ABSTRACT

GROWTH CHARACTERISTICS OF *Dunaliella primolecta* AND *Botryococcus braunii* IN FRUIT INDUSTRY WASTEWATER AND DEVELOPMENT OF A PROTOCOL TO GENERATE TRANSGEINIC ALGAE

In the present study, we studied the growth characteristics of two species of green algae; *Dunliella primolecta* and *Botryococcus braunii,* in fruit industry wastewater and also studied the COD removal from wastewater by these species. We found that both the species of algae could grow better in 25% (v/v) wastewater reaching the stationary phase when compared to other concentrations of wastewater. However, *Dunaliella primolecta* was found to be growing faster than *Botryococcus braunii,* reaching a concentration of $8x10^6$ cells/mL in 15 days. Also, it was found to remove 63% of COD from fruit industry wastewater obtained from Wawona Frozen Foods, CA. We also reported the first successful *Agrobacterium tumefaciens* method of transformation for the green algae *Dunaliella primolecta* using the binary vector pCAMBIA 1301 containing the genes coding for GUS (β-Glucuronidase) and hpt (hygromycin phospho-transferase). The developed transformation protocol would pave a way for easy manipulation of *Dunaliella primolecta* for the production of biofuels and other secondary metabolites.

Preethi Sarvabhowman
December 2010

# GROWTH CHARACTERISTICS OF *Dunaliella primolecta* AND *Botryococcus braunii* IN FRUIT INDUSTRY WASTEWATER AND DEVELOPMENT OF A PROTOCOL TO GENERATE TRANSGEINIC ALGAE

by

Preethi Sarvabhowman

A thesis

submitted in partial

fulfillment of the requirements for the degree of

Master of Biotechnology

in the College of Science and Mathematics

California State University, Fresno

December 2010

APPROVED

For the Department of Biology:

We, the undersigned, certify that the thesis of the following student meets the required standards of scholarship, format, and style of the university and the student's graduate degree program for the awarding of the master's degree.

Preethi Sarvabhowman
Thesis Author

Alejandro Calderon Urrea — Biology

Rick Zechman — Biology

Gour Choudhury — Food Science and Nutrition

For the University Graduate Committee:

Dean, Division of Graduate Studies

AUTHORIZATION FOR REPRODUCTION

OF MASTER'S THESIS

X I grant permission for the reproduction of this thesis in part or in its entirety without further authorization from me, on the condition that the person or agency requesting reproduction absorbs the cost and provides proper acknowledgment of authorship.

_____ Permission to reproduce this thesis in part or in its entirety must be obtained from me.

Signature of thesis author:_____

ACKNOWLEDGMENTS

I would like to extend sincere gratitude to Dr Alejandro Calderon Urrea, my thesis advisor and mentor, who extended intense support, guidance and advice in every step towards the completion of my thesis. I thank Dr Gour Choudhury for his help with obtaining the wastewater from Wawona Frozen Foods, CA and also for his support and encouragement throughout my work. I am very thankful to Dr Rick Zechman for his valuable inputs and suggestions. I express my thanks to Dr Davis W Cheng for his technical assistance that helped me completing my thesis. I would like to thank Dr Jason Bush for allowing me to store the algal cultures in the liquid nitrogen in his laboratory. I appreciate the help and assistance provided my lab colleagues and friends, in particular, Jagadeesh Yerneni, Alexander Guzzetta, Venu Polineni, Freddie, and all the present lab members. I am thankful to Glenda Polack for her support and friendship.

This work was supported by the RIMI Grant at CSU Fresno, several Faculty sponsored research awards from College of Science and Mathematics at CSU Fresno, and Research grant from Associated Students Inc at CSU Fresno, Graduate Student Research Merit Award at CSU Fresno.

I would like to extend my heartiest thanks to my parents whose encouragement and support helped me come and study in the US. A special thanks to my sister who has been my backbone and courage during all the hard times that I faced. I am very much grateful to all my friends who have been like a family supporting me throughout my stay in the US. I would like to extend my gratitude to each and everyone who helped me in finishing up my thesis.

TABLE OF CONTENTS

Page

Page

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

# 1. INTRODUCTION

In recent years, microalgae have attracted the attention of many scientists to investigate the potential of these organisms as a source of biodiesel. Biodiesel is a biodegradable, renewable, carbon-neutral and non-toxic fuel that consists of alkyl esters of fatty acid. It is the most appropriate alternative to the depleting petroleum fossil fuel that produces toxic gases like oxides of sulfur and carbon. However, the demand for diesel has been on a rise causing a steep raise in the price of the diesel. Hence biodiesel has recently been extracted from the rapeseed in Europe (Lang et al., 2001) and soybean in United States (Chisti, 2007) commercially. They have also been used as a blend with the fossil fuel like the B20 and B100 (Zhang et al., 2003). However, these crops have oil of just 5% of the total plant weight giving low productivity (Reijnders, 2008). Also the slow growth of plants does not fulfill the demand for biodiesel. Also, microalgae requires only 3% of the US cropping area as opposed to 61% of the US agricultural land required by the crop plants (Chisti, 2008). The *Chlorophycaeae* and *Bacillariophycaeae* are two known classes of algae that have the capacity to accumulate large amount of oil under certain environmental stressed conditions like nitrogen stressed condition (Gordillo et al., 1998). It has also been proved that these algae have the capacity to reduce the nitrogen, phosphorous and ammonia content of wastewater, thereby reducing the toxic levels in the wastewater. Hence we used two species of algae, *Dunaliella primolecta* and *Botryococcus braunii*, to study the growth of these promising species in wastewater obtained from the Wawona Frozen Foods, Clovis, CA.

Also, it was observed that increasing the accumulation of oil in the algae species would result in higher productivity and lower algal biodiesel cost. One of

the ways to increase the oil accumulation is to transform the algae with a gene capable of increasing lipid synthesis. Successful nuclear transformation has been done in different species of green algae by using glass beads, Silicon carbide (SiC) whiskers, electroporation and particle bombardment. These transformation protocols require sophisticated instruments, cell wall deficient mutants and some times poses health hazards. Also no protocol has been designed for transformation of *Dunaliella primolecta* and *Botryococcus braunii,* the promising algal species.

Hence, my research focused on studying the growth characteristics of the two species of algae, *Dunaliella primolecta* and *Botryococcus bruaunii*, in fruit wastewater. I also tried to develop a transformation protocol of *Dunaliella primolecta* using *Agrobacterium* mediated transformation.

## 1.1 Purpose of the Study

The overall purpose of the study is to increase the production of oil in the micro- algae for the further conversion into biodiesel. This study also aims in trying to find out ways to optimize the biodiesel production process. The specific objectives of my project is to study the growth rate of two selected strains of algae in wastewater obtained from the Wawona Frozen Foods, Clovis, CA and also develop a protocol to generate transgenic algae. The study is done to find out if the algae have the ability to grow in wastewater at a rate equal to or higher than that in the normal medium that contains all the required nutrients and chemicals for its growth. It is hypothesized that if the algae could grow faster in the wastewater it could be allowed to grow in the wastewater rather than in normal medium. This would decrease the cost of raw material required for the algae to grow, simultaneously utilizing algae to reduce the organic load in the wastewater. Once this study is conducted, we could develop a standard transformation protocol in

the algae that has the higher growth rate in the wastewater. The standard protocol could be further used for the transformation of the desired gene into the algae resulting in production of higher amount of oil. The transformed algae could then be allowed to grow in the wastewater, simultaneously achieving higher growth and higher oil production and wastewater remediation, thereby increasing the oil yield and reduction in the cost of the biodiesel.

# 2. BACKGROUND

## 2.1 Biodiesel

Biodiesels are mono-alkyl esters of fatty acid that are extracted from vegetable oils and animal fats (Li et al., 2008). They have received much attention in recent years due to depletion of fossil fuels and the fact that they are biodegradable, renewable and non-toxic (Lang et al., 2001). Biodiesel also results in reduced gaseous emissions ($CO_2$, nitrogen and sulfur oxides) causing decreased greenhouse gas and global warming effects. Biodiesel has commercially been extracted from the rapeseed in Europe (Lang et al., 2001) and soybean in United States (Chisti, 2007). They have been used as a blend with the fossil fuels like the B20 and B100 (Zhang et al., 2003).

Biodiesel can be produced by four different ways like direct use and blending, microemulsions, thermal cracking and transesterificaton (Ma and Hanna, 1999). Biodiesel produced from triglycerides by trans-esterfication reaction wherein three molecules of fatty acids are esterified with a glycerol molecule is the most common method. This reaction requires the presence of methanol and produces methyl esters of fatty acids; which are otherwise referred to as biodiesel. Industrial scale production of biodiesel requires 6 moles of methanol for every mole of triglyceride esterified, however theoretically 3 moles of methanol are sufficient (Chisti, 2007). The large amount of methanol ensures that the reaction is driven towards biodiesel production. This reaction is catalyzed by either alkali, acid or lipase enzyme. It has been found that the alkali-catalyzed reaction is 4000 times faster than the acid-catalyzed reaction (Chisti, 2007). Hence alkalis such as sodium hydroxide and potassium hydroxide (1% by weight of oil) and alkoxides such as sodium methoxide are commercially used for the production of biodiesel.

The reaction completes in about 90 min at 60 $^{0}$C under atmospheric pressure. The yield is found to be 98% on weight basis and biodiesel is recovered by repeated washing with water. The saponification reaction is prevented to reduce yield loss by using dry oil and alcohol.

The biodiesel industry has grown significantly over the past few years with production increasing from 1.9 million tons in 2004 to nearly 4.9 million tons in 2006 in the European Union (EU). In the United States, biodiesel production has expanded from 2 million gallons in 2000 to 250 million gallons in 2006 (Li et al., 2008). This expansion has also been observed in the developing countries like China, Brazil, Argentina, Indonesia and Malaysia. It is estimated that the biodiesel market would reach 37 billion gallons by 2016 (Li et al., 2008). It has been estimated that 0.53 billion $m^3$ of biodiesel would be required annually to replace all the transport fuel in the United States at the current rate of consumption. The present sources of biodiesel would require a minimum of more than 54% of the existing US cropping area to satisfy the demand (Li et al., 2008). Hence scientists have been searching for an alternative to crop plants as a source for biodiesel. Microalgae appeared to be the best alternative source of biodiesel production. Microalgae would require less than 10% of the cropping area to meet the demands of transport fuel, estimating that microalgae produces just 20% oil (by weight) in biomass (Li et al., 2008). Also, the use of microalgae does not require any compromise on food, fodder and other crop-derived products.

## 2.2 Microalgae

Microalgae are the diverse group of aquatic, photosynthetic, unicellular organism that have gained interest in commercial production of valuable compounds like therapeutic proteins, anticancer drugs, Polyunsaturated fatty acids

(PUFA), astaxanthin, β-carotene, and biofuels. Microalgae have the ability to survive in liquid cultures where they have access to light, carbon dioxide, and other nutrients. Some species survive both in autotrophic condition and heterotrophic condition by degrading substances like sugars. Algal growth would require essential elements like nitrogen, phosphorous, iron and in some cases silicon. Also, they utilize sunlight and carbon dioxide for oil accumulation, which is thought to be the most environmentally friendly way of producing lipids. It has been calculated by Grobbelaar that the approximate molecular formula of microalgae is $CO_{0.48}H_{1.83}N_{0.11}P_{0.01}$ (Grobbelaar, 2000). The practical methods of large-scale production are raceway ponds and tubular bioreactors. Large-scale production would use continuous culture during daylight and during night the feeding would cease.

The *Chlorophycaeae* and *Bacillariophycaeae* are two known classes of algae that have the capacity to accumulate large amount of oil under certain environmental conditions like nitrogen stressed conditions (Gordillo et al., 1998). The algae store oil mainly in the form of triacylglycerols (TAGs) that can be easily converted into biodiesel by trans-esterification reaction. The microalgae taxa that are reported to have the capacity to accumulate large quantities of lipids are *Chlorella* species, *Dunaliella* species, *Neochloris oleoabundans* and *Botryococcus braunii*. *Dunaliella* a halotolerant unicellular green algae lacking a cell wall has the capacity to tolerate extreme stress conditions (Tan et al., 2005). *Dunaliella* have an advantage over the other species in that they have less contamination problems. It has been found out that the algal species *Chlorella vulgaris* can be used as a supplement to rapeseed oil and can be used in an unmodified single cylinder diesel engine (Scragg et al., 2003). It was also observed that the emissions had decreased $NO_x$ and $CO_2$ but increased CO content. *Chlorella protothecoides*

grown under heterotrophic conditions are known to produce higher lipid content (55.20%) than in the cells grown under autotrophic conditions, which was close to 14.57% (Miao andWu, 2006). The biodiesel extracted from *Chlorella protothecoides* was found to comply within the limits established by ASTM (American Society for Testing and Materials) related to biodiesel quality.

## 2.3 Economics of Microalgal Biodiesel Production

Though the microalgal sources for biodiesel appear to be very promising, one of the major factors that hinder its commercial use is economics. The major factors that influence the cost of microalgae oil production are biomass productivity, lipid content and overall lipid productivity. It has been estimated that the cost of producing microalgal biomass is $0.50/kg in a 10,000 t capacity. However, assuming that the algae would contain 30 % of oil by weight, the cost of biomass for providing a liter of oil would be $1.50/L and the cost of the oil recovered from the same would be $2.80/L. This would be expensive when compared to the petrodiesel price, which was $0.49/L in 2006. Hence the price for microalgal oil should be reduced to practically $0.48/L to be a viable substitute for petrodiesel. This could be done either by using a bio-refinery based production strategy, improving capabilities of microalgae through genetic engineering techniques and advances in engineering of photo-bioreactors. It has been recognized by the US Department of Energy's Aquatic Species Program started in 1958 that the genetic engineering techniques apart from the species selection and optimal cultivation would have a great impact on economics of microalgal diesel production. The molecular engineering techniques could be used to increase the photosynthetic efficiency to enable increased biomass, enhance biomass growth rate, increase oil content in biomass and identify possible biochemical factors that

have an impact on the oil accumulation capacity of algae. Also molecular engineering techniques would allow one to have a direct control over the organism's cellular machinery. The use of bio-refinery concept and photo-bioreactor engineering could further be used to reduce the cost of biodiesel (Walker et al., 2005). However, a stable method of transformation needs to be developed to achieve the above said alternatives.

