ISOLATION, CHARACTERIZATION, METABOLITES PROFILING OF FRESHWATER MICROALGAE FROM WATERFALLS OF THIRUMOORTHY HILLS, TAMILNADU, INDIA

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Abstract

Microalgae are a common species found in freshwater areas as well as seashores. Microalgae are different from the nature of the microorganisms, differences such as morphology, physiology and the genetics of them. Microalgal samples were collected from Thirumoorthymalai Hills freshwater and are characterized by morphological and sequencing of 16s ribosomal DNA. The sequence revealed that four samples were *Leptolyngbya subtilis, Leptolyngbya sp, Oscillatoria tenuis,* and *Phormidium sp. based* on genetic similarity. These microalgae were cultivated and their bioactive compounds were analyzed by using GC-MS and FTIR, which shows that these Microalgae have vital bioactive metabolites which may have antimicrobial activity, anti-inflammatory activity, and anti-cancer activity.

Keywords: Microalgae, Freshwater, 16s RNA sequence, GC-MS

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Introduction

Microalgae are widely grown in both fresh and saltwater areas. These unicellular microorganisms' size ranges from 3-10µm. Microalgae consist of both prokaryotes and Eukaryotes [1, 24]. Cyanobacteria is a type of microalgae that possesses both bacterial and microalgae characteristics because of the presence of chlorophyll (chlorophyll a and chlorophyll b). Bioactive compounds such as fatty acids, amino acids, vitamins, and pigments. These bioactive metabolites from the microalgae play a vital role in developing new commercial products. Some species of microalgae were approved by FDA by the issuance of GRAS (Generally Recognized as Safe) [2].

Molecular characterization of the microalgae 16s rRNA widely employed to determine the nucleotide sequences of the microalgae. This molecular characterization is widely used to know about the evolution of these microalgae [3]. The microalgae species are photosynthetically more efficient than anyother organism and are efficient CO2 fixers. Each species of microalgae is efficient to produce different ratios of lipids, carbohydrates, and proteins. They are single cell photosynthetic organisms that use light energy nutrients and carbon sources to produce proteins, carbohydrates, lipids, and other valuable products. For example, the pigments from the microalgae widely used in dyes and PUFA's are used in nutritional supplements [4, 22].

Microalgae produce various metabolites as chemicals for their defense mechanism. The defense mechanism comes to act against predation, herbivores, and competition for space. Value-added products can be extracted from the microalgae biomass. Value-added products such as pigments, polysaccharides, triglycerides, fatty acids, and vitamins [5, 23]. Which are commonly used as bulk commodities and specialty chemicals in different industrial sectors. For example Pharmaceutical industry, Cosmetics, nutraceuticals, functional foods, aquaculture, biofuels. High-value added from the microalgae can be produced within a biorefinery model. Since the composition of the microalgae cell allows for the extraction of different co-products. Although specialty chemicals have higher revenue than bulk chemicals like oils as biofuel.

Discovery of drug has been developed greatly in the process of screening large numbers of pure organic compounds or crude extracts to provide new lead and large-scale screening will continue to play an crucial role in the procedure of developing the new drug. In recent times, microbes resistance against antibiotics have increased, due to this the need for novel antimicrobial compounds also increased [6, 25]. Currently, microalgae have been targeted as a source of bioactive compounds. Microalgae were producer of various antibiotic substances that are capable to inhibit bacteria, viruses, and fungi. Hence, the present study was focused on identifying the strains with novel bioactive compounds.

Materials and methods

Microalgal samples were collected from waterfalls of Thirumoorthy hills (Tamilnadu, India) freshwater rocks. The collected samples were brought to the laboratory for further investigations. Morphological analysis was done using Light microscope and it was named as C9, SA001, and 3T1, 2T2A.

