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RESEARCH ARTICLE

OCCURRENCE OF HYDROCARBON-PRODUCING GREEN ALGAL ISOLATES IN FRESH WATER BODIES OF TAMIL NADU

Bhuvanesh, N*., Sundaram, SP. and Balachandar, D.

Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore 641 003, Tamil Nadu, India.

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ABSTRACT

Three samples of green algal isolates were collected and purified from Sivagangai, Tamil Nadu, India. The genomic DNA from the the isolates was isolated using CTAB method and 18S rRNA gene of each isolate was amplified using primers CV1 and CV2. The variation in the DNA sequence was detected through Restriction fragment length polymorphism analysis. The phylogenetic placement of three isolates was investigated using sequences of the nuclear small subunit 18S rRNA gene and the results revealed that the isolated algal strains *viz*., ALAK 1, ALAK 2 and ALAK 3, were very close to *Actinistrum hanzschii, Clamydomonad* sp. and *Nannochloris* sp. respectively.

INTRODUCTION

Microphytes or microalgae are microscopic algae, typically found in freshwater and marine systems (Thurman, 1997). This colonial microalga is widespread in fresh and brackish waters of all continents (Chisti, 1980). The cosmopolitan nature of the algae is confirmed by the strains originating in the USA (Wolf *et al.* 1985a, b), Portugal, Bolivia, France, Ivory Coast, Morocco, Philippines, Thailand, and the West Indies (Metzger *et al.* 1985). These geographical regions belong to various climatic zones, including the continental, temperate, tropical, and alpine zones. It is also widely distributed in reservoirs at temperate, tropical and arctic latitudes (Tyson, 1995).

Microalgae comprise a vast group of photosynthetic, heterotrophic organisms which have an extraordinary potential for cultivation as energy crops. They are ubiquitously distributed throughout the biosphere and grow under the widest possible variety of conditions. Microalgae can be cultivated under aqueous conditions ranging from freshwater to situations of extreme salinity. They live in moist, black earth, in the desert sands and in all the conditions in between. Microalgae have been found living in clouds and are long known to be essential components of coral reefs. This wide span of ecological requirements plays a significant role in determining the range of metabolic products they produce (Bassam, 1998).

Due to specific characteristics such as its ubiquity, size, and low evolutionary rate, the small subunit ribosomal RNA (SSU rRNA) has proven to be an invaluable tool in molecular evolution. Its impact on phylogenetic studies has been tremendous and the molecule has accounted for numerous new insights in evolutionary biology (Woese, 1987; Sogin, 1989; Doolittle and Brown, 1994).

***Corresponding author:** *bhuva.agri@gmail.com*

Analyses of small subunit ribosomal RNA (rRNA) sequence data have facilitated clarification of phylogenetic relationships among microalgae (Gunderson *et al.* 1987, Wilcox *et al.* 1992). However, molecular based phylogenetic analysis of microalgal species is very much limited in India, as most of the diversity analysis relay on the morphological characterizations. Hence in the present work, we focused more on DNA-based phylogenetic analysis of microalgae occurring the fresh water bodies of Tamil Nadu, India.

MATERIALS AND METHODS

Isolation and purification of algal strains

The samples were collected from different fresh water bodies of Sivagangi, Tamil Nadu, India and cultured in modified Chu 13 medium (Largeau *et al.* 1980) by using enrichment technique. The algae were subjected to purification by serial dilution followed by plating. The individual colonies were isolated and inoculated into liquid medium (modified Chu 13 medium) and incubated at 25 ± 1ºC under 1.2 ± 0.2 Klux light intensity with 16:8 hrs light and dark cycle. The purity of the culture was ensured by repeated plating and by regular observation under microscope.

Molecular identification of isolated algal strains

The genomic DNA from the three isolates was isolated using the standard protocol of hexadecyl-trimethyl ammonium bromide (CTAB) method given by Melody (1997) with slight modifications. The genomic DNA of pure cultures were resuspended in 50 µl of TE buffer and stored at -20°C for future use.

