Towards Genetic Modification of Microalgae *Scenedesmus dimorphus and* *Chlorella spp.* using algae specific vector

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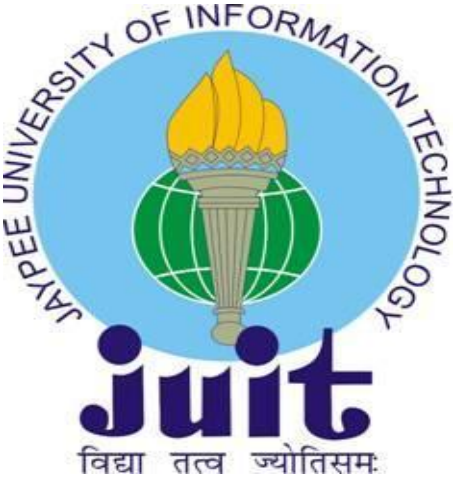

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Project report submitted in partial fulfillment of the degree of Bachelor of Technology

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CERTIFICATE

This is to certify that the work titled-"Towards genetic modification of microalgae- *Scenedesmus dimorphus and Chlorella spp."* submitted by **Samriti Singh, Shreya Singh and Shivangi Garg** in partial fulfillment for the award of degree of **Bachelor of Technology** in Biotechnology to Jaypee University of Information Technology, Waknaghat has been carried out under my supervision.

This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

Signature of Supervisor ……

Name of Supervisor …….

Designation ……

Date …….

ACKNOWLEDGEMENT

It is our great pleasure and privilege to work under the guidance of **Dr. Anil Kant** as my project guide who has been motivating and encouraging throughout the project and put in every possible effort to make the project a success.

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Signature of the students …… ……. …….

Name of the students …….. …… …….

Date ……...

Summary

Cultivation of microalgae for biofuels in general and oil production in particular is a promising approach but this technology still needs considerable research and development. Microalgae present in nature do not possess the desired qualities in much amount thus making them less potent for the production of biofuels. The objective of the study is to conduct the electroporation of the DGAT gene that catalyses the formation of Triacylglycerides from diacylglycerol and Acyl-CoA and is responsible for lipid accumulation in the microalgae *Scenedesmus dimorphus and chlorella spp*. and then checking if the transformation has occurred or not. We have used the pChlamy_1 vector which has gene DGAT for hygromycin resistance. Thus hygromycin is used as a selectable marker for checking the transformation. The concentration of hygromycin selected for *Scenedesmus dimorphus* was 40mg/l and 60mg/l was selected for *chlorella spp*. as this is the minimum concentration of the antibiotic at which the growth of the algae gets inhibited when plating of microalgae was done before transformation. The above protocol was applied in both the species of microalgae.

LIST OF SYMBOLS AND ACRONYMS

BG-11	-Blue green medium
CO_2	-Carbon dioxide
Ca_2^+	-Calcium ion
Mg_2^+	-Magnesuim ion
SO_4^{2-}	-Sulphate ion
Cl^-	-Chloride ion
NaCl	-Sodium Chloride
Na_2CO_3	-Sodium Carbonate
$NaNo_3$	-Sodium Nitrate
$CaCl_2$	-Calcium Chloride
$CaCl_2.2H_2O$	-Calcium Chloride Dehydrate
$MgSO_4.7H_2O$	-Magnesium SulphateHeptahydrate
$K_2HPO_4.7H_2O$	-Sodium Monohydrogen Phosphate Heptahydrate
H_3BO_3	-Boric acid
$MnCl_2.4H_2O$	-Manganese(II) Chloride Tetrahydrate
$ZnSO_4.7H_2O$	-Zinc SulphateHeptahydrate
$CuSo_4.5H_2O$	-Copper(II) Sulfate Pentahydrate
$CaCl_2.6H_2O$	-Calcium Chloride Hexahydrate
$Na_2MoO_4.2H_2O$	-Sodium Molybdate
EDTA	-Ethylene diamine tetra acetic acid
g/l	-Gram per liter
^oC	-Degree centigrade
V/V	-Volume by volume

mM	-Milli molar
r.p.m	-Revolutions per minute
TAG	-Triacylglycerol
GFP	-Green fluorescent protein
DHA	-Docosahexaenoic acid
DPA	-Diphenylamine
PUFA	-Polyunsaturated fatty acids

LIST OF FIGURES

1. INTRODUCTION

Biofuels are considered to be a reliable alternative source of energy in order to combat the developing energy crisis**.** They are mostly obtained by the trans-esterification of oils obtained from plants. Increasing energy demand and potential fossil fuel depletion has become most important concern for people throughout the world. Global warming is a major concern which is leading to many climatic changes. Possible solution for this is the use of renewable sources of energy like solar energy, biomass, wind energy etc. Biodiesel at present is extensively being used as alternative source of fuel for transportation. The component for the production of biodiesel is oil, which can be any oil i.e. sunflower oil, coconut oil etc. But if the oil which is being consumed is used for the production of biodiesel then what will be left for human consumptions.

Nowadays, oil from plants like *Jatropha crucas* is used for the production of biodiesel by the method of trans-esterification. This plant is used as its oil is not meant for human consumption. Alternatively microalgae prove to be promising in the field of biofuel production as the lower down the debate of food vs. fuel. The cultivation of the microalgae for biofuel production is very prominent these days. Unlike conventional oil seed crops, microalgae show immense potential due to their wide range of applications. Lipid from algal biomass is extracted, is converted to fatty acids by various refining methods; the fatty acids can further be processed into biodiesel by the above mentioned method of trans-esterification. Trans-esterification is the reaction of oil and alcohol in the presence of an alkali (KOH) to produce esters and glycerol.

Advantages of using microalgae are that it exhibits higher biomass production, higher photosynthetic efficiency, industrial CO_2 removal, faster growth, capability to grow in ponds as well as in fermentations units therefore, not requiring farmlands or forests. As compared to terrestrial biofuel crops like soyabean or corn, microalga production result in very less significant land footprint because of high yield of oil than any other crop. Microalga has low conservation value and can utilize water from salt aquifers which is not useful for drinking and agriculture. Cultivation of microalgae requires no external subsidy of herbicides or insecticides, eliminating any risk of generation of pesticides waste streams. Moreover, in comparision to fuels like

petroleum and diesel, the combustion of algal biofuel does not produce any oxide of sulphur ,produce a low amount of CO_2, and other harmful pollutants. Microalage biofuel contain safe, natural compounds that possess little or no harm to the environment.

