Screening, Morphological and Molecular Identification of Indigenous Microalgal Strains for Biodiesel Production

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Sustainability Statement Highlights:
- Utilizes Indigenous microalgae strains from fish farm of an institution in a Southwestern Nigeria, as a sustainable alternative to fossil fuels
- Reduces greenhouse gas emissions and pollutants, contributing to a cleaner environment.
- Microalgae-derived biodiesel is non-toxic and environmentally friendly.
- Enhances local economies and reduces dependency on fuel imports.
- Strains are well-adapted to local conditions, ensuring resilience and efficiency in energy production.

Abstract: In pursuing sustainable and eco-friendly energy sources, this study explores the potential use of microalgae as a biodiesel alternative to fossil diesel. Our study explores using an institution in a Southwestern Nigeria as a case study for the selection of suitable microalgae. Samples of water were collected from fishponds in the Western part of Nigeria. The technique of algae culturing was used for the isolation of microalgal strains. Microalgal screening for lipid capability production was carried out using Sudan black B and Volumetric Lipid Productivity (VLP). Morphological and molecular methods of morphological characteristics and 18S rRNA genes were used to identify strains with higher Lipid production. A total of l 36 microalgal were identified. Fifty strains were successfully isolated. Thirty-two out of the fifty showed black granules during primary screening with Sudan black B. 5, among 32 strains, had higher potential for production of lipids and identified as Scenedesmus obliquus-SeA, Chlorella vulgaris-ChA, Pediastrum duplex-PeD, Coelastrum morum-CoC, and Chlorococcum littorale-CiB. Scenedesmus obliquus-SeA with up to a 99% similarity index in comparison with data obtained from the NCBI database. Molecular identified strains of microalgae in this study are ideal and have good potential for fossil diesel replacement and the production of biodiesel.

Keywords: Isolation, Screening, Lipid production, Morphological, Molecular, Microalgae, Biodiesel

1. Introduction

Demands and utilization of energy across different human sectors, including usage at our various homes, are at a geometrical rate(Alves et al., 2015; Poizot & Dolhem, 2011; Prabhu et al., 2019). The present sources of energy and fuels all over the world are fossil energy from petroleum sources, which is associated with the release of poisonous gases, a layer of ozone depletion, and prediction by researchers revealed that it will soon be depleted (Ganesan et al., 2020; Shanmugam et al., 2020; Singh et al., 2011) The best replacement for diesel obtained through petroleum exploration is a type of diesel obtained from vegetable oils that has been described as biodiesel(Alves et al., 2015) (Ganesan et al., 2020). Presently, researchers across the globe are working on renewable and sustainable energy sources (Chisti, 2007; Günay et al., 2019). Therefore, vegetable esters of triacyl glycerides are considered a replacement for diesel of petroleum origin. Production processes involve catalyst-aided triacyl glyceride transesterification (Yang et al., 2019). These esters of methyl are regarded as diesel from biological sources. Aside from its sustainability characteristics, biodiesel is highly degraded through biological means, non-toxicity, and friendly to the environment with minimal emissions of carbon (Li et al., 2021; Adeyinka Sikiru Yusuff et al., 2021; Živković & Veljković, 2017). Also, they had a higher density of energy than that of the petroleum source of diesel (Adeyinka S. Yusuff et al., 2024; Adeyinka Sikiru Yusuff et al., 2021).

Dependence on diesel feedstock from biomass obtained through plants like Gossypium hirsutum, Calophyllum inophyllum, and Zea mays which are useful food crops, has impeded the security of foods and resulted in scarcity all over the world (Bawane et al., 2020; Mourshed et al., 2020; Veljković et al., 2018). Robustness, higher growth and productivity of biomass, use of non-arable land efficiently, and proliferation strength in wastewater are a few of the parameters that make microalgae generally acceptable in diesel production (Günay et al., 2019; Huang et al., 2019), as well as capacities to mitigate change in climate (Brindhadevi et al., 2021; Goh et al., 2019).

Microalgae are photosynthetic, oxygen-releasing autotrophs that are found growing in freshwater, marine water, and wastewater environment (Umar et al., 2021) Microalgae are a fractional group of other groups of organisms that are photosynthetic. Therefore, this makes available numerous genes from which strains with the potential for the production of biological diesel sources can be obtained. Different researchers have extensively studied the functionality of microalgae in the production of diesel (Kalsum et al., 2018; Khalaji et al., 2021; Li et al., 2021). However, appropriate microalgal isolate selection is one of the major problems hindering the commercial production of diesel (Khalaji et al., 2021)

Local adoption of traditional strains for diesel production is more advantageous because it ensures ecological security. Likewise, the climatic and ecological situation of the environment is well adapted to strains of native microalgae. Local adoption of traditional strains for diesel production is more advantageous because it ensures ecological security. Likewise, the climatic and ecological situation of the environment is well adapted to strains of native microalgae (Li et al., 2021). Appropriate selection of isolates with a higher content of lipids and productivity of biomass are the basic precursors for the manufacturing of diesel by microalgae (Huang et al., 2019). The first requirement towards algal isolate screening and eventual selection as ideal diesel replacement in production is pure culture isolation from the Indigenous environment.

A unique system for determining algal strains with the best qualitative and quantitative biodiesel is the physio-prospecting approach, which entails higher productivity of lipids for the commercial sustainability of the production of biofuel (Shin et al., 2018). Bioprospecting may result in the detection of novel or robust strains of microalgae with distinct and competitive biomass productivity and a wide range of lipid yields (Shin et al., 2018). Previous works in this field are mostly limited to characterization at the genus level, as problems are linked to arrays of strains isolated locally. Identification at the genus level is not enough to determine the identity of the strains due to the similarity and relatedness of isolates at the genus level.

