Vol 3 Issue 9 March 2014 ISSN No :2231-5063

International Multidisciplinary Research Journal

Golden Research Thoughts

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RNI MAHMUL/2011/38595 **ISSN No.2231-5063**

Golden Research Thoughts Journal is a multidisciplinary research journal, published monthly in English, Hindi & Marathi Language. All research papers submitted to the journal will be double - blind peer reviewed referred by members of the editorial board.Readers will include investigator in universities, research institutes government and industry with research interest in the general subjects.

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Golden Research Thoughts
ISSN 2231-5063
Impact Factor : 2.2052(UIF)
Volume-3 | Issue-9 | March-2014
Available online at www.aygrt.isrj.net

MOLECULAR CHARACTERIZATION AND FATTY ACID PROFILING OF INDIGENOUS MICROALGAE SPECIES WITH POTENTIAL FOR BIOFUEL PRODUCTION IN TAMIL NADU INDIA.

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Abstract:-Screening native algae for species with desirable traits gives a robust biological platform for Bioresource production. At present it is very important to screen the indigenous hyper-lipid producing microalgae for Biofuel application and should cultivate native microalgal strains that adapt to their local environment conditions. As part of pioneering efforts to assess the potential of native microalgae as biofuel feedstock in Tamil Nadu India, six microalgae (corresponding to the Phylum Chlorophyta) from a total of 25 isolated cultures were selected based on their morphology and ease of cultivation under our laboratory condition. Six strains were identified as *Scenedesmus armatus* FW005, *S. deserticola* FW006, *Chlorella vulgaris* PRR04, *S. obliquus* PRR02, *C. vulgaris* SE002 and *S.dimorphus* PRR05 based on 18s and LSU (D1-D2) rDNA sequence analysis. Among the six species, *C. vulgaris* PRR04 showed a higher biomass productivity 1.72 ± 0.06 g d wt L-1. The fatty acid compositions of the six species were studied and the major fatty acids are lauric acid, palmitic acid and oleic acid comprising of 5-35%, 16-54% and 3-34% of the total fatty acids. Oleic acid, occupied up to 34% of the total fatty acids in C. vulgaris SE002 which is an ideal component of biodiesel hence we suggest they might represent valuable resources for future research towards the regional development of the technology for microalgae-based biofuels.

Keywords: Biofuel, Freshwater, Indigenous Microalgae, 18s - LSU (D1-D2) rDNA, Fatty acids

INTRODUCTION:

Energy is essential for life and industry development, and the global economy actually runs on energy. Fossil fuels include about 88%of the global energy consumption, in which, oil, coal and neutral gas are the major fuels by 35%, 29% and 24% shares, respectively. The shares of nuclear energy and hydroelectricity are about 5% and 6% of the global primary energy consumption, respectively5,6. The rapid depletion of fossil fuels together with the uncertain global climate in the past decade has inevitably led to an increased commercial interest in renewable fuels. Biodiesel and bioethanol are viewed as attractive potential solutions to alleviate the existing dependence on petroleum-based fuels15,21. A microalga has been recognized as a promising alternative source for biodiesel-convertible lipids. They are a group of diverse photosynthetic organisms that can accumulate substantial amounts of lipids – up to 50% of dry cell weight in certain species7,26. In addition, microalgae also have certain advantages compared to other energy crops, including a high growth rate, short growth time, high biomass production, and low land use18.

Microalgae represent an exceptionally diverse but highly specialized group of microorganisms adapted to various ecological habitats. The wide variety of species and the Morphological similarity between some of them make necessary the combination of biochemical, Physiological and Morphological Characters to create a taxonomic classification8. Sometimes the result is a double classification of the same organisms in functions of the observer criteria, and it generates mistakes in the taxonomic assignation4, 9, 22. Nowadays, great emphasize is being put on such algal biofuel investigations that deal with the selection of best strain and its characterization which is predominantly missing in the algal biofuel research of developing countries, though there is a worldwide increasing interest in algae as an alternative clean, carbon neutral energy source. It has been widely accepted that the application of Molecular Markers as a useful tool in the detection, identification and characterization of Microalgae for potential biotechnological applications.