Inspite of, so many advantages of using microalgae there are various challenges to its use as a source of biofuel production. Main disadvantage of using microalga is that they tend to give lower yield of oil which limits the production of biodiesel. Thus, genetic manipulation is a solution in order to obtain ideal strains having different desired features. Transgenesis in microalgae is complicated and rapidly growing technology. Diverse methods have been develop for both nuclear and chloroplast transformation of microalgae. Nuclear transformation is primarily achieved by means of electroporation, agitation with glass beads or silicon carbide whisker. These protocols of transformation mainly use microalgae whose cell wall has been removed except for particle bombardment.

There are several disadvantages of direct gene delivery methods such as multiple copies of transgene insertion as well as a high degree of rearrangement at the site where insertion has taken place. The electroporation mediated genetic transformation has also overcome the majority of limitations encountered in other transformation processes. Electroporation is effective with nearly all type of cells and species, the amount of DNA required is smaller as compared to other methods used, usually non-chemical or biological method that does not seen to alter the biological structures or functions of the target cells and it is easy to perform and has high efficiency. Through this study we took the initiative to start the work on electroporation mediated genetic transformation of indigeniously isolated *Scenedesmus dimorphus* and *Chlorella spp.* using algae specific vector pChlamy_1.

The TAG biosynthesis pathway is relatively well understood in plants but not as such in microalgae. Type-2 DGAT proteins are present in different subdomains of endoplasmic reticulum, which indicates as enzymatic machinery for the formations of TAGs is located in endoplasmic reticulum. In triacylglycerol (TAG) biosynthesis pathway, diacylglycerol acyltransferase (DGAT) has been seen to be mainly responsible for lipid accumulation in microalgae. DGAT is also been seen as the target gene for enhancing lipid content via electroporation for generating more precursors of triacylglycerol accumulation. Thus, increasing

the lipid feedstock is the first step towards making algae an economically feasible source of biodiesel production.

This study was undertaken with following objectives:

1. Electroporated mediated genetic transformation of microalgae (*Scenedesmus dimorphus, Chlorella spp.* as shown in Fig 1 and 2*)*
2. To standardize the protocol for checking the transformants using hygromycin screening.

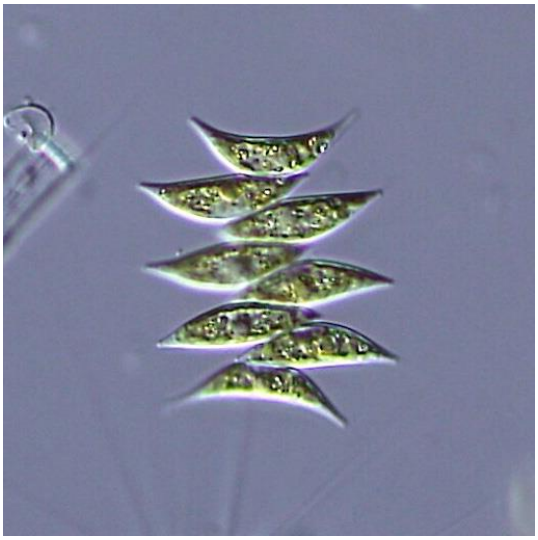


Fig 1: ***Scenedemus dimorphus***

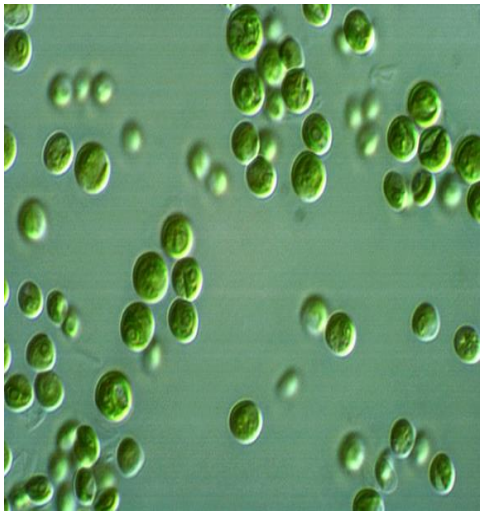


Fig 2: ***Chlorella spp.***

2. REVIEW OF LITERATURE

2.1 Characteristics of microalgae

Microalgae, known as one of the oldest living organism, are thallophytes that contain chlorophyll á' as there principle photosynthetic pigment and do not have a sterile casing of cells around the reproductive cells. They have a very high growth rate and can double their numbers in every few hours. They are able to convert solar energy better than higher plants due to their simple cellular structures. Microalgae can be cultivated with minimum requirements and they have to have high protein and lipid content which is further used for the production of biofuels or animal feeds. Moreover, algal biomass is rich in nutrients and can be used for dietary supplements to advance human health. Plastics, lubricants, fertilizers, cosmetics are also being produced by microalgae.

Nowadays, through wastewater treatment microalgae waste biofuel production is implemented which has major advantages. However, the main challenges with this technique include large scale production and harvesting of microalgae which allows the downstream processing of microalgae to produce biofuel and other bio-products with regard to harvesting, there should be control over auto-flocculation and bio-flocculation to improve the performance. There are several approaches used by companies and industries using clean water at laboratory.

Growing algae requires consideration of three primary nutrients that is carbon, nitrogen, phosphorus. Certain micronutrients such as silica, calcium, magnesium, iron, copper and cobalt are also required for its growth (1). Potential of algal biofuel without the use of wastewater is unlikely to be economically viable or provide positive energy (2).

Microalgae are of two basic types: prokaryotic and eukaryotic. Prokaryotics lack membrane-bound organelles and occur in cyanobacteria. Eukaryotics cells contain organelles. Microalgae can either be autotrophic or heterotrophic and are capable of fixing carbon dioxide efficiently. Many microalgae species have been able to use sodium carbonate and sodium

bicarbonates for cell growth. Additionally, direct uptake of biocarbonates by an active transport system has also been found (26). Algal cultures are susceptible to contamination by less desirable strains unless means of control are utilized (3). Monocultures of high lipid producing strains are likely to be out competed by faster growing species of microalgae and cyanobacteria. Growth, environment can be controlled by optimizing growth parameters that is temperature and pH.

2.2 Commercial applications of microalgae

Microalgae are rich in carbohydrate, proteins, enzymes and fibers. They contain vitamins like A, C, B1, B2, B6, niacin, calcium and magnesium. Being so rich in essential nutrients they serve s staple food in Japan, China, Korea. Commercially *Spirulina*, a powdered form of algae obtained from *Spirulina maxima* is now used as protein supplements. Algae are also added to snack food or drinks as nutritional supplements or as natural food colorants. Microalgae are rich in antioxidants (5), are rich with pigments like carotenoids (6). Components of algae are used in cosmetics as thickening agents, the main ones being *Arthrospira* and *Chlorella* (4). They are used for the production of anti-aging cream, peel-off masks and in sunscreen lotion. According to study, certain microalgae are polyphyletic and have a diverse amount of chlorophyll a containing microorganisms which are capable of oxygenic photosynthesis thus, making them quite protective towards ultraviolet radiations. Microalga is rich in PUFA, DHA, DPA (7). Microalgae contain beta-carotene which is anti-carcinogenic and is also effective in controlling cholesterol level thus, decreasing the risk of chronic heart diseases.

