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Isolation and Identification of Green Microalgae for Carbon Sequestration & Waste Water Treatment by Using PCR Studies

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Abstract*— Green micro algae are very simple unicellular algae, widely used in various physiological studies. Green microalgae, obtained from different brackish and fresh water sources within the state of Tamilnadu, Coimbatore and Erode region, were evaluated for their potential use in the production of biodiesel and carbon sequestration studies. The microorganisms were isolated and identified using genomic DNA, and 16S rRNA gene amplification followed by sequencing. The resultant sequences were compared with those available on the NCBI website database through the BLAST bioinformatics tool. The results showed high correlation with known nucleotide sequence identities at 99 % with Chlorella vulgaris.

Index terms— **Cholorella vulgaris, DNA Isolation, Microalgae, PCR ,16s rRNA.**

I. INTRODUCTION

The rising need for energy in developing nations is giving place to vehement competition for the world‘s decreasing energy resources [12]. The increased use of fossil fuels results in larger greenhouse gases (GHG) emissions and this is usually considered the main reason for global climate change [20]. Fossil fuels are the largest contributor of GHGs to the atmosphere (EIA, 2006). With the increase in anthropogenic GHG emissions due to the extensive use of fossil fuels for transport, new techniques for the development of electricity and thermal energy generation are needed. The goal of a 5.2 % reduction in GHG emissions worldwide from 1990 values was proposed during The Kyoto Protocol back in 1997 [19].

One hundred and ninety-three countries are currently part of this protocol, with the United States being the only remaining signatory nation that has not ratified it (Status of ratification of Kyoto Protocol, 2011). Greater use of bio-fuels, which compete and have partially displaced petroleum based fuels for use in transportation, could help meet that reduction in emissions objective [11].

In terms of CO2 reduction, first generation bio-fuels are said to have a limited performance and demand the use of large amounts of land and have now reached economic levels of production. Examples are food and oil crops (e.g. biodiesel from rapeseed oil, and ethanol from sugarcane, sugar beet, and corn) (FAO, 2008) as well as animal fats (FAO, 2007). Strong controversy surrounds the use of first generation biofuels, usually due to their negative impacts like contributing to the increase in food prices, deforestation and biodiversity losses; hence, the extent of their ultimate contribution to the reduction of GHG is frequently questioned [7].

The polymerase chain reaction (PCR) is a powerful and sensitive technique which amplifies specific DNA sequences exponentially through a three-step process done in multiple cycles. First, the double-stranded DNA template is denatured at a high temperature. Then, sequence-specific primers are annealed to the target sequence followed by the addition of a thermo stable DNA polymerase, such as Taq DNA polymerase.

This enzyme is responsible for extending the annealed primers, and doubling the amount of the original DNA sequence. The new product then becomes an additional template for subsequent cycles of amplification. These three steps are usually repeated in cycles 16 for 20 to 30 times, resulting in an increase of target DNA concentration of 105 to 109 times the original amount.

The use of PCR to obtain large amounts of a desired product can have both positive and negative aspects. If amplification is not successful, this can lead to the generation of many un desired products leading even to the exclusion of the target product. The opposite would be that no product may be amplified. In regards to optimization, several variables have been recognized to contribute to this effect [14]. Main optimization variables include magnesium (Mg2+) concentrations, buffer pH, and cycling conditions. Within cycling conditions, the annealing temperature is of utmost importance. The interdependence between variables adds difficulty to the situation. For instance, increasing the amount of deoxynucleotide triphosphates (dNTPs) lowers the concentration of free Mg2+available to exert an effect on polymerase function be caused NTPs directly chelate a proportional number of Mg2+ions

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Several additives and enhancing agents such asdimethyl sulfoxide (DMSO), N,N,N,-trimethylglycine (betaine), formamide, glycerol, non-ionic detergents, bovine serum albumin, polyethylene glycol and tetramethyl ammonium chloride can be included in PCR reactions with the aim of increasing yield, specificity and consistency [5].

Application of the Taguchi method , which focuses only on the main effects and two factor interactions, can eliminate the need of a full multivariate matrix analysis for each of the variables tested, which can become a burdensome and costly task. With this method, the size of the matrix can be trimmed down significantly and several key variables can be altered simultaneously [3].

