg g^{-1} AFDW] were mirrored in the percent contribution to Σtot FA of around 50 and 40% and 0.1%, respectively, at the control temperature of 35°C and control salinity (0 ppt) (**Fig. 4.5A**, **Appendix Table A4.22**). Low concentrations of Myristic acid (C14:0), Stearic acid (C18:0) and moderate levels of Oleic acid (C18:1) were also detected (**Tables 4.2 and 4.3**) contributing with 1.1, 0.6 – 1.0, and 2.8 – 5.4% to Σtot FA at control temperature and salinity, respectively (**Fig. 4.5B**, **Appendix Table A4.22**).

In general temperature stress of 45°C led to highest amounts of C16:0, C16:1, C17:0 in *ectABC*-transformed *S. elongatus* PCC7942, but percent contribution to Σtot FA was only highest at 45°C for C16:1 (47%, **Fig. 4.5A**, **Appendix Table A4.22**), whilst it was highest at 40°C (58%) for C16:0 (**Fig. 4.5A**, **Appendix Table A4.22**). Lowest amounts [mg g^{-1} AFDW] of these fatty acids were observed in the empty vector *pSyn_6* control (**Table 4.2**), but percent contribution to Σtot FA was highest for C16:0 (67%) at 45°C, whilst it was lowest for C16:1 (23%) at that temperature (**Fig. 4.5A**, **Appendix Table A4.22**). Salinity stress of 18

ppt had an even stronger effect with highest levels of C16:0, C16:1, C17:0 and C18:1 detected, but, dissimilar to temperature stress, the fatty acid profile of the empty vector control *pSyn_6* was not affected by salinity stress, except for C17:0, which was not detected (**Table 4.3**). While salinity stress did not affect percent contribution of C16:0 and C16:1 to Σtot FA for the *pSyn_6* empty control, C16:1 (43%), but not C16:0 percent contribution to Σtot FA was increased under salinity stress in the *ectABC*-transformant of *S. elongatus* PCC7942 (**Fig. 4.5A**, Appendix **Table A4.22**).

Table 4.2. Effect of temperature on total fatty acids, fatty acid profiles and levels of saturated-(SFA) and mono-unsaturated fatty acids (MUFA) in *ectABC*-transformed *S. elongatus* PCC7942 and the *pSyn_6* empty vector.

	***ectABC*-transformed *S. elongatus* PCC7942**			***pSyn_6* empty vector control**		
Fatty acid	**35°C**	**40°C**	**45°C**	**35°C**	**40°C**	**45°C**
C14:0	0.76 ± 0.19	0.62 ± 0.27	0.29 ± 0.03	0.35 ± 0.07	0.33 ± 0.07	0.37 ± 0.14
C16:0	30.4 ± 19.8	22.7 ± 10.8	32.7 ± 7.32	26.7 ± 12	24.4 ± 5.9	16.6 ± 8.2
C16:1	23.0 ± 19.6	13.9 ± 7.54	31.0 ± 6.45	23.3 ± 13.1	22.1 ± 9.4	5.65 ± 2.77
C17:0	0.03 ± 0.06	0.09 ± 0.08	0.13 ± 0.01	0.04 ± 0.08	0.04 ± 0.06	0
C18:0	0.44 ± 0.13	0.34 ± 0.04	0.38 ± 0.04	0.26 ± 0.07	0.29 ± 0.13	0.85 ± 0.31
C18:1	2.46 ± 0.67	1.50 ± 0.74	1.18 ± 0.20	1.30 ± 0.25	1.56 ± 0.36	0.99 ± 0.4
Σtot FA	57.2 ± 39.8	39.0 ± 19.2	65.6 ± 24.0	51.9 ± 25.1	48.7 ± 14.8	24.4 ± 11.8
ΣSFA	31.6 ± 19.9	23.7 ± 11	33.4 ± 7.4	27.3 ± 12.02	25.0 ± 5.8	17.7 ± 8.6
ΣMUFA	25.0 ± 19.9	15.3 ± 8.2	32.1 ± 6.6	24.6 ± 13.2	23.6 ± 9.1	6.61 ± 3.16

*Fatty acid profiles were not measured for wild type *S.elongatus* PCC7942 to minimise cost for sample analysis, as differences between the empty vector control (*pSyn_6*) and the *ectABC*-transformed strain is more meaningful.

Myristic acid (C14:0) and Oleic acid (C18:1) levels [mg g^{-1} AFDW] were highest for the *ectABC*-transformed *S. elongatus* PCC7942 at 35°C and 0 ppt salinity, which exceeded amounts and percent contribution to Σtot FA detected in the empty vector *pSyn_6* control at this temperature (**Table 4.2, Fig. 4.5B**, **Appendix Table A4.22**). Salinity and temperature negatively affected levels [mg g^{-1} AFDW] of C14:0, C18:0 and C18:1 (**Tables 4.2 and 4.3, Appendix Table A4.22**). Temperature, however, increased percent contribution to Σtot FA from 0.8 to 1.6, 0.6 to 3.6 and 2.8 to 4.2%, respectively in the *pSyn_6* empty vector control, whilst percent contribution to Σtot FA decreased in the *ectABC*-transformant of *S. elongatus* PCC7942 from 1.7 to 0.4, 1 to 0.6, and 5.4 to 1.8%, respectively (**Fig. 4.5B**, **Appendix Table A4.22**).

Salinity did not affect percent contribution to Σtot FA for these fatty acids in the *ectABC*-transformant and only increased percent contribution of C14:0 (0.8 to 1%) and C18:1 (2.8 to 3.3%) (**Fig. 4.5B**, **Appendix Table A4.22**). It needs to be noted that standard deviations were quite large for some fatty acids (**Tables 4.2 and 4.3, Appendix A4.22**), therefore, in order to determine, any significance of either

temperature or salinity on fatty acid profiles, univariate ANOVAs were carried out.

A Shapiro-Wilks test showed that log-transformed (log) C14:0, C18:0 and C18:1 and non-log-transformed C16:0, C16:1 and C17:0 were normally distributed for *pSyn_6* empty vector controls and *ectABC*-transformed *S. elongatus* PCC7942 at the different growth temperatures of 35 (permissible temperature), 40 and 45°C (**Appendix Table A4.14**). The Levene's test showed that variances were homogeneous, except for the minor content of C17:0 for the interactive effect of 'strain*temperature' ($F_{(5, 12)} = 5.69$; $p = 0.0064$), for log C14:0 ($F_{(1, 16)} = 5.03$; $p = 0.039$) and C18:0 ($F_{(1, 16)} = 11.44$; $p = 0.0038$) for the effect of 'strain' (**Appendix Table A4.15**). A factorial ANOVA showed that temperature had only a significant effect on C18:0 ($F_{(2, 12)} = 8.00$; $p = 0.0062$) and C18:1 ($F_{(1, 12)} = 21.6$; $p = 0.0006$), with significant interaction between temperature*strain on C18:0 contents ($F_{(2, 12)} = 8.054$, $p = 0.006$) but not on C18:1. In contrast, a significant effect of temperature and strain was detected for C14:0 ($F_{(2, 12)} = 4.2$; $p = 0.041$ and $F_{(1, 12)} = 8.29$, $p = 0.014$, respectively), and a significant interaction

of ‘strain*temperature’ was also observed ($F_{(2, 12)} = 5.198$; $p = 0.024$) (**Appendix Table A4.16**).

A Tukey’s post hoc analysis was performed on data where significant effects of temperature, strain or significant interactions were observed. C18:0 content was significantly affected in *pSyn_6* empty vector controls at 45°C, which were significantly different to itself at 35 and 40°C and *ectABC*-transformants at 40°C. Temperature, however, did not affect C18:0 contents of *ectABC*-transformants (**Appendix Table A4.17**). In contrast, C18:1 content was only significantly different between *pSyn_6* empty vector controls and *ectABC*-transformants at 45°C (**Appendix Table A4.17**). The significant effect of strain and temperature was driven by differences in *ectABC*-transformants between all three temperatures and the *pSyn_6* empty vector control at 45°C, while C14:0 contents were only significantly different between *ectABC*-transformants at 35°C and itself at 45°C, as well as *pSyn_6* at 35 and 40°C (**Appendix Table A4.17**).

Table 4.3. Effect of salinity on total fatty acids, fatty acid profiles and levels of saturated – (SFA) and mono-unsaturated fatty acids (MUFA) in *ectABC*-transformed *S. elongatus* PCC7942 and the *pSyn_6* empty vector control.

	***ectABC*-transformed *S. elongatus* PCC7924**		**pSyn-6 empty vector control**	
FA's	**0 Salinity**	**18 Salinity**	**0 Salinity**	**18 Salinity**
C14:0	0.76 ± 0.18	0.45 ± 0.13	0.35 ± 0.07	0.57 ± 0.24
C16:0	30.42 ± 19.75	39.44 ± 8.10	26.66 ± 11.91	26.12 ± 0.83
C16:1	23.04 ± 19.64	33.44 ± 12.67	23.33 ± 13.05	23.11 ± 1.23
C17:0	0.03 ± 0.06	0.12 ± 0.03	0.04 ± 0.08	0
C18:0	0.44 ± 0.13	0.53 ± 0.25	0.26 ± 0.06	0.28 ± 0.06
C18:1	2.46 ± 0.67	1.77 ± 0.28	1.30 ± 0.25	1.68 ± 0.40
Σtot FA	57.16 ± 39.75	75.75 ± 20.55	51.95 ± 25.11	51.76 ± 1.55
ΣSFA	31.66 ± 19.90	40.54 ± 8.164	27.32 ± 12.03	26.97 ± 0.66
ΣMUFA	25.50 ± 19.90	35.21 ± 12.396	24.63 ± 13.29	24.79 ± 0.91

*Fatty acid profiles were not measured for wild type *S.elongatus* PCC7942 to minimise cost for sample analysis, as differences between the empty vector control (*pSyn_6*) and the *ectABC*-transformed strain is more meaningful.

Except for C17:0 fatty acid content, which was present in minute quantities, data were normally distributed for the effect of salinity for *ectABC*-transformants and *pSyn_6* empty vector controls of *S. elongatus* PCC7942 (**Appendix Table A4.18**). The Levene's test for homogeneity of variance showed that data for the effect of salinity on fatty acid profiles had homogeneous variances, except for the interactive effect of 'strain*salinity' on C17:0 ($F_{(3, 8)} = 6.71$; $p = 0.014$), (**Appendix Table A4.19**). In contrast, an interactive effect of 'strain*salinity' ($F_{(1, 8)} = 7.51$; $p = 0.026$) was determined for C14:0 content, while neither 'strain' nor 'salinity' had a significant effect, and for C18:1 contents, only 'strain' had a significant effect ($F_{(1, 8)} = 6.3$; $p = 0.036$) (**Appendix Table A4.20**). No significant difference was determined in the Tukey's post hoc test for 'strain' or 'salinity' on C14:0 content, while C18:1 contents were only significantly different between *ectABC*-transformants and *pSyn_6* empty vector controls at a salinity of 0 ppt, confirming the effect of strain (**Appendix Table A4.21**).

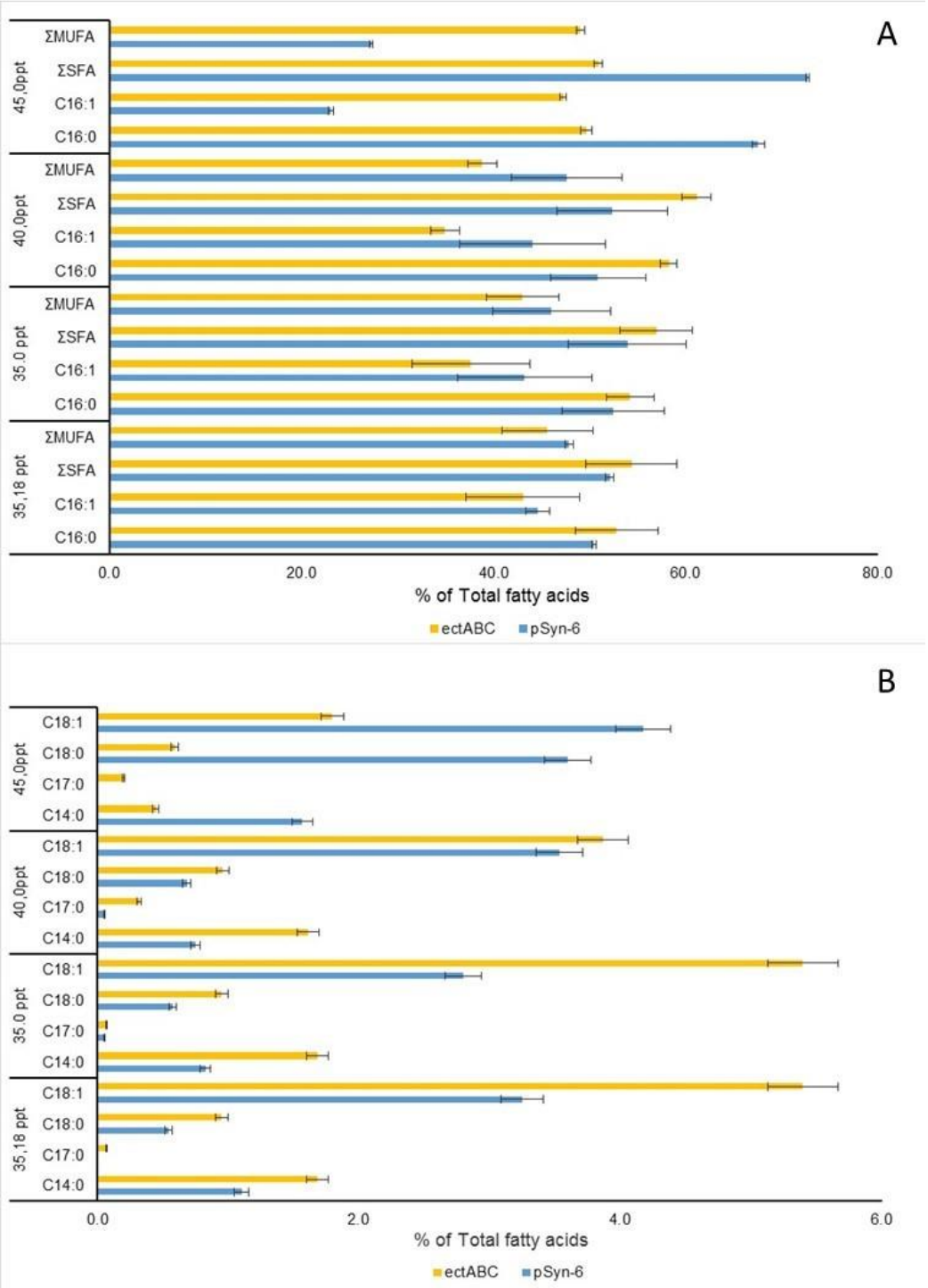


Figure 4.5. Effect of salinity and temperature on percent fatty acid of total fatty content for *ectABC*-transformed *S. elongatus* PCC7942 and the empty vector control *pSyn_6.*

4.3.5 Ectoine production

Overall intra- and extracellular and consequently total ectoine concentrations produced by *ectABC*-transformed *S. elongatus* PCC7942 were low, not exceeding 2.3 mg g^{-1} AFDW (**Table 4.4**). This was the result of low intracellular and extracellular ectoine concentrations, reaching 1.1 and 1.7 mg g^{-1} AFDW when placed under high temperature (45°C) and salinity stress (18 ppt), respectively (Table 2). Highest intra- and extracellular ectoine concentrations were achieved with salinity stress (**Table 4.4**), suggesting that salinity is a stronger inducer of ectoine expression compared to temperature in *ectABC*-transformed *S. elongatus* PCC7942.

Table 4.4. Effect of temperature and salinity on ectoine content and secretion in *ectABC*-transformed *Synechococcus elongatus* PCC7942

Salinity	Temperature	Intracellular ectoine	Extracellular ectoine	Total ectoine
[PPT]	[°C]	[mg g^{-1} AFDW]		
0	35	0.11 ± 0.03	0.5 ± 0.12	0.61 ± 0.1
18	35	0.60 ± 0.00	1.7 ± 0.42	2.30 ± 0.4
0	40	0.02 ± 0.00	0.5 ± 0.14	0.52 ± 0.1
0	45	0.24 ± 0.03	1.1 ± 0.28	1.34 ± 0.2

Ectoine expression were determined by HPLC chromatogram **(Figure 4.6)**. Empty vector control (*pSyn_6*) transformed *S. elongatus* PCC

7942 were used as a control where ectoine content was 0 [mg g-1 AFDW] (**please see Figure 4.6**).

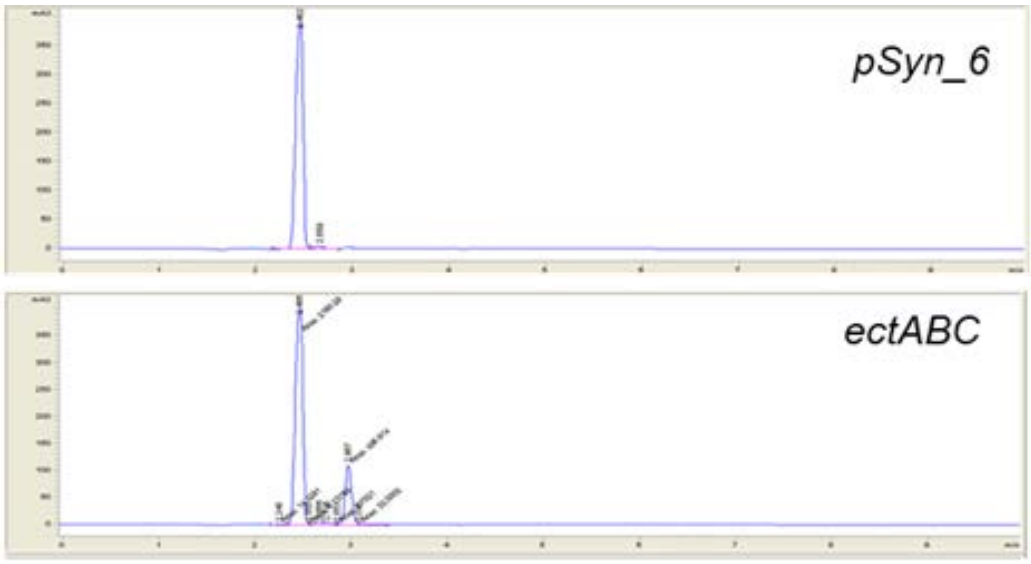


Figure 4.6 Quantification of ectoine using *ectABC* transfomed vs empty vector control (*pSyn_6*) containing *S. elongatus* PCC7942 by HPLC

Ectoine expression were determined by HPLC chromatogram **(Figure 4.6)**. Empty vector control (*pSyn_6*) transformed *S. elongatus* PCC7942 were used as a control where ectoine content was 0 [mg g-1 AFDW] (**please see Figure 4.6**). Data for all determined ectoine concentrations met homogeneity of variance (**Appendix Table A4.23**) and normality assumptions (**Appendix Fig. A4.4**) and an ANOVA confirmed that temperature significantly affected intra- and extracellular ectoine, and consequently total ectoine concentrations of *ectABC*-transformed *S.*

elongatus PCC7942 cultivated at 35, 40 and 45°C (**Appendix Table A4.24**). A Tukey's post hoc test determined that intracellular ectoine production was significantly different at all temperatures, while extracellular – and consequently total ectoine concentrations were significantly different at 45ºC compared to 40 and 35°C (**Appendix Table A4.25**). A Box and Whisker plot showed that intra-, extracellular and total ectoine concentrations were significantly higher for *ectABC*-transformants of *S. elongatus* PCC7942 at the salinity of 18 compared to ambient controls at a salinity of 0 ppt (**Appendix Fig. A4.5**). A t-test confirmed the significant effect of salinity on intra-, extracellular and total ectoine concentrations (DF: 4; $p < 0.0001$; DF: 4; $p = 0.0042$; and DF: 4; $p = 0.0013$, respectively).

4.4 Discussion

This study determined the effect of salinity (0, 18 and 36 ppt) and temperature (35, 40 and 45°C) on WT *S. elongatus* PCC7942, *pSyn_6* empty vector-containing *S. elongatus* PCC7942 and *ectABC*-transformed *S. elongatus* PCC7942. Effect of salinities and temperatures on growth performance, measured by ash- free weight, and revealed that *ectABC*-transformed *S. elongatus* PCC7942 tolerated

salinity stress upto 18 ppt and 45°C temperature stress. Similarly, population growth rate was higher for *ectABC*-transformed *S. elongatus* PCC7942 (0.141 ± 0.064) and more or less equal at all three temperatures at 0 ppt followed by growth rate at 18 ppt at permissible temperature 35°C. In addition, growth rate observed at permissible temperature at 36 ppt.was equal to the growth rate (0.143 ± 0.036) observed in WT *S. elongatus* PCC7942 at 18 ppt. This indicates that *ectABC*-transformation yielded some salinity-resilience to *S. elongatus* PCC7942. Ectoine-mediated salinity-resilience was also observed in agricultural crops of tobacco (Moghaieb et al., 2006) and tomato (Moghaieb et al., 2011) and some bacteria (Fallet et al., 2010; Guzmán et al., 2009; Nagata et al., 2008; Pastor et al., 2010) for bacteria for both salinity and temperature resilience (Calderon et al., 2004; Pastor et al., 2010). Our research demonstrated that combined temperature- and salinity-stress greatly affected growth performance of *ectABC*-transformants.

Total nitrite-corrected nitrate uptake was lower in *ectABC*-transformants at 18 ppt at permissible temperature (35°C) than WT *S. elongatus* PCC7942. This is similar to observed increase in nitrate uptake with

increasing salinity stress in wild-type *Anabaena torulosa* (Reddy et al., 1989). Higher N requirements were expected as a consequence of ectoine synthesis in response to increased salinity stress. Khmelenina et al. (2000) and Fernández et al. (2017) reported that supply of nitrogen was a crucial factor to achieve higher ectoine yields; thus the deviation from the expected outcome could be attributable to low levels of ectoine expression. Similarly, WT *S. elongatus* PCC7942 also exhibited increased phosphate uptake during both salinity (18 ppt) elevated temperature stress (45°C). In general, increased phosphate uptake has been reported for cyanobacteria and microalgae mainly associated with intracellular storage to aid survival during unfavourable stress conditions (Achbergerová & Nahálka, 2011; Mukherjee et al., 2015). In addition, compatible solute synthesizing moderate halophilic organisms are generally more nutrient demanding organism due to the fact that compatible solute accumulation or other growth factor or precursors synthesized slowly for for any other intermediate metabolism under high salt conditions (Javor, 2012; Ventosa et al., 1998).

Lipid contents significantly increased in both *ectABC*-transformed *S. elongatus* PCC7942 and empty vector controls at all temperatures (35, 40 and 45°C) and salinity stress 18 ppt at permissible temperature (35 °C). In general, salinity stress on microalgae increased lipid and fatty acid contents (Pandit et al., 2017). Our study showed a statistically significant increase in lipid contents under salinity stress of 18 ppt at permissible temperature (35°C) for *ectABC*-transformed *S.elongatus* PCC7942 at 45 °C. No change was observed between *ectABC*-transformed *S.elongatus* PCC7942 and empty vector controls for fatty acid contents at 35 and 40°C, however contents of total fatty acid and saturated fatty acids and mono unsaturated fatty acids significantly increased under salinity- (18 ppt at 35°C) and elevated temperature stress (45°C). It is generally observed that increase in salinity and temperature would normally increase lipid and fatty acid contents, repectively, as they play a vital role in maintaining membrane fluidity in cyanobacteria (Rajaram et al., 2014). Recent studies on *Chlorella vulgaris* and *Acutodesmus obliquus* reported increased lipids and fatty acid contents of Palmitic acid acid, Oleic acid and Linoleic acids with the increase in salinity stress ranging from 0.06 to 0.4 M NaCl (Pandit et al.,

2017), but none of the studies were reported for the effect of combined stress of salinity and temperature in cyanobacteria.

HPLC analysis validated ectoine production in *ectABC*-transformants.

Ectoine quantification by HPLC analysis determined that increase in ectoine expression was observed under salinity stress (**Table 4.4**). Similarly, cultivation at 45°C approximately doubled the expression of ectoin in *ectABC*-transformed *S. elongatus* PCC7942. Ectoine has been shown to play a physiological role in thermo-protection (Pastor et al., 2010). Results presented here, however, suggest that salinity stress is a stronger inducer of ectoine expression. In contrast, for *Halomonas elongata* DSM 3043 (otherwise known as *Chromohalobacter salexigens* DSM3043), ectoine yields did not increase in reposnse to temperature stress but thermo-protection was observed (Pastor et al., 2010). Ectoine synthesis solely depends on the availability of the precursor molecules oxaloacetate and L-aspartate which inititates ectoine expression from the TCA cycle (Cavalcanti et al., 2014; Saum & Müller, 2008). L-aspartate is a precursor molecule not only for ectoine synthesis but also for other amino acids such as glutamine and asparagine, repectively (Gutiérrez-Preciado et al., 2010). In the *ectABC*-transformed *S.*

elongatus PCC7942, synthesis of these other amino acids could have negatively impacted ectoine expression. Also foreign gene expression with model organism often do not reach the levels observed in the source organism (Gutiérrez-Preciado et al., 2010). Looking at ectoine as a potential high-value product, expression of the *ectABC*-transformed *S. elongatus* PCC7942 was low compared to other bacterial species, which either naturally synthesizie or were genetically modified (**Table 4.5**). Therefore, further studies are required to improve ectoine expression, if ectoine is targeted for high-value products from microalgae. Ectoine yileds would need to be improved by 2 orders (100-fold) of magnitude to be commercially viable (**Table 4.5**). Ectoine synthesis reported for several other heterotrophic bacteria rely on carbon (glucose) and nitrogen (sodium glutamate) supplies for the production of ectoine (**Table 4.5)**. Unlike heterotrophic bacteria, our study used natural photoautotrophic cyanobacterium *S. elongatus* PCC7942 require only sunlight, CO_2, water and other micro nutrients for growth and thus could avoid the cost for carbon, nitrogen and complex growth media in large scale environment (Lau et al., 2015). In addition to ectoine synthesis, there is a possibility that in the *ectABC*-transformed *S. elongatus*

PCC7942 synthesized ectoine could serve as a precursor molecule for hydroxyl ectoine production under high temperature stress, but this would require transformation with the *ectABCD* gene cassette (García-Estepa et al., 2006; Pastor et al., 2010). Given the low ectoine expression levels, expression levels of hydroxyl ectoine are also anticipated to be low. Due to time limitations, we recommend further research for inserting *ectABCD* into *S. elongatus* PCC7942 should nonetheless be conducted to identify whether *ectABCD* transformation show improved salinity- or temperature-resilience to the tolerance level observed in the extremophilic source organism. This recommendation is based on the fact that despite very low levels of ectoine expression, fairly impressive temperature- and salinity-tolerance levels were achieved for the *ectABC*-transformed *S. elongatus* PCC7942.

Table 4.5. Species naturally synthesize or genetically engineered for ectoine synthesis

Microrganism	Ectoine titre ($g\ L^{-1}$)	Specific production ($g.g^{-1}$ DCW)	Reactor System	Carbon Source	References
Brevibacterium epidermis DSM20659	8.0[a]	0.16	Fed-batch	Sodium glutamate, yeast extract	(Onraedt et al., 2005)
Brevibacterium sp. JCM6894	2.4[b]	0.15	Batch	Polypeptone yeast extract	(Nagata et al., 2008)
Chromohalobacter salexigens DSM3043	8.2[a]	0.54	Continuous with cell retention	Glucose	(Fallet et al., 2010)
Halomonas boliviensis	4.3[a], 0.74[b]	0.15	Two step fed-batch	Glucose and Sodium Glutamate	(Guzmán et al., 2009)
Halomonas elongata DSM2581[T]	7.4[b]	0.155	Batch	Glucose	(Sauer & Galinski, 1998)
Halomonas salina DSM 5928	6.9[a]	0.35, 0.22	Batch	Sodium glutamate	(Fallet et al., 2010)
Escherichia coli DH5α	5.94[a]	0.27	Batch	Glucose	(Schubert et al., 2007b)
Escherichia coli BW25113	25.1[a]	4.04	Whole-cell bio catalysis	Glucose	(He et al., 2015)

Escherichia coli W3110	25.1[a]	0.8	Fed-batch	Glucose	(Ning et al., 2016)
Synechococcus elongatus PCC7942 with *ectABC*	0.2 (35°C, 0ppt)[a] : 0.05 (35°C,0ppt)[b] 0.4 (40°C, 0ppt)[a] :0.01 (40°C,0ppt)[b] 0.1 (45°C, 0ppt)[a] : 0.1 (45°C,18ppt)[b] 0.6 (35°C,18ppt)[a] :0.2 (35°C,18ppt)[b]	0.001(35°C,0ppt) 0.001(40°C,0ppt) 0.001(45°C,0ppt) 0.003(35°C,18ppt)	Batch	Photoautotrop-hic	This study

Chapter 5

5 Evaluation of potential real term opportunities for large-scale cultivation of *ectABC* transformed *Synechococcus elongatus* PCC7942 for low-value high volume bio-products

5.1 Introduction

Bio-products from microalgae or cyanobacteria can contribute to the development of solutions for a range of problems facing the modern world, including freshwater scarcity, non- availability of agricultural land due to urbanization, and greenhouse gas emissions induced climate changes, each of which negatively affects agricultural productivity and food quality (Walsh et al., 2016). Microalgae or cyanobacteria represent a promising renewable resource that can be cultivated in saline/brackish/sea water or industrial wastewaters on non-arable land (Khan et al., 2018). Furthermore, greenhouse gas abatement by microalgae has been heralded as the potential solution to the world's energy security, due to their photosynthetic activity (Heimann et al., 2015b).

Whilst microalgae and cyanobacteria have the potential to remediate greenhouse gases and wastewater, the main aim of the algal industries

is to develop valuable products from the end biomass (Khan et al., 2018). High-value products such as b-carotene and astaxanthin from microalgae have potential market value worth expected to reach 1.53 billion USD by 2021 (Hu et al., 2018) whereas low-value products from microalgae such as animal feed and fuel are not currently economically viable in the market (Das, 2015). Production can be challenging due to the required volume of freshwater, climate induced salinity and temperature fluctuations and, most importantly, the lack of end biomass using industries adjacent to algal production facilities which can be the limiting factor for the newer development and further expansion of algal industries (Heimann et al., 2015a). Furthermore, unless when cultivating N_2-fixing cyanobacteria, all other algae require fertilisation, just like their terrestrial counterparts in order to sustain suitable biomass productivities (Heimann et al., 2015b). The cost of nitrate and phosphate for fertilization, CO_2 provision (unless a CO_2 emitter is co-located with the production facilities) and the technical difficulty to solubilise sufficient atmospheric CO_2, for their cultivation, as well as harvesting/ dewatering of the algal biomass adds significant costs and dramatically increases

the energy foot print of large-scale microalgal enterprises (Heimann et al., 2015b).