2.3 Microalgae and biofuel

The ideas of utilizing microalgae is not recent, but now it is taken seriously due to increase in price of petroleum and most considerably the rising concern about global warming which is connected with burning of fossil fuel. Microalga offers many potential advantages:

- They have the potential to produce 1000 to 4000 gallon/acre/year, which is higher than other oil crops.
- They do not compete with traditional agriculture because they are not traditional food.
- They require minimal growth conditions and a normal climate.

- They do not need any herbicides or pesticides for cultivation.
- They can be processed into biodiesel via trans-esterification, green diesel via direct catalytic hydrothermal conversion and catalytic upgrading.

They are many ways to convert microalga biomass to biofuel. Thus, microalgae can provide feedstock for renewable liquid fuels such as bioethanol and biodiesel. Studies reveal that green algae *Scenedesmus bijuga* has the highest biomass of 6.07g/ L and *Chlorella pyrenoidosa* was found to be best amongst oil producing algae.

2.4 Pchalmy_1 vector containing Type 2 DGAT gene

As microalgae possess shorter life cycle, perform photosynthesis and can rapidly grow, serve as attractive biofuel producers (8). One major concern that needs to be overcome before algal oil based biofuel production is the lack of strains with a high lipid content (9). According to study by James *et al* (10) lipid content in algae can be increased by blocking starch production or by nitrogen or phosphate starvations. Therefore, genetic engineering could offer potential solutions for generating a better algal strain with high lipid content (11).

TAG biosynthetic pathway is well understood in plants but not in algae. Type 2 DGAT genes are present in endoplasmic reticulum (12) which show enzymatic machinery for TAGs formation (13). In the *de novo* TAG biosynthetic pathway, diacylglycerol acyltransferase has been reported to be mainly responsible for lipid accumulation in the microalgae (14). DGAT has also been suggested as a target gene for enhancing lipid content *via* genetic engineering (9) to generate more precursors for triacylglycerol (TAG) accumulation (15)(16). Several types of biofuels can be produced from TAG-containing feedstock such as biodiesel (mono-alkyl esters) production by trans-esterification of the oil by hydrodeoxygenation of fat or oil. Thus, increasing the lipid feedstock is the first step towards making algae an economically feasible source for biofuel production.

The reaction catalyzed by DGAT enzyme is considered as a terminal and the only committed step in triglyceride biosynthesis; this is a key enzyme in neutral lipid biosynthesis (13). The reaction that is the conversion of glycerol to diaclyglycerol is catalyzed by DGAT

enzyme. DGAT catalyzes the formation of triacylglycerides from diacylglycerol and acyl-CoA and is responsible for lipid accumulation (17).

In *C. reinhardtii*, there are two types of DGAT, and transcripts encoding DGTT2 are reportedly present at lower levels under all favourable conditions. On the other hand, mRNA of the other homologue DGTT3 is present at low levels and only increases slightly under N deprivation (18), thus, not playing any major role in lipid accumulation. The single-celled algae that is *Chlamydomonas reinhardtii* has been proved to be best photosynthetic model organism to study transgene expression and different foreign genes have been expressed in Chlamydomonas (12). However, heterologous lipid biosynthetic genes have not been explored so far in this system. Therefore, this study is aimed at understanding the function of heterogeneous key enzyme involved in lipid biosynthesis. DGAT type 2 of *Brassica napus* (rapeseed) was introduced into *C. reinhardtii*.

2.5 Construction of the transformation vector

The pCHLAMY_1 is an expression vector which was procured from Life technologies, Vitrogen, USA. The size of the vector is 4283 base pair. It has hygromycin resistant gene (*aph 7)*. The six restriction sites namely BclI, HindIII, EcoRI, PstI, NcoI and BamHI were removed by changing codons of BnDGAT. The complete synthetic DNA cassette contained the BnDGAT2 gene labelled with 6XHIS and fused with KDEL-NOS-PolyA at the terminal 3′. The addition of carboxy-terminal amino acid sequence KDEL (Ays-Asp-Glu-Leu) at 3′end should help in the retention of DGAT type 2 in the endoplasmic reticulum (19). Promoter 35S derived the eGFP that is the reporter gene. The origin of GFP is the jellyfish *Aequorea Victoria* and eGFP is codon optimized for increasing the sensitivity of the reporter protein (21). Thus, complete cassette containing was synthesized and, cloned at Xba1 and Not1 restriction sites in the pCHLAMY_1 vector. It was bulked up by transforming into DH5α cells. The positive clones were selected on LB plates containing 100μg/mL ampicillin.