Ramganesh Selvarajan , Prakasam Velu and Elumalai Sanniyasi , "MOLECULAR CHARACTERIZATION AND FATTY ACID PROFILING OF INDIGENOUS MICROALGAE SPECIES WITH POTENTIAL FOR BIOFUEL PRODUCTION IN TAMIL NADU INDIA. ", Golden Research Thoughts | Volume 3 | Issue 9 | March 2014 | Online & Print

At present, it is very important to screen the indigenous hyper-lipid producing microalgae for biofuel application and should cultivate native microalgal strains that adapt to their local environment conditions. Indigenous strains have an inherent adaptability that may be the competitive edge required for open pond cultivation system. In addition they generally are able to adapt quickly to changes in environment and climate20, 35. Indigenous strains however require isolation, characterization, establishment of culturing conditions and determination of feasibility of production. Having this entire in mind we took this approach to characterize some indigenous microalgae that we isolated from local environmental fresh water samples and studied the aspects of their fatty acid profiles that could determine their suitability for use in the production of biodiesel.

MATERIALS & METHODS

Chemicals and reagents

FAME standards and Methylation catalysts (KOH–CH3OH; HCl in Methanol) were purchased from Sigma–Aldrich Pty. Ltd. All organic solvents (n-hexane, Chloroform and methanol) were analytical grade in the experiments.

Isolation and identification of microalgae

Water samples for indigenous microalgae isolation were collected aseptically from sites that appeared to contain algal growth in freshwater habitats at Tamil Nadu, India. The pH of the freshwater pond ranged from 7.85 – 8.25. Ten mL of water samples was transferred to a 500 mL conical flask containing 250 mL of sterilized Bold's Basal Medium (BBM)12 and then incubated on a rotary shaker at 27˚C and 150 rpm under continuous illumination using white fluorescent light at intensities of 40 µmol m-2 s-1 for three to four weeks.

Every two days, the flasks were examined for algal growth using an optical microscope, with serial dilutions being made in BBM from flasks showing growth. Subcultures were made by inoculating 50 µL culture solution onto Petri plates containing BBM solidified with 1.5% (w/v) of bacteriological agar. Petri plates were incubated at 26˚C under continuous illumination for two weeks. The purity of the culture was confirmed by repeated plating and by regular observation under a microscope. All the strains were preliminary identified by observation of morphological characters at the microscope and using taxonomic key approaches11 and confirmed using molecular markers.

DNA extraction, PCR amplification and sequencing

Cells were harvested from 100ml of liquid culture by centrifugation at 6000rpm for 10 min. Algal cells were frozen at -80˚C and homogenized in liquid nitrogen. The genomic DNA was extracted using a DN Plant Mini kit (Qiagen, USA) according to the Manufacturer instruction and Protocols. The DNA quality and quantity were determined by measuring the absorbance at 260nm and the 260/280nm and 234/260 ratios33 using a Spectrophotometer (Hitachi U-2900, Japan). DNA extractions were appropriately labeled and stored at -80˚C for subsequent works. Polymerase chain reaction (PCR) amplification of the LSU (D1-D2) coding region of the rDNA29 and 18S rRNA19, 27 gene was performed with a final volume of 50 ml using approximately 2 ml of genomic DNA, 0.2 mM of each deoxynucleotide, 2 mM MgCl2,1 U LC Taq DNA polymerase (Fermentas), 1x PCR buffer (Fermentas), 0.325 mM of Euk328F (5'-ACC TGG TTG ATC CTG CCA G-3') and Chlo 02R (5'-CTT CGA GCC CCC AAC TTT C-3') primers and LSU D1-D2F (5'-AGCGGAGGAAAAGAAACTA-3') and LSU D1-D2R (5'-TACTAGAAGGTTCGATTAGTC-3') and 400 ng of BSA (Fermentas). Aliquots (5 mL) of the reaction mixtures were analyzed by 1% horizontal agarose gel electrophoresis to confirm the presence of the product. PCR amplicon was purified with the PCR-MTM Cleanup System (Viogene). Sequencing was carried out with the BigDyeH Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) using the appropriate primers.

Phylogenetic analysis

Chromatograms obtained from the sequence were corrected manually with Chromas 1.45 software (Technelysium Pty Ltd). The generated sequences were compared to the GenBank nucleotide database using the Blast program1. The phylogenetic tree was constructed using the neighbor-joining (NJ) Kimura's two-parameter algorithm, as implemented within the MEGA4 program package30 after 100 rounds of bootstrap resampling.