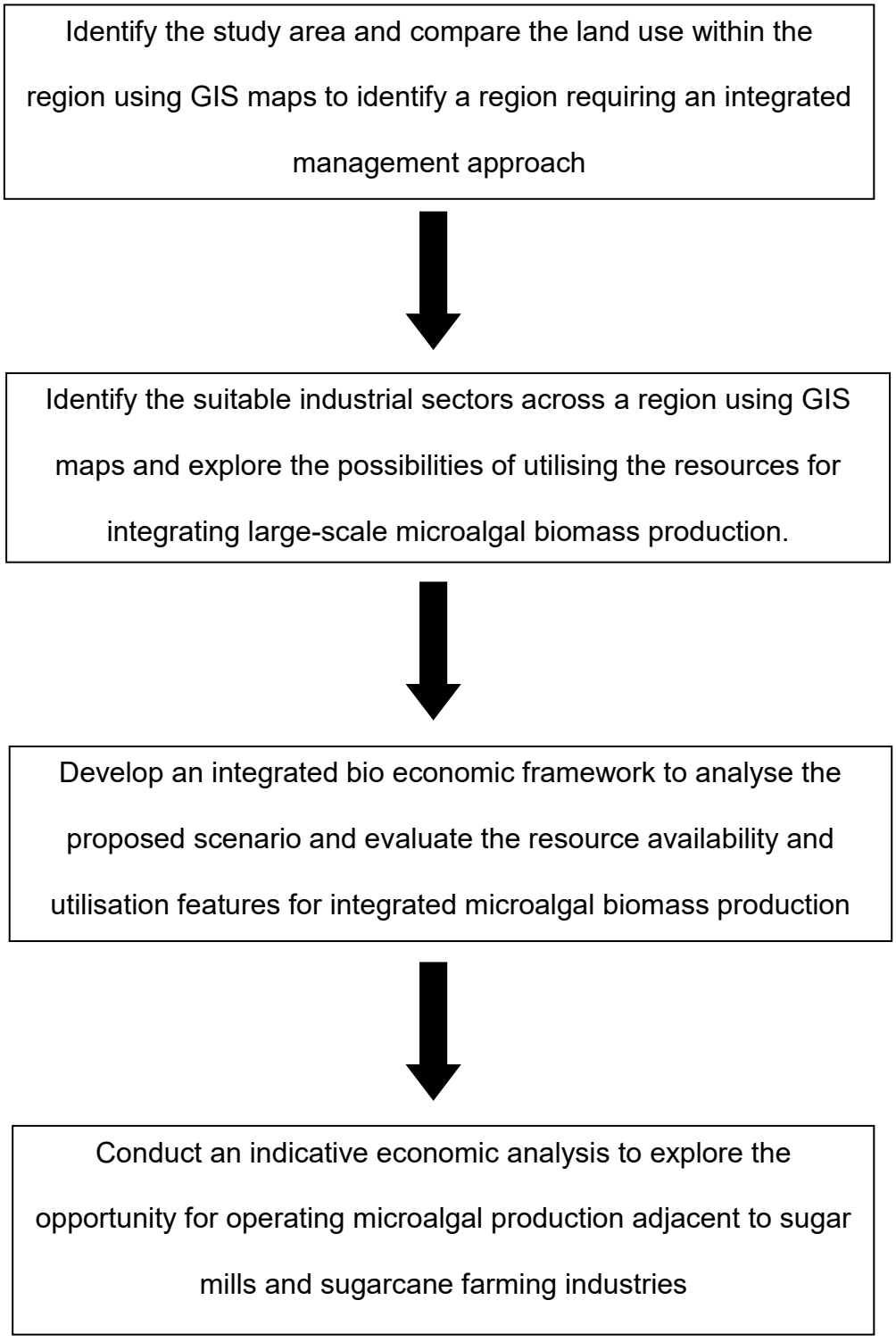
In order to overcome these problems, several reports have suggested that the co-location of algal production facilities with coal-fired power stations or other wastewater industries could potentially supply the flue gas containing CO_2 and nutrient rich wastewaters for sustainable biomass production (Lucas & Southgate, 2019; Raslavičius et al., 2018). This would minimise the cost involved in the production process mainly from water, nutrients and water associated energy demand. Furthermore, integration of microalgal production with end biomass using industries such as ethanol bio-refineries, cattle farms and related aquaculture facilities could significantly improve the economic performance and potentially reduce the costs associated with operation and production (Mayers et al., 2016).

These integrated farming approaches are an-old fashioned concept re-emerged in various production systems during the last 30 years to improve the sustainability of agri-aqua and reduce the environmental impacts (Vatsos et al., 2015). For example, integrated rice-fish farming have been adopted in many places in Asia for more than 1700 years

(Vatsos et al. 2015). In Queensland, sugar cane-microalgae farming model was developed to produce algal biomass for bio-fuel production (Sedghamiz, 2017). Similarly, microalgae or cyanobacterial biomass production integrated with aquaculture industries have been shown to provide nutrient rich wastewater resources for microalgal biomass production and the end biomass can be used as a feed ingredient in the aquaculture industry (Tossavainen et al., 2018; Velichkova et al., 2018). In addition, co-location of microalgal or cyanobacterial biomass production with aquaculture fish farming industries for aquaculture feed production would significantly improve the bio-product revenue to the microalgal industry and reduce the feed cost for aquaculture fish farming industries, thus providing notable benefits for both industries (Heimann et al., 2015b).

In this research, we have developed a framework model to illustrate the potential bio-economic benefits arising from algal biomass production integrated with sugar mills in Wet Tropics region of Queensland (see map at **Figure 5.1** for geographic location) for sustainable, successful and cost effective *ectABC* transformed *S. elongatus* PCC7942 biomass production.

5.2 Methodology



5.3 Study area

The selection of a site for microalgae or cyanobacteria biomass production co-location with other industries within northern Australia offers many advantages due to the availability of natural resources, including energy, and the opportunity to co-locate with a range of different types of agriculture. The north plays a major role in maintaining and growing Australia's successful economy (Australia, 2014). In addition, the north contains seven outstanding World Heritage Sites including the Great Barrier Reef, the Wet Tropics of Queensland, Kakadu national park, Uluru-Kata Tjuta national park and the Riversleigh Australian Fossil Mammal site (Australia, 2014).

The Great Barrier Reef (GBR) and it's catchments (**Figure 5.1**) form a region of great environmental and socio-economic significance (Deloitte, 2017). The economic value of the GBR alone has been valued at $56 billion and makes a $6.4 billion contribution towards Australian economy during 2015-2016 (Deloitte, 2017). However, the GBR is one amongst a number of world heritage sites around the world that are just a step away from being included in the 'in danger' list by UNESCO. The degradation of the GBR over last 27-year period has resulted in 50.7%

of coral loss, due to causes including an outbreak of Crown-of-Thorn Starfish (which eats coral and thrives in nutrient rich waters) and other climate induces changes such as global warming (Mohd Nazri, 2017). In response to this, Australia has developed a proactive approach built around the approach set out in the Reef 2050 Long- Term Sustainability Plan (Reef 2050 Plan) (Jarvis et al., 2017). The Reef 2050 Plan is a joint venture between Australian Federal government and the State government of Queensland and comprises a suite of different steps that seek to improve the health of the Reef including by limiting the nutrient, sediment and pesticide flows into the GBR lagoon (WQIP, 2018).

A key component of the Reef 2050 plan is a long term water quality improvement plan, which sets out a number of short and longer term targets, alongside steps designed to achieve these targets. These plans follow on from earlier government plans and programs, including the 2013 Reef quality plan (Reef, 2014). Unfortunately the targets to date have generally not been met. For example, water quality 2013 targets 50% nitrogen reduction target were not met (only 10 % between 2009 to 2013 due to the slow progress of implementing nutrient management plans by surrounding industries (Reef, 2014)). Recent report on Reef

2050 Water Quality Improvement plan 2017-2022 (WQIP, 2018) reiterated that current initiaties will not meet future water quality targets and thus changes in on-ground management, project design and evaluation systems are on urgent need (WQIP, 2018). As many of the significant threats to GBR are due to the level of nutrient run off by agriculture industries adjacent to GBR, it is important to prioritize the adjacent regions can be seen in the map (**Figure 5.1**) for an efficient nutrient managent approach to reduce the environmental impact associated with heavy loads of nutrient run off into GBR.This has led the current agri-aqua industries, natural resource management organisations and investors for future development adjacent to the GBR located across different regions within the GBR catchment to develop integrated projects to improve the quality of their discharged water. For example, major projects have been established within the Wet Tropics (Terrain, 2015) and within the Burdekin region (http://ldc.nqdrytropics.com.au/ldc-overview/).

A further recent initiative currently being developed for trial within the Wet Tropics region provides ‘Reef Credit’ incentives to the industrial landowners to develop integrated projects, which would generate

additional revenue to the industries and subsequent actions on recirculating waste waters will improve their discharge water quality (Greencollar, 2017). This is an innovative market based approach, which when fully implemented would operate similarly to a carbon credits scheme.

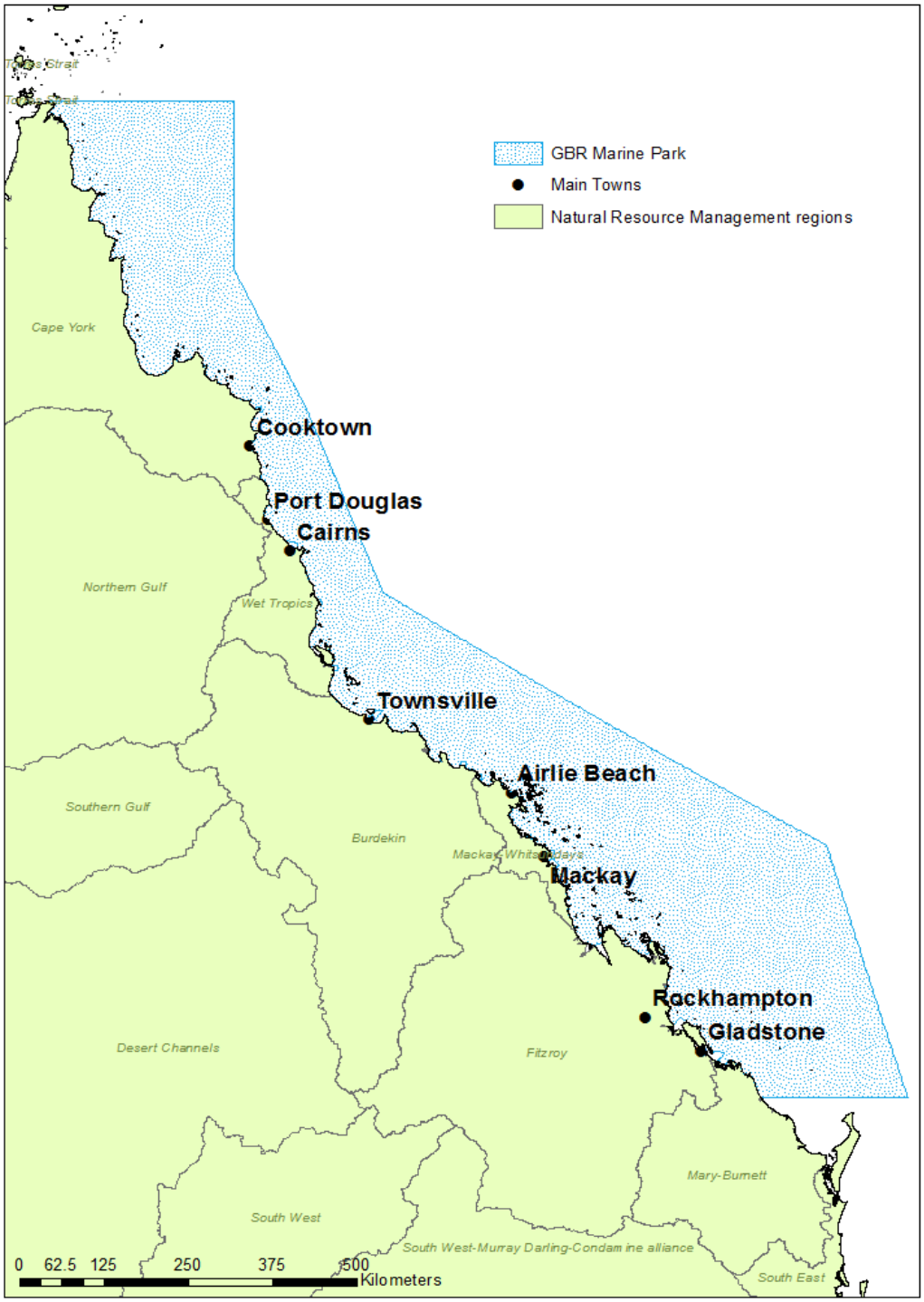


Figure 5.1. Great Barrier Reef catchments, major towns and natural resource management regions.

5.4 Land use, and subsequent environmental impacts of such uses, within the prirotized region.

Mohd Nazri (2017) prioritized the regions to be focused to reduce nutrient run-off into GBR. The Great Barrier Reef catchment management prioritized regions are listed in **Table 5.1** and their location can be seen in the map at **Figure 5.1**. Among identified regions, the Wet Tropics region were given first priority followed by Burdekin region (**Table 5.1**). Since 1999, significant changes in land use were observed in the Wet Tropics region. For example, the major land use changes were observed primarily from production forestry to nature conservation which account for 76% of total land use (DSITI, 2016). Apart from nature conservation, production forestry and resource protected areas, over 55% of the land within the Wet Tropics is used for agriculture production (dryland cropping which includes sugar cane production), 41% reported for cattle farming and grazing, 20% were used for horticulture and 3% reported for growing broad acre crops (ABS, 2010) (**Figure. 5.2**).

A map showing the distribution of land use across the region (using 2015 data[2]) is shown at **Figure.5.2.** Among these land uses, the nutrient discharge impacting water quality in the Wet Tropics region is associated primarily with sugarcane industries (Mohd Nazri, 2017). The current data shows that among the NRM regions adjacent to GBR, the Wet Tropics, Burdekin and Fitzroy regions contribute over 75% total nitrogen load, and over 55 % of phosphorous load was contributed by Burdekin and Fitzroy region from human activity (**Table. 5.2**). Comparing the loads of nutrient level and sediment flows to GBR catchments, Wet Tropics Region contribute heavy nutrient loads of total nitrogen 12151t, inorganic nitrogen 4437t and dissolved organic nitrogen 3870t per annum (**Table 5.2**). Total phosphorus 2184t and dissolved inorganic phosphate 341t per annum was contributed by Burdekin region followed by Wet Tropics region contributed 1656 t of total phosphate and 130 t of dissolved inorganic phosphate per annum (**Table 5.2**).

[2] Land use Wet Tropics 2015 data downloaded from (http://qldspatial.information.qld.gov.[2]au/catalogue/custom/detail.page?fid={0082B3DB-A37F-44A1-B4CD-FC9C70644CA7})

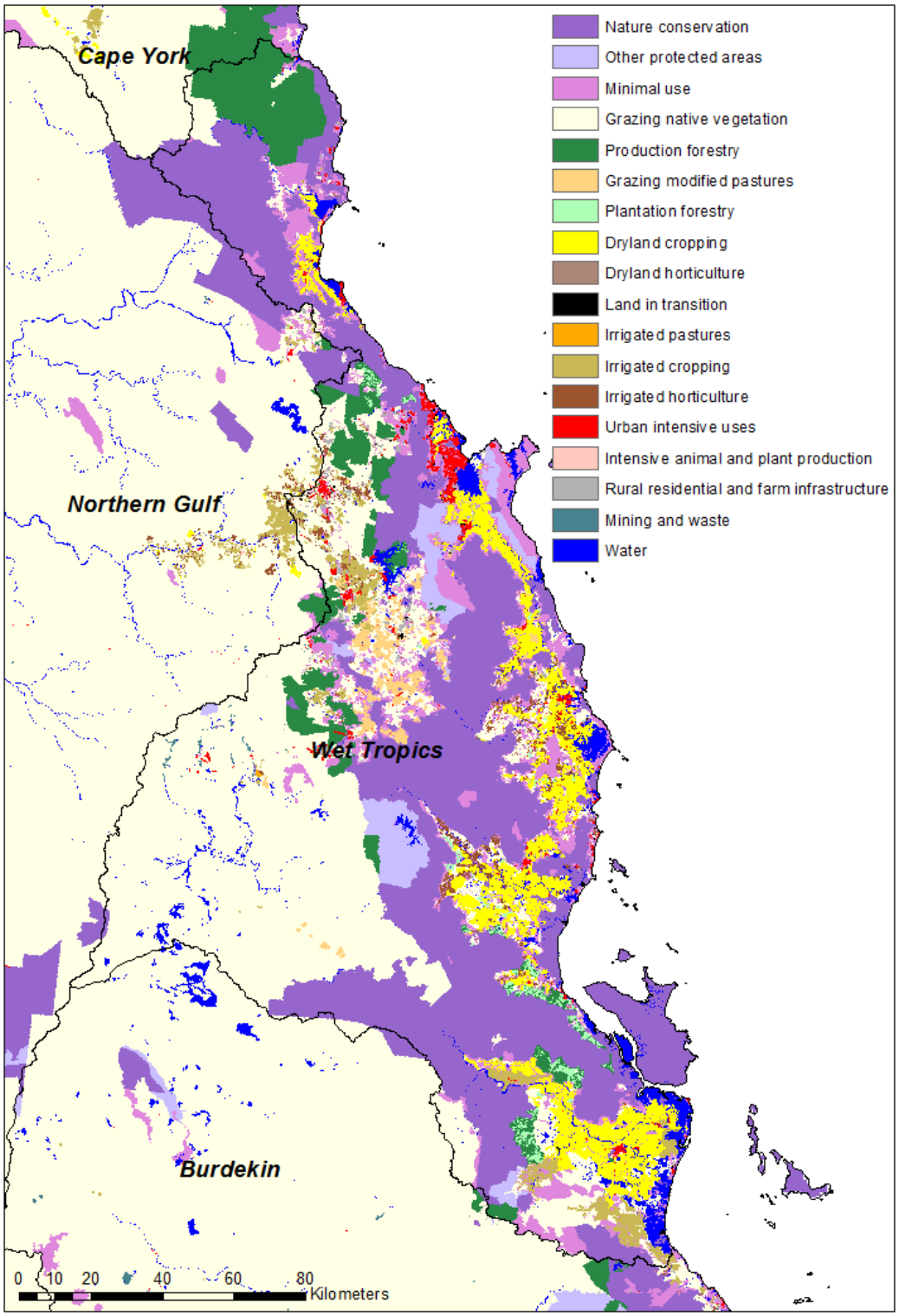


Figure 5.2. Land use in Wet Tropics natural resource management region

Table 5.1. Priorities for natural resource management regions adjacent to GBR

Relative Priority	Management Priorities		
	Region	Pollutant management	Land uses
1	Wet Tropics	Fertiliser nitrogen reduction	Sugarcane, Bananas
	Burdekin	Erosion management	Grazing
	Fitzroy	Erosion management	Grazing, cropping
2	Burdekin	Pesticide reduction in Burdekin and Haughton	Sugarcane
	Mackay Whitsunday	Pesticide reduction in all catchments	Sugarcane
	Burdekin	Fertiliser nitrogen reduction	Sugarcane
3	Makay Whitsunday	Fertiliser nitrogen reduction	Sugarcane
	Burnett Mary	Erosion management in all catchments	Grazing
	Wet Tropics	Pesticide reduction in all catchments	Sugarcane
	Fitzroy	Pesticide reduction in all catchments	Grazing, cropping
4	Burnett Mary	Eco-system monitoring is required as the corals and seagrass in this region is outside of Great Barrier Reef World Heritage Area.	
5	Cape York	Relatively low impacted area, Further assessment is required to maintain the current values of the region	

Adapted from (Mohd Nazri, 2017; Waters et al., 2014)

Table 5.2. Total nutrient loads from natural management regions of GBR

Site	NRM Regions	Area (Km^2)	TSS (kt/yr)	TN (t/yr)	DIN (t/yr)	DON	PN	TP	DIP	DOP	PP	PS II s
A	Cape York	42,988	429	5173	492	3652	1030	531	98	195	238	3
B	Wet Tropics	21,722	1219	**12151**	**4437**	3870	3844	1656	228	130	1297	8596
C	Burdekin	140,671	3976	**10110**	**2647**	3185	4278	2184	341	153	1690	2091
D	Mackay Whitsunday	8,992	511	2819	1129	950	739	439	132	35	271	3944
E	Fitzroy	155,740	1948	4244	1272	1790	1181	1093	278	56	759	579
F	Burnett Mary	53,021	462	2202	554	873	775	392	78	35	278	1528
G	GBR Total	423,134	8545	36699	10532	14320	11847	6294	1155	606	4532	16740

Adapted from (Mohd Nazri, 2017; Waters et al., 2014)

NRM regions: Natural Resource Management Regions; TSS: Total Suspended Solids; TN: Total Nitrogen; DIN: Dissolved In-organic Nitrogen; DON: Dissolved Organic Nitrogen; TP: Total Phosphate; DIP; Dissolved In-organic phosphate: DOP; Dissolved Organic Phosphate; PP; Pesticide pollutant; PSIIs: Photosystem II, Chlorophyll content

In the context of industrial-scale microalgal or cyanobacterial biomass production, nitrogen fertilizer can be supplied in the form of urea, ammonia, and ammonium phosphate, sodium or potassium orthophosphate or metaphosphate for phosphorous (Markou et al., 2014), with 100 ton of microalgal production requiring 10 t of nitrogen and 1 ton of phosphorous (Acien Fernández et al., 2018). The amount of dissolved inorganic nitrogen and dissolved organic nitrogen discharges are highest in Wet Tropics followed by Burdekin region (**Table 5.2)**. Although a number of the sugarcane farms adjacent to GBR have adopted the best management practices (BMP) for nutrients, there remains much scope for improvement. According to Reef (2016), BMP systems were being used by sugar cane farmers within the Wet Tropics for approximately 30% land regarding pesticide use, 16% for nutrient use and 52% for soil. Accordingly, the region could not meet 50% nitrogen reduction target by 2013 with only a 10% reduction reported (Mohd Nazri, 2017; Plan, 2014), and reported reductions since that date continue to report shortfalls against target (see[3] for shortfalls against target to 2015 to 2016 for pesticides, inorganic nitrogen and sediment). This information reveals that there is a significant requirement within the Wet Tropics for innovative new initiatives that could contribute to the existing efforts to reduce pesticides and nutrients in agricultural run off and hence improve water quality. Accordingly, this region offers a

[3] https://www.reefplan.qld.gov.au/measuring-success/report-cards/2016/#interactive-map

suitable location to co-locate algal farms adjacent to sugarcane or aquaculture farming industries, combining an established need for improved water quality with an already established presence of both of these industries.

5.5 Selection of industrial site for microalgal co-production

Large scale microalgal or cyanobacterial biomass production requires an integrated approach to enhance the development of the industry (Mayers et al., 2016; Salama et al., 2017). The Integration of both the supply chain and value chain management strategies was recommended for profitable microalgal biomass production (Solanki et al., 2012). The supply chain management requires identifying the policies and industrial regulation and process to supply the resources such as water, nutrients, CO_2 and energy for efficient operation of microalgal business. Linking value chain management would add extra value in distributing the products derived from supply chain inputs. In this research, the supply chain management includes policies on industries to reduce CO_2 emission and water quality improvement by limiting nutrient discharge as these provide advantages for developing or expanding microalgal industries in Wet Tropics region.

Several reports have suggested the integration of microalgal or cyanobacterial biomass production with coal gas fired power stations, for utilising the energy and waste flue gas for greenhouse gas remediation. However, this option is not applicable for the Wet Tropics region, as within the Wet Tropics the electricity supply is mainly derived from three hydroelectric power stations, which continue to produce renewable energy from the fast flowing rivers of Far North Queensland (Reef Assessment, 2015). The adjacent coal-fired power stations such as Tarong, Tarong North, Stanwell and Millerman are located far from the Wet Tropics and therefore out of the focus for this study. Hence, co-location with coal-fired power stations is not investigated within this study.

Furthermore, the literature study (chapter 2) identified that the co-location of microalgal farms with coal-fired power stations to utilize waste flue gas requires extremo-halophilic algae which can tolerate temperatures of more than 60°C. This is practically impossible for *ectABC* transformed *S. elongatus,* which tolerates temperatures up to 45°C. A further argument against co-location with coal fired power stations is that flue gas from coal-fired power stations contains high

concentration of SOx and NOx, which could be toxic to algae (Zhang, 2015). The USA based microalgal industry Seambiotic Ltd conducted several studies of utilizing coal-fired flue gas for microalgal or cyanobacterial cultivation and found out those SOx concentrations anything over 60 ppm is toxic to algae and inhibit the growth (Ben-Amotz, 2008). Flue gas containing SO_X concentrations are typically over 300-600 ppm from Australian coal-fired power stations, and would therefore require current pollutant removal technologies to remove SOx, NOx and particulate matter effectively (Haritos VS, 2012; Park et al., 2004).

Hence, we consider co-location with biomass fired energy generation instead. Biomass fired boilers are often advantageous over coal-fired power stations for CO_2 capture by microalgae (Lohrey & Kochergin, 2012). Biomass boilers have been shown to produce lower concentrations of SO_X and NO_X and thus are less toxic to algae (Lohrey & Kochergin, 2012). Therefore, flue gas utilisation from sugar mills offers a best option for CO_2 capture and delivery for large-scale microalgal biomass production across the Wet Tropics where significant sugar cane production takes place.

A further attraction offered by the Wet Tropics region, is that aquaculture industries offer the potential for large-scale microalgal or cyanobacterial cultivation targeting aquaculture feed production for adjacent prawn or fish farming industries or animal feed for existing cattle farms. Current Great Barrier Reef Marine Park Regulations 2000 demanding the zero net discharge from land based aquaculture industries (Mohd Nazri, 2017). The integration of microalgal or cyanobacterial biomass production with such industries has been less studied in Wet Tropics Region, and offers opportunities for future research into this important environmental/economic opportunity. Large scale cyanobacterial biomass cultivation integration with aquaculture industries have been shown to achieve 95-99% of nutrient removal from wastewater (Guo et al., 2013). However, most aquaculture industries are unlikely to supply or produce the required CO_2 and energy that would be required for development of a potential algal biomass production facility. As a result, co-location of microalgal or cyanobacterial biomass production with aquaculture industries in the Wet Tropics requires the additional co-integration of CO_2 emitting industries with the combined

aquaculture/algae facility to supply the resources such as CO_2 and energy for cost effective biomass production.

Prawn farming is a major aquaculture business in Wet Tropics which may be worthy of further consideration. This activity uses seawater, and thus the wastewater released from these industries is often too saline for cyanobacterial biomass production, ranging from brackish to sweater condition (15-35 pptppt). Our *ectABC* transformed *S. elongatus* PCC7942 have been shown to have a salinity tolerance of 18 ppt and thus is not suitable for co-locating with such sea water utilising prawn farming industries. However, sugar mills require large amount of freshwater for processing and discharge 50% freshwater input as effluent (Sahu, 2018). Morever, flue gas containing CO_2 extraction and energy supply from bagasse combustion bioilers is an added advantage for setting up microalgal business with sugar mills in Wet Tropics.

Given these factors, we hypothesize that our model integrating microalgal biomass production with sugar mills in the Wet Tropics region would be an ideal industrial combination to allocate and share the resources and infrastructure for industrial need, facilitated by the Wet

Tropics region providing the option of several sugar mills for integrating with microalgal biomass production (**Figure 5.3**).

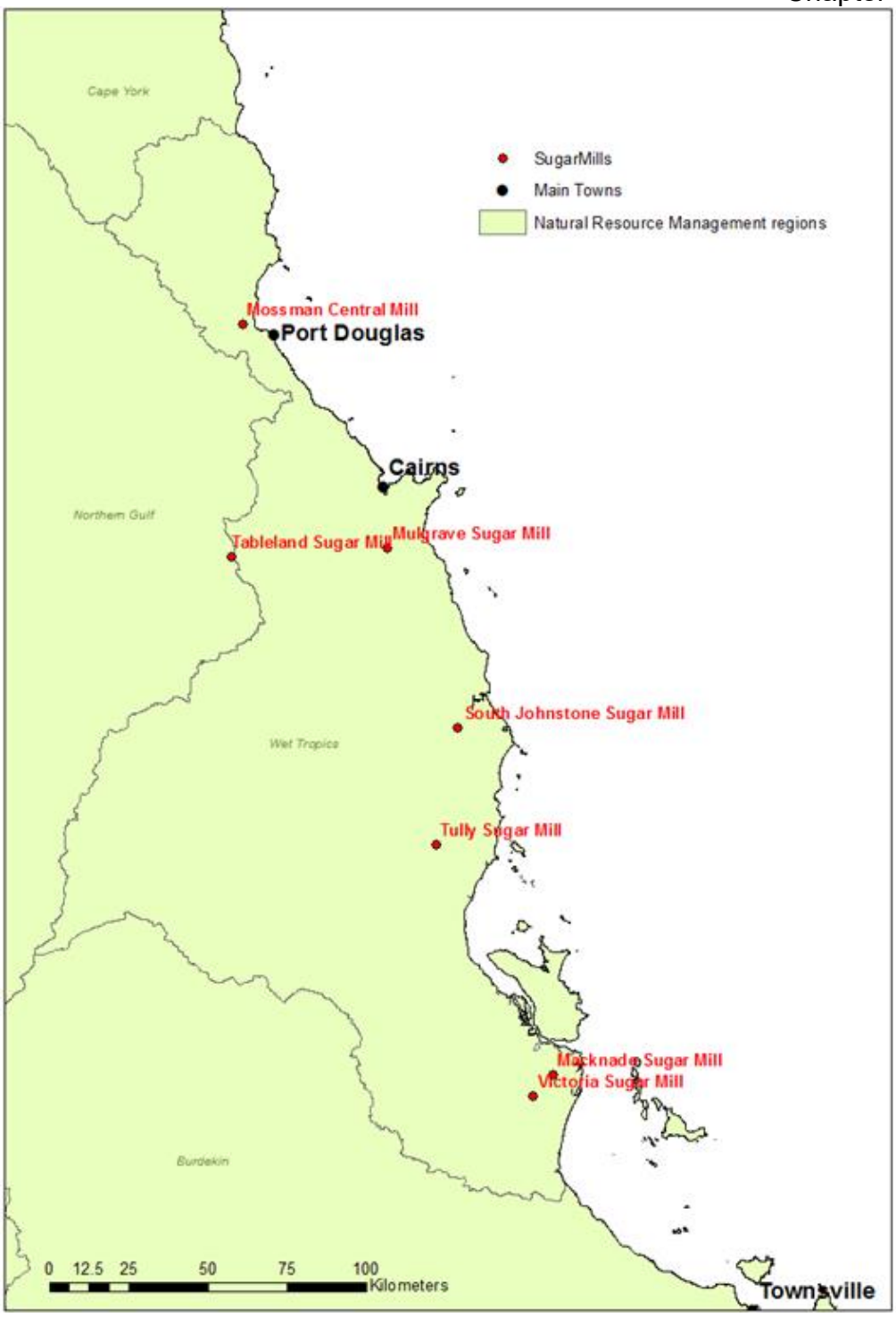


Figure 5.3. Sugar mills in Wet Tropics offering potential opportunities for co-locating microalgal biomass production

5.6 Wet Tropics Climatic Suitability for *ectABC* transformed *S. elongatus* mass cultivation

In order to co-locate *ectABC* transformed *S. elongatus* PCC 7942 biomass production with sugar mills in Wet Tropics, it is important to consider climatic factors such as temperature and rainfall (required to compensate the evaporative water loss). Accordingly we have assessed the typical climatic conditions across the Wet Tropics compared to the requirements for an algae-sugarcane co-located facility.