16s rRNA is the most commonly used genetic marker for the study of bacterial phylogeny and taxonomy Reasons for this include: (1) its presence in almost all bacteria as operons, no change in 16s rRNA gene‘s function has been observed over time and, the 16s rRNA gene‘s length (1,500 bp) is suitable for informatics purposes [8]. Erwin and Thacker (2008) characterized partial 16S rRNA and the entire 16S-23S internal transcribed spacer(ITS) sequences from Synechococcus spongiarumin an attempt to assess the phylogenetic utility of rRNA sequence data in resolving the phylogeny of sponge-associated bacteria. Their results showed that 16S rRNA sequences were highly conserved, exhibiting less than 1 % sequence divergence among symbiont clades; whereas, ITS gene sequences displayed a much higher variability than 16S rRNA sequences. In addition to molecular techniques based on PCR amplification targeting conserved regions inside the 16S rRNA gene [9] However, considering that several disagreements between traditional morphological classification and phylogenetic analysis still remain, the utilization of chemotaxonomic markers, such as lipids and their fatty acids, have been considered as complementary approaches since they provide data for taxonomic position assignment as well as some correlations with morphological properties of cyan bacteria [6]

In this study sample were collected from various region and obtained pure culture from strike plate method. The culture primilimnarly identified by using light microscope, sample isolated and identified by using DNA isolation and PCR studies.

II.MATERIALS AND METHODS

A. Serial Dilution, Streake Plate Method

The sample was taken and serial dilution was done. To this serially diluted culture, streak plates were done in MRS agar plates to obtain pure colonies (Figure-1).

B.DNA Purification

Ten µl of 3M sodium acetate solution (pH 5.2) and 275 µl of 95 % v/v ethanol were added to each sample‘s DNA, mixed by flipping and placed in dry ice for 15 min. The samples were then centrifuged for 15 min at 15,700 x g at 4°C (Hermle Z233 MK-2 High Speed Refrigerated Centrifuge) to remove the supernatant. The pellets were each washed once with 1 ml of 95% v/v cold ethanol and centrifuged for 5-6 mins. After supernatants were discarded,DNA pellets were placed in an oven (Isotemp Vacuum Oven Model 280A, Fisher Scientific, Pittsburgh, PA) at 37°C for 10 min to dry under a vacuum of -25 inches of Hg. After drying, 80 µl of TE buffer was added and the DNA concentration was determined with a ND-1000 Spectrophotometer (Nanodrop, Wilmington, DE). Two readings were taken per sample (2 µl).

C.PCR Amplication for 16s rRNA

The DNA was amplified using universal 16S rRNA primers A 8F and A 1492R (Ocimum Biosolutions Ltd) (PCR reaction was performed in a 50 µL containing 0.1 ng of template DNA, PCR Master Mixer, 10 pmol concentration of each primer and 0.025U of Taq DNA polymerase enzymes. The final volume was adjusted with sterilized Mili-Q water. A PCR thermocycler (Bio-rad) was used to amplify the reactions through an initial denaturation step consisting of 94^{o}C for 2 min followed by 25 cycles at 94^{o}C for 1 min, 52.3^{o}C for 1 min and with an extension of 72^{o}C for 1 min followed by an final extension temperature at 72^{o}C for 2 min. Amplified PCR products were stored at -20^{o}C for further purification and downstream application.

PCR primers for 16S r RNA amplification

Primers	Target for amplification	Oligonucleotide 5’-3’
A 8F	16S rRNA	AGAGTTTGATCCTGGCTCAG
A 1492R	16S rRNA	TACGGCTACCTTGTTACGACTT

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D. Agarose gel electrophoresis for PCR product

About 5 µl aliquot of PCR amplified product was loaded on 1.4% agarose in 1X TAE buffer at 50 V for 45 min and the PCR products were visualized in a UV Tran illuminator. Lambda DNA double digested with EcoR I and Hind III was used as a marker.

E. Fourier transforms infrared spectroscopy

The IR spectrum of dried algal biomass was recorded on Nicolet IR spectrometer at room temperature. The dried algal powder was blended with potassium bromide (KBr) powder, and pressed into tablets before measurement. A region of 4000–400 cm^{-1} was used for scanning.

III.RESULTS AND DISCUSSION

After growth for 2-3 weeks, cultures were heterogeneous at this stage. Colony homogenization was achieved by a serial dilution process. Samples were plated onto BG-11 for 2-4 weeks and observed under a light microscope (Figure-2). Isolated colonies were selected for this study based on rapid growth, morphological and color diversity, and homogeneity. They were tentatively identified as Chlorella vulgaris.