The 18S rRNA gene of each isolate was amplified using primers CV1 and CV2. The forward primer CV1 (5'TACCTGGTTGATCCTGCCAGTAG-3') and reverse primer CV2 (5'-CCAATCCCTAGTCGGCATCGT-3') (Sawayamo *et al.* 1995) were used for amplification of

18S rRNA gene (1000 bp). The 20 µl PCR reaction mixture contain DNA template 50 ng, 1xTaq buffer, 0.2 mM of each of dNTP mixture, 1 µM of each primers, 1.5mM $MgCl_2$ and 2U of Taq DNA polymerase (Bangalore Genei, India). PCR amplification was performed in a thermocycler (Eppendorf Master cycler, German) using conditions: initial denaturation at 95° C for 1 min, 35 cycles consisting of 95°C for 30 seconds (denaturation), 45°C for 30 seconds (annealing), 72°C for 2 min (primer extension) and final extension 72°C for 10 min (Senousy *et al.,* 2004). After the separation of the PCR products with 1.0 per cent agarose gel, it was viewed and photographed using Alpha imager TM1200 documentation and analysis system.

Restriction fragment length polymorphism analysis (RFLP)

The 5µl of amplified product of DNA from each isolate was digestion with 10U of *HaeIII* enzyme at 37°C for 3 h and the reaction was stopped by incubating at 37°C for 5 min. The resulting digested fragments were then separated by length through agarose gel electrophoresis using 1% agarose gel.

Phylogenetic analysis

The amplified 18S rRNA gene as described earlier were purified using PCR clean kit (Sigma GenElute^{TM} PCR clean-up kit, USA) according to the manufacturer's instruction. The purified PCR product was cloned into pT/Z vector and transformed in *E. coli* DH5α using TA cloning kit (Fermentas, USA) according to the instruction given by manufacturers. Sequencing reactions were performed using ABI prism terminator cycle sequencing ready reaction kit and electrophoresis of the products were carried out on an Applied Biosystems (Model 3100) automated sequencer. The phylogenetic placement of three isolates was investigated using sequences of the nuclear small subunit 18S rRNA gene by performing a similarity search against the GenBank database (website: http.www.ncbi.nih.gov/BLAST). The phylogenetic tree was constructed by neighbor-joining method of Saitou and Nei (1987) using MEGA 4.0 and the tree file was analyzed using treeview (Tamura *et al.* 2007).

Lipid and hydrocarbon content of microalgae

Hydrocarbon and lipid content of microalgal species were measured gravimetrically at 15 days interval when grown in Chu-18 medium at 30°C with 3000 lux light intensity for 12 h day / light cycle and expressed as dry weight percentage (Dayananda *et al.* 2005; Dayananda *et al.,* 2006).

RESULTS

The genomic DNA was obtained from all algal strains using cetyl-trimethyl ammonium bromide (CTAB) method. PCR analysis was done using a pair of universal primers (forward and reverse) which amplifies the 18S rRNA gene (Fig. 1). The presence of 18S rRNA gene was detected by partial amplification of gene using specific primers. The results confirmed the amplification of about 1000 bp size partial 18S rRNA gene in the algal isolates. Restriction digestion of the amplified product of DNA with HaeIII enzyme showed the strain level polymorphism in between the algal isolates studied (Fig. 2). The amplified 18S rRNA fragment about 1000 bp was ligated with T/A cloning vector by using T_4 DNA ligase. The cloned vector was transformed to *E. coli* DH5α strain. The recombinant and non-recombinant clones were screened

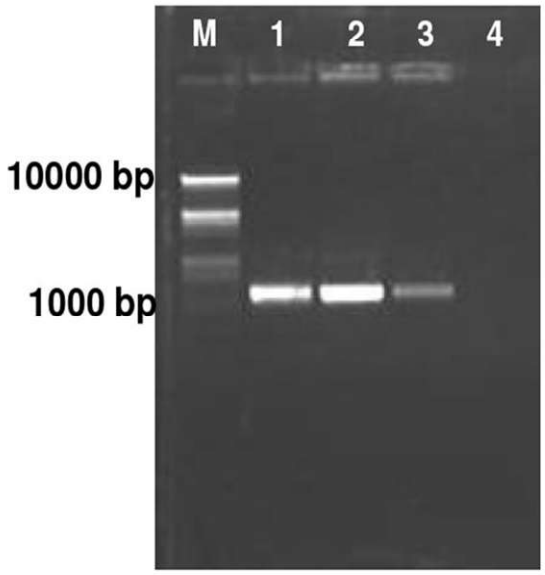

Fig.1. PCR amplification of 18S rRNA gene from three algal isolates. M – 1 kb DNA ladder; 1 – ALAK1; 2 – ALAK2; 3 – ALAK3; 4 – Negative control.

