# Biotechnological Interventions for Strain Improvement in *Scenedesmus dimorphus, Scenedesmus quadricauda* and *Chlorella spp*.

Enrollment No. - 111559

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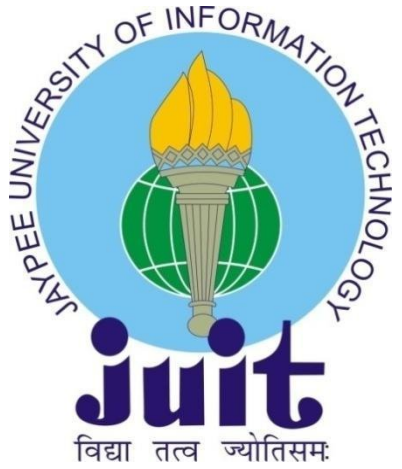


May 2015

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 —"Biotechnological Interventions for strain improvement in *Scenedesmus dimorphus, Scenedesmus quadricauda* and *Chlorella spp.*" submitted by **Shweta Tewari** 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 Dr. Anil Kant

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Date 27th May 2015

## Acknowledgement

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

Sincere thanks to Dr. Rajinder S. Chauhan, Dean, HOD, Biotechnology and Bioinformatics department, for being cooperative to the students of the department and providing relevant guidance in their endeavors.

I am also thankful and indebted to PhD scholar Mr. Aseem Chawla and Rakesh Singh Gaur who have been supervising me day and night and have been helping me at every odd hour acquainting me with the basics and subtleties of Molecular Biology, Microbiology and Genetic Engineering.

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## Summary

The cultivation of microalgae for biofuels in general and oil production in particular is not a near-term commercial prospect. This technology still needs considerable, long-term research and development. Microalgal strains present in nature possess only small number of desired qualities. Thus they are not sufficient for the performance demands for production of biodiesel. Genetic manipulation is the most plausible approach for obtaining microalgal strains which contain the desired key features. First objective of this study was to standardize protocol for *Agrobacterium* mediated genetic transformation of microalgae selected for the study. Hygromycin sensitivity test was done to figure out its minimum concentration for the selection of transformed algal cells of three microalgal species under this study. We have used the antibiotic hygromycin because the vector pCAMBIA 1301 has gene for hygromycin resistance. Thus, hygromycin resistance can be used as selectable marker for transformation experiments. The concentration selected for *Scnedesmus dimorphus* was 20 mg/L while for *Scenedesmus quadricauda* and *Chlorella spp.* it was 60 mg/L. These concentrations of hygromycin were selected because these were minimum concentrations in which no growth of microalgae was observed. The second objective was standardization of protocol for protoplast isolation. The isolated protoplasts were spherical in shape and when water was added to them, they burst as they had become permeable to water due to absence of cell wall. It was found that the best enzyme combination was 2% cellulase, 2% pectinase and 2% lysozyme. The mannitol concentration 0.6 M and incubation time 16 hrs were found to be best for protoplast isolation. This was applicable for all the three species.


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# List of symbols and acronyms

| | |
|---|---|
| BG-11 | -Blue green medium |
| GHGs | -Green House Gases |
| $CO_2$ | -Carbon dioxide |
| $Ca_2^+$ | -Calcium ion |
| $Mg_2^+$ | -Magnesium 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 |
| EDTA | -Ethylene diamine tetra acetic acid |
| MES | -2-(*N*-morpholino)ethanesulfonic acid |
| $gl^{-1}$ | -gram per liter |
| µm | -micro meter |
| ASTM | -American Society for Testing and Materials |
| ° C | -Degree centigrade |
| LAFC | -Laminar Air Flow Chamber |
| V/V | -volume by volume |
| mM | -milli molar |
| r.p.m. | -Revolutions per minute |
| TAG | -Triacylglycerol |

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# 1. Introduction

Increasing energy demand and potential fossil fuel depletion has become most important concern for people throughout the world. In addition, global warming and climatic changes possibly caused due to the emission of green house gases are becoming contemporary issues [1–3]. Possible solution is use of renewable energy sources like biomass, wind, solar, geothermal and hydropower. These are considered to decrease consumption of fossil fuels. Biomass is a renewable, carbon dioxide neutral and biodegradable source of energy with ample of resources, for instance, agricultural residue and waste, municipal solid waste, forestry waste, industrial waste, aquatic plants and terrestrial crops[4] . Among the available biofuel products from oil produced by terrestrial crops, biodiesel is at present an extensively used alternative fuel for transportation. However limited availability of land restricts the quantity of plant-based oil that can be generated for biodiesel production. Several microalgae as well as fungi species have capability of accumulating high lipid content that can be converted to many different forms of "drop-in" fuels like biodiesel [5–7]. Unlike conventional oilseed crops, microalgae show immense potential due to their wide range of applications. The algae biomass has potential to produce a variety of biofuels. Lipids from the algae biomass could be extracted and refined to fatty acids; the fatty acids can be further processed to produce biodiesel by transesterification. Gasification of the algal biomass by anaerobic digestion or thermal cracking can produce biogas. Carbohydrate fraction can be used for bioethanol production by direct fermentation. Pyrolysis or thermal degradation of biomass produces solid, liquid, and gaseous products. Anaerobic fermentation of biomass produces methane gas and direct combustion of biomass is used to generate power or syngas.

Advantages of using microalgae are that it exhibits higher biomass production, higher photosynthetic efficiency, industrial $CO_2$ removal, faster growth, and capability to grow in ponds as well as in fermentation units, therefore not requiring valuable farmlands and forests [8]. As compared to terrestrial biofuel crops like soybeans or corn, micro-algal production results in very less significant land footprint because of the higher oil yield from the microalgae than other oil crops [9]. Algae can also be cultivated on marginal lands not useful for ordinary crops, have low conservation value, and can utilize water from salt aquifers which is not useful for drinking or

agriculture [10]. Cultivation of algae requires no external subsidy of insecticides or herbicides, eliminating any risk of generating related pesticide waste streams. Moreover, in comparison to fuels like petroleum and diesel, the combustion of algal biofuel does not produce any oxides of sulfur, and produces a decreased amount of carbon monoxide, hydrocarbons which are left unburned and reduced release of harmful pollutants [11]. Algal biofuel contains safe, natural compounds that pose little to no environmental risk if spilled. When used in a bioreactor, harvested microalgae captures considerable amount of organic compounds together with heavy metal contaminants absorbed from wastewater streams which would else be directly discharged into surface and ground-water [9]. Moreover, using this process phosphorus, which is an important element but present in small amount in nature, can be recovered from the waste.

In spite of so many advantages of using microalgae there are various challenges to its use as a source for biofuel production. After searching a long time for suitable microalgal strains all through the world it has been concluded that such ideal strains are not likely to be present in nature. Most of the strains known till date have just one or only some characteristics. In contrast to crop plants which have been improved by means of breeding programs in order to meet certain essential traits, the availability of microalgae is restricted to strains which have been isolated. Thus genetic manipulation is a solution in order to obtain ideal strains having different desired features. [12-14].

Transgenesis in microalgae is a complicated and rapidly growing technology. Diverse methods have been developed for both nuclear and chloroplast transformation of microalgae. The nuclear transformations are primarily achieved by means of electroporation, agitation with glass beads or silicon carbide whisker. These protocols of transformation mainly use microalgae strains whose cell wall has been removed, except for particle bombardment. There are several disadvantages of direct gene delivery methods such as multiple copies of transgenes insertion as well as a high degree of rearrangement at the site where insertion has taken place. *Agrobacterium tumefaciens* mediated transformation has been known to overcome the majority of limitations encountered in these transformation processes. It provides stable integration at lower copy number and leads to lesser problems with transgene co-suppression and instability. The simplicity of the process, the ability to transfer comparatively large segment of DNA (up to 150 kb) with slight rearrangements, preferential incorporation of T-DNA into potentially transcribed regions and the integration of mostly single copy of the transgene(s) into plant chromosomes

have made this steadfast method for genetic transformation in dicotyledonous plants. There are few reports of *Agrobacterium* mediated genetic transformation mainly *Chlamydomonas reinhardtii* and few other green microalgae [41].

Through this study we took initiative to start work on *Agrobacterium* mediated genetic transformation of indigenously isolated *Scenedesmus spp*. and *Chlorella spp*. Protoplast isolation and culture is another biotechnological intervention through which genetic improvement of an organism is possible particularly if it cannot be directly bred as in case of algae. Protoplast isolation and cultivation have recently become of immense interest due to the potential use for a variety of strain improvement techniques such as fusion and uptake of different cell organelles and even genetic transformation. Thus another objective of this study was to investigate possibilities of protoplast isolation and culture of *Scenedesmus spp*. and *Chlorella spp*.

This study was undertaken with the following objectives:

1) Agrobacterium tumefaciens mediated genetic transformation of microalgae (*Scenedesmus dimorphus, Scenedesmus quadricauda* and *Chlorella spp*.)

2) To standardize the protocol for protoplast isolation in *Scenedesmus dimorphus*, *Scenedesmus quadricauda and Chlorella spp.*

# 2. Review of literature

## 2.1. Characteristics of microalgae

Microalgae, known as one of the oldest living organisms, are thallophytes that contain chlorophyll 'a' as their principal photosynthetic pigment and do not have a sterile casing of cells around the reproductive cells [15]. They have very high growth rate and can double their numbers every few hours, can be harvested daily and have the potential to produce a volume of biomass many times greater than that of most productive crops. They are capable of converting solar energy more efficiently than higher plants due to their simple cellular structure. Moreover as the cells grow in water, they can access water, $CO_2$ and several other nutrients more easily [16]. Algae store energy in the form of oils and carbohydrates which combined with their high productivity, can produce from 2,000 to as many as 5,000 gallons of biofuels per acre per year Microalgae can be cultivated to have a high protein and oil content which can be used to produce either biofuels or animal feeds, or both. In addition, microalgal biomass, which is rich in micronutrients, is already used for dietary supplements to advance human health. Microalgae can be cultivated to produce a variety of products for large to small markets: plastics, chemical feedstocks, lubricants, fertilizers, and even cosmetics.

Conventionally microalgae have been divided into various categories on the basis of their color. The present systems of classifying microalgae are according to the following main criteria: types of pigments, chemical nature of storage products and cell wall composition. Additionally, the following cytological and morphological features such as: occurrence of flagellate cells, organization of the flagella, scheme and path of nuclear as well as cell division, occurrence of an envelope of endoplasmic reticulum surrounding the chloroplast, and probable connection between the nuclear membrane and the endoplasmic reticulum are also taken into consideration [17]. Microalgae are of two basic types: prokaryotic and eukaryotic. Prokaryotic cells lack membrane-bounded organelles and occur in the cyanobacteria. The other microalgae are eukaryotic and contain organelles [18]. Microalgae can either be autotrophic or heterotrophic. They are capable of fixing carbon dioxide efficiently from diverse sources (e.g. the atmosphere, industrial exhaust gases, and soluble carbonate salts). Carbon dioxide fixation from atmosphere is perhaps the most basic method to sink carbon and is dependent on the mass transfer from air to

the microalgae during the process of photosynthesis. Numerous microalgal species have also been able to use carbonates like sodium carbonate and sodium bicarbonate for cell growth. A number of these species characteristically have elevated an extracellular carboanhydrase activity that is responsible to convert carbonate to free carbon dioxide in order to facilitate carbon dioxide assimilation. Additionally, the direct uptake of bicarbonate by an active transport system has also been found in several species [19].

## 2.2. Commercial applications of microalgae

Humans used microalgae for the first time 2000 years back when the Chinese used Nostoc in order to survive during famine, but, microalgal biotechnology only really started developing in the middle of the last century. Currently, there are several commercial applications of microalgae [20]. For example, (i) owing to their chemical composition, microalgae can be used to enhance the nutritional value of food as well as animal feed (ii) they play a vital role in aquaculture and (iii) they can be incorporated into cosmetics. Besides, they are cultivated as a source of highly valuable molecules. For instance, polyunsaturated fatty acid oils are used in infant formulas as well as nutritional supplements and pigments are important as natural dyes. Using transgenic mice fed with extracts from *Chlorella spp*. containing β-carotene and lutein, Nakashima *et al.* [21] claimed significant prevention of cognitive impairment. Likewise, carotenoids extracted specifically from *Chlorella ellipsoidea* and *Chlorella vulgaris* inhibited colon cancer development [22]. Furthermore, astaxanthin obtained from *Haematococcus pluvialis* decreased expression of cyclin D1, but increased that of p53 and some cyclin kinase inhibitors of colon cancer cell lines [23].