## 2.4 Genetic Engineering of Microalgae

Various DNA transfer techniques could be used to introduce the exogenous DNA into the algal genome. Few of these methods include biolistic bombardment, microinjection, electroporation of cells, agitation of cell wall deficient cells with DNA and glass beads and *Agrobacterium* mediated transformation. Successful nuclear transformation has been done in cell wall deficient mutants by vortexing with glass beads and wild type cells of *Chlamydomonas* by using Silicon carbide (SiC) whiskers (Dunahay, 1992) and electroporation (Shimogawara et al., 1998). While the glass bead method requires cell wall deficient organisms, SiC whiskers are difficult to purchase and can impose health hazard (León-Bañares et al., 2004). Particle bombardment method was first developed in plants and involves coating of DNA onto gold or tungsten particles and accelerating into target cells by helium driven gene gun. Highly successful nuclear transformation in diatoms like *Phaeodactylum tricornutum* (Apt et al., 1996), *Cyclotella cryptica* (Dunahay et al., 1995) and transient expression of lacZ gene in *Haemotococcus pluvialis* has been reported using this method (Teng et al., 2002). The particle bombardment method results in transformation with lower efficiency and also regeneration of the algae after exposure to pressure becomes tedious. Hence, *Agrobacterium mediated* transformation appears to be the next best alternative. Various transformation

protocols that include electroporation (Tan et al., 2005) glass bead method have been tried in *Dunaliella salina,* however no transformation protocols have been tried in *Dunaliella primolecta.* Also, there has been no work in *Agrobacterium* mediated transformation of any of the algae species that belong to the genus *Dunaliella.* Once the transformation has been done it is also necessary to regenerate the transgenic cell line from a single cell. Hence a method has to be devised for that. MMiCellEctor could be used for the same purpose. MMiCellEctor is an automatic device developed by MMI to isolate single rare cells from tiny sample sources for further genotypic and phenotypic characterization. The isolation of the single steps takes place in three steps; Cell recognition, cell acquisition and cell deposition. The inverted microscope from Olympus 1X81 allows in cell recognition. The automated microcapillary controlled by a high precision pump helps in the acquisition and deposition of the cells. This method could be used to isolate single cells so that a single transgenic line can be obtained.

## 2.5 Agrobacterium Mediated Transformation

*Agrobacterium tumefaciens* is a gram-negative pathogenic soil bacteria that causes crown gall disease in various monocotyledons plants by transferring its T-DNA from the Ti plasmid to the genome of the plant (Kumar et al., 2004). The low molecular weight phenolic compounds like acetosyringone (Gelvin, 2000) released from the wounded sites of the plant initiates the gene transfer. This procedure is relatively easy and one can transfer large segments of DNA of more than 150 kb with less rearrangement (Hamilton et al., 1996). One of the other main advantages of using *Agrobacterium* to transfer the required gene is that T-DNA is preferentially passed onto the plant genome that has an active transcription

activity. This method of transformation has been used in various dicotyledons plants, monocotyledons, fungi, HeLa cells (Talya et al., 2001) and some algae species like *Chlamydomonas reinhardtii* (Kumar et al., 2004)*, Haemotococcus pluvialis* (Kathiresan et al., 2009). Also *Agrobacterium* method of transformation requires just wild type cells whereas the other methods like glass bead and electroporation may require mutant for the gene transfer.

## 2.6 Wastewater Treatment by Microalgae

Another important environmental problem that needs to be handled efficiently is the large amounts of wastewater released from the various industries. For example, it is estimated by the Fresno, California officials that the amount of waste water released from the Wawona fruit processing industry will far exceed the handling capacity of the waste water treatment plant (80 million gallons per day) within 2010. Hence, the major solution is to find the alternative source for reducing the oxygen demand in wastewater. Algae are known to reduce the organic, nitrogen and phosphorous content of waste water (Sawayama et al., 1994). The wastewater treatment by algae are found to possess several advantages; 1) they rests on the principles of natural ecosystems and hence are environmentally friendly, 2) causes no secondary pollution as long as the biomass produced is reused and 3) allow efficient recycling of nutrients. The algae biomass could be used to produce a variety of secondary metabolites of economic importance, like fatty acids, pigments, stable isotopes and biofuels (Radmer andParker, 1994). The growth of *Botryococcus braunii* in secondarily treated sewage (STS) in a continuous bioreactor (Sawayama et al., 1994) and piggery waste water (An et al., 2003) resulted in the removal of nitrogen and phosphorous from the waste water, simultaneously producing hydrocarbon. *Chlorella vulgaris*

was also found to be growing well in the piggery waste water thereby removing ammonia (Yeoung-Sang Yun, 1997). *Chlorella vulgaris* and *Scenedesmus dimorphus* have found to remove 55% phosphorous from the agro-industrial wastewater after 216 hr. The removal efficiency of ammonia was however higher in *S. dimorphus* when compared to *Chlorella vulgaris* (González et al., 1997). The untreated wastewater has an increased level of organic compounds and decreased levels of dissolved oxygen, and toxic substances, which degrade the several aquatic ecosystems. Due to the inadequate supply of oxygen the control of high BOD/COD level becomes a major priority. Microalgae break down the organic compounds present in the wastewater and releases oxygen, thereby increasing the oxygen level. It was reported that algae releases oxygen at a rate of 0.48–1.85 kg $O_2$ $m^{-3}$ $d^{-1}$ in pilot-scale ponds or lab-scale tank photo bioreactors used in treating municipal or artificially contaminated wastewater (Muñoz andGuieysse, 2006). A mixed culture of algae comprised of *Chlorella, Chlamydomonas, Eudorina, Pandorina, Euglena, Nitzschia, Cyclotella* was found to remove 58% of COD level from the effluent obtained from paper industry after a period of 42 days (Tarlan et al., 2002).

The overarching goal of Dr Calderon's laboratory is to have increased production of bio-diesel from algae at a lower cost. To achieve this goal, we first need to select the strains of algae that have higher growth rate and also have increased oil production capacity. The algae strain possessing both the above said characteristics is difficult to obtain. Hence we argued that we must be able to select the strains of algae that have increased growth rate and further use genetic manipulation to increase the oil content. We must also be able to select a medium that does not result in greater cost. Hence we came up with specific objectives to the project.

## 3. SPECIFIC OBJECTIVES OF MY RESEARCH

1) To identify optimum conditions required for the growth of algae, *Dunaliella primolecta* and *Botryococcus braunii* in fruit industry wastewater by changing the growth parameters such as concentration of wastewater and estimating the growth rate of these species of algae.

2) To compare the amount of oil produced during the different growth stage of algae when grown in optimum conditions in wastewater.

3) To find the amount of COD removed from the wastewater by *Dunaliella primolecta* when grown in wastewater at the end of its log phase.

4) To develop protocols to generate the stable transformants of *Dunaliella promolecta* using *Agrobacterium* mediated transformation.

# 3. METHODOLOGY

## 3.1 Algae Strain and Wastewater

The *Dunaliella primolecta* (UTEX LB1000) and *Botryococcus braunii* (UTEX 572) strain were bought from the Culture Collection of Algae at the University of Texas at Austin (UTEX), USA. These strains were cultured in the medium conditions specified by the UTEX and were subsequently cryo-preserved in liquid nitrogen for long-term storage (as given in the section 3.9). The wastewater was obtained from a local fruit company, Wawona Frozen Foods, Clovis, CA.

## 3.2 Erdschreiber's Media and Modified Bold 3 M Media

The Erdschreiber's Media and Modified Bold 3 M media was prepared as specified by UTEX. The recipe for Erdschreiber's media and Modified Bold 3 M media is given in the appendix. The media was prepared as given in the appendix, stored at 4 $^{o}$C and was brought to room temperature a day before its use.

## 3.3 MS Supplemented with Salts (MSS) Media

To prepare MSS media; 4.3 g of MS Basal Salt Mixture (Phytotechnology lab, Cat # M519) and 20 g of Instant Ocean (Aquarium Systems Inc.) were mixed with 800 mL of double-distilled water using a magnetic stirrer. Once the solids were completely dissolved; the volume was brought to 1 L. The media was autoclaved at 121 $^{o}$C for 15 min. The media was then cooled and 2 mL of ampicillin (100 mg/mL) (Amresco Cat # 0339-25) and 1 mL of gentamycin (100 mg/mL) (ICN, Cat # 19007) were added under sterile conditions, inside a vertical laminar-flow biological cabinet (Labconco; Model # 3621Bio43726). The media was mixed well and stored at 4 $^{o}$C.

### 3.4 MSS Agar Medium and MSS Agar Medium Plates

To prepare MSS Agar media; 4.3 g of MS Basal Salt Mixture (Phytotechnology lab, Cat #M519), 20 g of Instant Ocean (Aquarium Systems Inc.) and 10 g of Agargel (Sigma, Cat #A3301) was added to 800 mL of distilled water and mixed well using a magnetic bar. The volume was later made to 1 L and autoclaved as previously described. The autoclaved MS agar medium was cooled down to around 50 $^{o}$C and 2 mL of ampicillin (100 mg/mL) (Amersco Cat # 0339-25) and 1 mL of gentamycin (100 mg/mL) (ICN, Cat # 19007) were added to it. The media was mixed well and approximately 20-25 mL of medium was poured into each 100 x 15 mm Petri dish (Fisher Scientific, Cat # 08-757-13). The medium was allowed to solidify for at least 15-20 min. The plates were repacked into an original Petri dish plastic bag (which was kept sterile) and the bag was sealed well. All these steps were performed under sterile conditions. The bag was stored at 4 $^{o}$C until the plates were used.

### 3.5 Hygromycin Stock Solution (50 mg/mL)

Under sterile conditions, 5 mL of sterile double distilled water was added into a 250 mg Hygromycin-B vial (MP Biomedicals Inc; Cat # 194170) and mixed well. The lid was closed tightly and the solution was transferred to 4 $^{o}$C.

### 3.6 Cefotaxime Stock Solution (500 mg/mL)

Under sterile conditions, 2 mL of sterile double distilled water was added into 1 g of Cefotaxime (Fisher Scientific; Cat # BP2951-1) and mixed well. The lid was closed tightly and the solution was stored at 4 $^{o}$C.

## 3.7 MSS Agar with Hygromycin and Cefotaxime Plates

The autoclaved MSS agar media was cooled down to around 50 $^{o}$C , and 1 mL of hygromycin stock solution and 1 mL of cefotaxime stock solution was added to 1 L of MS medium. The medium was mixed thoroughly, and approximately 20-25 mL of medium was poured into each 100 x 15 mm Petri dish (Fisher Cat # 08-757-13). The medium was allowed to solidify for at least 15-20 min. The plates were repacked into an original Petri dish plastic bag (which was kept sterile) and the bag was sealed well. These steps were performed under sterile conditions and the bag stored at 4 $^{o}$C.

## 3.8 Algae Maintenance and Growth

*D. primolecta* was maintained in Erbdschreiber's media as specified by UTEX. However for studying growth characteristics, *D. primolecta* was allowed to grow in MSS media. This media was chosen for propagation as it was earlier shown to maximize growth and provide axenic conditions (Santin-Montany· et al., 2007). *Botryococcus braunii* was allowed to grow in Modified Bold 3N medium. The liquid algal cultures were allowed to grow in a conical flask covered with sterile aluminum foils at 26 $^{o}$C under a 12:12 h light:dark cycle and a light intensity of 40 $\mu Em^{-2}s^{-1}$with continuous agitation.

## 3.9 Long-Term Storage

Both the species of algae were cryo-preserved for later use. The algae were allowed to grow in the respective maintenance media under optimum growth conditions until they reached a concentration of $10^6$ cells/mL (exponential growth phase). The cryovial was pre-chilled by placing it at 4 $^{o}$C at least a day before it is used for cryopreservation. 0.3 mL of 20 % methanol (Fisher, Cat #A411-4) was added to 0.9 mL of exponential phase grown culture and was mixed well in the

pre-chilled cryovial under subdued light condition; provided by wrapping the cryovial using aluminum foil. The cryovials were kept at -80 $^{o}$C for 3 h and were further transferred to liquid nitrogen canister for long term storage.

To revive cryopreserved algal cultures; 40-50 mL of water was pre-warmed to 37 $^{o}$C. The cryovial was removed from liquid nitrogen canister and was gently inserted into the water bath. It was agitated gently until the ice melted and centrifuged at 500 rpm for a minute to pellet the algal cells. The liquid was gently decanted and the cryovial was filled with fresh culture medium and left undisturbed for 2-3 min. The centrifugation was repeated and the liquid was removed as before. Fresh culture medium (1 mL) was added and the cells were allowed to suspend. This was further transferred to a larger volume (10 mL) and cultured under normal culturing conditions.

## 3.10 Wastewater Pretreatment

The wastewater obtained from the Wawona Frozen Foods, Clovis, CA was filtered through a Whatman filter paper (Fisher, Cat # 05-714-4) once to remove any solid debris present in them. The filtered water was sterilized by autoclaving at 121 $^{o}$C for 15 min. The sterilized wastewater was further diluted in the respective media. To keep the chemical composition of the control media unchanged; we used the diluted wastewater to make the experimental media conditions. That is, to make 25% diluted wastewater in the media; we added all the chemicals required to make the MSS media first and then made up the solution to one liter by adding 25% diluted wastewater instead of plain water that is used for the control. We prepared 50% and 75% diluted wastewater in a similar manner. Table 1 shows the characteristics of wastewater as analyzed by BSK Analytical Laboratories, Fresno, CA.