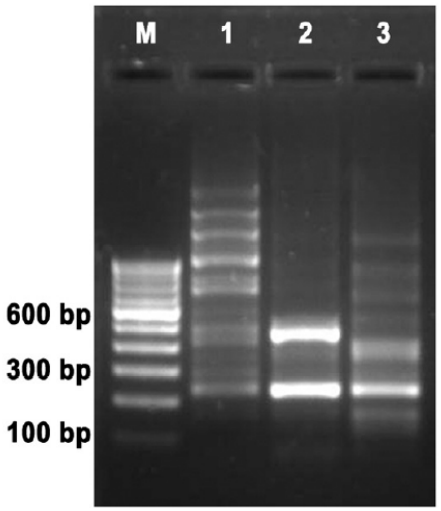

Fig. 2. Restriction pattern of 18S rRNA amplified from micro algal by *HaeIII* enzyme. M – 1 kb DNA ladder; 1 – ALAK1; 2 – ALAK2; 3 – ALAK3.

based on white colonies (transformed) and blue colonies (non-transformed). The plasmids were isolated from both transformed and non-transformed colonies and the presence of inserts was conformed by 1% agarose gel. Using the 18S rRNA genes amplified from algal isolates were sequenced and phylogenetic tree was constructed with different algal strains (the algal species, strain name and the accession number of the gene are presented in Fig.3). For the isolated algal strains, ALAK 1, ALAK 2 and ALAK 3, the phylogenetic tree showed that 18S rRNA gene was very close to *Actinastrum hantzschii, Clamydomonad* sp. and *Nannochloris* sp respectively. They form a very close clustering in neibhour joining phylogenetic tree of 18S rRNA (Fig. 3).

The total lipids and hydrocarbon content from the algal strains, the lipids were extracted according to the method of Bligh and Dyer and total lipids were measured gravimetrically. With respect to hydrocarbon content, the algal biomass of all the three strains were repeatedly extracted using hexane solvent and hydrocarbons were measured gravimetrically. From the Fig.4 it was inferred that strain ALAK 3 showed maximum lipid and hydrocarbon content during 60 days old cultures.

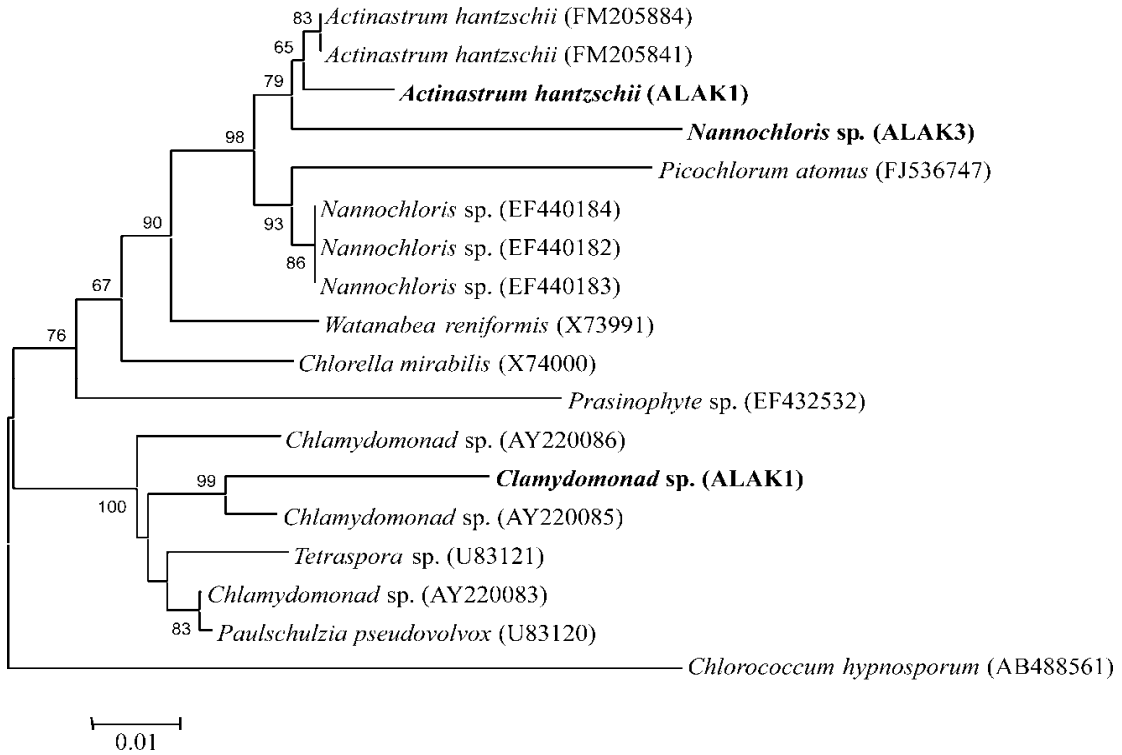

Fig.3. Phylogenetic tree of 18S rRNA sequence inferred with the neighbour-joining method. The scale bar is for 0.01 substitutions per site. Taxa shown in bold letters are the isolates ALAK 1, ALAK 2 and ALAK 3 whose 18S rRNA sequence were determined in this study. The boot-strap values of 500 and above are shown as per cent at the nodes.

