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Mixotrophic growth regime of novel strain *Scenedesmus* sp. DDVG I in municipal wastewater for concomitant bioremediation and valorization of biomass

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ABSTRACT

Availability of cost-effective nutrients for the commercial cultivation of microalgae is one of the major challenges. The present study investigated the feasibility of primary municipal wastewater (PMWW) collected from a local wastewater treatment plant in Minnesota, USA as an alternative to fresh-water microalgae growth media towards high-value bioenergy feedstock production. The novel strain *Scenedesmus* sp. DDVG I was cultivated in the PMWW under heterotrophic and mixotrophic modes in 250 mL Erlenmeyer flasks. The optimized cultivation mode was further scaled up to the 3-L bubbled bioreactor. The study confirmed the wastewater as a potential growth medium while *Scenedesmus* sp. DDVG I showed superior biomass productivity (0.069 g/L d), lipid yield (22.5%), and FAME content (22.04% in dry cell weight) over 10 days in the mixotrophic mode. The biomass also showed high essential amino acid content (159.8 mg/g DCW). The corresponding values for bioremediation efficiencies of chemical oxygen demand (COD) and total nitrogen (TN) by *Scenedesmus* sp. were 75.6% and 99.8% respectively. Meanwhile, ammonia nitrogen (NH_3–N) and total phosphorus (TP) were removed at up to 100% removal efficiencies. Analyses of fatty acid profile and different parameters that were required for biodiesel characterization revealed the high-quality nature of the *Scenedesmus*-derived oil. Overall, the study concluded effective bioremediation of municipal wastewater by *Scenedesmus* sp. DDVG I with the recovery of valuable resources from the biomass.

1. Introduction

Scarcity of freshwater and eutrophication become major threats due to excessive water requirements and increasing water pollution in the environment (Greenway, 2005). The effluent generated from anthropogenic activities (including cooking, washing, manufacturing, etc.) of residents, hospitals or institutions, etc., is considered municipal wastewater (Daverey et al., 2019). This wastewater had high concentrations of organic matter, phosphorus, nitrogen, etc., leading to the death of desirable flora and fauna (Chan et al., 2014). As per the US Environmental Protection Agency (EPA), the permissible limit for phosphorus (P) concertation in discharged effluents was 1 mg/L (US EPA, 2016). Conventional wastewater treatment processes such as trickling filters (Howell and Atkinson, 1976) and activated sludge (Sonune and Ghate, 2004) are not efficient enough and energy-intensive (Arbib et al., 2014). Hence, it is a major challenge to find out an effective and sustainable treatment method for the removal of excessive nutrients from municipal influents before water is recycled.

Moreover, increased population growth and industrialization resulted in global warming, depletion of fossil fuel reserves, and discharge of excessive pollutants into water bodies. The global energy demand would increase by 60% in 2030 (Ahmad et al., 2011). Hence, there is a dire need to find alternative renewable resources to preserve the environmental and energy security of the nation. Biodiesel is one of the alternative renewables to fossil fuels. Studies have been focused on finding a potential feedstock for biodiesel production. Microalgae emerged as a promising candidate owing to the fast growth rate, high oil content, great photosynthetic efficiency, mitigation of carbon dioxide (CO_2), and