**Table 1.**
Analysis of wastewater from Wawona Frozen Foods, Clovis, CA

| Analyte | Results |
|---|---|
| Biochemical Oxygen Demand (BOD) | 6700 mg/L |
| Calcium | 40 mg/L |
| Chromium (Hexavalent) | ND |
| Cobalt | ND |
| Conductivity – Specific (EC) | 1500 mho/cm |
| Copper | ND |
| Iron | 3.4 mg/L |
| Magnesium | 26 mg/L |
| Nickel | ND |
| Nitrate | 5.1 mg/L |
| Nitrogen | 120 mg/L |
| Phosphorous – Total | 21 mg/L |
| Potassium | 220 mg/L |
| Sodium | 260 mg/L |
| Total Suspended Solids (TSS) | 720 mg/L |
| Zinc | 0.30 mg/L |

### 3.11 Growth Studies

Different concentrations of wastewater (0%, 25%, 50% and 75%) were prepared using the method already described and the algae were allowed to grow in the conditions specified earlier. 100% v/v of wastewater was not used in the experiment as it was found to allow a lot of contamination due to heavy organic load. Also, 100% wastewater inhibited the growth of algae to a larger extent. The growth was monitored every 3 days for a period of 1 month by measuring the dry weight and cell count. Cell count was performed using Neubauer hemocytometer (Spencer Brightline, US Patent #2660.091). Three replicates were set up for each experiment and control.

### 3.12 Oil Measurement

Comparison of lipid content in the two species cells grown in 25% (v/v) of waste water was done by using the Oil Red O stain (Ramírez-Zacarías et al.,

1992). Oil Red O stain has been shown to selectively stain the triglycerides and other neutral lipids present in the cells. Working solution was made by dissolving 4.2 g of Oil Red O stain in 1200 mL absolute iso-propanol (Fisher, Cat #A419-1) and left overnight at room temperature. Solution was filtered through Whatman filter paper (Fisher Cat # 05-714-4) to remove any solid un-dissolved dye present and 900 mL of distilled water was added. The solution was left overnight at 4 $^{o}$C, filtered twice and stored at room temperature. Equal ratios of the working solution of Oil Red O stain and algal suspension were mixed and allowed for staining to take place for a period of 2 h. Color intensity of algal cells was measured by reading the absorbance at 510 nm in a spectrophotometer as Oil Red O stain bound to lipid bodies shows maximum absorbance peak at 510 nm.

### 3.13 Chemical Oxygen Demand (COD) Estimation

The wastewater samples were removed for COD estimation before and after the incubation of *D. primolecta* in different percentages of wastewater. The COD estimation was done in the samples using the CHEMetrics COD Vials Kit (Chemitrics; Cat # 10882). The samples were homogenized for 2 min in a blender and 2 mL of the samples were pipetted into a COD vial. The solutions were mixed well and were placed in a pre-heated digester block for 2 h at 150 $^{o}$C. The samples were allowed to cool and the absorbances were measured at 620 nm. The COD values were obtained from the standard graph.

### 3.14 Plasmid Construct and *Agrobacterium* Strain

The binary vector pCAMBIA1301 (Cambia, GenBank: AF234297.1), harboring a bacterial *hptII* gene driven by CaMV35S promoter and a *uidA* (gus) reporter gene under the control of CaMV35S (Figure 1), was mobilized into

*Agrobacterium tumefaciens* (strain C58C1). This strain was used for transformation in the further experiments.

### 3.15 Growth of *A. tumefaciens*

*A. tumefaciens* (strain C58C1) containing pCAMBIA 1301 vector was allowed to grow in 20 mL of sterilized LB broth until they reached an OD of A600 = 0.5 (usually overnight). The *A. tumefaciens* strain was allowed to grow in 100 mL conical flasks in a continuous shaker rotating at 227 rpm at 25 $^{o}$C under dark conditions.

### 3.16 Algae Transformation

Transformation was performed by co-cultivating *A. tumefaciens* containing the pCAMBIA1301 vector with *D. primolecta*. The co-cultivation was performed as follows. *D. primolecta* cells were allowed to grow in the MSS media until they reached a concentration of $10^6$ cells/mL. Around 10 mL of the culture solution was centrifuged at 3000 rpm for 3 min to separate out the algal cells and was plated in MSS agar medium. It was allowed to grow for a period of 2-3 days until a lawn of cells was observed. An overnight grown culture of *A. tumefaciens* in LB broth was pelleted and re-suspended in 1 mL liquid MSS media. 200 μL of the re-suspended cells was spread using a glass spreader onto the previously grown lawn of *D. primolecta* cells. It was further incubated for 48 h at 26 $^{o}$C under light intensity of 40 $\mu Em^{-2}s^{-1}$ and 12: 12 h light:dark cycle for transformation to take place. The incubated cells were later harvested by washing the plates with MSS media containing 500 mg/L cefotaxime (Agri Bio, Cat # 2000) and collecting the cells by centrifugation at 1000 rpm for 2 min. The cefotaxime treatment killed the *A. tumefaciens* cells. The cells were washed twice using centrifugation at 1000 rpm for 2 min. The pelleted cells were again spread on a MSS agar plate containing

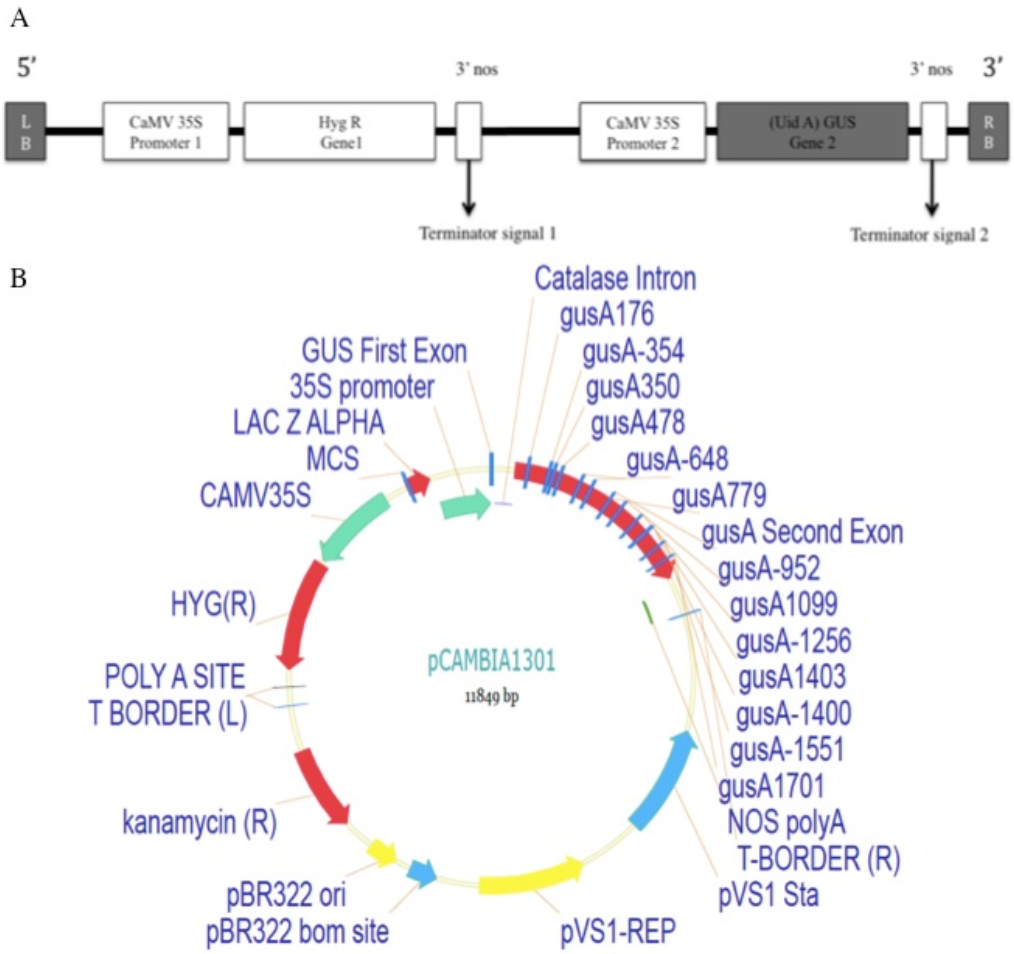



Figure 1. Linear map circular map of the T-DNA region of plasmid pCAMBIA 1301. (A) Linear map of the T-DNA region of the plasmid having the selectable marker gene *hpt (*hygromycin phosphotransferase), and GUS (-glucuronidase) as the reporter gene both driven by CaMV35S promoter. (B) Circular map of the plasmid pCAMBIA 1301 having the T-DNA region and a kanamycin gene, a bacterial selection marker.

50 mg/L of hygromycin and 500 mg/L of cefotaxime. Transformed colonies appeared in 10 days and individual colonies were allowed to propagate in a liquid culture for further molecular analysis. The transformed cells were further taken for further confirmatory analysis.

### 3.17 Detection of Reporter Gene

The transformed colonies obtained on the plates after co-cultivation were used for GUS histochemical staining. The cells were dissolved in the GUS stain (Gold Biotech, Cat # G128K2), incubated at 37 $^0$C for a period of 5 h and analyzed by microscopy. The presence of blue color in the cells confirmed the transformation event.

### 3.18 Analysis of -DNA Integration and Transgene Expression

Genomic DNA was isolated according to the manufacturer's instructions using the Promega genomic DNA extraction kit (Promega, Cat #A1125). Briefly, 10 mL of the algal cells that had grown to a density of around $10^6$ cells/mL was taken and centrifuged at 5000 rpm for 10 min. 600 µL of nuclei lysis solution was added and incubated at 65 $^0$C for 15 min. 200 µL of protein precipitation solution was added and vortex high speed for 20 sec. The solution was further centrifuged at 1600 g for 3 min and supernatant was transferred into a clean eppendorf tube. 600 µL of room temperature isopropanol was added and centrifuged at 16000 g for 3 min. The supernatant was removed and 600 µL of 70% ethanol was added. This was centrifuged again at 16000 rpm for 3 min and supernatant was removed. The tube was air-dried and 20 µL of nuclease free water was added and incubated at 65 $^o$C for 15 min. The genomic DNA was stored at -20 $^o$C.

The isolated genomic DNA was further analyzed by Polymerase Chain Reaction (PCR) using two sets of primers: one specific to the CaMV35S promoter and the other specific to GUS gene. The sequence of the PCR primers used is as follows:

Primer 1: 5'- CCAGCTATCTGTCAC-3'

Primer 2: 5'-CAACCACGTCTTCAAAGCAA-3'.

Primer 3: 5'-AACAGGTATGGAATTTCGCCGATTT-3'

Primer 4: 5'-TTTATCCTAGTTTGCGCGCTATATTT-3'

Primer 1 and 2 were specific to CaMV35S and primers 3 and 4 were specific to GUS gene. PCR reactions (BioRad, Model # PTC1148) were set up in 25 μL reaction volume as follows: 1μL of DNA template (approximate concentration of 5 μg/μL), 0.5 μL of each of the primers (approximate concentration of 25 mM), 1 μL of Taq Polymerase (Ambion, Cat # 2054), 1 μL of 20 μM $MgCl_2$ (Sigma, Cat # M1028), 0.5 μL of dNTP solution (5 mM of each dNTP) and 1 μL of 5X Green GoTaq PCR buffer (Promega, Cat # M7911) was used. The conditions used in the PCR reaction (for Primer 1 and Primer 2 set) was initial denaturation at 95 $^{o}$C for 3 min, denaturation at 95 $^{o}$C for 1 min, primer annealing at 42 $^{o}$C for 1 min, extension at 72 $^{o}$C for 1 min, cycle repetitions 30 times, final extension at 72 $^{o}$C for 1 min and 4 $^{o}$C forever. The PCR products were run on a 1% agarose gel and the bands were visualized. For the primer set 3 and 4; the PCR conditions were 95 $^{o}$C for 5 min for initial melting followed by 35 cycles of amplification with each cycle consisting of the following steps: 95 $^{o}$C for 1 min, 52 $^{o}$C for 1½ min and 72 $^{o}$C for 2½ min with a final extension at 72 $^{o}$C for 15 min. In each reaction, one positive control (pCAMBIA 1301 plasmid), a negative control (No DNA template), three lines of transformed cells and untransformed cells were used.

## 3.19 DNA Electrophoresis

### 3.19.1 0.5M EDTA (pH 8.0)

Disodium ethylenediaminetetraacetate·$2H_2O$ (Fisher; Cat # BP120-1) (186.1 g) was mixed with 800 mL of distilled water in a black-capped 1 L bottle. The mixture was stirred vigorously with a magnetic stirrer. The pH was adjusted to 8.0 with approximately 20 g of NaOH pellets (Fisher; Cat # S318-1), and then the volume was brought up to 1000 mL with distilled water. The bottle was loosely closed, the lid was covered with aluminum foil, and the solution was autoclaved.

### 3.19.2 10X TBE Electrophoresis Buffer

162 g of Tris Crystallized Free Base-Molecular Biology Grade (Fisher; Cat # BP152-500), 82.5 g of Boric Acid (Fisher; Cat #A74-3), and approximately 1,000 mL distilled water were transferred to a 2.5 L orange-capped bottle. The solid reagents were completely dissolved with a magnetic stirrer. 6 mL of 0.5M EDTA (pH 8.0) was added into the mixture and the total volume was adjusted to 1,500 mL. The cap was loosely placed and the lid was covered with aluminum foil. The labeled bottle was autoclaved.

### 3.19.3 1X TBE Electrophoresis Buffer

This solution was used as the DNA agarose gel running buffer. 200 µL of 10X TBE, 1,800 mL of distilled water, and 20 µL of a stock ethidium bromide solution (10mg/mL) were transferred to a 2 L plastic bottle. The solution was mixed thoroughly.