To advance and completely identify the strains of microalgae from genus to species level, molecular characterization of the strains is required. Therefore, the main target of this study is to search for new strains of microalgae from the local environment, screen the isolates for diesel production abilities, and finally identify the morphological and molecular characterizations of the screened isolates.

2. Methodology

2.1 Sampling and Sampling Methods

2.1.1 Sampling Location

This work was carried out in four selected earthen fish farm ponds from a tertiary institution in Southwestern Nigeria and fish ponds. The tertiary institution lies within Latitude and Longitude of 7.4417°N and 3.90000°E. The location of the fish ponds is 7°35'50''N Latitude and Longitude of 3°54'59''E.

2.1.2 Sample Collection

Samples of water were collected from the fish ponds in a tertiary institution in Southwestern Nigeria and fish ponds. The collection was done between 08.00 am to 12.00 noon. The plankton net shape of the cone method described by Bhosale et al (Patil et al., 2019) was used for microalgal sample collection. The net had a diameter of twenty centimetres in opening and twenty micrometres of silk bolting for the cloth of the net. At the base, the net was joined with a fifty millilitres vial for sample collection. To 1.5 to 2cm depth, the net was immersed and at 5 meters, it was towed. The collection vial content was poured into a 500 ml sterilized bottle for collection of samples. A solution of Iodine (Lugol's) of 2 ml was added to the sample for precipitation and preservation of species of algal within 8 hours (Mahadev, 2011).

2.2 Isolation and Purification of Microalgal Isolates

Sterilized media of Bold's Basa, Algae Culture, and Allen Blue-Green were used for algal isolation as well as purification (Wang et al., 2024). A sample of 10 ml of water was introduced to conical flasks of 500 ml containing 200 ml of media. At temperatures between 25°C to 37°C, media with inoculated samples were incubated under fluorescent cold white tubes with cycles of light and dark periods of 16hrs:8 min (Priya et al., 2015). For proper gaseous exchange and aeration, white cotton plugs (sterile) were used to cork the flasks (conical) and later at 100 rpm speed, they were placed on a rotary shaker REMI, RS-12R) for 3 weeks between 4 to 5 hours on daily basis. For the determination of the growth of microalgae, the light compound microscope was used to check the flasks at intervals of 48 hours. Dilutions (serial) were carried out on the above three stated media flasks with growth appearance. For strain isolation, petri dishes with agar prepared for the three-culturing media are inoculated with a solution of 50 µL culture. Petri plates were incubated under white tubes of cold fluorescent for two weeks at 27°C. Repeated plating and constant observation under a microscope were used to obtain the purity of the culture (Kaewkhaw et al., 2012; Tesař et al., 2024). The flowchart showing the processes involved after the sample has been collected is shown in Figure 1.
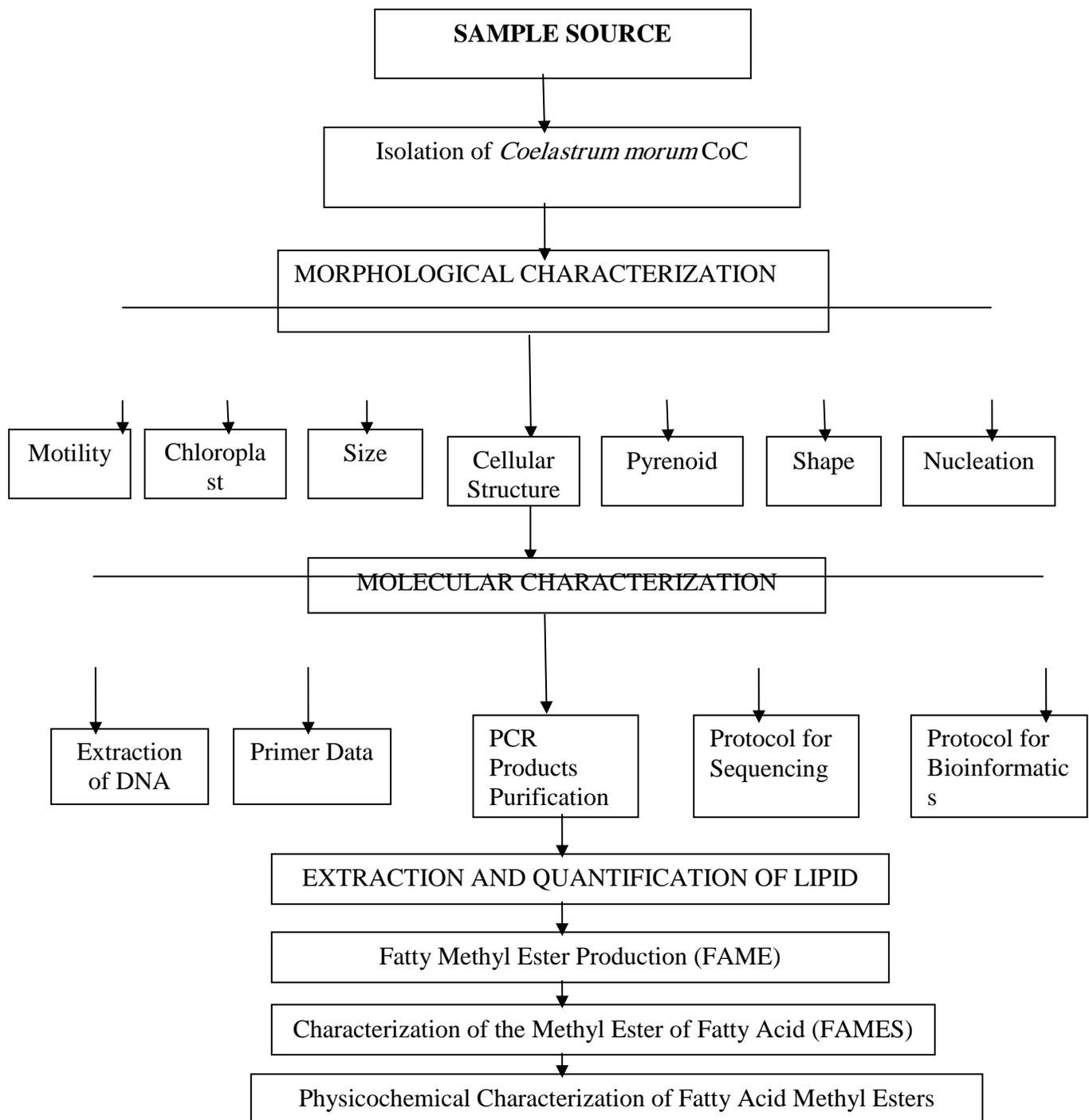
Figure 1: Flowchart Showing Process of Morphological and Molecular Characterization of the Isolates