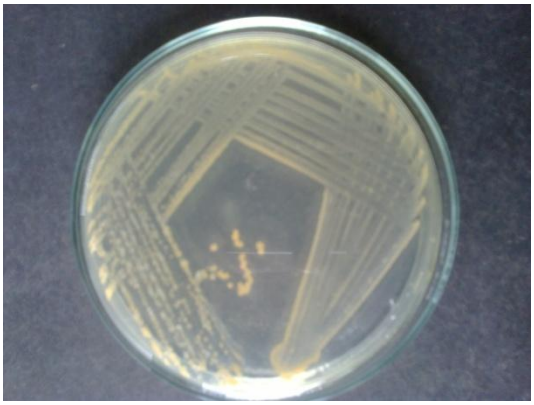

Fig-1 pure culture obtained from steake plate method.

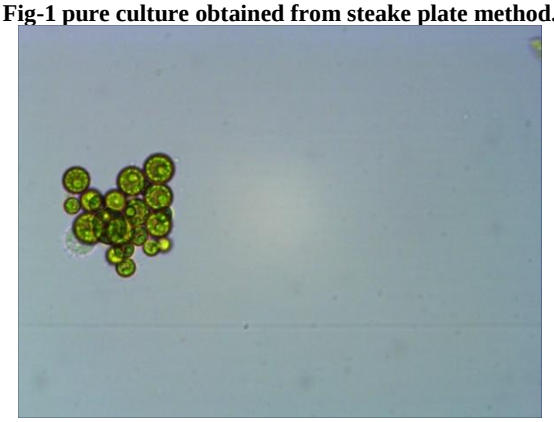

Fig-2 microscopic image of Cholorella vulgaris

First described in the late 1980‘s, PCR is one of the most widely used methods in molecular biology [14]. Several decades later, attempts for PCR optimization continued to be made to meet its specific objectives [4]. Templates known for being difficult to sequence include those with high guanine-cytosine (G/C) content, high adenine-thymine (A/T), as well as sequences with marked secondary structure or large regions of homopolymer [18]. In regards to sequences with high G/C content, several approaches have been taken to solve this problem and perhaps the most successful method for improving results is the inclusion of certain organic additives in the reaction mixture, such as DMSO, betaine, polyethylene glycol, glycerol and formamide [2].The effect DMSO exerts in the PCR amplification of some GC-rich sequences is a largely studied one [16, 17].

In this study, Using the designed primer pair, the 16S rDNA sequence of cultivated Chlorella was successfully amplified. Then, this PCR product (Figure 3) was cloned and sequenced, and the algal strain was determined to be Chlorella vulgaris when blasted on the NCBI database. According to the electrophoretic profiles, nine viral dsRNA bands were present in Chlorella cells. The sizes of these nine ds RNA segments ranged from 250 to 2500 bp, and the 150 segment 5 was much brighter than the others. Therefore, the segment 5 was selected to

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further analysis in our study.This information was then put into practice to positively identify an environmental strain of the Chlorella genus of freshwater unicellular green algae. When com- pared with both an internal standard and GenBank submissions, we gained a 97% match with the type strain for C. vulgaris and have identified this solvent-degrading and solvent-tolerant strain as such [1]. The genetic sequence we gained using these primers, although only W 600 bp in length, can be used successfully not only to positively identify the organism in question, but also to infer phylogenetic associations with similar accuracy to phylogenetic reconstructions involving the use of complete 16S rRNA gene sequences [13]. Thus, we have shown here, using various bioinformatics tools of a phototrophic eucaryote, that the primers originally designed by Nu«bel et al. for the use in cyanobacterial studies can be adapted for far greater use and benefit. They are ideal for not only biodiversity stud-ies into cyanobacterial communities, but can also be useful as a tool in the identification of phototrophic eucaryotes either for the positive identification of new species or for phylogenetic relatedness studies.