Harvested microalgae biomass was suspended in 500µl extraction buffer (200 mM Tris-Cl, pH 8.5, 250mM NaCl, 25mM EDTA, 0.5% SDS) and vortexed well. Then the resulting suspension was added with 350µl of ice-cold phenol: chloroform: isoamyl alcohol (25:24:1) and vortexed for a minute and subsequently centrifuged at 15000 rpm for 45 minutes. The aqueous phase was transferred to a fresh tube containing 1mg/ml final concentration of RNAse and incubated for 10 minutes at 37ºC. 350µl of ice-cold phenol: chloroform: isoamyl alcohol (25:24:1) was added to the solution, mix thoroughly, and centrifuge for 10 minutes at 15000 rpm. The supernatant was taken and 250µl of icecold isopropanol was added for DNA precipitation. 70% of ethanol was used to wash the nucleic acid pellet. Air-dried and suspended with 50µl TE buffer (10mM Tris-Cl, pH 8.0 &1mM EDTA) and stored at -20oC [7, 19, 20]. The sequences of the oligonucleotide primers used for PCR were listed in Table 1. Each DNA sample amplification was performed in a volume of 50 µl containing 1 µl of primer, 1 µl of 1.25 mM dNTPs, 1 (l (50ng) of DNA template and 1 µl of DyNAzyme DNA polymerase (Finnzymes, Finland). The buffer with enzyme was used according to the manufacturer's instructions. The amplifications were made with thermocycler (Applied Biosystems). After final reaction, amplified DNA was separated using lower melting point agarose (1.2%) (Sigma, USA. Among the seven isolates, the selected three isolates namely SA001, C9, 3T1, and 2T2A were subjected for their 16S rRNA gene sequence analysis. Primers CYA359 in combination with CYA781R(A) were used to specifically amplify the 18SrDNA fragments of green microalgae strain C9, SA001, 3T1, and 2T2A. The amplified PCR products were

purified using a QIA quick PCR purification kit (Qiagen GmBh, Germany) as recommended by the manufacturer. The sequences of the PCR products were determined by using the Big Dye Terminator Cycle Sequencing v2.0 kit on an ABI310 automatic DNA sequencer (Applied Biosystems, CA, USA) according to the manufacturer's instructions. The determined 18S rRNA gene sequences were deposited in the GenBank database [8,21].

The collected samples were cultivated using a BG-11 medium specifically for the cultivation of terrestrial microalgae. The composition of the BG-11 media. 1ml from trace metal mix was added to 1000ml of the medium. 0.8% of agar was added in the case of the BG-11agar medium. Made up to 1 liter with deionized water and sterilized at 15 psi for 15 minutes. Microalgae cells were harvested using centrifuged at 5000 rpm for 10 minutes. 2 grams of cells were harvested using centrifugation and the cell pellet was washed twice with distilled water. The biomass was collected after filtration. The biomass was lyophilized using free zone lyophilizer 6, lyophilization was carried out in -80°C to -86°C temperature[9, 26].

The methanol extracts of the powdered microalgae sample were analyzed for the presence of different compounds by GC-MS. GC-MS analysis of some of the potent volatile constituents present in the extracts was performed at The South Indian Textile Research Association (SITRA), Coimbatore, Tamil Nadu, India. GC analysis of the extract was performed using a GC-MS (Model; Thermo Trace GC Ultra) equipped with a DB-5MS fused silica capillary column (30m length x 0.25mm outside diameter x 0.25µm internal diameter) and GC interfaced to a Mass Selective Detector (MS-DSQ-II) with XCALIBUR software [10].

For GC-MS detection, an electron ionization system with an ionization energy of -70eV was used. Helium gas was used as a carrier gas at a constant flow rate of 1ml/min and the sample injected was 1µl; Injector temperature was 260ºC; Ion source temperature was 200ºC. The oven temperature was programmed from 70ºC to 260ºC at the rate of 6ºC/min, held isothermal for 1min, and finally raised to 260ºC at 10ºC/min. The interface temperature was kept at 260ºC. Total GC run time was 37.52 min. The relative percentage of the extract constituents was expressed as a percentage with peak area normalization. FT-IR analysis was used to identify the functional group of the microalgae [11].