2.3 Identification of Micro algal Species

A small pipette was used to take a drop from preserved pure culture and place it in the centre of the slide of the microscope. Tweezers were used to hold the cover of the glass on one side. To reduce the waves caused by bubbles of air, the cover of the glass was lowered onto the drop from pure culture placed on the slide. To avoid floating the glass cover freely, tissue paper was used to remove excess

water. Under objectives x10 and x40, the stage fixed slide of the microscope was examined simple light microscope. Available standards of the keys, literature, and other references that are comparative were used for the identification of microalgal pure cultures(Hatem & Al-Sultan, 2023; Oyelami et al., 2023; Sangapillai & Marimuthu, 2019)

2.4 Screening experiment for Biodiesel production

2.4.1. Preliminary screening of algae for lipids content

A smear of freshly growing culture of microalgae was stained with Sudan(Brindha et al., 2023; Prabhu et al., 2019). The stained slide was kept for 5 minutes at 37°C. Distilled water was used to remove stain excess and later counter-stained for 30 seconds with

safranin. Microscopic slides were washed with water that are distilled and dried through the air and finally observed under a digital compound microscope with high magnification (Motic BA 3100).

2.4.2 Screening of Micro-algal Isolates for Biodiesel Production

This was achieved through parameters of kinetic evaluation based on the phase of exponential growth described by the method of Nascimento et al. Nascimento (Nascimento et al., 2012) Production of Lipid efficiency by microalgae was established by the index of performance through dual roles of the content of lipids and productivity of biomass combined with productivity of lipid (Yeh & Chang, 2012). Conical flasks of 500 ml with a content (of 200 ml) of Bold's Basal Medium were used for the experimental setup involving initially screened microalgal strains (thirty-two). To separate flasks, 10 ml of exponentially growing pure culture of isolates from microalgae was inoculated and maintained at $25 \pm 2°C$ and $100\ rpm$ temperature and agitation speed respectively. The initial pH for incubation of the cells is 6.8 and 16:8h photoperiod under a fluorescent white tube of 2 feet(Cambridge University Press, 2002; Wu et al., 2024). After the stage of exponential growth (14 days), parameters of the kinetic were recorded for thirty-two distinct strains.

Concentration and Productivity of Biomass

Cells dry weight (g) of algal was obtained by measuring 10 ml of the experimental culture media on Whatman GF of filter paper type with 4.7 millimetres diameter, then with water (distilled) it was rinsed twice and for 24 hours, they were dried at 105°C. Concentration of biomass (g/l) was determined by equation (1) of Hempel et al. (Hempel et al., 2012) while another equation (2) by Hempel et al. 38 was used for the calculation of the Productivity of biomass $(P_{BIOMASS}, g/l/day)$.

Concentration of Biomass $(g/l) = M/V$ (1)

Where,

M = Weight of dry cell of micro alga

V = Culture of algal used volume

Productivity of Biomass $(P_{BIOMASS}, g/l/day) = \frac{X - X_o}{t}$ (2)

Where,

X = Biomass dry concentration at the end of the experiment

X_o = Dry biomass concentration at the initial stage of the experiment

t = duration of the batch run (day).

Extraction, Quantification of Total Lipid, and Estimation of Lipid productivity

To a gram of biomass of alga, $2\ ml$ and $1\ ml$ mixture of both methanol and chloroform was added in a ratio of 2 to 1 and for total dissolution of lipids; the mixture was kept at $37\ °C$ for 24 hours (Nascimento et al., 2012). The supernatant was then collected after centrifugation for 10 minutes at $300\ rpm$ (Bench Top, EBA 200). The procedure was repeated through the addition of chloroform $(2\ ml)$ to the pellets and was shaken properly. For 5 minutes, centrifugation was done again at $300\ rpm$ and this was preceded by the separation of supernatant. To supernatant, one percentage of potassium chloride $(2\ ml)$ was added and resulted in the formation of two distinct layers. The layer at the top that contain methanol cell was decanted while the bottom layer with chloroform and lipids extracted was collected into a new test tube. However, with $1\ ml$ of potassium chloride (of 1%), methanol methanol-contained layer at the upper part was extracted again with a similar procedure. This was followed by combination and evaporation of phase at the bottom (chloroform/lipids) and dried in an oven at $80\ °C$ for 24 hours. The content of lipid was expressed as the biomass of microalgae percentage (dry cell weight).