5.6.1 Rain fall suitability for combining algae production with sugar mills in Wet Tropics

To assess whether the rainfall levels across the region are sufficient to satisfy the requirements, we have calculated average rainfall based on data from 89 Bureau of Meteorology rain stations that are located within the Wet Tropics region and have been open and reporting data since 2000 (data sourced from http://www.bom.gov.au/). We have calculated the average annual rainfall across the years 2000 to 2017, to offer a representative picture of typical conditions that can be expected across the region, thus averaging out annual fluctuations across the period. Obviously, the data collected at a single rainstation reflects the data at that particular location, rather than across an area. Areal rainfall (the

amount of water that falls over a specified area in a specific time period) is frequently determined using Thiessen Polygons method (Thiessen, 1911), which creates a network of polygons around each of the rainstations, then assumes that any particular location within each polygon has the same rainfall as that of the rainstation closest to it - a form of interpolation widely used when considering rainfall across a large area.Thus, this is the method we have adopted within our analysis here. The results of the analysis can be seen in the map at **Figure 5.4**. Each polygon shown on the map is centred on a rainstation at the centre of it, and the entire polygon reflects the average rainfall measured at that rainstation. The map shows lower rainfall as the lighter coloured areas, and heavier rainfall as the darker colours, with the colour scale being based on each band having equal width. The lowest value on the scale of 590 reflects that that is the lowest annual rainfall received across the region, whilst the rainstation with the highest average rainfall reported 7838. All rainfall is reported in mm.

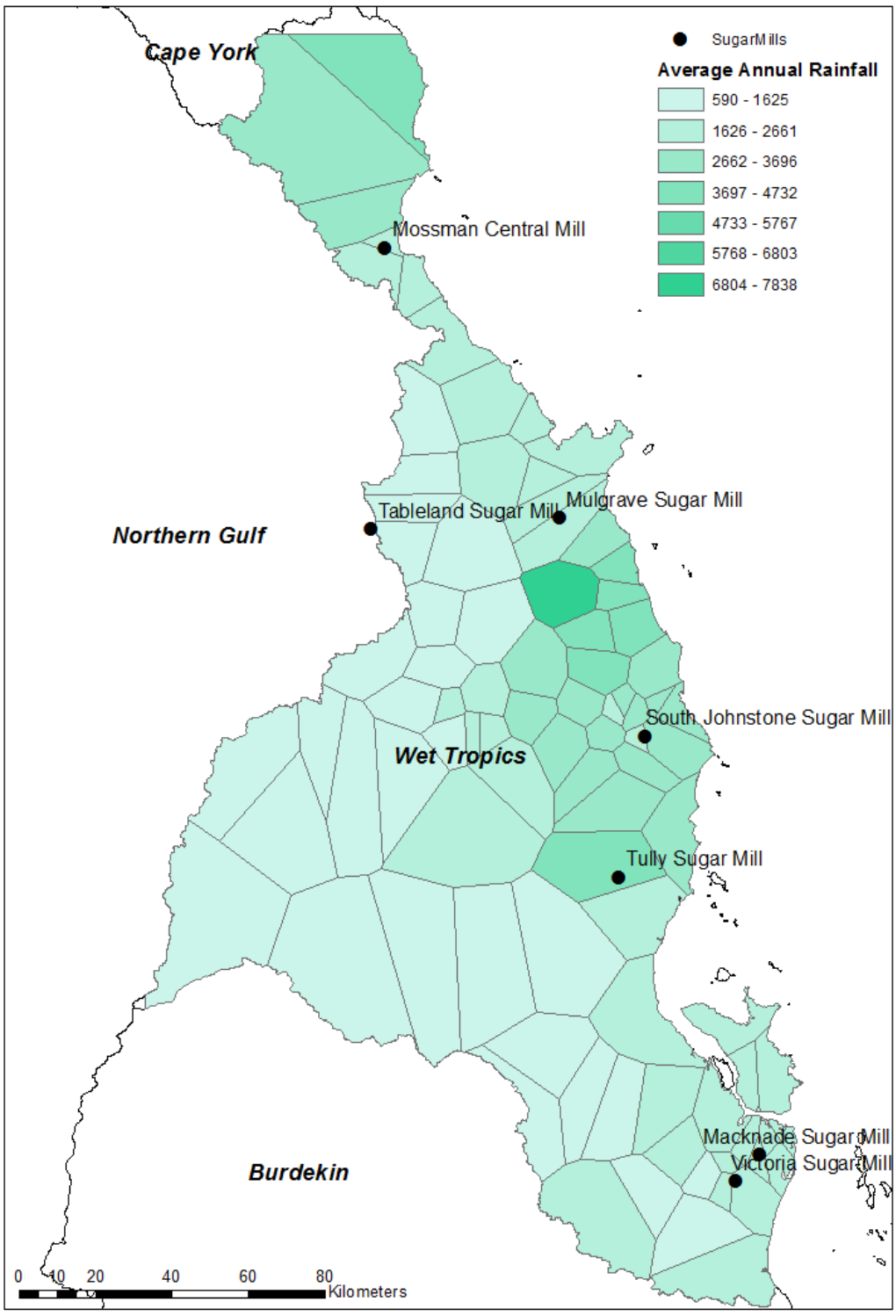


Figure 5.4. Average annual rainfall in Wet Tropics region

As can be seen from the average annual rainfall map (**Figure. 5.4**), the Wet Tropics region experiences significant rainfall each year, although this is concentrated mainly during the December – April wet season rather than being evenly distributed across the year. Indeed, the Wet Tropics is acknowledged to contain Australia's wettest towns (Bowley, 2014), with this title alternating between Tully and Babinda (which lies a little to the north of Tully). Accordingly, Tully sugar mill in Wet Tropics region receives high rainfall ranges between 2662 to 3696 mm **(Figure 5.4)**. Macknade and Victorial sugarmills falls into second category of receiving lower rain fall range between 1626-2661 mm followed by Tableland sugar mill range between 590 to 1625 mm **(Figure 5.4)**. Thus, our study reveals that Tully sugar mills offers an ideal location which allows us to create ample storage of water during high rain fall evets to compensate trans-evaporation and any other extreme events i.e, freshwater for cooling during extreme heat waves.

5.6.2 Temperature suitability at sugar mill locations within the Wet Tropics

Another important climatic factor to consider is temperature, the suitability of which was assessed across the Wet Tropics region, adopting a similar methodology to that used for assessing rainfall across

the region. Temperature calculations were based on data for 13 weather stations that have reported temperature data from 2001 to 2017, using stations within the WT and those within 100km of the boundary to ensure reasonable coverage across the region **(Figure. 5.5)**. We present our analysis in two ways. Using Thiessen Polygons (Thiessen, 1911) (similar to the rainfall map), the average number of days on an annual basis where temperatures exceed 30 degrees based on measures taken at particular weather stations, are shown at **Figure. 5.5**. Temperatures are not recorded in as many places as rainfall, hence less polygons. The map at **Figure 5.6** shows regions where at some point in the period 2001-2017 the maximum temperature has exceeded 35, 38 or 40ºC . As can be seen, there is nowhere across the region that hasn't reached at least the 35 degree threshold at some point across that time period.

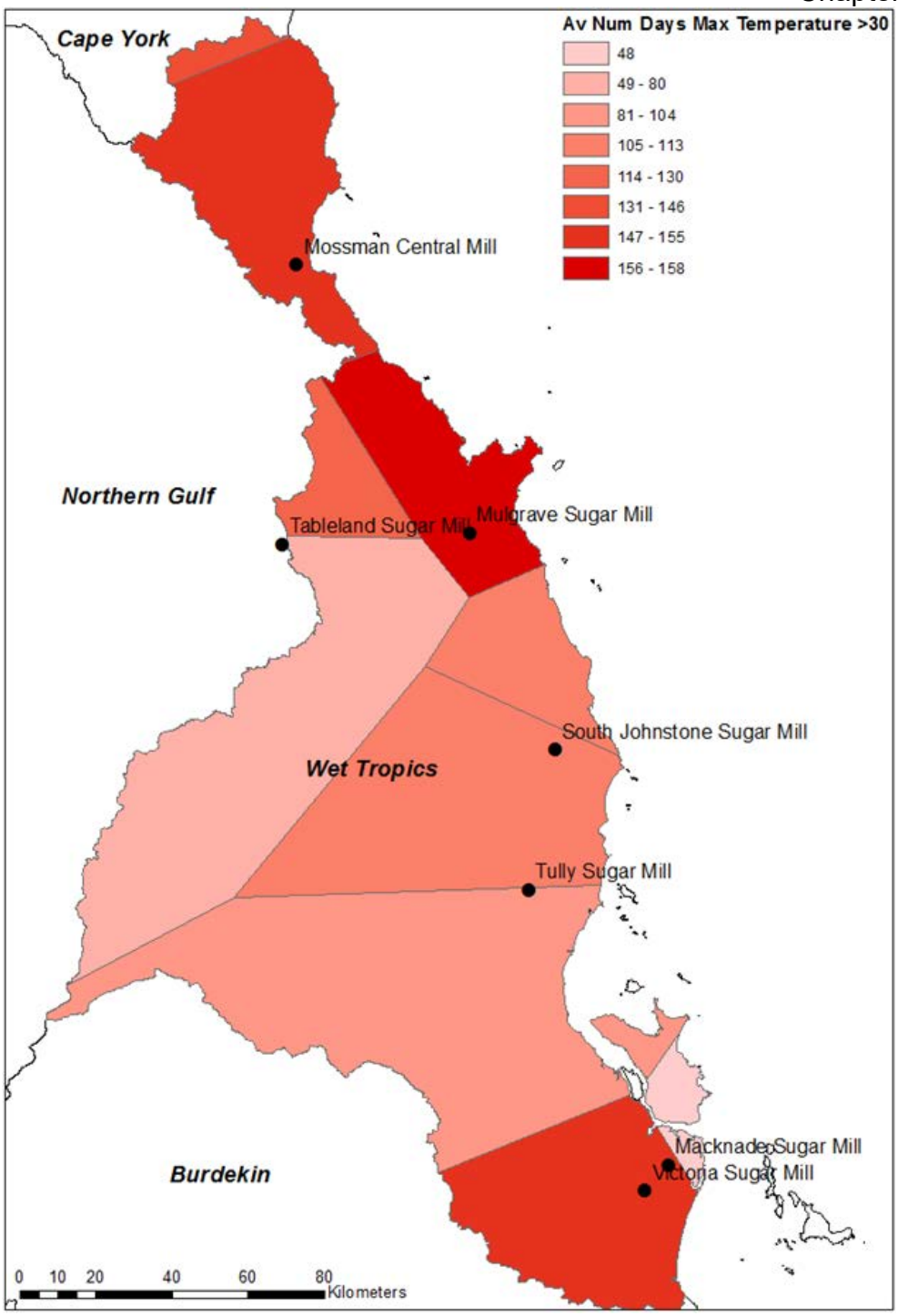


Figure 5.5. Wet Tropics regions average number of days per year where temperature exceeds 30ºC

As can be seen in the map (**Figure 5.5.**), we have calculated the average number of days per year when the maximum temperature exceeds 30ºC, which is an ideal growth condition for *ectABC-transformed S. elongatus* PCC7942 mass cultivation. All locations across the Wet Tropics has some days on average per year where the maximum temperature exceeds 30ºC, ranging from 48 days per year on average to 158 days (almost half the year) **(Figure. 5.5**).

In addition, we have also identified the regions where at some point in the period 2001-2017, the maximum temperature has exceeded 35 degrees, 38 or 40ºC **(Figure. 5.6)**. Our further analysis confirmed that nowhere across the region had experience maximum temperature in excess of 45ºC at any during this time period **(Figure. 5.6)**. This analysis revealed an additional suitability of the region for cultivating our *ectABC* transformed *S. elongatus* PCC7942 strain can tolerate of a higher degree of temperature to 45ºC **(Figure. 5.6).**

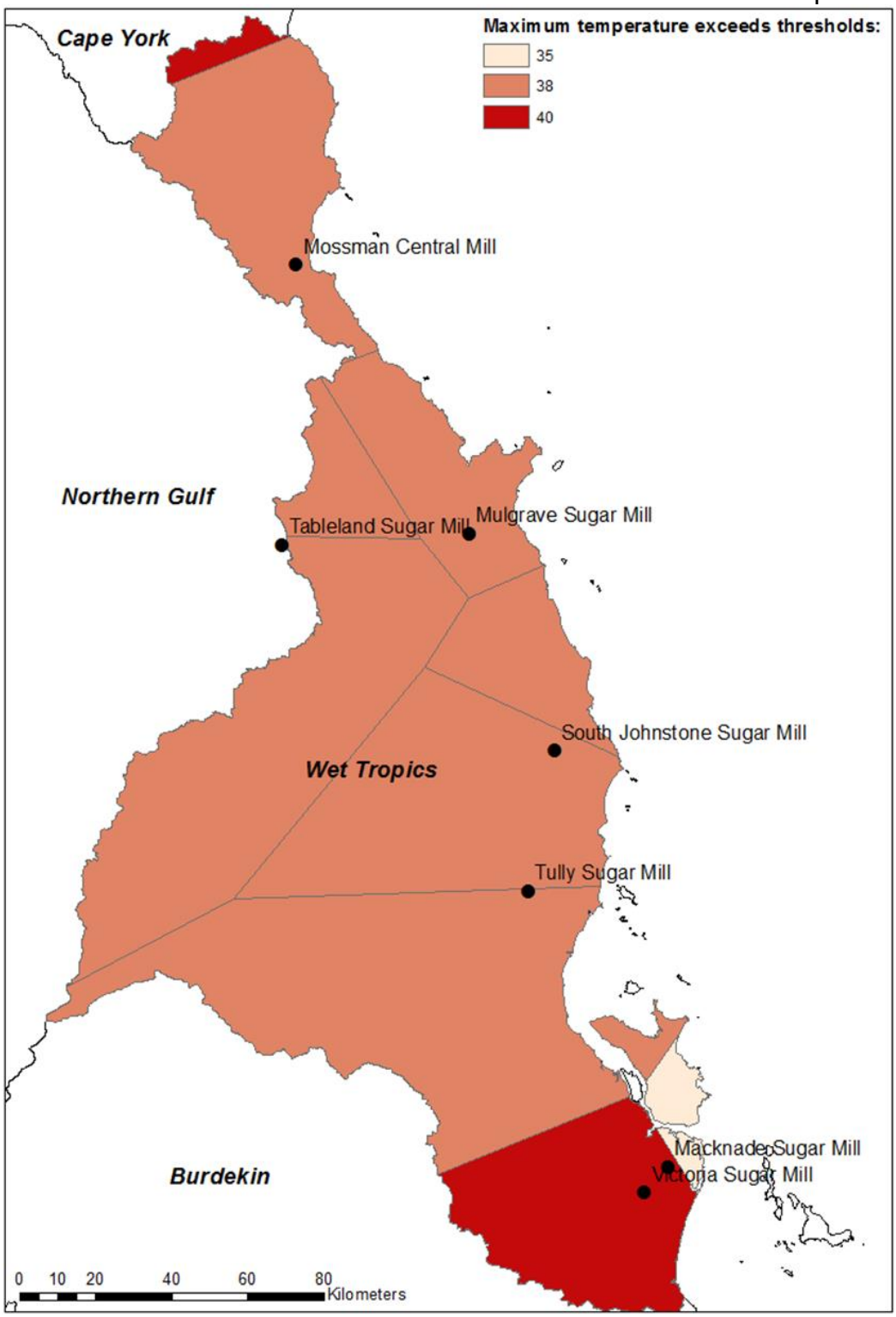


Figure 5.6. Wet Tropics region temperature threshold exceeds 35, 38 and 40ºC.

5.7 Framework to illustrate the proposed integrated bio-economic model co-locating microalgal biomass production with sugar mills

Microalgal mass cultivation requires a large volume of water and inorganic nutrients such as nitrate and phosphate, which are highly abundant in agricultural run-off and sugar mills discharge effluents (Lucas & Southgate, 2019). Recirculating sugar mill wastewater into microalgal cultivation would have mutual benefit to both parties to reduce the discharge nutrient and improve the sustainable biomass production **(Figure. 5.7)**. Since our *ectABC* transformed *S. elongatus* PCC7942 can tolerate salinities from 0 to 18 ppt, waste freshwater from sugar mills in Wet Tropics would be an option. In this research, we developed a sustainability framework to illustrate the proposed bio-economic model **(Figure 5.7)** of microalgal biomass production co-located with sugarcane processing sugar mills industries. Co-location of algal farms coupled with sugarcane processing industries would provide an added advantage to improve the water quality of generated wastewater during the process of sugarcane processing. The proposed process is set out below.

Crushing sugarcane generates a by-product called bagasse. Boiling bagasse generates heat energy and CO_2, the later can be a cheap carbon source for photosynthetic microalgal or cyanobacterial biomass production (Lohrey & Kochergin, 2012). Supply of industrial release CO_2 has the further advantage of avoiding the need to purchase large volume of CO_2 resulting in a significant cost reduction to algal biomass production. Sugarcane processing requires large amounts of freshwater and discharges 50% of the initial water volume as wastewater. For example, processing of one ton sugarcane requires 1500-2000 L of freshwater, which would generate 1000 L of wastewater (Sahu, 2018). The generated wastewater contains suspended solids, nitrogen, phosphates, calcium, chlorides, magnesium, oil and grease which can be directed to settlement ponds for pre-filtration to remove those excess suspended solids (Sahu, 2018). The settlement pond option has been established in the flow chart diagram **(Figure. 5.7)** as a remediation strategy for sugar mills and aquaculture wastewater prior to release into the environment. Settlement pond technologies are widely used as low-cost options for treating municipal, fish farm, and dairy farm wastewater (Lohrey & Kochergin, 2012). The pre-treated wastewater can then be

recycled into open pond cultivation of *ectABC transformed S. elongatus* PCC7942 for animal feed and high-value phycocyanin production.

After harvesting the end biomass, final cleaner water can be recycled into the sugarcane processing facilities. In addition, the sugar mills released waste gases (flue gas) can be recycled through pipeline into microalgal biomass production, which would be an added advantage in co-locating algal farms with sugarcane-industry. Furthermore, the energy required for dewatering and drying can be derived from bagasse combustion at sugar mills. The final dried biomass can be utilized for the production of animal feed and phycocyanin **(Figure. 5.7)**.

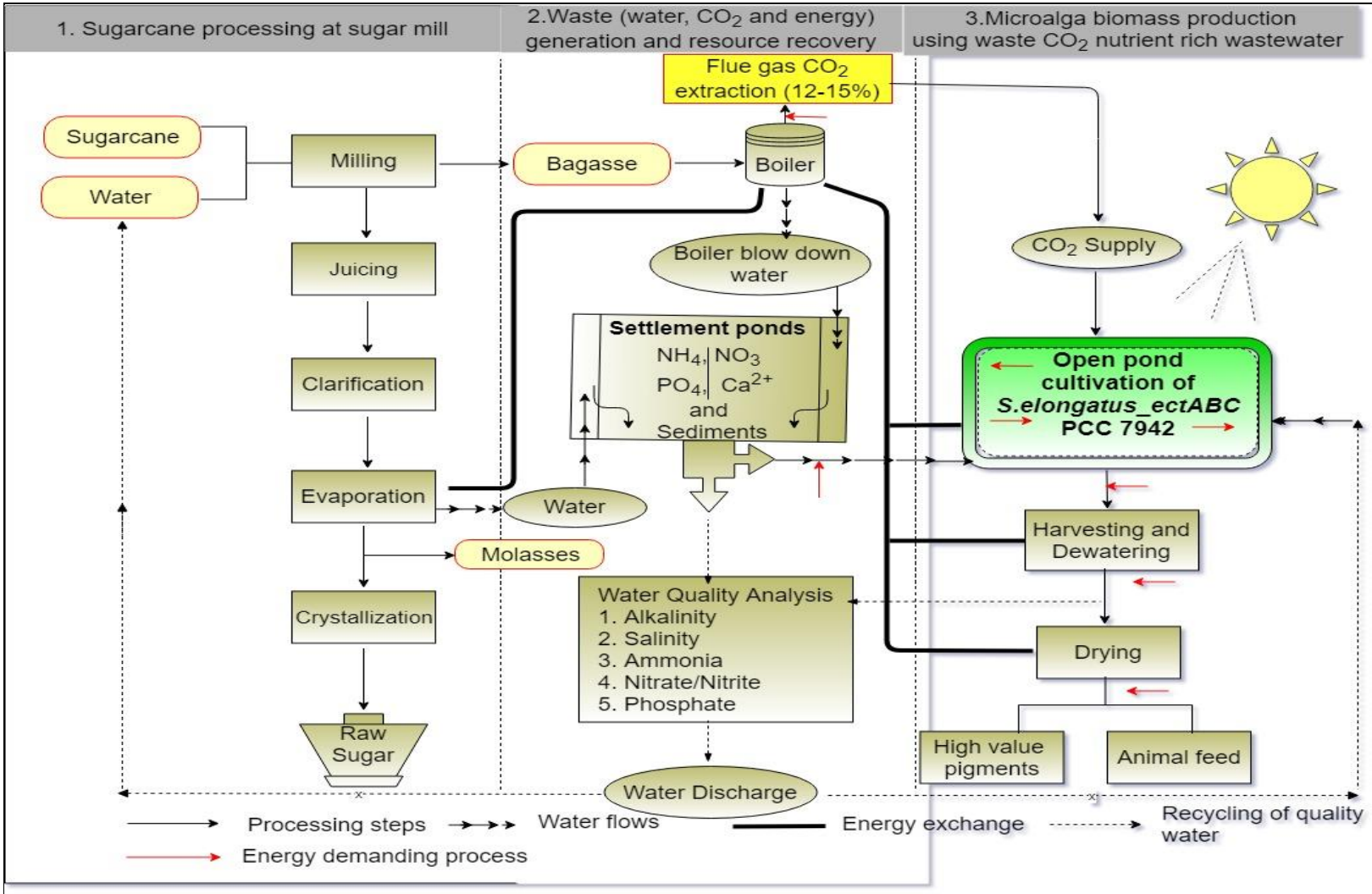


Figure 5.7. Framework for integrated bio-economic model of co-locating microalgal production with sugar mills

5.8 Resource evaluation from sugarcane and sugar industry

An Excel spreadsheet was developed to estimate waste resources generated such as water, CO_2 and energy resources for cost effective microalgal biomass production. Based upon our framework **(Figure. 5.7**), our model assumed a typical sugar mills processing 1,000,000 tons of sugarcane per annum. The operating season of sugarmill vary from 22-24 weeks in Australia. As our climatic suitability studies revealed Tully Sugar mill as an ideal partner for co-locating cyanobacterial biomass production, we focus on data relating to production levels at this mill. Tully sugar mill operates 24 weeks for 24 hours (http://www.tullysugar.com.au/index.php/products/operationalstatistics), and this facilty crushed 2,935,950 t of sugar cane during 2016. Therefore, our assumption of 1,000,000 ton processing sugarcane facility is cautious, and equates to 5,952 t per day. We have not sourced specific costs and production rates with in the Wet Tropics (an acknowledged limitation of this research), instead we have adopted a benefit transfer approach, whereby we have utilised appropriate values determined by other researcher in previous studies. The values of bagasse, sugar,

molasses and CO_2 productions rates included within are model are those suggested by previous research (Allen et al., 1997; Lohrey & Kochergin, 2012; OGTR, 2011; Sudhakar & Vijay, 2013). (**Table 5.3**).

Table 5.3. Values and assumption for resource estimation at sugar mills.

Parameters	Value	Reference
Sugarcane processed (tons/yr)	1,000,000	Own assumption
Mill operating season	24/168	Tully sugarmill
Mill capacity (tons/day)	5952	Own assumption
Sugar introduced with cane	13%	(Lohrey & Kochergin, 2012)
Bagasse wet wt (% on cane)	27.2	(OGTR, 2011)
Moisture content in bagasse	50%	(Lohrey & Kochergin, 2012)
Bagasse dry wt (% on cane)	13.6	(Allen et al., 1997; OGTR, 2011)
Excess bagasse	15%	(Lohrey & Kochergin, 2012)
Heating value of bagasse	9500 kj/kg	(Sudhakar & Vijay, 2013)
CO_2 produced (ton/ton of bagasse dry wt)	3.12	(Lohrey & Kochergin, 2012)
Molasses output (ton/ 100 ton of sugarcane)	2.6	(Allen et al., 1997; OGTR, 2011)
Wastewater generated at mill (ton/ 100 ton of sugarcane)	50.7	

On this literature findings, our estimates (**Table 5.4**) for 1,000,000 ton sugar cane processing sugar mill facility would have 500,000 t of water and 272,000 t of bagasse (**Table 5.4**). 24 weeks of sugar mills operation could supply the surplus amount of bagasse. Bagasse combustion generated flue gas could supply the 360,672 t of CO_2 during 24 weeks of operational season and remaining bagasse (15 % of total bagasse)

could generate 52,728 t CO_2 that can be used for microalgae or cyanobacteria cultivation when sugar mills are operational (**Table 5.4**). Similarly energy derived from bagasse would also be sufficient for sugar mill operation (24 weeks) and cyanobacterial cultivation throughout the year. Every year since 1997, the excess electricity (10MW) generated at Tully sugar mills is exported to the national grid which generates additional profit for the sugar industry. Australian Business Council for Sustainable Energy (BCSE) suggested that energy derived from sugar mills could meet 10-17% of Australia's electricity consumption by 2020 (Needham, 2011). However, the excess bagasse produced at Tully sugar mill during 24 weeks of operation are not used to convert electricity due to limitations resulting from their current infrastructure. Consequently, dumping of the left over bagasse on the land produces methane (a green house gas contributing to global climate change), which will be additional problem for the industry and environment within the Tully region, and more widely. Co-locating cyanobacterial production facilities, whose energy demands for operation through out the year could utilise the left over bagasse for generating more energy and CO_2

supply for cyanobacterial production, could thus avoid these problematic methane emissions.

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Table 5.4. Resource estimation at sugar mills for co-locating microalgal biomass production based upon costs and benefits transferred from previous research set out in Table 5.3

Parameters	Unit	Value	Calculated values	
Sugarcane crushed	tonnes/yr	1,000,000		
Cane processed	tonne/day	5,800		
Operating season	weeks/yr	24	168	days/yr
Cane composition				
Sugar introduced with the cane	(%)	13	130,000	tonnes/year
Fiber introduced with cane (Bagasse dry wt)	(%)	13.6	136,000	tones/year
Water introduced with cane	Litres/tonne	2,000	2,000,000,000	Litres/yr
Raw sugar produced	tonne/yr	1,430,000		
Molasses output		26,000		
Bagasse produced		272,000		
Heating value of bagasse	kj/kg with moisture	9,500	4750/ dry wt	
Bagasse compustion (tonnes to kg)		Bagasse combustion	tonnes to kg	
24 weeks season sugarcane processing		115,600	115,600,000	
Excess bagasse left after 168 days of mill operation		20,400	20,400,000	
Total (/yr)		136,000	136,000,000	
Energy				
24 weeks season sugarcane processing	kj/kg with moisture	113,100,000*9500	1,098,200,000,000	
Excess bagasse left after 168 days of mill operation		16,900,000*9500	193,800,000,000	
Energy Generated kj/kg	Total/yr		1,292,000,000,000	

Carbon dixiode			
24 weeks season sugarcane processing	3.12 ton CO2/ ton bagasse	113,100*3.12	360,672
Excess bagasse left after 168 days of mill operation		16,900*3.12	52,728
Carbon dixiode generated	Toal/yr		413,400
Waste water			
Waste water produced (50.7 ton/100 ton of sugarcane)			
Sugarcane input	Litres/tonne	1,000,000	500,000
Waste water generated	Total/yr		500,000

*Cane processing and operational assumptions were taken from operational statistics of Tully Sugar Mill located in wet tropics region

5.9 Cost benefit analysis for co-location microalgal production at sugar mills

In this analysis we provide a proof of concept for our method. A 10 ha facility has been assumed, including 8 ha cultivation surface area, which would have the capability of producing 450 t dry cyanobacterial biomass per annum. This assumption is based on recent report published by (Schenk, 2016). The assumed values for capital and operating costs for producing 450 t dry cyanobacterial biomass are also based on previously reported information (Moheimani, 2017; Schenk, 2016). Our frame work model indicated that the cyanobacterial biomass produced at sugar mill facilities could have been used as animal feed or high-value pigment phycocyanin. Among these animal feeds and pigments production are technically feasible options as demonstrated in (Schenk, 2016) and (Heimann et al., 2013) repectively. However, this study considered the energy demand associated with pigments extraction which will be detailed in our further studies.