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adaptation to extreme environments (Brennan and Owende, 2010; Chisti, 2007). The biomass production and oil content of microalgae depend on the accessibility of nutrients in the culture broth and the modes of cultivation. Cultivation of microalgae in heterotrophic and mixotrophic modes using organic and inorganic carbon sources could result in higher biomass and lipid content (Mitra et al., 2012). However, the cost of the organic substrate was expensive. Bhatnagar et al. (2011) verified the feasibility of utilizing wastewater as a substrate for microalgal growth in a mixotrophic mode. This suggested that the availability of nutrients in municipal wastewater could be an affordable substitute for the synthetic nutrient medium of microalgae. In addition, microalgae are rich in essential amino acids, pigments, and long-chain fatty acids which are indispensable for animal or aquaculture nutrition. A handful of studies had been done on microalgae-based wastewater treatment processes for the removal of nutrients from varieties of wastewater (Mishra and Mohanty, 2019; Wang et al., 2010; Azam et al., 2020; Malibari et al., 2018; Tsolcha et al., 2016; Pereira et al., 2019). However, microalgal-based wastewater treatment is still in its exploratory phase, is not extensively employed, and limited research has been conducted on original municipal wastewater without sterilization. Besides, to the best of our knowledge, there is no report of investigation on novel oleaginous *Scenedesmus* sp. DDVG I for sustainable wastewater remediation. This study was to explore the possibility of cultivating a new strain, *Scenedesmus* sp. DDVG I in original PMWW, its efficiency of nutrients removal from the wastewater. Moreover, the study might enrich the research on algal cultivation, effective waste management, and biomass generation, so establishing clean energy and the creation of high-nutritional feed.

This study aimed to evaluate the cultivation of novel *Scenedesmus* sp. DDVG I under heterotrophic and mixotrophic modes in municipal wastewater. The wastewater, which was derived from a local wastewater treatment plant (WWTP) in St. Paul, Minnesota, USA, was rich in phosphorus, nitrogen, and ammonium, making it a potential substrate for algal growth. Further, the condition that gave the higher biomass concentration was scaled up to a 3-L bubbled photobioreactor to achieve higher biomass productivity for evaluation of fatty acid methyl ester (FAME) profile, and biodiesel characteristics. The amino acid profile of the biomass was also analyzed for its animal feed application.

2. Materials and methods

2.1. Source of wastewater

Primary municipal wastewater (PMWW) used in this study was collected from a local Wastewater Treatment Plant (WWTP) in Saint Paul, Minnesota, USA. The visible solid particles were removed by sedimentation and filtration. The filtered wastewater was collected in a sterilized container and put under UV irradiation for about 45 min for disinfection. The characteristic of PMWW, including total solids (TS), total volatile suspended solids (TVSS), chemical oxygen demand (COD), pH, ammonia–nitrogen (NH_3–N), total nitrogen (TN), and total phosphorus (TP) were determined by following the standard methods of APHA (1995).

2.2. Microorganisms

A microalga strain, *Scenedesmus* sp. DDVG I (accession No. MN630585) was cultivated in a modified BG 11 medium (pH = 7) before transferred to the PMWW (Devi et al., 2021). The BG 11 medium was composed of (g/L): $NaNO_3$, 1.5; $MgSO_4.7H_2O$, 0.075; K_2HPO_4, 0.04; $CaCl_2$, 0.036; citric acid, 0.006; ammonium ferric citrate, 0.006; Na_2EDTA, 0.001; Na_2CO_3, 0.02; H_3BO_3, 2.86; Co $(NO_3)_2.6H_2O$, 0.05; $CuSO_4.5H_2O$, 0.08; $MnCl_2.4H_2O$, 1.81; $ZnSO_4.7H_2O$, 0.22. The inoculum was incubated at 25 °C in an orbital shaker at 150 rpm under a light intensity of 40.5 μmol m^{-2} s^{-1}.

2.3. Experimental setup for flask cultivation

To investigate the growth rate and biomass productivity, *Scenedesmus* sp. DDVG I was cultivated in mixotrophic and heterotrophic modes for ten days using PMWW. The experiment was performed in a 250 mL Erlenmeyer flask with 100 mL wastewater in different batches. An inoculum of 0.3 g/L was cultured in each 250 mL flask in both conditions. In the mixotrophic condition, the cultures were illuminated using white fluorescent lamps with an intensity of 40.5 μmol m^{-2} s^{-1} with 12 h:12 h (light: dark). While in heterotrophic mode, all the flasks were wrapped with aluminium foil to imitate the dark condition. A batch of blank containing wastewater medium but without the microalgal culture was set up simultaneously to observe any other microbial growth besides microalgae. All the studies were done in triplicate and were incubated at 27 ± 1 °C in an orbital shaker (150 rpm) for 10 days. Routine analyses including growth study and chlorophyll content were determined during the culturing to examine the favorable cultivation mode. At the end of the 10-day, the biomass was collected through vacuum filtration and dried. The favorable cultivation regime was selected and scaled up for enhanced biomass accumulation and biochemical analysis.