## 2.3. Microalgae as a potential source of biofuel

There are various ways in which microalgal biomass can be converted to energy sources. These can be categorized as biochemical conversion, direct combustion, thermochemical conversion and chemical reaction. Thus, microalgae can be utilized as feedstock for biofuels like biodiesel and bioethanol [24]. The idea of utilizing microalgae as biofuel feedstock is not recent, but it is now being taken seriously due to the increasing price of petroleum and more considerably the rising concern about global warming which is connected with burning of fossil fuels. The use of microalgae for production of biofuels offers various advantages over higher plants. These are as follows: (1) microalgae grow at greater rates than higher plants and are

capable of synthesizing and accumulating large quantities of neutral lipids. The average oil yield is reported between 1% and 70% but under certain conditions, some species can yield up to 90% of dry biomass weight [20]; (2) the yield of best oilseed crops could be greatly exceeded by oil yield per area of microalgae cultures as they are capable of all year round production; (3) microalgae require lesser water than terrestrial crops thus load on freshwater sources is reduced; (4) there is no need of herbicide or pesticide application in microalgae cultivation; (5) microalgae sequester $CO_2$ from flue gases released from fossil fuel-fired power plants as well as other sources, thereby decreasing emissions of the main greenhouse gas; (6) wastewater bioremediation by removal of ammonium , nitrate and phosphate from a range of wastewater sources like agricultural run-off, industrial and municipal wastewaters and concentrated animal feed operations [25]; (7) combined with their ability to grow under harsher conditions and their reduced needs for nutrients, microalgae can be cultivated in brackish/saline water/coastal seawater or on non-arable land easily and there is no competition for resources with conventional agriculture; (8) based on the microalgae species other compounds can also be obtained with important uses in various industrial sectors. They consist of a wide range of fine chemicals and bulk products like polyunsaturated fatty acids, pigments, natural dyes, antioxidants, polysaccharides, high-value bioactive compounds, and proteins [25].

It was estimated by Sheehan and his co-workers (1998) [26] that microalgal farming using 200,000 ha of land (less than 0.1% climately suitable lands in the U.S.) would allow the production of a quad (i.e., a quadrillion British thermal unit) of fuel in the form of biodiesel. Wu et al. (2007) [27] in China reported the production of biodiesel using *Chlorella protothecoides* at a scale of 11,000 L. They adopted a heterotrophic cultivation strategy which does not necessarily fulfill the mandate of microalgal farming to convert solar energy to biofuel. Instead, organic carbons such as glucose are used for fuel production. Recent trials in Italy using *Nannochloropsis spp.* in outdoor trials using nitrogen starvation strategy showed final lipid yields as high as 60%. Rodolfi et al. (2008) reported that 20 t/ha/yr lipid yield is realistically achievable in the Italian climate [28]. The Aquatic Species Program sponsored by the US Department of Energy estimated that algal oil yield of over 5,000 to 10,000 gallons per acre per year is possible compared with 50 to 100 gallons per acre per year for traditional oil crops such as soybean [29]. As discussd by Sydney et al. (2011) [30] *Botryococcus braunii* has ability to accumulate lipids without need for strict control of nitrogen levels. It would thus be expected to

attain 3300 kg lipids/ha/year if it were cultivated in 20 cm-deep lagoons of treated wastewater. This is 5-fold the productivity of soybean under identical conditions. In a study conducted by An-yue et al. (2011) [31] to identify some desirable algal strains for the production of algae-based biofuel, 43 green algal strains were successfully isolated from Chinese freshwaters, and then incubated in the laboratory bioreactors for the growth and oil accumulation investigations. Results indicated that highest biomass of 6.07 g/L was for green algae *Scenedesmus bijuga.* Green algae *Chlorella pyrenoidosa* was found to be one of the best oil producers among the selected species with the total lipid content of 51% of dry biomass. Taking the growth rates and the accumulations of intracellular lipids into the consideration, 10 strains were considered to have significant potential for biofuel applications. Guarnieri et al. (2011) [32] reported a comprehensive proteomic and transcriptomic investigation of lipid accumulation in the unsequenced green alga *Chlorella vulgaris*. This oleaginous species is known for its relatively fast growth rate, its value as a food supplement and a potential biofuel feedstock.

## 2.4. Strain improvement

Identification of a perfect and unchanged biofuel organism which fits into the established infrastructure for harvesting, extraction and purification is a possibility. A much more probable scenario is the identification of a number of species that each has one or a few of the traits which are desirable. These traits, when incorporated into a single strain can be sufficient to produce an economically viable production strain. Traits of microalgae that would significantly facilitate and improve the process of biodiesel production can be summarized into (i) rapid growth and, possibly, large size of cell for greater production of biomass, (ii) elevated lipid yield, (iii) the capability of secreting lipid into media, (iv) capability to adapt to environmental fluctuations and stress (v) the capability of forming flocs for trouble-free and low cost harvesting [33]. Some of major researches directed towards trait improvement or selection of microalgae with desired trait are discussed chronologically.

Benemann (1990) [34] observed that *Nannochloropsis*, a marine alga with high constitutive Triglyceride (oil) content, could be stressed with N limitation in batch culture to increase lipid productivity when light intensity was also increased. As evident form a study done by Schroda et al. (1999) [35] heat shock protein 70B (HSP 70 B) overexpressing Chlamydomonas exhibited greater photosynthetic efficiency because of the protection of

photosystem II. A genomic copy of HSP70B was cloned behind the fused Chlamydomonas HSP70A and ribulose bisphosphate carboxylase small subunit promoters. This promoter fusion exhibits high basal-level expression as well as inducibility by heat shock and light. Nakamoto etal. (2000) [36] demonstrated in their work that overexpression of chloroplast small heat shock protein (ch-sHSP) in *Synechococcus elongates* resulted in higher thermotolerance under light condition than the wild-type strain. A hexose symporter system from *Chlorella kessleri* was inserted specifically into strain Stm6glc4 of *Chlamydomona reinhardtii* to increase its hydrogen producing efficiency. This work was done by Doebbe et al. (2007) [37]. After this strain improvement it was able to efficiently produce hydrogen simultaneously via water photolysis and from external sugars. In an experiment done by Mussgnug et al. (2007) [38] LHC expression in transgenic *Chlamydomonas reinhardtii* was downregulated to increase the resistance to photooxidative damage and to enhance the efficiency of photosynthesis by 50%. This alteration allowed *Chlamydomonas reinhardtii* to tolerate photoinhibition. Several studies have shown metabolic shifts in starchless mutants of *Chlamydomonas reinhardtii* in favor of an overexpression of TAG. In a starchless mutant, Moellering et al. (2009) [39] inhibited the expression of myocardial lipid droplet proteins by RNAi. This increased the size of the lipid globules. This suggests that it is possible to improve the productivity of microalgae using lipid selection strategies. In expressing recombinant thioesterases to enhance the expression of shorter chain length fatty acids, Radakovits et al. (2010) [40] were able to improve the level of lauric and myristic acids in the diatom *Phaeodactylum tricornutum*. This creates an advantage for biofuel feedstock because biodiesel made from saturated short or medium chain length fatty acids has a relatively low cloud point and is resistant to oxidation. Recently, the phytoene synthase gene ( psy), the enzyme for the committing step in carotenoid synthesis, has been transformed in *Chlamydomonas reinhardtii* resulting in increased carotenoid accumulation [41].. Transgenic strains overexpressing psy from *Dunaliella salina* and *Chlorella zoofingensis* accumulated 2.6-fold and 2.2-fold more lutein (a commercially valuable carotenoid) than the controls, respectively [42]. Overexpression of homogentisate phytyltransferase vitamin E2 (VTE2), the first-committed step of tocopherol synthesis, led to higher protection against oxidative stress. This was concluded from a study by Li et al. (2012) [43]. *Chlamydomoas reinhardtii* strains were engineered to overproduce tocopherols by chloroplast transformation with non-codon optimized and codon optimized versions of the homogentisate phytyltransferase

vitamin E-2 from *Synchocystis* and by nuclear transformation with VTE-2 from *Chlamydomonas reinhardtii* , which resulted in 1.6-fold, 5-fold to 10-fold, and more than 10-fold increases in total tocopherol content respectively.

### 2.4.1. Methods for genetic transformation in microalgae

Several methods for microalgal gene transformation which have been developed were mainly based on *Chlamydomonas.* They alter the structure of cell wall of microalgae so that the passage of DNA through cell membrane is enabled. These methods are either enzymatic or mechanical [44, 45]. The glass bead method has been widely used for the transformation of *Chlamydomonas* since it is simple and highly efficient. Huge number of transformants can be obtained by simply vortexing the cells and DNA while making the use of glass beads in the presence of polyethylene glycol [46]. However cell wall must be removed by autolysin treatment before transformation or cell-wall less mutant should be used [46]. Therefore this method is presently restricted to *Chlamydomonas* as structures of cell wall of other microalgae are mostly not known. Efficient cell wall removal needs optimization of the transformation protocol. An alternative tool to glass bead is silicon carbide whisker. According to Dunahay (2006) [47], silicon carbide can be mixed and vortexed with the cells without the requirement of polyethylene glycol and removal of cell wall. Therefore, it can be utilized for transforming other microalgal species. However the limited supply and health related issues of silicon carbide make it less preferred in research.

After success of electroporation in bacteria, fungi, yeast and mammalian cells, it has been used for *Chlamydomonas* [48, 49]. The use of electric field enables the DNA fragment to enter the cells since the permeability of plasma membrane and its poration is enhanced. In comparison to the glass bead method, this method is highly efficient and can be utilized directly with intact cells [50]. Electroporation has been used for transformation of various species of microalgae. Several parameters like electrical voltage, concentration of salt or DNA, temperature are required to be verified in order to make transformation successful [51, 52]. In addition to physical method of transformation, gene gun or particle bombardment has also been used in microalgae like *Haematococcus* [53] and *Chlorella* [54].

*Agrobacterium* has been extensively used for genetic transformation in plants. It had recently shown to be useful in various other organisms like filamentous fungi [55], mammalian

cells [56] and in particular numerous microalgal species like *Chlamydomonas*, *Dunaliella salina* and *Haematococcus* [57, 58, 59]. In *Agrobacterium* mediated transformation the advantage is that fragment of DNA is transferred and is directly incorporated into the genome of microalgae and therefore provides stability to the transgene [58]. Moreover the transgene copy number is limited to only a few copies unlike the case of electroporation or particle bombardment where the copy number is high [45]. Low copy number insertion may be beneficial for stable gene expression as high copy number insertion sometimes result in gene silencing. The procedure is simple and low in cost; it needs only co-cultivation of microalgae and *Agrobacterium.*

Several successful attempts on genetic transformation of microalgae have been reported. Kumar et al. (2004) [60] reported that *Agrobacterium* mediated transformed *Chlamydomonas* cells were able to sustain hygromycin resistant phenotype for subsequent generations even when they were maintained in non selection media. Through a work done by Lerche et al. (2009) [61] stable nuclear transformation of *Gonium pectorale* was achieved using a heterologous dominant antibiotic resistance gene as a selectable marker. Heterologous 3'- and 5'-untranslated flanking sequences, including promoters, were from *Chlamydomonas reinhardtii* or from *Volvox carteri*. After particle gun bombardment of wild type *Gonium* cells with plasmid-coated gold particles the transformants were recovered. The transformants were able to grow in the presence of the antibiotic paromomycin [61]. Kathiresan and co-workers (2009) reported *Agrobacterium* mediated transformation in Haematococcus *pluvialis* [62]. The transformants were stable for more than 2.5 years. In 2011, in a study by Anila et al. *Dunaliella salina* was transformed efficiently with the help of Agrobacterium [59]. Southern blotting of DNA extracted from hygromycin resistant cells that were 15–18 months old established the presence of the integrated transgene in the DNA of *Dunaliella salina* [59]. Cheng et al. (2012) [63] developed a novel transformation approach for *Schizochytrium* (a marine microalga) using the *Agrobacterium tumefaciens* binary vector system. After co-cultivation of *Schizochytrium* with *Agrobacterium* harboring pCAMBIA2301 containing the neomycin phosphotransferase II (*NPT II*) gene as the selectable marker which confers resistance to antibiotic geneticin, the *Schizochytrium* transformants were successfully obtained on the geneticin containing plates. Though transformation has been performed successfully in few species still there are many obstacles to overcome. These are as mentioned below:

### 2.4.2. Bottlenecks in genetic engineering of microalgae and future challenges

Though there are several transformation procedures developed for microalgae, these have been developed on the basis of a few species typically *Chlamydomonas.* These methods cannot be used as it is for other species of microalgae without additional evaluation and optimization. Applicability of every method is different for different microalgal species and the appropriate method for every species must be verified. For instance, the glass bead method is extremely efficient for transformation of *Chlamydomonas* but the same method yields an awfully poor rate of transformation in other species [64]. Thus while obtaining new species, an optimized protocol for transformation needs to be developed. Conditions for selection particularly the antibiotic concentration are required to be examined carefully in order to ensure efficient selection of transformants.