Microalgal cultivation and growth kinetics

The Microalgal strains were cultivated in 250 mL Erlenmeyer flask containing 100mL Bolds Basal Medium (BBM) was inoculated with the cells (OD680 0.05) and incubated at 26˚C with shaking at 200 rpm under continuous illumination for four weeks. Algal growth was monitored by measuring daily changes in optical density at 680 nm with a spectrophotometer23.
Lipid extraction

Total lipid content was estimated from the cultivated indigenous freshwater microalgal biomass using a slightly modified method of Bligh and Dyer as described by Luyen HQ3, 16. The weight of the crude lipid obtained from each sample

was measured gravimetrically. Experiments were carried out in triplicate, and data are expressed as mean ±SD.

Fatty acid analysis

Cellular fatty acids were extracted, and methylation was performed according to Stead et al29. Nonadecanoic acid (Sigma- Aldrich) 500 mg L-1was added as an internal standard. Fatty acid methyl esters (FAME) were analyzed by gas-liquid chromatography (HP 5890 GC). The separation of FAMEs was performed using HP-1 dimethylpolysiloxane column, and 37-Component FAME Mix (Supelco) was used as a quality standard. Fatty acids were identified through comparison with the retention times and fragmentation patterns with those of the standards and it was expressed as a percentage of the total fatty acids identified in the oil.

RESULTS AND DISCUSSION

Energy security has become a national crisis and severe scientific attempts are being made to seek viable alternative source in the form of renewable energy to meet out the futuristic needs. In order to have energy security, Most of the developing countries including India are also committed to use renewable bioenergy sources to supplement its energy requirements2.

Isolation and Identification of Microalgae

Microalgae are one of the potential sources of biofuel feedstock due to several unique properties. According to Richmond24, photosynthetic algae represent a large and diverse group of organisms that have only a limited history of characterization and exploitation. Methods to screen for indigenous microalgal species have improved and can allow communities to prospect for algae suited to regional needs. In India, however, exploration of the potential of microalgae for biodiesel production is at its infancy and very few studies are available in the international literature 2,13. As a contribution to this regard, in the present work more than 25 algal cultures were isolated from different freshwater habitat in Tamil Nadu, India. Out of 25 cultures six green microalgal isolates such as FW005, FW006, PRR04, PRR02, SE002 and PRR05 were selected based on their morphological character which can also be tolerated an adverse condition and they could be successfully cultivated in pure form under our laboratory condition. Microscopic observation of selected indigenous algal isolates revealed its colony existence and purity (Fig. 1). The six isolated micro algal cultures of FW005, FW006, PRR04, SE002 and PRR05 genera were identified by the morphological examination under microscope based on their cell shapes. Nobel native strains can combine several desirable traits and be useful on a regional or broader scale. So far, a little information about the indigenous oil producing Microalgal strains were isolated and characterized in Tamil Nadu fresh water system. In this work, the molecular characterization of most habituated indigenous microalgae has been done and their fatty acid methyl ester composition has been clarified to identify the highly suitable strain for further exploration and commercial production, this could be a very valuable resource of as isolated algal species or as a genetic background or source of genes for selected traits in genetic engineering programs.

