ORIGINAL ARTICLE

Bioprospecting wild South African microalgae as a potential third-generation biofuel feedstock, biological carbon-capture agent and for nutraceutical applications

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Abstract

Microalgae are among the few biological resources studied that are found to possess vast biotechnological potential. This study isolated, identified and investigated two wild green microalgal species with substantial potential as a bioresource and climate change mitigation importance. Two isolates, *Chlorella sorokiniana* and *Tetradesmus reginae* were cultivated in selected artificial media under laboratory conditions. The isolates were analysed for nutrient consumption, biomass productivity, CO_2 biosequestration rate, elemental composition and fatty acid methyl profiles/composition. The outcome showed maximum daily biomass productivity of 0.128 ± 0.003 and 0.2 ± 0.004 g L^{-1} for *C. sorokiniana* and *T. reginae*, respectively. CO_2 biosequestration rate of *T. reginae* was the highest among the isolates, indicating that it can act as a biological climate change mitigation agent. Moreover, *T. reginae* recorded a significantly higher ($p < 0.05$) total lipid and carbohydrate content than *C. sorokiniana*. The C/N ratio for *T. reginae* was significantly higher than the C/N ratio for *C. sorokiniana*. *Tetradesmus reginae* also demonstrated the ability to produce a considerable quantity of omega-3 oils; hence, the species is of nutraceutical importance. Furthermore, *T. reginae* demonstrated maximal carbohydrate content and is therefore considered a potential feedstock for bioethanol production. *Chlorella sorokiniana*, on the other hand, showed a remarkable ($p < 0.05$) protein content making it a potential source for human food and animal feed supplement. Finally, the two isolates met both European and American quality biodiesel standards with exceptional cetane (CN) and iodine numbers (IV).

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1 Introduction

Microalgae are simple prokaryotic or eukaryotic organisms found in nearly every environment, especially the aquatic ecosystem. Microalgae are essential primary producers in the ecosystem due to their ability to harness solar energy for photosynthesis. Studies have shown that when compared to higher plants, algae have one of the most efficient photosynthetic and biomass productivity rates [1, 2], and this results in an elevated biomass build-up. Other studies also reported that most microalgal species have great potential and value as bio-resources and climate mitigation agents [3, 4]. To determine optimal use for more than 1,000,000 microalgal species, on-going investigations are conducted globally to analyse their primary metabolites (proteins, carbohydrates, lipids, fatty acids, amino acids, vitamins and sterols) and useful secondary metabolites (including allelochemicals,

toxins and hormones) [5–7]. The economics of microalgal cultivation, where all that is needed is low-cost water and atmospheric CO_2, has piqued researchers' interest even more. Moreover, they can grow on non-arable land; hence, the competition for land for food crop cultivation is eliminated. Nevertheless, a nutrient-rich medium is necessary for microalgae cultivation [3].

Recent reports on alarming levels of atmospheric CO_2 and resultant climate change-related issues due to combustion of fossil fuel for energy has been a cause for concern among global leaders [8]. For example, 2020 data indicates atmospheric CO_2 levels were about 412 ppm, almost twice the figure recorded during pre-industrial times [9]. Increasing atmospheric CO_2 levels have several negative consequences, including rise in sea level, species extinction, food security risks and threats to ecosystem functioning and biodiversity survival [10]. Therefore, the world is currently in search of a sustainable carbon mitigation technology and carbon–neutral or carbon-negative alternative energy source to fossil fuel, whose source might even be depleted soon. Currently, researchers have identified microalgae-based technologies as having an efficient photosynthesis mechanism capable of effectively biosequestering CO_2 from a point source into a biomass that could be considered a potential source of biofuels, a promising but currently expensive alternative to fossil fuels [11–13]. Research data compiled by Sidney et al. [12] on carbon fixation rates by species of microalgae, whilst assessing the role of microalgae technologies in the global carbon market, revealed that genera—*Anabaena*, *Chlorococcum*, *Spirulina*, *Chlorella*, *Botryococcus* and *Haematococcus* have the greatest carbon fixation potential.

Currently, South Africa generates 90% of its domestic energy from coal resources with deleterious environmental implications, which has called for the intensification of research into sustainable energy development from indigenous renewable resources [14]. Though South Africa is considered semi-arid, its distinguishing geological and geographical features promote biodiversity including algae [15, 16]. This could be an essential lead to exploring more renewable energy options, especially third-generation biofuels, to tackle climate change problems thoroughly, energy deficit and sustainable growth in the country. Moreover, indigenous microalgal species that contain economic quantities of protein and omega oils in their biomass can be harnessed as raw material for the animal feed, human food and food supplement industries.

This study focused on the bioprospecting of native microalgal species that can potentially provide commercially relevant and sustainable raw materials for the biofuel and nutraceuticals industry in the Southern Africa region. Wild microalgae species were obtained from a South African freshwater wetland and assessed for their potential to accumulate carbohydrate and lipids of commercial value.

Thus, the objective of this work was to isolate and identify local wild microalgae using molecular barcoding approach. Furthermore, isolates of interest were cultivated in artificial media for the assessment of its biomass production, nutrient utilisation, elemental and biochemical composition, CO_2 biosequestration efficiencies and fatty acid profiles.

2 Materials and methods

2.1 Microalgae sample collection, isolation and unialgal stock culture maintenance

Algae-rich water samples were collected from a freshwater site with a global positioning system (GPS) coordinates 26.09′04.4″S, 28.03′19.4″E in Killarney Country Club, Johannesburg, Gauteng Province, South Africa, in August 2019. Composite samples from the surface water at depths between 10 and 100 cm were collected in well-labelled 100 mL sampling bottles. Sample collection was conducted at prevailing conditions of 20 °C and pH 8.6 between 10 and 11 am and samples were transported to the laboratory on ice. The field samples were serially diluted and added to sterilised modified Blue-Green 11 (BG-11) medium [17] and Bold's basal medium (BBM) [18] in a ratio of 1:1 (v/v) in 50 mL conical flasks to promote microalgae growth. Cultures were grown in a Labcon 3081U shaking incubator (Labcon, South Africa) maintained at a temperature similar to that of the collection site under low light of 1000 lx and 14 h/10 h light/dark photoperiod for 10 days.

Modified BG-11 and BBM agar plates were prepared by dissolving 15 g bactoagar per litre (L) of deionised water in a 2 L conical flask and autoclaving the resulting solution (121 °C for 15 min). The molten agar was then mixed at a temperature of 60 °C with the same volume of either sterilised BG-11 medium or BBM medium slowly whilst swirling to ensure uniform mixing. The mixtures were poured into Petri dishes, allowed to solidify and stored (in an inverted position) at 4 °C [19]. Mixed microalgal colonies were cultured on sterilised agar-modified BG-11 medium and BBM medium plates, respectively. The plates were incubated at a temperature of 22 ± 3 °C with continuous illumination, and single colonies were repeatedly subcultured until unialgal isolates were obtained with the aid of light micrograms at every stage. Eight unialgal colonies were isolated and labelled UJEAS1, UJEAS2, UJEAS3, UJEAS4, UJEAS5, UJEAS6, UJEAS7 and UJEAS8. Colonies of unialgal strains from the agar plates were transferred to 100 mL conical flasks containing 60 mL media (either BG-11 or BBM) and cultivated in a Labcon 3081U shaking incubator (Labcon, South Africa) at a speed of 150 rpm for 10 min with a temperature of 25 ± 3 °C, 2000–2500 lx using Digital Lux Meter AS803 (Arco Science & Technology Ltd,

China) lighting condition and 14/10 photoperiod for 8 days to produce the stock cultures meant for morphological and genetic analysis as well as subsequent inoculum.

2.2 Morphological analysis of microalgae isolates

The preliminary identity of microalgal isolates were established using morphological methods. Briefly, labelled isolates were viewed under the LEICA DM500 light microscope, and the respective images were captured with a LEICA ICC50W camera (Leica Microsystems, USA) at $400 \times$ magnification. Culture contamination was regularly checked by comparing the microscopic images with the isolates in the stock cultures. The isolates were further subjected to molecular analysis and identification of the species.

