



# Assortment of Native Microalgae for Improved Biomass and Lipid Production on Employing Vegetable Waste as a Frugal Cultivation Approach for Biodiesel Application

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## Abstract

Enhanced biofuel production strategies from microalgae by employing affordable bio-waste usage are fetching significance, nowadays. This study examines the effect of VWE for enhanced biomass from new indigenous microalgal isolates, *Asterarcys* sp. SPC, *Scenedesmus* sp. KT-U, *Scenedesmus* sp. KTWL-A, *Coelastrum* sp. T-E, and *Chlorella* sp. TWL-B. The growth of microalgae in VWE-treated growth media showed considerable increase (1.14–2.3 folds) than control medium (without VWE). Further, two effective native microalgae were selected based on growth in VWE treatment, biomass productivity, and TAG accumulation through statistical clustering analysis. Mixotrophic batch cultivation of *Scenedesmus* sp. KT-U and *Asterarcys* sp. SPC cultivated using VWE treatment in the optimum concentration had produced significant average increase in BP (1.8 and 1.4 folds, respectively) than control (without VWE). Whereas in the lipid production phase, there was a noticeable increase in lipid yield in VWE-treated cells of lipid phase ( $231.8 \pm 17.9$  mg/L and  $243.5 \pm 25$  mg/L) in *Scenedesmus* sp. KT-U and *Asterarcys* sp. SPC, respectively, than in control ( $140.5 \pm 28$  mg/L and  $166.4 \pm 23$  mg/L) with considerable TAG accumulation. Thus, this study imparts strain selection process of native microalgae based on vegetable waste usage for improved yield of biomass and lipid amenable for cost-effective biodiesel production.

## Abbreviations

VWE	Vegetable waste extract	GHG	Greenhouse gas
FAO	Food and Agriculture Organization	FAME	Fatty acid methyl esters
$\mu\text{L}/\text{mL}$	Microliters per milliliter	TLC	Thin Layer Chromatography
$\mu\text{g}/\text{mL}$	Micrograms per milliliter	DNA	Deoxyribonucleic acid
mEq/L	Milliequivalents per liter	ITS	Internal Transcribed Spacer
mmol/L	Millimoles per liter	PCR	Polymerase chain reaction
mg/L	Milligrams per liter	Chl a	Chlorophyll a
DCW	Dry cell weight	LC	Lipid content
TDS	Total dissolved solids	BP	Biomass production
TAG	Triacylglycerol		
PCA	Principal Component Analysis		
MMT	Million metric tons		

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## Introduction

Globally food loss and waste levels are high. For instance, in developing countries around 15% to 50% of all fruit and vegetable wastages occur during post-harvest stage, whereas in Switzerland, around 47% of all vegetables produced are wasted in the food supply chain, and in Germany, fruits and vegetable wastages account for 43% of all household waste [1]. It is worth mentioning that, India produces large amount of municipal solid waste (MSW), and it is projected that about 300 million tons per annum of MSW will be generated in the near future [2]. Among the waste generated in India, the biodegradable portion is higher than the estimate

of 51% [3]. Vegetable waste occupies the bulk portion of the biodegradable wastes generated from processing, packaging, and post-harvest losses including rotten vegetables, peels and scrapped portions [4]. The segregation and utilization of these biodegradable—municipal solid waste from hotels and houses are the major highlights of Solid waste management rules notified by Union Ministry of Environment, Forest, and Climate Change [5]. Substantially, the global production of vegetable waste has been estimated to be 70.2 MMT [6]. In fact, an estimated average of 6.03 million tons of unavoidable vegetable waste has been generated from the consumption of fresh vegetables in households of European Union [7]. It was reported that the vegetable wastes are rich in nutrients, however on dumping these biodegradable wastes in land-filling site generate GHG emissions such as methane emissions [8]. Recently, a comprehensive estimate proposes that the annual global methane emissions are around 570 million tons [9]. Therefore, recycling and proper utilization of vegetable waste for bioenergy applications paves the way for energy generation which can also gratifies the environmental concern [10]. It is worth mentioning that the procurement of vegetable waste from hotels, restaurants, big kitchens or food industries is easier. Biowastes such as vegetable waste is considered as one of the best raw materials to obtain energy (biogas, bioelectricity, and biofuels) due to high organic composition with good biodegradable nature, and most importantly, vegetable wastes have the potential to enhance the microbial growth due to its high protein, vitamins, minerals and other trace elements [11].

Microalgae are the potent candidates for biodiesel production owing to its capability of higher lipid accumulation. Moreover, microalgae are highly efficient in waste and CO<sub>2</sub> remediation [12, 13]. Biodiesel (FAME) can be produced from lipids of oleaginous microalgal feedstock [14]. Transesterification is the most common reaction employed for the conversion of lipids/oil into biodiesel [15, 16]. In addition to biodiesel production, microalgal biomass are the potent feedstock for the production of bioethanol, biogas, carotenoids, and other value-added chemicals [13]. Nevertheless, the cost of production of microalgae is one of the major hindrances to commercial production [17]. Combinatorial approaches for microalgal biomass cultivation and waste utilization are considered to be sustainable for microalgae-based biofuel production which facilitate microalgal cultivation in a cost-effective manner [13, 18–20]. Subsequently, growth media for microalgae has been produced using biowastes such as kitchen effluent [21], waste molasses [22], food waste [23], rice husks [24], dairy waste [25], etc., for frugal biomass cultivation. Mixotrophic cultivation using biodegradable waste is one of the suitable cultivation mode for overall enhanced lipid yield appropriate for biodiesel application with economic viability as well [26]. In fact, anaerobically digested kitchen waste-based media

for *Chlorella sorokiniana* SDEC-18 and *Scenedesmus* sp. SDEC-8 [27], fruit waste hydrolysates for *C. vulgaris* had shown increased biomass and lipid production [28]. Moreover, growth medium derived from diluted vegetable waste hydrolysate, and diluted fruit waste hydrolysate had increased the biomass growth in *Chlorella vulgaris* [28]. This kind of cultivation system coupled with waste utilization improves resource recovery and the economics of sustainable bioenergy production from microalgae [13, 29]. But at the same time, reports on imparting vegetable waste in the process of screening of new indigenous microalgal isolates are scarce.

Therefore, this current study was aimed to evaluate five new indigenous microalgal isolates for biodiesel application through statistical clustering analysis based on the key parameters such as (i) growth in various VWE concentrations, (ii) biomass productivity, and (iii) TAG production. Thus, the purpose of this study is to enable the strain selection process as well as ascertaining the suitability of new indigenous microalgal isolates for mixotrophic cultivation employing vegetable wastes. Furthermore, TAG accumulation, biomass, and lipid yield of VWE-treated biomass of lipid phase and the control cells (untreated) of the selected indigenous microalgal isolates were examined under batch cultivation.