3.19.4 10X Loading Dye

25 µL of Glycerol (Fisher; Cat # BP299-1), 1mL of 20 mM EDTA, 0.5 mL of 10mM Tris-HCl (pH 7.4: Amresco Inc.; Cat # 0234-1), 0.1 g of bromophenol-blue (Sigma; Cat # B5525), and 0.1 g of xylene cyanide (ICN Biomedical Inc. Cat # 806801) were mixed with distilled water, for a final volume of 50 mL, in a 50 mL centrifuge tube (Corning; Cat # 430290). The mixture was stored at room temperature.

3.19.5 1% Agarose DNA Gel

One gram of Agarose I (Pierce Prod#17852) was added to 100 mL of 1X TBE for a 1% Agarose gel. The gel was prepared in a 250 mL orange-capped bottle. The mixture was heated in a microwave oven first for 1 min, then for additional 30 sec periods until the agarose was completely melted. The agarose solution was cooled down and poured into a casting gel try (Bio-Rad; Cat # 170-4486 or 170-4469) with appropriate combs. The combs were removed after the agarose solidified. The wells were washed and the samples were loaded. The gel was allowed to run at 80 V until the dye front reached ¾th of the gel.

## 3.20 Confirmation of GUS Gene Using Southern Blotting

Southern blotting was used to confirm the presence of GUS gene in the transformed cells.

### 3.20.1 Restriction Digestion of DNA Samples

The DNA isolated from the transformed cell lines were single restricted digested using the enzymes *EcoR*I (Promega, Cat # R601A) and *Hind*III (Promega, Cat # R604A). The restriction digestion setup were done in 20 µL reaction volume consisting of 5 µL of DNA (0.5 µg/µL DNA), 1 µL of respective

buffers (Buffer H (Promega, Cat # R008A) for *EcoR*I and Buffer E (Promega, Cat # R005A) for *Hind*III), 1 μL of either *EcoR*I or *Hind*III and 13 μL of water. The reaction mixture was incubated at 37 $^{o}$C for 12 h. The restricted digested samples were run on a 1% agarose gel at 50 V for 1½ hour. The gel was further used for transfer onto the nylon membrane.

3.20.2 Preparation of 20 X SSC buffer

To prepare 20 X SSC, 262.95 g of NaCl (Fisher, Cat # S271-1) and 132.3 g of Sodium Citrate (Fisher, Cat # BP 327-1) was made upto 1.5 L using water and pH was adjusted to 7 using HCl. The solution was poured into a 2 L orange capped bottle and lid was covered with aluminum foil. It was further autoclaved and stored at room temperature.

3.20.3 Nylon Membrane Equilibration

The nylon membrane (Osmonics Inc., Cat # NB0HY00010) was first cut in the same dimensions as the agarose gel. The membrane was slightly placed over the top of the Milli-Q-Water in a shallow tray and allowed to get wet on just one side. Once one of the sides of the membrane was wet, the tray was gently shaken so as to have the membrane completely get immersed in the water. The membrane was transferred to another tray that contained the 20 X SSC buffer. The membrane was allowed to equilibrate for 5 min in the buffer.

3.20.4 Processing the Gel

The gel was placed on the UV trans-illuminator (Fisher, Model # 614A) and sharp holes were made using a needle corresponding to the bands obtained on the 1 kb DNA ladder. The holes were filled with ink by rubbing ink against the gel. The gel was now carefully transferred to a depurination solution consisting of

0.25 N HCl (Fisher, Cat # A114-212) to nick the DNA and incubated for 15 min and then rinsed using Milli-Q-water. The gel was further allowed to incubate in the denaturing solution consisting of 0.5 M NaOH (Fisher, Cat # S318-1) and 1.5 M NaCl (Fisher, Cat # S271-1) for 30 min to denature the DNA and further rinsed with Milli-Q-Water. The gel was finally incubated in the neutralization solution consisting of 1 M Tris-CL pH 8, 1.5 M NaCl for 30 min and then used for blotting.

3.20.4 Capillary Blot Assembly

A tray was filled with 1000 mL of 20 x SSC and a glass plate was suspended across the sides of the tray. Two sheets of Whatman 3MM filter paper (Fisher, Cat # 05-714-4) was wet in 20 x SSC and was laid across the support with the ends soaking in the 20 x SSC in the tray. The gel was placed on the filter paper wicks carefully and the equilibrated membrane was placed on the top of the gel. A strip of parafilm (Pechiney, Cat # PM 999) was placed along each edge of the gel. Three sheets of Whatmann 3MM filter paper cut to the size of the membrane was wet in 20 x SSC and placed on the top of the membrane. The trapped bubbles were rolled out completely from the gel, membrane and filter paper layers with a pipette. A 10 – 20 cm high stack of the absorbent material was placed on top of the filter paper and a glass plate was placed on top of the stack. Then a 250 – 300 g weight was placed on top of the plate to evenly distribute the downward force. The DNA transfer from the gel to the membrane was allowed to take place for 12 h and the filter paper and absorbent material were slowly removed. The blot was lifted slowly from the gel with a pair of sterile forceps and rinsed in 6 x SSC to remove any loose particles of agarose. The blot was finally allowed to dry and further fixed using UV light. For fixation, the blot was placed on a clean filter paper with DNA bound side facing upward and a hand held UV light source (UltraViolet

Products Inc., Model # UVS-54) was placed on the blot at a distance of 2 cm and exposed for a minute. The blot was carefully taken out and stored at room temperature until use.

3.20.5 DNA Probe Preparation

3.20.5.1 Amplification of GUS gene. A series of PCR using GUS specific primers were performed to obtain a high concentration of GUS gene. The first PCR reaction was set up in 25 μL reaction volume as follows: 1μL of pCAMBIA 1301 plasmid DNA template (approximate concentration of 5 μg/μL), 0.5 μL of each of the primers 3 and 4 (approximate concentration of 20 μM), 1 μL of Taq Polymerase, 1 μL of 25 mM $MgCl_2$ (Sigma, Cat # M1028), 0.5 μL of dNTP solution (5 mM of each dNTP) and 1 μL of 5X Green GoTaq PCR buffer (Promega, Cat # M7911) was used. The PCR conditions were 95 $^oC$ for 5 min for initial melting followed by 35 cycles of amplification with each cycle consisting of the following steps: 95 $^oC$ for 1 min, 52 $^oC$ for 1½ min and 72 $^oC$ for 2½ min with a final extension at 72 $^oC$ for 15 min. The second PCR followed the first and was set up in a 100 μL reaction volume as follows: 1 μL of PCR product from the first PCR, 2 μL of each of the primers 3 and 4 (approximate concentration of 20 μM), 1 μL of Taq Polymerase, 10 μL of 25 mM $MgCl_2$ (Sigma, Cat # M1028), 2 μL of dNTP solution (5 mM of each dNTP) and 10 μL of 5X Green GoTaq PCR buffer (Promega, Cat # M7911) was used. The same reaction conditions were used as the previous one. The products of both the PCR reactions (450 bp) were checked using a 1% agarose gel. The two PCR reaction products were mixed and Qiaquick gel extraction was performed for DNA cleanup from the PCR mix.

3.20.5.2 DNA cleanup using Qiaquick gel extraction. The DNA cleanup was done using Qiaquick Gel Extraction kit (Qiagen, Cat # 28-704). The PCR reaction products were made up to 500 µL using DNase RNase free PCR grade water (ICN, Cat # 821-739). To this, 300 µL of Buffer QG and 100 µL of isopropanol were added. The QIAquick spin column was placed in a 2 ml collection tube and the sample was applied to the column. It was centrifuged at high speed for 1 min. The flow through was discarded and the column was placed in the same collection tube. 500 µL of Buffer QG was added to the column and centrifuged again for 1 min. 750 µL of Buffer PE was added for washing and centrifuged for 1 min The flow through was discarded and column was centrifuged again for a min at 13,000 rpm. The column was now placed in a clean 1.5 mL microcentrifuge tube and 30 µL of elution buffer was added to the centre of the QIAquick membrane and the column was allowed to stand for a min. The set up was then centrifuged for 1 min and analyzed using 1% agarose gel. The concentration of the purified DNA was found out using Biophotometer (Eppendorf, Model # 6131-24877) and was diluted so that the concentration was 10 ng/µL.

3.20.5.3 Preparation of Labeled Probe. The preparation of labeled probe and detection were done using Gene Images™ AlkPhos Direct™ labeling (GE Healthcare, Cat # RPN 3680) and detection system (GE Healthcare, Cat # RPN 3682). 20 µL of cross-linker solution was mixed with 80 µL of the water supplied to give the working concentration. 10 µL of the diluted DNA (10 ng/µL) was placed in a microcentrifuge and denatured by heating for 5 min in a vigorously boiling water bath. The DNA was immediately allowed to cool on ice for 5 min and the contents were gently spun to collect at the bottom of the tube. 10 µL of the

reaction buffer was added to the cooled DNA and mixed thoroughly. 2 μL of labeling reagent was added and mixed thoroughly. After this, 10 μL of the cross-linker working solution was added and mixed thoroughly. The microcentrifuge was spun briefly to collect the contents at the bottom of the tube. The reaction was incubated for 30 min at 37 $^{o}$C. The probe was used immediately, kept on ice or stored in 50% (v/v) glycerol for long-term storage.

### 3.20.6 Hybridization

The required volume of AlkPhos Direct hybridization buffer was heated to 55 $^{o}$C and the prepared blots were placed in the buffer for pre-hybridization for 15 min at 55 $^{o}$C in a hybridization oven (Fisher, Model # FBHI10). The prepared labeled probe was added to the buffer used for pre-hybridization step (at a concentration of 10 ng/mL) and hybridized at 55 $^{o}$C overnight in a hybridization oven.

### 3.20.7 Post Hybridization Stringency Washes

The primary wash buffer was heated to 55 $^{o}$C. The blot was carefully transferred to this solution and washed for 10 min at 55 $^{o}$C with gentle agitation. A further wash was performed using primary wash buffer at 55 $^{o}$C for 10 min. The blots were placed in a clean container and the secondary wash buffer was added. The blots were placed in this container and washed for 5 min at room temperature. A second wash was performed using secondary wash buffer for 5 min.

### 3.20.8 Signal Generation and Detection

The excess secondary wash buffer was drained and the blot was placed with the sample side up on a clean, non-absorbent, flat surface. It was made sure that

the blots did not dry out. The detection reagent was pipetted on the blots and left for 2-5 min. The excess detection reagent was drained off touching the corner of the blot onto the non-absorbent surface. The blot was wrapped in a SaranWrap and was placed in the film cassette (Fisher, Model # FBXC 810) with DNA side up. A sheet of autoradiography (Kodak, Cat # 165-1496) was placed on top of the blot under dark conditions and the cassette was closed. It was exposed overnight at room temperature. The film was further removed and developed. The X-ray film was checked for GUS specific bands.

### 3.21 Alternative Isolation of Single Cells for Obtaining Single Transgenic Lines

Single transgenic cells were also isolated using MMI Cellector (Molecular Machines and Industries, MI). The cells were collected using a 20 μm capillary in the scrape position controlled by a high precision pump and were deposited on a 4-cell chamber slide. They were allowed to grow in a 4-cell chamber, until a reasonable growth was obtained. These cells were further cultivated in the growth media and were frozen in liquid nitrogen.

### 3.22 Statistical Analysis

The experiments were repeated three times in this study. The mean from the three experiments were calculated. The error bars were drawn in the graph based on the calculation from the standard deviation.

# 4. RESULTS

The two species of green algae, *Dunaliella primolecta* and *Botryococcus braunii,* were obtained from UTEX The Culture Collection of Algae, University of Texas at Austin. The species of algae were propagated and cryopreserved for further use. The wastewater was obtained from the Wawona Frozen Foods, Clovis, CA and was diluted in the respective media so as to obtain desired concentration (25%, 50% and 75% dilution). These species of algae were allowed to grow in the diluted wastewater and the growth characteristics were studied for a period of one month. The oil production rate was also studied for both the species of algae when grown in the concentration that supports the maximum growth. The algae with higher growth rate were chosen as the model for transformation experiments as well as COD reduction rate studies. A transformation protocol was developed and was confirmed by morphological and genetic analysis.

## 4.1. Growth Characteristics of *Dunaliella primolecta* and *Botryococcus braunii* in Fruit Industry Wastewater

*Dunaliella primolecta* and *Botryococcus braunii* were grown in the wastewater that was diluted at different concentrations (25%, 50% and 75%) as mentioned in the materials and methods section. The cell weight and the cell number were found till the algae reached a static growth phase.