2.4. Bioreactor study

The scale-up cultivation was conducted mixotrophically with a 3 L bioreactor (BR) (Biostat A plus, Sartorius, USA), with a 2 L working volume as illustrated in Fig. S1(a) of Electronic Supplementary Material (ESM). The base plate of the reactor was connected with a sparger to provide bubble aeration. The aeration was achieved by compressed air from an air pump (Tetra Whisper AP 150, 2.5 W) through the sparger. The bubbling air-flow rate was controlled by a flow meter (Cole Parmer, USA) at the rate of 80 mL/min. The white fluorescent lamps were set up on different sides of the reactor for the distribution of light evenly. The light intensity provided was 40.5 μmol m^{-2} s^{-1} at 12 h:12 h (light: dark) photoperiod. The bioreactor was connected to a pH probe and a temperature probe for monitoring pH and temperature regularly. A sample collecting port was installed at the top of the reactor, and 5 ml of culture was taken every second day to analyze the growth and removal of nutrients (COD, TP, TN, and NH_3–N). At the end of the 10-day, the biomass was collected through vacuum filtration and dried. Dried biomass was later used for biochemical analysis.

2.5. Analytical method

2.5.1. Growth determination

The culture growth was measured in terms of the total solids (TS) and total volatile solids (TVS), which represent biomass concentration and were determined according to the standard methods (APHA, 1995). The biomass concentration for the growth profile of the microalgae was determined as the total biomass minus the biomass in wastewater that was not inoculated with algae (or blank condition). Specific growth rate (μ, d^{-1}) and biomass productivity (BP, mg/L· d) were estimated according to the following equations [1] and [2] (Ye et al., 2018) respectively.

$$\mu = \left[\ln(X_f) - \ln(X_i)\right] / \Delta t \quad [1]$$

$$BP = (X_f - X_i) / \Delta t \quad [2]$$

where X_f and X_i were biomass concentrations at the period 'Δt' (d) during the exponential phase.

For water quality analysis, the cultures were collected at regular intervals and subsequently centrifuged at 6000 rpm for 10 min. The supernatant was filtered through a 0.45 μm membrane and the filtrate was used for the determination of COD, TP, TN, and NH_3–N. The nutrient removal efficiency (%) was calculated with the following

formula [3] where C_i and C_f are the initial and the final concentration of pollutants.

$$Nutrient\ removal\ efficiency = \left[\left(C_i - C_f\right) / C_i\right] 100 \quad [3]$$

2.5.2. Chlorophyll content

Chlorophyll-a (Chl-a) was determined according to the following method and equation [4] (Pruvost et al., 2011). Briefly, one mL of microalgal culture was centrifuged at 10,000 rpm for 10 min. The cell pellet was lysed with 1.5 mL of methanol by incubating in a water bath at 45 °C for 30 min. Then the sample was centrifuged at 10,000 rpm for 10 min, and the absorbances at 652 nm and 665 nm were measured using Hach DR 5000 Spectrophotometer (Hach, 2008).