## Materials and Methods

### Chemicals and Reagents

Analytical grade chemicals (minimum assay  $\geq 98\%$ ) were used. The following are the list of chemicals, sodium nitrate (NaNO<sub>3</sub>), sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>), disodium magnesium EDTA (Na<sub>2</sub>MgEDTA), calcium chloride (CaCl<sub>2</sub>·2H<sub>2</sub>O), ferric chloride (FeCl<sub>3</sub>), ammonium sulfate (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, citric acid (C<sub>6</sub>H<sub>8</sub>O<sub>7</sub>), magnesium sulfate (MgSO<sub>4</sub>·7H<sub>2</sub>O), dipotassium hydrogen phosphate (K<sub>2</sub>HPO<sub>4</sub>), boric acid (H<sub>3</sub>BO<sub>3</sub>), manganese chloride (MnCl<sub>2</sub>·4H<sub>2</sub>O), Zinc sulfate (ZnSO<sub>4</sub>·7H<sub>2</sub>O), Copper sulfate (CuSO<sub>4</sub>·5H<sub>2</sub>O), Cobaltous chloride (CoCl<sub>2</sub>·6H<sub>2</sub>O), Sodium molybdate (NaMoO<sub>4</sub>·2H<sub>2</sub>O); TEN buffer (10 mmol/L Tris–HCl, 10 mmol/L Na<sub>2</sub>EDTA, 150 mmol/L NaCl); SDS-EB buffer (2% SDS, 400 mmol/L NaCl, 40 mmol/L Na<sub>2</sub>EDTA, 100 mmol/L Tris–HCl); phenol, chloroform, hexane, diethyl ether, isoamyl alcohol, isopropanol, ethanol, Bovine Serum Albumin (BSA), Coomassie brilliant blue-G250, Carbinol/ Methanol, 85% orthophosphoric acid, D-glucose; 3,5-dinitrosalicylic acid (DNSA), sodium potassium tartrate, potassium sulfate, sodium acetate, glacial acetic acid, silver nitrate, gum acacia, sulfuric acid, hydrochloric acid, disodium hydrogen phosphate, ammonium chloride, barium

chloride, ammonium oxalate, ammonium hydroxide, methyl red, potassium permanganate, and sodium hydroxide.

### Sampling, Isolation and Growth of Microalgae

Fresh water sample was collected for the isolation of indigenous microalgae from Sular lake, Coimbatore, Tamil Nadu, India (11.0295 N 77.1208 E). Further, soil samples were collected from Tamil Nadu Agricultural University (TNAU) campus, Coimbatore, Tamil Nadu, India (11.0129 N 76.938 E); wetland TNAU, Coimbatore, Tamil Nadu, India (11.0018 N 76.9242 E & 11.0022 N 76.9247 E). Each sample was serially diluted, inoculated on modified BG-11 agar medium [30], and incubated at 25 °C for 14–20 days. The plates were maintained under the average light intensity of 3500 lx (Lux meter: HTC-LX101A, India) with the alternate photoperiod of light: dark cycle-10:14 h. The purity of the isolates was monitored under light microscope. Based on the pure colonial existence, the microalgal isolates, namely SPC (from Sular lake), KT-U (from TNAU campus, Coimbatore), KTWL-A, T-E, and TWL-B (from wetland, TNAU) of the above-mentioned geographical locations were cultured, and maintained as pure cultures in modified BG-11 medium (pH 7.0 ± 0.2) under the average light intensity of 3500 lx in Erlenmeyer's flask with alternate photoperiod (light: dark cycle-10:14 h) at 28 ± 3 °C. The pure microalgal isolates were monitored under light microscope (Labomed, Germany) with 1000X magnification.

### Molecular Identification, Phylogeny and Taxonomy

Genomic DNA was extracted from the pure microalgal isolates. Briefly, cell pellets were collected; washed with TEN buffer; resuspended in 150 µL ice cold water, and vortexed for two min. Further, 350 µL SDS-EB buffer was added; cells were lysed through agitation using tissue lyser (Qiagen, Germany) at a frequency of 28 s<sup>-1</sup> for 10 min, and incubated for 3 min. After cell lysis, genomic DNA was then extracted following phenol: chloroform method. DNA amplification of partial 18S rRNA /ITS regions were performed using genomic DNA as the template by PCR. The ITS1-5.8S-ITS2 region was amplified with the following primer sequences 5'-ACCTAGAGGAAGGAGAAGTCGTAA-3', 5'-TTCCCTCGCTTATTGATATGC-3' as forward and reverse primer, respectively [31], whereas the partial small subunit 18S rRNA region was amplified with the following primers, 5'-TCTAGAGCTAATACGTGCG -3', 5'-GTGGTGCCCTTCGTCAAT-3' as forward and reverse primer, respectively [this study]. PCR products were sequenced by Sanger's dideoxy method of sequencing and the sequences were subjected to Nucleotide BLAST analysis. Phylogenetic trees were constructed (phylogeny test by neighbor joining tree method) with 1000 bootstrap replications using MEGA 6 software

using the multiple sequence alignment (Clustal W) of the ITS1- 5.8 S- ITS2 regions (*Asterarcys* sp. SPC, *Scenedesmus* sp. KT-U, *Scenedesmus* sp. KTWL-A, *Coelastrum* sp. T-E) and partial small subunit 18S rRNA regions (*Chlorella* sp. TWL-B, *Scenedesmus* sp. KTWL-A) with the existing *Asterarcys* sp., *Scenedesmus* sp., *Coelastrum* sp., and *Chlorella* sp. The sequences of small subunit rRNA (partial 18S rRNA) and ITS1-5.8S-ITS2 regions of the above-mentioned indigenous microalgal isolates were submitted to GenBank (Accession nos. MN508368.1; MN450293.1; MN472749; MN472748.1; MN508367, and MN450294) ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). The taxonomic lineage in the hierarchical arrangement, i.e., Superkingdom, Kingdom, Phylum, Class, Order, Family, Genus of the indigenous microalgal isolates were retrieved from NCBI Taxonomy.

### Preparation of VWE and its Characterization

Vegetable wastes were collected from the kitchen of a local hostel at Coimbatore, Tamil Nadu, India. Briefly, onion peels, brinjal waste, garlic peels, and pumpkin wastes were dried in oven at 60 °C; ground; mixed in the ratio of 2:1:0.1:1, respectively (20 g powdered waste in 200 mL of distilled water); autoclaved twice (at 121 °C for 20 min at 15 psi); filtered, and this aqueous extract (VWE) was used for the waste supplementation studies. The total dissolved solids (TDS) present in VWE were calculated by gravimetric method. The protein concentration present in VWE (mg/mL) was estimated using bovine serum albumin standard calibration curve by Bradford reaction, at 595 nm [32]. The amount of reducing sugar present in VWE (mg/mL) was quantified using the standard glucose graph by DNSA method [33]. The spectrum of dried VWE was obtained from FT-IR spectroscopy (FT/IR-6800 type A) and the interpretation of functional groups present in the sample was performed by ID Expert software. Moreover, Ca, Mg, Na, K, Cl, and S content were estimated for ground water and VWE [34].