DNA Extraction and PCR amplification

Morphological data are frequently unreliable when used to identify green microalgae to species level. The morphological heterogeneity of the alga makes the microscopic examination highly difficult23. Metzger and Largeau 17 reported that for algae within each chemical race and for the same strain, morphology could vary in relation to age and culture condition. Nuclear and organelle genomes have different rate of variability and they are reflected in conserved sequences of closely related species which happens in nuclear than in plastid genes36. The gene encodes for the rRNA molecule are good candidates for detection because they are present in high copy numbers, and the sensitivity of their detection. Therefore, we isolated total DNA and PCR-amplified rRNA (18s & LSU) gene to confirm our morphology-based species identifications. PCR amplification of the genomic DNA of the microalgal isolates with the LSU rDNA (D1-D2) and 18s primers revealed efficient amplification. The primers used to amplify the LSU (D1-D2) region successfully amplified DNA from the PRR04, PRR02, SE002 and PRR05 microalgal cultures. While no amplification was detected using LSU rDNA primer pairs, the DNA was successfully amplified with 18s primers. Based on the 18s and LSU rDNA (D1- D2) sequences, we concluded that microalgal isolates FW005, FW006, PRR04, PRR02, SE002 and PRR05 were closely related to Scenedesmus armatus, S. deserticola, Chlorella vulgaris, S. obliquus, C vulgaris and S.dimorphus based on 100%, 99%, 98%, 97%, 95 and 98% sequence similarities, respectively. The DNA sequences were published in the NCBI databases (accession numbers are provided in Table 1). The lengths of the 18s and LSU rDNA (D1-D2) regions of the six species of microalgae, their specific accession numbers and the nearest identifiable match present in the GenBank nucleotide database are shown in Table 1. Identification of the six microalgal strains was also supported by the results from the phylogenetic analysis of the 18s and LSU rDNA (D1-D2) sequence. In the 18s phylogram (Fig. 2), FW005 and FW006 which were identified as S. armatus and S.deserticola and they were clearly grouped with the microalgal strain S. armatus FR865727 and S.deserticola AY510464 (Table 1) respectively. Whereas the LSU rDNA sequences of isolates PRR04, PRR02, SE002 and PRR05 confirmed their identification as C. vulgaris, S. obliquus, C vulgaris and S. dimorphus; they had sequence similarities of 98%, 97%, 95 and

98% and the LSU rDNA (D1-D2) phylogram (Fig 3) showed they were clearly grouped with the microalgal strain C. vulgaris AB237642, S. obliquus AF183452, C vulgaris AB237642 and S.dimorphus FR865725, respectively. In our Phylogenetic analysis all the selected strains were clearly grouped with the microalgal strain to the nearest identifiable match present in the GenBank nucleotide database. The LSU rRNA gene has a higher evolutionary rate, as compared to the SSU rRNA gene28 and should be a better molecular tool for the discrimination of closely related species using short diagnostic sequences.

Growth Kinetics of Selected Microalgae

Under suitable environmental conditions and sufficient nutrients, microalgae can grow profusely. Their biomass usually doubles within 3.5- 24 h during the exponential growth phase6. In this study, under the same laboratory condition, the net growth rates differed among the examined species (Fig. 4). The average specific growth rates of S. armatus FW005, S. deserticola FW006, C. vulgaris PRR04, S. obliquus PRR02, C vulgaris SE002 and S.dimorphusPRR05 were 0.88 ± 0.04, 1.53 ± 0.10, 3.79 ± 0.19, 3.18± 0.10, 2.41 ± 0.17 and 1.92 ± 0.09 days-1, respectively. Thus, some of these strains might be suitable feedstocks for bioethanol or biogass production5. The highest growth rate was recorded in C. vulgaris PRR04 after 21 days of incubation was 3.79 ± 0.19 compared with an initial reading of 0.32 ± 0.07. At the same time S. obliquus PRR02 showed the growth rate 3.18 ± 0.10 compared with an initial reading of 0.32 ± 0.06 at OD 680 nm. Algal growth is directly affected by the various environmental factors such as availability of nutrients, light, the stability of pH, temperature and the initial inoculum density14. But only some strains have an inherent adaptability to adapt quickly to changes in the environment and other conditions. In our study the selected candidates showed higher biomass productivity (Table 1) which is suitable for high density culture and open pond cultivation. Our result clearly indicates that C. vulgaris PRR04 is suitable for high-density culture and S. obliquus PRR02 can also be taken in the consideration of large cultivation. These results may also aid in the ease of culturing an indigenous algae at large scale due to shorter acclimation period.