The Content of Lipid (C$_{LIPID}$) was measured from equation (3) described by Da Ros et al (Da Rós et al., 2012).

$$C_{LIPID} = \frac{W_L}{W_A} \times 100$$ (3)

Where,

$W_L\ (g)$ = Extracted Lipid weight

$W_A\ (g)$ = Dry cell of biomass of Microalgae

Productivity of Lipid, L_P in $(mgl^{-1}day^{-1})$ was estimated through equation (4) by multiplying the value of the content of lipids by the productivity of biomass measured for each species of microalgae (Da Rós et al., 2012).

$$L_P(mgl^{-1}day^{-1}) = \frac{P_{BIOMASS} \times L_{LIPID} \times 1000}{100}$$ (4)

Where,

$P_{BIOMASS}$ = Productivity of Biomass

L_{LIPID} = Content of Lipid

2.5. Identification of Microalgal by Morphological and Molecular Characterization

2.5.1. Morphological characterization of algal isolates

Based on characteristics (shape, size, cellular structure i.e. unicellular/multicellular, chloroplast, pyrenoid, motility, and nucleated, characterization of isolates was successful through the identity of the morphology (of the algal strains) with the aid of a light compound microscope.

2.5.2 Characterization morphological

The motility, chloroplast, size, cellular structure (unicellular/multicellular), pyrenoid, Shape, and nucleation are morphological traits to be examined using a compound light microscope.

2.5.3 Characterization of the molecular structure

The Extraction of Deoxyribonucleic Acid (DNA)

DNA from the genome was retrieved from microalgae utilizing the Matrix Genomic isolation kit from Insta GeneTM. Following the kit's instructions, the approach indicated below was used. Microalgae colonies were selected and placed within the confines of a microfuge tube with 1 ml of water that is free of contaminants. The supernatant was decanted after centrifugation at $10,000-12,000$ rpm for 1 minute. The pellet was incubated for 15 minutes at 56 °C with 200 ml of Insta Gene matrix. After vortexing for 10 seconds at high speed, then at 100 °C for 8 minutes, the tube was lowered in a boiling water bath. The gene fragment of universal primers was amplified using the MJ ResearchPTC-225 Peltier Thermal Cycler and the 18S Ribosomal RNA (rRNA) ITS Region.

Primer Data

In a twenty (20) liter Polymerase Chain Reaction (PCR) solution, one (1) liter of template DNA was added. The PCR reaction was performed under the following circumstances with the primers 18S-C[a] (5'-TGATCCTTCYGCAGGTTCAC-3') /18S-D[a](5'-ACCTGGTTGATCCTGCCAG-3') /18S C-2[b](5'-ATTGGAGGGCAAGTCTGGT-3') /18S D-2[b](5'-ACTAAGAACGGCCATGCAC-3'): For the first time, denaturation at 94°C "for" two (2) minutes, then thirty-five (35) amplification cycles at 90°C for forty-five (45) seconds, 50°C for sixty (60) seconds, and 72°C for sixty (60) seconds, for ten (10) minutes stretch at 72°C "the very least It is possible to amplify DNA fragments. In the PCR, include both a positive and negative control.

PCR Products Purification

PCR primers that haven't been integrated and Deoxyribonucleotide triphosphate (dNTPs) were taken out of PCR products with the help of the PCR Cleaning kit from montage (Millipore). The PCR product sequence was generated using 18S-Ca/18S-Da/18S C-2b/18S D-2b primers. AmpliTaq® DNA polymerase (FS enzyme) and ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits were used for the sequencing (Applied Biosystems).

Protocol for Sequencing

Using 18s Ribosomal RNA (rRNA) universal primers, each template was subjected to a single run of sequencing. Employing ethanol precipitation, unincorporated terminators yielded a labeled fluorescent fragment. The materials were electrophoresed in an ABI 3730xl sequencer after being suspended in distilled water (Applied Biosystems).

Protocol for Bioinformatics

The NCBI blast similarity search tool was used to blast the rRNA sequence. After phylogenetic analysis of the sequence that is closely linked to the blast results sequence, alignment of several sequences was done. Multiple sequence alignments were achieved with the help of "The MUSCLE 3.7" program (Edgar, 2004). The program G blocks 0.91b were used to align aligned sequences. To remove the poorly aligned sites and divergent regions, the G blocks (which reduce alignment noise) were used (Talavera et al., 2007). Finally, the phylogeny was investigated at LRT using the PhyML 3.0 tool and the HKY85 substitution model. With simulated data, PhyML has been proven to be at least as accurate as other phylogeny tools while also being one order of magnitude faster. The stress was rendered with the Tree Dyn 198.3 software (Dereeper et al., 2008).

3. Results and discussion

3.1 Isolation of microalgal isolates

Microalgae are essential biological resources in the present systems of energy that are renewable with special emphasis on biological sources of diesel production. The major bottleneck to produce biodiesel commercially is the isolation of appropriate strains of microalgae with higher biomass and the production of optimum lipids. Microalgae are regarded as largely untapped as well as unused resources; therefore, the best approach for getting the best strains with high quantity and best quality of lipid is bioprospecting for active species from natural sources (Shin et al., 2018; Singh et al., 2011). Through a light microscope, one hundred and thirty-six genera of microalgae were identified while fifty strains were isolated successfully.