### Effect of VWE at Different Concentrations on Biomass Growth

The indigenous microalgal isolates, *Asterarcys* sp. SPC, *Scenedesmus* sp. KT-U, *Chlorella* sp. TWL-B, *Scenedesmus* sp. KTWL-A, and *Coelastrum* sp. T-E with the initial inoculum of 0.68 mL [containing DCW of 3 mg, 4.9 mg, 1.7 mg, 3.5 mg, and 2.5 mg, respectively] were inoculated in culture tubes containing 20 mL of control growth medium (modified BG-11 media made using ground water instead of distilled water). After 5 days of growth, VWE was added to the cultures at different VWE concentrations of 5 µL/mL, 10 µL/mL, 15 µL/mL, and 20 µL/mL growth media. The control growth media was maintained in the experiment without VWE addition which was served as control, i.e., untreated. Independent

triplicate culture tubes were maintained for each condition. To estimate the growth of microalgal biomass in control (untreated) and VWE-treated cultures, Chl a ( $\mu\text{g/mL}$ ) was estimated from the cell pellets harvested at regular intervals for up to 20 days of cultivation, based on the following equation [35].

$$\text{Chl a } (\mu\text{g/mL}) = [16.5169 \times \text{OD}_{665} - 8.0962 \times \text{OD}_{652}] \quad (1)$$

### Analysis of Biomass and TAG Accumulation

To assess the production of biomass, the pure biomass of the five indigenous microalgal isolates, *Asterarcys* sp. SPC, *Scenedesmus* sp. KT-U, *Chlorella* sp. TWL-B, *Scenedesmus* sp. KTWL-A, and *Coelastrum* sp. T-E were cultivated with the initial inoculums of DCW,  $30.5 \pm 0.5$  mg;  $34 \pm 1.0$  mg;  $17.5 \pm 1.5$  mg;  $33.5 \pm 2.5$  mg; and  $23.5 \pm 3.5$  mg, respectively in 250 mL Erlenmeyer's flasks containing 50 mL of modified BG-11 media made using distilled water (complete nutrient condition) for 25 days. Biomass was harvested in late log phase after 25 days of cultivation from the day of inoculation; washed; dried; weighed gravimetrically, and the BP was expressed as DCW (mg/L) [it is worth mentioning that BP was calculated after excluding the dry weight of respective initial inoculum sizes]. To get crude lipid extracts, wet pellets of equal biomass concentration from the above-mentioned cells of complete nutrient condition (cultivated for up to 25 days from the day of inoculation) was suspended in 1 mL of chloroform–methanol (2:1, v/v) [36]; agitated in tissue lyser (Qiagen, Germany) at a frequency of 17 per second for 10 min; centrifuged, and supernatant was collected. To assess TAG accumulation under N starvation, nutrient stress condition was created by culturing the early log phase cells of microalgae in N starved medium (modified BG-11 medium without  $\text{NaNO}_3$ ) for 15 days and total crude lipid was extracted from these cells as mentioned above. Then the crude lipid extracts were separated by TLC using developing solvent (hexane; diethyl ether; acetic acid in the ratio of 70:30:1, respectively) (v/v). TLC plate (Merck, Germany) was sprayed with 10% copper sulfate (in 8% phosphoric acid) (w/v); charred at  $150^\circ\text{C}$  for 12 min, to develop the bands of lipid moieties and reference TAG standard. To analyze relative TAG production among five isolates, intensities of each TAG band were documented; measured using Image J software, and (i) relative TAG production under complete nutrient condition and (ii) relative TAG band production under N starvation were represented in terms of relative TAG band intensity (as percentage distribution among five indigenous microalgae).

### Validation of VWE Treatment-Mixotrophic Batch Cultivation of *Scenedesmus* sp. KT-U and *Asterarcys* sp. SPC Using VWE

The biomass of indigenous microalgal isolates, *Scenedesmus* sp. KT-U and *Asterarcys* sp. SPC with the initial inoculum of DCW, 82 mg and 80 mg, respectively, were cultivated as batch cultures in 500 mL Erlenmeyer flask containing 250 mL of VWE-treated growth media (modified BG-11 medium was made using ground water instead of distilled water; adjusted to  $\text{pH } 7.0 \pm 0.2$ ; sterilized by autoclaving, and VWE at a concentration of  $10 \mu\text{L/mL}$  was added into it). Control growth media (without VWE addition) was also maintained. After 25 days of biomass growth, BP (mg/L) was estimated for control and VWE treatment (represented as Ctrl and VWE, respectively) as biomass phase. To allow lipid induction i.e., as a lipid phase condition, VWE-treated cells of biomass phase was harvested, washed, cultured in N starved media for 25 days, and these VWE-treated cells of lipid phase were represented as VWE-II. On the other hand, control cells of lipid phase were the control cells (without VWE) with the total incubation time was 50 days, represented as Ctrl-II. Total crude lipid was extracted from Ctrl-II and VWE-II using chloroform: methanol (2:1, v/v), and the crude LC (%) was then calculated gravimetrically [36]. Lipid yield (mg/L) of *Scenedesmus* sp. KT-U and *Asterarcys* sp. SPC at lipid phase conditions was then estimated from LC and BP [37, 38]. Further, the crude lipids were separated through TLC as mentioned above.

### Statistical Analysis

All the above experiments were performed in independent repeat experiments (duplicates/triplicates). Data were analyzed through student t test through a statistical tool (<http://vassarstats.net/>). Statistical Clustering analysis using PCA was carried out for the following six parameters evaluated for the five microalgae (i) biomass fold increase [in terms of Chl a] in VWE treatment at  $10 \mu\text{L/mL}$  concentration, relative to the control [without VWE] (ii) biomass fold increase in VWE treatment at  $15 \mu\text{L/mL}$  concentration, relative to the control (iii) biomass fold increase in VWE treatment at  $20 \mu\text{L/mL}$  concentration, relative to the control (iv) BP (mg/L) (v) relative TAG production under complete nutrient condition (% distribution), and (vi) relative TAG production under N starvation (% distribution). PCA was carried out by applying unit variance scaling to the data. Singular value decomposition with imputation was used to calculate the principal components (PC), and clustering heat maps were generated using correlation distance and average linkage using a web-based statistical tool, ClustVis [39].