Table 5.5. Microalgal facitlity capital and operating costs values and assumptions

Microalgal farm inputs	Unit	Cost (AUD)
Land acquisition if not co-located[a]	$/ha	21,427.09
Open raceway pond construction[b]	$/ha	51,469
Water requirement if not co-located [c]	$	1,768,549
CO2 purcahse if not co-located [d]	$	576,800
Energy demand for flue gas supply if co-located[d]	$/yr	134,640
CO_2system[d]	$/ha	9,756
Water and nutrient supply system[d]	$/ha	22,995
Harvesting and Dewatering system[d]	$/ha	18,308
Energy demand for cultivation, dewatering and drying[d]	$/yr	15,948
Total depreciable fixed capital[d]	$	2,619,894
Standardized indirect capital costs		
Engineering fees[d]	15% capital	392,984
Contingency[d]	5% capital	130,995
Working capital[d]	5% capital	130,995
Operating costs		
Labour –Plant manager/ Supervisor[d]	2 person ($)	235,502.28
Labour - Engineer[d]	2 person ($)	175,257.50
Labour- Lab analyst[d]	2 person ($)	131,443.14
Labour- Administration[d]	2 person ($)	125,966.34
Labour- Technician/ Pond operator[d]	2 person ($)	104059.14
Maintenance and Insurance[d]	10% of total capital	261989.35
Biomass derived potential income for cyanobacteria		
Animal feed[e]	$/ha yr^{-1}	39,163.6
Phycocyanin[e]	$/ha yr^{-1}	1174907.74

Source:[a] (Griffin & Batten, 2009); [c](Doshi, 2017);[d](Moheimani, 2017);[e](Heimann et al., 2015a); ;

All the costs were inflation adjusted to 2019 AUD value[4] and thus summarised in the **table 5.5.** Analysis of these costs between those that may or may not be avoidable by co-location are shown in the table S6 in supplementary materitals. Biomass derived products such as animal feed and high-value c-phycocyanin generated income assumptions were based on the report produced by (Heimann et al., 2013) (Table 5.5). By utilising these estimates of costs and volumes of production that could be generated from co-locating a cyanobacterial biomass production facility with a sugar cane facility within the Wet Tropics we estimated a simple cost-benefit analysis to calculate the potential net present value of such a facility.

Many development decisions have a time dimension, with different costs and benefits arising at different time points (Tietenberg, 2014; Turner, 1994). Thus, a method is required to evaluate benefits and costs occurring at different points in time. This is facilitated by utilising the concept of present values, which allows us to incorporate the time value of money and to compare dollars today to dollars in some future period by translating everything back to its current worth. Simplistically, the costs and the income or other benefits that arise in each time period first

[4] As of close 6/3/19 https://www.xe.com/currencyconverter/convert/?Amount=1&From=USD&To=AUD)

needs to be estimated, and then the present value of each flow is calculated by using a discount rate to determine the current value of each future flow. Capital costs, investment in working capital, sales values and operating costs based on previous research (**Table 5.5**). The overall net present value of the project can then be determined by summing the present values of all of these various predicted future cash flows (**Supplementary table S5.1**).

In mathematical form, the net present value of a net benefit, $B, received n years from now is $\$B_n/(1 + r)^n$ where r is the discount rate. Thus, the net present value of a stream of net benefits {B0. . . Bn} received over a period of n years is the sum from time i = 0 until year n of $\$B_i/(1 + r)^i$. To decide whether a development project should go ahead, we apply the decision rule that if NPV > 0 then the project should be accepted, as the value of the present and future flow of benefits exceeds the value of the current and future flows of costs. Accordingly we have presented our calculation using 5%, and repeated the calculation using a lower rate of 3% and a higher rate of 10% (**Supplementary table S5.2**). This sensitivity analysis revealed that the financial viability of the model was not impacted by the choice of

discount rate (**Supplementary table S5.3**). Cyanobacterial co-production with sugar mills integrated facility has been assumed to have a 25 year useful life, with no site remediation works required at the end of this period. Sales and distribution costs have been assumed to amount to 50% of sales revenue.

Our indicative estimations, based on the assumptions and data above, indicate that the project is financially viable, and thus we recommend that a more detailed evalution should be prepared as part of future research on this topic. A summary of the results of our indicative estimation are set out in Table 5.6, whilst the full calculations can be found at supplementary section **(table S5.1, 5.2 and 5.3)**.

Table 5.6 Summary of indicative estimate of financial viability of co-integrated micro-algal sugar cane facility

Initial development expenditures	
Capital costs	1,487000
Initial investment in working capital	74000
Annual revenues and expenditures	
Revenue from sales of pigments (c-phycocyanin)	392000
Revenue from sales of biomass	11,749000
Operating costs	3,417000
Sales and distribution costs	6,070000
Net inflow per year	2,653000
NPV at 5% discount rate	31,534000

When estimating the NPV, beyond estimating the various cash inflows and outflows generated by the project, a further key decision relates to the discount rate to use within the calculation. This rate should reflect: (i) the time preference for money – individuals assign less weight to a benefit or cost in the future than to a benefit or cost now; and (ii) the opportunity cost of capital – if the capital was not used for the project being evaluated it could be used for something else instead. Having selected a rate perceived to be appropriate, say 5%, it is recommended that sensitivity analysis be conducted using alternate discount rates, enabling us to determine how sensitive the calculation is to the choice of

rate. The lower the rate, the more the estimate reflects the impact of flows occurring further into the future, whilst a higher rate places stronger emphasis on the present time and the near future.

5.10 Discussion:

This study presented a concept of how available resources at a sugarcane mill can be utilized to address the current main bottleneck of large scale micralgal or cyanobacterial cultivation and biomass production and to meet the energy requirement for drying the biomass. For our indicative proof of concept for the framework presented here, it has been shown that the energy contained in 15% excess bagasse is sufficient to supply all of the energy required for biomass production and drying the algae, which is about 50% of total process energy requirements. Using current algae production and harvesting technologies, co-location of an algal or cyanobacterial biomass production facility with a sugarcane mill can improve the overall economic feasibility of algae bio-energy projects by having available resources, infrastructure, and established markets for the produced biodiesel and algal meal. A study on determining the advantages of co-locating algae production with sugarcane mills has shown that the

integrated facility could signicantly reduce 50% total production cost by utilising the following waste products: CO_2, water, and energy (Lohrey & Kochergin, 2012).

Climate suitablility for our *ectABC transformed S. elongatus* PCC7942 was assessed. GIS modelling revealed that the Wet Tropics region has an average maximum temperature that exceeds 30ºC for half a year and there are some regions where temperature exceed 35, 38 and 40ºC. Interestingly, our analysis determined that nowhere in the region has an average temperature reached 45ºC from 2001 to 2017. Thus the Wet Tropics region sugar mills are an ideal industry for co-locating *ectABC* transformed *S. elongatus* PCC7942 that has a proven tolerance limit to 45 ºC (**Chapter 4**, **Figure 4.1**)

The Wet Tropics was also considered a suitable region due to the presence of established agricultural infrastructure and markets. Our assumptions on values and parameters for 1,000,000 t sugarcane processing sugar mill have been shown to provide sufficient resources for a 10 ha cultivation faciltity to produce 450 t dry cyanobacterial biomass for the production of high-value c-phycocyanin and low- value animal feed. Recognising that it is unrealistic to assume that 100% of

cyanobacterial biomass production will be sold each year, a sensitivity analysis was conducted to determine the minimum volume of production required for the facility to remain finacially viable that is to have an estimated NPV in excess of zero. For this analysis we held all capital and operating costs equal - that is we assumed the same scale of facility was developed, and the same volume of cyanobacterial pigments and biomass were produced each year. The revenue from sales of pigments and biomass was then reduced within the model, to reflect the outcome that only a proportion of all the cyanobacterial biomass produced was actually sold. Sales and distribution costs were estimated at 50% of the revised sales revenue, recognising that these costs would reduce if a lower volume were actually sold. Again a discount rate of 5% was used, and an estimated useful life of 25 years, and any potential revenue from Reef Credits was excluded. Based on this analysis, (shown a Table A5 in Appendix) we find the co-located facility to be financially viable provided that at least 58% of the cyanobacterial biomass produced at the facility can be sold each year. Further research is required to ascertain whether the potential market is such that there is demand for this level of production.

We also find that significant reductions in the total production costs are available by co-locating with sugar mills. Indeed, this initial analysis suggests that up to 82% of the operating costs of a stand alone facility could be avoided by fully exploiting all of the potential benefits resulting from this co-location (Appendix Table A.4).

This analysis also excludes the likely future benefits that could arise from participation in the Reef Credit scheme currently under development, generating an additional income stream for the co-located facility.

Overall, our framework, and proof of concept analysis, indicates that the development of cyanobacterial production faciltiies co-located with adjacent industries have a useful role to play in helping solve (in part) current modern world problems of freshwater scarcity, and GHG emissions whilst creating jobs and generating incomes for rural communities in Australia.

Chapter 6

6 General discussion, limitations of this research, future research directions and conclusion

6.1 Introduction

Increasing CO_2 emissions are a global problem and the reduction thereof is essential to attempt to limit global temperature increases to 1-1.5°C (Allen et al., 2019). Likewise, freshwater constraints are a challenge under climate change, particularly in arid countries (Goddard & Al-Abri, 2019). Consequently, there is pressure for policies to be adopted by governments around the world to aim to reduce CO_2 emissions and freshwater use (Fekete et al., 2015; Holland et al., 2015). There is also increasing evidence that climatic instability reduces agriculture productivity (Wang et al., 2018), which, due to expected population increases to >9 billion people worldwide, challenges food security (FAO, 2017). Microalgae cultivation for renewable bioresource production for bio-product development offers integrated opportunities that reduce CO_2 emissions and remediate nutrient-rich wastewater, if production is co-located with source industries (Senate, 2016). Temperature and salinity levels, however, challenge production and these factors are extremely difficult to control in outdoor commercial-

scale cultivation (Linares et al., 2017; Mallick et al., 2016). This research aimed to examine if ectoine transformation could yield a sufficiently temperature- and salinity-tolerant cyanobacterium that could enable commercial cultivation in hitherto unsuitable areas and climates for the generation of large-volume, low-value bio-products, such as animal feed and bio-fertilisers.

6.2 Thesis outcomes and summary of key research findings

6.2.1 Summary of chapter 2 – Literature findings on economic challenges associated with co-locating microalgal biomass production with other industries.

My research used insights from published data to better understand the economic challenges that arise in commercial-scale microalgal production. The overall aim of my literature study was to provide a pathway for improving salinity- and temperature-resilience in cyanobacteria. My key findings from the literature were that extremophilic organisms synthesize ectoine in response to extreme salinity and temperature stress. Ectoine-transformed tomato and tobacco plants showed increased salinity tolerance, while transfection of the green freshwater microalgae with the *ectA* and *ectC* gene yielded no

positive outcomes, as it targeted the chloroplast relying on an *ectB* equivalent enzyme.

Whilst addressing these knowledge gaps in the lietarure on the inter-relationship of *ectA*,*ectB* and *ectC* genes for ectoine synthesis, the specific research question was set out to close these knowledge gaps by *ectABC* gene cassete transformation into freshwater cyanobacterium *S. elongatus* PCC7942 was investigated.

6.2.2 Summary of chapter 3 – Whether *ectABC* gene cassette transformation was successfully achieved into freshwater cyanobacterium *S. elongatus* PCC7942

The specific research question was set out to investigate whether transformation of *ectABC* gene cassette could successfully integrate into cyanobacteria. Therefore, I chose to transfer the *ectABC* gene cassette from the salinity- and temperature-tolerant bacterium *Halomonas elongata* DSM3043 into the freshwater cyanobacterium *Synechococcus elongatus* PCC7942 to examine whether this would yield a more salinity- and temperature-tolerant cyanobacterium.

The current policies on developing GMO strains and OGTR regulations allowed me to work with the freshwater cyanobacterium *Synechococcus*

elongatus PCC7942, as it is exempt from bans on genetic modification at laboratory scale. The availability of species-specific vectors and established transformation protocols facilitated *ectABC* gene cassette-transformation into *S. elongatus* PCC7942. My research successfully codon-optimised the *ectABC* gene cassette to overcome codon-bias in *S. elongatus* PCC7942, which yielded temperature- and salinity-tolerant *ectABC*-transformants.

6.2.3 Summary of chapter 3 –Could *ectABC* gene cassette transformation yield temperature and salinity resilient cyanobacteria by enabling ectoine expression

Specifically, the *ectABC*-transformed *S. elongatus* PCC7942 tolerated temperatures of 45ºC and still showed significant growth at a salinity of 18 ppt at a permissible temperature of 35°C, conditions that were not tolerated by either the *pSyn_6* empty vector (the control transformed *S. elongatus* PCC7942 - lacking the *ectABC* gene cassette) or the WT (untransformed) *S. elongatus* PCC7942.

As the economic viability of commercial-scale microalgae or cyanobacterial biomass production is tightly linked to meeting the requirements for nitrogen and phosphate, phosphate and nitrate uptake

was examined. The *ectABC*-transformant required more nitrogen during the fast growth period to cope with increases in salinity and temperature stress, possibly for ectoine synthesis. This indicates that mass cultivation of *ectABC*-transformed *S. elongatus* PCC7942 would require increased nitrogen fertilisation if the cultivation environment experiences significant increases in temperature and salinity stress.

Salinity and temperature stress significantly improved lipid and fatty acid contents which was expected in response to temperature stress, as fatty acids are the main driver to balance membrane fluidity of the cell (Bergmann et al., 2013). Ectoine expression in the *ectABC*-transformed *S. elongatus* PCC7942 was confirmed by HPLC and mass spectrometry. Although significant improvement in temperature tolerance was achieved, ectoine quantifcation indicated that increased salinity induced more ectoine expression than increased temperature. In contrast to increased nitrogen requirements of the *ectABC*-transformed *S. elongatus* PCC7942, phosphate requirements were unaffected, unlike the *pSyn_6* empty vector-transformed *S. elongatus* PCC7942 and WT control *S. elongatus* PCC7942.

6.2.4 Summary of chapter 5- whether *ectABC* transformed *S. elongatus* PCC7942 suitable for industrial co-location for achieving sustainable and successful biomass production.

Salinity- and temperature- tolerant *ectABC*-transformed *S. elongatus* PCC7942 suitable for production that is co-located with industries producing nitrogen-rich waste waters. Accordingly, my research investigated the feasibility for co-locating the *ectABC*-transformed *S. elongatus* PCC7942 culture with adjacent industries where this integrated approach could improve the associated water quality of the industry. My key findings determined that the Wet Tropics offers a suitable location for *ectABC*-transformed *S. elongatus* PCC7942 culture co-located with sugar mills, suggesting that such a production facility has the potential to be financially viable. The integration of salinity- and temperature-tolerant cyanobacterial biomass production (using the already existing open pond cultivation strategy) paves a clear pathway to generate cleaner water and animal feed for adjacent aquaculture facilties who currently rely on feed from external suppliers. Such co-location would alleviate capital and operating expenditure on energy, CO_2 and water, so it has the potential to reduce GHG emissions - essential for sustained economic growth of the regional population.

Furthermore, the demonstrated integrated cultivation platform is significantly more water-efficient than are traditional commercially implemented open pond designs, making it applicable to environments where water resources are scarce. This is a significant advance beyond existing cultivation methods.

6.3 Research limitations and policy implications on *ectABC* transformed *S. elongatus* PCC7942 commercial development

My research was constrained by several limitations. While the *ectABC*-transformed *S. elongatus* PCC7942 yielded some salinity tolerance, tolerance, levels matching those of *Halomonas elongata* DSM3043 were not achieved, limiting production to water sources with no more than 18 ppt. This limited salinity tolerance is potentially due to the low levels of ectoine expression achieved, which needs to be improved by 2 orders of magnitude in order to compete with other commercial ectoine-producing organisms, if high-value ectoine production is desired for co-product development. Another hurdle that would need to be addressed in order to take this research to an applied level is the amendment of policies and legislations, requiring approval from various departments, which will be a time-consuming process (**Table 6.1**). Zhang et al. (2016) reported

that wild microalgal strains from the local environment are particularly suitable for open-pond systems, especially when wastewater is used as a substrate for growth. However, the operation of aquaculture industry is a high risk and capital-intensive industry requires permission and approval for wastewater recycling and cultivating cyanobacterial GMO's in open environment and recycling of wastewater from other related aquaculture facilties, which limits the development and expansion of aquaculture industries in Australia.

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Table 6.1 Legislations and policy implications aquaculture industry operation and cyanobacterial GMO's cultivation

Activities	Legislation Acts	Governing and certifying bodies
Development and approval for operational works in freshwater areas for water extraction, effluent discharge, recycling and protection of coral habitats	Waste reduction and recycling act 2017 Sustainable planning act 2009 Environment Protection (Waters et al.) policy 2009	Environment and Heritage Protection, Fisheries Queensland
Permission for take of species or collection from wild environment	Environmental Protection and Biodiversity Conservation Act 1999 Great Barrier Reef Marine Park Act 1975	Commonwealth Department of the Environment and Energy and GBRMPA
Relocation of aquatic animals or industry one place to other	Fisheries Act 1994	Fisheries Queensland
Importation of aquatic species outside Australia	Quarantine Act 1908	Department of Agriculture and Water Resources
Food Safety if the product is for human consumption	Food Act 2006, Food Production(Safety) Act 2000 General Fisheries Permit and Fisheries Act 1994	Safe food Queensland health, Fisheries Queensland
Dealing with GMO''s in laboratory environment if the species are not under exemption	Gene Technology regulations 2001 Gene Technology ACT 2000	Office of Gene Technology Regulator
GMO'S Field trial and risk mitigation approval	Gene Technology regulations 2001 Gene Technology ACT 2000	Office of Gene Technology Regulator and Department of Agriculture, Land and Water Resources
GM food safety	Australia New Zealand Food Standards Act 2003	Australia New Zealand Food Standards

Current policies and legistations are significant constraints for any newer development in the aquaculture industries (Mohd Nazri, 2017). In addition, our research identified that the generation of nutrient-laden wastewaters from aquaculture industries cause significant amount of nutrients and sediments flows into GBR. Great Barrier Reef Marine Park regulations 2000 is very specific to aquaculture industries to discharge on zero net nutrient discharge into GBR (Mohd Nazri, 2017) which was identified one of the main reasons for the absence of aquaculture industry expansion and development in Queensland and Australia (Mohd Nazri, 2017). While co-location of *ectABC*-transformed *S. elongatus* PCC 7942 would help to combat this constraint for aquaculture production at moderate salinity levels, existing policies and legislation would disallow this approach, which emphasises that policy review and change is required for Australia.

Finally, a limitation of the economic analysis investigating the feasibility of co-locating cyanobacterial biomass production with sugar mills within the Wet Tropics region of northern Queensland is that this analysis is based on the benefit transfer approach, utilising benefits and costs identified in other studies conducted in other locations. Thus, the

outcomes of our case study region are indicative only, and need to be fine-tuned by utilising costs and benefits sourced specifically within the target (Wet Tropics) region. Furthermore, the level of demand for the cyanobacterial biomass and pigments produced by the hypothesized production facility has not been explored; instead the indicative model developed by this research has been used to estimate the required level of sales for the project to be financially viable. Finally, the research has identified the potential for additional revenue to be generated by Reef Credits, but has not quantified the value of these potential revenue streams.

6.4 Future research directions

As ectoine could yield a high-value co-product, low levels of ectoine expression could be tackled in further studies focusing at molecular level using different promoter sequences to improve *ectABC* gene expression. The moderate levels of salinity-tolerance achieved in the *ectABC*-transformed *S. elongatus* PCC7942 could potentially be overcome by inserting *ectD* along with *ectABC*, which may yield resilience to the extent of the source organism's tolerance (*Halomonas elongata*

DSM3043). This would yield hydroxyl-ectoine as a potential product, which is of equally high value in the cosmetics industry (Czech et al., 2018).In addition, further research on integrating cyanobacterial biomass production that is co-located with sugar mills and aquaculture industries. Our frame work model (**Figure 6.1**) indicated the opportunities for both sugar mills and algal industry for direct selling of molasses and cyanobacterial biomass as a feed to all growth stages of bivalves, fish and crustaceans respectively (**Figure 6.2**). In Aquaculture operation either fish or prawn farming, feed is a major stand-alone factor increases the production cost i.e., typically 50-60 percent of total production costs in aquaculture systems (Hasan, 2010). Similarly, molasses from sugar mills could serve as carbon source which enhances the bacterial growth and thus limiting harmful algal bloom by controlling inorganic nitrogen and pH in aquaculture fish or prawn ponds (Willett & Morrison, 2006). Molasses and aquatic feed expenditures can be overcome by using molasses from co-located sugar mill and farm made feed from cyanobacterial biomass production which are affordable, accessible by rural farmers due to minimum resource input in own feed

.

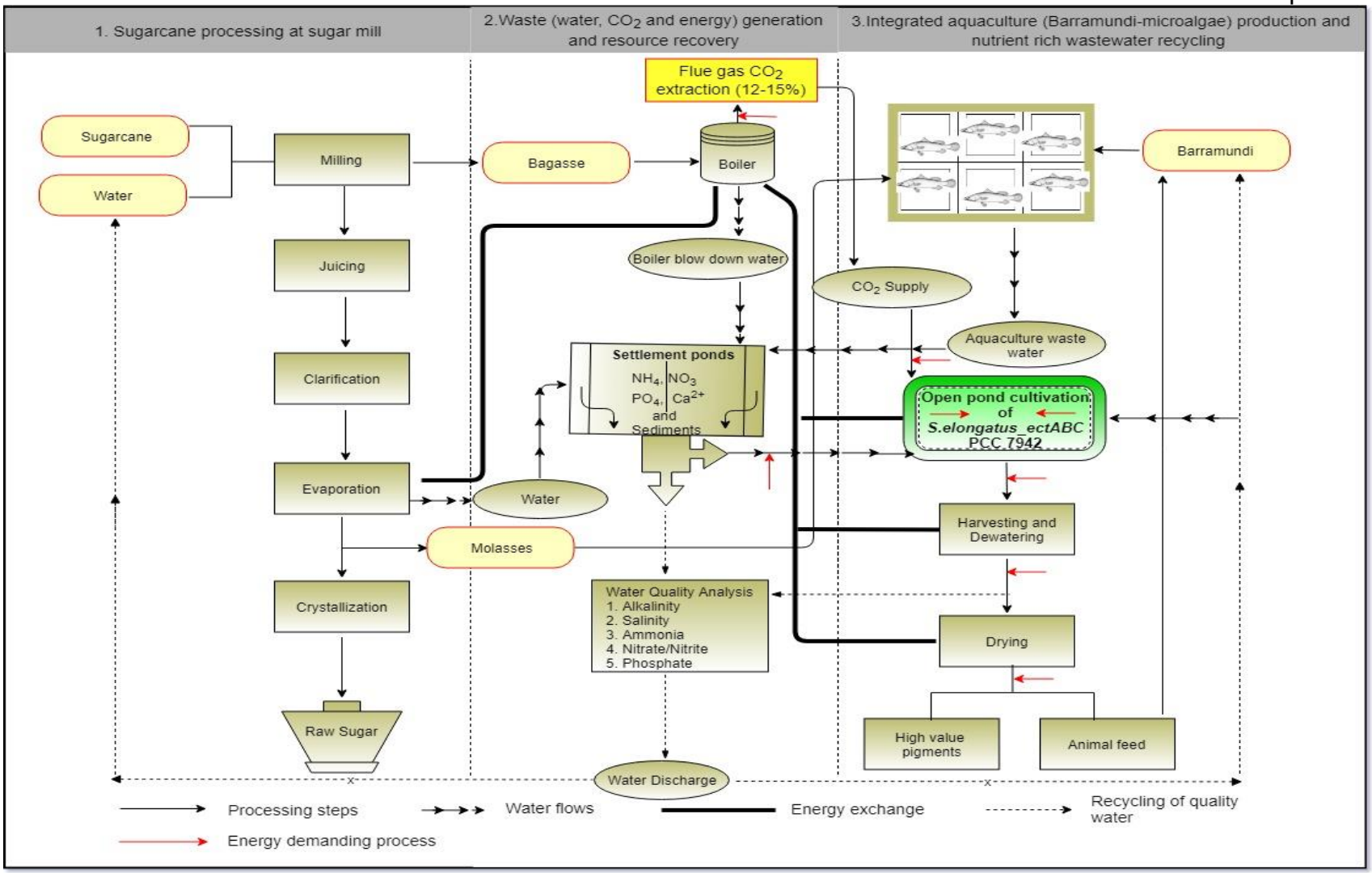

Figure 6.1. Framework for integrated bio-economic model of co-locating microalgal production with sugar mills

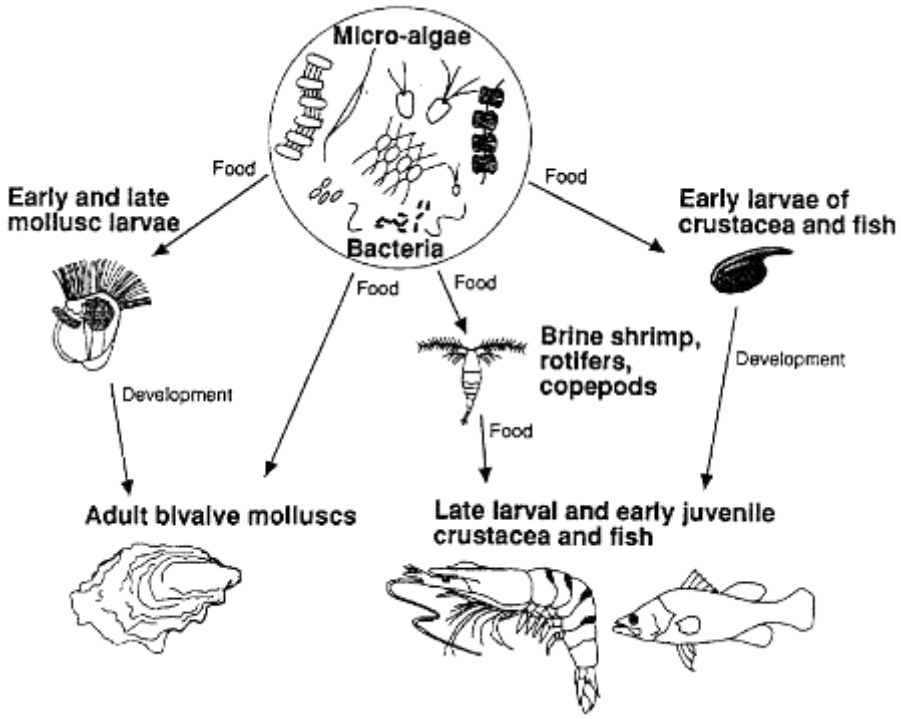

Figure 6.2. Traditional uses of microalgae or cyanobacteria as a feed in Aquaculture (http://www.fao.org)

Several studies reported that feed choice and feed management practices have a significant impact on the economic performance of a production system (Hasan, 2010). Cyanobacterial biomass production for aquatic feed has been widely suggested and researched as a sustainable way to solve the global energy, food and environmental crisis and the current competition for resources (e.g., land and water). Overall, this research addresses an important area of research important for transitioning to a bio-economy and implementing environmentally and

economically sustainable production of renewable low value high volume bio products such as animal feed and some high value neutraceuticals, specifically.

In addition to sugarmill, co-location of end biomass using related aquaculture industries could significantly reducing costs of transporting the biomass to feed industries (Benemann, 2013; Heimann et al., 2015b; Mandal & Mallick, 2012).While examining these integration feasibilities, it is also important to determine the techno-economics for larger scale production, in order to attract current industrialists or investors to invest in achieving profitable biomass production.

Future reseach would usefully expand the economic analysis presented here to reflect actual costs and benefits (both price and customer demand) within the Wet Tropics region, and to determine likely levels of demand for the biomass and pigments produced; these are non-trivial tasks that would be required as the next step towards developing a robust business case for such a development.

6.5 Final remarks and thesis conclusions

In my research, I have successfully transformed a codon-optimised *ectABC* gene cassette into *S. elongatus* PCC7942 *ectABC* gene transformation significantly improved resilience to both salinity and temperature stress. Thus the *ectABC*-transformed *S. elongatus* PCC7942 can be cultivated in the tropics. Salinity stress-dependent ectoine expression suggested that the *ectABC*-transformed *S. elongatus* PCC7942 can be cultivated in a brackish wastewater stream which significantly reduces freshwater demand for cultivation. Nitrogen demand of the *ectABC*-transformed *S. elongatus* PCC7942 could be met in the Wet Tropics region where nitrogen is highly abundant in discharge wastewater from agri-aquacultrue industries. Based on the available data, the nascent microalgal industry is developing slowly, mainly due to required investment for production facilities and infrastructure, especially for low-value- high-volume bio-products such as fuel, feed, and fertiliser.

The take home message of my research to commercial producers of nascent algal biomass producing industries is that they should aim to generate and develop integrated cultivation with adjacent industries to achieve significant reductions in operation and production costs. The

indicative analysis presented here suggests that such a commercial production facility could be financially viable. In addition, my thesis demonstarated that integrating cyanobacterial cutivation with sugar mills could also significantly improve cyanobacterial biomass production and recirculating wastewater, which would reduce the environmental impact associated with heavy loads of nutrient discharge into the GBR. Also setting up a large-scale business with *ectABC*-transformed *S. elongatus* PCC7942 could significantly contribute to achieving the emissions reduction target of 28-30% by 2030 and the target of the Reef 2050 water Quality Improvement Plan under the Reef 2050 Long-Term Sustainability plan to protect Australia's most prestigious World Heritage Site, the GBR.