3.2 Preliminary Screening

The technique of Sudan black B staining was adopted during the examination of the uniqueness of functionality of bioenergy for isolates concerning the possession of cellular lipids. The method has been identified and used by previous scientists as a good tool for lipid production screening and detection of microorganisms (oleaginous)(Niehus et al., 2018; Patel et al., 2019; Poizot & Dolhem, 2011) , hence it's adopted in this study. Black granules showing intracellular lipid were observed in preference to low lipid pink colour lipid production for thirty-two (32) of the fifty strains of microalgae (Table 1). The recorded characteristics were linked to the quantity-dependent levels of phospholipids production and the reaction of triacylglycerols in structures of cells of microalgae with the dye (Sudan Black B) to elicit or produce black colour in a dye-lipid complex(Refaat et al., 2023) (Singh et al., 2011). An observed black granule in this study shows the presence of lipid, that can serve as a diesel production starting material. Diesel production potentials through the lipid-producing capacity of strains of microalgae are not established through content of lipid alone but rather through other parameters. Therefore, more than half of strains of microalgae that was isolated successfully with neutral intracellular lipids as shown in Table 1, and detected by Sudan Black B which were further subjected to the screening test.

Table 1: Preliminary Microalgal Strains Screening for Biodiesel Production (BP)

Microalgal Isolate	Microalgal Isolates Visualized as black granules (+)
Spirulina (SiA)	+
Chlamydomonas (CaA)	+
Ankistrodesmus (AkA)	+
Monoraphidium (MoA)	+
Coelastrum (CoB)	+
Pediastrum (PeA)	+
Closterium (CtA)	+
Chlamydomonas (CaD)	+
Pediastrum (PeB)	+
Chlorococcum (CiA)	+
Crucigenia (CrA)	+
Chlorella (ChA)	+
Volvox (VoA)	+
Closterium (CtB)	+
Scenedesmus (SeB)	+
Chlorella (ChB)	+
Chlorococcum (OSK)	+
Spirogyra (SpA)	+
Coelastrum (CoA)	+
Scenedesmus (SeA)	+
Crucigenia (CrC)	+
Coelastrum (UiD)	+
Chlorella (ChC)	+
Scenedesmus (SeD)	+
Chlorococcum (CiD)	+
Spirogyra (SpD)	+
Ankistrodesmus (AkD)	+
Pediastrum (UiF)	+
Chlamydomonas (CaD)	+
Coelastrum (CoC)	+
Microcystis (MiC)	+
Chlorella (ChD)	+

Note: + is an indication of higher lipid bodies for Biodiesel Production

3.3 Secondary Screening

For a further qualitative sequence of lipid production analysis, the screening that entails specific lipid and biomass volumetric productivity by the test strains was applied for the determination of exactitude levels of production among earlier screened strains (Nascimento et al., 2012; Oyelami et al., 2023)

The concentration of biomass for the experimented isolates varies from 0.55 ± 0.02 to 7.28 ± 0.20 (g/l). The result shows that Scenedesmus sp SeA had the highest concentration of biomass (7.28 ± 0.20 g/l), followed by Chlorella sp. ChA (6.07 ± 0.13 g/l) while the lowest concentration of biomass (0.55 ± 0.02 g/l) was observed for Crucigenia sp. CrC (Table 2).

The range of 0.04 to 0.52 (gl^{-1}day^{-1}) was recorded for the productivity of biomass, Scenedesmus sp. SeA had the highest productivity of biomass (0.52 gl^{-1}day^{-1}) followed by 0.43 gl^{-1}day^{-1} observed for Chlorella sp. ChC and the least productivity of biomass with the value of 0.04 gl^{-1}day^{-1} was found in Crucigenia sp. CrC (Table 2).

The production of lipids is within the values of 0.16 ± 0.01 and 3.99 ± 0.19 (g/l) for the isolates, with 3.99 ± 0.19 g/l as the highest concentration of lipid for Scenedesmus sp SeA, then 2.94 ± 0.03 g/l found in Chlorella vulgaris ChC and finally the least value (0.16 ± 0.01 g/l) observed in Crucigenia sp. CrC (Table 2).

The content of lipid for the thirty-two strains of microalgae falls within values of 16.32 ± 0.57 and 62.76 ± 1.51 %, the highest content of lipids with value of 62.76 ± 1.51 % recorded for Chlorococcum sp. ClB, next to this value is 54.86 ± 2.57 % found in Scenedesmus sp. SeA and the lowest content of lipid (16.32 ± 0.57 %) examined in Closterium sp. CtA (Table 2).

Scenedesmus sp. SeA recorded 285.25±13.38 as the highest productivity of lipid, preceded by Chlorella sp ChA with a value of 208.03±2.18 for volumetric lipids productivity, Pediastrum sp. PeD Coelastrum sp CoC and Chlorococcum sp. CiB had volumetric lipid productivities of 186.27±3.86, 175.14±1.77, and 163.49±3.90 respectively (Table 2).

Thirty-two isolates showed high dependency of species for volumetric productivity while five of the rest isolates namely, Scenedesmus sp SeA, Chlorella sp ChA, Pediastrum sp PeD, Coelastrum sp CoC and Chlorococcum sp CiB showed distinct and remarkable capability for higher volumetric productivity of the lipid (Table 2).

In relatedness, Azeez et al., (Oyelami et al., 2023) reported maximum concentration of biomasses in Scenedesmus and Chlorella sp (7.04 ± 0.05 g/l and 5.55 ± 0.04 g/l), high lipids concentration (3.62 ± 0.03 g/l and 2.52 ± 0.04 g/l) and lipid content ($51.43 \pm 0.84\%$ and $45.38 \pm 1.67\%$) from fish ponds in Owode, Ede, Osun State. In a similar vein, the highest productivity of lipids with the value of 257.15 ± 5.29 was observed for Scenedesmus sp. isolated from the pond (Fish) in Owode, Osun State, Nigeria (Oyelami et al., 2023).

A biomass species-based productivity of lipids, when microalgae were examined for biodiesel experimental production, was reported by Nascimento et al. (Nascimento et al., 2012). Recently, high productivity of lipids was determined in a strain of Scenedesmus sp. R-16 when cultivated medium that contains glucose (Ren et al., 2013).