## Results

### Molecular Identification and Phylogenetic Analysis of the Indigenous Microalgal Isolates

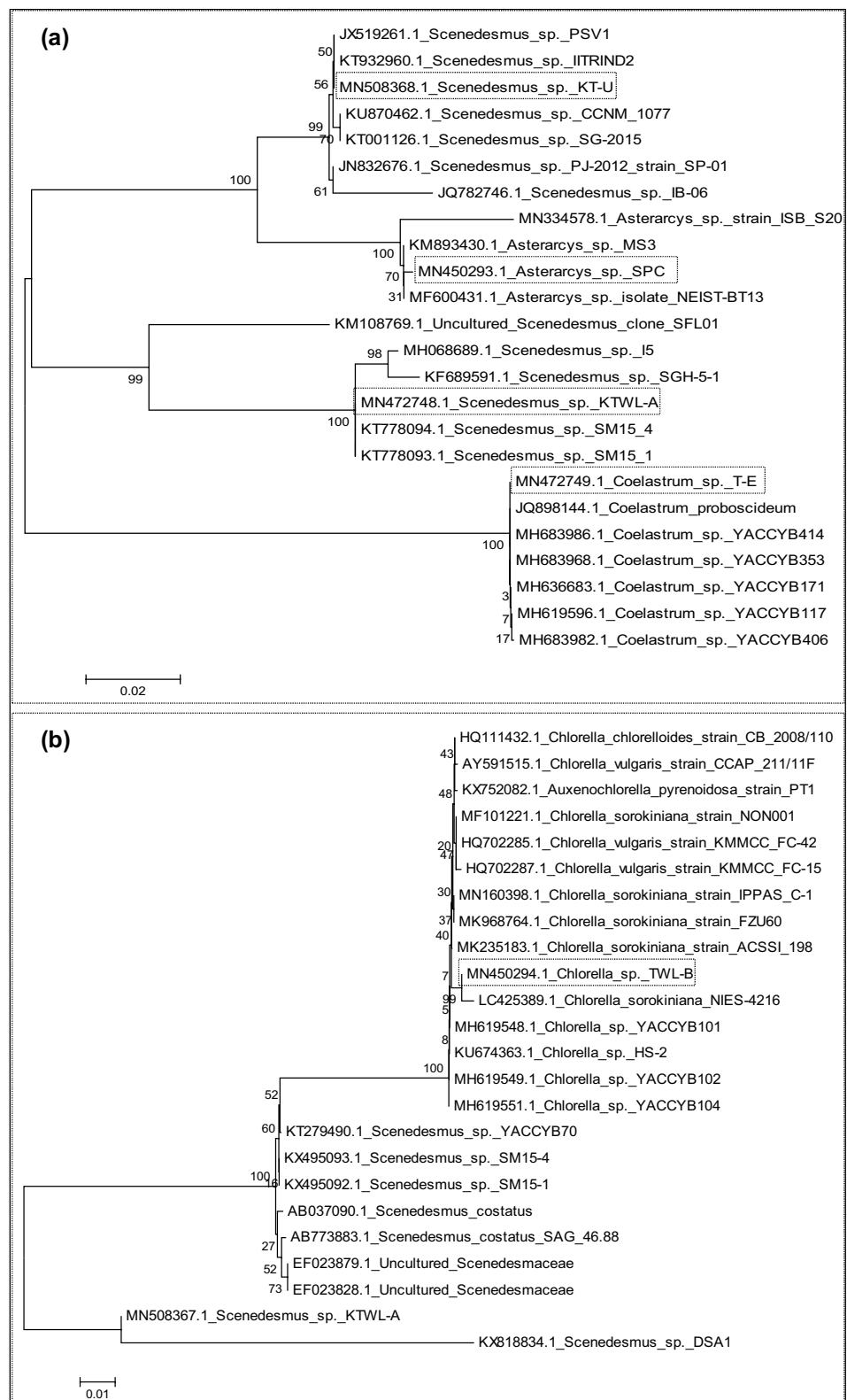
Five freshwater microalgae prevailing predominantly in the lake and wet soil of the Coimbatore regions (of the above-mentioned geographical locations), Tamil Nadu, India were isolated based on their pure colonial existence. Morphological identification by microscopic analysis showed that the isolates, SPC, KT-U, T-E, and KTWL-A were solitary to cluster of few cells of coenobium, oval to ellipsoidal cell shape that resembles Scenedesmaceae-like microalgae [40], whereas, TWL-B cells were smaller, spherical, smooth, and mostly found solitary which were similar to *Chlorella* [41]. All the isolates were unicellular green eukaryotic microalgal cells devoid of spines or flagella of Phylum: Chlorophyta. Microscopic images of the pure microalgal isolates were given in the Supplementary Fig. 1. Moreover, the isolates SPC, KT-U, T-E, and KTWL-A were further confirmed by ITS1-5.8S-ITS2 gene region sequencing and identified as *Asterarcys* sp. (maximum identity 99%; similarity to *Asterarcys* sp. MS3), *Scenedesmus* sp., (maximum identity 99%; similarity to *Scenedesmus* sp. CCNM 1077); *Coelastrum* sp. (maximum identity 99%; similarity to *Coelastrum proboscideum*) and *Scenedesmus* sp. (maximum identity 99%; similarity to *Scenedesmus* sp. SM 15\_4), respectively. Whereas microalgal isolate TWL-B was identified as *Chlorella* sp. (maximum identity 99%; similarity to *Chlorella* sp. YACCYB105 based on partial 18S rRNA sequence analysis). To further confirm the identity of the indigenous isolates, phylogenetic analysis was performed with the other related microalgal species revealing definite clade (Fig. 1). The indigenous microalgal isolates, KT-U, SPC, T-E, KTWL-A, and TWL-B were submitted to GenBank with the accession numbers: MN508368.1; MN450293.1; MN472749; MN472748.1, and MN450294, respectively. Further, the taxonomic lineages of the microalgal isolates are designated in the hierarchical arrangement, i.e., Superkingdom, Kingdom, Phylum, Class, Order, Family, Genus, respectively, as Eukaryota, Viridiplantae, Chlorophyta, Chlorophyceae, Sphaeropleales, Scenedesmaceae, *Scenedesmus* (NCBI:txid2617489) for *Scenedesmus* sp. KT-U; (II) taxonomic lineage of *Coelastrum* sp. T-E is determined as Superkingdom: Eukaryota, Kingdom: Viridiplantae, Phylum: Chlorophyta, Class: Chlorophyceae, Order: Sphaeropleales, Family: Scenedesmaceae, Genus: *Coelastrum* (NCBI:txid2613536); (III) taxonomic lineage of *Chlorella* sp. TWL-B is determined as Superkingdom: Eukaryota, Kingdom: Viridiplantae, Phylum: Chlorophyta, Class: Trebouxiophyceae, Order: Chlorellales, Family:

Chlorellaceae, Genus: *Chlorella* (NCBI:txid2609506); (IV) taxonomic lineage of *Asterarcys* sp. SPC is as follows, Superkingdom: Eukaryota, Kingdom: Viridiplantae, Phylum: Chlorophyta, Class: Chlorophyceae, Order: Sphaeropleales; Family: Scenedesmaceae, Genus: *Asterarcys* (NCBI:txid2609505), and the (V) taxonomic lineage of *Scenedesmus* sp. KTWL-A is designated as Superkingdom: Eukaryota, Kingdom: Viridiplantae, Phylum: Chlorophyta, Class: Chlorophyceae, Order: Sphaeropleales, Family: Scenedesmaceae, Genus: *Scenedesmus* (NCBI:txid2613535).