Cell Biomass, Lipid Content and Lipid Productivity

Neutral lipids are produced by a large group of microalgae isolated from diverse aquatic environments. These lipids are favorable candidates for conversion to biodiesel32. Many microalgae species can be induced to accumulate substantial quantities of lipids26, thus contributing to a high oil yield. Lipid productivity takes into an account both in the lipid concentration within the cells and the biomass produced by these cells and is therefore it acts as a good indicator of the potential costs of liquid biofuel production. For analyzing cell biomass and lipid production profile, the six indigenous algal cultures were grown under a 12 h light and 12 h dark photoperiod in 250 mL flask at 25 ˚C in a stationary condition with hand shaking twice a day, after 21days of incubation algal cells were harvested tested for their lipid production by evaluating biomass productivity (Table 2). Biomass productivities (g dwt L-1) of 1.08 ± 0.07; 1.51 ± 0.05; 1.72 ± 0.06; 1.57 ± 0.03; 1.46 ± 0.02 and 1.53 ± 0.06 were found for S. armatus, S. deserticola, C. vulgaris, S. obliquus, C. vulgaris and S.dimorphus respectively. The total lipid contents for the microalgae cultured in this study ranged from 19 - 34% of the dry weight (Table 2). S. obliquus PRR02 showed the highest lipid content where as S.dimorphus PRR05 showed the lowest lipid content. The lipid productivity of S. obliquus PRR02 was highest at 0.53 ± 0.04 g L-1, when compared with the other microalgal species (Fig 5). . In this study the percentage of Lipid content of selected strains ranged from 19-34% of the dry biomass weight, were quite good without any limiting conditions. However, previous studies have demonstrated that some Chlorella and Scenedesmus species can produce more lipids under certain conditions10,25.

Fatty acid composition

Biodiesel consists of largely of fatty acid methyl esters which are produced by the transesterification of biologically derived lipids33. Differences in chemical and physical properties among biodiesel fuels can be explained largely by the fuels Fatty acid profiles. Therefore, Fatty acid composition of six selected strains of microalgae S. armatus FW005, S. deserticola FW006, C. vulgaris PRR04, S. obliquus PRR02, C vulgaris SE002 and S.dimorphusPRR05 were primarily esterified and the major fatty acid composition of each isolate was determined through Gas Charomatographic analysis . The fatty acid profiles of the isolates indicated the presence of lauric (C12:0), myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), Heptadecanoic acid (C17:0), stearic acid (C18:0), oleic acid (C18:1n9c), linoleic acid (C18:2n6c), α – linolenic acid (C18:3n3) and γ - linolenic acid (C18:3n6) (Table. 3). The major fatty acids are lauric acid, palmitic acid and oleic acid comprising of 5-35%, 16-54% and 3-34 % of the total fatty acids respectively, whereas other fatty acids existed as minor fatty acids. Palmitic acid occupied 46% and 54% in S.deserticola and S.obliquus respectively. Oleic acid, occupied up to 34% of the total fatty acids in C. vulgaris SE002. S.armatus showed 49% of Linoleic acid of their total fatty acids. The most common fatty acid methyl esters present in biodiesel are palmitic acid, stearic acid, oleic acid, linoleic acid, and linolenic acid14, which were also the major fatty acids synthesized in the six indigenous microalgal species isolated in this study (Table 3).According to Kaur et.al13 prolonged cultivation of microalgal cultures leads to an increased synthesis of the total saturated and monounsaturated fatty acids of Scenedesmus and Desmodesmus spp. In our study ,the selected indigenous strains showed higher saturated fatty acids (Table 3), which is ideal for biodiesel production whereas high proportion of poly-unsaturated fatty acids is not suitable for

biodiesel due to its potential oxidation tendency and the commission of Europe laid down rule to limit poly-unsaturated fatty acid component to less than 12% (http:// www.biofuelsystems.com/specification.htm). Based on these results, high monounsaturated oleic acid ratio seems to be a distinctive feature. Higher oleic acid increases the oxidative stability of fuel enabling longer storage31 and decrease the CFPP of fuel allowing it to be used in cold regions32. Among the tested strains, C. vulgaris SE002 showed highest oleic acid content and more over the presence of oleic acid showed in all the strains, this acid content making it the most suitable for quality biodiesel production.

CONCLUSION

A concerted scientific effort in regionally-based phycoprospecting for indigenous microalgae with advantageous characteristics will increase the rate and application of sustainable biotechnological solutions. The objective of this research work is to generate a primary effort for ascertaining the feasibility of large scale indigenous microalgal biodiesel production using promising cultures isolated from native fresh water system that have high oil content. The result of this experiment can serve as useful reference for selection of potential indigenous microalgal candidate for further assessment of the techno-economical feasibility of developing a microalgae-based industry in the region.