$$Chlorophyll - a = 16.5169A_{665} - 8.0962A_{652} \quad [4]$$

2.5.3. Amino acids profile

The amino acid content of the biomass was determined based on the following protocol (Barnharst et al., 2021). Fifty mg of each dried and ground sample was hydrolyzed with 1.0 mL of 6.0 M HCl in a 2 mL sealed centrifuge tube at 110 °C for 24 h. Each tube was purged with pure nitrogen before hydrolysis to avoid the oxidation of certain amino acids (cysteine and methionine). The derivatization of amino acids in each sample and standards were done by 9-fluorenyl-methyl chloroformate (FMOC) and ortho-phthalaldehyde (OPA) with the autosampler (G1329A, Agilent Technologies) before injection (Sun et al., 2021). Further, the amino acids were quantified using HPLC-DAD (1200 Infinity Series, Agilent Technologies) equipped with a C18 column (4.6 × 250 mm, 3.5 μm) (Agilent Technologies). For detection, two mobile phases were used, A: 10 mM Na_2HPO_4, 10 mM $Na_2B_4O_7$, 5 mM NaN_3 with pH 8.2, and B (by volume): acetonitrile, methanol, ultra-pure water in the ratio of 4.5: 4.5: 1. The flow rate was 1.5 mL/min. The injection volume of the sample was 40 μL and the amino acids in each sample were separated within a 40 min retention time at a column temperature of 40 °C. The essential amino acid ratio (EAAR) of the sample was calculated with the following formula [5]. Based on the EAAR, the protein quality was evaluated.

$$EAAR = EAA / TAA \quad [5]$$

where *EAA* is the essential amino acids and *TAA* is the total amino acids.

2.5.4. Lipid estimation and transesterification of fatty acid

The total lipids were extracted using a modified Bligh and Dyer method (Bligh and Dyer, 1959). The extracted lipid was transesterified to obtain FAME with the following method (Morrison and Smith, 1964). Briefly, 14–16 mg of extracted lipid was mixed with a 1 mL solution of BF_3–methanol (10% v/v, Sigma Aldrich). The sample was incubated at 90 °C for 30 min in a water bath. After incubation, FAME was extracted by adding 2 mL of hexane and 2 mL of water, the upper phase was recovered and the esters were evaporated to dryness under a nitrogen stream. Finally, hexane (chromatography grade) was added to obtain a final volume of 1 mL. The FAME compositions were analyzed by the GC-FID (PerkinElmer Clarus 590). The GC-FID was equipped with a BPX-70 column (film thickness 0.25 μm, and 50 m × 0.22 mm i.d.) and hydrogen was used as the carrier gas at a flow rate of 2 mL/min on split mode. The initial column temperature was set as 100 °C for zero min and gradually increased to 240 °C at 3 °C/min rate 10 min. The injector and FID temperatures were set at 250 °C. The FAME compositions of microalgal lipid were compared with the FAME standard (Supelco 37 component FAME mix) and literatures.

2.5.5. Evaluation of biodiesel quality

The biodiesel quality can be calculated from the parameters of the biodiesel with empirical equations [6] to [18] (Bagul et al., 2017; Sergeeva et al., 2017). All the parameters were calculated based on the composition and content of individual fatty acids in the biomass of the microalgae.

The degree of unsaturation (DU) was calculated with the following equation [6]:

$$DU = \sum_{i=0}^{n} MUFA_i + \sum_{i=0}^{n} PUFA_i \quad [6]$$

where $MUFA_i$ and $PUFA_i$ are contents of the individual monounsaturated fatty acid and polyunsaturated in the sum of total fatty acids, respectively.

The saponification value (SV, mg KOH/g oil) was calculated by using the following equation [7]:

$$SV = \sum_{i=0}^{n} (560\ w_i)/M_i \quad [7]$$

where, w_i is the content of the individual fatty acid in the sum of total fatty acids, and M_i is the molecular weight of the fatty acid (or its methyl ester).

The iodine value (IV, g I_2/100 g) was calculated with the equation [8]:

$$IV = \sum_{i=0}^{n} (254\ N\ w_i)/M_i \quad [8]$$

where N is the number of double bonds in the fatty acid molecule.

The cetane number (CN) was estimated with the equation [9]:

$$CN = \sum_{i=0}^{n} w_i\ \varphi_i \quad [9]$$

where φ_i is the cetane number of the individual FAME which can be calculated with the following formula:

$$\varphi_i = -7.8 + 0.302\ M_i - 20\ N$$

The long-chain saturated factors (LCSF) can be determined with the formula [10]

$$LCSF = 0.1\ w_{C16:0} + 0.5\ w_{C18:0} + 1\ w_{C20:0} \quad [10]$$

where, $w_{C16:0}$, $w_{C18:0}$ and $w_{C20:0}$ is the weight content of C16:0, C18:0 and C20:0, respectively.