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8 Supplementary Materials

S 1. *Halomonas elongata* DSM3043 genes for ectoine synthesis, *ectA, ectB* and *ectC* genes (GenBank accession no: **AJ011103.2**)

***Ect A* gene**(Diaminobutyric acid-acetyltransferase)

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  1 ATGACGCCTACAACC GAGAACTTCACGCCT TCCGCCGATCTGGCC CGCCCGAGCGTTGCC GACACGGTCATCGGC
  1  M  T  P  T  T   E  N  F  T  P   S  A  D  L  A   R  P  S  V  AD  T  V  I  G
 76 AGCGCGAAGAAAACC CTCTTCATCCGTAAG CCCACGACCGACGAC GGGTGGGGCATTTAC GAACTCGTCAAGGCA
 26  S  A  K  K  T   L  F  I  R  K   P  T  T  D  D   G  W  G  I  Y   E  L  V  K  A
151 TGCCCGCCACTGGAC GTCAACTCGGGGTAC GCCTATCTGCTGCTT GCCACCCAGTTCCGC GATACCTGCGCCGTG
 51  C  P  P  L  D   V  N  S  G  Y   A  Y  L  L  L   A  T  Q  F  R   D  T  C  A  V
226 GCTACGGACGAAGAA GGGGAGATCGTCGGT TTCGTCTCCGGCTAC GTCAAACGCAACGCA CCGGACACCTACTTT
 76  A  T  D  E  E   G  E  I  V  G   F  V  S  G  Y   V  K  R  N  A   P  D  T  Y  F
301 CTATGGCAAGTCGCC GTCGGCGAGAAAGCG CGGGGCACGGGCCTG GCGCGACGCCTCGTC GAAGCCGTGCTGATG
101  L  W  Q  V  A   V  G  E  K  A   R  G  T  G  L   A  R  R  L  V   E  A  V  L  M
376 CGCCCCGGCATGGGC GATGTCCGCCATCTC GAAACTACCATCACG CCCGACAATGAGGCA TCGTGGGGACTGTTC
126  R  P  G  M  G   D  V  R  H  L   E  T  T  I  T   P  D  N  E  A   S  W  G  L  F
451 AAGCGCCTTGCCGAT CGCTGGCAAGCCCCC CTGAACAGCCGTGAA TACTTCTCCACGGGG CAGTTGGGTGGCGAG
151  K  R  L  A  D   R  W  Q  A  P   L  N  S  R  E   Y  F  S  T  G   Q  L  G  G  E
526 CACGATCCGGAAAAT CTCGTCCGTATCGGC CCGTTCGAGCCGCAA CAAATTTGA
176  H  D  P  E  N   L  V  R  I  G   P  F  E  P  Q   Q  I  *
```

***Ect B* gene** (Diaminobutyrate-2-oxoglutarate transaminase)

```
  1 ATGCAGACCCAGATT CTCGAACGCATGGAA TCCGAAGTCCGGACC TATTCACGTTCTTTT CCTACCGTTTTCACT
  1  M  Q  T  Q  I   L  E  R  M  E   S  E  V  R  T   Y  S  R  S  F   P  T  V  F  T
 76 GAAGCCAAGGGCGCG CGCCTGCATGCCGAG GACGGCAACCAGTAC ATCGATTTTCTCGCC GGCGCCGGCACGCTC
 26  E  A  K  G  A   R  L  H  A  E   D  G  N  Q  Y   I  D  F  L  A   G  A  G  T  L
151 AACTACGGTCACAAC CACCCCAAGCTCAAG CAGGCACTGGCCGAT TACATCGCCTCCGAT GGCATCGTCCATGGT
 51  N  Y  G  H  N   H  P  K  L  K   Q  A  L  A  D   Y  I  A  S  D   G  I  V  H  G
226 CTGGACATGTGGAGC GCGGCCAAGCGCGAC TATCTGGAAACCCTC GAAGAGGTGATCCTC AAGCCGCGTGGCCTG
 76  L  D  M  W  S   A  A  K  R  D   Y  L  E  T  L   E  E  V  I  L   K  P  R  G  L
301 GATTACAAGGTTCAT CTGCCGGGCCCGACG GGCACCAATGCCGTG GAAGCCGCCATTCGA CTGGCGCGCAACGCC
101  D  Y  K  V  H   L  P  G  P  T   G  T  N  A  V   E  A  A  I  R   L  A  R  N  A
376 AAGGGTCGTCACAAC ATCGTCACCTTCACC AACGGATTCCATGGC GTGACCATGGGCGCG CTGGCCACCACCGGC
126  K  G  R  H  N   I  V  T  F  T   N  G  F  H  G   V  T  M  G  A   L  A  T  T  G
451 AATCGCAAGTTCCGT GAAGCCACCGGCGGT ATCCCGACCCAGGGC GCCAGCTTCATGCCG TTCGATGGCTACATG
151  N  R  K  F  R   E  A  T  G  G   I  P  T  Q  G   A  S  F  M  P   F  D  G  Y  M
526 GGCGAGGGCGTCGAC ACCCTGAGCTACTTC GAGAAACTGCTCGGC GACAACTCCGGTGGT CTCGACGTTCCCGCG
176  G  E  G  V  D   T  L  S  Y  F   E  K  L  L  G   D  N  S  G  G   L  D  V  P  A
601 GCCGTGATCATCGAG ACGGTGCAGGGCGAG GGCGGTATCAATCCG GCCGGCATCCCGTGG CTGCAGCGCCTGGAA
```

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  201  A   V   I   I   E     T   V   Q   G   E     G   G   I   N   P     A   G   I   P   W     L   Q   R   L   E
  676 AAGATCTGCCGCGAT CACGACATGCTGCTG ATCGTCGACGACATT CAGGCCGGCTGCGGT CGTACGGGCAAGTTC
  226  K   I   C   R   D     H   D   M   L   L     I   V   D   D   I     Q   A   G   C   G     R   T   G   K   F
  751 TTCAGCTTCGAGCAT GCCGGCATCACGCCG GACATCGTCACCAAC TCCAAGTCCCTGTCG GGTTTCGGCCTGCCG
  251  F   S   F   E   H     A   G   I   T   P     D   I   V   T   N     S   K   S   L   S     G   F   G   L   P
  826 TTCGCGCATGTGCTG ATGCGCCCGGAACTG GATATCTGGAAGCCC GGCCAGTACAACGGC ACGTTCCGTGGTTTC
  276  F   A   H   V   L     M   R   P   E   L     D   I   W   K   P     G   Q   Y   N   G     T   F   R   G   F
  901 AACCTGGCCTTCGTC ACGGCCGCCGCCGCG ATGCGTCACTTCTGG AGCGACGACACCTTC GAGCGCGACGTTCAG
  301  N   L   A   F   V     T   A   A   A   A     M   R   H   F   W     S   D   D   T   F     E   R   D   V   Q
  976 CGCAAGGGCCGTGTG GTCGAGGATCGCTTC CAGAAGCTTGCCAGC TTCATGACCGAGAAA GGGCATCCGGCCAGC
  326  R   K   G   R   V     V   E   D   R   F     Q   K   L   A   S     F   M   T   E   K     G   H   P   A   S
 1051 GAGCGTGGCCGTGGC CTGATGCGTGGCCTG GACGTCGGTGACGGC GACATGGCCGACAAG ATCACCGCACAAGCG
  351  E   R   G   R   G     L   M   R   G   L     D   V   G   D   G     D   M   A   D   K     I   T   A   Q   A
 1126 TTCAAGAACGGGCTG ATCATCGAGACATCC GGCCATTCAGGCCAG GTGATCAAGTGCCTT TGCCCGTTGACCATT
  376  F   K   N   G   L     I   I   E   T   S     G   H   S   G   Q     V   I   K   C   L     C   P   L   T   I
 1201 ACCGACGAAGACCTC GTCGGCGGCCTGGAC ATCCTCGAGCAGAGC GTCAAGGAAGTCTTC GGTCAAGCCTAA
  401  T   D   E   D   L     V   G   G   L   D     I   L   E   Q   S     V   K   E   V   F     G   Q   A   *
```

***Ect C* gene** (Ectoine Synthase)

```
    1 ATGATCGTTCGTAAC CTGGAAGAATGCCGC AAGACCGAGCGCTTC GTCGAAGCCGAAAAC GGCAACTGGGACAGC
    1  M   I   V   R   N     L   E   E   C   R     K   T   E   R   F     V   E   A   E   N     G   N   W   D   S
   76 ACCCGTCTGGTGCTG GCCGACGACAACGTC GGTTTCTCGTTCAAC ATCACCCGCATTCAT CCGGGTACCGAGACG
   26  T   R   L   V   L     A   D   D   N   V     G   F   S   F   N     I   T   R   I   H     P   G   T   E   T
  151 CATATCCATTACAAG CATCACTTCGAGGCG GTTTTCTGCTACGAA GGCGAAGGCGAAGTC GAAACGCTGGCCGAT
   51  H   I   H   Y   K     H   H   F   E   A     V   F   C   Y   E     G   E   G   E   V     E   T   L   A   D
  226 GGCAAGATCCATCCC ATCAAGGCCGGCGAC ATGTACTTGCTCGAT CAGCACGACGAGCAC CTGCTGCGCGGCAAG
   76  G   K   I   H   P     I   K   A   G   D     M   Y   L   L   D     Q   H   D   E   H     L   L   R   G   K
  301 GAAAAAGGCATGACC GTGGCATGCGTGTTC AATCCGGCGCTGACG GGCCGCGAAGTGCAC CGTGAAGACGGTTCC
  101  E   K   G   M   T     V   A   C   V   F     N   P   A   L   T     G   R   E   V   H     R   E   D   G   S
  376 TACGCACCGGTCGAT TGA
  126  Y   A   P   V   D     *
```

S 2. *pSyn_6* vector gene sequences composition.

RP4/RK2 *bom* site(1-304)

CTGGTTGGCTTGGTTTCATCAGCCATCCGCTTGCCCTCATCTGTTACGCCGGCGGTAGCCGGCCAGCCTC
GCAGAGCAGGATTCCCGTTGAGCACCGCCAGGTGCGAATAAGGGACAGTGAAGAAGGAACACCCGCTCGC
GGGTGGGCCTACTTCACCTATCCTGCCCGGCTGACGCCGTTGGATACACCAAGGAAAGTCTACACGAACC
CTTTGGCAAAATCCTGTATATCGTGCGAAAAAGGATGGATATACCGAAAAAATCGCTATAATGACCCCGA
AGCAGGGTTATGCAGCGGAAGATCGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACT
GAGCGTCAGAC

pUC origin(362-977)

CCCGTATAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTG
CTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTT
CCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTTCTTCTAGTGTAGCCGTAGTTAGGCC
ACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGC
CAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCG
GGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTAC
AGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAG
GGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGG
TTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACG
CCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTGTGCTGGGCCC

NS1a(neutral site 1a)(1049-1847)

CAATGCCTTCTCCAAGGGCGGCATTCCCCTGACTGTTGAAGGCGTTGCCAATATCAAGATTGCTGGGGAAGA
ACCGACCATCCACAACGCGATCGAGCGGCTGCTTGGCAAAAACCGTAAGGAAATCGAGCAAATTGCCAAG
GAGACCCTCGAAGGCAACTTGCGTGGTGTTTTAGCCAGCCTCACGCCGGAGCAGATCAACGAGGACAAAA
TTGCCTTTGCCAAAAGTCTGCTGGAAGAGGCGGAGGATGACCTTGAGCAGCTGGGTCAAGTCCTCGATAC
GCTGCAAGTCCAGAACATTTCCGATGAGGTCGGTTATCTCTCGGCTAGTGGACGCAAGCAGCGGGCTGAT
CTGCAGCGAGATGCCCGAATTGCTGAAGCCGATGCCCAGGCTGCCTCTGCGATCCAAACGGCCGAAAATG
ACAAGATCACGGCCCTGCGTCGGATCGATCGCGATGTAGCGATCGCCCAAGCCGAGGCCGAGCGCCGGAT
TCAGGATGCGTTGACGCGGCGCGAAGCGGTGGTGGCCGAAGCTGAAGCGGACATTGCTACCGAAGTCGCT
CGTAGCCAAGCAGAACTCCCTGTGCAGCAGGAGCGGATCAAACAGGTGCAGCAGCAACTTCAAGCCGATG
TGATCGCCCCAGCTGAGGCAGCTTGTAAACGGGCGATCGCGGAAGCGCGGGGGGCCGCCGCCCGTATCGT
CGAAGATGGAAAAGCTCAAGCGGAAGGGACCCAACGGCTGGCGGAGGCTTGGCAGACCGCTGGTGCTAAT
GCCCGCGACATCTTCCTGCTCCAGAAGTCTAGATAATCCCTAGCGATCGCAAGTCCAAAGGTTGTCTACA
ATCAATATCCAAGCATCAAAAAGCGCCCCATTCGAGGCGCTTTTTGATTATTCAGACTGCTGTAATTCCG
GCAATTAGG

Spectinomycin resistance gene(1970-2980)

TTATTTGCCGACTACCTTGGTGATCTCGCCTTTCACGTAGTGGACAAATTCTTCCAACTGA
TCTGCGCGCGAGGCCAAGCGATCTTCTTCTTGTCCAAGATAAGCCTGTCTAGCTTCAAGTATGACGGGCT
GATACTGGGCCGGCAGGCGCTCCATTGCCCAGTCGGCAGCGACATCCTTCGGCGCGATTTTGCCGGTTAC
TGCGCTGTACCAAATGCGGGACAACGTAAGCACTACATTTCGCTCATCGCCAGCCCAGTCGGGCGGCGAG
TTCCATAGCGTTAAGGTTTCATTTAGCGCCTCAAATAGATCCTGTTCAGGAACCGGATCAAAGAGTTCCT
CCGCCGCTGGACCTACCAAGGCAACGCTATGTTCTCTTGCTTTTGTCAGCAAGATAGCCAGATCAATGTC
GATCGTGGCTGGCTCGAAGATACCAGCAAGAATGTCATTGCGCTGCCATTCTCCAAATTGCAGTTCGCGC
TTAGCTGGATAACGCCACGGAATGATGTCGTCGTGCACAACAATGGTGACTTCTACAGCGCGGAGAATCT
CGCTCTCTCCAGGGGAAGCCGAAGTTTCCAAAAGGTCGTTGATCAAAGCTCGCCGCGTTGTTTCATCAAG
CCTTACGGTCACCGTAACCAGCAAATCAATATCACTGTGTGGCTTCAGGCCGCCATCCACTGCGGAGCCG
TACAAATGTACGGCCAGCAACGTCGGTTCGAGATGGCGCTCGATGACGCCAACTACCTCTGATAGTTGAG
TCGATACTTCGGCGATCACCGCTTCCCTCATAATGTTTAACTTTGTTTTAGGGCGACTGCCCTGCTGCGT
AACATCGTTGCTGCTCCATAACATCAAACATCGACCCACGGCGTAACGCGCTTGCTGCTTGGATGCCCGA

GGCATAGACTGTACCCCAAAAAAACAGTCATAACAAGCCATGAAAACCGCCACTGCGCCGTTACCACCGC
TGCGTTCGGTCAAGGTTCTGGACCAGTTGCGTGAGCGCAT

Spectinomycin promoter gene(2981-3114)

ACGCTACTTGCATTACAGCTTACGAACCGAACAGGCTTATGTCCACTGGGTTCGTGCCTTCATCCGTTTCCACGG
TGTGCGTCACCCGGCAACCTTGGGTAGCAGCGAAGTCGAGGCATTTCTGTCCTGGCTGGCT
psbA promoter(3117-3339)

ATTTAGCGTCTTCTAATCCAGTGTAGACAGTAGT
TTTGGCTCCGTTGAGCACTGTAGCCTTGGGCGATCGCTCTAAACATTACATAAATTCACAAGTTTTCGT
TACATAAAAATAGTGTCTACTTAGCTAAAAATTAAGGGTTTTTTACACCTTTTTGACAGTTAATCTCCTA
GCCTAAAAAGCAAGAGTTTTTAACTAAGACTCTTGCCCTTTACAACCTC

Ribosome binding site(3340-3346)

GAAGGAGCGTCAGATCTCATATG
RBS *BglII* *NdeI*
6xHis tag(3363-3380)

CACCACCACCATCACCAC

TEV recognition site(3381-3401)

GAAAACCTGTACTTTCAGGGCAAGCTTCGAATTCCCGGATCCGCGGTACCA
HindIII *EcoRI* *BamHI* *KpnI*
V5 Epitope(3432-4373)

GGCAAACCCATCCCCAACCCCCTGCTGGGCCTGGATAGCACCGGTGGTGGT
6xHis tag(3483-3500)

CACCACCACCATCACCACTAG
STOP
rrnB transcriptional termination region(3522-3679)

TGCCTGGCGGCAGTAGCGCGGTGGTCCCACCTGACCCCATGCCGAACTC
AGAAGTGAAACGCCGTAGCGCCGATGGTAGTGTGGGGTCTCCCCATGCGAGAGTAGGGAACTGCCAGGCA
TCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTT

NS1b(Neutral site 1b)(3680-4457)

CTCGAGTCCCTGCTCGTCACGCTTTCAGGCA
CCGTGCCAGATATCGACGTGGAGTCGATCACTGTGATTGGCGAAGGGGAAGGCAGCGCTACCCAAATCGC
TAGCTTGCTGGAGAAGCTGAAACAAACCACGGGCATTGATCTGGCGAAATCCCTACCGGGTCAATCCGAC
TCGCCCGCTGCGAAGTCCTAAGAGATAGCGATGTGACCGCGATCGCTTGTCAAGAATCCCAGTGATCCCG
AACCATAGGAAGGCAAGCTCAATGCTTGCCTCGTCTTGAGGACTATCTAGATGTCTGTGGAACGCACATT
TATTGCCATCAAGCCCGATGGCGTTCAGCGGGGTTTGGTCGGTACGATCATCGGCCGCTTTGAGCAAAAA
GGCTTCAAACTGGTGGGCCTAAAGCAGCTGAAGCCCAGTCGCGAGCTGGCCGAACAGCACTATGCTGTCC
ACCGCGAGCGCCCCTTCTTCAATGGCCTCGTCGAGTTCATCACCTCTGGGCCGATCGTGGCGATCGTCTT
GGAAGGCGAAGGCGTTGTGGCGGCTGCTCGCAAGTTGATCGGCGCTACCAATCCGCTGACGGCAGAACCG
GGCACCATCCGTGGTGATTTTGGTGTCAATATTGGCCGCAACATCATCCATGGCTCGGATGCAATCGAAA
CAGCACAACAGGAAATTGCTCTCTGGTTTAGCCCAGCAGAGCTAAGTGATTGGACCCCCACGATTCAACC
CTGGCTGTACGAATAAGGTCTGCATTCCTTCAGAGAGACATTGCCAT GCCG

S 3. *ectABC* gene cassette sequence without Codon Optimization

ACGACCGACGACGGGTGGGGCATTTACGAACTCGTCAAGGCATGCCCGCCACTGGACGTCAACTCGGGGTACGCC
TATCTGCTGCTTGCCACCCAGTTCCGCGATACCTGCGCCGTGGCTACGGACGAAGAAGGGGAGATCGTCGGTTTC
GTCTCCGGCTACGTCAAACGCAACGCACCGGACACCTACTTTCTATGGCAAGTCGCCGTCGGCGAGAAAGCGCGG
GGCACGGGCCTGGCGCGACGCCTCGTCGAAGCCGTGCTGATGCGCCCCGGCATGGGCGATGTCCGCCATCTCGAA
ACTACCATCACGCCCGACAATGAGGCATCGTGGGGACTGTTCAAGCGCCTTGCCGATCGCTGGCAAGCCCCCCTG
AACAGCCGTGAATACTTCTCCACGGGGCAGTTGGGTGGCGAGCACGATCCGGAAAATCTCGTCCGTATCGGCCCG
TTCGAGCCGCAACAAATTTGAGGATCCCCTCGAAGGAGCGTAAAATCTAATATGCAGACCCAGATTCTCGAACGC
ATGGAATCCGAAGTCCGGACCTATTCACGTTCTTTTCCTACCGTTTTCACTGAAGCCAAGGGCGCGCGCCTGCAT
GCCGAGGACGGCAACCAGTACATCGATTTTCTCGCCGGCGCCGGCACGCTCAACTACGGTCACAACCACCCCAAG
CTCAAGCAGGCACTGGCCGATTACATCGCCTCCGATGGCATCGTCCATGGTCTGGACATGTGGAGCGCGGCCAAG
CGCGACTATCTGGAAACCCTCGAAGAGGTGATCCTCAAGCCGCGTGGCCTGGATTACAAGGTTCATCTGCCGGGC
CCGACGGGCACCAATGCCGTGGAAGCCGCCATTCGACTGGCGCGCAACGCCAAGGGTCGTCACAACATCGTCACC
TTCACCAACGGATTCCATGGCGTGACCATGGGCGCGCTGGCCACCACCGGCAATCGCAAGTTCCGTGAAGCCACC
GGCGGTATCCCGACCCAGGGCGCCAGCTTCATGCCGTTCGATGGCTACATGGGCGAGGGCGTCGACACCCTGAGC
TACTTCGAGAAACTGCTCGGCGACAACTCCGGTGGTCTCGACGTTCCCGCGGCCGTGATCATCGAGACGGTGCAG
GGCGAGGGCGGTATCAATCCGGCCGGCATCCCGTGGCTGCAGCGCCTGGAAAAGATCTGCCGCGATCACGACATG
CTGCTGATCGTCGACGACATTCAGGCCGGCTGCGGTCGTACGGGCAAGTTCTTCAGCTTCGAGCATGCCGGCATC
ACGCCGGACATCGTCACCAACTCCAAGTCCCTGTCGGGTTTCGGCCTGCCGTTCGCGCATGTGCTGATGCGCCCG
GAACTGGATATCTGGAAGCCCGGCCAGTACAACGGCACGTTCCGTGGTTTCAACCTGGCCTTCGTCACGGCCGCC
GCCGCGATGCGTCACTTCTGGAGCGACGACACCTTCGAGCGCGACGTTCAGCGCAAGGGCCGTGTGGTCGAGGAT
CGCTTCCAGAAGCTTGCCAGCTTCATGACCGAGAAAGGGCATCCGGCCAGCGAGCGTGGCCGTGGCCTGATGCGT
GGCCTGGACGTCGGTGACGGCGACATGGCCGACAAGATCACCGCACAAGCGTTCAAGAACGGGCTGATCATCGAG
ACATCCGGCCATTCAGGCCAGGTGATCAAGTGCCTTTGCCCGTTGACCATTACCGACGAAGACCTCGTCGGCGGC
CTGGACATCCTCGAGCAGAGCGTCAAGGAAGTCTTCGGTCAAGCCTAAAAGCTTCCTCGAAGGAGCGTAAAATCT
AATATGATCGTTCGTAACCTGGAAGAATGCCGCAAGACCGAGCGCTTCGTCGAAGCCGAAAACGGCAACTGGGAC
AGCACCCGTCTGGTGCTGGCCGACGACAACGTCGGTTTCTCGTTCAACATCACCCGCATTCATCCGGGTACCGAG
ACGCATATCCATTACAAGCATCACTTCGAGGCGGTTTTCTGCTACGAAGGCGAAGGCGAAGTCGAAACGCTGGCC
GATGGCAAGATCCATCCCATCAAGGCCGGCGACATGTACTTGCTCGATCAGCACGACGAGCACCTGCTGCGCGGC
AAGGAAAAAGGCATGACCGTGGCATGCGTGTTCAATCCGGCGCTGACGGGCCGCGAAGTGCACCGTGAAGACGGT
TCCTACGCACCGGTCGATTGAACCGGTCGATGGTGGTGGTCACCACCACCATCACCACTAGAGTACT

S 4 Codon optimized *ectA*, *ectB* and *ectC* genes and corresponding amino acid sequences after codon optimization to *S. elongatus* PCC7942

