SOMATIC HYBRIDIZATION OF SELECTED MICROALGAL SPECIES BY PROTOPLAST FUSION-AN ATTEMPT TO GET DESTINED ALGAL CROP FOR COMMERCIAL BIOFUEL PRODUCTION

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INTRODUCTION
With the rapid decrease in fossil fuel reserves, the increasing demand of energy, there is a worldwide urge to develop renewable platforms for fuel production. Though, there are many methods for the production, the third generation energy production from photosynthetic microorganisms such as microalgae is gaining major importance these days, because of its high surface productivity and cultivation in non-arable land, fresh, brackish, sea or even waste water depending on species (Beer, et al., 2009; Scott, et al., 2010; Wijffels and Barbosa, 2010) and also for accumulation of up to 60% oil per dry weight under stress conditions (Chisti, 2007). Use of microalgae for biofuel production, its commercialization and current status has been reviewed by many researchers (Greenwell et al., 2010; Darzins et al., 2010, Tahani et al., 2013). Some microalgal strains which are tested for the production of biofuel are Chlamydomonas reinhardtii in which oil content can reach 50% of dry biomass (Li, et al., 2010), Ostreococcus tauri whose physiology is not understood properly (personal communication F.Y Bouget), Phaeodactylum tricornutum which can accumulate significant amount of lipids under silicon absence (Sheehan, 1998), Nannochloropsis in which the homologous recombination has been demonstrated with high efficiencies for N. gaditana (Kilian, et al., 2011). The strains which are tested for genetic improvement are Chlamydomonas mutants (Tolletter, et al., 2011), Phaeodactylum tricornutum...
(Radakovits, et al., 2011) and *Chlamydomonas* (Yohn, 2011; Boyle, et al., 2012). But, slow growth, high production cost, non-optimal fatty acid composition of micro algal lipids and arrest of cell growth during fatty acid accumulation due to stress are the main hurdles of these strains in Industrial level for large scale production of biodiesel ((Grima, et al., 2003; Siaut, et al., 2011). Therefore, there is no “Algal Crop” destined for large scale biofuel production till date and intensive research efforts are needed in both strain development and technology innovations. Decoupling oil synthesis from arrest of cell division and harnessing the complexity of lipid metabolism are the main biotechnological challenges in this area. The one way to produce hyper oil accumulator algal species is through selection or breeding. However, conventional breeding is not suitable for algae strains since sexual cycles are lacking or poorly defined for most species (Yonghua and Gilles, 2013). Therefore, the present study is aimed on somatic hybridization of selected micro algae species by protoplast fusion to get the new strain and to estimate its biofuel yield.

**OBJECTIVES:**

Establishment of algal culture which has high lipid or triglyceride content by optimizing the growth conditions for their rapid growth and multiplication. Media tested with or without phyto regulators like; Glycerol, auxins and cytokinins in various combinations for rapid multiplication of microalgae. Development of pure cultures of the selected species and subject them to protoplast isolation by removing the cell wall using various concentrations and combinations of macerozymes, pectinase, cellulases to find the best digestion combination. Development of new hybrid algal strain through somatic hybridization with the strains with higher oil content using different methods and to standardize the accurate protocol for successful fusion. Subjecting the hybrids for lipid extraction and quantification of lipid and biodiesel production and analyze the quality and compare with each strain.
METHODOLOGY:

MATERIALS AND METHODS

A. SAMPLE COLLECTION

Collected water sample from KR Puram, Banswadi and Varthur Lake as well as stagnant water for isolation of algae. *Chlorella vulgaris* was purchased from central marine fisheries and Research institute. The samples were inoculated in BG-11 media and Bold’s basal media. These cultures were used for protoplast isolation, fusion and lipid extraction.

B. TOTAL LIPID EXTRACTION

The total lipid content was extracted using Folch’s method.

1. The algal samples of 5g were washed with water and the fibres were separated using forceps.
2. The samples were dried in a hot air oven at 60ºC for 20 minutes.
3. To the dried samples 8ml of 2:1 chloroform-methanol (v/v) mixture was added.
4. The sample was ground to fine paste using mortar and pestle.
5. The mixture was transferred to 10ml centrifuge tubes.
6. To the tubes 2ml of 0.73% NaCl water solution was added.
7. The contents were centrifuged at 350g for 2 minutes.
8. The phases were separated and the lower phase consisting of lipids was recovered for analysis.

C. PROTOPLAST ISOLATION

The algal samples were subjected to protoplast isolation.