In a related study, Chlorococcum sp., Chlorella sp., and Scenedesmus sp. were noticed with higher biomass and content of lipids required for the production of biodiesel when they were screened among other microalgae isolates from the brackish water of the coast habitat of Odisha in India (Jena et al., 2012).

Also, Chlorella vulgaris and Scenedesmus obliquus were identified as the best strains in the productivity of lipid (Chisti, 2007). Chlorococcum spp, Scenedesmus sp, Pediastrum spp, and Coelastrum spp have been established as viable potential lipids of algal sources with good lipid possession for conversion to diesel (Goswami et al., 2011; Maity et al., 2014).

Table 2: Secondary screening of algal isolates for biodiesel production (BP)

S/N	Microalgal Isolates	Isolate Code	Biomass Concentration (g/l)	Biomass Productivity Pdwt (gl^{-1}day^{-1}D=14)	Total Lipid Concentration (g/l)	Lipid Content Lc;%dwt	Volumetric Lipid Productivity Pdwt x Lc x1000/100
1	*Spirulina sp*	SiA	*1.45±0.01	0.10	0.31±0.02	21.38±1.13	21.38±1.13
2	*Scenedesmus sp*	SeC	4.43±0.02	0.32	1.90±0.04	42.82±0.87	137.01±2.78
3	*Chlamydomonas sp*	CaA	0.98±0.02	0.07	0.18±0.03	18.71±2.54	13.10±1.78
4	*Chlorella sp*	ChD	4.25±0.03	0.30	1.16±0.07	27.29±1.67	81.87±5.02
5	*Ankistrodesmus sp*	AkA	3.20±0.03	0.23	0.65±0.03	20.42±0.81	45.63±2.30
6	*Monoraphidium sp*	MoA	3.08±0.02	0.22	0.62±0.02	20.24±0.65	44.52±1.12
7	*Microcystis sp*	MiC	1.36±0.03	0.10	0.42±0.02	31.13±1.25	31.13±1.25
8	*Coelastrum sp*	CoB	3.00±0.22	0.21	1.36±0.03	45.22±1.03	94.97±2.17

9	*Pediastrum sp*	PeA	4.76±0.02	0.34	1.55±0.03	32.63±0.52	110.94±1.63
10	*Closterium sp*	CtA	4.39±0.03	0.31	0.72±0.03	16.32±0.57	50.59±1.77
11	*Coelastrum sp*	CoC	3.36±0.03	0.24	0.93±0.03	28.35±0.22	65.03±3.78
12	*Chlamydomonas sp*	CaD	2.17±0.09	0.16	0.43±0.02	19.66±0.79	31.45±1.26
13	***Pediastrum sp.***	**PeD**	**˃5.84±0.03**	**0.42**	**2.59±0.05**	**44.35±0.92**	**186.27±3.86**
14	*Ankistrodesmus sp*	AkD	3.44±0.02	0.25	0.81±0.02	23.45±0.54	58.64±1.23
15	*Pediastrum sp*	PeB	4.62±0.14	0.33	1.21±0.10	25.88±1.65	85.40±5.46
16	*Spirogyra sp*	SpD	1.55±0.04	0.11	0.51±0.03	32.69±1.69	35.96±1.87
17	*Chlorococcum sp*	ClA	0.84±0.01	0.06	0.17±0.02	19.84±2.02	11.90±1.21
18	*Crucigenia sp*	CrA	1.32±0.04	0.09	0.41±0.02	30.81±1.91	27.43±1.31
19	***Chlorella sp.***	**ChA**	**6.07±0.13**	**0.43**	**2.94±0.03**	**48.38±0.51**	**208.03±2.18**
20	*Chlorococcum sp*	CiD	3.07±0.02	0.22	1.09±0.03	35.61±0.81	78.35±1.78
21	*Scenedesmus sp*	SeD	3.51±0.08	0.25	1.19±0.02	33.99±0.49	84.99±1.21
22	*Chlorella sp*	ChC	3.89±0.07	0.28	1.77±0.28	45.42±7.14	127.17±20.00
23	*Volvox sp*	VoA	1.05±0.02	0.08	0.30±0.02	28.89±1.62	23.11±1.29
24	***Coelastrum sp.***	**CoC**	**5.79±0.30**	**0.41**	**2.47±0.03**	**42.72±0.43**	**175.14±1.77**
25	*Closterium sp*	CtB	2.45±0.04	0.18	0.50±0.02	20.27±0.84	36.49±1.51
26	*Scenedesmus sp*	SeB	3.99±0.01	0.29	2..09±0.11	52.46±2.82	152.14±8.19
27	*Chlorella sp*	ChB	3.83±0.17	0.27	1.06±0.02	27.59±0.46	74.49±1.20
28	*Crucigenia sp*	CrC	0.55±0.02	0.04	0.16±0.01	29.09±1.49	11.64±0.59
29	***Chlorococcum sp.***	**CiB**	**3.70±0.22**	**0.26**	**2.33±0.06**	**62.76±1.51**	**163.49±3.90**
30	*Spirogyra sp*	SpA	0.68±0.01	0.05	0.18±0.01	26.47±1.20	13.24±0.60
31	*Coelastrum sp*	CoA	3.74±0.07	0.27	1.28±0.07	34.14±1.19	92.17±5.15
32	***Scenedesmus sp.***	**SeA**	**7.28±0.20**	**0.52**	**3.99±0.19**	**54.86±2.57**	**285.25±13.38**

* Data are presented as $Mean \pm SD$ of results in triplicate
˃Values in bold font are higher than other values on the same column.