One of the main barriers in microalgal transformation is how to get significant transgene expression in microalgae. At the level of transcription, regulatory elements present in the expression cassette are essential for correct and efficient expression of gene. Promoters existing for microalgae are presently limited in number. Thus promoter sequences from one organism could offer insufficient recognition in various other organisms and as a result there is low or no transcription.

An additional chief problem which has no solutions currently is that transgene expression is unstable. The reason for this could be that there is no incorporation of the transgene into the chromosome of algae and the transgene is replicated episomally. Several earlier studies have shown that the expression of transgene was suppressed following their maintenance in non-selective media for a long duration of time. The silencing effects may be because of several aspects of epigenetic controls which are associated with the response of virus invasion, transposable element or transgene found in eukaryotic system. This prohibits the practicality of growing transgenic microalgae in a large scale or an open pond [65]. This is because adding antibiotics in a large-scale culture is not considered cost-effective. Moreover the transgene expression in transgenic cells will steadily be suppressed or eliminated at later stages [66].

### 2.4.3. Protoplast isolation and culture

Microalgal cell walls are complex and poorly understood [67]. The variation in cell walls of different species as well as variations observed in a single strain grown under dissimilar conditions can be remarkable. Therefore it is difficult to predict which of the compounds will be present in any one strain. For example while some Chlorella species have only a single microfibrillar layer others have two layers with the microfibrillar layer proximal to the cytoplasmic membrane and a mono or trilaminar outer layer [68]. The cell walls of various green microalgae are known to have rigid wall components embedded within a more plastic polymeric matrix. This matrix contains uronic acids, rhamnose, arabinose, fucose, xylose, mannose, galactose, and glucose. The rigid, trifluoroacetic acid resistant cell wall is either glucosamine or a glucosemannose polymer [69].

While mutation and selection techniques are conventionally used for strain improvement, genetic manipulations such as protoplast fusion and DNA-mediated transformation are considered to be more effective [70]. These procedures require the efficient isolation and regeneration of protoplasts. Induction of naked protoplast formation from microalgae has been a matter of concern and interest for a long time. Although several methods are available for the release and regeneration of protoplasts no single method is universally applicable.

Various parameters need to be standardized for optimization of protocol for protoplast isolation. First parameter which must be analyzed is concentration of cell-wall degrading enzymes. The concentration of enzymes is critical for complete protoplast release. A growth inhibition screen demonstrated that cellulase, chitinase, lysozyme, pectinase, sulfatase, b-glucuronidase, and laminarinase had the broadest effect across the various *Chlorella* strains tested and also inhibited *Nannochloropsis* and *Nannochloris* strains [70]. According to a study on *Dendrobium crumenatum,* the protoplasts obtained from the isolation solution containing 2% (w/v) pectinase and cellulase were spherical in shape and well separated. In contrast, the protoplasts obtained from the protoplast isolation solution containing 1% (w/v) pectinase and cellulase were aggregated. The yield of protoplast by using 2% (w/v) enzymes (pectinase and cellulase), was more than 2-fold higher than that from 1% (w/v) enzymes isolation solution. This study suggested that 2% of both enzymes used were more suitable for protoplast releasing [71].

Another parameter is concentration of osmotic stabilizer which is also very important in protoplast isolation. Appropriate concentration of osmoticum can prevent protoplasts from bursting or shrinking. In general, the concentration of osmoticum used for protoplast isolation could be within the range of 1.0 M. For instance, 0.4 M sorbitol was the optimum concentration used to obtain high yield of protoplast for winged bean, $6.5\times10^6$ protoplasts/g fresh weight of explants [73] and 0.6 M mannitol; and 0.6 M sorbitol was the suitable osmotic stabilizers for obtaining good protoplasts yield of Antarctic sea ice algae [74]. Sorbitol and mannitol are popular osmoticums use for protoplast isolation.

Third parameter to be analyzed is incubation time. After a study on *Chlorella protothecoides*, the results showed that incubation time was an important factor influencing the yield of protoplasts. The density of protoplasts increases with extended time and reaches to maximum when treated for 16 hrs. The incubation time required for releasing protoplasts could also be influenced by the enzyme concentration and compositions of the protoplast isolation solution used. The longer incubation time is required when the concentration of enzymes is lower [75].

# 3. Materials and methods

The culture isolates of *Scenedesmus dimorphus*, *Scenedesmus quadricauda* and *Chlorella spp*. isolated in our lab previously (Gour and Kant et al. 2015) were used in all the experiments in this study.

## 3.1. Culture conditions

BG11 media (1000 ml) was taken into three, 1500 ml Erlenmeyer conical flasks and was autoclaved. In sterile condition (in LAFC), 10% of culture (Strain no. 12 of *Scenedesmus dimorphus*, strain no. 19 of *Scenedesmus quadricauda* and strain no. 16 of *Chlorella spp.)* was used to inoculate 1000 ml of media. Light intensity used for culturing the microalgae was between 3000-5000 Lux. Temperature was in the range of 18-25° C. pH of the 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

In order to prepare this media 1 ml of each stock solution (composition as given in table no. 1) was taken. $Na_2CO_3$ (0.02 gm) and $NaNO_3$ (1.5 gm) were also added in 1 liter of distilled water and autoclaved. Stock 1, 2 and 3 should be filter sterilized or autoclaved and transferred to 4 °C cold room for storage.

### 3.2.2. Luria broth

Preparation of this media required suspension of 20 gm powder in 1L distilled water. It was autoclaved for 15 minutes at 121˚C to sterilize.

## 3.3. Hygromycin sensitivity test

Hygromycin sensitivity test was done to get its minimum concentration which is detrimental to the growth of the microalgal species under study. These hygromycin concentrations would be used for selection of transformants. The algae were cultured in both solid and liquid BG-11 media supplemented with hygromycin. Concentrations of hygromycin used were 10mg/ml, 20mg/ml, 40mg/ml, 60mg/ml and 80mg/ml. When plated on the solid media, 50µl algal culture was spread on the Petri plates while for liquid media inoculum 10% (V/V) was added to the test tubes containing liquid media. The Petri plates as well as the test tubes were incubated at 18-25 °C temperature under 3000-5000 lux light intensity and 16:8 hours (light: dark) cycle. Plates were observed daily to check whether growth was present or not. 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. Isolation of vector

Binary vector pCAMBIA1301 was isolated from *E. coli* using alkaline lysis method. Three alkaline lysis solutions were used and their composition was as follows:

Alkaline lysis solution I:

50 mM Glucose

25 mM Tris-Cl (pH 8.0)

10 mM EDTA (pH 8.0)

Alkaline lysis solution II (freshly prepared):

0.2 N NaOH

1% (w/v) SDS

Alkaline lysis solution III:

5 M Potassium acetate, 60 ml

Glacial acetic acid, 11.5 ml

Distilled water, 28.5 ml

Luria broth medium was inoculated with a single colony of *E. coli* and was incubated overnight with vigorous shaking. 1.5 ml of the culture was poured into a microfuge tube and centrifuged at 7000 rpm for 30 seconds at 4˚C. The medium was removed by aspiration leaving the bacterial pellet. The bacterial pellet was resuspended in 100 µl of ice-cold alkaline lysis solution I by vigorous vortexing. 200 µl of freshly prepared alkaline lysis solution II was added and the contents were mixed by inverting the tube several times. After this 150 µl of ice-cold alkaline lysis solution III was added and the contents were mixed by inverting the tubes several times. It was stored on ice for 3-5 minutes. The bacterial lysate was centrifuged at 7000 rpm for

5 minutes at 4˚C and the supernatant transferred to a fresh tube. Equal volume of chloroform: isoamyl alcohol solution was added and mixed by vortexing. It was then centrifuged at 7000 rpm for 2 minutes at 4˚C and the upper layer transferred to a fresh tube. Nucleic acid was precipitated from the supernatant by adding 2 volumes of ethanol at room temperature. The solution was mixed by vortexing and allowed to stand for 2 minutes. The precipitated nucleic acid was collected by centrifugation at 7000 rpm for 5 minutes at 4˚C. Supernatant was removed by aspiration and the tube was made to stand in an inverted position on a paper towel to drain away the fluid. 1 ml of 70% ethanol was added to the pellet. The tube was inverted several times. DNA was recovered by centrifugation at 7000 rpm for 2 minutes at 4˚C. All of the supernatant was again removed by gentle aspiration and open tube stored at room temperature until the ethanol had evaporated. The nucleic acid was dissolved in 50 µl of TE.

### 3.4.2. Preparation of competent *Agrobacterium* cells

This was done to make the cells more likely to incorporate foreign DNA. Competent cells were prepared using ice cold $CaCl_2$ method. 50 ml LB medium was prepared and autoclaved. Overnight grown *Agrobacterium* culture (500 µl) was added to the media. It was grown overnight at 28˚C on a rotating shaker. Prior to inoculation, antibiotics kanamycin (50 mg/ml), gentamycin (25 mg/ml) and rifampicin (25 mg/ml) were added to LB. 45 ml of chilled culture was transferred to a pre chilled centrifuge tube and centrifuged at 4000 rpm for 10 minutes at 4˚C. The supernatant was removed and the pellet resuspended in 5.0 mL ice cold 20 mM calcium chloride, holding the tube in ice while working. It was then stored at -20˚C.

### 3.4.3. Transformation of *Agrobacterium tumefaciens*

This was done to introduce the isolated binary vector into the competent *Agrobacterium* cells. *Agrobacterium* was transformed using the vector pCAMBIA 1301. 10 µl of the isolated vector was added to 100 µl of competent cells. It was kept on ice for 10 minutes. Heat shock treatment was given by keeping it at 42˚C for 45 seconds. It was again kept on ice for 10 minutes. Finally, PCR analysis was done to validate if the vector had been inserted in *Agrobacterium*.