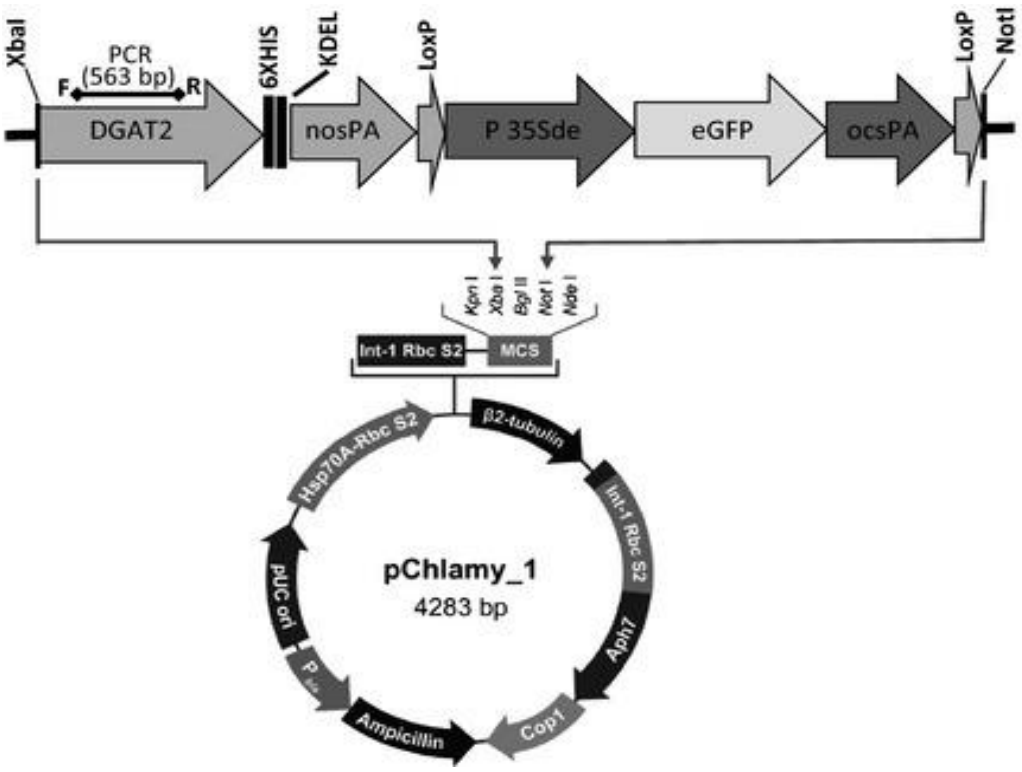


Fig 3: Construction of pChlamy_1 vector

2.6 Methods for genetic transformation

Different methods used for microalgae gene transformation have been developed were mainly based upon *Chlamydomonas*. They have the capability to alter the cell wall structure of the microalgae so that the passage of DNA is enabled through the cell membrane. These methods of direct passage of DNA are either enzymatic or mechanical (28, 29). The method such as glass bead has been widely used for the transformation of *Chlamydomonas* as it is very simple and highly efficient method. By simply vortexing the algal cell and DNA, huge numbers of transformants are obtained by using the glass beads in the presence of polyethylene glycol (30). Hence, this method of glass beads is not applied to *Chlamydomonas* as the cell wall structures of other microalgae are mostly unknown. To get transformation done efficiently there is need to optimize the protocol to eliminate the cell wall structure. Silicon carbide whisker method can act as an alternative tool for glass beads method. According to Dunahay (31), the mciroalage is

mixed with silicon carbide and vortexed well without any usage of polyethylene glycol and cell wall removal. Therefore, this method can be used for other algal species transformation. However, silicon carbide method also has disadvantages of limited supply and health related issues.

Agrobacterium has been extensively used for genetic transformation in plants. It prove to be useful in organisms like filamentous fungi (32), mammalian cells (33) and in certain microalgae species like *Chlamydomonas, Himatococuus* (34). The advantage of using *Agrobacterium* mediated transformation is that the fragment of DNA is directly transported and incorporated into the genome of microalgae thus, providing stability to the transgene (34). Low copy number insertion may be beneficial for stable gene expression as high copy number insertion sometime leading to gene splicing. This procedure is simple and low in cost; it needs co-cultivation of microalgae and *Agrobacterium.*

There have been many successful attempts of genetic transformation of microalgae. Report showed that this method of gene transfer transformed *Chlamydomonas* and its cells were able to sustain hygromycin-resistant phenotype for subsequent generation even when they were maintained in non-selection media. *Agrobacterium* mediated plant transformation via floral dip is widely used technique. The goal of this protocol is to produce transgenic flax. However, the transformation of floral dip is not reported in algae.

There are certain disadvantages associated with *Agrobacterium* mediated cell transformation:

- Many food crops cannot be transformed due to limitation of host range.
- Monocots are mostly not infected by Agrobacterium because wounded monocots do not secrete acetosyringone which a phenolic compound which acts as inducer od agrobacterium infections.
- Cells and tissues which can regenerate are difficult to transform.
- Embryonic cells might not target to T-DNA transfer.
- Stability of gene transfer sometimes is not proper.
- Integration of T-DNA is relatively tedious process.

Keeping in mind the various disadvantages associated with *Agrobacterium* mediated gene transfer. Our project aims at electroporation of gene Type 2 DGAT in *Scenedesmus dimorphus* and *Chlorella spp.* for enhancement of lipid production as DGAT catalyzes the formation of triacylglycerides from diacylglycerol and acyl CoA and is responsible for lipid accumulation.

Electroporation uses electrical pulse for the introduction of new species into cells. Main challenge of introducing a gene into cell is breaking the cell wall of the cell(22). The electric pulse open ups the cell membrane, causing temporary channels for a gene to enter. If the conditions are maintained properly the temporary channels return to its original state. Some main advantages for electroporation are its efficiency, requirement of less DNA concentration, it is less tedious and can work on various cell types (23).

2.7 Screening of transformants

Gus Assay

In order to identify the transformed algal cells they were grown on selective media. Currently available substrate for histochemical localization of beta-glucuronidase activity in cells is 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc). Beta-glucuronidase, an enzyme from the bacterium *Escherichia coli* is used in this technique. The function of this enzyme is to transform colorless substrate into colored products thus, providing transformed cells a different phenotype.

Hygromycin sensitivity test

The minimum concentration of hygromycin was observed to check the transformed and untransformed algae. The transformed algae were inoculated in inhibitory concentration of hygromycin to check its growth and confirm the transformation of algae.

3. MATERIALS & METHODS

The isolates of *Scenedesmus dimorphus and Chlorella spp.* revived in the lab were used in all the experiments in this study.

3.1. Culture conditions

BG11 media (500ml) was taken into three, 150 ml conical flasks and was autoclaved.10% of culture (Strain no.12 of *Scenedesmus dimorphus* and strain no 16 of *Chlorella spp*.) was used to inoculate 500ml of media. Light intensity used for culturing the microalgae was between 3000-5000 Lux. Temperature was in the range 18-25^{0}C, pH of media was 7.5. Humidity was 40-45% and photoperiod 16:8 hour (light cycle: dark).

3.2. Media used in experiments

3.2.1. BG-11 media

For the preparation of this media 1ml of each stock solution (composition as given in table no.1) was taken. Na_2CO_3 (0.02gm) and $NaNO_3$ (1.5gm) were also added in 1L of distilled water and was autoclaved. Stocks 1, 2 and 3 were filter sterilized and transferred to 4^{0}C cold room for the storage.

3.2.2. Luria broth

To prepare this media 20gm luria broth was dissolved in 1L distilled water. It was then autoclaved for 15 minutes at 121^{0}C for sterilization.

3.2.3. Revival of algal cultures

Glycerol stocks of previously preserved microalgae were taken. They were brought at room temperature without thawing. In an autoclaved eppendorf, 1ml of BG11 media was taken and 500ul of microalgae were revived. The cultures were kept at 25^{0}C and growth was observed within two weeks after frequent shaking.

3.2.4. Maintenance of the algal cultures

150ml of BG-11 media was taken in a 250 ml flask.1.5% agar powder was dissolved in a 250ml flask and was autoclaved. Approximately, 25ml of BG-11 media was poured in 4 petri plates. Approximately, 5ul of culture was spread on to each petri plate and kept at 25^0C for optimal growth of the microalgae.

3.2.5 Sub-culturing of the culture

150ml of BG 11 media was taken in an autoclaved flask. 50 ml of previously revived culture was mixed with 150 ml of the media. It was then kept at 25^0C for growth followed by frequent shaking.