2.3 Molecular identification and phylogeny

2.3.1 Genomic DNA extraction

Genomic DNA was extracted from the respective unialgal stock cultures with Quick-DNA™ Plants/Seed DNA Mini-Prep Kit (Zymo Research-USA) according to manufacturer's instructions. The extracted DNA was stored at -20 °C for further use.

2.3.2 Polymerase chain reaction and sequencing

Polymerase chain reaction for each sample was targeted at the nuclear genome, internal transcribed spacer 2 (ITS2) region [20, 21]. The primers used to amplify the ITS2 region included forward 5′-AGGAGAAGTCGTAACAAG GT-3′ and reverse 5′-TCCTCCGCTTATTGATATGC-3′ [22]. Polymerase chain reaction (PCR) was conducted in a BIO-RAD T100™ Thermal Cycler (Bio-Rad Laboratories, USA) at an initial denaturation of 95 °C for 4 min followed by 35 cycles of denaturation at 95 °C for 2 min, annealing at 55 °C for 30 s and extension at 72 °C for 5 min and a final extension at 72 °C for 10 min. The integrity of all PCR products was assessed by viewing the PCR products under UV light on a 1.4% agarose gel stained with ethidium bromide (EtBr). All PCR products were sequenced by a commercial service provider, Inqaba Biotechnical (Pty) Ltd, Pretoria, South Africa. BioEdit v 7.2 (Informer Technologies, Inc.) was used to analyse the forward and reverse reads and to generate consensus sequences.

2.3.3 Phylogenetic analysis

To establish the phylogeny of the isolated microalgae, basic local alignment search tool for nucleotides (BLASTn) was employed to compare ITS 2 sequences of the isolates with the existing National Centre for Biotechnology Information (NCBI) database sequences to identify closest homologues. Molecular Evolutionary Genetics Analysis version 7.0 (MEGA 7) [23] software was used to perform multiple sequence alignment of related microalgae species from the NCBI database and isolate sequences. The MEGA 7 software was subsequently employed to generate phylogenetic trees using the maximum-likelihood (ML) algorithm with the Tamura-Nei model option [24], and branch reliabilities were evaluated with a bootstrap of 1000 replicate for the identification of the unknown microalgae isolates.

2.4 Microalgae cultivation and analytical determinations

Unialgal cultures were batch cultivated in triplicate in BG-11 or BBM growth media under autotrophic growth conditions for 16 days. Parameters such as pH, temperature and residual nitrate-nitrogen (nitrate–N) levels in the cultures were monitored every 48 h. Prior to cultivation, unialgal stock cultures were taken during the exponential growth phase, which was at day 8, centrifuged at the conditions stated at Sect. 2.1 and washed twice with deionised water. The pasty unialgal cells were then reconstituted in deionised water and finally inoculated in 1 L conical flasks containing either BG-11 or BBM ratio 1:10 (algal cell inoculum: culture nutrient medium). This culminated into an initial culture optical densities of 0.052 and 0.015 for UJEAS4 and UJEAS8, respectively. The cultures were initially sparged with pure CO_2 at the flow rate of 1 L min^{-1} for 20 s and subsequently bubbled with filtered air (0.04% CO_2) with no pH control throughout the culture period. Controls were set up in the same manner, with only the growth media and no microalgal species in each case. The culture pH and temperature were monitored with Ohuas Starter300 instrument (Ohaus Corporation, USA). The analysis for residual nitrate–N concentration in the culture was conducted using the colorimetric method after nitration of salicylic acid as developed by Cataldo et al. [25]. Briefly, 10 mL microalgae culture was centrifuged at 4500 rpm for 12 min and the supernatant collected. A sample aliquot of 50 μL was pipetted into a clean tube with 200 μL salicylic acid-H_2SO_4 mixture (5%, w/v) and mixed thoroughly by agitation. It was followed by a 10-min incubation at room temperature. Then 2 mL NaOH (4 M) was added to the solution and the mixture was shook and incubated for 20 min at room temperature. A Cary 60 UV–Vis (Agilent Technologies, USA) spectrophotometer was used to measure the absorbance at 410 nm. To assess nitrate–N levels in the sample, the absorbance readings achieved were back-plotted on a previously constructed nitrate calibration curve ($y = 0.0132x - 0.0022$; $R^2 = 9938$). The blank sample was treated with 96% H_2SO_4 without salicylic acid, followed by 4 M NaOH as described above.

2.5 Cell density, growth kinetics and CO$_2$ biosequestration rate

Microalgae biomass measurements were taken every 48 h. Briefly, 10 mL of well-mixed microalgae culture was aseptically weighed in a dried pre-weighed falcon tube using analytical Ohaus balance to 3 decimal places (Ohaus Corporation, USA). The culture was centrifuged at 7000 rpm for 10 min after which the supernatant was carefully discarded. The residual biomass was washed twice with deionised water, kept at −20 °C and subsequently moved to −80 °C for 30–50 min before freeze-drying. Frozen samples were dried with lyoQuest freeze drier at (Telstar, Japan) stable operating conditions of −54.8 °C and 0.287 nBar for 24 h. The net biomass weight was determined after re-weighing the dried samples according to Eq. (1):

$$DWB = B − F \tag{1}$$

where B and F represents the gross biomass weight and falcon tube weight respectively whilst DWB is the net dry weight biomass of the isolates.

Microalgae culture aliquots (2 mL) were collected every 48 h and diluted with BG-11 or BBM medium to a maximum optical density (OD) of less than 1.0. The OD was determined at a wavelength of 750 nm using Eppendorf BioSpectrometer® kinetic (Eppendorf, Germany), and the instrument was blanked with sterilised BG-11 or BBM. The actual OD was determined by multiplying the spectrometric results with the dilution factor.

The respective microalgal biomass productivity (BP), specific growth rate (μ) and CO$_2$ biosequestration rate (R_{CO2}) were determined according to Eqs. (2), (3) and (4) below:

$$BP \ (g \ day^{-1}) = \frac{DWB_2 − DWB_1}{t_2 − t_1} \tag{2}$$

$$\mu (day^{-1}) = \frac{\ln(DWB_2/DWB_1)}{(t_2 − t_1)} \tag{3}$$

$$R_{CO_2} (gCO_2 \ L^{-1} \ day^{-1}) = C_{avg} \times BP \times \left(\frac{MW_{CO_2}}{MW_C}\right) \tag{4}$$

where DWB is the dry weight biomass at the time of measurement (t_2) and the beginning of the culture (t_1). MW_{CO_2} and MW_C represent the molecular weights of CO$_2$ and C (elemental), respectively. C_{avg} is the mean elemental carbon content of the respective microalgae biomass determined by an elemental analyser.

2.6 Microalgal biomass harvesting, biochemical and elemental analysis

All unialgal cultures were harvested after 16 days with a centrifuge at 7000 rpm for 10 min and freeze-dried.

2.6.1 Total lipid and carbohydrate content and fatty acid methyl esters measurement

Microalgae carbohydrate was determined by phenol–sulfuric acid method of Dubois et al. [26] with glucose as a standard. Ten milligrams (mg) of freeze-dried powdered microalgae biomass was reconstituted in an 8-mL deionised water, vortexed, resulting in a concentration of 1.25 mg/mL. One millilitre of aliquot was transferred into a clean tube. One millilitre of 50 g L^{-1} phenol solution and 5 mL concentrated H$_2$SO$_4$ (96–98%) were added in turns and heated for 5–7 min at 90 °C. The resultant solution was then cooled at room temperature (RT) for 15 min and measured at a wavelength of 490 nm with Cary 60 UV–Vis (Agilent Technology, USA) spectrophotometer using various glucose standards concentrations. The carbohydrate (glucose) content was then computed from the pre-calibrated glucose standard curve ($y = 0.9044x − 0.0392$; $R^2 = 9978$).