### 3.4.4. Co-cultivation of *Agrobacterium* with algae

Co-cultivation was done so that Agrobacterium could infect the algae and transfer its T-DNA through this the gene for hygromycin resistance can be incorporated into algal genome. *Agrobacterium* was inoculated in LB medium and kept overnight in a rotary shaker until $O.D_{600}$ was between 0.8-1.2. The bacterial and algal culture was harvested by centrifugation at 7000 rpm for 30 minutes. The supernatant was discarded whereas the algal and bacterial pellet obtained were mixed and resuspended in 5 ml BG-11 media containing 100 µM acetosyringone. It was incubated for 4 days at 25˚C. The procedure of co-cultivation was done as shown in Fig. 1

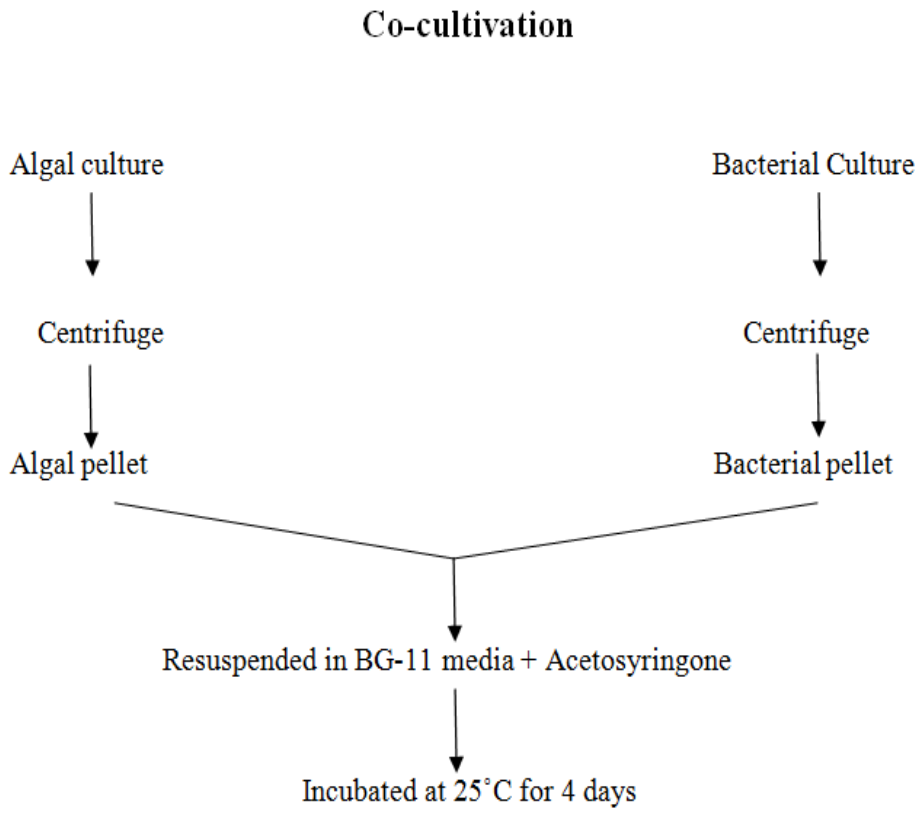


Figure 1: Co-cultivation of algal cells with *Agrobacterium tumefaciens.*

### 3.4.5 Selection of transformed algal cells:

This experiment was done to select the algal cells which have been transformed. After 4 days of co-cultivation cells were harvested and washed with BG-11 media containing 500mg/L cefotaxime via mild vortexing and centrifuging for 2 min (twice). After washing, the algal cells were plated on solid BG-11 media in the manner shown in fig 2.

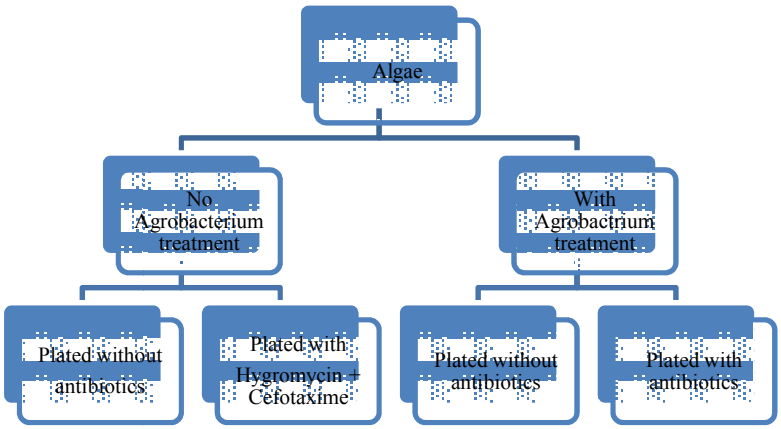


Figure 2: Plating of algal cells for selection of transformants.

The algal cells treated with *Agrobacterium* as well as without treated cells were taken. Both the types of cells were plated in two ways. They were plated on media supplemented with hygromycin and on media without hygromycin.

## 3.5. Standardization of protocol for protoplast isolation

This experiment was done in order to remove the cell-wall of the selected microalgal species by using cell-wall digesting enzymes. Firstly enzyme solution was prepared which contained 20 mM MES (pH 5.7), the required concentrations of enzymes being used, mannitol, 20mM KCl. The solution was warmed at 55˚C for 10 minutes. It was cooled to room temperature and 10 mM $CaCl_2$ was added. The enzyme solution was filter sterilized before use. Algal cultures were harvested at log phase by centrifugation. Pellet was resuspended in 25 mM Tris-Cl containing D-mannitol and cell wall degrading enzymes. Algal cells were incubated with enzymes for appropriate duration of time. Then the cells were centrifuged at 300g for 2 minutes. The deposit was resuspended in 1.5 M sugar solution

to separate protoplast. After being centrifuged at 300g for 3 minutes, the protoplast layer was transferred into 1 ml of 25 mM Tris-Cl containing D-mannitol for further analysis.

Different parameters were tested for protocol standardization. These parameters were:

1) Enzyme combinations - While preparing the enzyme solution the combinations and the concentrations of enzymes were varied. Three enzyme combinations which were tested to obtain the combination best suited for protoplast isolation are as under:

a) Combination 1: 2% cellulose, 2% pectinase and 2% cellulase
b) Combination 2: 1% cellulase, 1% pectinase and 1% cellulase
c) Combination 3: 2% cellulase and 1% pectinase

Once the enzyme combination had been found it was selected for further experiments.

2) Mannitol concentration: The concentration of the osmoticum was varied to determine which concentration gave maximum yield of the protoplast. Different mannitol concentrations used while preparing the enzyme solution were- 0.4 M, 0.5 M, 0.6 M, 0.7 and 0.8 M. The concentration of the osmoticum which gave highest yield was used for further experiments of standardization.
3) Incubation time: In order to select the time which is most appropriate for protoplast isolation and at which the protoplast yield is highest but the protoplasts are not damaged, various time durations were tested. Different incubation time were- 14 hrs, 16 hrs, 18 hrs and 20 hrs.

### 3.5.1Verification of isolated protoplast

Formation of osmotically labile cells (protoplasts) was examined by adding 0.1 ml of protoplast suspension to 0.9 ml of water and then counting disrupted cells under the microscope with the help of a haemocytometer. Protoplast yield was calculated for all the parameters for each species.

**Protoplast yield**

It was calculated using the formula:

$$\frac{\text{No. of isolated protoplasts per ml}}{\text{Initial no. of cells in control}} \times 100$$

Table 1: Components of BG 11 media

| Stock | Chemical | 1 X (g/L) | 1000 X (mg/L) |
|---|---|---|---|
| 1 | Na2MG EDTA | 0.1 | 100 |
| | Ferric ammonium | 0.6 | 600 |
| | citrate | 0.6 | 600 |
| | Citric Acid.H2O | 3.6 | 3600 |
| | CaCl2. 2H2O | | |
| 2 | MgSo4.7 H2O | 7.5 | 7500 |
| 3 | K2HPO3.3 H2O | 4.0 | 4000 |
| | (OR K2HPO4) | 3.05 | 3050 |
| 4 | H3BO3 | 2.86 | 28.6 |
| | MnCl2. 4 H2O | 1.81 | 18.1 |
| | ZnSO4. 7 H2O | 0.222 | 2.22 |
| | CuSO4.5 H2O | 0.079 | 0.79 |
| | CoCl2.6 H2O | 0.050 | 0.5 |
| | NaMoSO4.2 H2O | 0.391 | 3.91 |
| | (or MoO4) | 0.018 | 0.18 |

# 4. Results and discussions

## 4.1. Hygromycin sensitivity test

### 4.1.1. Hygromycin sensitivity in solid media

Growth of *Scenedesmus dimorphus* in solid BG-11 medium containing different hygromycin concentrations was monitored. Cells did not grow in solid medium containing hygromycin concentrations higher than 10 mg/l. Lowest concentration of hygromycin which did not allow growth of *Scenedesmus dimorphus* was found to be 20 mg/l (Fig. 3). Therefore solid BG-11 medium with 20 mg/l hygromycin was chosen as the selection medium.

*Scenedesmus quadricauda* was able to tolerate higher hygromycin concentrations as compared to *Scenedesmus dimorphus.* This was evident from Fig. 4 where cells were seen on the plates with hygromycin concentration 10 mg/l, 20 mg/l and 40 mg/l. No growth was observed on the plates containing concentration of antibiotic beyond 40 mg/l. This means that this microalga can resist the effect of the antibiotic hygromycin till the concentration 40 mg/l but beyond this concentration the growth of algae gets restricted. So 60 mg/l concentration was chosen to be used for selection of transformed cells in further experiments.

*Chlorella spp*. was also found to be insensitive to the hygromycin concentrations 10 mg/l, 20 mg/l, 40 mg/l (Fig. 5) but sensitive to the concentrations 60 and 80 mg/l. Microalgal growth was not observed on plates with the hygromycin concentrations higher than 40 mg/l. The minimum concentration of hygromycin required to inhibit the growth of un-transformed *Chlorella spp*. cells was found to be 60 mg/l. Anila et al. (2011) [59] reported that 75mg/l hygromycin results in complete killing of control *Dunaliella bardawil* in solid TAP medium which contains 0.2M NaCl. In *Chlamydomonas reinhardtii* 10 mg/l concentration of hygromycin in TAP agar media was found to completely inhibit its growth. This was evident from a study done by Pratheesh et al. (2012) [75].

### 4.1.2. Hygromycin sensitivity in liquid media

The number of cells/ml of *Scenedesmus dimorphus* was found to increase from 1st to 10th day cultured in media with no as well as in medium with hygromycin at concentration of 10 mg/l & 20 mg/l. On the other hand, the cell density decreased or there was no substantial increase when Scenedesmus dimorphus was cultured in media supplemented with hygromycin at 40 to 80 mg/l concentration. This indicates that hygromycin at concentration below 40 mg/l did not affect the growth of algae much (as evident from Fig. 6a) but at higher concentrations it affected the growth and multiplication of cells. At this concentration number of cells/ml on 1st day was $11.25x10^6$ then it decreased to $10.4x10^6$ on 4th day. On 7th day it was $9.11x10^6$ and on 10th day it was $9.6x10^6$. Since there is a decrease in number of cells/ml so we recommend 40 mg/l concentration of hygromycin to be used for selection of transformants.

When cell count was done for *Scenedesmus quadricauda*, it was observed that the number of cells/ml increased from 1st day to 10th day in media without hygromycin. Same was observed for concentrations 10 mg/l, 20 mg/l, 40 mg/l. But in media with hygromycin concentrations 60 mg/l and 80mg/l number of cells/ml were found to decrease from 1st day to 10th day (Fig. 6b). On 1st day the number of cells/ml was $11.75x10^6$. It reduced and was found to be $11.4x10^6$ on 4th day and $8.9x10^6$ on 7th day. It decreased further in the subsequent days and was $7.6x10^6$ on the 10th day. This shows that the hygromycin concentration below 60 mg/ml did not affect the growth of algae to a large extent but above that it retarded the algal growth. Thus, 60 mg/l concentration of hygromycin should be used for transformation experiments.

*Chlorella spp*. was also found tolerant to hygromycin at concentrations 10 mg/l, 20 mg/l, 40 mg/l but was affected when tested for higher concentrations (Fig. 6c). In media containing 60mg/l hygromycin concentration, the number of cells/ml as counted on 1st, 4th, 7th and 10th days were $18.25x10^6$, $11.7x10^6$, $11.63x10^6$ and $10.8x10^6$ respectively. As in this concentration the number of cells decreased with passage of days, the dose of hygromycin for *Chlorella spp*. will also be 60 mg/l. A hygromycin concentration of 5 mg/l completely inhibited growth of *Chlamydomonas reinhardtii* cells in TAP liquid media as reported by Pratheesh et al. [75].

## 4.2. Genetic transformation

Isolation of vector pCAMBIA 1301 was done using the alkaline lysis method and competent *Agrobacterium* cell were prepared which were transformed using the previously isolated vector. Analysis of transformation was done by PCR using hptII specific primers. If transformation had taken place, the hptII gene present in the vector would be amplified otherwise it will not be amplified. Positive result was obtained which means the vector was successfully inserted in *Agrobacterium* and it had been transformed (Fig. 7).

## 4.3. Selection of transformed algal cells

Once the *Agrobacterium* has been transformed with pCAMBIA it was co-cultivated with the microalgae. After this step the washed microalgal cells were plated on solid BG-11 media. Algal growth was observed on all the plates which did not contain hygromycin. On the plates containing hygromycin, ideally growth should have been observed for algae which were plated after co-cultivation with *Agrobacterium*. But, no growth was observed on any plate containing hygromycin. From this we can conclude that current *Agrobacterium* mediated transformation method is not favorable for the transformation of *Scenedesmus dimorphus* (Fig. 8), *Scenedesmus quadricauda* (Fig. 9) and *Chlorella spp.* (Fig. 10) and further experimentation is required with changes in variables and the degree of the experimental protocol.