The cold flow plugging properties (CFPP, °C) was calculated with the following equation [11]:

$$CFPP = 3.1417\ LCSF - 16.477 \quad [11]$$

The cloud point (CP, °C) was determined with the following formula [12]:

$$CP = 0.526\ w_{C16:0} - 4.99 \quad [12]$$

The pour point (PP, °C) was calculated with the formula [13]:

$$PP = 0.571\ w_{C16:0} - 12.24 \quad [13]$$

The oxidative stability (OS, h) was determined with the following equation [14]:

$$OS = 117.9295/[w_{C18:2} + w_{C18:3} + 2.5905] \quad [14]$$

where, $w_{C18:2}$ and $w_{C18:3}$ is the weight content of C18:2 and C18:3, respectively.

The higher heating value (HHV, MJ/kg) was calculated with the equation [15]:

$$HHV = \sum_{i=0}^{n} w_i\ \delta_i \quad [15]$$

where δ_i is the higher heat value of the individual FAME obtained from the formula, $\delta_i = 46.19 - 1794/M_i - 0.21\ N$

The flash point (FP, °C) was calculated with the formula [16]:

$$FP = 23.36 \sum_{i=0}^{n} w_i C_i + 4.854 \sum_{i=0}^{n} w_i N \quad [16]$$

where C_i is the number of carbons of the individual FAME.

The density (ρ_i, g/cm^3) was calculated with the formula [17]:

$$\rho_i = \sum_{i=0}^{n} w_i \rho_i \quad [17]$$

where. $\rho_i = 0.8463 + 4.9/M_i + 0.0118\,N$

Kinematic viscosity (η, mm^2/s) was calculated with the following equation [18]:

$$\eta = \exp\left(\sum_{i=0}^{n} w_i \ln(\eta_i) \right) \quad [18]$$

where, $\ln(\eta_i) = -12.503 + 2.496 \ln(M_i) - 0.178\,N$

2.6. Statistical analysis

All the experiments were performed in triplicates and the mean values with standard deviations were estimated. The variance in the means of the different groups was analyzed by Welch's two-sample *t*-test and ANOVA followed by Tukey's HSD. In all the tests, 5% level ($p < 0.05$) was considered significant. All the statistical analyses were done with the R-statistical package.

3. Results and discussion

3.1. Wastewater characterization

The physicochemical parameters of PMWW and the average data of the measurement are summarized in Table 1. The PMWW has a pH of 7.0 ± 0.01 which is favorable for the cultivation of microalgae without any pH adjustments. The PMWW showed the presence of COD (484.8 ± 8.5 mg/L), NH_3–N (40.2 ± 1.1 mg/L), TN (44.9 ± 1.9 mg/L), and TP (7.9 ± 0.5). Wang et al. (2010) used the primary municipal wastewater collected from the Metropolitan WWTP for the growth of *Chlorella* sp. The study showed the presence of 40.65 ± 0.07 mg/L of TN and 5.66 ± 0.08 mg/L of TP, which is comparable to the nutrient content of the wastewater used in this study.

3.2. Mixotrophic and heterotrophic cultivation of Scenedesmus sp. DDVG I in batch culture with PMWW

The biomass concentrations and chlorophyll content attained at regular intervals under heterotrophic and mixotrophic modes are illustrated in Fig. 1a and Fig. 1b, respectively. In mixotrophic mode, the biomass concentration of *Scenedesmus* sp. DDVG I increased significantly (p = 0.001) from 0.36 ± 0.00 g/L to 1.5 ± 0.04 g/L after 2 days of inoculation. After 8 days, *Scenedesmus* sp. attained its stationary phase with a biomass concentration of 3.27 ± 0.12 g/L. At the end of the culture period (10-day), the biomass concentration achieved by *Scenedesmus* sp. DVVG I was 3.4 ± 0.13 g/L. The maximum biomass productivity and lipid content achieved by *Scenedesmus* sp. were 0.065 ± 0.01 g/L d and 21.9%, respectively (Table 2). During the mixotrophic mode, the growth of unwanted microbes (like bacteria or protozoa) in the blank condition was insignificant when examined under microscopy. The occurrence of higher Chl-a content in the mixotrophic cells further confirmed the photosynthetic activity of the microalgal cells. The Chl-a content increased from 0.62 ± 0.1 mg/L to 5.46 ± 0.7 mg/L significantly ($p < 0.001$) over two days. The highest Chl-a reached up to 10.2 ± 0.16 mg/L over the 10 days cultivation period.