The total lipid was determined with a single-step method developed by Axelsson and Gentilia [27]. Briefly, 10 mg freeze-dried microalgae biomass was reconstituted in 5 mL methanol-chloroform (1:2 v/v) and vortexed until the microalgae biomass was evenly distributed in the mixture. The mixture was sonicated briefly, after which 2 mL of 0.9% NaCl solution was added and finally centrifuged at 7000 rpm for 10 min. Microalgae biomass debris formed a thin layer between the upper organic phase and the lower lipid phase. A Pasteur pipette was then used to gently remove the lower lipid phase into a 15-mL dried pre-weighed glass vial. The residue was re-extracted twice with 2.0 mL methanol-chloroform (1:1, v/v) as indicated above. The solvent in the vial was oven-dried at 60 °C until a constant lipid weight was gained and the percentage total lipid content determined by gravimetric method according to Eq. (5):

$$\% \ Microalgal \ Lipid_{total} = \frac{W_{lipid \ extracted}(mg)}{W_{microalgal \ biomass}(mg)} \times 100 \tag{5}$$

where $W_{lipid \ extracted}$ and $W_{microalgal \ biomass}$ are weight of the microalgal biomass and lipid extracted in milligrammes, respectively.

Fatty acid methyl esters (FAME) were prepared by direct transesterification method developed by Lepage and Roy [28] modified by Van Wychen and Laurens [29] and analysed with a high-resolution gas chromatograph-mass spectrometer (GC–MS) (Agilent Technologies, USA).

Briefly, 0.2 mL chloroform–methanol (2:1, v/v) was added to 5 mg freeze-dried powdered microalgal biomass contained in a 1.5-mL GC–MS vials. This was followed by the addition of 0.025 mL of 10 mg/mL methyl tridecanoate (internal standard) and 0.3 mL of 0.6 M methanolic-HCl, respectively, and vortexed. The vial was heated at 85 °C for 1 h and cooled at RT subsequently. One millilitre hexane was added to the cooled mixture, vortexed and allowed to stand for 1 h at RT to enable proper phase separation. The organic (upper) phase was later collected and analysed with an Agilent Technologies 7890B GC system (B.02.04.2) equipped with mass spectrometer with following operating conditions capillary column-DB-WAX 30 m×0.25 mm×0.25 µm; oven temperature—100 °C for 1 min, 25 °C/min up to 200 °C and hold for 5 °C/min up to 250 °C and hold for 7 min; carrier gas flow—1 mL/min helium; sample injection—1 µL at 10:1 split ratio. The FAME components were separated and detected based on their retention time.

Biodiesel quality parameters, including the saponification number (SN), the iodine number (IV) and the cetane number (CN), were calculated in accordance with Zhou et al. [30] and represented by Eqs. (6), (7) and (8), respectively.

$$SN = \sum \left(560 \times \% \, RFAME_C\right)/MW_C \tag{6}$$

$$IV = \sum \left(254 \times db \times \%FAME_C\right)/MW_C \tag{7}$$

$$CN = 46.3 + \left(\frac{548}{SN}\right) - (0.225IV) \tag{8}$$

where % $RFAME_c$ and MW_c are the relative percentage abundance of individual FAME components and their molecular weights, respectively.

2.6.2 Elemental constituents and protein estimation

The chemical constituent of microalgal biomass was determined with calibrated Flash 2000 organic elemental analyser (Thermo Fisher Scientific Inc, Japan). The calibration standards included cystine (N = 11.64%, C = 30.10%, 5.02%, S = 26.62%), BBOT (N = 6.54%, C = 72.59%, 6.06%, S = 7.43%), methionine (N = 9.35%, C = 40.26%, 7.37%, S = 21.46%) and sulfanilamide (N = 16.24%, C = 41.81%, 4.72%, S = 18.68%). Briefly, about 2 mg dried sample was weighed in a tin foil cup and combusted at 950 °C with pure oxygen and helium as a carrier gas. Percentage nitrogen was then multiplied by 4.78 to obtain the crude protein content of the dry microalgal biomass [31]. The 4.78 factor was used in this work to estimate the protein content because it has been frequently used in related studies as a reliable factor for such conversions, especially with regards to green microalgae

[32–34]. The percentage of carbon, hydrogen and sulphur were also analysed.

2.7 Statistical analysis

Triplicate studies were performed unless otherwise stated and values were expressed as mean ± standard deviation. Data were subjected to T-test at significance $p \leq 0.05$ with Excel (Microsoft Office 2016; Microsoft; USA) and IBM Statistical package for Social Sciences (SPSS version 20.0 for windows; IBM Corporation; USA) and represented graphically with OriginLab software (Version 8.5 for Windows; OriginLab Corporation; USA). Standard deviation (SD) values are depicted in the graphs as bars.

3 Results and discussion

3.1 Isolation and identification of microalgal strains

In order to obtain microalgal strains with novel properties, bioprospecting of native microalgae from unexplored biodiverse aquatic environments is essential. Thus, composite freshwater samples were taken from Killarney Country Club, Johannesburg, Gauteng Province-South Africa, which is new to algae research in South Africa. This sampling site was chosen based on the microalgal abundance on the "Golf-hazard" water at that particular time of the year.

The sample was collected when the water was at a temperature of 20 °C and pH 8.6 which was noted to be within the optimal conditions for microalgae growth [35] and between 10 and 11 am when most of the species are expected to have come up to the water surface. Microalgae isolation is a core requirement for obtaining monocultures, and represents the major initial hurdle towards selecting species with novel qualities. Nevertheless, isolating these microalgal strains from their natural habitats may demand the use of various nutrient media with different compositions to ensure the optimal growth of specific strains for complete recovery [36]. Our study used only BG-11 and BBM media for this purpose since these media are reportedly capable of supporting the growth of most microalgal species from different natural habitats [36–38]. A total of 4 microalgal isolates out of the initial 8 survived on both the agar-based media and their respective liquid media, but the other four failed to do so in the liquid media. These four isolates include 3 (UJEAS1, UJEAS4 and UJEAS7), which were successfully cultured in BG-11 media, whilst the remaining 1 (UJEAS8) was adequately supported with BBM media. Of the 4 isolates, we considered 2 for downstream analysis based on their initial promising growth rates and their ability to remain buoyant in their respective nutrient media, BG-11 media thrived-UJEAS4 and BMM media

thrived-UJEAS8. In addition, UJEAS4 and UJEAS8 selected for confirmatory molecular identification and further culturing to evaluate their capabilities to remove nitrogen, fix CO_2 as well as their potential for use as raw materials in biofuel and nutraceutical industries.

The light microscopic examination of the selected microalgal species revealed distinct morphologies indicated by a difference in cell structure and size. Images captured during the morphological analysis with the light microscope are represented in Fig. 1a and b with some key observed features of UJEAS4 (Fig. 1a) as having spherical shaped cells with no flagella and the average sizes varied between 3 and 4 and 5 and 6 μm, which is a common feature of the genus *Chlorella* [4, 39, 40].

Whilst the cells of the isolate-labelled UJEAS8 (Fig. 1b) were spindle shaped with acute cell poles, typical of genus *Acutodesmus (Scenedesmus)* [41]. The light microgram of UJEAS8 also showed 2- to 4-celled coenobia with no-mucilage coverings, alternates on two levels and ranges in sizes between 4 and 14 and 7 and 17 μm on the average.

Nevertheless, conclusions drawn with morphological analysis alone can be ambiguous, inadequate and misleading because of the highly publicised phenomenon of analogous morphology predominant among the diverse categories of microalgae [42]. Hence, ITS2 sequencing was employed in this study as one of the reliable barcoding techniques for identifying the microalgal isolates to a precise and accurate species level. The amplified ITS2 genes of both UJEAS4 and UJEAS8 isolates were above 780 base-pairs (bp) in size. UJEAS4 and UJEAS8 isolates were further found to relate to *Chlorella sorokiniana* with similarities > 97% and *Acutodesmus reginae* with > 99%, respectively, when their sequences were subjected to BLASTn analysis. Previous studies have reported that

homology levels of 97–100% can be used to establish the identity of sample species or strains with reported species in the NBCI database, especially when the *E*-values are < 0.00 [43–46].

A subsequent phylogenetic analysis shown in Fig. 2a, b confirmed that UJEAS4 is closely related to *Chlorella sorokiniana* [39, 47] whilst UJEAS8 was *Acutodesmus reginae* [21] with bootstrap values of 69% and 95%, respectively.