ACKNOWLEDGEMENTS

The authors thank Tama´s Felfoldi for helpful discussions toward molecular characterization and Head of the Department, Presidency College for his kind support

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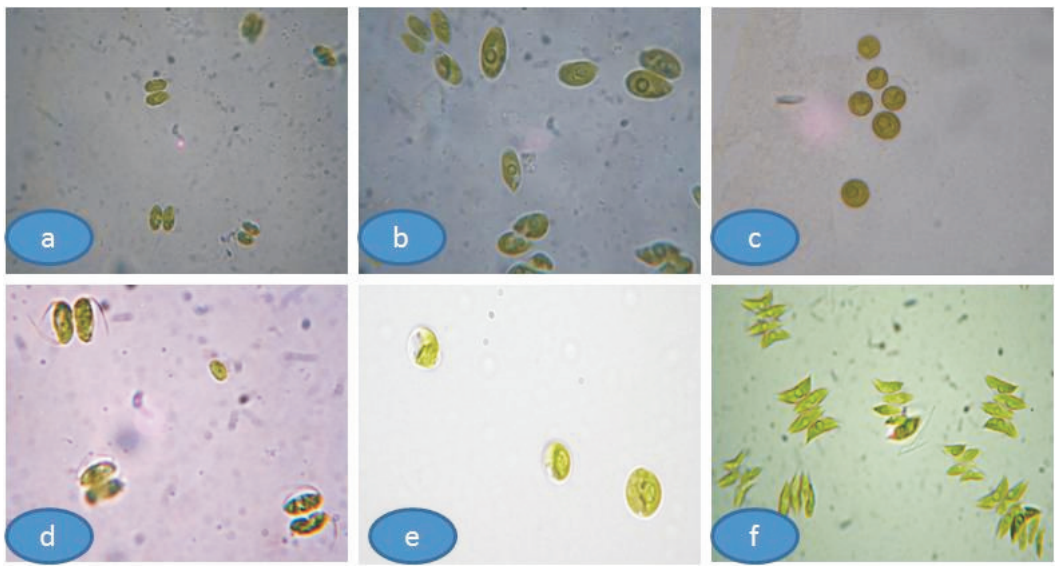
Figure Legends

Figure 1 - Light Microscopic images of Selected Microalgal Species



a-FW_{005}, b- FW_{006}, c- PRR_{04}, d- PRR_{02}, e- SE_{002}, f- PRR_{05} Unique scale bar – 10 µm.

Figure 2 Phylogenetic tree showing the relationships among 18s sequences of isolate FW005 and FW006 and the most similar sequences retrieved from NCBI nucleotide database