### 4.1.1. Growth rate study of Dunaliella primolecta

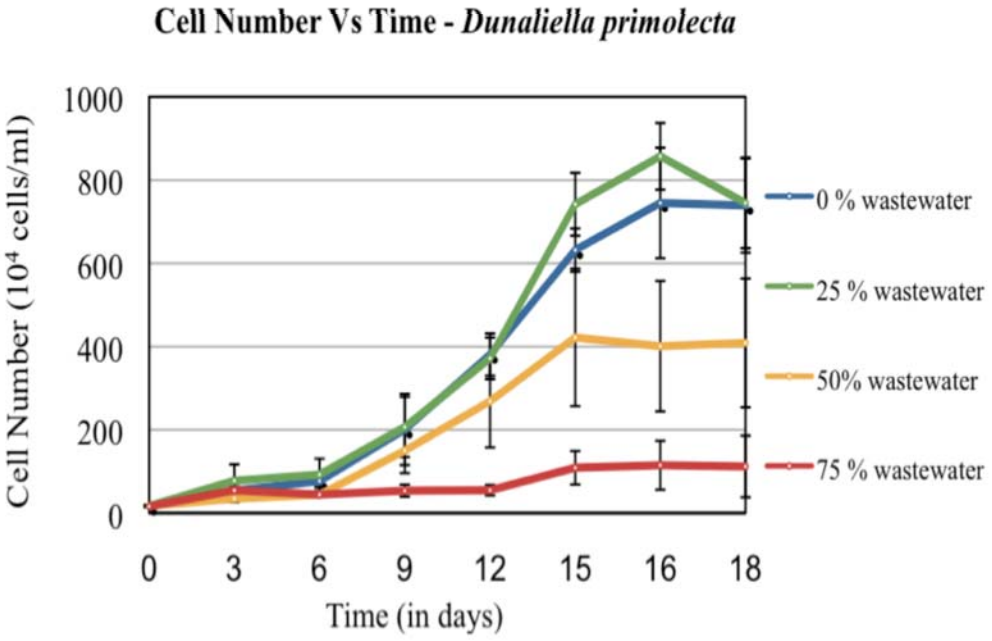
*Dunaliella primolecta* grew faster in wastewater that was 25 % (v/v) diluted using the medium. This could be easily seen from the cell number and the cell weight studies. This species of algae reached a log phase in 6 days and stationary phase in 15 days (Figure 2). The cell reached a maximum concentration

in 18 days. The concentration of cells at the end of 18 days was found to be around $8x10^6$ cells/mL at 25% (v/v) diluted wastewater and in control it was found to be $7x10^6$ cells/mL. The weight of the cells at the end of 18 days period was 1.4 g/L for 25% (v/v) diluted wastewater and 1.2 g/L for control. The cells were however found to be growing very slowly in 75% (v/v) diluted wastewater. The growth rate of *Dunaliella primolecta* in different percentages of wastewater is given in the Table 2. As seen, it is clear that the 25% wastewater supports the growth slightly better than the control, however the 50% and 75% diluted wastewater has a slower growth rate.

### 4.1.2 Growth rate study of *Botryococcus braunii*

*Botryococcus braunii* also had a higher growth rate in 25% (v/v) diluted wastewater when compared to the control media. However, *Botryococcus braunii* reaches the log phase only in 15 days and stationary phase in 27 days. The maximum concentration reached by the cells at the end of 27 days was $3x10^6$ cells/mL in 25% (v/v) wastewater during which the weight of the cells was 1.5 g/L. In the control cells, the maximum concentration reached by the cells was $1.5x10^6$ cells/mL and the weight of the cells was 1.2 g/L (Figure 3). It can be seen from the graph that the 50% and 75% wastewater does not support the growth of the cells. Interestingly, though the 50% and 75% diluted wastewater had slower growth rate, the weight of the cells were found to be comparable to the control and the 25% (v/v) diluted wastewater. The growth rate of *Botryococcus braunii* was found to be 0.362 cells/day during the exponential phase in the cells grown in 25% (v/v) diluted wastewater, whereas in control cells it was 0.125 cells/day.

A




B

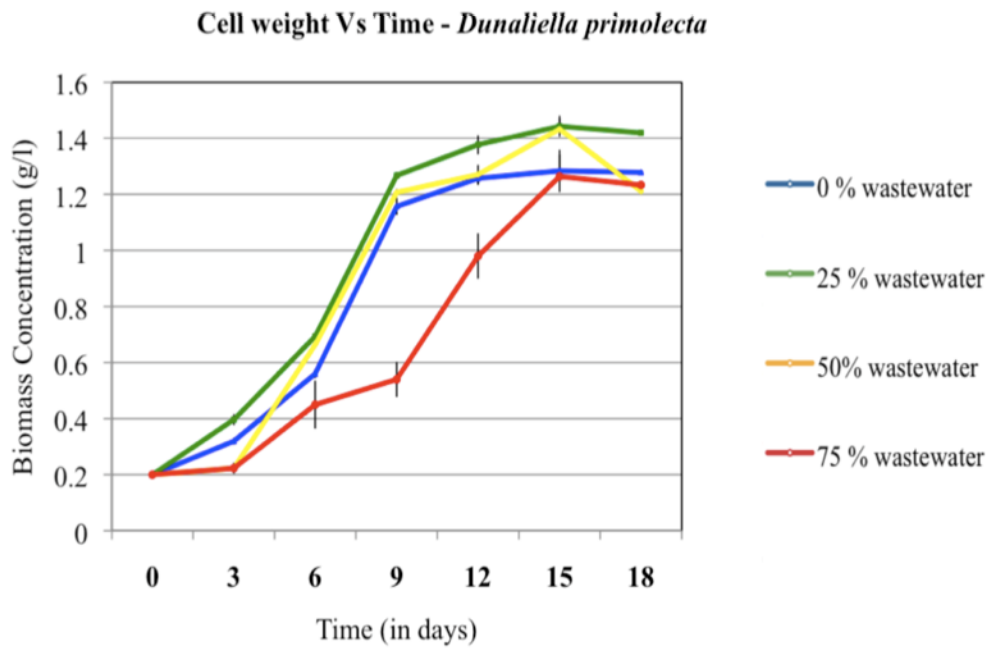



Figure 2. Cell growth study of *Dunaliella primolecta* in different percentages of wastewater. A. Cell number vs time B. Cell weight vs time. Experiments were repeated three times.

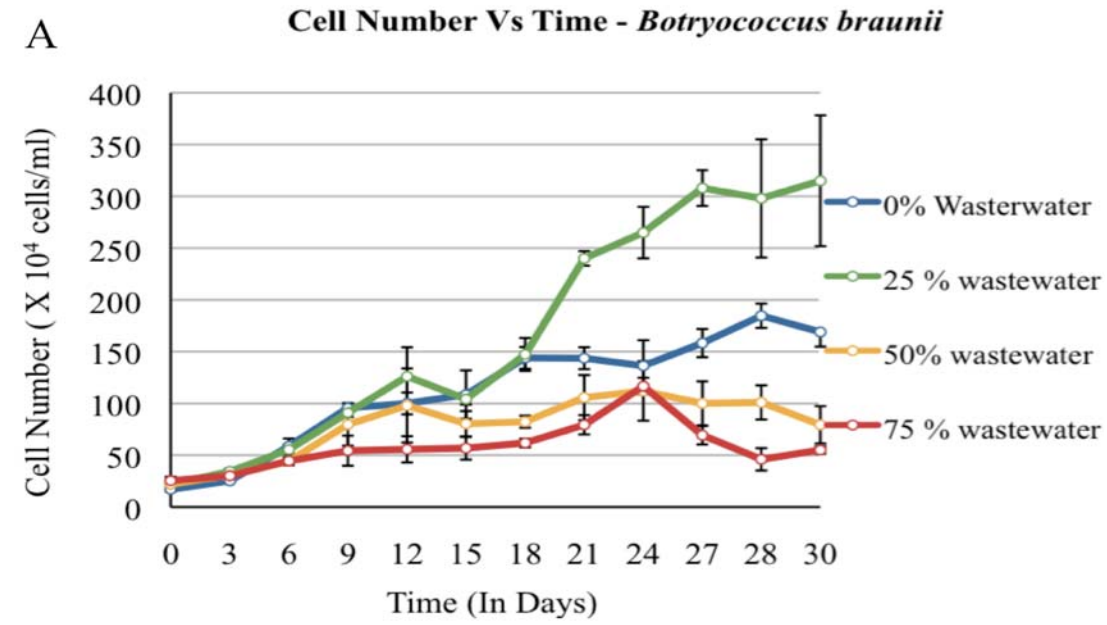
**Table 2.**
Comparison of the growth rate of the two species of algae (*D. primolecta* and *B. braunii)* during their exponential phase in different percentages of wastewater

| Algal Species | Growth rate of cells in different percentages of wastewater (cells/day) in the exponential phase. | | | |
|---|---|---|---|---|
| | Control Cells | 25% Wastewater | 50% Wastewater | 75% Wastewater |
| *Dunaliella primolecta* | 0.9±0.12 | 1.23±0.14 | 0.88±0.08 | 0.38±0.02 |
| *Botryococcus braunii* | 0.125±0.03 | 0.36±0.13 | 0.07±0.02 | 0.06±0.01 |

It is clear from the figure that *Dunaliella primolecta* grows faster than *Botryococcus braunii* in 25% (v/v) diluted wastewater (Figure 4). The growth rate of *D. primolecta* was 1.23 cells/day at 25% dilution; however for *B. brauni* the growth rate was found to be 0.36 cells/day during the exponential phase. This meant that *D. primolecta* grew approximately 250% times faster that *B. braunii* when grown in 25% dilution. The cell concentration that it reached at the end of the log phase was also 50% more than *B. braunii.* However the weight of the cells at the end log phase and the beginning of stationary phase was 1.5 g/L for both the algal species.

## 4.2. Comparison of the Oil Production in *Dunaliella primolecta* and *Botryococus braunii* When Grown in 25 % (v/v) Diluted Wastewater.

It is clearly seen from the Figure 5 that the oil production in *Botryococcus braunii* far exceeds that in *Dunaliella primolecta.* The amount of oil produced in *Botryococcus braunii* when it reaches the log phase can be easily compared to the amount of oil produced by *Dunaliella primolecta* at the end of its log phase (Figure 5). Although the cell concentration of *Botryococcus braunii* is less when compared to *Dunaliella primolecta,* the weight of the cells are higher, and it is perhaps due to the increased oil accumulation.



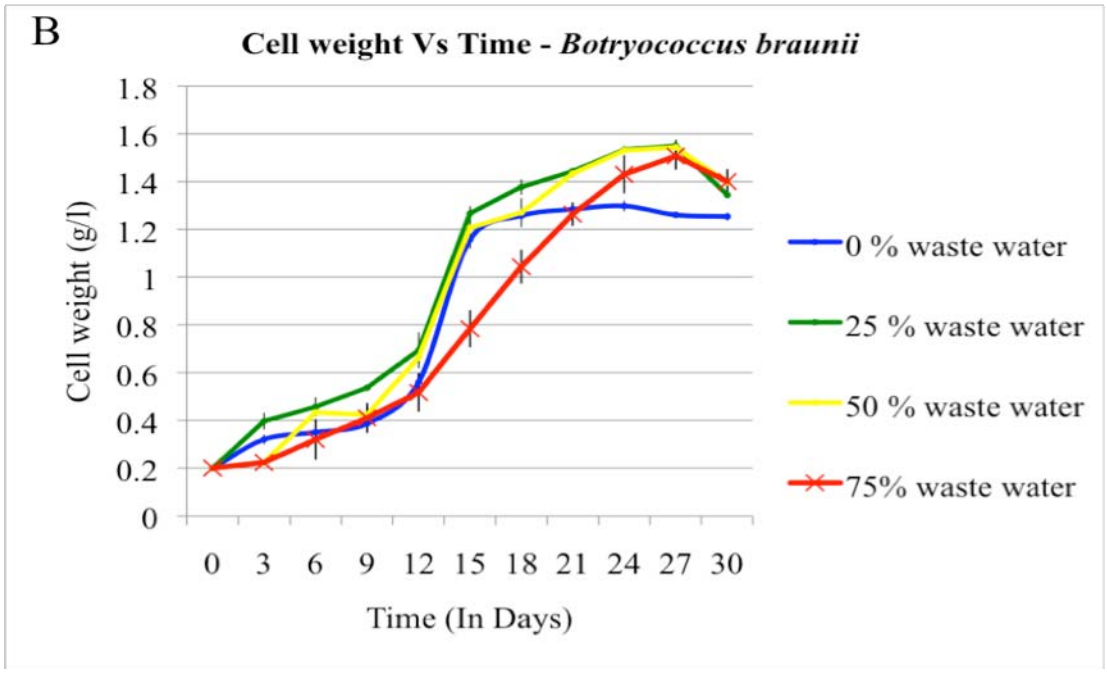




Figure 3. Cell growth study of *Botryococcus braunii* in different percentages of wastewater. (A. Cell number vs time b. Cell weight vs time. Experiments were repeated three times.)

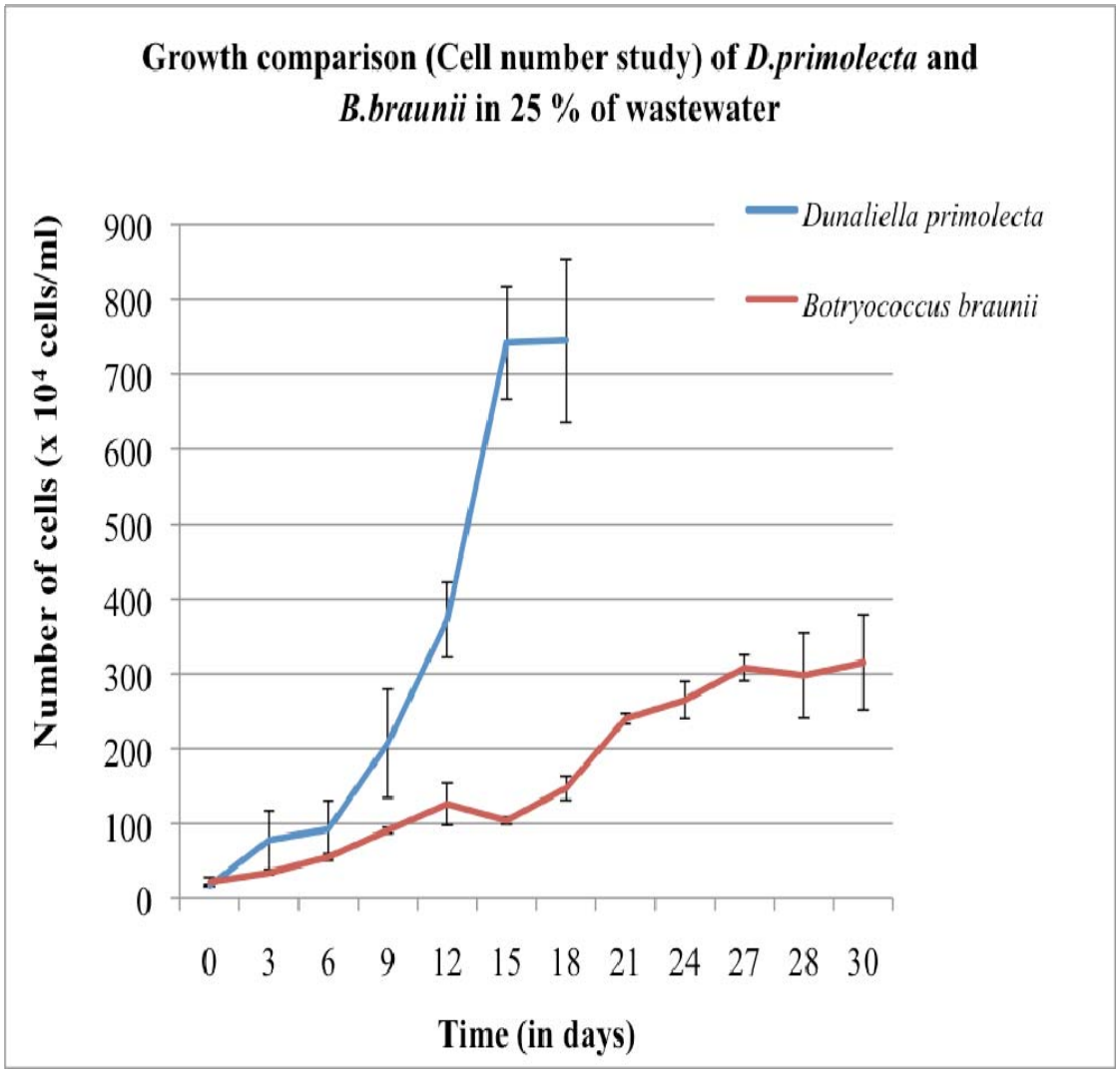



Figure 4. Growth comparison of *D. primolecta* and *B. braunii* in 25 % wastewater.