### Effect of VWE at Different Concentrations on Biomass Growth

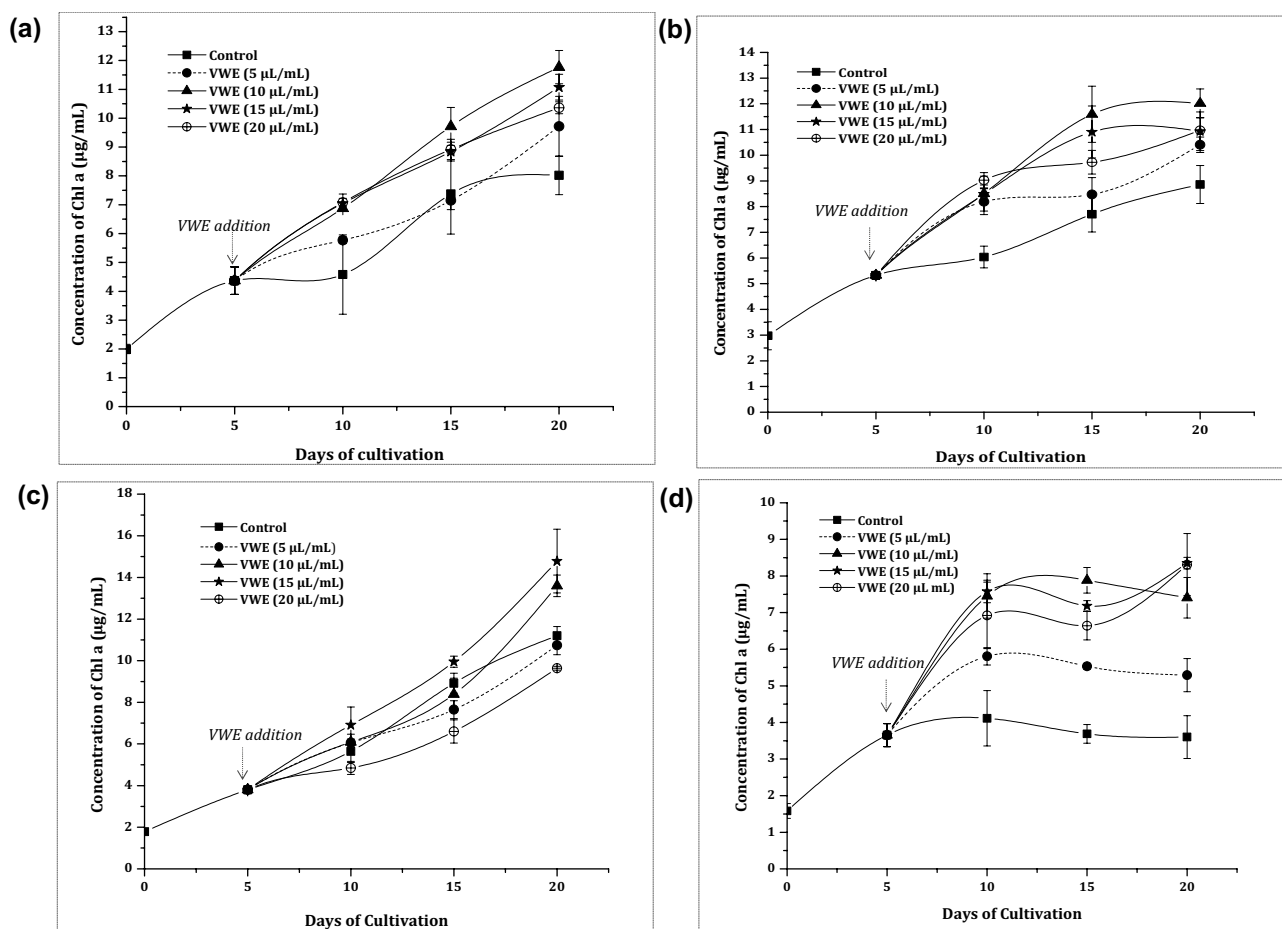
To study the influence of VWE in the production of microalgal biomass for the above-mentioned new native microalgal isolates, different concentrations of VWE (5  $\mu\text{L}/\text{mL}$ , 10  $\mu\text{L}/\text{mL}$ , 15  $\mu\text{L}/\text{mL}$ , and 20  $\mu\text{L}/\text{mL}$  of growth media) were added and the biomass growth was estimated in terms of Chl a concentration at regular interval till 20 days of cultivation (Fig. 2). The chlorophyll a is the primary chlorophyll pigment and the biomass growth of green microalgae is directly proportional to Chl a concentration [42]. Subsequently, this study revealed that the VWE supplementation at the concentrations of 10  $\mu\text{L}/\text{mL}$ , 15  $\mu\text{L}/\text{mL}$ , and 20  $\mu\text{L}/\text{mL}$  had resulted in increased biomass growth when compared to control (without VWE) and VWE supplementation at the concentration of 5  $\mu\text{L}/\text{mL}$ . However, the optimum increase in Chl a for all the five isolates was observed at either 10  $\mu\text{L}/\text{mL}$  or 15  $\mu\text{L}/\text{mL}$  concentration (Fig. 2). In case of *Scenedesmus* sp. KT-U, considerable increase in biomass concentrations ( $1.47 \pm 0.09$  and  $1.38 \pm 0.07$  folds) was observed in VWE supplementations of 10  $\mu\text{L}/\text{mL}$  and 15  $\mu\text{L}/\text{mL}$ , respectively, than the control (untreated). *Coelastrum* sp. T-E had also shown considerable increase in biomass concentration of  $1.36 \pm 0.08$  and  $1.23 \pm 0.11$  folds than control, respectively, in 10  $\mu\text{L}/\text{mL}$  and 15  $\mu\text{L}/\text{mL}$  VWE supplementation. *Chlorella* sp. TWL-B and *Asterarcys* sp. SPC also exhibited considerable increase in Chl a content with VWE supplementation. The results of this study clearly depicted that VWE provided as supplementation to the inorganic growth media encouraged the microalgal biomass growth, which was revealed through increased Chl a for most of the indigenous isolates, *Scenedesmus* sp. KT-U, *Coelastrum* sp. T-E, *Chlorella* sp. TWL-B, and *Asterarcys* sp. SPC (Fig. 2). On the other hand, *Scenedesmus* KTWL-A displayed mild increase on Chl a concentration with VWE treatment (at the VWE concentration of 15  $\mu\text{L}/\text{mL}$ ) than control (untreated). The effect of VWE at different concentrations on biomass growth (in terms of Chl a) of *Scenedesmus* sp. KTWL-A was given in supplementary Fig. 2. The physico-chemical properties of VWE and ground water used

**Fig. 1** Phylogenetic tree constructed by neighborhood joining method. **a** Sequences containing ITS1-5.8S-ITS2 region. **b** Sequences containing 18S rRNA region. Bootstrap values are indicated as percentage at the nodes of the tree (with 1000 bootstrap replications). The distances between the indigenous microalgal isolates to the other related species are being measured through the scale bar



for media preparation are given in Table 1. It is noteworthy to mention that ground water with modified BG-11 nutrients was used as the control growth media in this study to substantiate the suitability of these native strains for large scale

cultivation. The physiochemical analysis of ground water showed considerable quantities of elements such as Ca, Mg, Na, K, Cl, and S (Table 1). In terms of nutrient aspects of VWE, the extract used had enriched organic C and organic



**Fig. 2** Effect of VWE treatment at various concentrations on the biomass growth of indigenous microalgal isolates in terms of Chl a concentration ( $\mu\text{g/mL}$ ) at regular intervals, **a** *Scenedesmus* sp. KT-U, **b** *Coelastrum* sp. T-E, **c** *Chlorella* sp. TWL-B, **d** *Asterarcys* sp. SPC.

