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Genetic identification of new microalgal species from Epe Lagoon of West Africa accumulating high lipids

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ABSTRACT

The green microalgal strains are favorable alternatives for producing biodiesel. In spite of the worldwide awareness on microalgae-derived oil to potentially replace the conventional fossil fuel, information on potential microalgal strains from Nigerian lagoons is scarce. In view of this, environmental samples were collected from the Epe Lagoon, Nigeria to assess the biodiversity of microalgae and identify robust strains for biodiesel production. The field samples were collected and genomic DNA was isolated from the monoclonal cultures. Microalgal species were identified based on the PCR amplified DNA sequences of 18S ribosomal DNA. The sequences of PCR products from the unknown samples were compared with the DNA database. Using the phylogenetic maximum-likelihood trees, the green algal strains *Desmodesmus* sp., *Desmodesmus costato-granulatus*, *Tetradesmus* sp. and *Chlorella* sp. were identified, while two strains belonging to the Chlorococcaceae and Family Selenastraceae need further validation. Microalgal species morphology was validated using electron microscopy. Lipid analysis was carried out using Fatty Acid Methyl Ester (FAMEs) analysis, Gas Chromatography and Mass Spectrometry (GC–MS). The strain of the Family Selenastraceae produced the maximum amount of poly-unsaturated fatty acids (56%), which has potential for food processing and other bio-based products; while *Tetradesmus* sp. with 35.16 mg (FAMEs per gram of dry cell weight) had a balanced lipid composition (44% saturated fatty acid, 39% mono-unsaturated fatty acid, 15% poly-unsaturated fatty acids) making it a good candidate for exploitation as a biodiesel feedstock.

1. Introduction

Lagoons are coastal bodies of shallow water formed where low-lying rock, sand or coral presents a partial barrier to the open sea and are common features along the West African coast [48]. The coastline of South Western Nigeria is made up of nine lagoons and ecological studies within these lagoons have documented the presence of different divisions of algae, which include majorly the Bacillariophyta, Chlorophyta, Cyanophyta, Euglenophyta and Chrysophyta [1,2,35,38,49]. The Epe Lagoon in Nigeria is a freshwater ecosystem with a total surface area of 234 km^2 [31] and is connected to two other lagoons, the Lekki Lagoon to the East and Lagos Lagoon to the West. Artisanal fishing is the mainstay of the inhabitants and the lagoon supplies fish and fisheries to most part of Nigeria. Previous research work on the ecology of planktonic algae in Epe Lagoon include studies on the seasonal changes in phytoplankton composition in relation to environmental factors [36] and a report on the periphyton community on submerged aquatic macrophytes [37]. However, basic information on the microalgal species existing in Epe Lagoon and their potential use as biodiesel feedstock has not been documented despite the Nigerian government's consideration on introducing the biofuel technology in Nigeria using algae as a feedstock. Microalgae accumulate neutral lipids, in the form of triacylglycerides (TAGs) [8,17], which can be converted to biodiesel through a simple process of trans-esterification [13,19]. Therefore, many laboratories around the world are engaged in screening more oleaginous algal strains that are rich in lipids and testing their suitability at a commercially viable scale.

The genetic identification of microorganisms uses molecular technologies to evaluate the specific regions of the genome and distinctively determine which genus or species a microorganism belong. This technique has made it possible to study the genetic structure of populations of microorganisms such as microalgae [20] as well as identify and classify phenotypically plastic organisms [39]. Molecular techniques have successfully been utilized to identify other algae taxa [3,14,42,53]. Chloroplast (*rbcl*, *tuf*A and 23S) and nuclear genes (18S rDNA, *nu*ITS1 and *nu*ITS2) have been used in the DNA barcodes of the green algae [9,22]. However, the 18S rDNA marker has been used successfully in genera and species identification among the green algae [26,51,52] including

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microalgae taxa *Tetranephris brasiliense*, *Ankistrodesmus gracilis*, *Desmodesmus subspicatus*, *Chlorella sorokiniana* and *Monoraphidium dybowskii*. The 18S gene contains highly conserved regions with variable parts which allow species-level identification [27].