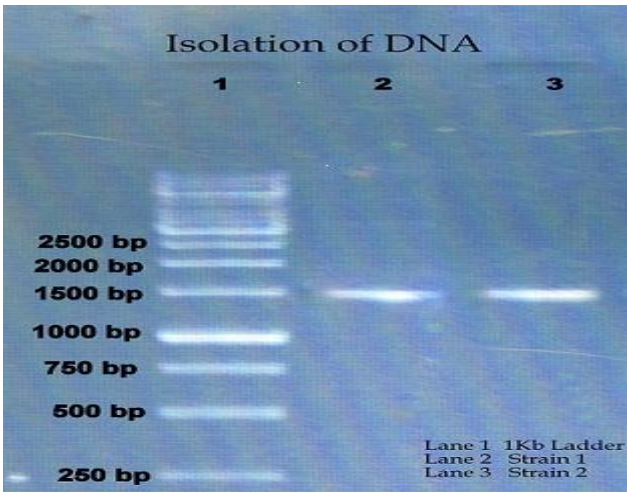

Fig-3 PCR Result

FTIR spectra (Figure-4) in relation to specific groups. Each peak assigned a functional group. The molecular assignments of bands are based on published data phytoplankton, bacteria and other biological materials. In this study chlorella vulgaris protein spectra characterized by strong peaks 1656 cm^{-1} (amide I) and 1536 cm^{-1} (amide II). These bands were due primarily to C=O stretching vibration and a combination of N-H and C-H Stretching vibrations in amide complexes. Lipid and carbohydrates were characterized by strong vibrations the C-H 2925cm^{-1},C-O-C of polysaccharides at 1079cm^{-1},1047cm^{-1}respectively.while carbohydrates are the strongest absorbers between 1200 and 1000 cm^{-1}.Several other classes of compounds, such as nucleic acids have functional groups with absorption bands in the same region of the spectrum. The strongest peaks 1536 and 1422 shows that bending modes of methyl groups of protein. The peak 1243 shows carboxylic acid present in the algae. In this study, the close correlation between the peaks and the existence of with band 2 suggested that lipid content very high and also carbohydrate, nucleic acid also present in chlorella vulgaris.

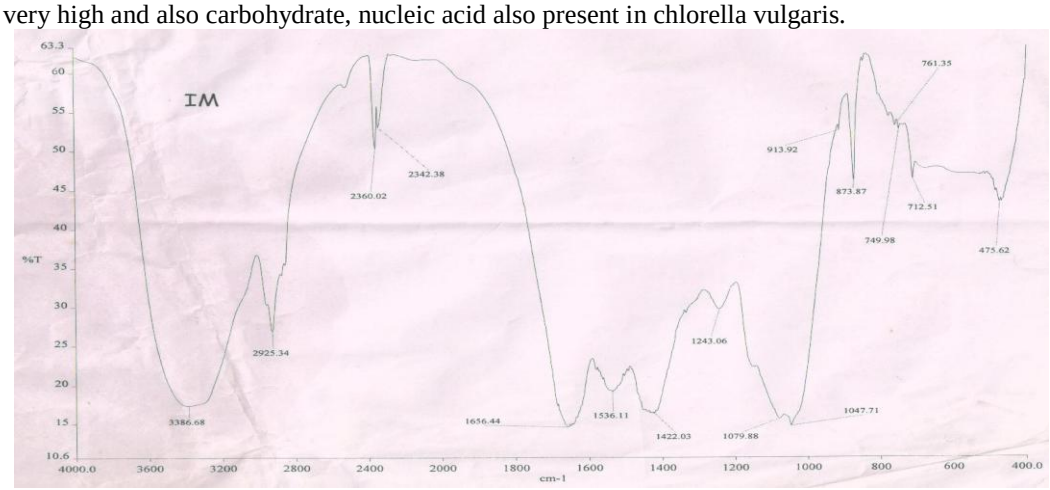

Fig-4 FTIR Results

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IV CONCLUSION

In this study Bio-mass growth rate, Isolation and Characterization of micro algae by utilizing hostel waste water medium reveals that.

- Lipid-producing micro algae species were isolated, using microscopic and PCR analysis, the culture identified as Chlorella Vulgaris.
- Its simple and rapid technique
- Algal species FTIR spectroscopy determination shows that high amount of protein, carbohydrate, nucleic acid were present in the Chlorella Vulgaris
- In this 'post-genomics age', it is vital to link biochemical data obtained from type strains to environmental strain studied using information present within public databases, to aid in the transition towards full structure-to-function studies. In harnessing this information, the pathway to improved biotechnological processes will be considerably shortened if common features between potentially useful microorganisms can be identified and categorized a priori.

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