## 4.4. Protoplast isolation

In some cells, cell wall was completely removed while in others it was removed partially. The cells whose cell-wall had been removed had become spherical in shape. Since cell wall maintains the shape of the cell, in its absence the original shape of the cell could not be maintained. The osmotically labile cells were found to burst or were about to burst. The reason for this is that due to removal of cell wall, the cell became permeable and water can easily enter the cell since it is hypotonic with respect to the cytoplasm. Therefore, the cells would burst. This verified that protoplast had been isolated because only those cells would burst which lack the cell wall. The images of isolated protoplast for *Chlorella spp*. (Fig. 11), *Scenedesmus dimorphus* (Fig. 12), *Scenedesmus quadricauda* (Fig. 13) have been compared with the original cells.

### 4.4.1. Effect of enzyme combination and concentration on protoplast yield

The protoplast yield in *Chlorella spp*. was found to be highest (20.17%) for enzyme combination 1 followed by 11.34% for enzyme combination 2 (Table 2, Fig. 14a). The result obtained indicates that the enzyme combination 1 i.e., cellulase 2%, pectinase 2% and lysozyme 2% was best among different combinations tested. Similar trend of protoplast yield was recorded in case of *Scenedesmus dimorphus* and *Scenedesmus quadricauda* in this study. Although protoplast yield was higher in both these species than that of *Chlorella spp*. when compared with respective treatments of enzyme combinations. The yield of *Scenedesmus quadricauda* protoplast was 61.54%, 33.60% & 27.69% (Table 3, Fig. 14b) and that of *Scenedesmus dimorphus* was 49.20%, 46.54% & 40.16% (Table 4, Fig. 14c )when enzyme combination 1, 2 and 3 were used for protoplast isolation. According to a study on *Dendrobium crumenatum*,by Tee et al. the protoplasts was successfully isolated using enzyme solution containing 2% (w/v) pectinase and cellulase [71].

The overall trend of data indicates that enzyme combination 1 was best for protoplast isolation across all these species. The data also indicate that protoplast isolation, as indicated by percentage of protoplasts isolated, was easier in case of *Scenedesmus quadricauda* followed by *Scenedesmus dimorphus* with tested enzyme combinations compared to *Chlorella spp*. This also in a way throws light on the cell wall composition of these species. It may be concluded that cell walls of *Scenedesmus quadricauda* and *Scenedesmus dimorphus* may consist primarily of cellulose and pectin fraction whereas that of *Chlorella spp*. is more complex. However, further investigations may be required to validate this conclusion.

### 4.4.2. Effect of osmoticum concentration on protoplast yield

Different concentrations of mannitol were used for this experiment. In *Chlorella spp*. 0.6 M was found to assist the isolation of maximum protoplast yield 40.22% followed by 0.5 M mannitol concentration which yielded 38.23%. The protoplast yield further reduced for 0.7 M, 0.8M and 0.4 M concentration of mannitol. (Fig. 15a, Table 5). *Scenedesmus quadricauda* also responded somewhat in the same way with highest yield of 57.31% at 0.6 M followed by 30.38%, 25.12%, 20.12% and 18.85 % for 0.5M, 0.7M, 0.8M & o.4 mannitol concentrations respectively. (Fig. 15b, Table 6). In *Scenedesmus dimorphus* yield of 15.16% was observed at

0.6 M mannitol concentration. This was maximum yield. Minimum was observed at 0.8M and was 5.32% (Fig. 15c,Table 7). Results showed that use of mannitol at 0.6 M concentration as an osmoticum in the enzyme solution together with enzyme combination 1 provided maximum protoplast yield. There was a significant difference in the yield at all concentration levels with highest yield in *Scenedesmus quadricauda* as compared to the other two species in the study. Protoplast yield for *Chlorella spp.* was more than that of *Scenedesmus quadricauda*. 0.6 M sorbitol concentration has been found suitable for good protoplast yield of Antarctic sea ice algae as discussed by Liu and co-workers (2006) in their study [73].

Since the cell wall composition varies among species, it may be assumed that osmoticum at different concentrations may have different effects on various species. The extremely low yield of protoplast in *Scenedesmus dimorphus* as compared to the other two species in the study, may suggest that mannitol is not a suitable osmoticum for this microalgae. As protoplasts yield was fairly well for *Scenedesmus quadricauda* and *Chlorella spp*., it can be concluded that mannitol can be used for successful isolation of protoplast from *Scenedesmus quadricauda* and *Chlorella spp*.

### 4.4.3. Effect of incubation period on protoplast yield

Among the different incubation time combined with enzyme combination 1 and 0.6 M mannitol, 16 hrs of enzyme action upon the *Chlorella spp*. provided the highest (28.57%) protoplast yield (Fig. 16a, Table 8). Incubation periods longer than 16 hours resulted in a decrease in yield of isolated protoplasts caused by increased membrane instability. *Scenedesmus quadricauda* and *Scenedesmus dimorphus* also followed the same pattern with highest yield of 36.18% (Fig. 16b, Table 9) and 15.16% (Table 10, Fig. 16c) respectively for 16 hrs incubation time. Supporting this are results from work done by Lu et al. (2012) [74] on *Chlorella protothecoides* where 16 hrs was chosen as the best incubation time for protoplast isolation. We can draw an inference that the incubation time less than 16 hrs was not sufficient for the enzymes for removing the cell wall while when incubated for 16 hrs, the protoplast yield was maximum and cell wall was removed efficiently. When the digestion time exceeded 16 hrs, the cells were damaged. So, in order to maintain a balance between maximum protoplast yield and preventing cells from damage, 16 hrs incubation time was selected as the most suitable for isolation of protoplasts.

# Figures

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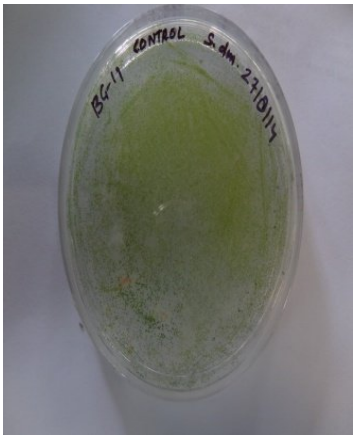

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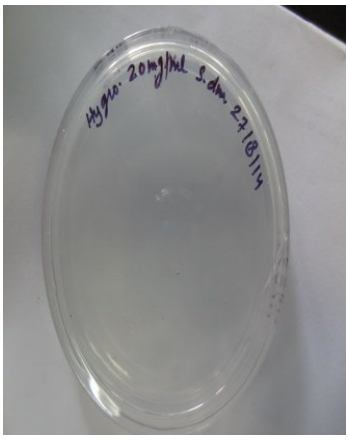

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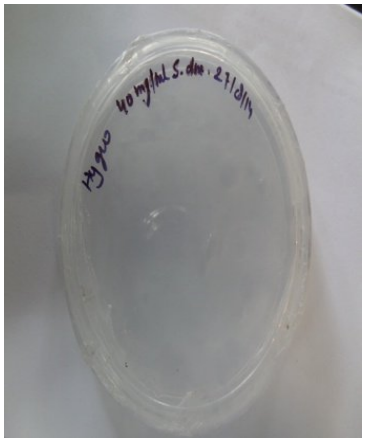

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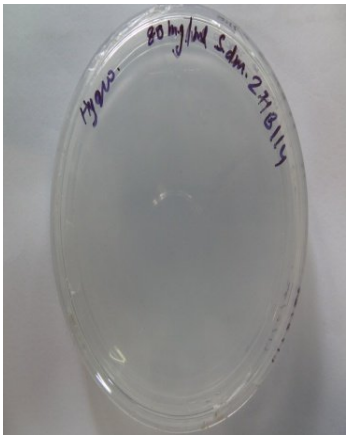

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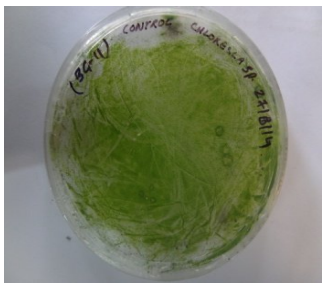
Fig 3: Hygromycin antibiotic sensitivity test of *Scenedesmus dimorphus*



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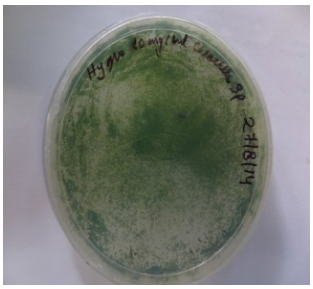

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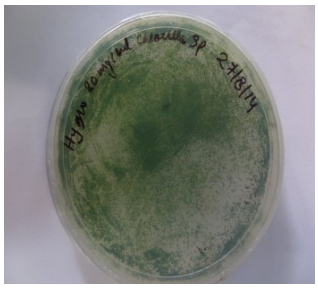

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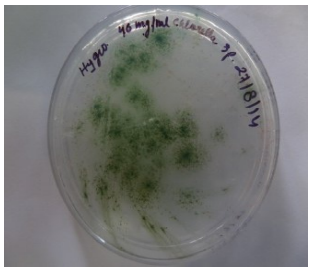

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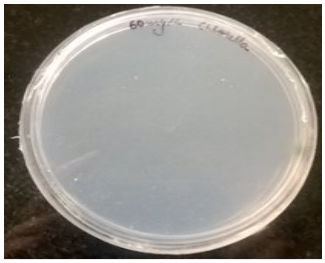

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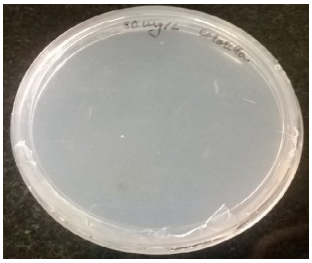

**80mg/L**

Fig 4: Hygromycin antibiotic sensitivity test of *Chlorella spp.*

**CONTROL**

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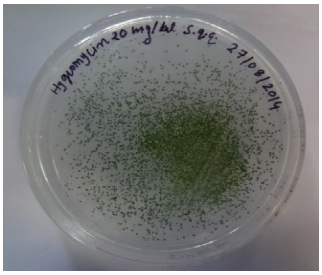

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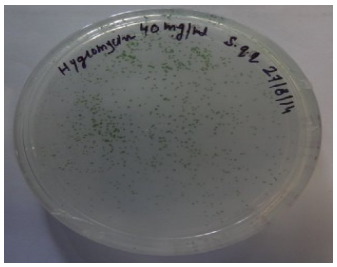

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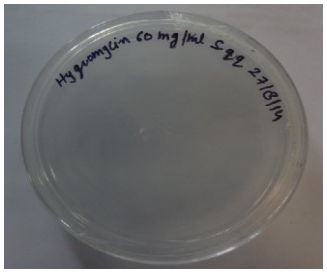

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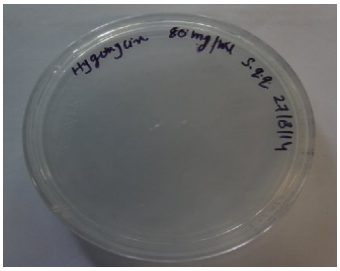
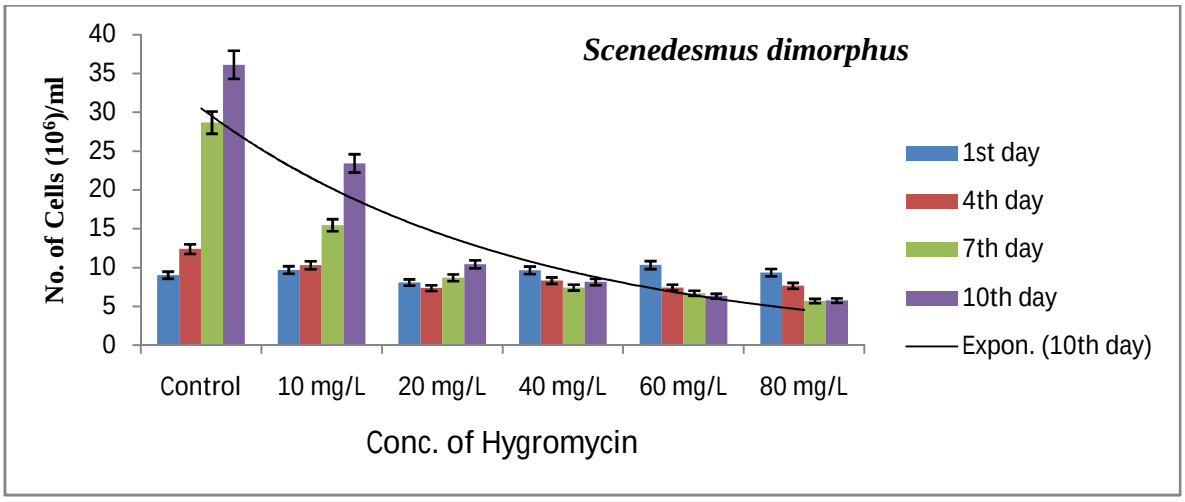