**Table 1** Physicochemical properties of VWE and ground water

Parameters	VWE	Ground water
pH	$4.6 \pm 0.2$	$7.45 \pm 0.02$
TDS (mg/mL)	$44.7 \pm 0.7$	<1
Reducing sugar content (mg/mL)	$19.6 \pm 0.9$	nd
Protein content (mg/mL)	$8.5 \pm 1.6$	nd
Ca (mEq/L)	nd (not detected)	0.21
Mg (mEq/L)	80	0.27
Na (mEq/L)	nd	0.09
K (mEq/L)	nd	0.01
Cl (mEq/L)	400	0.4
S (mEq/mL)	5.416	0.0056

N content substantiated by the presence of high reducing sugar ( $19.6 \pm 0.9$  mg/mL) and protein ( $8.5 \pm 1.6$  mg/mL) content, respectively, with the noticeable levels of nutrients such as Cl, Mg, and S (Table 1). The functional group

Control represents growth media without VWE. i.e., untreated. Data represent the mean of independent triplicate experiments and the error bar denotes the standard error

analysis of VWE by FT-IR spectrum substantiated the presence of C-C, C=O, -OH, C-O, C-H, -NH<sub>3</sub>, -NH<sub>2</sub> absorption peaks corresponding to sugars and proteins; in addition to the absorption peaks corresponding to the functional groups, C-Cl, P, and S were also detected (Supplementary Fig. 3A). Further, nutrient utilization from VWE by the microalgae was substantiated from the peak shifts and peak intensity variations from the FT-IR spectra of media containing fresh/unused VWE, and used VWE (Supplementary Fig. 3B). The nutrient-rich aspect of VWE had increased the biomass growth of microalgae evident through increase in Chl a concentration with VWE treatment than control (untreated) (Fig. 2).

### Clustering Analysis of Parameters Influencing Biodiesel Production Employing Vegetable Waste

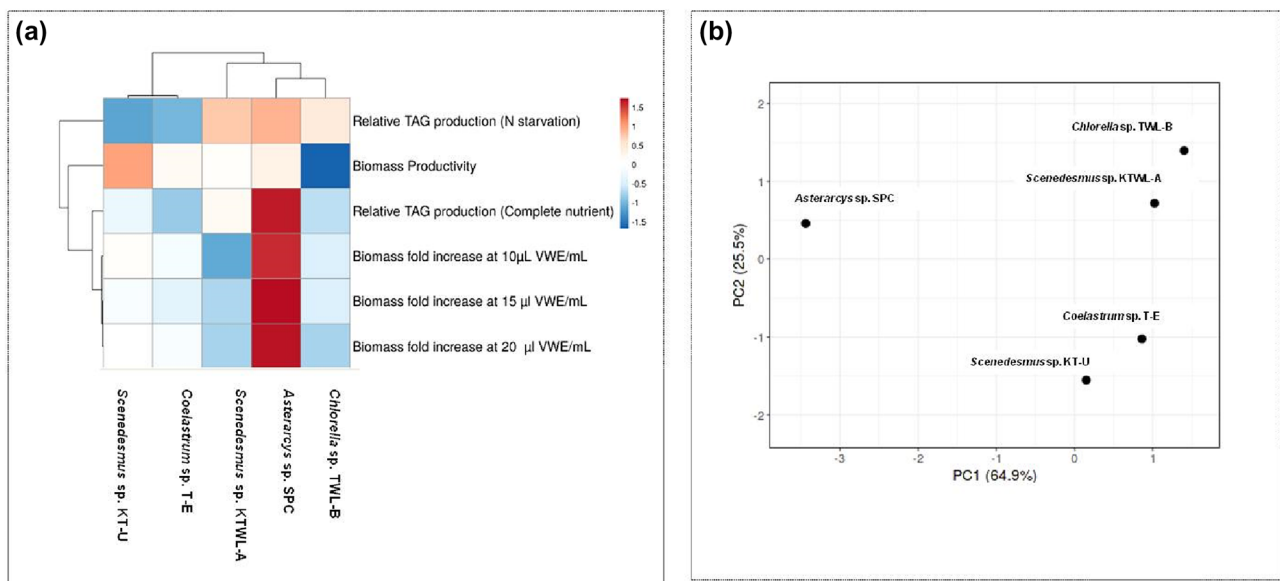
To identify suitable isolates for biodiesel production in the perspective of vegetable waste usage ability, critical

parameters of five indigenous microalgae such as biomass growth in VWE, biomass, and TAG accumulation were determined. The results revealed maximum BP of  $734.4 \pm 53$  mg/L in *Scenedesmus* sp. KT-U under complete nutrient culture conditions employed, among five indigenous microalgal isolates (Supplementary Table 1). The relative TAG level for mentioned five microalgae under N starvation showed higher TAG in *Asterarcys* sp. SPC, *Scenedesmus* sp. KTWL-A, and *Chlorella* sp. TWL-B (Supplementary Fig. 4) (Supplementary Table 1). Further, PCA performed for the above-mentioned parameters for five indigenous microalgal substantiated the influence of the most variances, PC1 and PC2 at 64.9% and 25.5%, respectively (Fig. 3b). Clustering heat maps generated through the analysis clearly depicted that the indigenous microalgae, *Scenedesmus* sp. KTWL-A and *Chlorella* sp. TWL-B exhibited less positive influence in biomass growth with VWE treatment, when compared to other isolates (Fig. 3a). At the same time their relative TAG accumulation under N starvation is higher among other strains, next to *Asterarcys* sp. SPC (Supplementary Table 1). In this study of TAG accumulation under N starvation (for 15 days), *Scenedesmus* sp. KT-U exhibited less TAG production among all the isolates; however, prolonged N starvation for 25 days caused higher TAG accumulation in *Scenedesmus* sp. KT-U (Supplementary Fig. 5). Pertaining to biodiesel production ability, all the five indigenous microalgae are potent candidates that can

be appropriately cultivated through suitable cultivation strategies. At the same time, it is important to observe that the more positive influence was obtained for *Asterarcys* sp. SPC in terms of all the parameters considered, especially based on its growth in bio-waste i.e., in VWE supplementation. Also, *Scenedesmus* sp. KT-U had shown positive influence by better growth response with VWE treatment (Fig. 3). Hence the indigenous microalgal isolates, *Asterarcys* sp. SPC and *Scenedesmus* sp. KT-U were used for further evaluation of biomass, lipid, and TAG production in mixotrophic batch cultivation in two phases, viz. Biomass phase and Lipid phase.