It is important to note that the genus of isolate UJEAS8 has been under review in recent years. *Acutodesmus* was previously considered a subgenus of *Scenedesmus* until Tsarenko and Petlevanny [48], asserted that *Scenedesmus* and *Acutodesmus* are paraphyletic and must be upgraded to a genus. Hegewald et al. [49] further validated this and pushed for the acceptance of *Acutodesmus* as a genus through their work. Later, Wynne and Hallan [50] challenged the name *Acutodesmus* describing it as illegitimate and hence made a case for the name *Tetradesmus* which was earlier proposed by Smith [51] and hence *Tetradesmus* became the current accepted genus name. However, the algological community currently considers the genus names *Scenedesmus*, *Acutodesmus* and *Tetradesmus* as heterotypic synonyms, with *Scenedesmus* as the basionym. UJEAS8 was grouped among Chlorophyta (phylum), Chlorophyceae (class), Sphaeropleales (order) and Scenedesmaceae (family) *Tetradesmus* (genus). Therefore, our study will subsequently refer to UJEAS8 or *Tetradesmus (Acutodesmus) reginae* as *Tetradesmus reginae*. UJEAS4 shares the same phylum with isolate UJEAS8 (which qualifies them as green algae) but differs in subsequent classifications as follows with unique characteristics: Trebouxiophyceae (class), Chlorellales (order), Chlorellaceae (family) and *Chlorella* (genus).

Fig. 1 Light micrographs of the two wild Southern Africa microalgal isolates in this study **a** UJEAS4 and **b** UJEAS8. Magnification: 400×
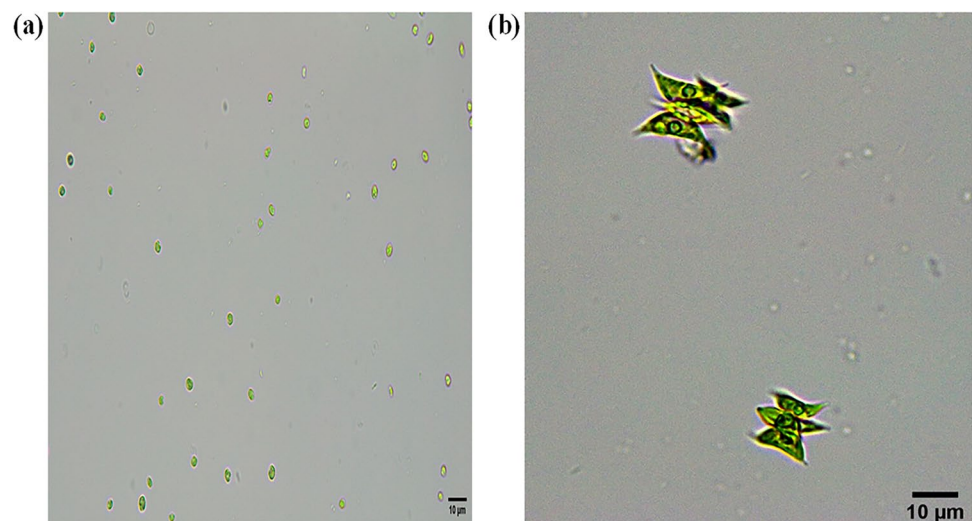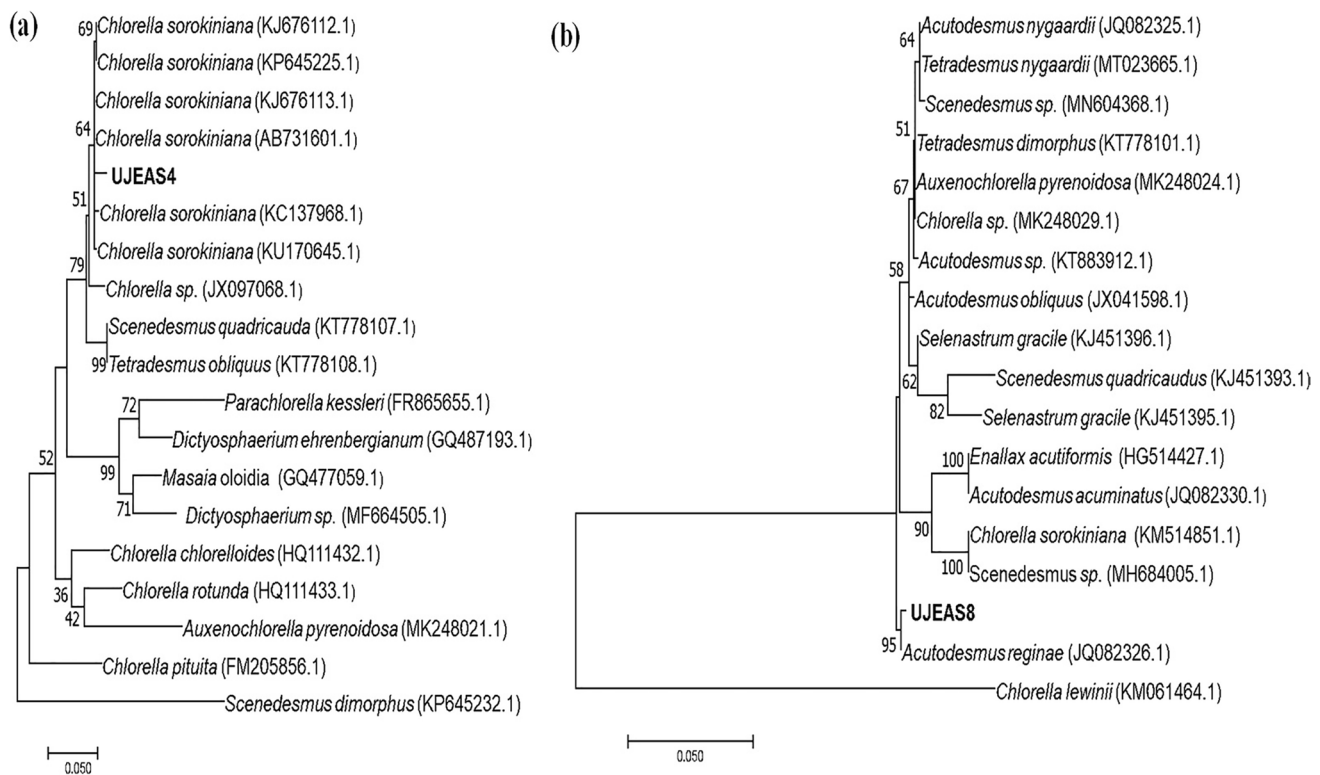
Fig. 2 Phylogenetic tree of microalgal isolates **a** UJEAS4 and **b** UJEAS8 based on ITS2 sequence constructed by ML algorithm. Bootstrap numbers below 50 were not shown on the tree. The numbers on nodes indicate bootstrap values after 1,000 replicates expressed in percentages

3.2 Biomass productivity, growth kinetics and CO$_2$ biosequestration rates

High microalgal growth rate, rapid biomass accumulation and excellent carbon fixation rates are crucial requirements for selecting a particular microalgae species as a potential bioresource with climate change mitigation capabilities [52, 53]. Therefore, our work assessed each microalgal isolate cultivated in their respective BG-11 and BBM media for their biomass accumulation abilities, growth kinetics and CO$_2$ biosequestration rates and presents the results in Fig. 3.

It can be inferred in Fig. 3 that the growth pattern of the two microalgal species is expected to be in line with the four conventional phases, namely, lag, exponential, stationary and declining phases but the first two phases were notable. This observation could be due to the fact that the isolates' growth culture was stopped prior to significant nutrient stress being experienced by the algal cells. Even so, whilst the nitrate levels in the culture medium did decrease and became completely consumed by the end of the culture period as it was in the case of *T. reginae*; the isolate might still have been thriving on intracellular nitrogen reserves [54]. The measured dry wet biomass (DWB) and the optical cell densities at 750 nm of all monocultures also showed

a linear regression association per Eqs. (9) and (10) and respective strong R^2 values.cy4

$$\textbf{a}\ DWB_{C.sorokiniana} = 1.8923\ (OD_{750}) + 0.2058; R^2 = 0.9912 \tag{9}$$

$$\textbf{b}\ DWB_{T.reginae} = 1.2571(OD_{750}) + 0.1289; R^2 = 0.9872 \tag{10}$$

Our observations revealed that there was a significant difference ($p < 0.05$) in biomass productivity, specific growth rate and doubling times among the isolates under cultivation (Table 1).