ectA Gene

```
      Met His His His His His His Glu Asn Leu His Met Thr Pro Thr
1     ATG CAC CAC CAC CAC CAT CAC GAA AAC CTG CAT ATG ACC CCC ACC
1     ATG CAC CAC CAC CAT CAC CAC GAA AAC CTG CAT ATG ACG CCT ACA

      Thr Glu Asn Phe Thr Pro Ser Ala Asp Leu Ala Arg Pro Ser Val
46    ACC GAA AAC TTT ACC CCC AGC GCT GAT CTG GCT CGC CCT AGC GTG
46    ACC GAG AAC TTC ACG CCT TCC GCC GAT CTG GCC CGC CCG AGC GTT

      Ser Ala Lys Lys Thr Leu Phe Ile Arg Lys Pro Thr Thr Asp Asp
91    AGC GCC AAA AAA ACC CTG TTT ATC CGC AAA CCC ACC ACC GAT GAT
91    AGC GCG AAG AAA ACC CTC TTC ATC CGT AAG CCC ACG ACC GAC GAC

      Gly Trp Gly Ile Tyr Glu Leu Val Lys Ala Cys Pro Pro Leu Asp
136   GGC TGG GGC ATC TAC GAA CTG GTG AAA GCC TGC CCT CCC CTG GAT
136   GGG TGG GGC ATT TAC GAA CTC GTC AAG GCA TGC CCG CCA CTG GAC

      Val Asn Ser Gly Tyr Ala Tyr Leu Leu Leu Ala Thr Gln Phe Arg
181   GTG AAC AGC GGC TAC GCC TAC CTG CTG CTG GCC ACC CAG TTT CGC
181   GTC AAC TCG GGG TAC GCC TAT CTG CTG CTT GCC ACC CAG TTC CGC

      Asp Thr Cys Ala Val Ala Thr Asp Glu Glu Gly Glu Ile Val Gly
226   GAT ACC TGC GCT GTG GCC ACC GAT GAA GAA GGC GAA ATC GTC GGC
226   GAT ACC TGC GCC GTG GCT ACG GAC GAA GAA GGG GAG ATC GTC GGT

      Phe Val Ser Gly Tyr Val Lys Arg Asn Ala Pro Asp Thr Tyr Phe
271   TTT GTG AGC GGC TAC GTG AAA CGC AAC GCT CCC GAT ACC TAC TTT
271   TTC GTC TCC GGC TAC GTC AAA CGC AAC GCA CCG GAC ACC TAC TTT

      Leu Trp Gln Val Ala Val Gly Glu Lys Ala Arg Gly Thr Gly Leu
316   CTG TGG CAG GTC GCC GTG GGC GAA AAA GCT CGC GGT ACG GGT CTG
316   CTA TGG CAA GTC GCC GTC GGC GAG AAA GCG CGG GGC ACG GGC CTG

      Ala Arg Arg Leu Val Glu Ala Val Leu Met Arg Pro Gly Met Gly
361   GCT CGC CGC TTG GTG GAA GCC GTG TTG ATG CGC CCT GGC ATG GGT
361   GCG CGA CGC CTC GTC GAA GCC GTG CTG ATG CGC CCC GGC ATG GGC

      Asp Val Arg His Leu Glu Thr Thr Ile Thr Pro Asp Asn Glu Ala
406   GAT GTG CGC CAC CTG GAA ACC ACC ATC ACC CCT GAT AAC GAA GCC
406   GAT GTC CGC CAT CTC GAA ACT ACC ATC ACG CCC GAC AAT GAG GCA

      Ser Trp Gly Leu Phe Lys Arg Leu Ala Asp Arg Trp Gln Ala Pro
451   AGC TGG GGC TTG TTT AAA CGC CTG GCC GAT CGC TGG CAG GCT CCC
451   TCG TGG GGA CTG TTC AAG CGC CTT GCC GAT CGC TGG CAA GCC CCC

      Leu Asn Ser Arg Glu Tyr Phe Ser Thr Gly Gln Leu Gly Gly Glu
496   CTG AAC AGT CGC GAA TAC TTT AGC ACG GGT CAG CTG GGT GGC GAA
496   CTG AAC AGC CGT GAA TAC TTC TCC ACG GGG CAG TTG GGT GGC GAG
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      His Asp Pro Glu Asn Leu Val Arg Ile Gly Pro Phe Glu Pro Gln
541   CAC GAT CCG GAA AAT CTC GTC CGT ATC GGC CCG TTC GAG CCG CAA
541   CAC GAT CCC GAA AAC CTG GTG CGC ATC GGT CCC TTT GAA CCC CAG

      Gln Ile  *
586   CAG ATT TAG
586   CAA ATT TGA
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ectB gene:

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      Met Gln Thr Gln Ile Leu Glu Arg Met Glu Ser Glu Val Arg Thr
1     ATG CAG ACC CAG ATC CTG GAA CGC ATG GAA AGC GAA GTG CGC ACC
1     ATG CAG ACC CAG ATT CTC GAA CGC ATG GAA TCC GAA GTC CGG ACC

      Tyr Ser Arg Ser Phe Pro Thr Val Phe Thr Glu Ala Lys Gly Ala
46    TAC AGC CGC AGC TTT CCC ACC GTG TTT ACC GAA GCC AAA GGC GCT
46    TAT TCA CGT TCT TTT CCT ACC GTT TTC ACT GAA GCC AAG GGC GCG

      Arg Leu His Ala Glu Asp Gly Asn Gln Tyr Ile Asp Phe Leu Ala
91    CGC CTG CAC GCC GAA GAT GGC AAC CAG TAC ATC GAT TTT CTG GCT
91    CGC CTG CAT GCC GAG GAC GGC AAC CAG TAC ATC GAT TTT CTC GCC

      Gly Ala Gly Thr Leu Asn Tyr Gly His Asn His Pro Lys Leu Lys
136   GGC GCT GGC ACC CTG AAC TAC GGC CAC AAC CAC CCC AAA CTG AAA
136   GGC GCC GGC ACG CTC AAC TAC GGT CAC AAC CAC CCC AAG CTC AAG

      Gln Ala Lys Ala Asp Tyr Ile Ala Ser Asp Gly Ile Val His Gly
181   CAG GCC CTG GCC GAT TAC ATT GCC AGT GAT GGC ATC GTG CAC GGC
181   CAG GCA CTG GCC GAT TAC ATC GCC TCC GAT GGC ATC GTC CAT GGT

      Leu Asp Met Trp Ser Ala Ala Lys Arg Asp Tyr Leu Glu Thr Leu
226   CTG GAT ATG TGG TCG GCT GCC AAA CGC GAT TAC CTG GAA ACC CTG
226   CTG GAC ATG TGG AGC GCG GCC AAG CGC GAC TAT CTG GAA ACC CTC

      Glu Glu Val Ile Leu Lys Pro Arg Gly Leu Asp Tyr Lys Val His
271   GAA GAA GTG ATC CTG AAA CCT CGC GGT CTG GAT TAC AAA GTG CAT
271   GAA GAG GTG ATC CTC AAG CCG CGT GGC CTG GAT TAC AAG GTT CAT

      Leu Pro Gly Pro Thr Gly Thr Asn Ala Val Glu Ala Ala Ile Arg
316   CTG CCT GGT CCC ACC GGC ACC AAC GCC GTG GAA GCC GCT ATT CGC
316   CTG CCG GGC CCG ACG GGC ACC AAT GCC GTG GAA GCC GCC ATT CGA

      Leu Ala Arg Asn Ala Lys Gly Arg His Asn Ile Val Thr Phe Thr
361   CTG GCT CGC AAC GCC AAA GGT CGC CAC AAC ATC GTG ACC TTT ACC
361   CTG GCG CGC AAC GCC AAG GGT CGT CAC AAC ATC GTC ACC TTC ACC

      Asn Gly Phe His Gly Val Thr Met Gly Ala Leu Ala Thr Thr Gly
406   AAC GGC TTT CAC GGC GTG ACC ATG GGT GCC CTG GCC ACC ACC GGC
406   AAC GGA TTC CAT GGC GTG ACC ATG GGC GCG CTG GCC ACC ACC GGC

      Asn Arg Lys Phe Arg Glu Ala Thr Gly Gly Ile Pro Thr Gln Gly
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       Ala Ser Phe Met Pro Phe Asp Gly Tyr Met Gly Glu Gly Val Glu
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 496   GCC AGC TTC ATG CCG TTC GAT GGC TAC ATG GGC GAG GGC GTC GAC

       Thr Leu Ser His Phe Glu Lys Leu Leu Gly Asp Asn Ser Gly Gly
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 541   ACC CTG AGC TAC TTC GAG AAA CTG CTC GGC GAC AAC TCC GGT GGT

       Leu Asp Val Pro Ala Ala Val Ile Ile Glu Thr Val Gln Gln Glu
 586   CTG GAT GTT CCC GCT GCC GTG ATC ATC GAA ACC GTG CAG GGC GAA
 586   CTC GAC GTT CCC GCG GCC GTG ATC ATC GAG ACG GTG CAG GGC GAG

       Gly Gly Ile Asp Pro Ala Gly Ile Pro Trp Leu Gln Arg Leu Glu
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 631   GGC GGT ATC AAT CCG GCC GGC ATC CCG TGG CTG CAG CGC CTG GAA

       Leu Ile Cys Arg Asp His Asp Met Leu Leu Ile Val Asp Asp Ile
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       Gln Ala Gly Cys Gly Arg Thr Gly Lys Phe Phe Ser Phe Glu His
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 721   CAG GCC GGC TGC GGT CGT ACG GGC AAG TTC TTC AGC TTC GAG CAT

       Ala Gly Ile Thr Pro Asp Ile Val Thr Asn Ser Lys Ser Leu Ser
 766   GCT GGC ATT ACC CCT GAT ATC GTG ACC AAC AGC AAA AGC CTG AGC
 766   GCC GGC ATC ACG CCG GAC ATC GTC ACC AAC TCC AAG TCC CTG TCG

       Gly Phe Gly Leu Pro Phe Ala His Val Leu Met Arg Pro Glu Leu
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 811   GGT TTC GGC CTG CCG TTC GCG CAT GTG CTG ATG CGC CCG GAA CTG

       Asp Ile Trp Lys Pro Gly Gln Tyr Asn Gly Thr Phe Arg Gly Phe
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 956   AGC GAC GAC ACC TTC GAG CGC GAC GTT CAG CGC AAG GGC CGT GTG

       Val Glu Asp Arg Phe Gln Lys Leu Ala Ser Phe Met Thr Glu Lys
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 991   GTC GAG GAT CGC TTC CAG AAG CTT GCC AGC TTC ATG ACC GAG AAA

       Gly His Pro Ala Ser Glu Arg Gly Arg Gly Leu Met Arg Gly Leu
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        Phe Lys Asn Gly Leu Ile Ile Glu Thr Ser Gly His Ser Gly Gln
1126    TTT AAA AAC GGC CTG ATC ATT GAA ACC AGT GGC CAT AGT GGC CAG
1126    TTC AAG AAC GGG CTG ATC ATC GAG ACA TCC GGC CAT TCA GGC CAG

        Val Ile Lys Cys Leu Cys Pro Leu Thr Ile Thr Asp Glu Asp Leu
1171    GTG ATC AAA TGC CTG TGT CCC CTG ACC ATC ACC GAT GAG GAT CTG
1171    GTG ATC AAG TGC CTT TGC CCG TTG ACC ATT ACC GAC GAA GAC CTC

        Val Gly Gly Leu Asp Ile Lys Glu Gln Ser Val Lys Glu Val Phe
1216    GTT GGC GGT CTG GA  ATC CTG GAA CAG AGC GTG AAA GAA GTG TTT
1216    GTC GGC GGC CTG GAC ATC CTC GAG CAG AGC GTC AAG GAA GTC TTC

        Gly Gln Ala Stop*
1261    GGC CAG GCC TAG
1261    GGT CAA GCC TAA
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ectC Gene

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        Met Ile Val Arg Asn Leu Glu Glu Cys Arg Lys Thr Glu Arg Phe
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1       ATG ATC GTT CGT AAC CTG GAA GAA TGC CGC AAG ACC GAG CGC TTC

        Val Glu Ala Glu Asn Gly Asn Trp Asp Ser Thr Arg Leu Val Leu
46      GTC GAA GCC GAA AAC GGC AAC TGG GAT AGC ACC CGC TTG GTG CTG
46      GTC GAA GCC GAA AAC GGC AAC TGG GAC AGC ACC CGT CTG GTG CTG

        Ala Asp Asp Asn Val Gly Phe Ser Phe Asn Ile Thr Arg Ile His
91      GCC GAT GAT AAC GTG GGC TTT AGC TTT AAC ATC ACC CGC ATC CAC
91      GCC GAC GAC AAC GTC GGT TTC TCG TTC AAC ATC ACC CGC ATT CAT

        Pro Gly Thr Glu Thr His Ile His Tyr Lys His His Phe Glu Ala
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136     CCG GGT ACC GAG ACG CAT ATC CAT TAC AAG CAT CAC TTC GAG GCG

        Val Phe Cys Tyr Glu Gly Glu Gly Glu Val Glu Thr Leu Ala Asp
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181     GTT TTC TGC TAC GAA GGC GAA GGC GAA GTC GAA ACG CTG GCC GAT

        Gly Lys Ile His Pro Ile Lys Ala Gly Asp Met Tyr Leu Leu Asp
226     GGC AAA ATC CAC CCC ATC AAA GCT GGC GAT ATG TAC CTG CTG GAT
226     GGC AAG ATC CAT CCC ATC AAG GCC GGC GAC ATG TAC TTG CTC GAT

        Gln His Asp Glu His Leu Leu Arg Gly Lys Glu Lys Gly Met Thr
271     CAG CAC GAT GAA CAT CTG CTG CGC GGT AAA GAA AAA GGC ATG ACC
271     CAG CAC GAC GAG CAC CTG CTG CGC GGC AAG GAA AAA GGC ATG ACC

        Val Ala Cys Val Phe Asn Pro Ala Leu Thr Gly Arg Glu Val His
316     GTG GCC TGC GTG TTT AAT CCC GCT CTG ACG GGT CGC GAA GTG CAC
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316     GTG GCA TGC GTG TTC AAT CCG GCG CTG ACG GGC CGC GAA GTG CAC

        Arg Glu Asp Gly Ser Tyr Ala Pro Val Asp Stop*
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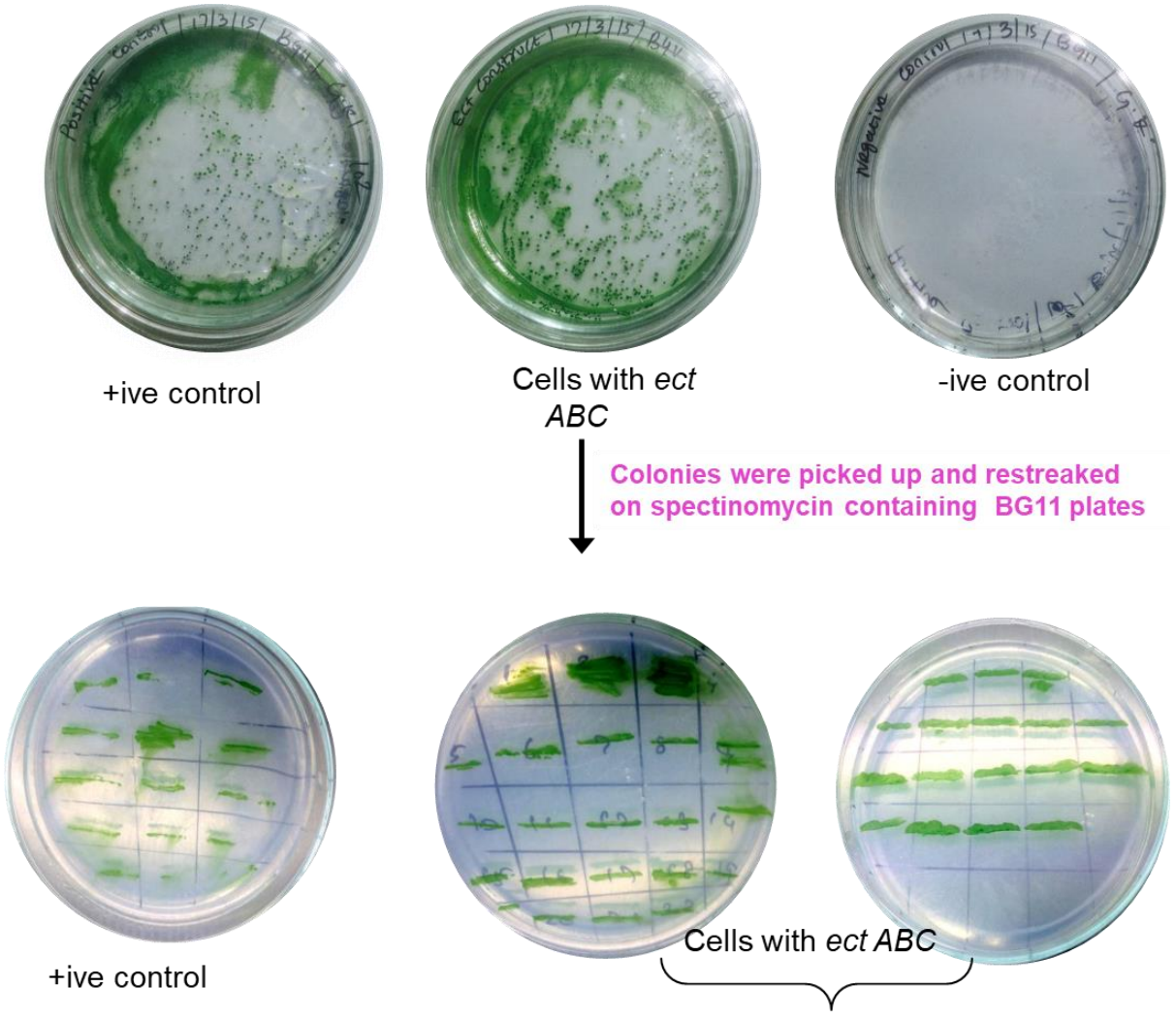
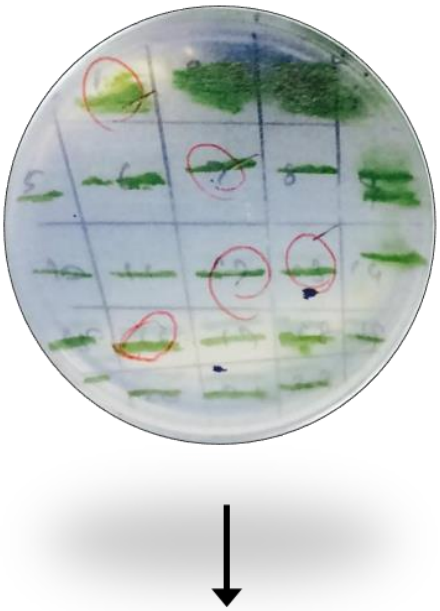


FIgure S 1. Selection of *ectABC* and empty vector control (*pSyn_6*) recombinants on spectinomycin containing agar plates.



94 °C 2 min	52.5 °C 1 min	72 °C 1 min
94 °C 1 min		72 °C 7 min

X 34 cycles

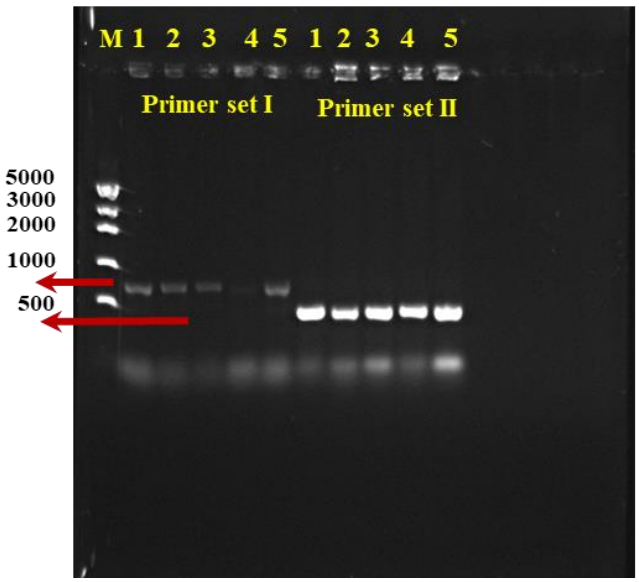


FIgure S 2. Secondary screening of *ectABC* transformants for *ectA&B* and ect*C*

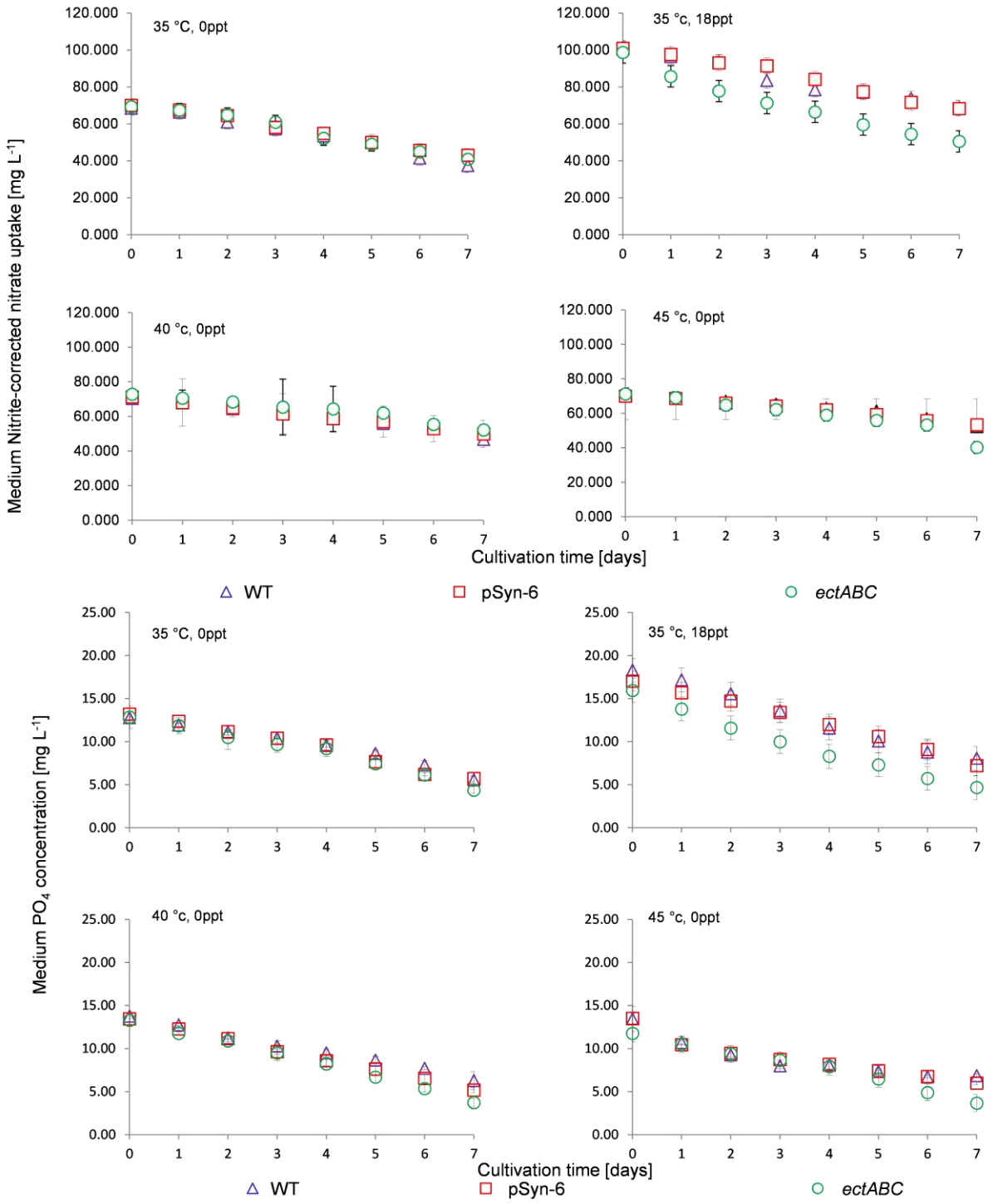


FIgure S 3. Medium nitrite-corrected nitrate and phosphate concentrations of cultures of WT, pSyn-6 and *ectABC*-transformants of *S. elongatus* PCC7942 at cultivation temperatures of 35, 40 and 45°C at a salinity of 0 ppt and at 18 ppt for 35°C

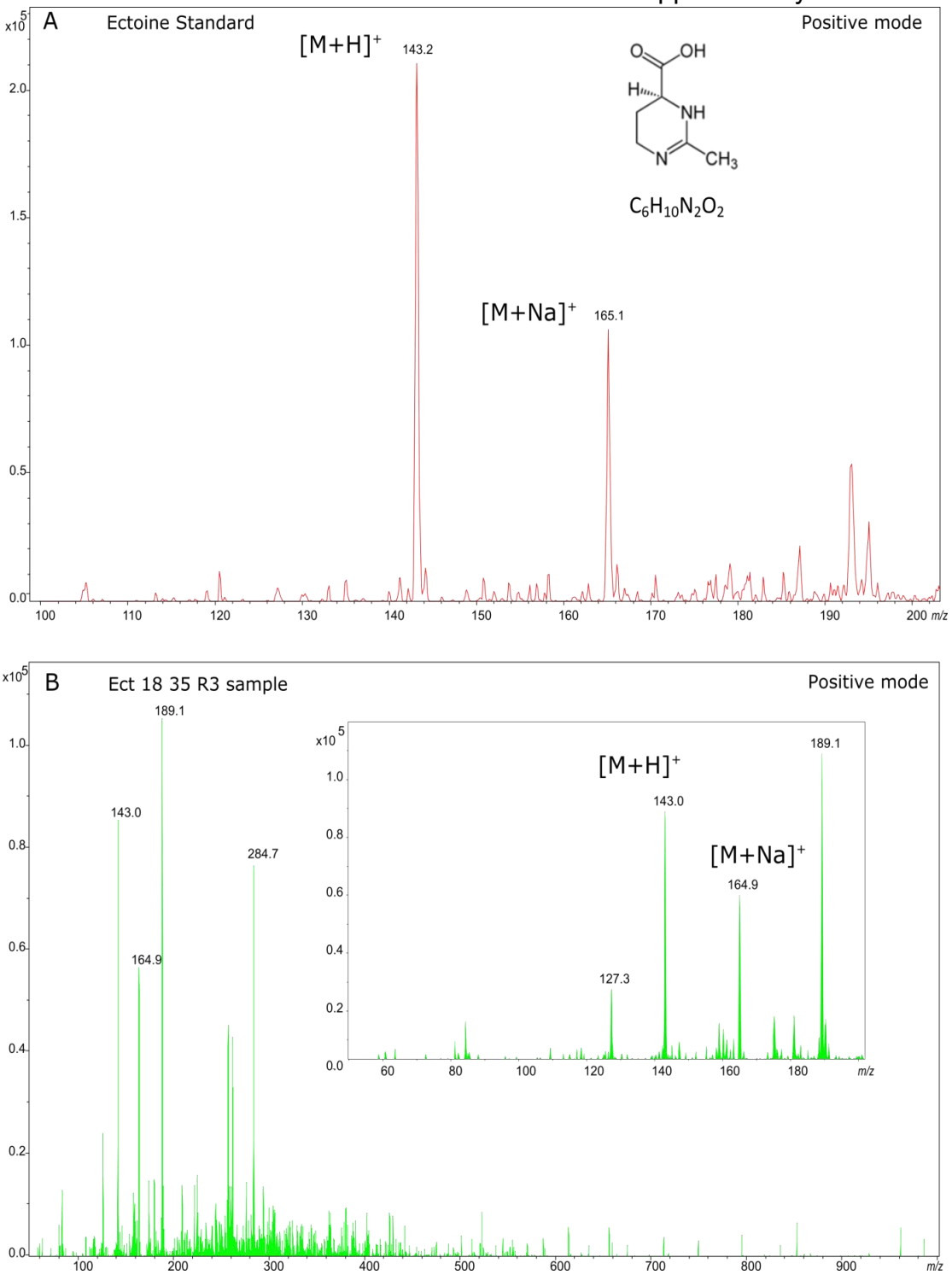


FIgure S 4. LC-MS spectrograms for standard ectoine from Sigma and intracellular ectoine from *ectABC* transfromants under salinity stress 18 ppt at 35ºC (positive mode).

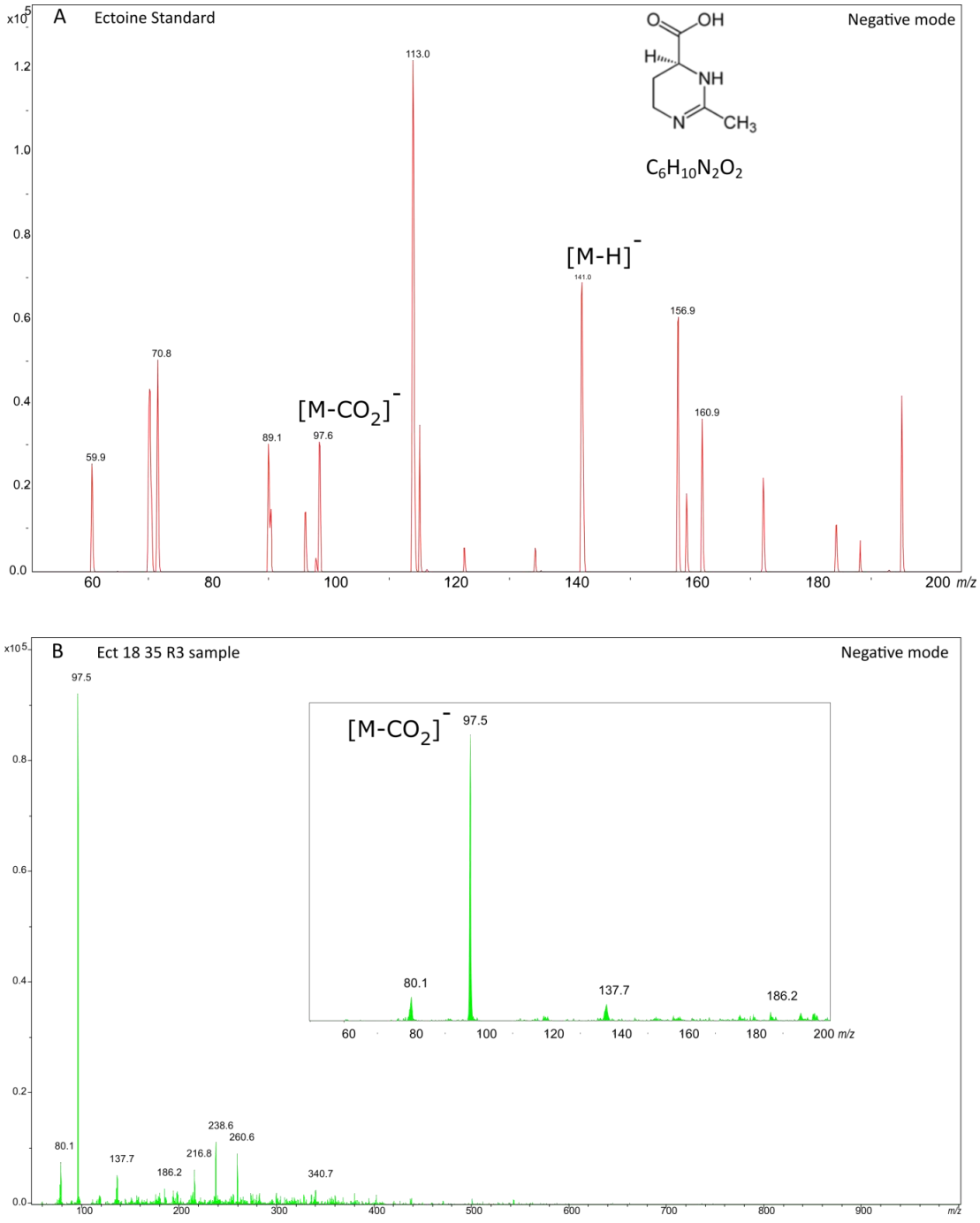


FIgure S 5. LC-MS spectrograms for standard ectoine from Sigma and intracellular ectoine from *ectABC* transfromants under salinity stress 18 ppt at 35ºC (negative mode)

5. Economic evaluation – 'proof of concept' of co-location framework

Table S5. 1. Annual cash flows

Cost or revenue stream	Impact of co-location on cost	Start of project – time zero	Repeated annually for years 1 to 20
		$	$
Direct capital costs	Avoidable	- 214,271	
	Not avoidable	- 1,025,293	
Indirect capital costs	Avoidable	- 42,854	
	Not avoidable	- 205,059	
Working capital		- 74,374	
Proceeds of selling biomass			391,636
Proceeds of selling pigments			11,749,077
Operating costs	Avoidable		- 2,790,013
	Not avoidable		- 626,901
Sales & distribution costs			- 6,070,357
Net flow per year		**- 1,561,851**	**2,653,443**

Table S5. 2. Calculated Net Present Value at 5% and sensitivity analysis based on alternate discount rates

NPV based on discount rate	Discount rate	NPV ($)
	5%	31,533,950
Sensitivity analysis to change in discount rate		
If lower rate	3%	37,955,866
If higher rate	10%	21,039,464

Table S5. 3. Economic valuation of benefits of colocation

Co-location benefits	**$**
Reduced capital costs	257,125
Reduced operating costs each year	2,790,013

9 Appendix

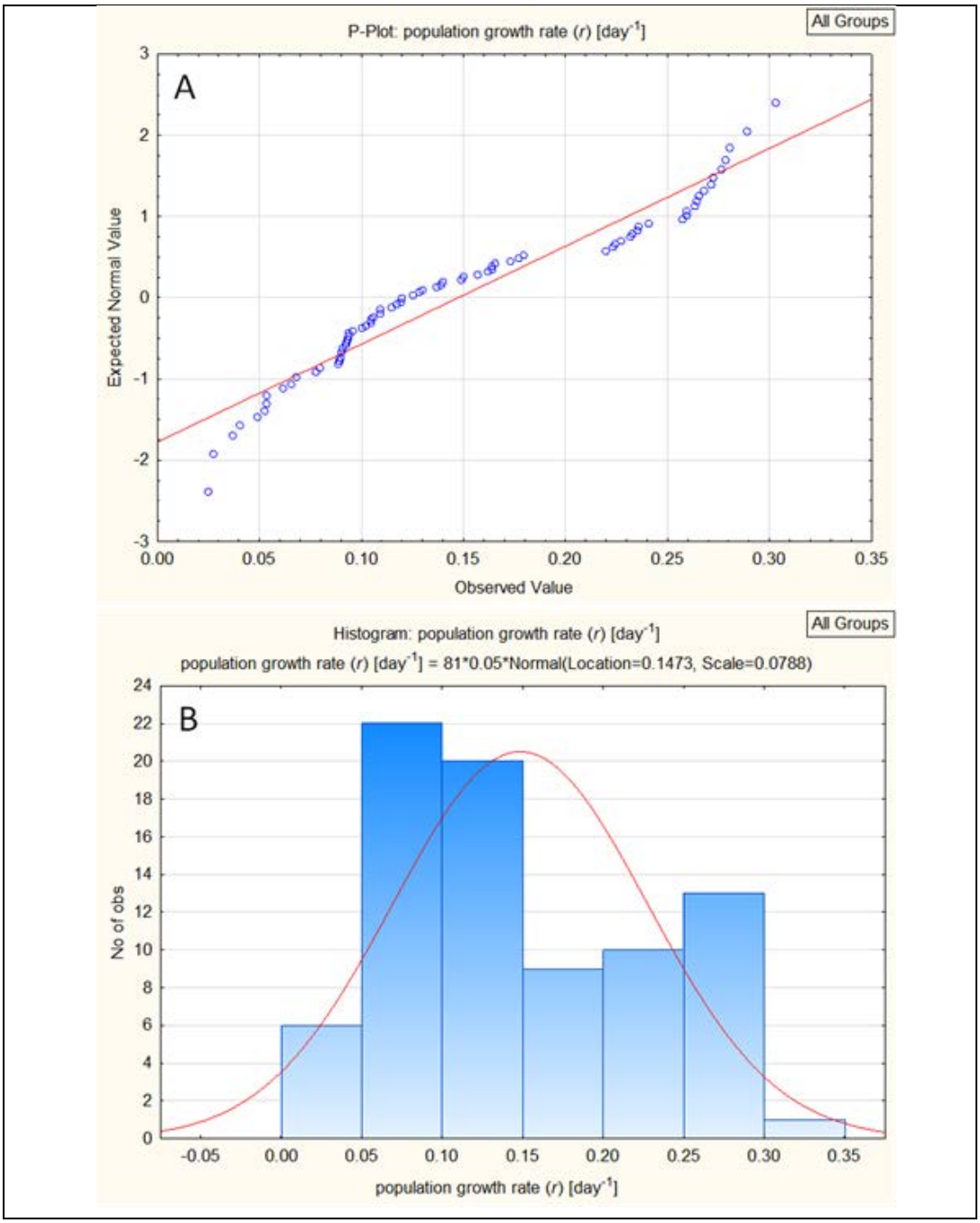


Figure A4 1. P-P plot (a) and distribution histogram (b) of population growth rate (r) of *S. elongatus* PCC7942 for all groups (salinity (0, 18 and 36 ppt), temperature (35, 40 and 45°C) and strain (WT, *pSyn_6* and *ectABC*).

Table A4 1. Tests of Homogeneity of Variances;
Effect: Strain*"Salinity [ppt]"*"Temperature [°C]"

	Hartley	Cochran	Bartlett	df	p
population growth rate (*r*) [day^{-1}]	715.3045	0.209200	36.86013	26	0.076948

Table A4 2. Univariate test of significance of the effect of ectoine transformation (strain), salinity and temperature for population growth rate of *S. elongatus* PCC7942

Effect	SS	Degrees of Freedom	MS	F	p
Intercept	1.758134	1	1.758134	7988.745	0.000000
Strain	0.038358	2	0.019179	87.147	0.000000
Salinity [ppt]	0.288401	2	0.144201	655.230	0.000000
Temperature [°C]	0.106461	2	0.053230	241.872	0.000000
Strain*Salinity [ppt]	0.009998	4	0.002499	11.357	0.000001
Strain*Temperature [°C]	0.005730	4	0.001432	6.509	0.000238
Salinity [ppt]*Temperature [°C]	0.016859	4	0.004215	19.151	0.000000
Strain*Salinity [ppt]*Temperature [°C]	0.018626	8	0.002328	10.579	0.000000
Error	**0.011884**	**54**	**0.000220**		

Table A4 3. *Tukeys Post Hoc analysis for significance of the effect of ectoine transformation (strain), salinity and temperature for population growth rate of S. elongatus PCC7942*

Cell No.	Strain	Salinity [ppt]	Temperature [°C]	{1}	{2}	{3}	{4}	{5}	{6}	{7}	{8}	{9}	{10}	{11}	{12}	{13}	{14}	{15}	{16}	{17}	{18}	{19}	{20}	{21}	{22}	{23}	{24}	{25}	{26}	{27}
1	Wild type	0	35		0.153996	0.000202	0.000202	0.000202	0.000202	0.000202	0.000202	0.000202	1.000000	0.759853	0.000202	0.000202	0.000202	0.000202	0.000202	0.000202	0.000202	0.891245	1.000000	1.000000	0.473339	0.000202	0.000202	0.000202	0.000202	0.000202
2	Wild type	0	40	0.153996		0.000202	0.003480	0.000202	0.000202	0.000202	0.000202	0.000202	0.432952	0.999999	0.001135	0.034972	0.000202	0.000202	0.000202	0.000202	0.000202	0.000365	0.025717	0.042946	1.000000	0.000202	0.000202	0.000205	0.000202	0.000202
3	Wild type	0	45	0.000202	0.000202		0.003905	1.000000	0.001779	1.000000	0.876479	0.997520	0.000202	0.000202	0.012250	0.000456	0.912201	0.003562	0.999999	1.000000	0.000342	0.000202	0.000202	0.000202	0.000202	0.862713	1.000000	0.437941	0.999450	0.973628
4	Wild type	18	35	0.000202	0.003480	0.003905		0.018917	0.000202	0.000453	0.000203	0.000213	0.000202	0.000289	1.000000	1.000000	0.000203	0.000202	0.068430	0.000495	0.000202	0.000202	0.000202	0.000202	0.000202	0.000621	0.719583	0.000501	0.979009	0.000221	0.000205
5	Wild type	18	40	0.000202	0.000202	1.000000	0.018917		0.000452	0.999897	0.551410	0.928886	0.000202	0.000202	0.053743	0.001805	0.614827	0.000770	1.000000	0.999942	0.000220	0.000202	0.000202	0.000202	0.000202	0.990310	0.999947	0.792519	0.965732	0.778985
6	Wild type	18	45	0.000202	0.000202	0.001779	0.000202	0.000452		0.018813	0.518606	0.155592	0.000202	0.000202	0.000202	0.000202	0.456465	1.000000	0.000243	0.016628	1.000000	0.000202	0.000202	0.000202	0.000202	0.000202	0.016351	0.000202	0.110029	0.302290
7	Wild type	36	35	0.000202	0.000202	1.000000	0.000453	0.999897	0.018813		0.999215	1.000000	0.000202	0.000202	0.001161	0.000214	0.999711	0.036000	0.989630	1.000000	0.002454	0.000202	0.000202	0.000202	0.000202	0.000202	0.358662	1.000000	0.089739	1.000000	0.999991
8	Wild type	36	40	0.000202	0.000202	0.876479	0.000203	0.551410	0.518606	0.999215		1.000000	0.000202	0.000202	0.000205	0.000202	1.000000	0.679501	0.257414	0.998779	0.152121	0.000202	0.000202	0.000202	0.000202	0.009229	0.998708	0.001280	1.000000	1.000000
9	Wild type	36	45	0.000202	0.000202	0.997520	0.000213	0.928886	0.155592	1.000000	1.000000		0.000202	0.000202	0.000249	0.000203	1.000000	0.251518	0.687572	1.000000	0.028065	0.000202	0.000202	0.000202	0.000202	0.060451	1.000000	0.009406	1.000000	1.000000
10	pSyn-6	0	35	1.000000	0.432952	0.000202	0.000202	0.000202	0.000202	0.000202	0.000202	0.000202		0.971805	0.000202	0.000203	0.000202	0.000202	0.000202	0.000202	0.000202	0.562080	0.999966	0.999998	0.832094	0.000202	0.000202	0.000202	0.000202	0.000202