3.3. Hygromycin sensitivity test

3.3.1. For solid media

Hygromycin sensitivity test was done to get its minimum concentration which is detrimental to the growth of the microalga species. The microalga species selected were *Scenedesmus dimorphus* and *Chlorella spp*. They were cultured in both solid and liquid BG-11 media containing antibiotic hygromycin. Concentrations of hygromycin used were 20mg/l, 40mg/l, 60mg/l, 80mg/l. For the solid media, 100µl algal culture was spread on the Petri plates. The Petri plates were incubated at 18-25 °C temperature under 3000-5000 lux light intensity and 16:8 hours (light: dark) cycle. Growth was observed on daily basis on the plates.

3.3.2. For liquid media

The microalga was cultured in liquid BG-11 media containing antibiotic hygromycin. Different hygromycin concentrations used were 20mg/l, 40mg/l, 60mg/l and 80mg/l. For the liquid media, 5µl of algal culture was dispensed in test tube containing different hygromycin

concentrations were incubated at 18-25^{o}C temperature under 3000-5000 lux light intensity and 16:8 hours (light: dark) cycle. On daily basis, the growth was observed in the test tubes.

Cell count was done for the liquid media on the 1st, 4th, 7th and 10th day using Neubauer haemocytometer under the microscope.

No. of cells/ml was calculated using the formula

$$\text{Cons. Of cells} = \frac{\text{No. of cells, counted}}{\text{Chamber connted} \times \text{Volume of chamber}} \times \frac{\text{Volume of sample dilution}}{\text{Volume of original mixture in sample}}$$

3.4. Genetic transformation

3.4.1. Maintaining the vector

Master plate containing DGAT (di-acyl transferase) gene was obtained. This gene catalyzes the formation of triglycerides from diacylglycerol and acyl-CoA. 150ml of Luria Bertani agar was prepared in two separate 500mL flasks. 1ml of 100mg/ml kanamycin was added to one flask and 1ml of 50mg/ml ampicillin was added to the other flask. 75ml media containing kanamycin was poured in three petri plates respectively and 75 ml media containing ampicillin was poured in another three petri plates. The plates were left to solidify. A colony of the bacteria from the master plate was streaked on to all six plates containing different antibiotic resistance. Plates were sealed with parafilm and kept at 37^{0}C to observe culture's resistance towards the two antibiotics. The plates were stored at 4^{0}C the next day. (Fig. 4)

Fig. 4: *E.coli* containing vector having DGAT gene

3.4.2. Preparation of glycerol stocks of the *E.coli*

50% and 70% glycerol stocks were prepared by dissolving 50 ml and 70 ml glycerol in 50 ml and 30 ml distilled water respectively in 250mL flasks and autoclaved. It was then kept at 37^0C overnight to check its stability. 70ml of Luria Bertani broth was prepared in four 250mL flasks and autoclaved. A colony of *DGAT* gene incorporated *E.coli* was inoculated in the broth. Two 250mL flasks were inoculated with kanamycin resistant gene and two 250mL flasks with ampicillin gene. It was kept at shaking incubation at 37^0C overnight. 1ml of the broth was taken in 12 autoclaved eppendorfs. It was centrifuged at 13000 rpm for 1 minute. The supernatant was discarded and pellet was retained. 1ml of 50% glycerol was dissolved in three eppendorfs containing kanamycin resistant culture and in three eppendorfs containing ampicillin resistant culture. 1ml of 70% glycerol was dissolved in three eppendorfs containing kanamycin resistant culture and in three eppendorfs containing ampicillin resistant culture. The cultures were properly mixed with glycerol. The cultures were initially kept at 4^0C for a day, then at -20^0C the next day and lastly stored at -80^0C. (Fig. 5)

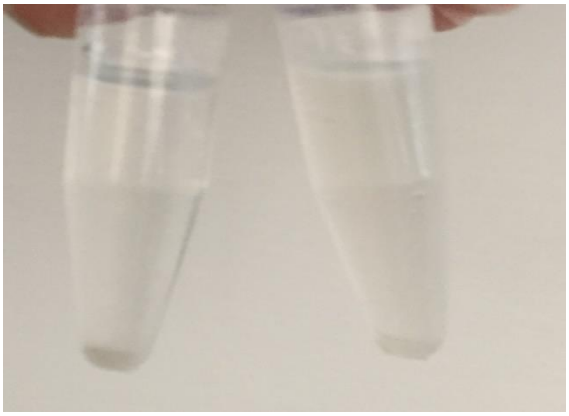


Fig. 5: 50%, 70% glycerol stocks

3.4.3. Buffer preparation for plasmid isolation

The following buffers were prepared for the isolation of plasmid.

Alkaline Lysis Solution I:

50mM glucose

25mM Tris-Cl (pH 8.0)

10mM EDTA (pH 8.0)

Solution I was prepared from standard stocks in batches of approximately 100 mL. It was then autoclaved for 15 minutes at 15 psi (1.05 kg/cm2) on liquid cycle, and stored at 4°C for plasmid preparation.

Alkaline Lysis Solution II:

0.2N NaOH (freshly diluted from a 10 N stock)

1% (w/v) SDS

Solution II was prepared fresh and used at room temperature for plasmid preparation.

Alkaline Lysis Solution III:

5M potassium acetate-60.0 mL

Glacial acetic acid-11.5 mL

H2O-28.5 mL

The resulting solution was 3 M with respect to potassium and 5 M with respect to acetate. The solution was stored at 4 ^{0} C and was transferred to an ice bucket just before use.

3.4.4. Plasmid isolation

- 1.5 ml of culture was taken in vial and was centrifuged at 8000rpm/3min/4^{0}C.
- The supernatant was discarded and add 20ul of alkaline solution 1 was added into the pellet.
- It was incubated for 5 min. and vortexed and mixed well by inverting.
- 50ul of alkaline solution 2 was added and mixed well.
- It was then incubated for 5mins.
- 40ul of solution 3 was added. It was then inverted immediately and incubated on ice for 30min.
- It was centrifuged at 12000 rpm for 15 min at 4^{0}C.
- The supernatant was taken and Rnase A was added i.e. 0.7ul.
- It was then incubated for 2 hours at 37^0.
- Equal volume of chloroform isoamyl alcohol was added.
- It was then vortexed for 90 sec.
- It was then centrifuged at 13000 rpm (RT-20mins).
- The aqueous layer was taken and the above three steps were repeated.
- 150ul of isopropanol was added and incubated 20 min at -20^{0}C.
- It was centrifuged at 13000 rpm at 4^{0}C for 20 min.
- It was the washed with 70% ethanol and dried completely.
- 30ul of autoclaved distilled water was added to the vial.

3.4.5 Gel electrophoresis

- Weighed 1gm of agarose and added to 100ml 1X TAE buffer.