T. reginae, which was cultured in BBM out-performed the BG-11-cultivated *C. sorokiniana*, in the mentioned growth parameters. *Tetradesmus reginae* showed a specific growth rate (μ) of 0.20 ± 0.009/day with a shorter doubling time (d2) of 3.5 ± 0.158 days whilst the μ for *C. sorokiniana* was lower at 0.14 ± 0.015/day with a relatively longer d2 of 4.8 ± 0.544/ day. This growth kinetics eventually culminated in biomass productivity of 0.2 ± 0.004 g L^{-1} day^{-1} for *T. reginae* and 0.128 ± 0.003 g L^{-1} day^{-1} for *C. sorokiniana*. Thus, *T. reginae* stands a better chance as a potential bioresource between the two, since biomass productivity is a key selection factor in that regard. In addition, our study recorded maximum biomass concentrations of 3.32 ± 0.051 and 2.21 ± 0.038 g L^{-1}
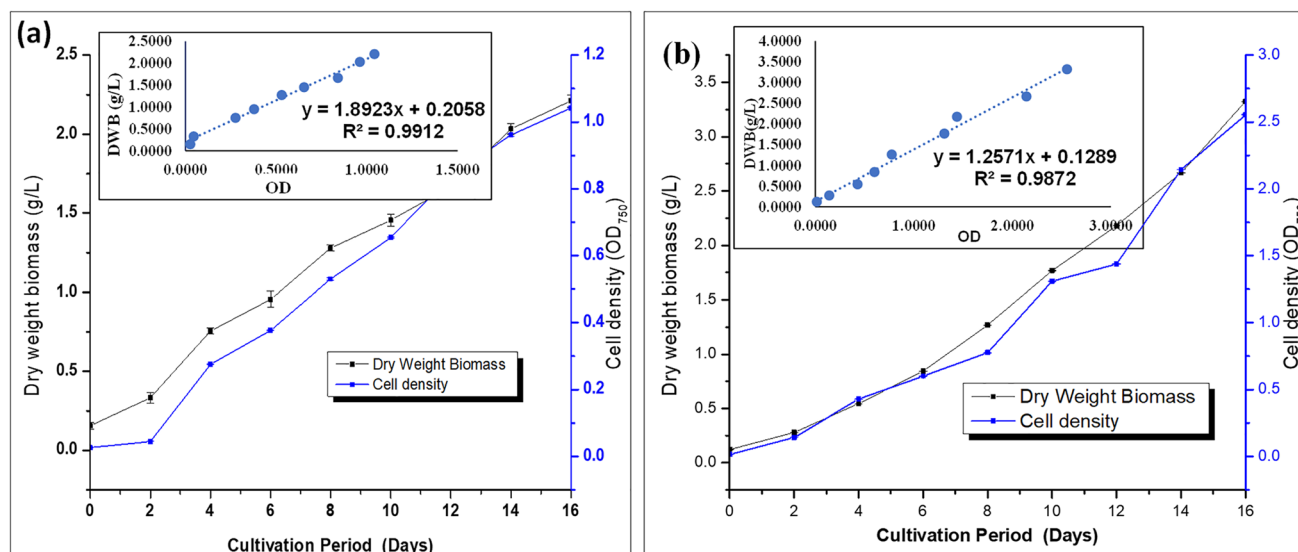
Fig. 3 Linear relation between dry weight microalgae biomass and respective optical cell densities for **a** *C. sorokiniana* and **b** *T. reginae*

Table 1 Biomass production, growth kinetics and CO_2 biosequestration rate of microalgal isolates

Microalgal strain	Culture medium	Culture duration (days)	Max. biomass conc (g L^{-1})	Biomass productivity, BP (g L^{-1} day^{-1})	Specific growth rate, μ (day)	Biomass doubling time, d_2 (days)	CO_2 sequestration rate (gCO_2 L^{-1} day^{-1})
C. sorokiniana	BG-11	16	2.21 ± 0.038	0.128 ± 0.003	0.14 ± 0.015	4.8 ± 0.544	23.05 ± 0.711
T. reginae	BBM	16	3.32 ± 0.051	0.200 ± 0.004	0.20 ± 0.009	3.5 ± 0.158	31.52 ± 0.600

for *T. reginae* and *C. sorokiniana,* respectively. Though the biomass concentration for *C. sorokiniana* might appear in this study to be relatively lower, it was two times higher than what Lugo et al., [55] reported for the same species for unsupplemented BG-11, under uncontrolled pH and air bubbling. This could be attributed to an optimum CO_2, nitrate-nitrogen and light exposure in the current study. A similar argument could be made for *T. reginae* when its biomass concentrations are compared to other species belonging to the *Scenedesmus/Acutodesmus* genus in other studies under similar conditions [56, 57]. The microalgae isolates were reported to tolerate a high temperature up to 37 °C and could also thrive in a heterotrophic environment [58, 59]. Even though, culture medium and conditions as well as each microalgal strain's nutrient requirement could also influence the differences between each microalgae productivity [60], this was not our study's focus, and there is a need to further explore this to ensure higher growth rates and improved metabolite production.

The CO_2 biosequestration rates of the two microalgal isolates were also found to be significantly different with *T. reginae* biofixing dissolved CO_2 at the rate of 31.52 ± 0.600 gCO_2 L^{-1} day^{-1} followed by 23.05 ± 0.711 gCO_2 L^{-1} day^{-1} for *C. sorokiniana,* respectively. Compared

with high-performing CO_2-fixing microalgae reported in literature [12], the isolates demonstrated reasonably high biosequestration rates but our studies did not assess their CO_2 tolerance rates. Notwithstanding that fact, our isolates may be considered potential excellent CO_2 mitigation options in reducing the current global high atmospheric CO_2 levels if cultivated under optimal conditions.

3.3 Nutrient consumption and pH variations

The observed rates of nitrogen consumption *vis-a-vis* pH variation in the cultures every 48 h for 16 days is presented in Fig. 4a, b.

Generally, there was a positive correlation between biomass productivity and the variations in culture pH and a negative correlation between culture pH and nitrate–N consumption or depletion in both cultures under the photoautotrophic cultivation period. In other words, changes in the culture pH, was a sign that nitrate-nitrogen was getting assimilated by the microalgal species, resulting in biomass accumulation [61, 62]. However, the initial concentration of nitrate–N in the microalgal cultures could impact the biomass output of the two isolates. This observation could explain why *T. reginae* with an initial culture nitrate–N