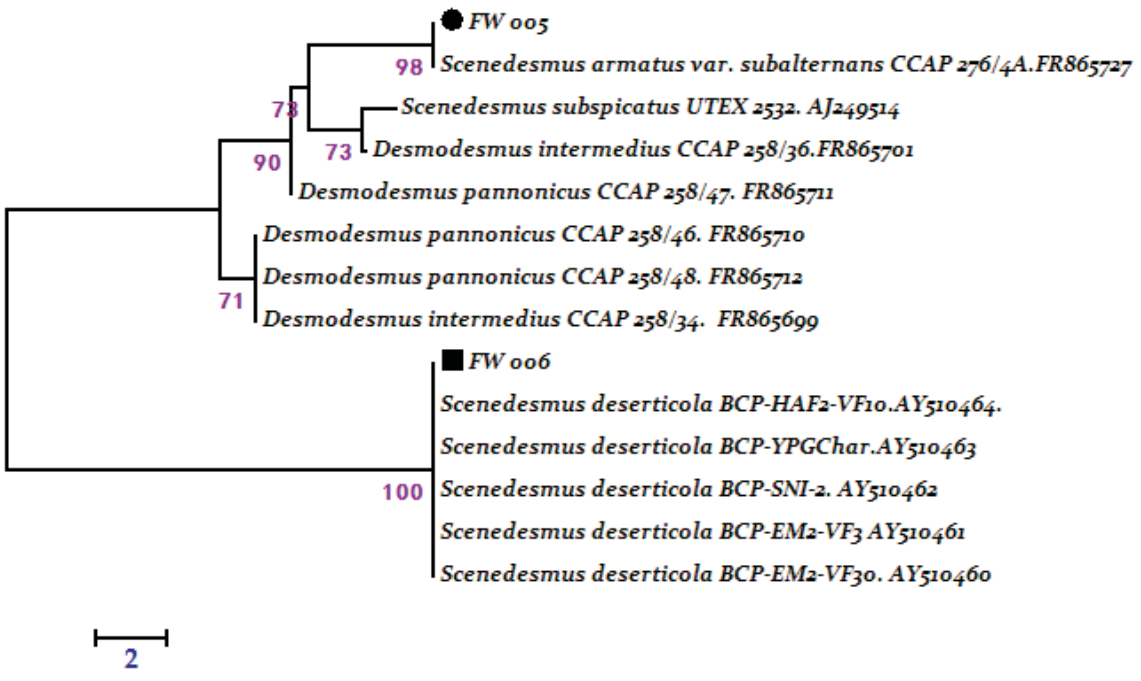


Figure 3 - Phylogenetic tree showing the relationships among LSU rDNA D1-D2 sequences of isolate PRR002, PRR04, SE002 and PRR05 and the most similar sequences retrieved from NCBI nucleotide database