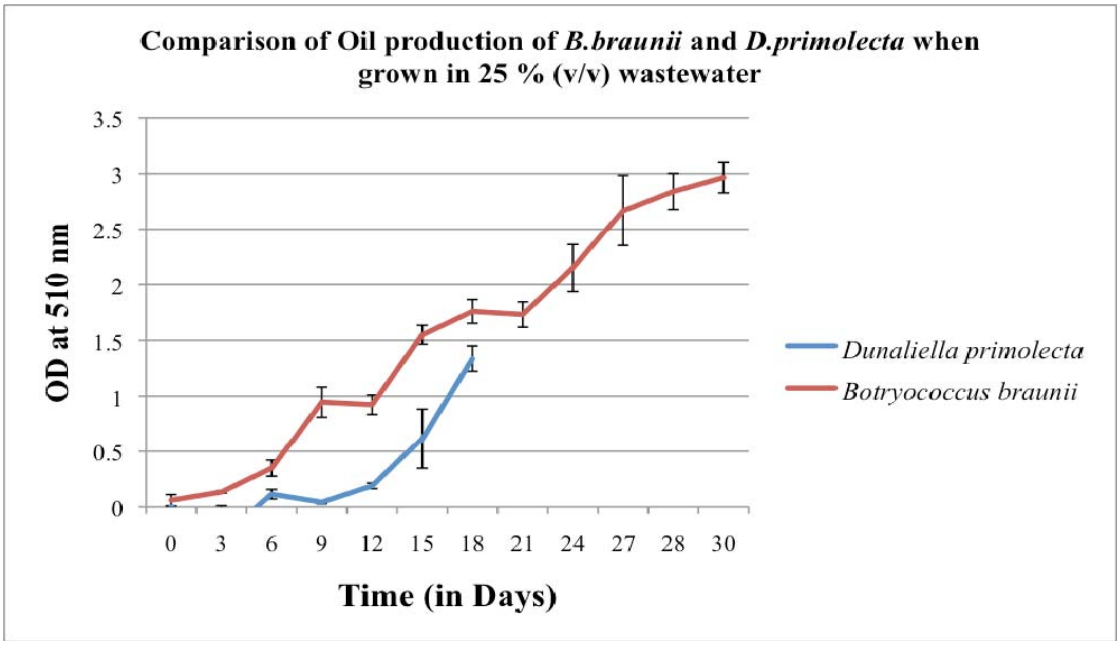



Figure 5. Comparison of oil production of *D. primolecta* and *B. braunii* when grown in 25% wastewater.

## 4.3 Efficiency of COD Removal from Wawona Frozen Foods, Inc Wastewater by *Dunaliella primolecta*

COD removal efficiency of *Dunaliella primolecta* from the wastewater obtained from Wawona Frozen Foods, Clovis, CA was studied, when the algae was allowed to grow in different percentages of wastewater. It was seen that the decrease in COD was around 63 % when this species was allowed to grow in 25 % wastewater, whereas the decrease in wastewater when grown in 50 % and 75 % (v/v) wastewater was on an average 35 % (Figure 6). This could be attributed to the fact that 25 % (v/v) wastewater supported more algae growth than the other percentages of wastewaters, thereby reducing COD level to higher extent.

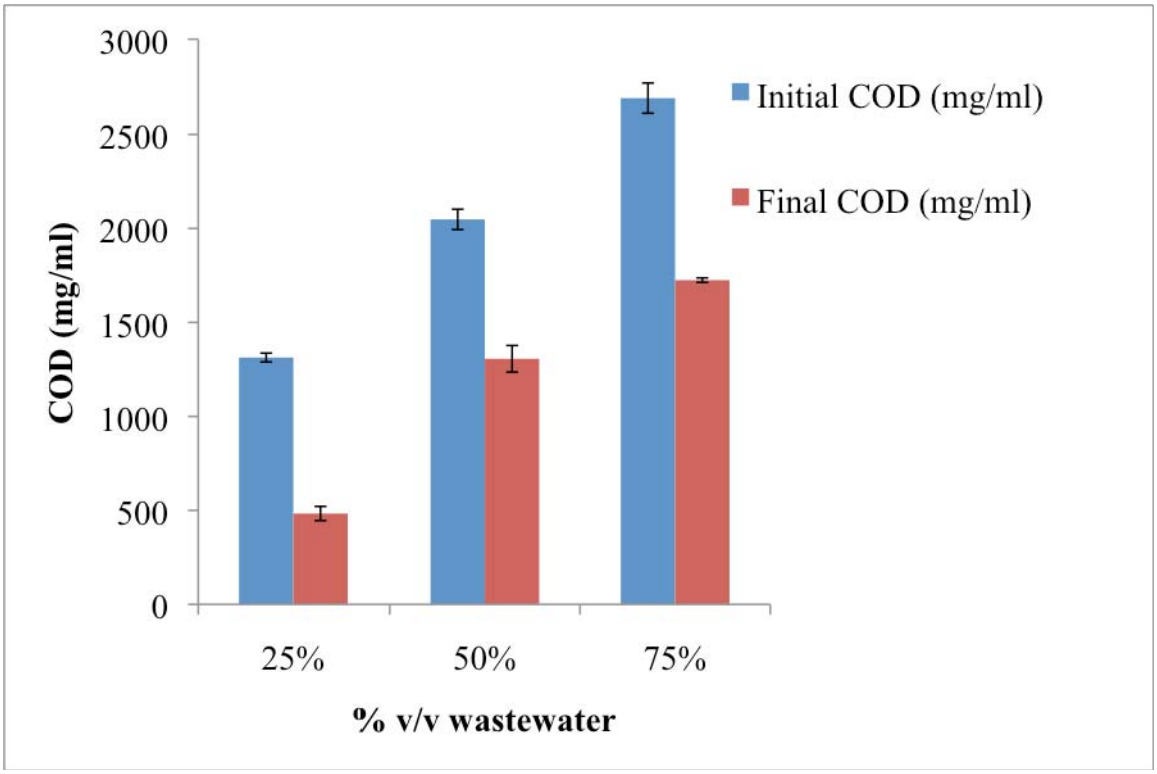



Figure 6. COD reduction by *D. primolecta* when grown in different percentages of wastewater.

## 4.4 Transformation of *Dunaliella primolecta* Using Agrobacterium-mediated Transformation

From the earlier study it was found that *Dunaliella primolecta* has a higher growth rate when compared to *Botryococcus braunii* in both control media and also in 25 % (v/v) diluted wastewater. Hence it was proposed that *Dunaliella primolecta* should be used as a model organism that could be transformed to produce more amount of oil. However a stable method of transformation was not established for this species of algae. We therefore established a protocol for stable transformation using *Agrobacterium tumefaciens.* We used *A. tumefaciens* Ti-plasmid-based binary vector pCAMBIA 1301harbouring the *hpt* gene and *uidA* (GUS) gene to transform *Dunaliella primolecta* by co-cultivation. DNA transfer events were studied by monitoring the transgenes associated with T-DNA,

observing the hygromycin resistance phenotype ($Hyg^R$) and assaying for β-glucuronidase activity (GUS gene expression). We obtained several $Hyg^R$ colonies following selection in agar containing 100 mg/Lof hygromycin confirming transformation event (Figure 7). It was also found that the transformed cells after allowing them to grow in the selection medium for 48-72 h had a different morphology when compared to the control cells. The control cells were oval and long whereas the transformed cells were circular and round. Also these transformed cells could tolerate the osmotic stress when dissolved in distilled water for a period of 1 h (Figure 8).

Figure 7. Confirmation of transformation by testing for $Hyg^R$ in the algae. *D. primolecta* cells were grown in media containing100 mg/ L hygromycin. Growth was observed in transformed cells (left panel) and no growth was observed in untransformed cells (right panel).

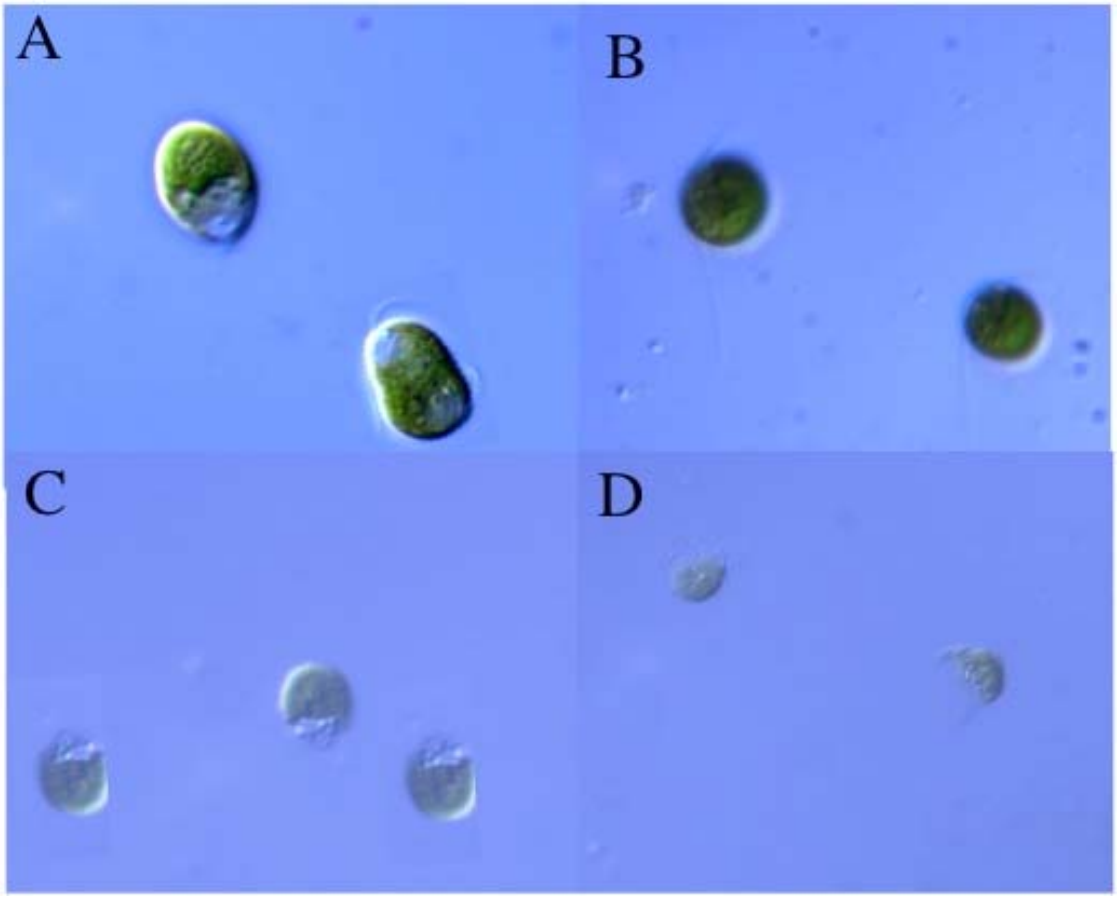



Figure 8. Morphological observation of transformed cells and untransformed cells. (A) Transformed cells when grown in MSS media. (B) Untransformed cells when grown in MS media. The response of the transformed cells (C) and untransformed cells (D) when subjected to osmotic stress (distilled water treatment)

## 4.5 Detection of GUS Gene Expression

After 72 h of transformation, cells of *Dunaliella primolecta* were collected for histochemical staining analysis. Some of the cells had the blue color in them which confirmed the transformation event. Most of these cells had partial blue color, suggesting a low expression of GUS gene in the transformed cells. Few cells that grew in hygromycin containing media did not show any traces of blue color. This indicated that either the GUS gene had got integrated into a region, which was inactive, or there was a rearrangement that had occurred during the integration of T-DNA into the algal genome.