Fig. 4 Biomass yields and nitrate–N consumption with corresponding culture pH variations and their respective controls of **a** *C. sorokiniana* and **b** *T. reginae*
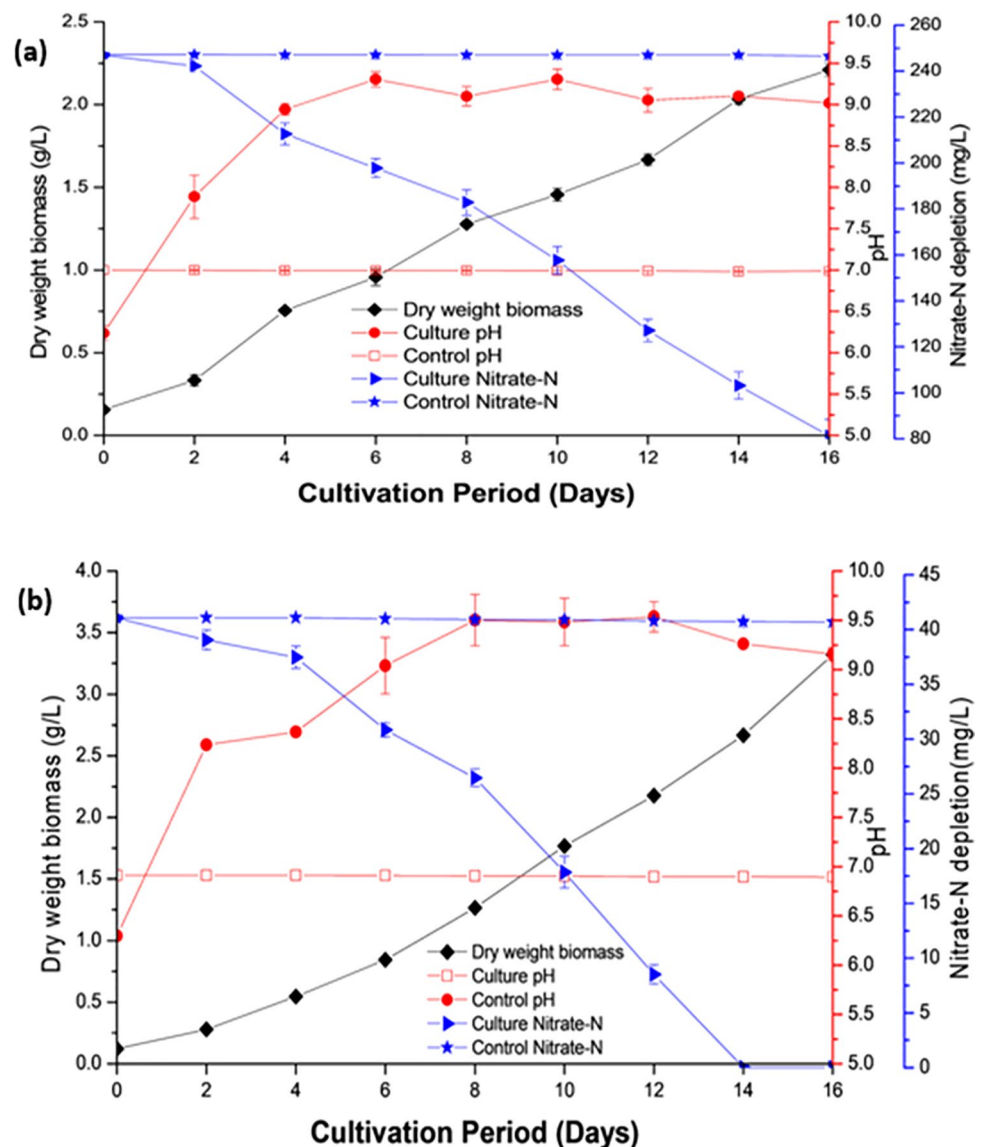
concentration of about 41 mg L^{-1} (in BBM) recorded higher maximum biomass output in comparison to *C. sorokiniana* with initial culture nitrate–N levels of around 247 mg L^{-1} each (in BG-11). Though earlier reports on initial culture nitrate–N levels' have an effect on microalgal growth, maybe contrary, there is a strong suggestion that an increase in initial culture nitrate–N levels stimulate nitrogen reductase and nitrite reductase activities [63]. This may lead to ammonium accumulation and its related toxic effect on microalgal cells with an eventual reduction in cell growth or increase in cell death [64]. Whilst Wang and Lan [65] showed in their study that increasing initial culture nitrate–N levels (218 mg L^{-1}) reduced microalgae cell growth of green alga *Neochloris oleaobundans*, Ogbonna et al. [66] reported no significant change in green microalgal species, *C. sorokiniana* and *Spirula platensis* with an initial culture nitrate–N levels of 700 mg L^{-1}. It is also very important to situate this argument

in the broader context of other prevailing culture conditions (pH, light intensity, temperature, CO$_2$ and photoperiod) and the physiology of the microalgal cells used which in most cases are not exactly the same.

The culture pH in this study was not controlled after initial sparging with pure CO$_2$ gas and continuous air bubbling to supply 0.34% CO$_2$ (in air) and to prevent the microalgae species under cultivation from clinging to the walls of the bioreactors. At the end of the 16-day cultivation period, culture pH ranged from 6.24 to 9.10 and 6.30 to 9.54 for *C. sorokiniana* and *T. reginae*, respectively. This could be explained based on the source of available nitrogen (nitrate–N) and the absence of excess-CO$_2$ for microalgal photosynthesis. The observation is consistent with the findings of Wang and Curtis [67], which suggested that nitrate–N consumption in microalgal cultures could increase the culture pH due to the production of excess hydroxyl

(OH^-) ions during its assimilation. The above-mentioned process can be expressed with Eq. (11).

$$NO_3^- + 3H_2O \rightarrow NH_4^+ + 2O_2 + 2OH^- \tag{11}$$
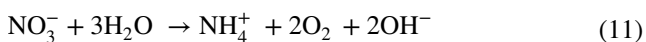
The pH profiles of the microalgal isolates under cultivation in our study were, however, within their tolerance pH ranges reported in the literature, but their respective biomass productivity could have improved if pH was regulated at an optimal level. Ahmad et al. [68] confirmed this assertion when he recorded high biomass productivity (38 to 41 cells mL^{-1} day^{-1}) for a BBM cultivated *Scenedesmus* sp. at pH 7.5–8.0. Qui [69] also established that a pH 6 is suitable for *C. sorokiniana*'s maximum biomass productivity (0.140 ± 0.004 g L^{-1} day^{-1}).

3.4 Biochemical composition, elemental composition and fatty acid methyl ester profile
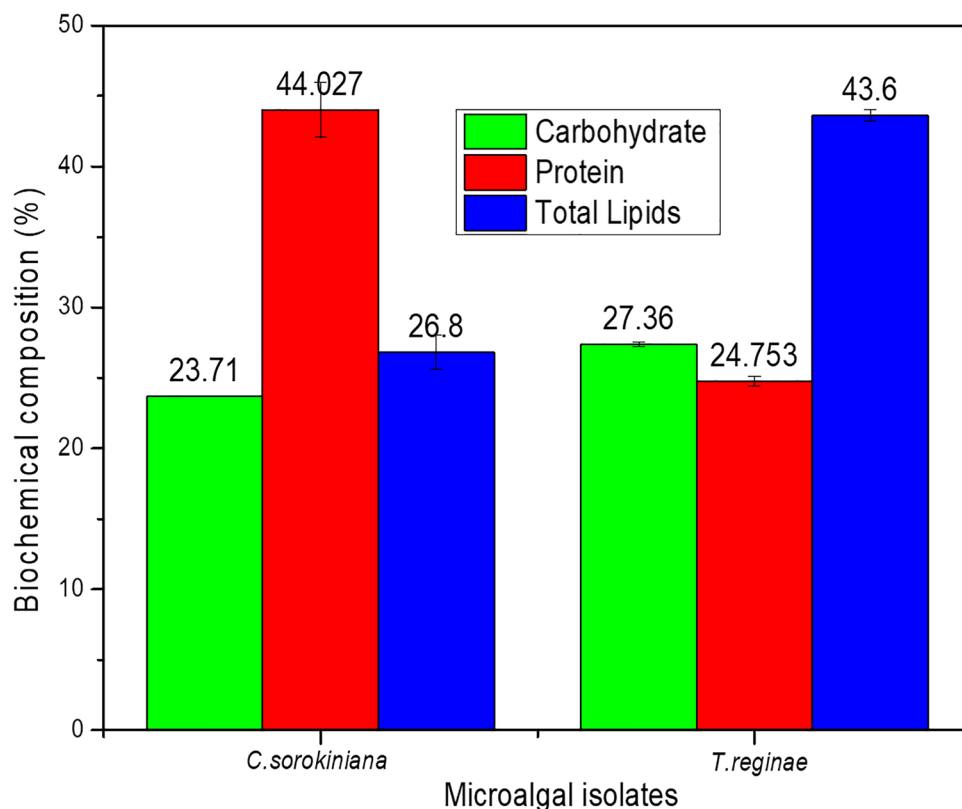
The isolates' biochemical composition reflects on the composition of the culture medium and the species type [70]. The composition of dry weight biomass carbohydrate, protein and total lipid, as well as fatty acid methyl ester (FAME) profile and elemental composition of the two isolates, are illustrated in Figs. 5 and 6 and Table 2.