1. Different species of Micro algal cells from the maintained cultures were centrifuged at 900 g for 5 min,
2. The cell pellet was suspended in 25 mM Tris buffer (pH 6.0) containing 0.6M D-mannitol.
3. The cell-wall-degrading enzymes such as cellulase (1%) macerozyme (2%) pectinase (1%) were added to the mixture to get the optimum digestion conditions.
4. Each treatment was kept at 30C for 12 hrs and 24hrs, then the cells were centrifuged at 300 g for 2 min
5. The supernatant was discarded and the pellet was suspended in 1.5M sugar solution to separate the protoplasts.
6. After being centrifuged at 300 g for 3 min, the viable protoplast layer was transferred into 1ml of 25mM Tris buffer (pH 6.0) containing 0.6M D-mannitol for further analysis.

D. PROTOPLAST FUSION

Polyethylene glycol (PEG) treatment.

1. *Vaucheria* and *Chlorella vulgaris*; *Spirogyra adnate* and *Chlorella vulgaris*; *Spirogyra adnate* and *Vaucheria* were mixed.
2. 3 ml of the above mixed solution was dispersed and allowed to settle at the bottom of a Petri dish (5 min).
3. To the above solution, PEG solution (3 ml) containing 0.2M glucose, 10mM CaCl, 0.7M KH$_2$PO$_4$ and 0.125mM PEG (MW-4000), pH 5.8, was added and kept aside for 30 minutes
4. Dilution of PEG was initiated by adding, in droplets, 5 ml of the eluting medium.

**Eluting Medium:** (a) 100mM glycine, 0.3M glucose, pH 10-5; (b) 100mM CaCl, 0.3M glucose. Solutions (a) and (b) were mixed in equal proportions just before use. A further 10 ml eluting medium was added after 10 min.

5. The protoplasts were washed five times with A1 medium containing 0.6M NaCl, with interval of 5 minutes between each wash. These cells were then resuspended in the culture medium and incubated in shaker overnight at 100 rpm at 25 ºC.

RESULTS

- Algal species *Spirogyra adnate* and *Vaucheria* was isolated from the water samples and confirmed by observing under microscope. Maximum cell wall digestion was obtained under 24hrs treatment with cell wall degrading enzymes.
- Out of the various combinations tried for fusion of protoplast, *Spirogyra adnate* and *Chlorella vulgaris* was successfully fused and the hybrid acquired few common characters from both the parent. As the hybrid acquired the pigmentation similar to that of *Chlorella vulgaris* and the morphological characters like the cellular structure is similar to that of *Spirogyra adnate*, observed under microscope for the characteristics in comparison with the original cell structures.
- The cells were subjected to stress conditions like increase in carbon dioxide level in the culture condition and also few sets of cultures were subjected to depleted media and
observed for the yield of lipid content, the cells produced slight higher amount of lipid as compared to that of normal strains.
DISCUSSION:

- Protoplast induction for spirogyra species has been reported since 1980, the enzymatic digestion methods used to get protoplasts have been successfully in many (Braun and Aach 1975). Even then, due to the complexity of the cell wall structures or chemical composition, protoplast isolation systems were difficult to develop for some species (Yamada and Sakaguchi 1982; Rosen et al.1985). Since cell wall complexity is the major factor influencing the deficiency, selection of the appropriate degrading enzymes is the key to isolating protoplasts.

- If the cell density is not too high, photosynthesis is still possible. While actual growth is limited under these conditions, cells are still able to fix CO₂ and accumulate photo-assimilates in the form of starch or lipids that have important storage functions for survival under unfavourable conditions (e.g. low nutrients, micro-oxic conditions, anaerobiosis, low/high light or dry, hot or cold conditions). Algae that accumulate lipids in large amounts are therefore often found where environments and microclimates alter frequently between optimal growth conditions and survival under suboptimal conditions. Under normal growth conditions most algal species have a lipid content of ~10–30% dry weight. However, during nitrogen depletion the cells stop dividing and the storage products continue to accumulate at approximately the same rate as in non-nitrogen limited cells with the result that dry weight lipid (hydrocarbon) contents can double or triple.
SCOPE FOR FUTURE WORK:

- Further study on critical factors for all the strains, would help to develop the protocol for large scale production in industries for obtaining biodiesel or any essential components.
- The hybrid variety obtained would be used in industries to produce biofuels.
- To check for the adaptability of the hybrid strain developed in the natural environment and effect of these strains on the natural habitat without causing any adverse effect with other natural habitats.
- They can be processed into a broad spectrum of products including biodiesel via transesterification, green diesel and gasoline replacements via direct catalytic hydrothermal conversion, and catalytic upgrading, and bioethanol via fermentation, methane via anaerobic digestion, heat via combustion, bio-oil and biochar via thermochemical conversion, and high protein animal feed.
- Polyunsaturated fatty acid oils are tested for use as nutritional supplements and pigments are important as natural dyes.
- Stable isotope biochemicals help in structural determination and metabolic studies.
- The hybrid variety so obtained could be an avenue for further researchers to reduce the production cost by slight modifications.