In this study, six green microalgal species were identified using 18S ribosomal DNA marker. In addition to genetic identification, electron microscopy was used to confirm the identity of the strains. The total lipid extracted from microalgal species was transesterified for Fatty Acid Methyl Esters (FAMEs) analysis and GC–MS was used to assess the fatty acid composition in isogenic algal cultures from the Epe Lagoon, Nigeria for biofuel applications.

1.1. Materials and methods

1.1.1. Sample collection and growth conditions

Natural algal strains were isolated from Epe Lagoon, Lagos, Nigeria (latitudes 03°50′–04°10′N and longitudes 005°30′–005°40′E) and cultivated in the laboratory using the BG-11 medium under the following culture conditions. 1 ml of environmental samples was added to 250 ml Erlenmeyer flasks containing 100 ml BG-11 liquid medium (Broth, Himedia). The medium was prepared by dissolving 1.627 g BG-11 broth in 1 l of distilled water; autoclaved at 121 °C after adjusting the pH to 7.3 with HCl (0.5 N). An aliquot of 750 μl of filter-sterilized trace metal solution was added to the medium after cooling. The components of the trace metals solution were H_3BO_3 (2.86 g/l), $MnCl_2 \cdot 4H_2O$ (1.81 g/l), $ZnSO_4 \cdot 7H_20$ (0.222 g/l), $Na_2MoO_4 \cdot 2H_20$ (0.039 g/l), $CuSO_4 \cdot 5H_2O$ (0.079 g/l) and $CoCl_2 \cdot 6H_2O$ (0.0494 g/l). Monoclonal cultures were obtained and purified by subjecting samples to successive rounds of streaking across BG-11 plates containing antibiotics. The antibiotics used to obtain axenic cultures include Ampicillin (100 mg/ml), Kanamycin (50 mg/ml), and Rifampicin (25 mg/ml). Samples were maintained in axenic conditions by adding 1 ml of antibiotic in 1 l of BG-11 culture medium. Isolates were maintained in a liquid medium and kept for constant shaking on orbital shaker at 150 rpm, a 16 h light and an 8 h dark photoperiod under 7000 lux light intensity.

1.1.2. Molecular characterization and phylogenetic analysis

Total genomic DNA was extracted from 1 g fresh cultures using CTAB method [16] and the conserved DNA region corresponding to 18S rDNA was amplified. The 18S primers used for the PCR amplification are NS1 - 5′-GTAGTCATATGCTTGTCTC-3′ and NS6 - 5′-GCATCACAGACCTGTTAT TGCCTC-3′. PCR amplification was carried out in a 20 μl reaction containing 10 mM dNTP, 5 pmol of each primer, 10× Taq buffer, 0.5 U of Taq polymerase (Real Biotech Corporation, Delhi) and 25 ng template DNA. The conditions for PCR amplification are an initial denaturation for 3 min at 95 °C, followed by 35 cycles of denaturation for 30 s at 95 °C, primer annealing for 30 s and extension for 30 s at 72 °C. The sizes of PCR products were approximately between 719 bp and 900 bp. DNA sequencing was carried out by Invitrogen Bioservices India Pvt. Ltd. (Gurgoan, India) and checked for similarity against other publically available sequences using Basic Local Alignment Search Tool (BLAST) algorithm [5]. DNA sequences were aligned automatically using the ClustalW [45] alignment algorithm under default parameters using Molecular Genetics Analysis (MEGA) 6.06 software [44]. The DNA sequences were compared by BLAST analysis to identify homologous taxa available on the NCBI DNA database. Phylogenetic trees were derived using maximum-likelihood algorithms [43]. The robustness of the topologies for the maximum-likelihood trees was estimated through Tamura-Nei model and bootstrap analysis [18] was based on 1000 re-samplings of the sequences. Gap/missing data treatment was completed by deletion. *Elliptochloris bilobata* (KT253169) was used as an outgroup to define the root of trees.