### Biomass, Lipid, and TAG Production Under Mixotrophic Batch Cultivation Using VWE

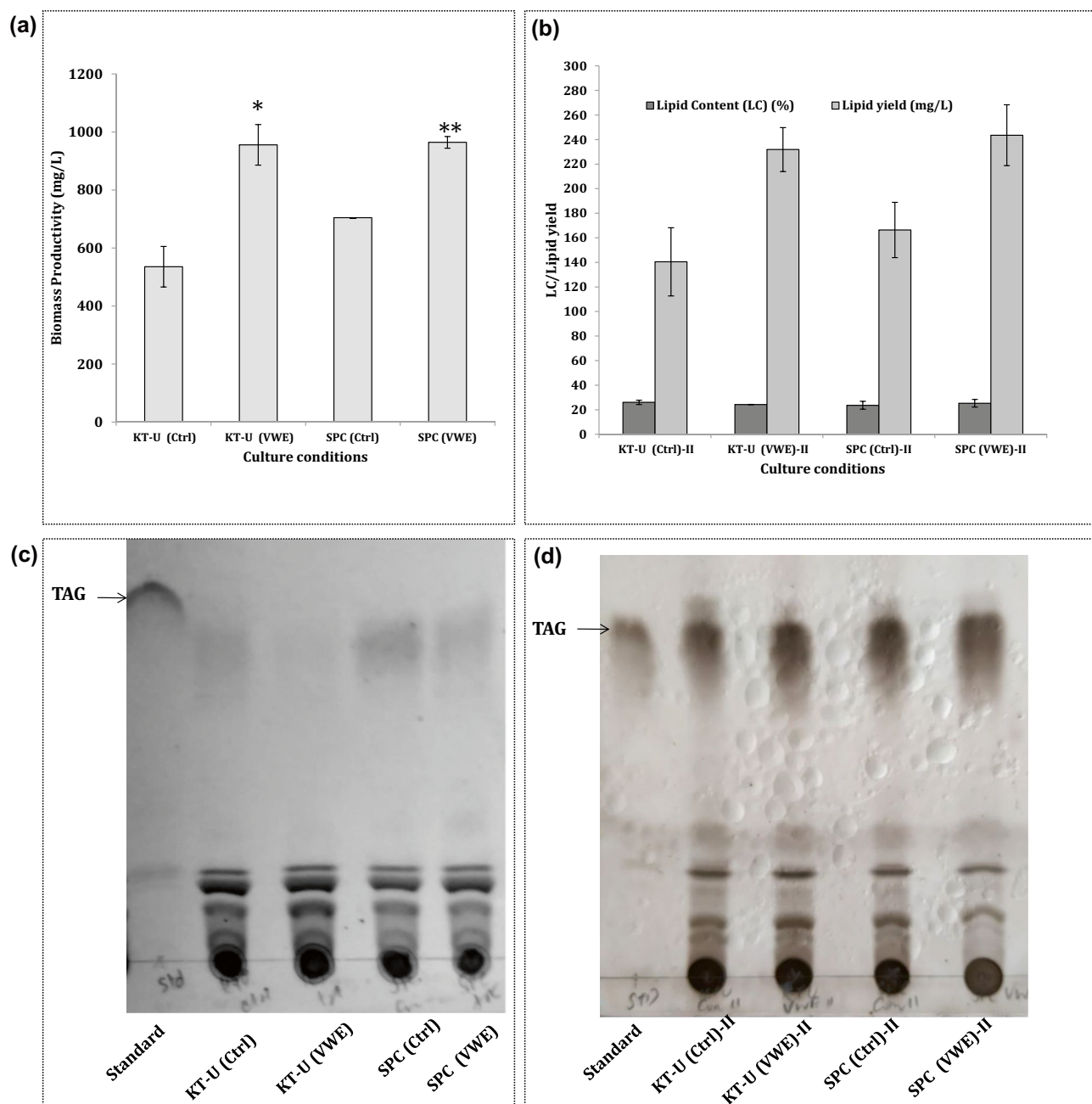
Biomass yield of *Scenedesmus* sp. KT-U and *Asterarcys* sp. SPC was validated in batch culture of 25 days of cultivation in control (untreated) and VWE-treated media conditions. The results showed significant increase in BP ( $956 \pm 70$  mg/L &  $964 \pm 70$  mg/L) for both *Scenedesmus* sp. KT-U and *Asterarcys* sp. SPC, respectively, in the VWE-treated growth media [at the standardized VWE concentration of  $10 \mu\text{L/mL}$  growth media] than in control (untreated) ( $535 \pm 70$  mg/L and  $704 \pm 0.4$  mg/L, respectively) (Fig. 4a). It is clear that the less TAG accumulation occurred in biomass phase during VWE treatment (Fig. 4c), since the nutrients in VWE are assimilated for biomass growth evident



**Fig. 3** PCA for five indigenous microalgal isolates for the following six parameters. (i) relative increase in biomass (in folds) upon VWE addition (at  $10 \mu\text{L/mL}$ ); (ii) relative increase in biomass upon VWE addition (at  $15 \mu\text{L/mL}$ ); (iii) relative increase in biomass upon VWE addition (at  $20 \mu\text{L/mL}$ ); (iv) biomass productivity (mg/L) under com-

plete nutrient condition; (v) relative TAG production under complete nutrient condition (as % distribution), and (vi) relative TAG production under N starvation (as % distribution) (Culture conditions are detailed in “Materials and Methods” section). **a** Clustering heat map, **b** PC 1 and PC2 are the principal components





**Fig. 4** Validation of VWE treatment: **a** Biomass productivity (mg/L) of *Scenedesmus* sp. KT-U, *Asterarcys* sp. SPC under control (untreated) [represented as *KT-U (Ctrl)* & *SPC (Ctrl)*, respectively] and VWE-treated conditions [represented as *KT-U (VWE)* and *SPC (VWE)*, respectively]. **b** Total crude LC (%) and total lipid yield (mg/L) of *Scenedesmus* sp. KT-U, *Asterarcys* sp. SPC during lipid phase under control [represented as *KT-U (Ctrl)-II* & *SPC (Ctrl)-II*,

respectively] and in VWE-treated cells subjected to N starvation for 25 days [represented as *KT-U (VWE)-II* & *SPC (VWE)-II*, respectively]. [Culture conditions employed are detailed in “Materials and Methods” section]. Data represent the mean of independent duplicate experiments and the error bars denotes the standard error (\* $P < 0.05$ ; \*\* $P < 0.01$ ). **c** Separation of lipids by TLC in BP phase. **d** Separation of lipids by TLC in lipid phase

through significant increase in Chl a concentration (supplementary Table 2), and increased biomass yield (Fig. 4a). Higher Chl a content in green microalgae is negatively correlated to storage lipid accumulation level, on the other hand, N depletion nutrient status allow for storage lipid