Results and discussion

In recent years, microalgae have gained interest in producing valuable molecules ranging from therapeutic proteins to biofuels. Nutritional and medical applications are most suitable for these organisms because many biomolecules expressed by microalgae are generally regarded as safe (GRAS) for human consumption [12]. Although numerous species produce useful compounds naturally, these unicellular organisms are also well suited for genetic manipulation and high-throughput analysis. So, due to the advantages of microalgae, this work was chosen. The sample was collected from the freshwater areas in the Thiru Moorthy hills. The location, type, and appearance of the microalgae sample are given in Table 1. The physicochemical parameters of the aquatic environment are responsible for biodiversity and enrichment of phytoplankton and primary producers, which are designing the food web process. The environmental factors which favor the growth of the green microalgae include high nutrient concentration and light availability. The physicochemical parameters of the water samples were analyzed to know the nature of the environment of the isolated strains.

Table 1 Sample Name, Type and Sample Collection Site Latitude and Longitude

S.No	Sample	Appearance	Type	Latitude	Longitude
1	C9	Green Color	Water microalgae	10.4858 N	77.178 E
2	SA001	Green Color	Water microalgae	10.9132 N	77.369 E
3	3T1	Green Color	Water microalgae	28.1422 N	75.8737 E
4	2T2A	Green Color	Water microalgae	10.5408 N	76.9822 E

The microalgae isolate was viewed under Light Microscope, and the picture is shown in Figure 1. The morphology of microalgae can be observed by using a light microscope [13]. In this technique, the microalgal cells were mounted on the glass slide and viewed under a light microscope at ambient temperature.

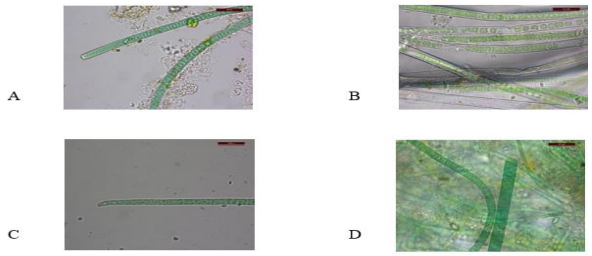

Figure 1 Microscopic Observation of microalgal isolates (A – C9, B – SA001, C - 2T2A, D – 3T1)

The microalgae isolate was mass cultivated using a BG-11 medium (Figure 2) specific for terrestrial microalgae. The isolation of the microalgae can be done by subculturing method [14]. Frequent sub-culturing leads to the isolation of microalgae and makes the subculture the pure culture. The biomass of the microalgae isolate. The growth rate of microalgae is normally measured by the total amount of accumulated biomass per unit and volume, and microalgae should be maintained at a particular temperature, light, subsequent oxygen, and sufficient nutrient input [15].

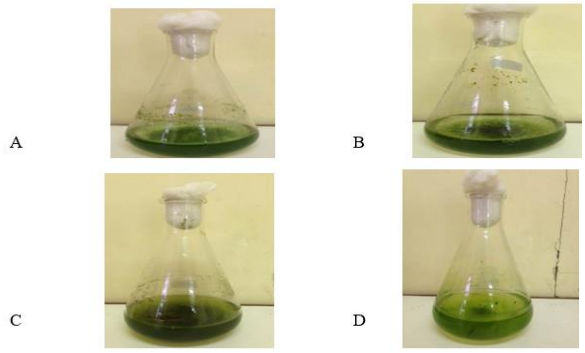

Figure 2 Biomass Cultivation of Microalgal isolates (A – C9, B – SA001, C - 2T2A, D – 3T1)

The identification and molecular characterization of microalgae isolates were done by the 16s rRNA sequence method (Figure 3). The harvesting of the microalgal biomass can be done by several methods such as sedimentation, centrifugation, filtration, ultrafiltration, sometimes with an additional flocculation step or combination of flocculation and flotation. In the present study, the centrifugation method was used to harvest the microalgal biomass because of the easiest and most effective way to approach the harvesting of microalgae biomass [16]. Apart from the morphological observation, the microalgal identification can be done by comparing the 16s rRNA sequence with the known DNA sequences to identify the isolate [17].