As revealed in Fig. 5, *T. reginae* recorded a significantly higher ($p < 0.05$) percentage value (of dry biomass) for total

lipid ($43.60 \pm 0.40\%$) and carbohydrate ($27.36 \pm 0.17\%$) when compared to the same biochemical components of *C. sorokiniana* (i.e., $26.80 \pm 1.20\%$ and $23.71 \pm 0.02\%$, respectively). This study also revealed that *C. sorokiniana* had a significantly higher ($p < 0.05$) total protein value of $44.03 \pm 1.94\%$ in comparison to $24.75 \pm 0.36\%$ for *T. reginae*. This observation suggests that either of the isolates could be selected based on a particular biochemical component need. For example, *T. reginae* is a potential biodiesel and bioethanol source, whilst *C. sorokiniana* is a food and feed supplement potential.

Nevertheless, the content analysis (Fig. 5) of *C. sorokiniana* showed a relatively balanced biochemical composition compared to the results of Berrejeb et al. [71] for the same species cultivated in BG-11 under similar conditions. The remarkable total lipid and carbohydrate content of *T. reginae* is the first reported, based on the authors' knowledge when cultivating BBM species. The relatively high total lipid value recorded for *T. reginae* could be attributed to the low nitrogen content (approx. 47 mg L^{-1}) in its culture medium (BBM), which depleted around the 11–12th day of cultivation as compared to the high nitrogen levels (about 247 mg L^{-1}) in culture medium (BG-11) of *C. sorokiniana*. This assertion is consistent with the conclusions from another related study that nutrients, especially exhaustion of nitrogen, trigger cascades of biochemical activities that lead to lipid accumulation in most green microalgal strains [72].

Fig. 5 Biochemical composition of microalgal isolates *C. sorokiniana* and *T. reginae*

Fig. 6 Elemental composition of microalgal isolates *C. sorokiniana* and *T. reginae*
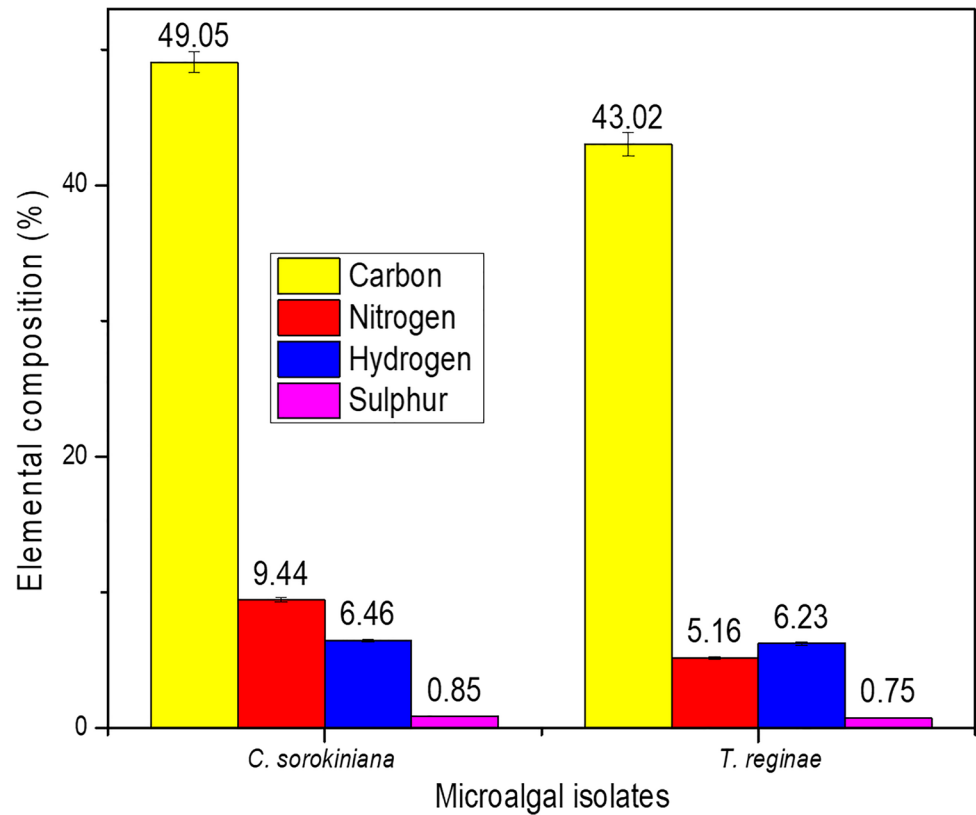
Table 2 Biochemical and elemental composition of microalgae isolates

Microalgal strain	Culture medium	Biochemical content (%)			Elemental content (%)			
		Carbohydrate	Protein	Total lipids	Carbon	Nitrogen	Hydrogen	Sulphur
C. sorokiniana	BG-11	23.71 ± 0.02	44.03 ± 1.94	26.80 ± 1.20	48.99 ± 0.56	9.21 ± 0.41	6.45 ± 0.08	0.85 ± 0.012
T. reginae	BBM	27.36 ± 0.17	24.74 ± 0.36	43.60 ± 0.40	42.98 ± 0.60	5.18 ± 0.08	6.21 ± 0.09	0.75 ± 0.004

Sharma et al. [73] confirmed our observation when they cultivated *Chlorella* sp. with four different media including M_4N, Fog, Bold's basal and BG-11 but found that the species in BBM produced relatively higher lipids. Sharma and colleagues also related their results to BBM's low nitrogen content, which led to early depletion [73], as a result, a stressed condition developed, which encouraged the development of more lipids. A recent report by Gonclaves et al. [74] suggested that one possible mechanism to explain the observed phenomenon is the decrease in the cellular contents of the microalgal thylakoid membrane during nitrogen starvation. This leads to the activation of the enzyme, acyl hydrolase and subsequent phospholipid hydrolysis resulting in the extracellular increase of the β-oxidation precursor, acyl-CoA fatty acid content. At the same time, diacylglycerol acyltransferase, the enzyme responsible for converting acyl-CoA to triacylglycerol (TAG), is also activated. Based on the relatively high protein, total lipid and carbohydrate contents, it can be suggested that both isolates in this study

are excellent bioresources. Figure 6 illustrates the percentage total carbon (C), nitrogen (N), hydrogen (H) and sulphur (S) of the dry isolates' biomass.

The *T*-test analysis reveals a remarkable difference ($p < 0.05$) among C, N, H and S for all isolates. However, this study focuses on the biomass C and N content and the C/N ratio for each isolate since the ratio can give credence to some suggested lipid accumulation mechanisms of total biomass lipid, even though the subject has not fully been explored for microalgae biomass. In simple terms, limited nitrogen availability with simultaneous sufficient organic carbon in cultures could promote the accumulation of algal lipids [75]. The usefulness of the C/N ratio as a measure for the lipid content and the condition of individual microalgae is based on the assumption that the C/N ratio and the lipid content within and among microalgae species will differ predictably under a wide range of conditions. The approximate calculated C/N ratios of *C. sorokiniana* and *T. reginae* from our study were 5 and 8, respectively. *Tetradesmus reginae*

primary carbon product (sugar) biochemical pathway may have been altered to favour significant lipid synthesis (Fig. 5) as a result of the low nitrate-nitrogen available to the cells in its culture medium (BBM) as compared to that of *C. sorokiniana*. This observation is coherent with similar research findings by Ananthi et al. [76], which suggests that the higher the C/N ratio for a particular microalgae culture, the more likely the species would accumulate lipid.

Algal biomass was harvested after the 16-day cultivation period and analysed for fatty acid methyl esters with in situ transesterification method and a high-resolution GC–MS. The results obtained are presented in Table 3.

FAME, fatty acid methyl ester; *SFA*, saturated fatty acid; *MUFA*, mono-unsaturated fatty acid; *PUFA*, poly-unsaturated fatty acid; *SN*, saponification number; *IV*, iodine value; *CN*, cetane number.

This study showed that *C. sorokiniana's* fatty acid methyl ester profile contained 5 components with their relative abundances ranging from 4.7 to 35.7% whilst that of

T. reginae ranged between 0.8 and 29.8% with 12 components. Palmitic acid and linolenic acid, methyl esters were observed in appreciable abundance in both green microalgal isolates. In contrast to Sharma and colleagues' [73] results, in which BG-11 cultivated *Chlorella* sp. showed significant levels (15–33%) of oleic acid, our study found no oleic acid, an essential fatty acid in green algae. However, it should be noted that Sharma's study used organic carbon source (sucrose, glucose, sodium acetate and glycerol) whilst our study relied on CO_2 as the sole carbon source. Nevertheless, this observation presents a research opportunity aimed at filling the gap identified.