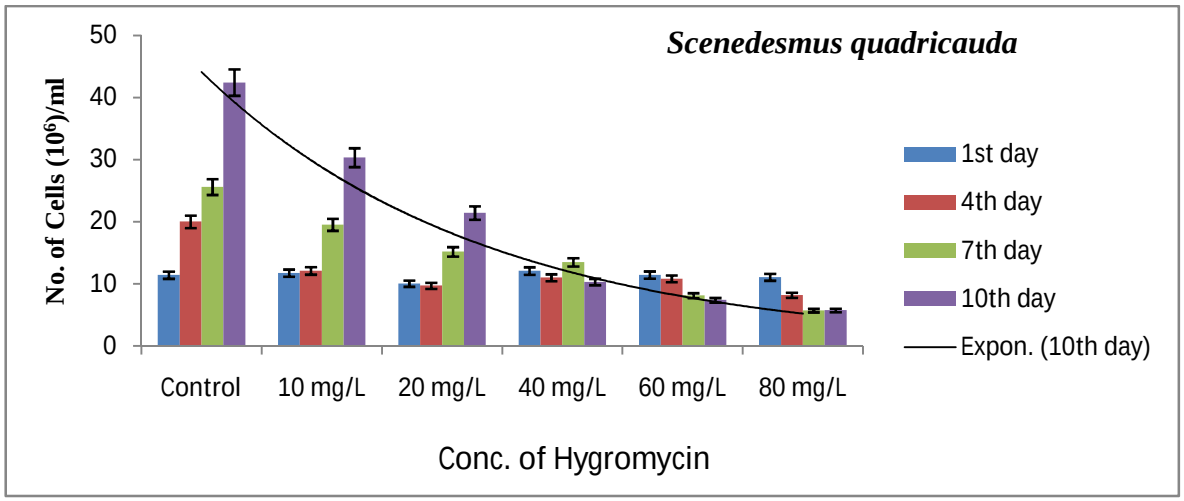
**80mg/L**

Fig 5: Hygromycin antibiotic sensitivity test of Scenedesmus *quadricauda*

a)




b)




c)

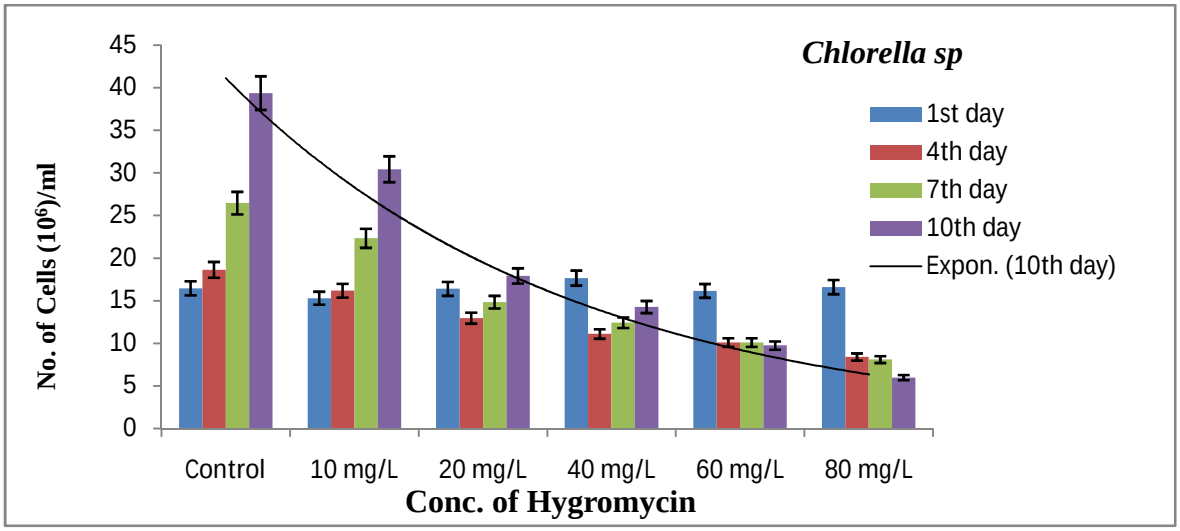



Fig 6: Growth response of *Scenedesmus dimorphus* (a), *Scenedesmus quadricauda*(b) and *Chlorella spp.*(c) measured as no. of cells/ml of culture in different concentration of hygromycin

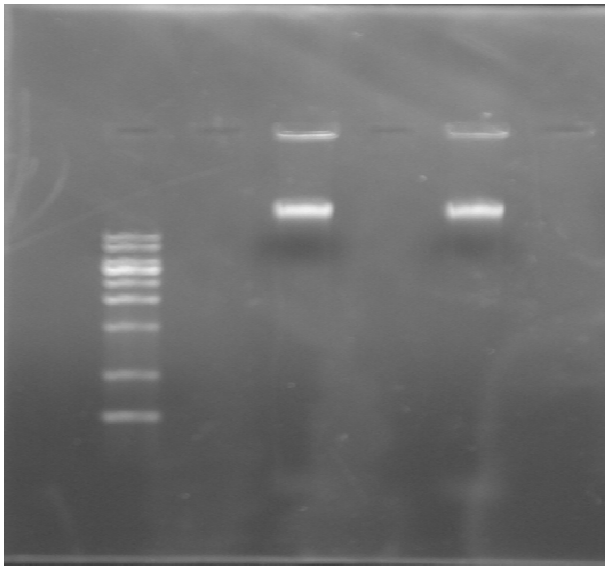


Fig 7: PCR analysis of plasmid DNA from transformed *Agrobacterium*

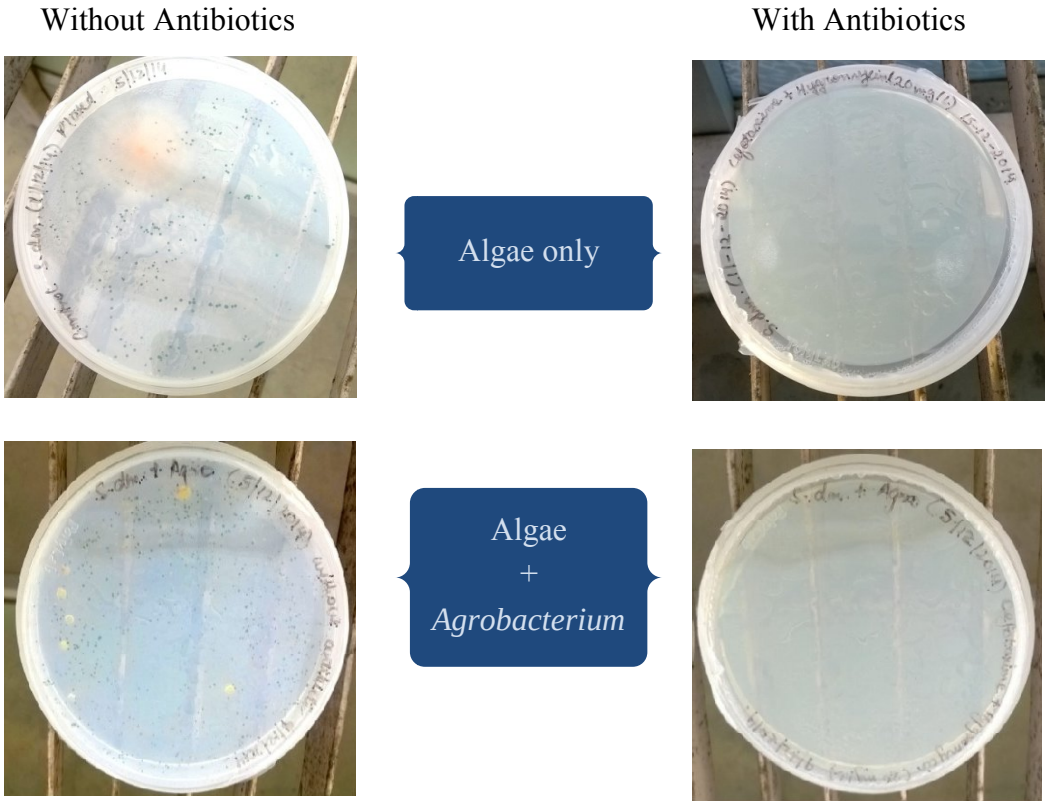



Fig 8: Selection of transformed algal cells (Scenedesmus *dimorphus*)

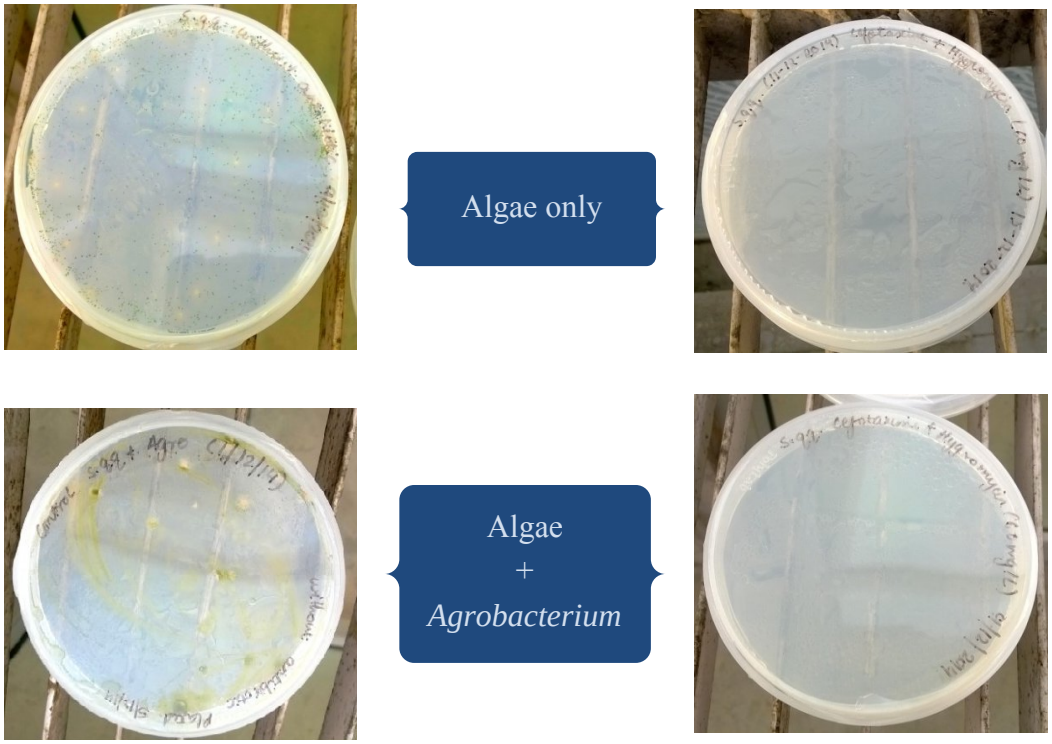



Fig 9: selection of transformed algal cells (Scenedesmus *quadricauda*)