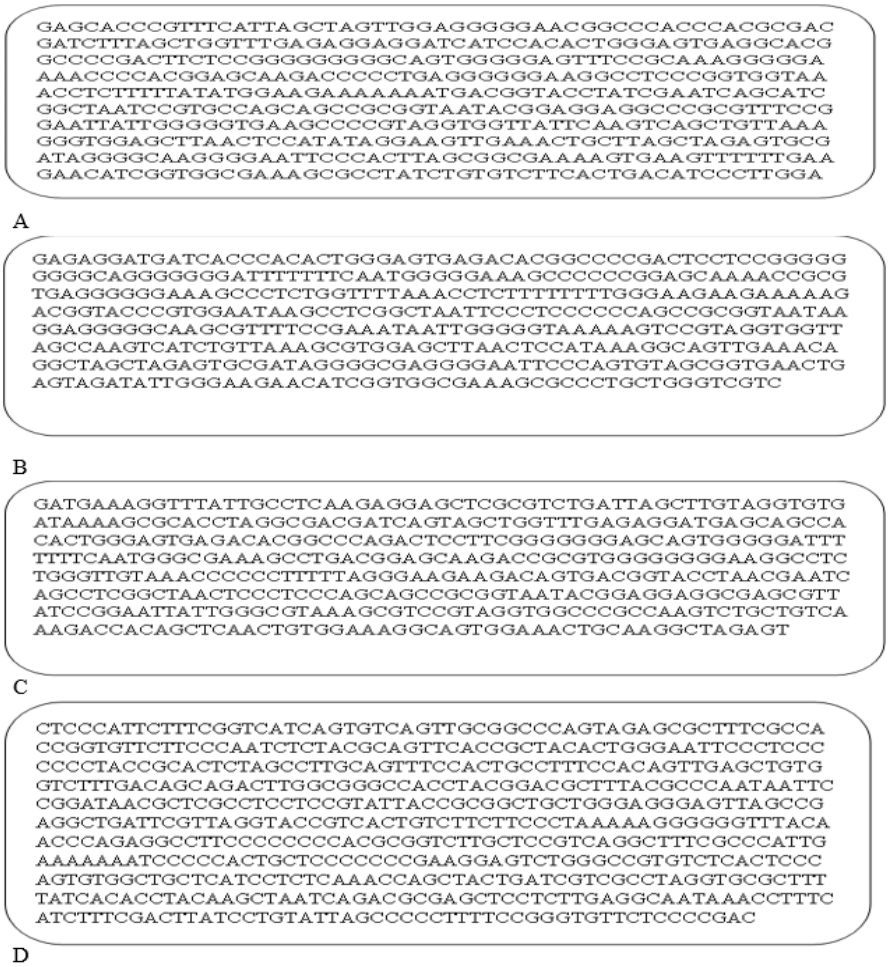

Figure 3 16s rRNA sequence of microalgal isolates (A – C9, B – SA001, C - 2T2A, D – 3T1)

In this study, 16s rRNA profiling was done to identify the microalgal isolate. After the molecular identification, the sequences were blasted in NCBI, which reveals similar organisms such SA001- *Leptolyngbya subtilis* (98%), C9 - *Leptolyngbya sp* (98%), 3T1 - Oscillatoria tenuis(98%), and 2T2A - *Phormidium sp*(98%). The partial sequence of the 16srRNA gene obtained for microalgae isolates was submitted to genebank through the BankIt program, at the NCBI site.

The accession number of the strains were shown in Table 2.