## 4.6 PCR Analysis of Transformants

The DNA isolated from the transformed cells and the control cells were used for PCR analysis to confirm the presence of exogenous gene in *Dunaliella primolecta.* Two different sets of primers were used for the analysis: one specific to 35S promoter and the other specific to GUS gene. The PCR results from the primers specific to 35S promoter showed positive results (Figure 9A). Due to the false positive results obtained in the control cells, we had to use another set of primers specific to GUS gene. The expected 450 bp bands were observed in transformed cultures when the GUS specific primers were used (Figure 9B). However few longer bands were observed in another set of transformed culture that showed a negative result in GUS staining. This suggested that the T-DNA was rearranged during the integration into the algal genome. Also since these cultures were not from single line of cells, multiple types of rearrangement were obtained.

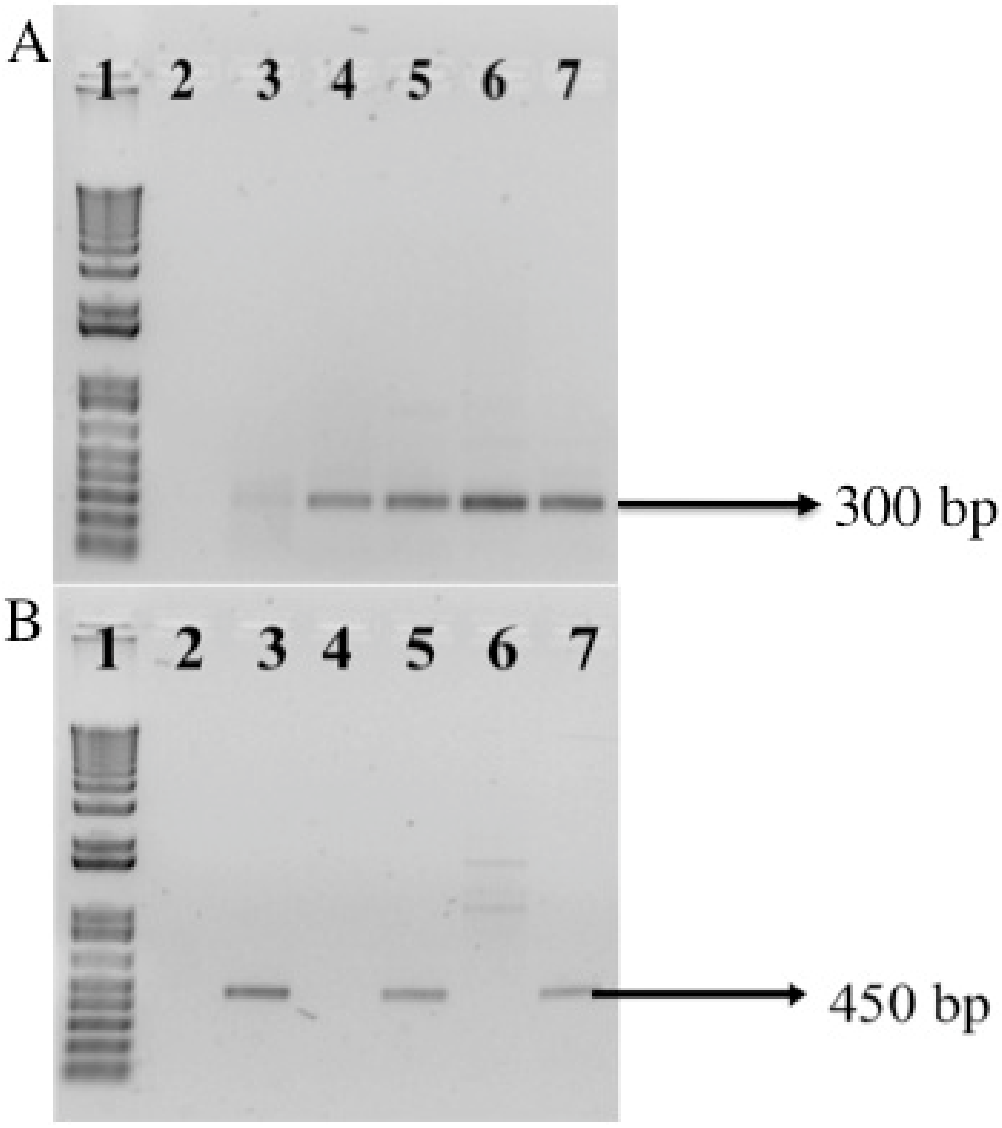



Figure 9. PCR analysis using 35S and GUS specific primers. 9A. PCR analysis using 35S specific primers Lane 1: 1kb DNA ladder, Lane 2: Negative control; Lane 3: Untransformed cells (Non-specific binding-False Positive); Lane 4: Positive control (pCAMBIA1301); Lanes 5-7: Transformed cells from three independent transformation event. 9B. PCR analysis using GUS specific primers -- Lane 1: 1kb DNA ladder, Lane 2: Negative control; Lane 3: Positive control (pCAMBIA1301); Lane 4: Untransformed cells, Lanes 5-7: Transformed cells from three independent transformation event.

## 4.7 Southern Blot Analysis of Transformants

Southern blot analysis was done on the DNA from the transformed and untransformed cells. The GUS gene was labeled for its use as the probe. We found that GUS specific bands were observed in the transformed cells. After hybridization, a band of approximately 10 kb was found in transformed cells and was absent in untransformed cells (Figure 10).

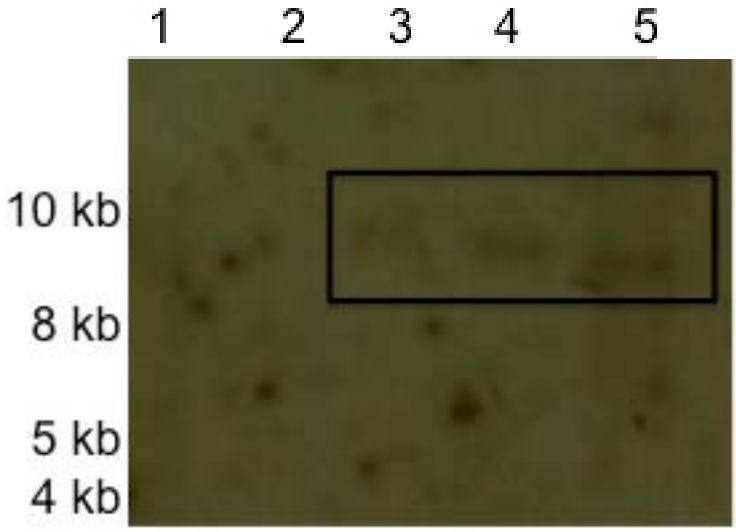



Figure 10. Southern blot analysis of genomic DNA from transformed *D. primolecta.*
Genomic DNA from transformed and untransformed cells were digested with EcoRI and hybridized with GUS sequence. No hybridization bands were visible in wild type algae (Lane 1 and 2) and bands approximately 10 kb size was obtained in lane 3,4 and 5 (Transformed cells).

## 4.8 Isolation of Single Cells of Transgenic Algae Using mmi CellEctor

The cells were isolated using CellEctor (Figure 11) and were allowed to grow in a 4-cell chamber plate until a desirable growth was achieved.

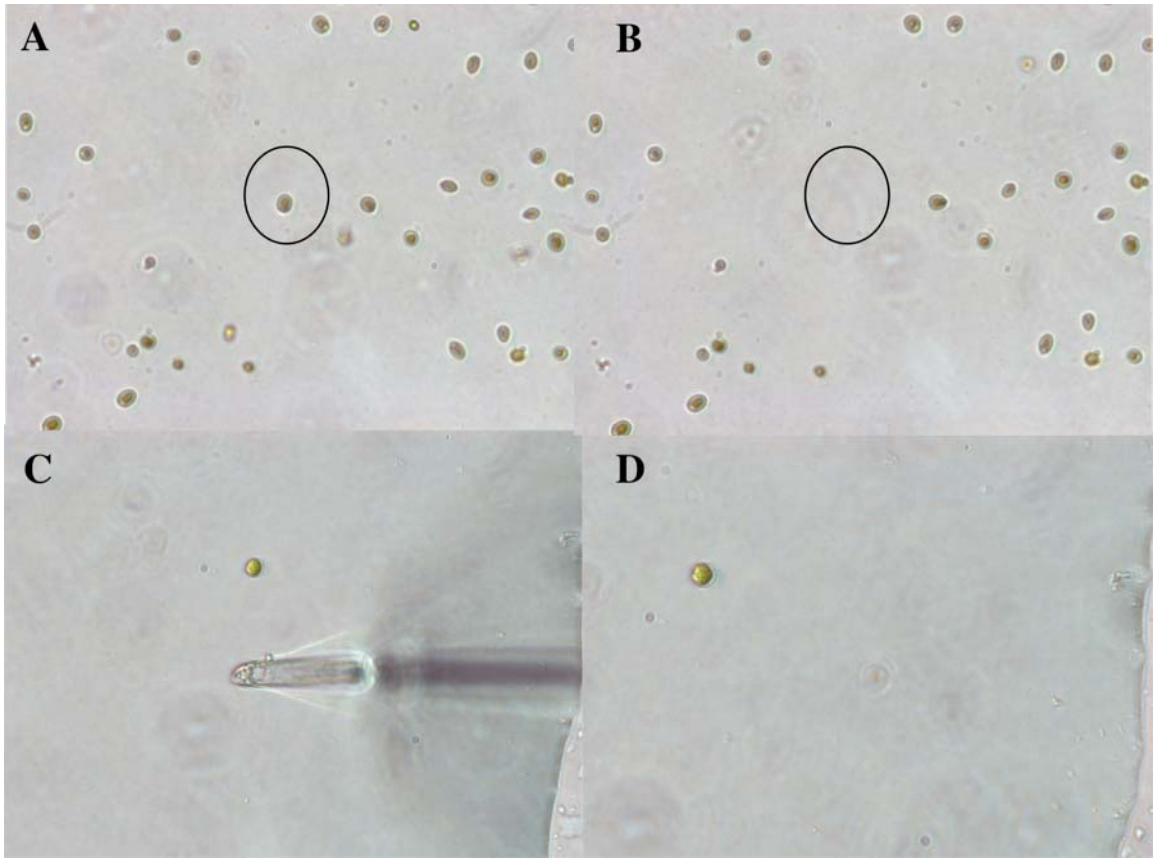



Figure 11. Isolation of cells using MMI CellEctor.
Panel A and B are the sample slide. The dark circle shows the cell that was picked up by the capillary; Panel C and D are the deposit slide; which shows the delivery of the picked cells on the 4-cell chamber slide.

# 5. DISCUSSION

*Dunaliella primolecta* and *Botryococcus braunii* are two well-known species of green algae that are promising for biodiesel production. In the present study we tried to understand ways to reduce the cost of biodiesel production from algae. We reasoned that the ways to decrease the cost of producing microalgal biodiesel would be to improve the capabilities of microalgae through genetic engineering, reducing the cost involved in raw material procurement and increasing the growth rate of algae (Chisti, 2007). We argued that changing the conditions necessary for growth might be tedious and not practically possible. However, we could increase the oil production in the algae that have a comparatively higher growth rate and also reduce the cost necessary for the procurement of raw materials for the growth of algae. Baring this in mind, we selected two strains of green algae, *Dunaliella primolecta* and *Botryococcus braunii,* that are presently the two promising source of green algae for biodiesel production. We chose to use wastewater from the Wawona frozen foods, Clovis, CA. We started studying the growth of *Dunaliella primolecta* and *Botryococcus braunii* in fruit industry wastewater that was obtained from Wowana frozen foods. We found that *Dunaliella primolecta* reached a cellular density of $8X10^6$ cells/ml in a period of 18 days in MS media supplemented with 25% (v/v) wastewater. It was found out earlier that marine microalgae could grow axenically in culture in minimal media containing synthetic salts reaching a concentration of around or below $10^6$ cells/ml. However, this could be further increased by addition of Algal-I (Santin-Montany· et al., 2007). In such a media the cellular concentration reached was greater than $60X10^6$ cells/ml. We used just the plain wastewater without the addition of any other growth enhancers to study the growth rate, since our focus

was to reduce the raw material cost. We had obtained the final growth of 1.5 $gL^{-1}$ of *Botryococcus braunii* in 25% v/v wastewater, which related to the previous study done where the dry weight achieved was around 2 $gL^{-1}$ after 6 weeks (Dayananda et al., 2007). In this study the algae was grown in BG-11 media. It was also observed that *Botryococcus braunii,* when grown in piggery wastewater from the rural areas of Korea grew faster reaching a concentration of 8 $gL^{-1}$ in 8 days as opposed to 2 $gL^{-1}$ in modified Chu13 medium in 2 weeks (An et al., 2003). It was clearly observed that the *Dunaliella primolecta* could grow in wastewater faster than *Botryococcus braunii.* Hence we had selected *Dunaliella primolecta* as the model organism for genetic engineering to increase oil production. We had also checked to see if *Dunaliella primolecta* could reduce the COD content in wastewater. It was seen that the algae when grown to a concentration of $8X10^6$ cells/mL could reduce 63% of COD and bring the COD of wastewater to 480 mg/L COD. COD removal study was performed because it was found that the pretreated wastewater obtained from Wawona Frozen Foods, Clovis, CA had low phosphorous and nitrogen level, which was at 110 mg/L and 20 mg/L respectively. However, the COD level was as high as 6000 mg/L on an average.

At present no protocol has been designed for genetic engineering of *Dunaliella primolecta.* However, particle bombardment method (Tan et al., 2005;Tan C, 2005) and electroporation (Dayananda et al., 2007;Geng D, 2003;Sun et al., 2005) have been tried with *Dunaliella salina*. It was earlier seen that the transformation efficiency using electroporation was much lower than particle bombardment (Tan et al., 2005)However, the particle bombardment resulted in the physical damage of the cells. Hence we had used *Agrobacterium* to transform *Dunaliella.* It was also observed that the transformation frequency and the stability of the gene in subsequent generations have been limiting factors in all methods of

transformation except in *Agrobacterium*-mediated transformation (Tzfira andCitovsky, 2006). Also, no work has been presently done to transform *Dunaliella* using *Agrobacterium.*

We found out that *Agrobacterium* could be used for the successful transformation of *Dunaliella primolecta.* We were able to perform the transformation even in the absence of acetosyringone, the inducer of *Agrobacterium* infection. This method was found to be simple and reproducible. We used the vector pCAMBIA 1301 that had genes for hygromycin resistance and GUS expression that could be used as a reporter. PCR analysis indicated that the t-DNA was transferred from the vector into the algae cells. This was confirmed by the 450 bp PCR product that was obtained from the transformed cells that was similar to the positive control cells when primers specific to GUS gene were used. However, out of three independent transformation events, we had obtained one transformation event in which the T-DNA had undergone some kind of rearrangements before integration.