11	pSyn-6	0	40	0.759853	0.999999	0.000202	0.000289	0.000202	0.000202	0.000202	0.000202	0.000202	0.971805		0.000222	0.001672	0.000202	0.000202	0.000202	0.000202	0.000202	0.000202	0.005786	0.296746	0.407987	1.000000	0.000202	0.000202	0.000202	0.000202	0.000202
12	pSyn-6	0	45	0.000202	0.001135	0.012250	1.000000	0.053743	0.000202	0.001161	0.000205	0.000249	0.000202	0.000222		0.999989	0.000207	0.000202	0.167542	0.001317	0.000202	0.000202	0.000202	0.000202	0.000202	0.000304	0.914007	0.001339	0.998938	0.000286	0.000215
13	pSyn-6	18	35	0.000202	0.034972	0.000456	1.000000	0.001805	0.000202	0.000214	0.000202	0.000203	0.000203	0.001672	0.999989		0.000202	0.000202	0.007546	0.000216	0.000202	0.000202	0.000202	0.000202	0.000202	0.005956	0.225714	0.000217	0.637544	0.000203	0.000202
14	pSyn-6	18	40	0.000202	0.000202	0.912201	0.000203	0.614827	0.456465	0.999711	1.000000	1.000000	0.000202	0.000202	0.000207	0.000202		0.617264	0.304770	0.999529	0.123980	0.000202	0.000202	0.000202	0.000202	0.012066	0.999498	0.001640	1.000000	1.000000
15	pSyn-6	18	45	0.000202	0.000202	0.003562	0.000202	0.000770	1.000000	0.036000	0.679501	0.251518	0.000202	0.000202	0.000202	0.000202	0.617264		0.000302	0.032020	1.000000	0.000202	0.000202	0.000202	0.000202	0.000203	0.031513	0.000202	0.184751	0.444018
16	pSyn-6	36	35	0.000202	0.000202	0.999999	0.068430	1.000000	0.000243	0.969630	0.257414	0.687572	0.000202	0.000202	0.167542	0.007546	0.304770	0.000302		0.992370	0.000205	0.000202	0.000202	0.000202	0.000202	0.999910	0.992690	0.968450	0.783610	0.459504
17	pSyn-6	36	40	0.000202	0.000202	1.000000	0.000495	0.999942	0.016628	1.000000	0.998779	1.000000	0.000202	0.000202	0.001317	0.000216	0.999529	0.032020	0.992370		0.002158	0.000202	0.000202	0.000202	0.000202	0.385032	1.000000	0.099697	1.000000	0.999983
18	pSyn-6	36	45	0.000202	0.000202	0.000342	0.000202	0.000220	1.000000	0.002454	0.152121	0.028065	0.000202	0.000202	0.000202	0.000202	0.123980	1.000000	0.000205	0.002158		0.000202	0.000202	0.000202	0.000202	0.000202	0.002121	0.000202	0.018229	0.067561
19	ectABC	0	35	0.891245	0.000365	0.000202	0.000202	0.000202	0.000202	0.000202	0.000202	0.000202	0.562080	0.005786	0.000202	0.000202	0.000202	0.000202	0.000202	0.000202	0.000202		0.998730	0.993596	0.001626	0.000202	0.000202	0.000202	0.000202	0.000202
20	ectABC	0	40	1.000000	0.025717	0.000202	0.000202	0.000202	0.000202	0.000202	0.000202	0.000202	0.999956	0.296746	0.000202	0.000202	0.000202	0.000202	0.000202	0.000202	0.000202	0.998730		1.000000	0.123523	0.000202	0.000202	0.000202	0.000202	0.000202
21	ectABC	0	45	1.000000	0.042946	0.000202	0.000202	0.000202	0.000202	0.000202	0.000202	0.000202	0.999998	0.407987	0.000202	0.000202	0.000202	0.000202	0.000202	0.000202	0.000202	0.993596	1.000000		0.186624	0.000202	0.000202	0.000202	0.000202	0.000202
22	ectABC	18	35	0.473339	1.000000	0.000202	0.000621	0.000202	0.000202	0.000202	0.000202	0.000202	0.832094	1.000000	0.000304	0.005956	0.000202	0.000202	0.000202	0.000202	0.000202	0.001626	0.123523	0.186624		0.000202	0.000202	0.000202	0.000202	0.000202
23	ectABC	18	40	0.000202	0.000202	0.862713	0.719583	0.950310	0.000202	0.358662	0.009229	0.060451	0.000202	0.000202	0.914007	0.225714	0.012066	0.000203	0.999910	0.385032	0.000202	0.000202	0.000202	0.000202	0.000202		0.388677	1.000000	0.088514	0.024823
24	ectABC	18	45	0.000202	0.000202	1.000000	0.000501	0.999947	0.016351	1.000000	0.998708	1.000000	0.000202	0.000202	0.001339	0.000217	0.999498	0.031513	0.992690	1.000000	0.002121	0.000202	0.000202	0.000202	0.000202	0.388677		0.100991	1.000000	0.999981
25	ectABC	36	35	0.000202	0.000205	0.437941	0.979009	0.792519	0.000202	0.089739	0.001260	0.009406	0.000202	0.000202	0.998938	0.637544	0.001640	0.000202	0.968450	0.099597	0.000202	0.000202	0.000202	0.000202	0.000202	1.000000	0.100991		0.014719	0.003465
26	ectABC	36	40	0.000202	0.000202	0.999450	0.000221	0.965732	0.110029	1.000000	1.000000	1.000000	0.000202	0.000202	0.000286	0.000203	1.000000	0.184751	0.783610	1.000000	0.018229	0.000202	0.000202	0.000202	0.000202	0.088514	1.000000	0.014719		1.000000
27	ectABC	36	45	0.000202	0.000202	0.973628	0.000205	0.778985	0.302290	0.999991	1.000000	1.000000	0.000202	0.000202	0.000215	0.000202	1.000000	0.444018	0.459504	0.999983	0.067561	0.000202	0.000202	0.000202	0.000202	0.024823	0.999981	0.003465	1.000000	

Approximate Probabilities for Post Hoc Tests; Error: Between MS = 0.00022, df = 54

Table A4 4. Effect of temperature on homogeneity of variance for nutrient uptake in WT, *pSyn_6* and *ectABC*-transformants of *Synechococcus elongatus* PCC7942

	Tests of Homogeneity of Variances (nutrient and temperature.sta) Effect: "Temperature [°C]" and "Strain*Temperature [°C]"				
	Hartley	Cochran	Bartlett	df	p
Log NO_2-corrected NO_3 uptake	7.574499	0.784666	10.16281	2	0.006211
Log NO_2-corrected NO_3 uptake	18.77007	0.231331	4.988936	8	0.758758
	Tests of Homogeneity of Variances (nutrient and temperature.) Effect: "Strain"; "Temperature [°C]" and "Strain*Temperature [°C]"				
	Hartley	Cochran	Bartlett	df	p
Log PO_4 uptake	2.495273	0.498326	1.575376	2	0.454895
Log PO_4 uptake	3.093567	0.495147	2.091380	2	0.351449
Log PO_4 uptake	4633.295	0.618869	21.95706	8	0.004996

Table A4 5. Factorial ANOVA of the effect of temperature and strain on nitrite-corrected nitrate uptake of WT, *pSyn_6* and *ectABC*-transformants of *S. elongatus* PCC7942

	Univariate Tests of Significance for Log NO_2-corrected NO_3 uptake (nutrient and temperature.sta) Sigma-restricted parameterization Effective hypothesis decomposition				
Effect	SS	Degr. of	MS	F	p
Intercept	86.75682	1	86.75682	26728.20	0.000000
Strain	0.19060	2	0.09530	29.36	0.000003
Temperature [°C]	0.06537	2	0.03268	10.07	0.001304
Strain*Temperature [°C]	0.08993	4	0.02248	6.93	0.001689
Error	0.05518	17	0.00325		

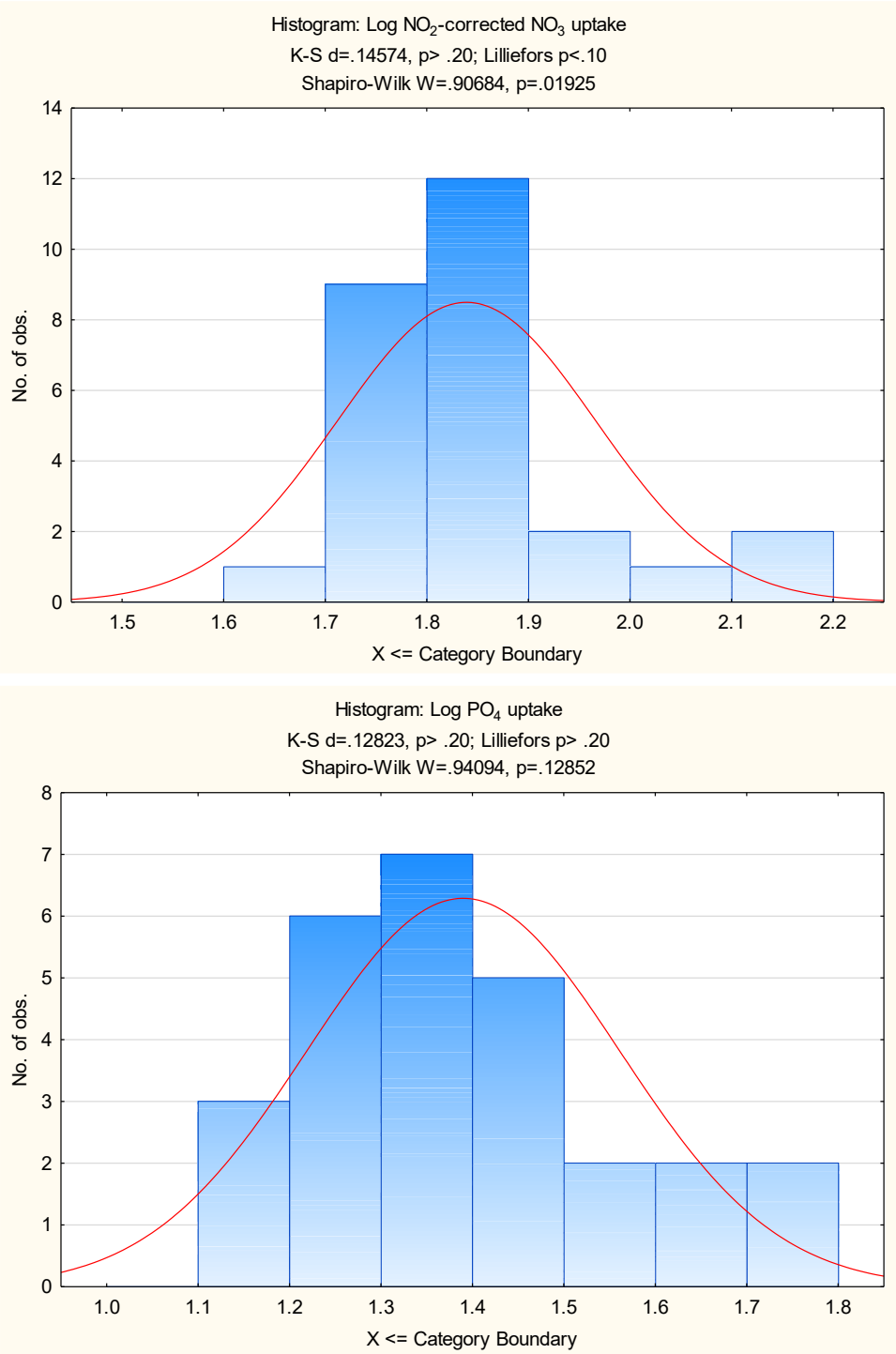
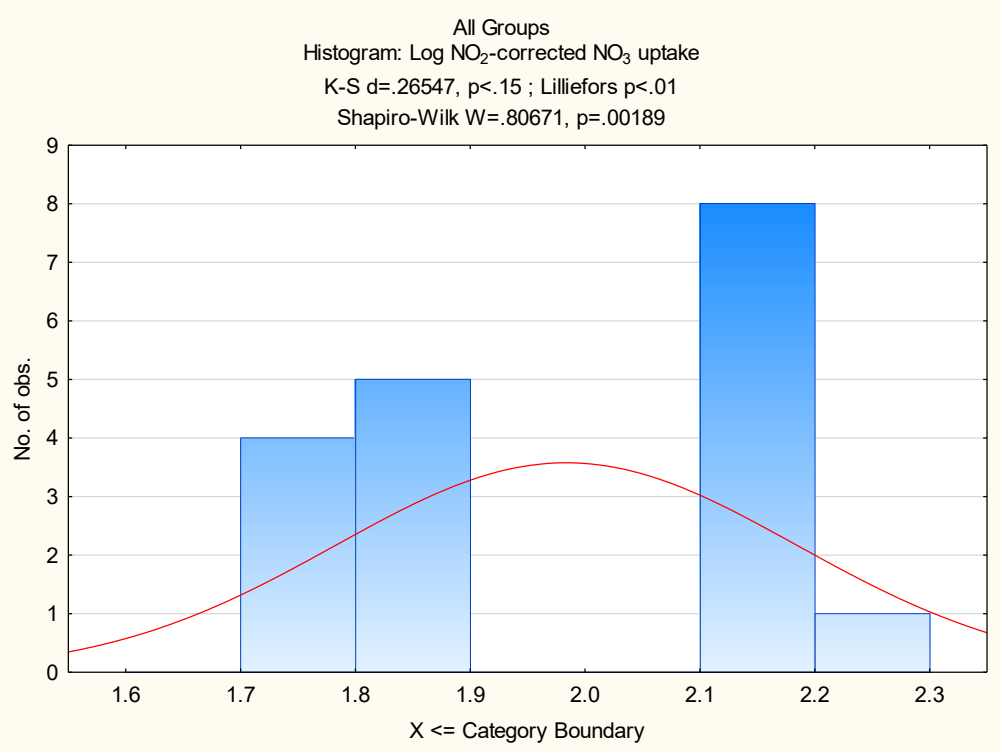


Figure A4 2 Analysis of effect of temperature on normality of nutrient uptake for WT, empty vector control (*pSyn_6*) and *ectABC*-transformed *S. elongatus* PCC7942**.**



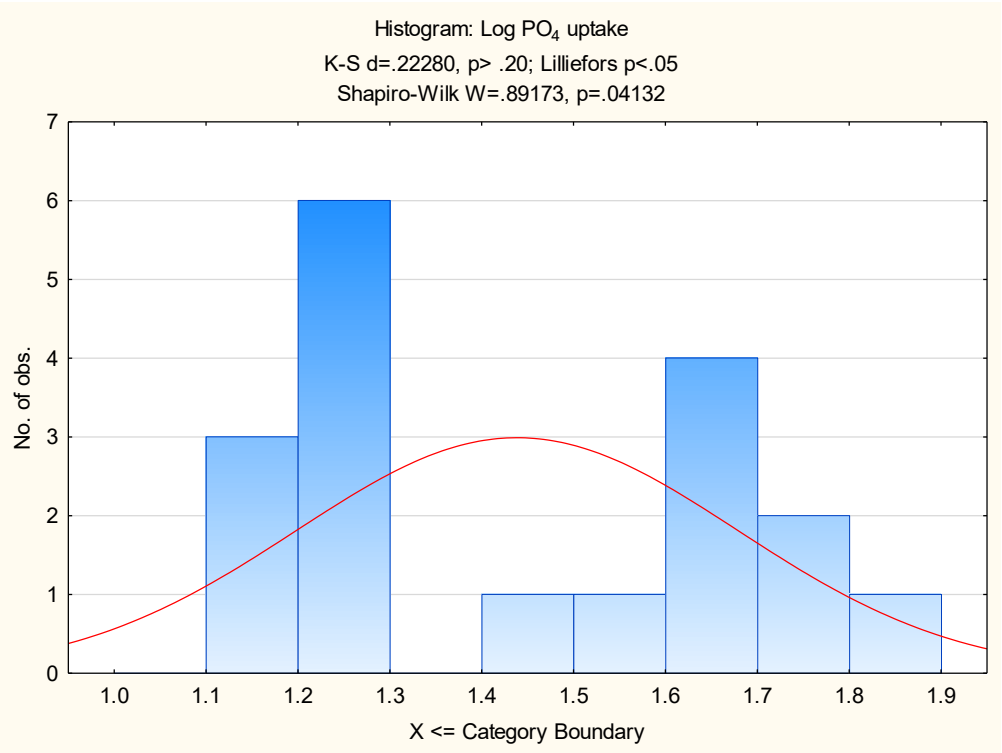


Figure A4 3. Analysis of effect of salinity on normality of nutrient uptake for WT, empty vector control (*pSyn_6*) and *ectABC*-transformed *S. elongatus* PCC7942

Table A4 6. Tukeys Post Hoc analysis for the effect of temperature and strain on nitrite-corrected nitrate uptake of WT, *pSyn_6* and *ectABC*-transformants of *S. elongatus* PCC7942

Tukey HSD test; variable Log NO_2-corrected NO_3 uptake (nutrient and temperature.sta)
Approximate Probabilities for Post Hoc Tests
Error: Between MS = .00325, df = 17.000

Cell No.	Strain	Temperature [°C]	{1}	{2}	{3}	{4}	{5}	{6}	{7}	{8}	{9}
1	Wild type	35		1.000000	0.001451	0.607477	0.566322	0.999980	0.531009	0.347803	0.083086
2	Wild type	40	1.000000		0.001249	0.658120	0.611763	0.999906	0.581487	0.390394	0.096986
3	Wild type	45	0.001451	0.001249		0.000201	0.000255	0.002949	0.000196	0.000187	0.000180
4	pSyn-6	35	0.607477	0.658120	0.000201		0.999998	0.390578	1.000000	0.999905	0.907171
5	pSyn-6	40	0.566322	0.611763	0.000255	0.999998		0.375842	1.000000	1.000000	0.988402
6	pSyn-6	45	0.999980	0.999906	0.002949	0.390578	0.375842		0.327341	0.195273	0.040599
7	ectABC	35	0.531009	0.581487	0.000196	1.000000	1.000000	0.327341		0.999992	0.944478
8	ectABC	40	0.347803	0.390394	0.000187	0.999905	1.000000	0.195273	0.999992		0.991975
9	ectABC	45	0.083086	0.096986	0.000180	0.907171	0.988402	0.040599	0.944478	0.991975	

Table A4 7. Effect of salinity on homogeneity of variance for nutrient uptake in WT, *pSyn_6 and ectABC*-transformants of *Synechococcus elongatus* PCC7942

Tests of Homogeneity of Variances (nutrient and salinity.sta)
Effect: "Salinity [ppt]"; "Strain" and "Salinity*Strain"

	Hartley	Cochran	Bartlett	df	p
Log NO_2-corrected NO_3 uptake	8.854707	0.898526	7.594578	1	0.005854
Log NO_2-corrected NO_3 uptake	1.992996	0.444244	0.535491	2	0.765103
Log NO_2-corrected NO_3 uptake	24.83911	0.578443	5.394433	5	0.369661

Tests of Homogeneity of Variances (nutrient and salinity.sta)
Effect: "Salinity [ppt]" and "Salinity*Strain"

Log PO_4 uptake	2.386300	0.503125	0.915509	2	0.632703
Log PO_4 uptake	3.689663	0.786765	3.003422	1	0.083089

Table A4 8. Factorial ANOVA of the effect of salinity and strain on nitrite-corrected nitrate uptake of WT, *pSyn_6* and *ectABC*-transformants of *S. elongatus* PCC7942

Univariate Tests of Significance for Log NO_2-corrected NO_3 uptake (nutrient and salinity.sta)
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of	MS	F	p
Intercept	70.83241	1	70.83241	71602.70	0.000000
Strain	0.02113	2	0.01056	10.68	0.002168
Salinity [ppt]	0.63945	1	0.63945	646.40	0.000000
Strain*Salinity [ppt]	0.01248	2	0.00624	6.31	0.013417
Error	0.01187	12	0.00099		

Table A4 9. Tukeys Post Hoc analysis for the effect of salinity and strain on nitrite-corrected nitrate uptake of WT, *pSyn_6* and *ectABC*-transformants of *S. elongatus* PCC 7942

	Tukey HSD test; variable Log NO_2-corrected NO_3 uptake (nutrient and salinity.sta)Approximat							
Cell No.	Strain	Salinity [ppt]	{1}	{2}	{3}	{4}	{5}	{6}
1	Wild type	0		0.000159	0.040367	0.000159	0.001215	0.000159
2	Wild type	18	0.000159		0.000159	0.894659	0.000159	0.983176
3	pSyn-6	0	0.040367	0.000159		0.000159	0.306429	0.000159
4	pSyn-6	18	0.000159	0.894659	0.000159		0.000159	0.998710
5	ectABC	0	0.001215	0.000159	0.306429	0.000159		0.000159
6	ectABC	18	0.000159	0.983176	0.000159	0.998710	0.000159	

Table A4 10. Factorial ANOVA of the effect of temperature and strain on phosphate uptake of WT, *pSyn_6* and *ectABC*-transformants of *S. elongatus* PCC7942

Univariate Tests of Significance for Log PO_4 uptake
(nutrient and temperature.sta)
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of	MS	F	p
Intercept	49.50373	1	49.50373	4766.670	0.000000
Strain	0.08385	2	0.04192	4.037	0.036771
Temperature [°C]	0.35023	2	0.17512	16.862	0.000092
Strain*Temperature [°C]	0.14747	4	0.03687	3.550	0.027918
Error	0.17655	17	0.01039		

Table A4 11. Tukeys Post Hoc analysis for the effect of temperature and strain on phosphate uptake of WT, *pSyn_6* and *ectABC*-transformants of *S. elongatus* PCC 7942

Tukey HSD test; variable Log PO_4 uptake (nutrient and temperature.sta)
Approximate Probabilities for Post Hoc Tests
Error: Between MS = .01039, df = 17.000

Cell No.	Strain	Temperature [°C]	{1}	{2}	{3}	{4}	{5}	{6}	{7}	{8}	{9}
1	Wild type	35		0.318727	0.000794	1.000000	0.923519	0.046061	0.956974	0.999554	0.781086
2	Wild type	40	0.318727		0.082969	0.247264	0.989207	0.962609	0.917582	0.635302	0.994087
3	Wild type	45	0.000794	0.082969		0.000615	0.028198	0.481583	0.006591	0.002167	0.016809
4	pSyn-6	35	1.000000	0.247264	0.000615		0.869585	0.033203	0.911928	0.997194	0.686662
5	pSyn-6	40	0.923519	0.989207	0.028198	0.869585		0.614794	0.999999	0.996399	1.000000
6	pSyn-6	45	0.046061	0.962609	0.481583	0.033203	0.614794		0.332083	0.132172	0.592511
7	ectABC	35	0.956974	0.917582	0.006591	0.911928	0.999999	0.332083		0.999530	0.999892
8	ectABC	40	0.999554	0.635302	0.002167	0.997194	0.996399	0.132172	0.999530		0.974344
9	ectABC	45	0.781086	0.994087	0.016809	0.686662	1.000000	0.592511	0.999892	0.974344	

Table A4 12. Factorial ANOVA of the effect of salinity and strain on phosphate uptake of WT, *pSyn_6* and *ectABC*-transformants of *S. elongatus* PCC7942

Univariate Tests of Significance for Log PO_4 uptake
(nutrient and salinity.sta)
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of	MS	F	p
Intercept	37.25272	1	37.25272	12993.12	0.000000
Strain	0.06541	2	0.03271	11.41	0.001677
Salinity [ppt]	0.84479	1	0.84479	294.65	0.000000
Strain*Salinity [ppt]	0.03543	2	0.01771	6.18	0.014300
Error	0.03441	12	0.00287		

Table A4 13. Tukeys Post Hoc analysis for the effect of salinity and strain on phosphate uptake of WT, *pSyn_6* and *ectABC*-transformants of *S. elongatus* PCC7942

Tukey HSD test; variable Log PO_4 uptake (nutrient and salinity.sta)
Approximate Probabilities for Post Hoc Tests
Error: Between MS = .00287, df = 12.000

Cell No.	Strain	Salinity [ppt]	{1}	{2}	{3}	{4}	{5}	{6}
1	Wild type	0		0.000159	0.999424	0.000163	0.934399	0.000346
2	Wild type	18	0.000159		0.000159	0.038791	0.000159	0.001020
3	pSyn-6	0	0.999424	0.000159		0.000161	0.989430	0.000270
4	pSyn-6	18	0.000163	0.038791	0.000161		0.000160	0.263907
5	ectABC	0	0.934399	0.000159	0.989430	0.000160		0.000206
6	ectABC	18	0.000346	0.001020	0.000270	0.263907	0.000206	

Table A4 14. Shapiro Wilk test for normality test for the effect of temperature on total lipid and fatty acids of *pSyn_6* (empty vector control) and *ectABC*-transformants of *S. elongatus* PCC7942

Variable	**W**	**p**
Log total lipid	0.8952	*0.04743*
Total fatty acids	0.94609	0.36708
Log C14:0	0.89116	*0.0404*
C16:0	0.93731	0.26054
C16:1	0.91843	0.12127
C17:0	0.69978	*0.00008*
Log C18:0	0.95466	0.50262
Log C18:1	0.96894	0.77756
ΣSFA	0.93712	0.25854
ΣMUFA	0.92751	0.1755

Table A4 15. Levene's test of homogeneity of variance for the effect of temperature, strain and temperature*strain interaction on total lipids and fatty acids of *pSyn_6* (empty vector control) and *ectABC*-transformants of *S. elongatus* PCC7942

	Levene's Test for Homogeneity of Variances (fatty acid stats effect of temperature.sta) Effect: Strain*"Temperature [°C]" Degrees of freedom for all F's: 5, 12			
	MS	MS	F	p
Log total lipids	0.0006	0.0003	2.083657	0.138071
Total Fame [mg g^{-1} AFDW]	177.8409	100.1684	1.775419	0.192453
Log C14:0	0.0038	0.0022	1.753106	0.197217
C16:0 [mg g^{-1} AFDW]	39.5631	22.9594	1.723178	0.203809
C16.1 [mg g^{-1} AFDW]	58.0713	21.9305	2.647973	0.077513
C17:0 [mg g^{-1} AFDW]	0.0021	0.0004	5.692517	0.006431
Log C18:0	0.0058	0.0023	2.539406	0.086342
Log C18:1	0.0043	0.0026	1.673361	0.215317
ΣSFA [mg g^{-1} AFDW]	39.2179	23.8007	1.647765	0.221500
ΣMUFA [mg g^{-1} AFDW]	56.6116	23.4809	2.410960	0.098290
	Levene's Test for Homogeneity of Variances (fatty acid stats effect of temperature.sta) Effect: Strain Degrees of freedom for all F's: 1, 16			
	MS	MS	F	p
Log total lipids	0.00003	0.0022	0.01200	0.914118
Total Fame [mg g^{-1} AFDW]	98.64023	147.2978	0.66967	0.425189
Log C14:0	0.03546	0.0070	5.02970	0.039432
C16:0 [mg g^{-1} AFDW]	55.67254	33.6947	1.65226	0.216946
C16.1 [mg g^{-1} AFDW]	0.49966	34.3996	0.01453	0.905572
C17:0 [mg g^{-1} AFDW]	0.00099	0.0007	1.33779	0.264402
Log C18:0	0.11209	0.0098	11.43523	0.003805
Log C18:1	0.01309	0.0063	2.08305	0.168236
ΣSFA [mg g^{-1} AFDW]	57.18622	34.5407	1.65562	0.216502
ΣMUFA [mg g^{-1} AFDW]	0.17211	38.4486	0.00448	0.947485
	Levene's Test for Homogeneity of Variances (fatty acid stats effect of temperature.sta) Effect: "Temperature [°C]" Degrees of freedom for all F's: 2, 15			
	MS	MS	F	p
Log total lipids	0.0039	0.0007	5.522638	0.015948
Total Fame [mg g^{-1} AFDW]	145.5516	135.9295	1.070787	0.367540
Log C14:0	0.0124	0.0100	1.246028	0.315775
C16:0 [mg g^{-1} AFDW]	41.7763	35.6657	1.171330	0.336757
C16.1 [mg g^{-1} AFDW]	46.5271	25.5888	1.818258	0.196313
C17:0 [mg g^{-1} AFDW]	0.0003	0.0002	1.682721	0.219116
Log C18:0	0.0064	0.0074	0.862448	0.442037
Log C18:1	0.0017	0.0049	0.348828	0.711088
ΣSFA [mg g^{-1} AFDW]	41.6497	35.4870	1.173662	0.336078
ΣMUFA [mg g^{-1} AFDW]	44.0686	29.0984	1.514470	0.251714

Table A4 16. Factorial ANOVA for the effect of temperature, strain and temperature*strain interaction on total lipids and fatty acids of *pSyn_6* (empty vector control) and *ectABC*-transformants of *S. elongatus* PCC7942

	Univariate Tests of Significance for (fatty acid stats effect of temperature.sta) Sigma-restricted parameterization Effective hypothesis decomposition					
Effect	SS	Degr. of	MS	F	p	
Intercept	78.62948	1	78.62948	48146.57	0.000000	Log total lpids
Strain	0.02272	1	0.02272	13.91	0.002876	
Temperature [°C]	0.11772	2	0.05886	36.04	0.000008	
Strain*Temperature [°C]	0.01202	2	0.00601	3.68	0.056657	
Error	0.01960	12	0.00163			
Intercept	41182.34	1	41182.34	78.74276	0.000001	Total fatty acids
Strain	676.66	1	676.66	1.29381	0.277549	
Temperature [°C]	410.44	2	205.22	0.39239	0.683796	
Strain*Temperature [°C]	2053.30	2	1026.65	1.96301	0.182998	