Table 2 Accession Number of the Microalgal isolates

Microalgal Isolates	NCBI Accession Number	Strain Name
SA001	KY817318	SAR 1
C9	KY817319	SAR 2
3T1	KY817320	SAR 3
2T2A	KY863515	SAR 4

Based on biomass cultivation, the C9 strain was found to be promising and further metabolites extraction and characterization were done with it. An analytical method such as GC-MS analysis can be used to characterize and identify the natural compounds in the microalgae. This result almost supported us for the Leptolyngbya sp. characterization, and identification of the natural compounds [18]. Ç-HIMACHALENE is the compound that shows a higher retention time of 36.53 from GC-MS analysis (Table 3 and Figure 4).

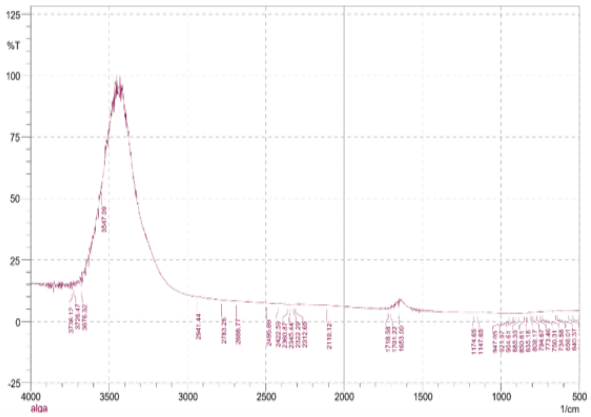

Figure 4 GC – MS analysis of SAR 2 Strain (C9)

Table 3 GC – MS analysis of SAR 2 Strain (C9)

S.No	Compound Name	Retention Time
1.	Dodecane(CAS)	6.82
2.	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	19.71
3.	Hexadecanoic acid, methyl ester (CAS)	21.76
4.	14,17-Octadecadienoic acid, methyl ester	25.55
5.	9á-Acetoxy-3,5à,8-trimethyltricyclo[6.3.1.0(1,5)]dodec-3-ene	30.84
6.	2,5-Bis(10-hexyldibenzothiazine-3-yl)thiophene	32.80
7.	ç-HIMACHALENE	36.53

The lyophilized culture C9 (SAR 2) was analyzed by FTIR to identify the functional group of natural bioactive compounds present in the microalgal sample. The intensive peak of the natural compound is 3547.09 cm-1. The peak relevance to the Alcohol/Phenol O-H stretch (Figure 5).

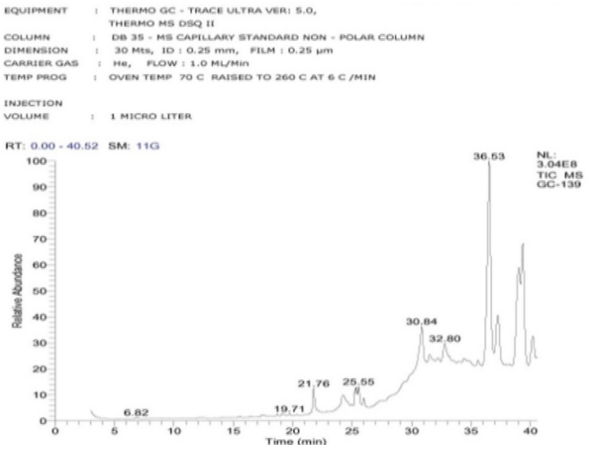

Figure 5 FTIR Analysis of SAR 2 Strain (C9)

Conclusions

Based on the results obtained from the present study it is concluded that there is a vast scope for the probable utilization of these microalgae isolates for the production of bioactive natural compounds. These isolates were found to be rich in novel bioactive compounds that may have various functions. There is a need for further study of the identified compounds and their activities in the treatment and prevention of various diseases, in addition to an ongoing search for other, as yet undetected, metabolites.

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