- Kept the conical flask in the oven till a clear solution was obtained.
- As it cooled down two (50-55^{0}C), ethidium bromide was added.
- The solution was poured into a gel caster ad comb was placed.
- Electrophoretic chamber was filled with buffer and the solidified gel along with caster was placed in it.
- The samples were mixed with loading dye and loaded into the wells.
- Electrodes were connected and gel was run at 100V till the loading dye has moved 2/3 of gel's length.
- Gel was removed and observed in gel doc system.

3.4.6. Transformation using electroporation

This was done to increase the permeability of the cell membrane allowing the gene to be introduced into the cell.

The cell cultures were added to two 50mL tarsons and were chilled on ice before the addition of 10% Tween-20 solution. The solution was added at 1/2000(v/v) for the pelleting of algal cells. The cells were then collected by centrifugation at 8000rpm at 4^{o}C for a time period of 5 minutes. The cells were then resuspended in BG-11 media to obtain a final density of 1X10^8 and 4X10^8 cells per ml. The above procedure was carried out in ice. Then, under standard conditions, 10ug/mL of previously isolated plasmid was added to the tarsons. This cell suspension was further transferred to two disposable electroporation cuvettes with 4-mn gap. These cuvettes were then immersed in a water bath to maintain a specific temperature i.e. 20^{o}C to 25^{o}C. An electric pule of 2400v/cm was applied to the sample using GTE-10 electroporation model. Following this, the cuvettes were removed from the apparatus and incubated at 25^{o}C in a water bath for atleast 5 minutes.

4.6.7. Plating of the electroporated cells

After electroporation, the electroporated microalgae cells were plated onto solid BG-11 media containing hygromycin concentration 40mg/l for *Scenedesmus dimorphus* and 60mg/l for *Chlorella spp.* The plates were incubated at 18-25^{0}C temperature under 3000-5000 lux light

intensity and 16:8 hours (light: dark) cycle to check whether the transformed cells are growing in this hygromycin concentrations.

4.7. Screening of the transformed cells

After checking the growth in the plates containing hygromycin concentration i.e. 40mg/l and 60mg/l for *Scenedesmus dimorphus* and *Chlorella spp.* respectively, the algae colonies were inoculated in 250ml flasks containing 50ml liquid BG-11 media with different hygromycin concentrations i.e. 40mg/l, 60mg/l and 80mg/l for *Scenedesmus dimorphus* and 60mg/l and 80mg/l for *Chlorella spp.* The flasks were incubated at 18-25^{0}C temperature under 3000-5000 lux light intensity and 16:8 hours (light: dark) cycle and were observed on daily basis.

Table 1: Components of BG-11 media

Stock	Chemical	1 X (g/L)	1000X (mg/L)
1	Na_2Mg EDTA	0.1	100
	Ferric Ammonium	0.6	600
	Citrate	0.6	600
	Citric acid.H_2O	3.6	3600
	$CaCl_2.2H_2O$		
2	$MgSo_4.7H_2O$	7.5	7500
3	$K_2HPO_3.3H_2O$	4.0	4000
	(or K_2HPO_4)	3.05	3050
4	H_3BO_3	2.86	2860
	$MnCl_2.4H_2O$	1.81	1810
	$ZnSO_4.7H_2O$	0.222	222
	$CuSO_4.5H_2O$	0.079	79
	$CoCl_2.6H_2O$	0.050	50
	$NaMoSo_4.2H_2O$	0.391	391
	MoO_4	0.018	18

4. RESULTS AND DISCUSSIONS

4.1. Hygromycin sensitivity test

4.1.1. Hygromycin sensitivity test in solid medium

Different hygromycin concentrations were monitored for the growth of *Scenedesmus dimorphus* in solid BG-11 medium. Plates containing hygromycin concentration higher than 20mg/l did not show any cell growth. Concentration of 40mg/l hygromycin was chosen as the selection medium for *Scenedesmus dimorphus* as this is the lowest concentration of hygromycin in solid BG-11 medium in which the growth of the algae was inhibited. (Fig. 6)

Growth of *Chlorella spp.* in solid BG-11 medium containing hygromycin concentrations 20mg/l and 40mg/l, was found to be insensitive but sensitive to the concentration of 60mg/l and 80mg/l. 60mg/l was chosen as the selection medium for *Chlorella spp.* (Anila et al) (35) reported that 75mg/l hygromycin concentration result in complete inhibition of *Dunaliella bardawil* in solid TAP medium. (Fig. 7)

4.1.2. Hygromycin sensitivity in liquid media

The number of cells/ml of *Scenedesmus dimorphus* was found to be increased from 1st to 10th day. Culture with media having hygromycin at concentration of 20mg/l and 40mg/l. Control with BG-11 media was also observed. It was observed that the cell density gradually decreased in media with hygromycin concentration of 60mg/l and 80mg/l. This indicates that hygromycin concentration below 60mg/l did not affect the growth of algae but higher concentration did. At this particular concentrations the number of cells/ml on 1st day was 10.25×10^6 and then got decreased to 8.4×10^6 on 4th day, 9.11×10^6 and on 10th day it came down to 6.5×10^6. Since, there was decrease in cell count so 60mg/l concentration of hygromycin in liquid BG-11 is used as selective medium for selection transformants. (Fig. 8)

Chlorella spp. was found insensitive to hygromycin at concentration 20mg/l, 40mg/l and 60mg/l but was sensitive when tested in higher concentration. In media containing 80mg/l hygromycin concentration, the number of cells/ml as counted on 1^{st}, 4^{th}, 7^{th}, 10^{th} days were 18.25×10^6, 12.02×10^6, 11.24×10^6 and 10.4×10^6. The number of cells decreased gradually at this concentration thus the dose of hygromycin for *Chlorella spp*. would be 80mg/l. According to a study by Pratheesh et al. (36), growth of *Chlamydomonas reinhardtii* is completely inhibited at hygromycin concentration of 5mg/l. (Fig. 9)

4.2. Islolation of plasmid

The pChlamy_1 vector was obtained from International Center for Genetic Engineering and Biotechnology, New Delhi by Dr. Shashi Kumar (38). The plates containing the vector were maintained and plasmid was isolated thereafter. Positive result of isolation of plasmid was successfully observed. The concentration of plasmid came out to be 12µg/ml. (Fig. 10)

4.2.1. Preparation of Algae for electroporation

Before preparation of algae culture for electroporation the cell density was calculated. The cell density came out to be 6.5×10^6 for *Scenedesmus dimorphus* and 10.04×10^6 for *Chlorella spp.* The algal culture was treated with 2% cellulose for cell wall was breakage. It was then centrifuged and the pellet was suspended in BG-11 medium. For electroporation the algae cell count should be 4.5×10^6 for Scenedesmus dimorphus and 6.9×10^6.