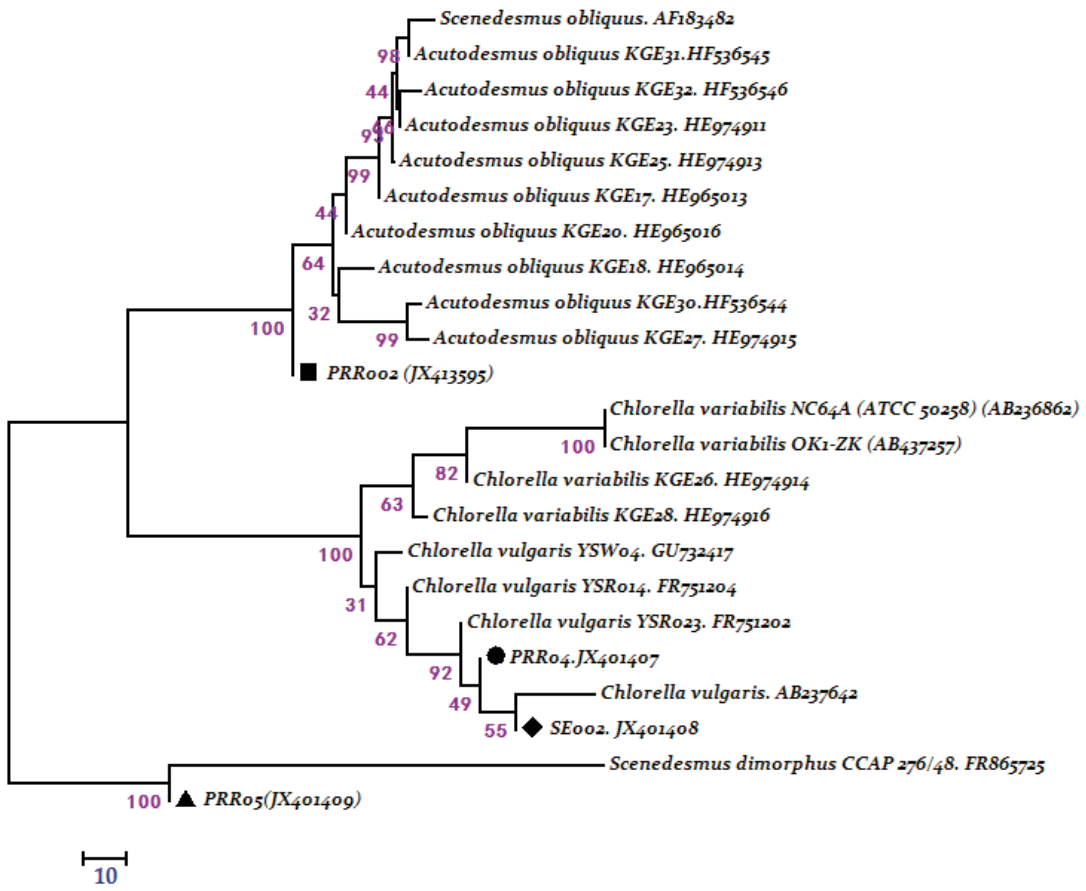
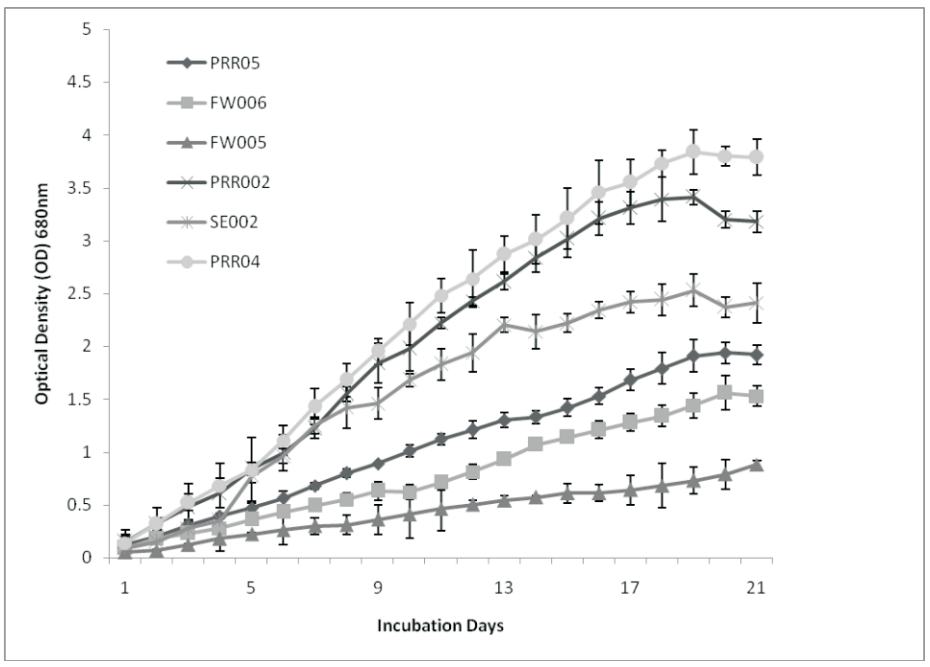
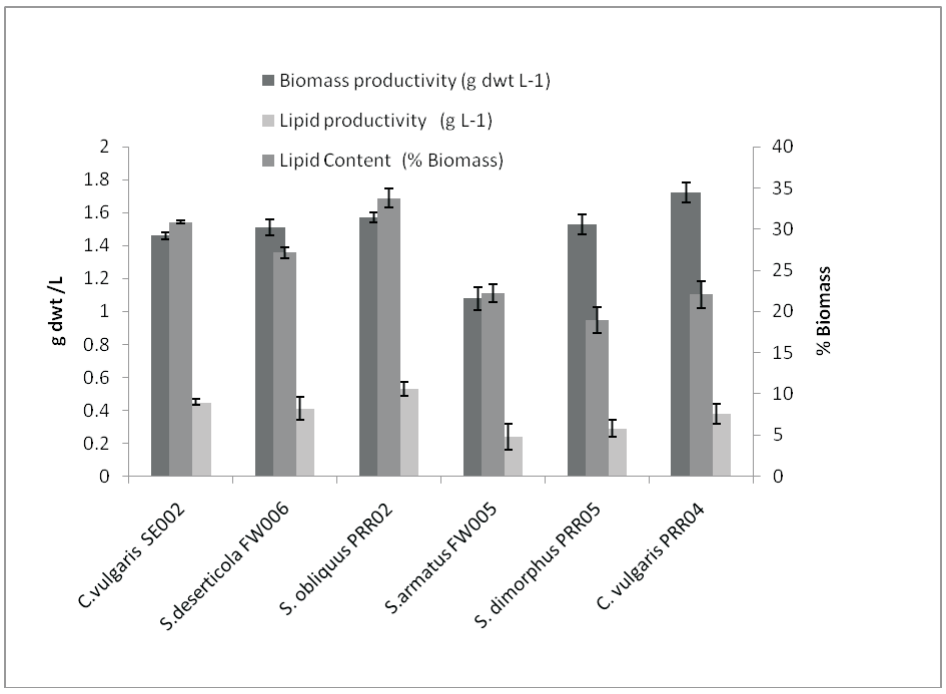


Figure 4 - Growth curves of six microalgal species cultivated in a batch experiment



Experiments were carried out in triplicate

Figure 5



Experiments were carried out in triplicate