3.4 Morphological identity of screened microalgal strains

As part of measure to link specificity in specie with productivities of biomass and lipid, identification of species of microalgae is necessary. The shape of Isolate PeD is circular with small size with spherical, flat, and one thick layer colony in a multicellular of plate-like pattern arrangement. The cells are made up of a parietal and cup-like shaped chloroplast with a single pyrenoid. They had smooth and planktonic cell walls with immobile cells (Table 3). The stated morphologies were noted through microscopic observation and in long run confirm the strain as *Pediastrum sp*. In similarity, aforementioned characteristics of *Pediastrum sp* were reported from a related strain isolated from fish farm ponds in Owode Ede, Osun State, Nigeria by Azeez *et al*., (Oyelami et al., 2023).

Isolate ChA is made up of immotile and small unicellular cells with spherical and small round shape together with a single, parietal, cup-shaped chloroplast. In colour, they are dark or light green with smooth but thin and planktonic cell walls. The strain ChA was observed as *Chlorella sp* after a morphological study with aid of a simple light microscope (Table 3). Recently, researchers have mentioned similar features (shape, size, and chloroplast of the cell) for *Chlorella sp*(Oyelami et al., 2023; Sero et al., 2021)

Isolate CoC cells is small in size with a cubical shape and a parietal with a single chloroplast that has a single pyrenoid. The appearance of the cell wall is smooth, planktonic, and without flagella (Table 3). Morphological assessment under light microscope revealed similarity with *Coelastrum sp*. This was contrary to findings from standard keys and other citations used for comparison.

A cell of Isolate CiB is small, wide, and spherical. With the age, the walls thickened. Chloroplast contains lateral pores, a hollow sphere with a single pyrenoid. Cells have two nuclei: planktonic and non-motile (Table 3). The above morphological characteristics are related to *Chlorococcum sp* as we have it in standard keys and other references(Oyelami et al., 2023; Sero et al., 2021)

Isolate SeA occurred in solitary form with pointed ends and an oval shape. Cells contain a nucleus, plate-like with a single parietal chloroplast. The colony is not motile with a single pyrenoid. A combination of 4–16 cells formed long linear cells with green colour (Table 3). After microscopic examination, the strain was confirmed to be *Scenedesmus sp*. cell arrangement and shape of the cells show close similarity and relatedness with *Scenedesmus sp*. (Oyelami et al., 2023; Sero et al., 2021)

3.5 Molecular Characterization

Tentative representation is given to isolates by identification of microalgal through the presumption method, because of difficulties in functional differentiation of green algal morphology; the stated method reliability is not full. Nucleated physiology is the unique and distinctive characteristic of the isolates. They varied from uni-nucleation, bi-nucleation, and multi-nucleation with possession of different patterns of chloroplast. Perceived heterogeneity situation among strains as noticed was given by characteristics observed through a microscope (Abou-Shanab et al., 2011). This was also attested by Abou-Shanab *et al.*(Abou-Shanab et al., 2011) that stated that difficulty and tediousness in identification through microscope was as result of heterogeneity of algal morphology.

A blast search through the National Centre for Biotechnology Information (NCBI) gene bank confirmed the strain PeD as *Pediastrum duplex*. 99% similarity mark with *Pediastrum duplex* strain CCMA_UFSCar 055 was attained with the genetic sequence of the 18S rRNA of *Pediastrum duplex* PeD on comparison (Figure 1). Upon comparison of isolate ChA 18S rRNA sequence to the same stored sequences in the bank of the gene of NCBI, the strain was identified as *Chlorella vulgaris*. The identified *Chlorella vulgaris* is closely related to *Chlorella vulgaris* strain SAG 30.80 by 99% (Figure 1).

Analysis of 18S rRNA as well as a search of NCB data gene base through the blast showed the close relatedness between *Coelastrum morum* CoC and *Coelastrum morum* strain SAG217-5 (Figure 1). A similarity was established between Isolate CiB and *Chlorococcum littorale* gene for strain: MBIC10280 after blast search in National Centre for Biotechnology Information (Figure 2). Blast search through a gene bank of the National Centre for Biotechnology Information (NCBI) after 99% similarity was achieved when 18S rRNA genetic sequence of *Scenedesmus obliquus* SeA marked with *Scenedesmus obliquus* strain LU33 through comparison (Figure 1).

Levels of homology with 97-100% have been reported recently by scientists for determination of sample species identity with species already mentioned in the National Centre for Biotechnology Information (NCBI) database (Ferro et al., 2018)

Table 3: Morphological characteristics of screened microalgal isolates

Isolate code	Shape	Size	Unicellular/ Multicellular	Planktonic/ Benthic	Chloroplast	Pyrenoid	Motility	Nucleated	Isolate name
UIF	Circular	Small	Unicellular	Planktonic	Parietal Cup-shaped	Single	Non-motile	Multinucleated	*Pediastrum sp*
UIA	Spherical	Small	Unicellular	Planktonic	Parietal	Single	Non-motile	Uni-nucleated	*Chlorella sp.*
UID	Cubical	Small	Unicellular	Planktonic	Parietal	Single	Non-motile	Uni-nucleated	*Coelastrum sp.*
OSK	Spherical	Small	Unicellular	Planktonic	Parietal Hollow sphere	Single	Non-motile	Bi-nucleated	*Chlorococcum sp.*
OSC	Ovoid	Small	Unicellular	Planktonic	Parietal Plate-like	Single	Non-motile	Uninucleate	*Scenedesmus sp*