The experiments in this thesis prove that *D. primolecta* grows faster in 25% (v/v) fruit industry wastewater, simultaneously reducing 63% of COD levels from the wastewater. It also confirms that though *B. braunii* produces more lipid body than *D. primolecta,* the latter organism proved to be a better candidate for the oil production due to its faster growth. Also, this thesis provides an original strategy for transforming *D. primolecta* using *A. tumefaciens,* an easy and stable method of transformation.

# 6. CONCLUSION

*Dunaliella primolecta* and *Botryococcus braunii* are the two well-known species of green algae that could potentially be used for the biodiesel production. However, *D. primolecta* was found to be a better suitable candidate as it grew faster in wastewater than *B. braunii.* Also, *D. primolecta* was capable of reducing 63% of COD level in the wastewater, reducing the COD to around 500 mg/L. This wastewater could be resused again further reducing the COD to levet. Though *B. braunii* was found to be producing higher amount of oil, its slow growth rate might impede its use towards the commercialization. We also were able to develop a successful transformation protocol for *D. primolecta* using *A. tumefaciens.* We found that, *D. primolecta* could easily be transformed by *A.tumefaciens* even without the presence of inducer molecule. However, further studies have to be done to find out a gene required for increased oil production. This gene could be used to transform algae using our developed protocol.

# REFERENCES

# REFERENCES


An J-Y, Sim S-J, Lee JS, Kim BW, 2003. Hydrocarbon production from secondarily treated piggery wastewater by the green alga Botryococcus braunii. J. Appl. Phycol. 15, 185-91.

Apt K, Grossman A, Kroth-Pancic P, 1996. Stable nuclear transformation of the diatomPhaeodactylum tricornutum. Mol. Gen. Genet. 252, 572-79.

Chisti Y, 2007. Biodiesel from microalgae. Biotechnol. Adv. 25, 294-306

Chisti Y, 2008. Response to Reijnders: Do biofuels from microalgae beat biofuels from terrestrial plants? Trends Biotechnol. 26, 351-52.

Dayananda C, Sarada R, Usha Rani M, Shamala TR, Ravishankar GA, 2007. Autotrophic cultivation of Botryococcus braunii for the production of hydrocarbons and exopolysaccharides in various media. Biomass Bioenerg. 31, 87-93.

Dunahay TG, Jarvis EE, Roessler PG, 1995. Genetic Transformation of the diatoms Cyclotella cryptica and *Navicula saprophila.* J. Phycol. 31, 1004-12.

Gelvin SB, 2000. Agrobacterium and Plant Genes Involved in T-DNA Transfer and Integration. Ann. Rev. Plant Phys. 51, 223-56.

Geng D WY, Wang P, Li W, Sun Y, 2003. Stable expression of hepatitis B surface antigen gene in *Dunaliella salina* (Chlorophyta). J. Appl. Phycol. 15, 451-56.

González LE, Cañizares RO, Baena S, 1997. Efficiency of ammonia and phosphorus removal from a colombian agroindustrial wastewater by the microalgae Chlorella vulgaris and Scenedesmus dimorphus. Bioresource Technol. 60, 259-62.

Gordillo F, Goutx M, Figueroa F, Niell F, 1998. Effects of light intensity, CO2 and nitrogen supply on lipid class composition of Dunaliella viridis. J. Appl. Phycol. 10, 135-44.

Grobbelaar JU, 2000. Physiological and technological considerations for optimising mass algal cultures. J. Appl. Phycol. 12, 201-06.

Hamilton CM, Frary A, Lewis C, Tanksley SD, 1996. Stable transfer of intact high molecular weight DNA into plant chromosomes. PNAS 93, 9975-79.

Kathiresan S, Chandrashekar A, Ravishankar GA, Sarada R, 2009. Agrobacterium-mediated transformation in the green algae *Haemotococcus Pluvialis(Chlorophyceae, Volvocales)*. J. Phycol. 45, 642-49.

Kumar SV, Misquitta RW, Reddy VS, Rao BJ, Rajam MV, 2004. Genetic transformation of the green alga--Chlamydomonas reinhardtii by Agrobacterium tumefaciens. Plant Sci. 166, 731-38.

Lang X, Dalai AK, Bakhshi NN, Reaney MJ, Hertz PB, 2001. Preparation and characterization of bio-diesels from various bio-oils. Bioresource Technol. 80, 53-62.

León-Bañares R, González-Ballester D, Galván A, Fernández E, 2004. Transgenic microalgae as green cell-factories. Trends Biotechnol. 22, 45-52.

Li Q, Du W, Liu D, 2008. Perspectives of microbial oils for biodiesel production. Appl. Microbiol Biot. 80, 749-56.

Ma F, Hanna MA, 1999. Biodiesel production-A review. Bioresource Technol. 70, 1-15.

Miao X, Wu Q, 2006. Biodiesel production from heterotrophic microalgal oil. Bioresource Technol. 97, 841-46.

Muñoz R, Guieysse B, 2006. Algal-bacterial processes for the treatment of hazardous contaminants: A review. Water Res. 40, 2799-815.

Radmer R, Parker B, 1994. Commercial applications of algae: opportunities and constraints. J. Appl. Phycol. 6, 93-98.

Ramírez-Zacarías JL, Castro-Muñozledo F, Kuri-Harcuch W, 1992. Quantitation of adipose conversion and triglycerides by staining intracytoplasmic lipids with oil red O. Histochem. Cell Biol. 97, 493-97.

Reijnders L, 2008. Do biofuels from microalgae beat biofuels from terrestrial plants? Trends Biotechnol. 26, 349-50.

Santin-Montany· I, Sandin-Espaoa P, Garcia Baudin JM, Coll-Morales J, 2007. Optimal growth of Dunaliella primolecta in axenic conditions to assay herbicides. Chemosphere 66, 1315-22.

Sawayama S, Inoue S, Yokoyama S, 1994. Continuous culture of hydrocarbon-rich microalga Botryococcus braunii in secondarily treated sewage. Appl. Microbiol Biotechnol. 41, 729-31.

Scragg AH, Morrison J, Shales SW, 2003. The use of a fuel containing Chlorella vulgaris in a diesel engine. Enzyme Microb. Tech. 33, 884-89.

Shimogawara K, Fujiwara S, Grossman A, Usuda H, 1998. High-Efficiency Transformation of Chlamydomonas reinhardtii by Electroporation. Genetics 148, 1821-28.

Sun Y, Yang Z, Gao X, Li Q, Zhang Q, Xu Z, 2005. Expression of foreign genes in Dunaliella by electroporation. Mol. Biotechnol. 30, 185-92.

Talya K, Tzvi T, Yoram K, Yedidya G, Colin D, Vitaly C, 2001. Genetic transformation of HeLa cells by Agrobacterium. PNAS 98, 1871-76.

Tan C, Qin S, Zhang Q, Jiang P, Zhao F, 2005. Establishment of a micro-particle bombardment transformation system for Dunaliella salina. J. Microbiol. 43, 361-65.

Tarlan E, Dilek FB, Yetis U, 2002. Effectiveness of algae in the treatment of a wood-based pulp and paper industry wastewater. Bioresource Technol. 84, 1-5.

Teng C, Qin S, Liu J, Yu D, Liang C, Tseng C, 2002. Transient expression of lacZ in bombarded unicellular green alga Haematococcus pluvialis. J. Appl. Phycol. 14, 497-500.

Tzfira T, Citovsky V, 2006. Agrobacterium-mediated genetic transformation of plants: biology and biotechnology. Curr. Opin. Biotech. 17, 147-54.

Walker TL, Collet C, Purton S, 2005. Algal transgenics in the genomic era. J. Phycol. 41, 1077-93.

Yeoung-Sang Yun SBLJMPC-ILJ-WY, 1997. Carbon Dioxide Fixation by Algal Cultivation Using Wastewater Nutrients. J. Chem. Technol. Biot. 69, 451-55.

Zhang Y, Dubé MA, McLean DD, Kates M, 2003. Biodiesel production from waste cooking oil: 1. Process design and technological assessment. Bioresource Technol. 89, 1-16.

# APPENDIX

## 6.1. Preparation of 1 L of Pasteurized Sea Water

20 g of Instant Ocean (Aquarium Systems Inc.) was added to 700 mL of distilled water and was mixed well using a magnetic stirrer till it salts dissolved. The solution was made upto 1 L using distilled water and transferred to a orange-capped bottle. It was then pasteurized for 45 min at $93^0$C. The pasteurized content was allowed to cool for 24 h at room temperature. The pasteurization process was repeated for the second day at the same temperature and time. The solution was again allowed to cool and stored at $4^0$C.

## 6.2. Preparation of PIV metal solution

To prepare 1 L of PIV metal solution, the following components were added in the same order to 700 mL of distilled water and mixed well using magnetic stirred. The solution was made upto 1 L using water and transferred to orange-capped bottle and autoclaved. It had to be made sure that $Na_2EDTA.2H_2O$ is fully dissolved before the other components were added.

**Table A1.**
Constituents of PIV Metal Solution

| Component | Amount |
|---|---|
| $Na_2EDTA.2H_2O$ (Sigma, Cat # ED225) | 750 mg |
| $FeCl_3.6H_2O$ (Sigma, Cat # 1513) | 97 mg |
| $MnCl_2.4\ H_2O$ (Baker, Cat # 2540) | 41 mg |
| $ZnCl_2$ (Sigma, Cat # Z0152) | 5 mg |
| $CoCl_2.6\ H_2O$ (Sigma, Cat # C3169) | 2 mg |
| $Na_2.MoO_4.2H_2O$ (Baker, Cat # 3764) | 4 mg |

## 6.3. Preparation of Soilwater: GR+Medium

To 200 mL of distilled water; 1 tsp of green house soil and 1 mg of $CaCO_3$ was added and transferred to a 250 ml orange-capped bottle. It was pasteurized for 2 consecutive days, 3 h on each day at $95^0$C followed by cooling to room

temperature. The medium was stored at 40C and brought to room temperature before use.

6.4. Preparation of Vitamin$B_{12}$

To 200 mL of distilled water, 2.4 g of HEPES buffer (Sigma; Cat #H-3375) and the pH was adjusted to 7.8. 27 mg of Vitamin B12 (Sigma; Cat #V-6629) and was allowed to dissolve completely. It was stored in dark at $4^0$C.

6.5. Preparation of Erdbschreiber's media

To 700 ml of pasteurized seawater, 12 ml of PIV metal solution, 195 mg of $NaNO_3$ (Fisher; Cat # BP360-500), 18 mg of $Na_2HPO_4.7H_20$ (Sigma; Cat # S-9390), 50 ml of soilwater: GR+media and 1 ml of Vitamin $B_{12}$ was added and mixed well; under sterile conditions. The solution was made upto 1 L using pasteurized seawater. The medium was stored in the refrigerator at $4^0$C.

6.6 Preparation of Modified Bold 3M Media

To 850 ml of distilled water; the following components were added in the same order as given in the table.

**Table A2.**
Preparation of Modified Bold 3M Media

| Component | Amount (mL/L) | Stock solution (g/400 mL) |
|---|---|---|
| $NaNO_3$ (Fisher; BP360-500) | 30 | 10 |
| $CaCl_2.2H_2O$ (Sigma; C-3881) | 10 | 1 |
| $MgSO_4.7H_2O$ (Sigma; 230391) | 10 | 3 |
| $K_2HPO_4$ (Sigma; P3786) | 10 | 3 |
| $KH_2PO_4$ (Sigma; P0662) | 10 | 7 |
| NaCl (Fisher; S271-500) | 10 | 1 |
| PIV Metal solution | 6 | |
| Soilwater: GR+Medium | 40 | |
| Vitamin $B_{12}$ | 1 | |

The volume was made upto 1 L using distilled water and transferred to an orange-capped bottle. The media was autoclaved and stored at $4^0$C. It was brought to room temperature before use.

# California State University, Fresno

Non-Exclusive Distribution License
(to make your thesis available electronically via the library's eCollections database)

By submitting this license, you (the author or copyright holder) grant to CSU, Fresno Digital Scholar the non-exclusive right to reproduce, translate (as defined in the next paragraph), and/or distribute your submission (including the abstract) worldwide in print and electronic format and in any medium, including but not limited to audio or video.

You agree that CSU, Fresno may, without changing the content, translate the submission to any medium or format for the purpose of preservation.

You also agree that the submission is your original work, and that you have the right to grant the rights contained in this license. You also represent that your submission does not, to the best of your knowledge, infringe upon anyone's copyright.

If the submission reproduces material for which you do not hold copyright and that would not be considered fair use outside the copyright law, you represent that you have obtained the unrestricted permission of the copyright owner to grant CSU, Fresno the rights required by this license, and that such third-party material is clearly identified and acknowledged within the text or content of the submission.

If the submission is based upon work that has been sponsored or supported by an agency or organization other than California State University, Fresno, you represent that you have fulfilled any right of review or other obligations required by such contract or agreement.

California State University, Fresno will clearly identify your name as the author or owner of the submission and will not make any alteration, other than as allowed by this license, to your submission. **By typing your name and date in the fields below, you indicate your agreement to the terms of this distribution license.**

Preethi Sarvabhowman

Type full name as it appears on submission

December 7, 2010

Date