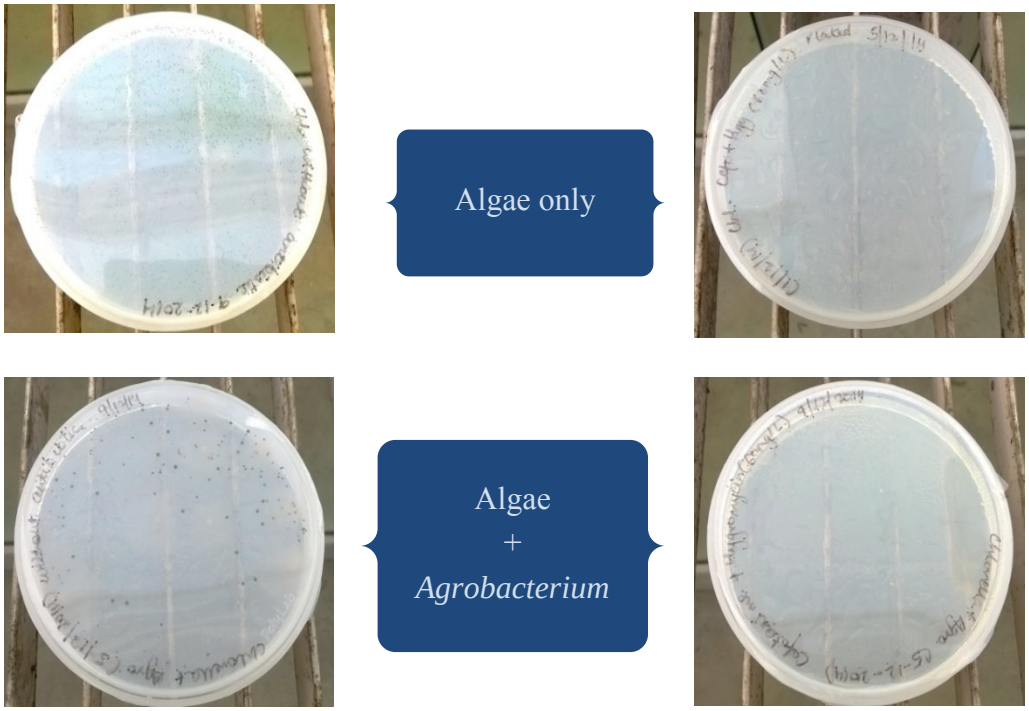
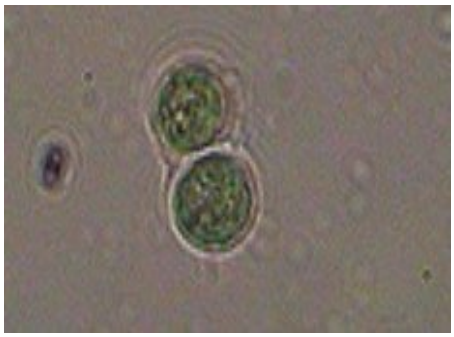
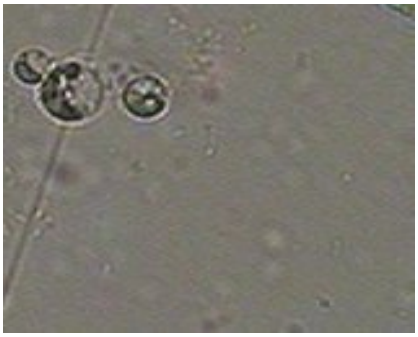


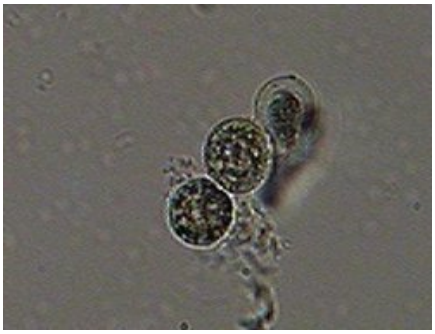

Fig 10: Selection of transformed algal cells (*Chlorella spp.*)





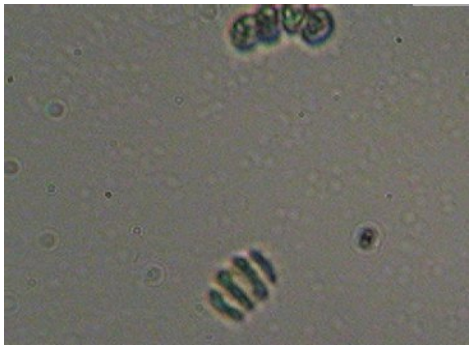
Control Protoplast

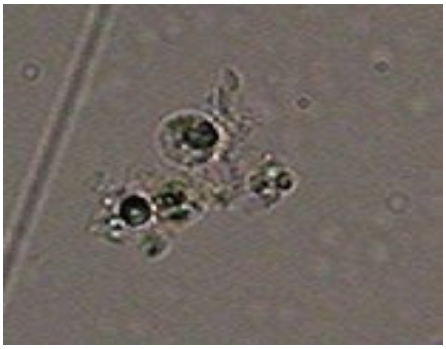
Fig 11: Comparison of original algal cell with isolated protoplast (*Chlorella spp.*)



Control Protoplast

Fig 12: Comparison of original algal cell with isolated protoplast (*Scenedesmus dimorphus*)

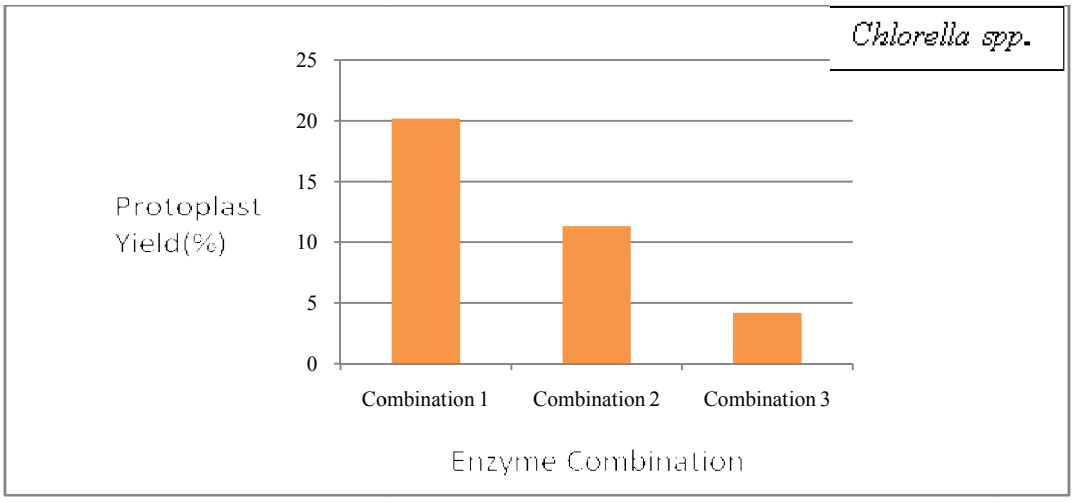




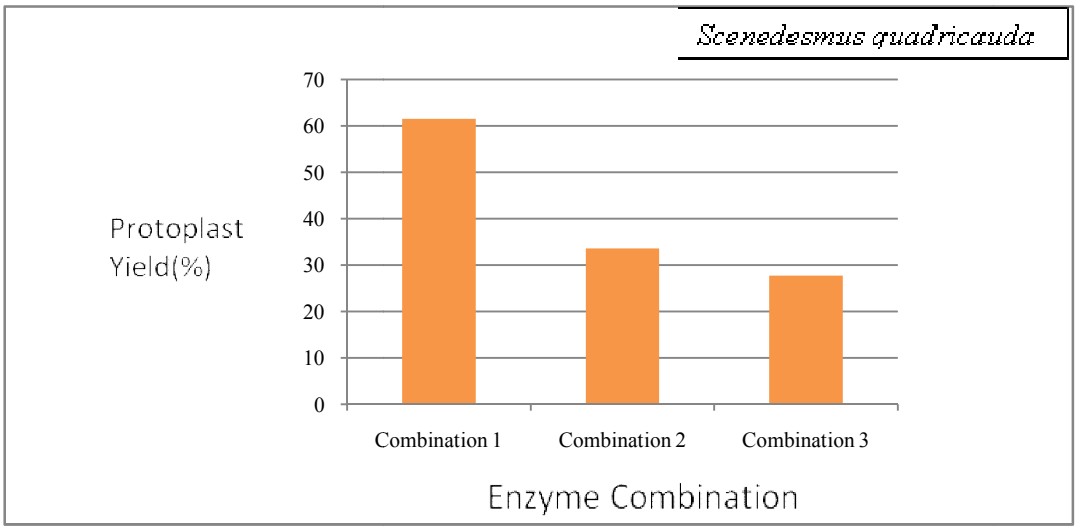
Control Protoplast

Fig 13: Comparison of original algal cell with isolated protoplast (*Scenedesmus quadricauda*)

a)




b)




c)

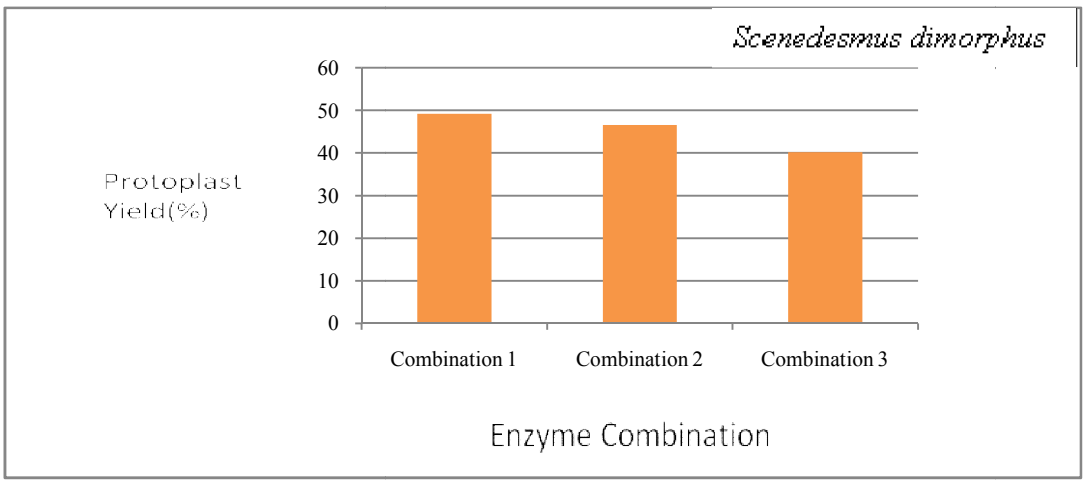
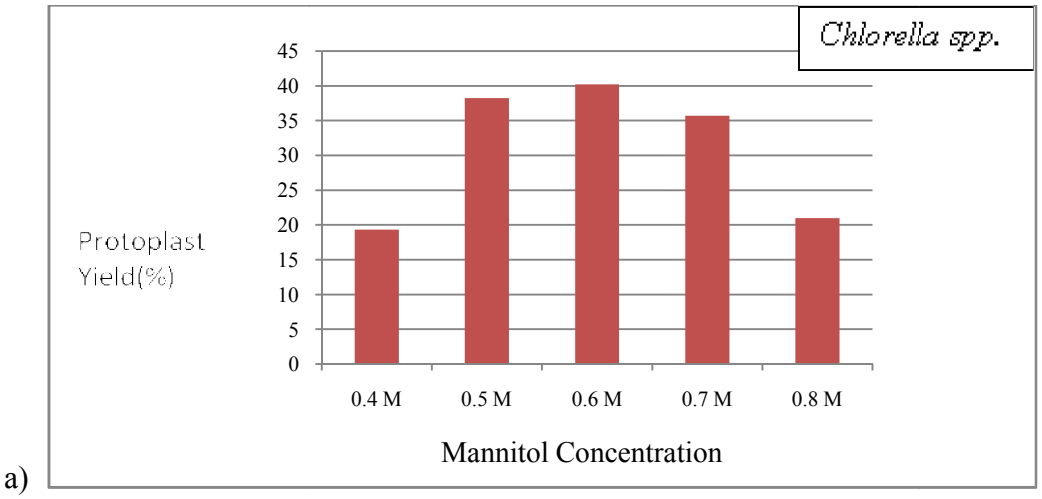
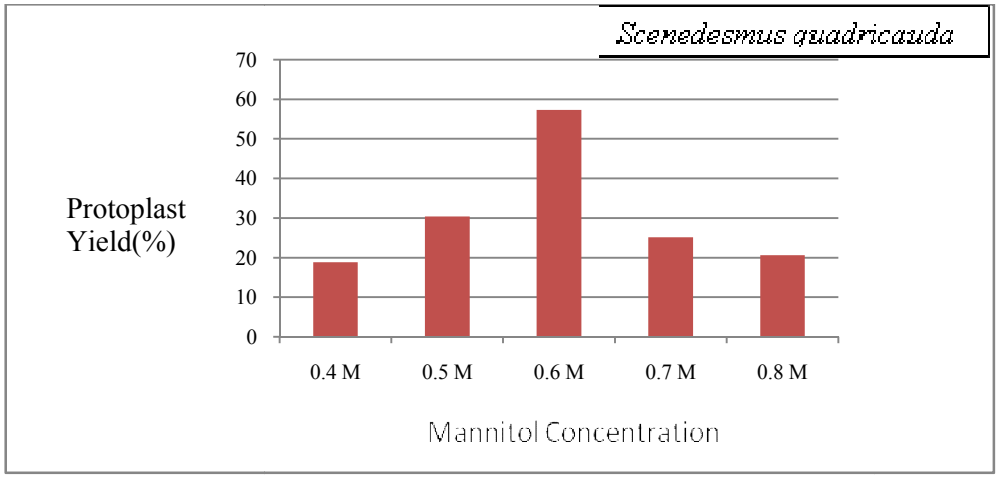