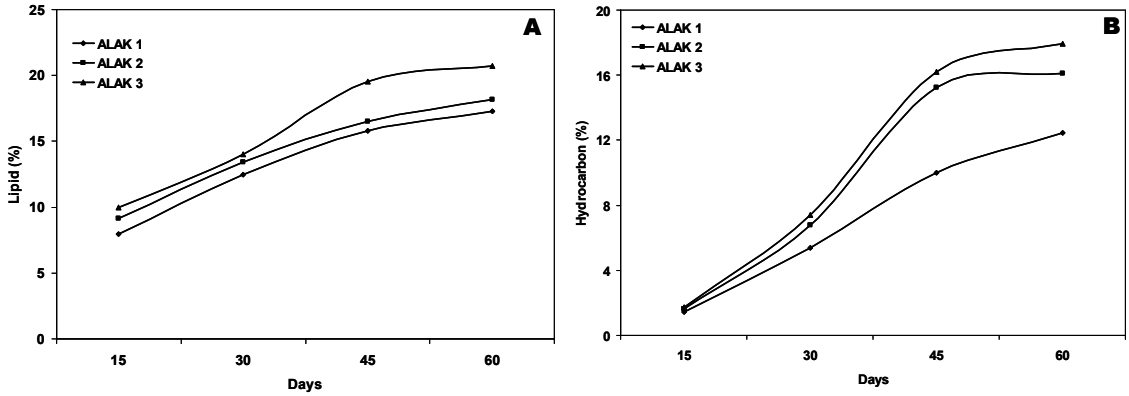

Fig.4. Lipid (A) and hydrocarbon (B) accumulation by three micro algal isolates (ALAK-1; ALAK-2; ALAK3) from fresh water ponds of Sivagangai district of Tamil Nadu. The cultures grown in Chu-18 medium at 30°C with 3000 lux light intensity for 12 h day / light cycle were used.

DISCUSSION

The diversity of eukaryotic microorganisms is generally considered to be lower than that of many other organisms (Fawley *et al.* 2004).The occurrence of green algae in fresh water bodies is earlier reported by several workers (Banerjee *et al.,* 2002; Metzger and Largeau, 2005). Molecular techniques allow an assessment of the validity of the morphological species concept for common microalgae and ultimately niche partitioning and distribution of these organisms. For many types of microorganisms, the gene most commonly employed for diversity studies is the small-subunit ribosomal RNA gene (16S rDNA in prokaryotes and 18S rDNA in eukaryotes) (Linder *et al.* 2000). Very less work has been done in molecular charecterization and studies of green algae. Analyses of small subunit ribosomal RNA (rRNA) sequence data have facilitated the phylogenetic relationships among microalgae (Gunderson *et al.* 1987 and Wilcox *et al.* 1992). In the present study the algal strains *viz.,* ALAK 1, ALAK 2 and ALAK 3 strains were purified and authenticated by PCR. The well-characterised colonies were subjected to further authentication by detection of 18S rRNA gene. Analyses of

small subunit ribosomal RNA (rRNA) sequence data have facilitated the phylogenetic relationships among microalgae (Gunderson *et al.* 1987 and Wilcox *et al.* 1992). Conclusions from the molecular studies of phylogeny depend on the quality of the sequence alignment and the method of tree construction (Friedl, 1997, Chapman *et al.* 1998). The sequence analysis supports this phylogenetic position of the alga by bootstrap method. In this study, from the phylogenetic tree inferred from 18S rRNA gene sequences showed that all the three isolated algal strains were belong to the class Chlorophyceae. For the isolated algal strains, ALAK 1, ALAK 2 and ALAK 3, the phylogenetic tree showed that 18S rRNA gene was very close to *Actinastrum hantzschii, Clamydomonad* sp. and *Nannochloris* sp respectively. They form a very close clustering in phylogenetic tree of 18S rRNA by neighbor-joining method. The present study confirmed the species richness of microalgae through 18S rRNA sequencing and phylogenetic analysis, capable of accumulating lipids in their cells which can be further exploited for biofuel production.

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