accumulation with decreased Chl a [43], since the nitrogen-rich chlorophyll compounds could serve as intracellular nitrogen pool to facilitate microalgal cellular growth and enhance biomass [44]. Moreover, nitrogen starved microalgae can serve as an appropriate biodiesel feedstock due

to substantial amounts of TAG possessing optimum fuel properties [45]. Subsequently, as a lipid phase, VWE-treated microalgae of biomass phase was subjected to N starvation and analyzed for lipid production after 25 days in this study. It is remarkable to mention that the control cells at that time had completed 50 days of cultivation period (represented as Ctrl-II, control of lipid phase). The results showed noticeable increase in lipid yield in VWE-treated cells of lipid phase ( $231.8 \pm 17.9$  and  $243.5 \pm 25$  mg/L) in *Scenedesmus* sp. KT-U and *Asterarcys* sp. SPC, respectively, than in control ( $140.5 \pm 28$  mg/L and  $166.4 \pm 23$  mg/L) cells of lipid phase (Fig. 4b). This increase corresponds to average increase of lipid production of 1.65 and 1.46-folds, respectively, with more or less equivalent lipid content (%) and relative TAG accumulation level in lipid phase for both control and N starved-VWE-treated microalgae. It is because the control cells at that time of harvest had entered lipid phase, due to 50 days of growth. This is also evident with the increase in TAG band intensity in the control with prolonged culturing time of 50 days [Fig. 4d represented as KT-U (Ctrl) –II and SPC (Ctrl)-II] than in control of biomass phase with culturing time of only 25 days [Fig. 4c represented as KT-U (Ctrl) & SPC (Ctrl)]. This study shows indigenous microalgal cultivation in two phases with vegetable waste usage for the biomass phase led to benefit of significant enhancement in BP and concomitant lipid yield in lipid phase.

## Discussion

Technoeconomic studies have clearly shown that algal bio-fuel production through the inclusion of nutrients from the available waste resources along with multiple microalgal production technologies are needed for gaining the economic feasibility of microalgal production [46]. Assessment of microalgal biomass growth improvement via vegetable waste supplementation is one of the most reliable approach of cheaper cultivation mode. Subsequently, in this current study, evaluation of biomass growth of new five indigenous microalgal isolates performed under VWE treatment showed improved biomass growth. Likewise, food waste hydrolysate as growth medium in heterotrophic microalgae, *Schizochytrium mangrovei* and *Chlorella pyrenoidosa* showed increased production of biomass than conventional medium, and the produced biomass was rich in carbohydrates, lipids, proteins, saturated and polyunsaturated fatty acids [23]. In yet another study, usage of rice husk solution at 6 mL/L concentration in growth medium produced enhanced microalgal biomass in case of *Phaeodactylum tricornerutum* with higher Chl c content ( $8.48 \pm 0.13$  µg/mL) than control ( $6.25 \pm 0.99$  µg/mL) [24]. In another study, vegetable waste growth media for *Chlorella vulgaris* (CPCC 90) showed positive effect in increasing lipid and carbohydrate content,

though decreased biomass was obtained than control [11]. It was also reported that the lesser production of biomass of *Chlorella vulgaris* (CPCC 90) in the vegetable waste media than control inorganic base medium was probably due to the lesser nutrient assimilation efficiency of complex organic molecules by microalgae than inorganic carbon and nitrogen source [11]. This suggests the proper and efficient bio-waste pretreatment so as to derive less complex nutrient molecules for facilitating better nutrient assimilation by the microalgal cells. Hence in this study VWE was prepared through thermal hydrolysis by autoclaving twice for better nutrient recovery. The better nutrient assimilation ability from VWE by the microalgae was substantiated by FT-IR and physiochemical analyses in this study. Moreover, based on the statistical clustering analysis of critical parameters such as (i) growth in VWE, (ii) biomass productivity, and (iii) TAG accumulation of five new indigenous microalgal isolates, *Asterarcys* sp. SPC and *Scenedesmus* sp. KT-U were selected. This paved the way for strain selection employing vegetable waste in this current study. These selected microalgal isolates were cultivated under mixotrophic batch cultivation employing VWE at the standardized concentration. Increased lipid yield was obtained from VWE-treated cells of lipid phase ( $231.8 \pm 17.9$  mg/L and  $243.5 \pm 25$  mg/L) in *Scenedesmus* sp. KT-U and *Asterarcys* sp. SPC, respectively, than in control ( $140.5 \pm 28$  mg/L and  $166.4 \pm 23$  mg/L). Likewise, in a study on cultivation in food wastewater effluent in *Scenedesmus bijuga* had improved lipid production in the range of 13.81–15.59 mg/L/day which was higher than in synthetic medium [47]. It was reported that, anaerobically digested effluent from kitchen waste (ADE-KW) for the cultivation of *Chlorella sorokiniana* SDEC-18 and *Scenedesmus* SDEC-8 showed optimal BPs of 0.42 g/L and 0.55 g/L, respectively, with 1/15 diluted ADE-KW [27]. It was reported that in microalgae, *Chlorella vulgaris*, in addition to increased lipid content, higher carbohydrate content was obtained in diluted vegetable waste medium than control [11]. Increase in lipid production was obtained in *Chlorella* SDEC-11 and *Scenedesmus* SDEC-13 in ADW-KW added with brassinolide [21]. It is important that the nutrient-rich biowastes such as agricultural wastes, food wastes, vegetable wastes, and fruit wastes are to be exploited effectively for the microalgal cultivation that can pave the way for feasible improved production with proven efficient nutrient recovery [28, 48, 49].

## Conclusion

New indigenous microalgae identified as *Asterarcys* sp. SPC, *Scenedesmus* sp. KT-U, *Scenedesmus* sp. KT-WL-A, *Coccolobastrum* sp. T-E, and *Chlorella* sp. TWL-B had showcased improved biomass growth in VWE-treated media with at

least one of the VWE concentration. Mixotrophic cultivation of the selected microalgae, *Scenedesmus* sp. KT-U and *Asterarcys* sp. SPC cultivated using VWE treatment in the optimum concentration of 10 µL/mL growth medium during biomass phase had produced increased biomass (1.8 and 1.4 folds, respectively) than control (medium without VWE). Enhanced lipid yield of  $231.8 \pm 17.9$  mg/L and  $243.5 \pm 25$  mg/L in *Scenedesmus* sp. KT-U and *Asterarcys* sp. SPC, respectively, were obtained with considerable TAG accumulation. Thus, this study employed new indigenous microalgae which imparted vegetable waste in the strain assortment and cultivation process for enhanced lipid yield for biodiesel application.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s00284-021-02643-1>.

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**Author Contributions** Conceptualization, RK; Investigation, RK, NA, and RG; Methodology and data analysis, RK and NA; Writing of the original draft, RK; Reviewing, Editing & Supervision, RK and RG. All authors have read and approved the published version of manuscript.

## Declarations

**Conflict of Interest** Authors declare no conflict of interest.

**Ethical Approval** This article does not contain any studies with human participants or animals performed by any of the authors.

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