1.1.3. Microscopy

The morphological features and cellular details of algal cells were studied by observing algal cells under a light/fluorescence microscope (Nikon Eclipse, Japan). Taxonomic classification of species was based on the earlier descriptions by some authors [7,24,25,28,29,46]. Further studies were carried out using scanning electron microscopy (SEM) method which involved fixation of algal cells in 2.5% gultaraldehyde, post fixing in 1% Osmium tetraoxide, dehydration in ethanol, drying with air dryer, mounting on specimen stub, coating with sputter coater gold coating unit (POLARON SC7640, UK.) and imaging under SEM (Carl Zeiss EVO 40 used at 20 kV, UK.)

1.1.4. Growth kinetics and lipid analysis

Microalgae strains were cultivated in triplicate (100 ml BG-11) and growth was studied by measuring daily changes in optical density (OD) at 750 nm via UV-VIS spectrophotometer (Amersham Biosciences Ultrospec 3100 pro). Data were expressed as the means and standard deviations from triplicate determination. Moreover, the intracellular lipid bodies of algal cells were also visualized daily via Nile red (9-diethlyamino-5H-benzo(*a*)-phenoxazine-5-one) staining. 1 ml aliquot of the cultures was taken daily and centrifuged at 13,000 rpm for 2 min, and the pellet was re-suspended in 1 ml of 20% DMSO solution and vortexed for 10 min at room temperature. The pellet was further re-suspended in 1 ml of distilled water and vortexed for 2–3 min before adding 3 μl Nile red stain solution (50 μg/ml) and incubated in the dark for 10 min at room temperature. Stained cells were visualized under the fluorescent microscope (Nikon Model 50i) via UV light with emission and excitation at 575 nm and 530 nm, respectively [11].

1.1.5. Determination of neutral lipid

The analysis of neutral lipid production was estimated by culturing algal cells under a 16 h light and an 8 h dark photoperiod at 25 °C in 60 ml BG-11 medium with continuous shaking on an orbital shaker for 16 days. Two separate aliquots of fresh algal suspensions (volume 200 μl) were used for the determination of neutral lipid; one aliquot was stained with 50 μl Nile red solution (15 μg/ml) while the other was left unstained, and both samples were incubated in the dark for 10 min. The fluorescence of both samples was measured on a Multimode SpectraMax spectrophotometer (Molecular Devices, USA) at excitation and emission wavelengths of 485 nm and 552 nm respectively. The optical density of the unstained algal sample was read at 750 nm. The difference in fluorescence intensity of Nile red was estimated by subtracting the auto-fluorescence of algal cells from the fluorescence intensity of Nile red alone in the solution [32] and the lipid yield was calculated using following equation:

$$\text{Fluorescence unit/OD of cells} = (x - y)/z$$

where:

x	fluorescence value of stained algae cells
y	fluorescence value of unstained algae cells
z	OD_{750} (optical density at 750 nm).

The optical density of the cell suspension used for this analysis was 0.30 (OD_{750}). Data were expressed as the means and standard deviations from triplicate determination.

1.1.6. Lipid extraction, transesterification and FAME quantification

For lipid production analysis, the primary inoculum was prepared by cultivating each microalgal strain in 100 ml BG-11 medium for six days until an OD reading of 0.6 was recorded at 750 nm. The primary cultures were then diluted in triplicate (200 ml BG-11) and allowed to grow for 15 days under a 16 h light and an 8 h dark photoperiod at 25 °C with continuous shaking on an orbital shaker. Algal cells were harvested by centrifugation on the 15th day. Lipid was extracted from wet algal biomass (100 mg dry mass equivalent) using wet lipid extraction procedure [41]. Transesterification was performed by adding 2 ml of Hexane to every 10 mg of lipid and using methanolic KOH (200 μl of