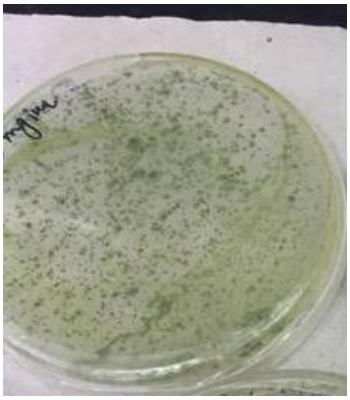
4.2.2. Electroporation

After taking the concentration of algae and plasmid for electroporation, the electroporation process was performed. After the process, the culture of *Scenedesmus dimorphus* and *Chlorella spp.* was plated in solid BG-11 media containing hygromycin concentration of 40mg/l for scenedesmus dimorphus and 60 mg/l for chlorella spp. The growth was observed in the plates after incubation at 18-25^{0}C. (Fig. 11)

4.2.3. Screening of transformants

The colonies of algal culture grown in plates after electroporation was inoculated in inhibitory concentration of hygromycin i.e. 40mg/l, 60mg/l, 80mg,l for *Scenedesmus dimorphus* and 60mg/l, 80mg/l for *Chlorella spp.*. The growth was seen in 40mg/l, 60mg/l and 80mg/l for *Scenedesmus dimorphus* (Fig. 12) and for *Chlorella spp.* growth was seen in 60mg/l and 80mg/l (Fig. 13).

5. FIGURES



CONTROL

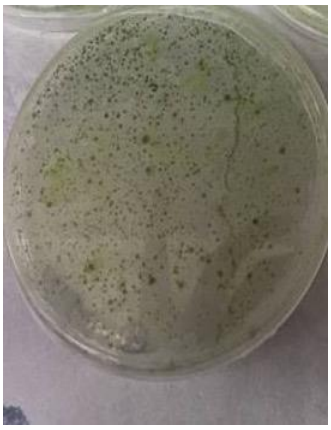
20mg/l

40mg/l

60mg/l

80mg/l

Fig. 6: Hgromycin anitibiotic sensitivity test of *Scenedesmus dimorphus* in solid medium



CONTROL

20mg/l

40mg/l

60mg/l

80mg/l

Fig. 7: Hygromycin antibiotic sensitivity test of *Chlorella spp.* in solid medium

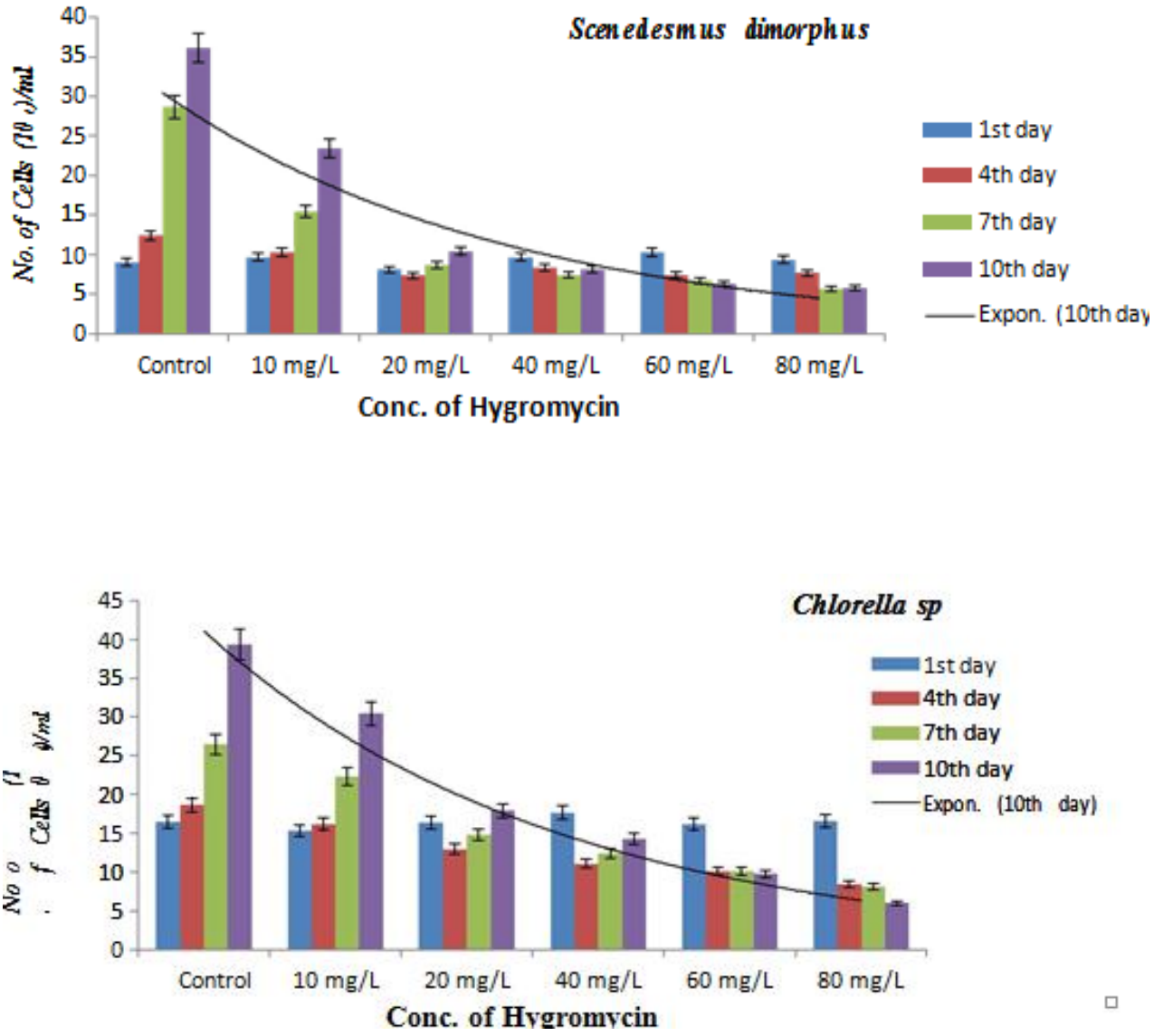
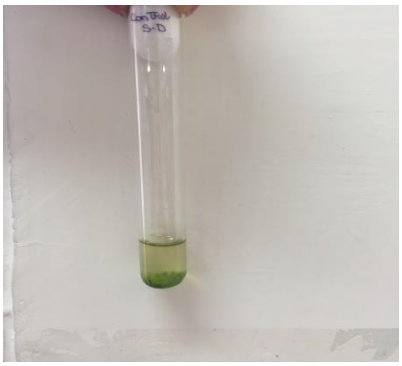
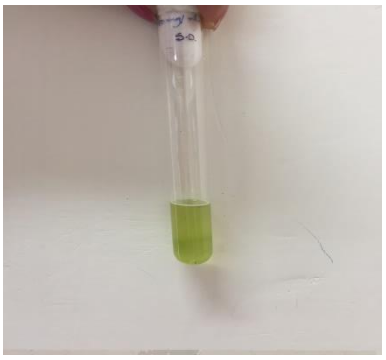