Error	6275.98	12	523.00			
Intercept	2.640045	1	2.640045	180.8745	0.000000	Log C14:0
Strain	0.121017	1	0.121017	8.2911	0.013845	
Temperature [°C]	0.122728	2	0.061364	4.2042	0.041328	
Strain*Temperature [°C]	0.151750	2	0.075875	5.1983	0.023659	
Error	0.175152	12	0.014596			
Intercept	11765.62	1	11765.62	87.82414	0.000001	C16:0
Strain	164.43	1	164.43	1.22736	0.289632	
Temperature [°C]	83.20	2	41.60	0.31054	0.738770	
Strain*Temperature [°C]	251.15	2	125.57	0.93734	0.418550	
Error	1607.62	12	133.97			
Intercept	7077.602	1	7077.602	56.55138	0.000007	C16:1
Strain	141.417	1	141.417	1.12995	0.308710	
Temperature [°C]	101.879	2	50.940	0.40702	0.674485	
Strain*Temperature [°C]	924.235	2	462.117	3.69240	0.056275	
Error	1501.842	12	125.153			
Intercept	0.055556	1	0.055556	17.36111	0.001307	C17:0
Strain	0.015022	1	0.015022	4.69444	0.051099	
Temperature [°C]	0.002678	2	0.001339	0.41840	0.667338	
Strain*Temperature [°C]	0.014744	2	0.007372	2.30382	0.142312	
Error	0.038400	12	0.003200			
Intercept	3.141856	1	3.141856	202.7634	0.000000	Log C18:0
Strain	0.000123	1	0.000123	0.0079	0.930495	
Temperature [°C]	0.248180	2	0.124090	8.0083	0.006174	
Strain*Temperature [°C]	0.249594	2	0.124797	8.0539	0.006055	
Error	0.185942	12	0.015495			
Intercept	0.371844	1	0.371844	21.60082	0.000563	Log C18:1
Strain	0.051344	1	0.051344	2.98263	0.109788	
Temperature [°C]	0.154447	2	0.077224	4.48600	0.035095	
Strain*Temperature [°C]	0.072777	2	0.036388	2.11384	0.163512	
Error	0.206572	12	0.017214			
Intercept	12643.44	1	12643.44	91.85564	0.000001	ΣSFA
Strain	174.50	1	174.50	1.26775	0.282203	
Temperature [°C]	84.71	2	42.35	0.30770	0.740770	
Strain*Temperature [°C]	226.26	2	113.13	0.82190	0.462888	
Error	1651.74	12	137.64			
Intercept	8188.668	1	8188.668	63.01688	0.000004	ΣMUFA
Strain	163.914	1	163.914	1.26142	0.283350	
Temperature [°C]	125.787	2	62.894	0.48401	0.627838	
Strain*Temperature [°C]	917.813	2	458.907	3.53157	0.062219	
Error	1559.328	12	129.944			

Table A4 17. Tukey's post hoc analysis for the effect of temperature, strain and temperature*strain interaction on total lipids and fatty acids of *pSyn_6* (empty vector control) and *ectABC*-transformants of *S. elongatus* PCC7942

Cell No.	Strain	Temperature [°C]	{1}	{2}	{3}	{4}	{5}	{6}
Tukey HSD test; variable Log total lipids (fatty acid stats effect of temperature.sta) Approximate Probabilities for Post Hoc Tests Error: Between MS = .00163, df = 12.000								
1	pSyn-6	35		0.885352	0.000772	0.877977	0.001727	0.000283
2	pSyn-6	40	0.885352		0.003501	1.000000	0.009216	0.000832
3	pSyn-6	45	0.000772	0.003501		0.003607	0.989134	0.907399
4	ectABC	35	0.877977	1.000000	0.003607		0.009510	0.000852
5	ectABC	40	0.001727	0.009216	0.989134	0.009510		0.610776
6	ectABC	45	0.000283	0.000832	0.907399	0.000852	0.610776	
Tukey HSD test; variable Log C14:0 (fatty acid stats effect of temperature.sta) Approximate Probabilities for Post Hoc Tests Error: Between MS = .01460, df = 12.000								
Cell No.	Strain	Temperature [°C]	{1}	{2}	{3}	{4}	{5}	{6}
1	pSyn-6	35		0.999903	0.999990	0.049426	0.256665	0.964835
2	pSyn-6	40	0.999903		0.998949	0.033927	0.184835	0.991871
3	pSyn-6	45	0.999990	0.998949		0.062567	0.312351	0.931765
4	ectABC	35	0.049426	0.033927	0.062567		0.894210	0.013188
5	ectABC	40	0.256665	0.184835	0.312351	0.894210		0.075737
6	ectABC	45	0.964835	0.991871	0.931765	0.013188	0.075737	
Tukey HSD test; variable Log C18:0 (fatty acid stats effect of temperature.sta) Approximate Probabilities for Post Hoc Tests Error: Between MS = .01550, df = 12.000								
Cell No.	Strain	Temperature [°C]	{1}	{2}	{3}	{4}	{5}	{6}
1	pSyn-6	35		0.999747	0.003569	0.305205	0.847791	0.547275
2	pSyn-6	40	0.999747		0.005509	0.432357	0.942943	0.702737
3	pSyn-6	45	0.003569	0.005509		0.134013	0.023957	0.061121
4	ectABC	35	0.305205	0.432357	0.134013		0.894904	0.995938
5	ectABC	40	0.847791	0.942943	0.023957	0.894904		0.992149
6	ectABC	45	0.547275	0.702737	0.061121	0.995938	0.992149	
Tukey HSD test; variable Log C18:1 (fatty acid stats effect of temperature.sta) Approximate Probabilities for Post Hoc Tests Error: Between MS = .01721, df = 12.000								
Cell No.	Strain	Temperature [°C]	{1}	{2}	{3}	{4}	{5}	{6}
1	pSyn-6	35		0.973724	0.813249	0.190279	0.998904	0.998229
2	pSyn-6	40	0.973724		0.412364	0.502789	0.998964	0.856114
3	pSyn-6	45	0.813249	0.412364		0.025447	0.612462	0.956605
4	ectABC	35	0.190279	0.502789	0.025447		0.321912	0.100797
5	ectABC	40	0.998904	0.998964	0.612462	0.321912		0.966871
6	ectABC	45	0.998229	0.856114	0.956605	0.100797	0.966871	

Table A4 18. Shapiro-Wilk normality test for total lidids and fatty acids_effect of salinity on total lipids and fatty acids of *pSyn_6* (empty vector control) and *ectABC*-transformants of *S. elongatus* PCC7942

Variable	W	p
Log total lipid	0.7791	*0.00547*
Total fatty acids	0.91436	0.24254
C14:0	0.87834	0.08347
C16:0	0.92801	0.35953
C16:1	0.93448	0.43001
C17:0	0.72193	*0.00138*
Log C18:0	0.86073	0.04993
C18:1	0.91005	0.21365
ΣSFA	0.92637	0.3432
ΣMUFA	0.93352	0.41888

Table A4 19. Levene's test for homogeneity of variances for total lipids and fatty acids_effect of salinity on total lipids and fatty acids of *pSyn_6* (empty vector control) and *ectABC*-transformants of *S. elongatus* PCC7942

	Levene's Test for Homogeneity of Variances (fatty acid stats_effect of salinity.sta) Effect: Strain*"Salinity [ppt]" Degrees of freedom for all F's: 3, 8			
	MS	MS	F	p
Log total lipid	0.000106	0.000048	2.206460	0.164971
Log total Fame [mg g^{-1} AFDW]	0.023062	0.009721	2.372408	0.146176
C14:0 [mg g^{-1} AFW]	0.0085	0.0042	2.047047	0.185879
C16:0 [mg g^{-1} AFDW]	100.4953	27.6685	3.632120	0.064207
Log C16.1 [mg g^{-1} AFDW]	0.035372	0.011521	3.070128	0.090901
C17:0 [mg g^{-1} AFDW]	0.0019	0.0003	6.705882	0.014167
Log C18:0	0.0000	0.0021	0.004912	0.999484
Log C18.1 [mg g^{-1} AFDW]	0.001359	0.001791	0.759171	0.547743
ΣSFA [mg g^{-1} AFDW]	102.4716	28.6308	3.579064	0.066270
Log ΣMUFA [mg g^{-1} AFDW]	0.030997	0.010526	2.944896	0.098621
	Levene's Test for Homogeneity of Variances (fatty acid stats_effect of salinity.sta) Effect: Strain Degrees of freedom for all F's: 1, 10			
	MS	MS	F	p
Log total lipid	0.001400	0.000228	6.135791	0.032707
Log Total Fame [mg g^{-1} AFDW]	0.030565	0.011900	2.568514	0.140092
C14:0 [mg g^{-1} AFW]	0.0049	0.0167	0.292072	0.600725
C16:0 [mg g^{-1} AFDW]	161.0747	34.2256	4.706262	0.055227
Log C16.1 [mg g^{-1} AFDW]	0.050581	0.015413	3.281809	0.100144
C17:0 [mg g^{-1} AFDW]	0.0006	0.0010	0.555951	0.473058
Log C18:0	0.0000	0.0020	0.000000	1.000000
Log C18.1 [mg g^{-1} AFDW]	0.002247	0.003928	0.572089	0.466875
ΣSFA [mg g^{-1} AFDW]	161.5133	35.3283	4.571777	0.058214
Log ΣMUFA [mg g^{-1} AFDW]	0.036941	0.013660	2.704240	0.131108
	Levene's Test for Homogeneity of Variances (fatty acid stats_effect of salinity.sta) Effect: "Salinity [ppt]" Degrees of freedom for all F's: 1, 10			
	MS	MS	F	p
Log total lipid	0.0009	0.0001	7.000825	0.024484
Log total Fame [mg g^{-1} AFDW]	0.021593	0.008942	2.414859	0.151240
C14:0 [mg g^{-1} AFW]	0.0126	0.0124	1.015717	0.337301
C16:0 [mg g^{-1} AFDW]	48.3960	33.8306	1.430541	0.259266
C16.1 [mg g^{-1} AFDW]	43.0789	30.8791	1.395084	0.264877
C17:0 [mg g^{-1} AFDW]	0.0002	0.0004	0.349607	0.567460
Log C18:0	0.0000	0.0016	0.018419	0.894738
Log C18.1 [mg g^{-1} AFDW]	0.016843	0.004731	3.560215	0.088520
ΣSFA [mg g^{-1} AFDW]	47.6478	35.3235	1.348897	0.272443
Log ΣMUFA [mg g^{-1} AFDW]	0.040289	0.008471	4.756123	0.054167

Table A4 20. Factorial ANOVA on effect of salinity on total lipids and fatty acids of *pSyn_6* (empty vector control) and *ectABC*-transformants of *S. elongatus* PCC7942

	Univariate Tests of Significance for (fatty acid stats_effect of salinity.sta) Sigma-restricted parameterization Effective hypothesis decomposition					
Effect	SS	Degr. of	MS	F	p	
Intercept	57.32104	1	57.32104	201017.2	0.000000	Log total lipid
Strain	0.00989	1	0.00989	34.7	0.000366	
Salinity [ppt]	0.43889	1	0.43889	1539.1	0.000000	
Strain*Salinity [ppt]	0.00140	1	0.00140	4.9	0.057581	
Error	0.00228	8	0.00029			
Intercept	41990.25	1	41990.25	63.75084	0.000044	Total FAM
Strain	639.67	1	639.67	0.97116	0.353253	
Salinity [ppt]	253.96	1	253.96	0.38557	0.551915	
Strain*Salinity [ppt]	264.48	1	264.48	0.40154	0.543984	
Error	5269.30	8	658.66			
Intercept	3.401412	1	3.401412	124.4265	0.000004	C14:0
Strain	0.063247	1	0.063247	2.3136	0.166736	
Salinity [ppt]	0.005258	1	0.005258	0.1923	0.672580	
Strain*Salinity [ppt]	0.205098	1	0.205098	7.5027	0.025485	
Error	0.218694	8	0.027337			
Intercept	11280.55	1	11280.55	75.41652	0.000024	C16:0
Strain	218.95	1	218.95	1.46378	0.260863	
Salinity [ppt]	53.80	1	53.80	0.35967	0.565285	
Strain*Salinity [ppt]	68.60	1	68.60	0.45864	0.517362	
Error	1196.61	8	149.58			
Intercept	7946.002	1	7946.002	44.26191	0.000160	C16:1
Strain	75.545	1	75.545	0.42081	0.534707	
Salinity [ppt]	77.711	1	77.711	0.43288	0.529055	
Strain*Salinity [ppt]	84.496	1	84.496	0.47067	0.512067	
Error	1436.179	8	179.522			
Intercept	0.028033	1	0.028033	11.21333	0.010101	C17:0
Strain	0.008533	1	0.008533	3.41333	0.101863	
Salinity [ppt]	0.001200	1	0.001200	0.48000	0.508032	
Strain*Salinity [ppt]	0.012033	1	0.012033	4.81333	0.059555	
Error	0.020000	8	0.002500			
Intercept	3.968848	1	3.968848	400.9203	0.000000	Log C18:0
Strain	0.000000	1	0.000000	0.0000	1.000000	
Salinity [ppt]	0.003545	1	0.003545	0.3581	0.566131	
Strain*Salinity [ppt]	0.000000	1	0.000000	0.0000	1.000000	
Error	0.079195	8	0.009899			
Intercept	38.91290	1	38.91290	209.2307	0.000001	C18:1
Strain	1.17242	1	1.17242	6.3040	0.036327	
Salinity [ppt]	0.07376	1	0.07376	0.3966	0.546419	
Strain*Salinity [ppt]	0.86361	1	0.86361	4.6435	0.063291	
Error	1.48785	8	0.18598			
Intercept	11998.32	1	11998.32	78.94928	0.000020	ΣSFA
Strain	240.78	1	240.78	1.58436	0.243621	
Salinity [ppt]	54.65	1	54.65	0.35957	0.565336	
Strain*Salinity [ppt]	64.00	1	64.00	0.42111	0.534566	
Error	1215.80	8	151.98			
Intercept	9097.034	1	9097.034	50.02117	0.000105	ΣMUFA
Strain	95.540	1	95.540	0.52534	0.489228	
Salinity [ppt]	72.996	1	72.996	0.40138	0.544061	
Strain*Salinity [ppt]	68.275	1	68.275	0.37542	0.557077	
Error	1454.909	8	181.864			

Table A4 21. Tukey's post hoc analysis on the effect of salinity on total lipid, C14:0 and C18:1 of *pSyn_6* (empty ector control) and *ectABC*-transformants of *S. elongatus* PCC7942

Cell No.	Tukey HSD test; variable Total Lipid [mg g^{-1} AFDW] (fatty acid stats_effect of salinity.sta) Approximate Probabilities for Post Hoc Tests Error: Between MS = 39.500, df = 8.0000					
	Strain	Salinity [ppt]	{1}	{2}	{3}	{4}
1	pSyn-6	0		0.000231	0.426052	0.000231
2	pSyn-6	18	0.000231		0.000231	0.000332
3	ectABC	0	0.426052	0.000231		0.000231
4	ectABC	18	0.000231	0.000332	0.000231	
Cell No.	Tukey HSD test; variable C14:0 [mg g^{-1} AFW] (fatty acid stats_effect of salinity.sta) Approximate Probabilities for Post Hoc Tests Error: Between MS = .02734, df = 8.0000					
	Strain	Salinity [ppt]	{1}	{2}	{3}	{4}
1	pSyn-6	0		0.417154	0.065448	0.867801
2	pSyn-6	18	0.417154		0.540775	0.824334
3	ectABC	0	0.065448	0.540775		0.190426
4	ectABC	18	0.867801	0.824334	0.190426	
Cell No.	Tukey HSD test; variable C18.1 [mg g^{-1} AFDW] (fatty acid stats_effect of salinity.sta) Approximate Probabilities for Post Hoc Tests Error: Between MS = .18598, df = 8.0000					
	Strain	Salinity [ppt]	{1}	{2}	{3}	{4}
1	pSyn-6	0		0.711427	0.043754	0.571198
2	pSyn-6	18	0.711427		0.197305	0.994017
3	ectABC	0	0.043754	0.197305		0.274957
4	ectABC	18	0.571198	0.994017	0.274957	

Table A4 22. Effect of temperature and salinity on percent contribution of fatty acids and fatty acid groups to total fatty acid content in *ectABC*-transformed and *pSyn_6* empty vector controls of *S. elongatus* PCC7942

		pSyn-6		ectABC				pSyn-6		ectABC	
		Mean	**Stdev**	**Mean**	**Stdev**			**Mean**	**Stdev**	**Mean**	**Stdev**
35,18 ppt	**C16:0**	50.5	0.2	52.8	4.3	**35,18 ppt**	**C14:0**	1.1	0.5	1.7	0.8
	C16:1	44.6	1.2	43.1	5.9		**C17:0**	0.0	0.0	0.1	0.1
	ΣSFA	52.1	0.4	54.4	4.7		**C18:0**	0.5	0.1	1.0	0.4
	ΣMUFA	47.9	0.4	45.6	4.7		**C18:1**	3.3	0.8	5.4	2.4
35.0 ppt	**C16:0**	52.5	5.3	54.3	2.5	**35.0 ppt**	**C14:0**	0.8	0.6	1.7	0.8
	C16:1	43.2	7.0	37.6	6.2		**C17:0**	0.1	0.1	0.1	0.1
	ΣSFA	54.0	6.1	57.0	3.8		**C18:0**	0.6	0.3	1.0	0.4
	ΣMUFA	46.0	6.1	43.0	3.8		**C18:1**	2.8	1.0	5.4	2.4
40,0ppt	**C16:0**	50.9	4.9	58.3	0.9	**40,0ppt**	**C14:0**	0.8	0.4	1.6	0.1
	C16:1	44.1	7.6	35.0	1.5		**C17:0**	0.1	0.1	0.3	0.3
	ΣSFA	52.4	5.8	61.2	1.5		**C18:0**	0.7	0.5	1.0	0.3
	ΣMUFA	47.6	5.8	38.8	1.5		**C18:1**	3.5	1.9	3.9	0.1
45,0ppt	**C16:0**	67.6	0.7	49.7	0.6	**45,0ppt**	**C14:0**	1.6	0.1	0.4	0.0
	C16:1	23.1	0.3	47.3	0.4		**C17:0**	0.0	0.0	0.2	0.0
	ΣSFA	72.7	0.2	50.9	0.4		**C18:0**	3.6	0.4	0.6	0.1
	ΣMUFA	27.3	0.2	49.1	0.4		**C18:1**	4.2	0.4	1.8	0.1

Table A4 23. Homogeneity of variance of effect of temperature on intracellular, extracellular and total ectoine production of *ectABC*-transformed *S. elongatus* PCC7942

Levene's Test for Homogeneity of Variances
(ectoin 0 ppt stats.sta)
Effect: "Temperature [°C]"
Degrees of freedom for all F's: 2, 6

	MS	MS	F	p
Intracellular ectoine [mg g^{-1} DW]	0.000037	0.000164	0.226256	0.804021
Extracellular ectoine [mg g^{-1} DW]	0.006859	0.004584	1.496364	0.297016
Total ectoine [mg g^{-1} DW]	0.007955	0.004924	1.615688	0.274571

Table A4 24. One-way ANOVA on the effect of temperature on intra- and extracellular and total ectoine concentrations of *ectABC*-transformants of *S. elongatus* PCC7942

	Univariate Tests of Significance for Intracellular ectoine [mg g^{-1} DW] (ectoin 0 ppt stats.sta) Sigma-restricted parameterization Effective hypothesis decomposition				
Effect	SS	Degr. of	MS	F	p
Intercept	0.134200	1	0.134200	220.7642	0.000006
Temperature [°C]	0.073894	2	0.036947	60.7788	0.000104
Error	0.003647	6	0.000608		
	Univariate Tests of Significance for Extracellular ectoine [mg g^{-1} DW] (ectoin 0 ppt stats.sta) Sigma-restricted parameterization Effective hypothesis decomposition				
Effect	SS	Degr. of	MS	F	p
Intercept	4.636844	1	4.636844	188.0649	0.000009
Temperature [°C]	0.551022	2	0.275511	11.1744	0.009481
Error	0.147933	6	0.024656		
	Univariate Tests of Significance for Total ectoine [mg g^{-1} DW] (ectoin 0 ppt stats.sta) Sigma-restricted parameterization Effective hypothesis decomposition				
Effect	SS	Degr. of	MS	F	p
Intercept	6.348720	1	6.348720	221.8358	0.000006
Temperature [°C]	0.982907	2	0.491453	17.1723	0.003289
Error	0.171714	6	0.028619		

Table A4 25. Tukey's post hoc analysis on the effect of temperature on intra- and extracellular and total ectoine concentrations of *ectABC*-transformants of *S. elongatus* PCC7942

	Tukey HSD test; variable Intracellular ectoine [mg g^{-1} DW] (ectoin 0 ppt stats.sta) Approximate Probabilities for Post Hoc Tests Error: Between MS = .00061, df = 6.0000			
Cell No.	Temperature [°C]	{1}	{2}	{3}
1	35		0.011920	0.001579
2	40	0.011920		0.000276
3	45	0.001579	0.000276	
	Tukey HSD test; variable Extracellular ectoine [mg g^{-1} DW] (ectoin 0 ppt stats.sta) Approximate Probabilities for Post Hoc Tests Error: Between MS = .02466, df = 6.0000			
Cell No.	Temperature [°C]	{1}	{2}	{3}
1	35		0.930485	0.012671
2	40	0.930485		0.018843
3	45	0.012671	0.018843	
	Tukey HSD test; variable Total ectoine [mg g^{-1} DW] (ectoin 0 ppt stats.sta) Approximate Probabilities for Post Hoc Tests Error: Between MS = .02862, df = 6.0000			
Cell No.	Temperature [°C]	{1}	{2}	{3}
1	35		0.954540	0.006490
2	40	0.954540		0.004919
3	45	0.006490	0.004919	

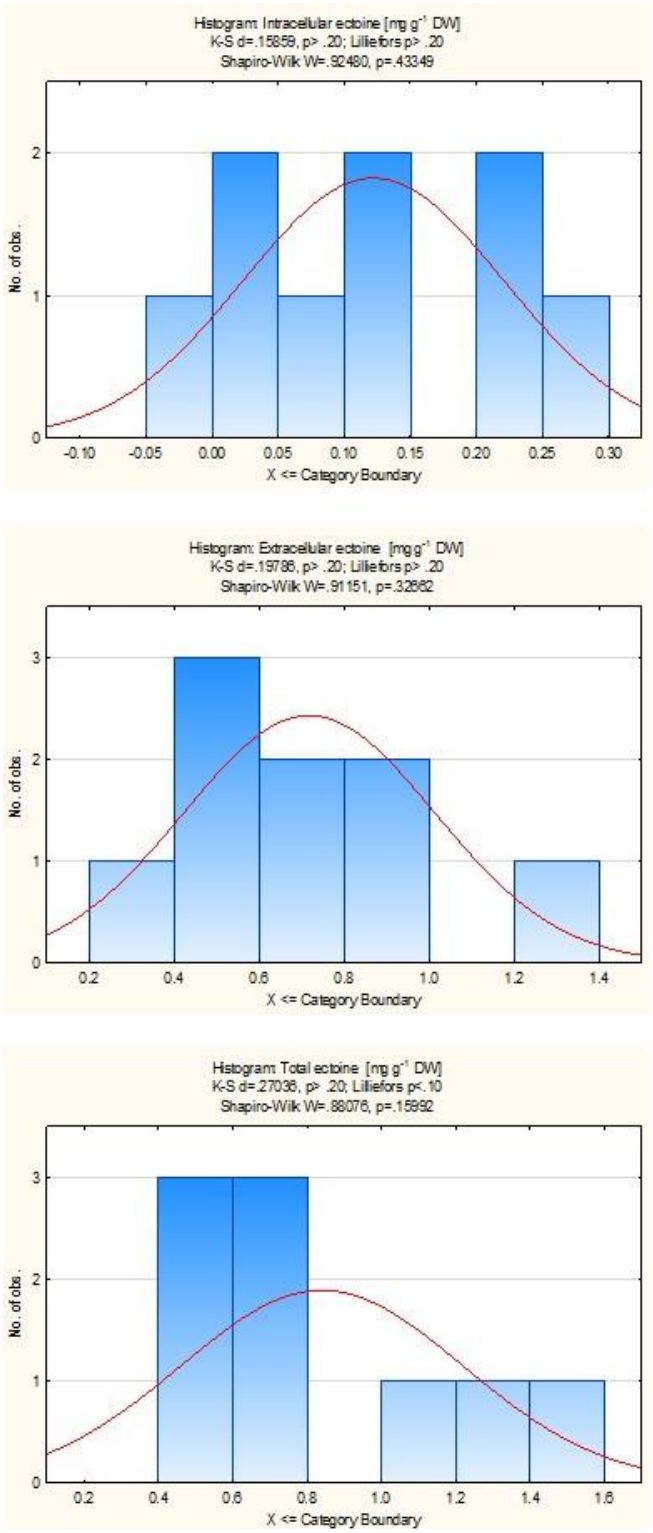


Figure A4 4. Shaprio-Wilk test for normality for the effect of temperature on intracellular, extracellular and total ectoine content in *ectABC*-transformed *S. elongatus* PCC7942

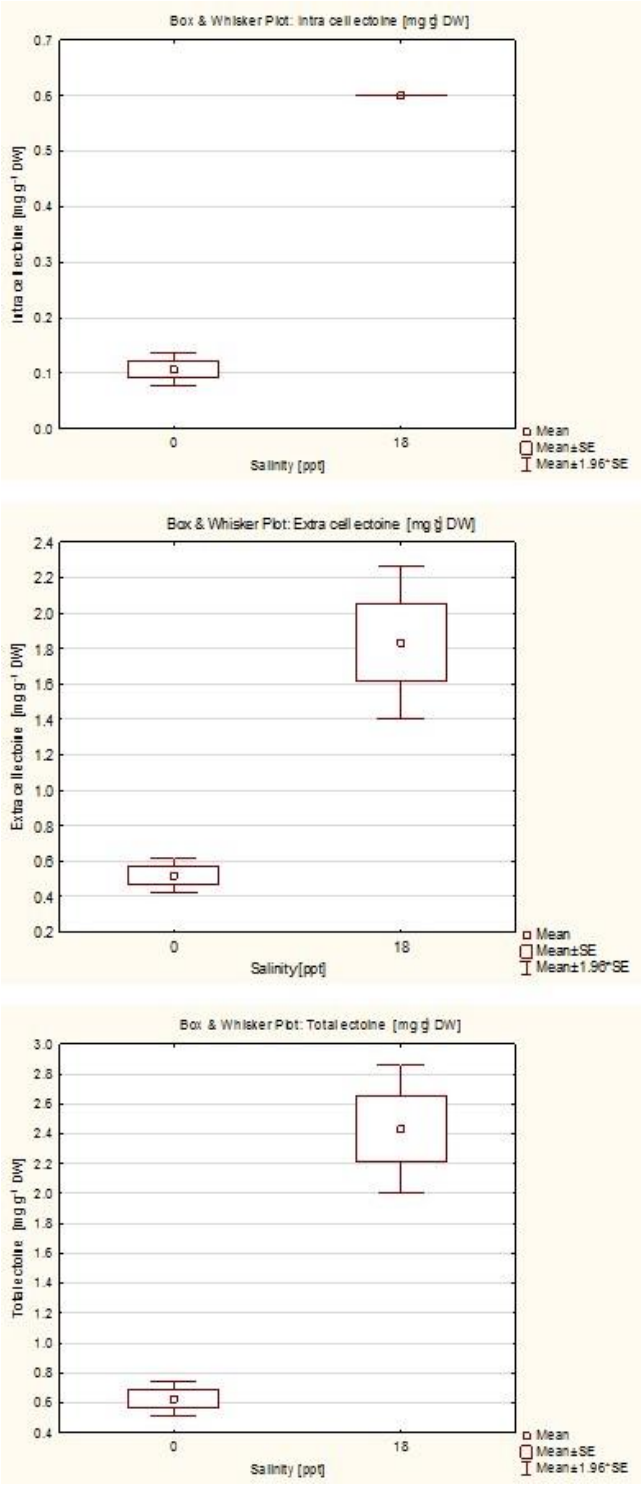


Figure A4 5. Box and Whisker plots of intracellular and extracellular and total ectoine content for *ectABC*-transformants of *S. elongatus* PCC7942 cultivated at salinities of 0 and 18 ppt