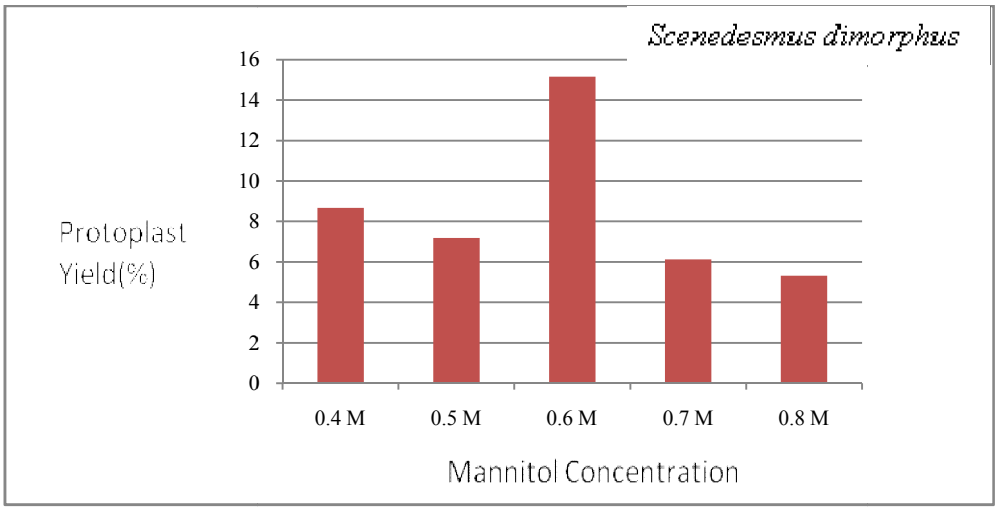

Fig 14: Protoplast yield in response to different enzyme concentrations (*Chlorella spp.*(a), *Scenedesmus quadricauda* (b), *Scenedesmus dimorphus* (c))




a)



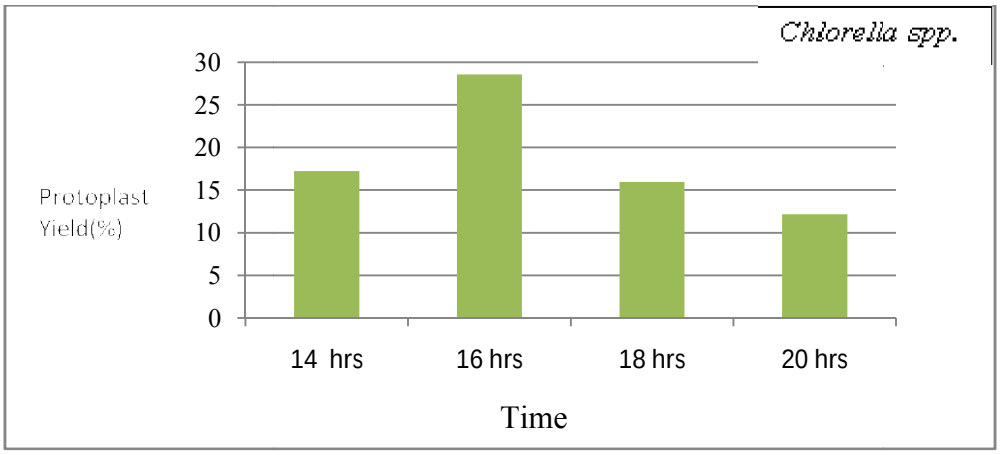

b)



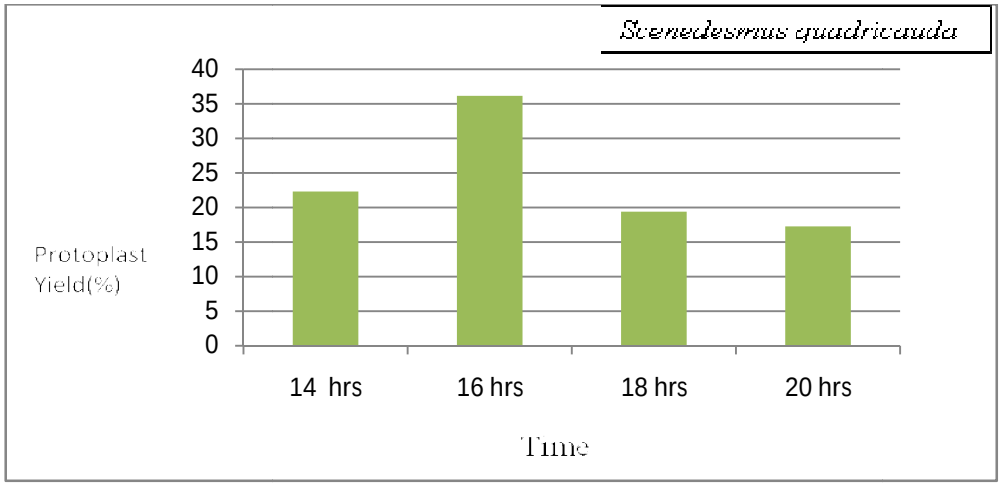

c)

Fig 15: Protoplast yield in response to different mannitol concentrations (*Chlorella spp.*(a), *Scenedesmus quadricauda* (b), *Scenedesmus dimorphus* (c))

a)




b)




c)

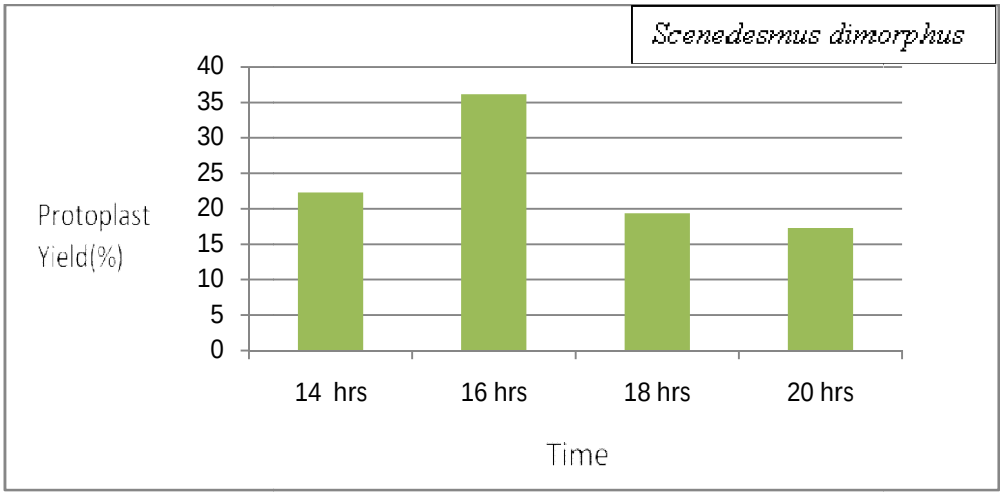



Fig 16: Protoplast yield in response to different durations of incubation time (*Chlorella spp.*(a), *Scenedesmus quadricauda*(b), *Scenedesmus dimorphus* (c))

# Tables

Table 2: Protoplast yield in response to different enzyme concentrations (*Chlorella spp*.)

| | Cellulase | Pectinase | Lysozyme | No. of protoplasts/ml ($10^5$) | Protoplast Yield (%) |
|---|---|---|---|---|---|
| Combination 1 | 2% | 2% | 2% | 12.00 | 20.17 |
| Combination 2 | 1% | 1% | 1% | 6.75 | 11.34 |
| Combination 3 | 2% | 1% | - | 2.50 | 4.20 |

Table 3: Protoplast yield in response to different enzyme concentrations (*Scenedesmus qq.*)

| | Cellulase | Pectinase | Lysozyme | No. of protoplasts/ml ($10^5$) | Protoplast Yield (%) |
|---|---|---|---|---|---|
| Combination 1 | 2% | 2% | 2% | 40.00 | 61.54 |
| Combination 2 | 1% | 1% | 1% | 21.80 | 33.60 |
| Combination 3 | 2% | 1% | - | 18.00 | 27.69 |

Table 4: Protoplast yield in response to different enzyme concentrations (*Scenedesmus dm.*)

| | Cellulase | Pectinase | Lysozyme | No. of protoplast/ml ($10^5$) | Protoplast Yield (%) |
|---|---|---|---|---|---|
| Combination 1 | 2% | 2% | 2% | 9.25 | 49.20 |
| Combination 2 | 1% | 1% | 1% | 8.75 | 46.54 |
| Combination 3 | 2% | 1% | - | 7.50 | 40.16 |

Table 5: Protoplast yield in response to different mannitol concentrations (*Chlorella spp*.)

| Mannitol concentration | No. of protoplast/ml ($10^5$) | Protoplast yield (%) |
|---|---|---|
| 0.4 M | 11.50 | 19.32 |
| 0.5 M | 22.75 | 38.23 |
| 0.6 M | 23.00 | 40.22 |
| 0.7 M | 21.25 | 35.71 |
| 0.8 M | 12.50 | 21.00 |

Table 6: Protoplast yield in response to different mannitol concentrations (*Scenedesmus qq.*)

| Mannitol concentration | No. of protoplast/ml ($10^5$) | Protoplast yield (%) |
|---|---|---|
| 0.4 M | 12.25 | 18.85 |
| 0.5 M | 19.75 | 30.38 |
| 0.6 M | 37.25 | 57.31 |
| 0.7 M | 16.25 | 25.12 |
| 0.8 M | 13.00 | 20.64 |

Table 7: Protoplast yield in response to different mannitol concentrations (*Scenedesmus dm.*)

| Mannitol concentration | No. of protoplast/ml ($10^5$) | Protoplast yield (%) |
|---|---|---|
| 0.4 M | 1.63 | 8.67 |
| 0.5 M | 1.35 | 7.18 |
| 0.6 M | 2.85 | 15.16 |
| 0.7 M | 1.15 | 6.12 |
| 0.8 M | 1.00 | 5.32 |

Table 8: Protoplast yield in response to different duration of incubation time (*Chlorella spp*.)

| Incubation Time (hrs) | No. of protoplast/ml ($10^5$) | Protoplast yield (%) |
|---|---|---|
| 14 | 10.25 | 17.23 |
| 16 | 17.00 | 28.57 |
| 18 | 9.50 | 15.96 |
| 20 | 7.25 | 12.18 |

Table 9: Protoplast yield in response to different duration of incubation time (*Scenedesmus qq.*)

| Incubation Time (hrs) | No. of protoplast/ml ($10^5$) | Protoplast yield (%) |
|---|---|---|
| 14 | 14.50 | 22.31 |
| 16 | 23.52 | 36.18 |
| 18 | 12.60 | 19.38 |
| 20 | 11.23 | 17.27 |

Table 10: Protoplast yield in response to different duration of incubation time (*Scenedesmus dm.*)

| Incubation Time (hrs) | No. of protoplast/ml ($10^5$) | Protoplast yield (%) |
|---|---|---|
| 14 | 1.625 | 8.64 |
| 16 | 2.850 | 15.16 |
| 18 | 2.170 | 11.54 |
| 20 | 1.280 | 6.81 |

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