Fig. 8: Growth response of *Scenedesmus dimorphus* and *Chlorella spp.* respectively measured as no. of cells/ml of culture in different concentration of hygromycin

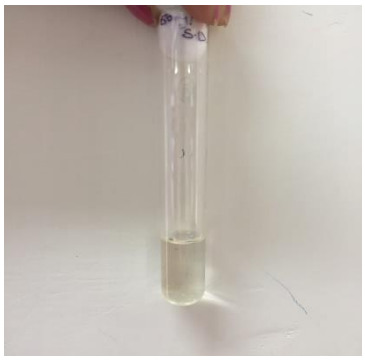


CONTROL

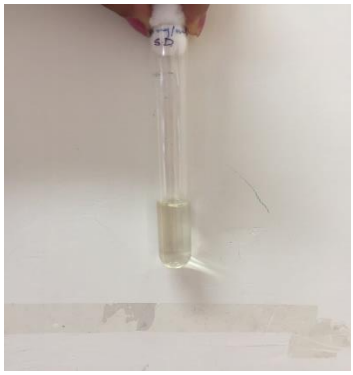
20mg/l



40mg/l

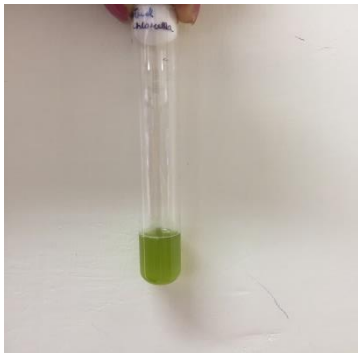


60mg/l



80mg/l

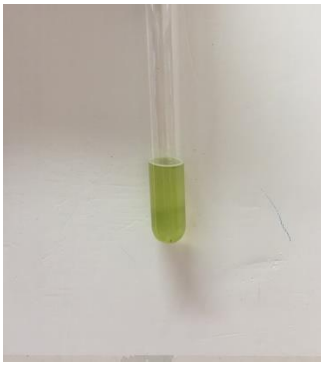
Fig. 9: Hygromycin antibiotic sensitivity test of *Scenedesmus dimorphus* in liquid medium



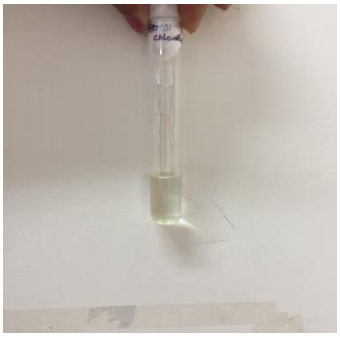
CONTROL

20mg/l

40mg/l



60mg/l



80mg/l

Fig. 10: Hygromycin antibiotic sensitivity test of *Chlorella spp.* in liquid media

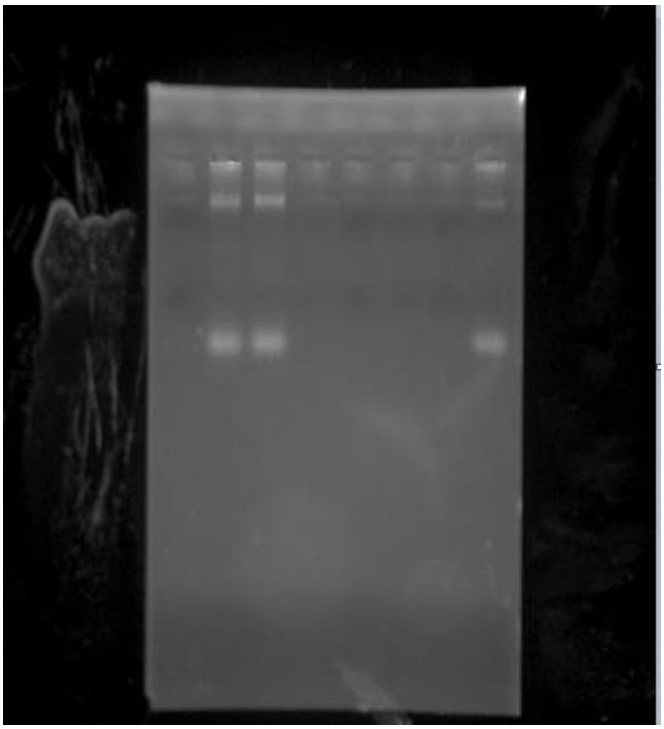


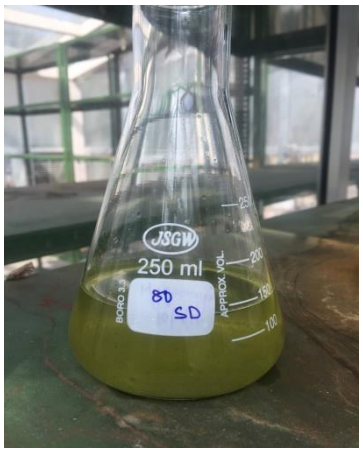
Fig. 11: Plasmid isolation for genetic transformation

Fig. 12: Electroporated cells at 40mg/l and 60mg/l i.e. inhibitory hygromycin concentration for *Scenedesmus dimorphus* and *Chlorella spp.*

20mg/l

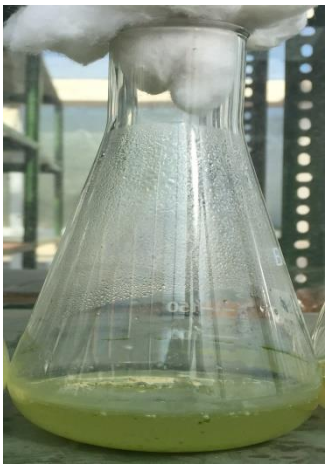
40mg/l

60mg/l



80mg/l

Fig. 13: Growth of electroporated cells in inhibitory concentration of hygromycin for *Scenedesmus dimorphus*



20mg/l

40mg/l

60mg/l

80mg/l

Fig. 14: Growth of electroporated cells in inhibitory concentration of hygromycin for *Chlorella spp.*

CONCLUSION

Our work describes the integration of gene DGAT in microalgae *Scenedesmus dimorphus and Chlorella spp.* for the enhanced production of lipid which would further lead to the efficient production of biofuels. In our thesis we listed the various issues which led to usage of microalgae for biofuel production, food versus fuel being the main reason.

Through our research we found out that electroporation is an efficient technique for transfer of gene DGAT in microalgae, still certain standardization of the protocol is needed in order to obtain better results of transformation which not only increases algal growth but lipid production as well.

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