Note: The isolates codes were changed from UIF, UIA, UID, OSK and OSC to PeD, ChA, CoC, CiB and SeA for *Pediastrum sp, Chlorella sp, Coelastrum sp, Chlorocuccum sp and Scenedesmus sp*
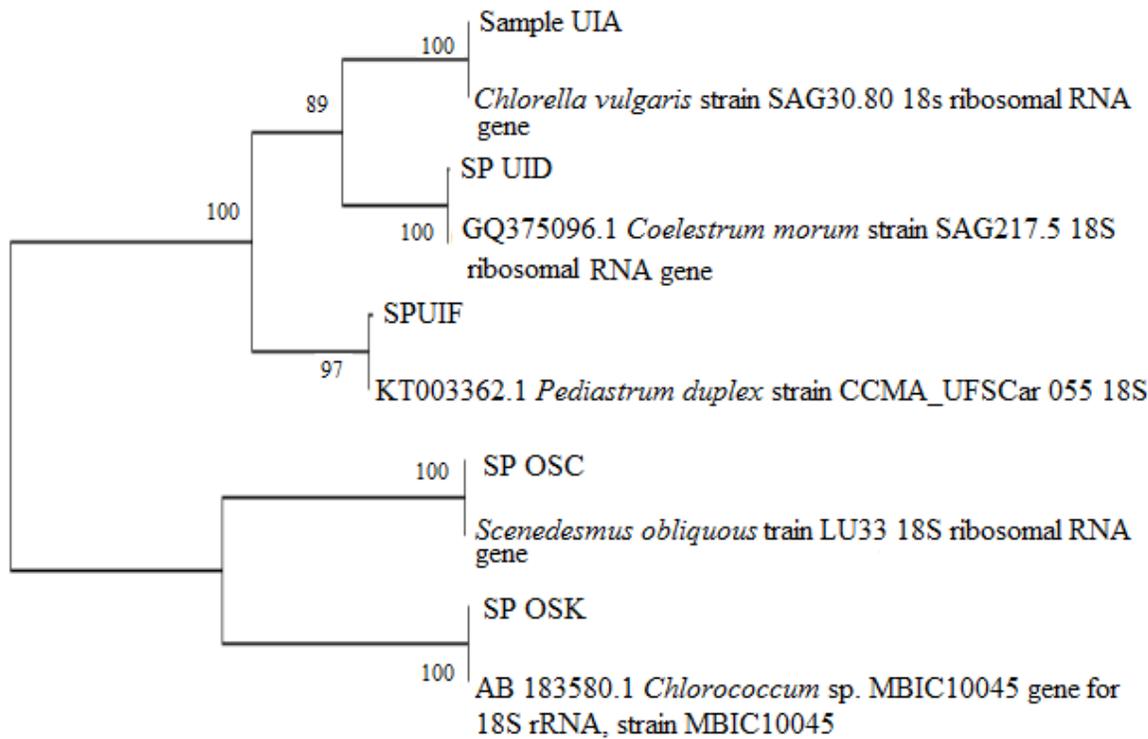
Figure 2: The phylogenetic tree of *Microalgal isolates* (*Chlorella vulgaris* UIA, *Coelastrum morum* UID, *Pediastrum duplex* PeD, *Scenedesmus obliquus* OSC and *Chlorococcum littorale* OSK based on the 18S rRNA sequence comparison of related isolates.

Note: The isolates codes were changed from UIF, UIA, UID, OSK and OSC to PeD, ChA, CoC, CiB and SeA for *Pediastrum duplex, Chlorella vulgaris, Coelastrum morum, Chlorocuccum littorale and Scenedesmus obliquus*

4. Conclusion

In this research, one hundred and thirty-six genera of microalgae were identified while fifty strains were isolated successfully from fishponds of fish farms in a tertiary institution from Southwestern Nigeria and fish ponds. The isolates were screened for biodiesel production capabilities through primary screening (Sudan B Black) and Secondary screening (Volumetric Lipid Productivity). Out of the 50 strains, thirty-two species were selected based on black granules with black colouration showing quantity-dependent phosphor levels, and intracellular lipids were used for lipid potential estimation at the initial level. Five isolates were finally selected during advanced screening through the productivity volume of lipids where an exactitude level of lipid production was measured using lipids' content and biomass productivity.

The five strains of microalga with efficiency in higher production of lipids were initially known through morphological characterization (motility, chloroplast, size, cellular structure unicellular/multicellular, pyrenoid, Shape, and nucleation) and later identified as *Scenedesmus obliquus* SeA, *Chlorella vulgaris* ChA, *Pediastrum duplex* PeD, *Coelastrum morum* CoC and *Chlorococcum littorale* CiB on the approach of molecular identification using 18S rRNA genes with up to a 99% similarity index in comparison with data obtained from NCBI database.

This research directly supports the Sustainable Development Goals (SDGs), SDG 7: Affordable and Clean Energy by exploring sustainable alternatives for biodiesel production. It also aligns with SDG 13: Climate Action, as biodiesel from microalgae reduces greenhouse gas emissions. Furthermore, it contributes to SDG 12: Responsible Consumption and Production by promoting the sustainable use of biological resources for energy generation. Additionally, the local focus on enhancing biofuel production from indigenous strains aids SDG 9: Industry, Innovation, and Infrastructure, fostering local energy resilience and innovation in biotechnology.

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Ethics Statements

This study, titled "Screening, Morphological and Molecular Identification of Microalgal Strains for Biodiesel Production", was carried out in line with ethical guidelines for environmental and microbiological research. Microalgal samples were collected with appropriate permissions and minimal environmental impact. No endangered or protected species were involved. All laboratory work followed biosafety protocols to prevent contamination or harm to the environment.

The research complies with relevant national and international regulations, including the Nagoya Protocol, ensuring responsible use of genetic resources. There are no conflicts of interest, and all data is accurately reported without fabrication or manipulation.

This manuscript reflects the collective effort of all authors, who have contributed significantly to the study.

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