Saturated fatty acids (SFAs) have been described in numerous studies as biological tools for biodiesel production and microalgae are rich sources of these compounds [4]. The percentage of SFA content for *C. sorokiniana* and *T. reginae* corresponds to $35.73 \pm 1.13\%$ and $29.79 \pm 1.06\%$, respectively. Interestingly, palmitic acid is the highest and only measured SFA in the FAME profile for each isolate.

Table 3 Relative FAME component abundance and biodiesel quality indicators of microalgal isolates cultivated in artificial media

Microalgae FAME profile	Chain	RT (min)	Formula	MW (g mol^{-1})	Relative FAME component abundance (%)	
					C. sorokiniana (BG-11)	*T. reginae* (BBM)
(11Z,14Z,17Z)-Methyl icosa-11,14,17-trienoate	C20:3 cis 11,14,17 (n-3)	18.85	$C_{21}H_{36}O_2$	320.5	0	10.04 ± 0.46
11,14,17-Eicosatrienoic acid, methyl ester	C20:3 (n-3)	18.84	$C_{21}H_{36}O_2$	320.5	0	10.62 ± 0.10
11-Octadecenoic acid, methyl ester	C18:1	18.77	$C_{19}H_{36}O_2$	296.5	0	2.11 ± 0.10
9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)- (linolenic acid, methyl ester)	C18:3 (n-3)	20.76	$C_{19}H_{32}O_2$	292.5	12.54 ± 5.03	11.02 ± 0.03
9,12-Octadecadienoic acid (Z,Z)-(linoleic acid)	C18: 2 cis 9,12	22.28	$C_{19}H_{34}O_2$	294.5	4.09 ± 0.85	0
9,12-Octadecadienoic acid, methyl ester, (E,E)- (linolelaidic acid, methyl ester)	C18: 2 trans 9,12	18.71	$C_{19}H_{34}O_2$	294.5	5.24 ± 0.66	0
9-Octadecenoic acid (Z)-, methyl ester (oleic acid methyl ester)	C18: 1 cis 9	14.48	$C_{19}H_{36}O_2$	296.5	0	0
Hexadecanoic acid, methyl ester (methyl palmitate)	C16:0	16.97	$C_{17}H_{34}O_2$	284.5	35.73 ± 1.14	29.79 ± 1.06
9,12-Hexadecadienoic acid, methyl ester	C16:2	16.70	$C_{17}H_{30}O_2$	266.4	4.69 ± 1.39	0
Methyl 4,7,10,13,16,19-docosahexaenoate (cervonic acid)	C22:6 (n-3)	16.66	$C_{23}H_{34}O_2$	342.5	0	0.77 ± 0.07
13-Octadecenoic acid, methyl ester	C18:1	18.83	$C_{19}H_{36}O_2$	296.5	0	6.57 ± 0.76
trans-13-Octadecenoic acid, methyl ester	C18:1 trans 13	18.84	$C_{19}H_{36}O_2$	296.5	0	2.36 ± 0.04
cis-13-Octadecenoic acid, methyl ester	C18:1 cis 13	18.84	$C_{19}H_{36}O_2$	296.5	0	4.51 ± 0.03
12,13-Octadecadienoate, methyl ester	C18: 2	18.77	$C_{19}H_{34}O_2$	294.5	0	2.89 ± 0.14
8,11,14-Heptadecatrienoate, methyl ester	C17:3	18.84	$C_{18}H_{30}O_2$	278.4	0	7.4 ± 2.46
4,7,10,13-Hexadecatetraenoic acid, methyl ester	C16:4	16.63	$C_{17}H_{26}O_2$	264.4	0	4.81 ± 0.2
Phytyl, 2-methylbutanoate	C24:1	18.48	$C_{25}H_{48}O_2$	380.6	22.26 ± 4.46	0
	SFA (%)				35.73 ± 1.13	29.79 ± 1.06
	MUFA (%)				34.81 ± 9.50	27.34 ± 1.02
	PUFA (%)				14.02 ± 2.9	35.76 ± 3.35
	SN				122	146
	IV				36	75
	CN				83	67

Poly-unsaturated fatty acids (PUFAs), on the other hand, are of special nutraceutical importance for the treatment and prevention of several ailments and prominent among them are docosahexaenoic acids (DHAs) and eicosapentaenoic acids (EPAs) [77]. These compounds were mainly found in fish oil because the fishes bioaccumulated them through the consumption of phytoplankton including microalgae in the food chain, making fish an important human nutritional source [78]. However, there is now a significant challenge associated with the sustainability of fish source; fortunately, some microalgae have been reported to contain most of these critical DHAs and EPAs in abundance and could be cultivated autotrophically for economic use [79]. This study, however, found only *T. reginae* to contain EPA isomers (11,14,17-eicosatrienoic acid, (Z,Z,Z)- and 11,14,17-eicosatrienoic acid, methyl esters) with an appreciable percentage abundance of 10.04 ± 0.46 and 10.62 ± 0.10 and DHA (4,7,10,13,16,19-docosahexaenoic acid, methyl ester) with a low % abundance of 0.77 ± 0.07. This indicates that, at optimal culturing conditions, *T. reginae* could be considered a sustainable omega-3 oil source.

The quality of potential microalgae biodiesel was measured based on the biofuel properties, such as cetane number (CN), iodine number (IV) and saponification number (SN). These parameters are strongly affected by the size of the carbon chain and the number of double bonds in the fatty acids of the feedstock oil. Cetane number is associated with long saturated fatty acid chains of carbon and enables the assessment of engine performance. A high CN guarantees good properties of ignition/combustion and minimises white smoke formation. Iodine number is a measure of a fatty material's gross unsaturation. Therefore, a lower degree of microalgal oil unsaturation results in a higher CN and a lower IV. Saponification number gives information on the average relative molecular mass of fatty acids [71]. It should be noted that these criteria have been commonly used to determine the quality of biodiesel, although they have not been sufficiently studied for algal oils to date to the best of our knowledge. The SN, IV and CN were determined from the FAME profile and presented in Table 3. The calculated critical indicators (IV and CN) varied among the isolates. The study shows that all calculated IV values were within the European Standards (EN)14,214 maximum standard of 120 and CN values above the lowest acceptable limits of 51 and 47 for EN 14,214 and American society for testing and materials(ASTM) D6751-08, respectively [80]. When the sum of saturated fatty acids was greater than the unsaturated fatty acids, high CN values were achieved. Based on the above, the green microalgal isolates are potential excellent sources of quality biodiesel. However, despite these positive indicators which may make algal cultivation as a third-generation biofuel feedstock as magnificent option, application at an industrial scale is still lagging. This may be ascribed to negative attributes of the technology including the cost intensive process of algal mass culture with potential minimal biodiesel yield [81]

4 Conclusion

In this study, we isolated, identified and investigated two microalgal species, *C. sorokiniana* and *T. reginae* collected from Killarney Golf Course, Johannesburg-South Africa. Our work demonstrated that both isolates could be cultivated in artificial media for economic production of biomass, CO_2 biosequestration and nutraceutical compounds and are potential sources for bioethanol and biodiesel production. The study further revealed that *T. reginae* remarkably outperformed ($p < 0.05$) *C. sorokiniana* with regard to biomass production rate, total lipids and carbohydrate production, CO_2 biosequestration rate and C/N ratio. In addition, *T. reginae* contained a promising level of omega-3 oils. *Chlorella sorokiniana* was found to have a significant level of total protein. There was also a significant difference between the BBM and BG-11 media, making BBM a better medium for cultivating microalgae species for commercial value. From an economic point of view, the cost of preparing BBM is relatively lower than the preparation of the same quantity of BG-11 due to the chemical composition requirements. Hence, it makes economic sense to use BBM. Our study has shown that even though both isolates can be used as bioresources, *T. reginae* cultivated photoautotrophically in BBM has been proven to perform excellently as a bioresource and for CO_2 fixation. It is also interesting to conclude that both isolates met both European and American quality biodiesel standards with exceptional IV and CN values.

Declarations

Conflict of interest The authors declare no conflict of interest.

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