In heterotrophic mode, *Scenedesmus* sp. persisted in the lag phase over 3 days. No particular exponential phase was observed over the 10 days culture duration. The maximum biomass concentration achieved by the *Scenedesmus* sp. in heterotrophic mode was 0.71 ± 0.05 g/L after 4 days. Thereafter, the growth declined slowly up to day 10 of the study. The biomass productivity and lipid yield at the end of the culture period were 0.021 g/L d and 8.7%, respectively. However, the blank condition in heterotrophic mode showed a minor population of bacterial growth. A similar observation was reported by Khan et al. (2016) stating that the heterotrophic mode favored the growth of microbes. They further stated that the heterotrophic mode provided a dark fermentation environment for bacteria thus inducing the growth. The Chl-a content in the heterotrophic mode decreased gradually from 0.44 ± 0.09 mg/L to 0.40 ± 0.09 mg/L during the first two days. The Chl-a further declined to the lowest concentration of 0.10 ± 0.04 mg/L over 10 days in heterotrophic cultivation. Furthermore, *Scenedesmus* sp. in mixotrophic mode showed a higher specific growth rate of 0.16 d^{-1} (p = 0.001) as compared to the specific growth rate (0.06 d^{-1}) of heterotrophic mode. This suggested that the heterotrophic mode of cultivation is less efficient for *Scenedesmus* sp. as compared to the mixotrophic. The result behind the slower activity of *Scenedesmus* sp. DDVG I in heterotrophic mode could be related to the non-biodegradability of organic matter for utilization by the lesser cell densities (Ruiz et al., 2014). Cid et al. (1992) reported that the uptake of organic nutrients by photosynthetic microalgae was greatly influenced by light. In a mixotrophic mode, catabolism of organic matter during photosynthesis coupled with aerobic respiration provides higher energy required for the greater cell densities (Mitra et al., 2012). A similar study was reported by Daneshvar et al. (2019) that the mixotrophically grown *Tetraselmis suecica* showed higher biomass concentration (0.58 g/L) and chlorophyll content (11.70 mg/L) as compared to heterotrophic growth with dairy wastewater. Likewise, *Chlorella vulgaris* JSC-6 could achieve higher biomass growth (3.96 g/L) under mixotrophic mode as compared to the heterotrophic growth (2.35 g/L) with swine wastewater (Wang et al., 2015). They further reported that the presence of light is a limiting parameter supporting the higher availability of organic matter to microalgal growth in a mixotrophic mode. The present study clearly suggested that heterotrophic cultivation of *Scenedesmus* sp. in PMWW is less efficient, compared to the mixotrophic mode. Thus, the further scale-up study was focused only on the mixotrophic mode to evaluate enhanced productivity with an increase in culture volume.

Table 1
Physicochemical characteristics of the PMWW. All measurements were performed in triplicate, and the results are expressed as mean values ± standard deviations (SD).

Parameter	PMWW
TS (mg/L)	245 ± 3.7
TVSS (mg/L)	23 ± 0.03
COD (mg/L)	484.8 ± 8.5
NH_3–N (mg/L)	40.2 ± 1.1
TN (mg/L)	44.9 ± 1.9
TP (mg/L)	7.9 ± 0.5
pH	7.0 ± 0.01

3.3. Mixotrophic growth of Scenedesmus sp. DDVG I in 3 L bioreactor

The scale-up cultivation of *Scenedesmus* sp. DDVG I in mixotrophic mode using PMWW to a 3 L bioreactor is illustrated in Fig. S2(a) and Fig. S1(b) of ESM. The growth in terms of biomass concentration over 10 days cultivation period is shown in Fig. 1c. The growth curve in the bioreactor study illustrated a similar trend to the mixotrophic growth curve of the flask. The initial biomass concentration (0.18 ± 0.04 g/L) increased significantly (p = 0.001) up to 1.27 ± 0.13 g/L after day 2. After day 4 and day 8, the biomass concentration increased up to 2.12 ± 0.2 g/L and 3.21 ± 0.03 g/L, respectively. At the end of cultivation (day 10), the biomass concentration, biomass productivity, and lipid content

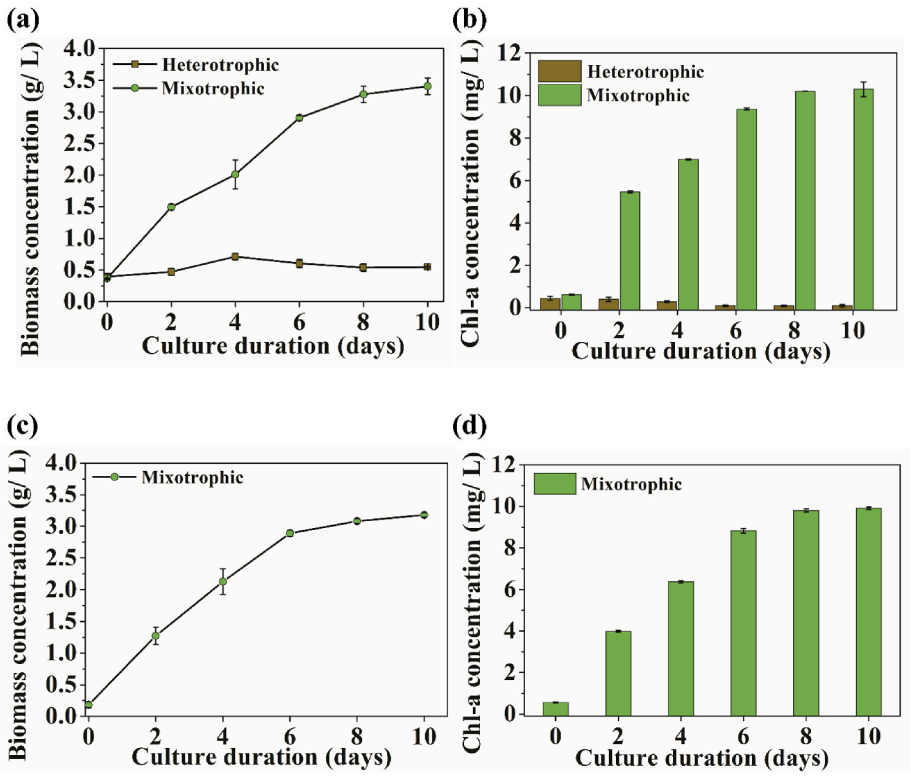

Fig. 1. (a) Heterotrophic and mixotrophic growth curves of *Scenedesmus* sp. DDVG I in PMWW for 10 days cultivation period; (b) Chlorophyll-a content of *Scenedesmus* sp. DDVG I under heterotrophic and mixotrophic modes; (c) Mixotrophic growth curve of *Scenedesmus* sp. DDVG I in the 3-L bioreactor for 10 days cultivation period., and (d) Chlorophyll-a content of *Scenedesmus* sp. DDVG I in the 3-L bioreactor.

Table 2
Comparison of mixotrophic and heterotrophic cultivation of *Scenedesmus* sp. DDVG I in PMWW in terms of lipid content, biomass productivity, and specific growth rate after 10 days of cultivation.

Growth condition	Specific growth rate (μ, d^{-1})	Biomass productivity (g/L. d)	Lipid content (% DCW)
Heterotrophic	0.06	0.021	8.7
Mixotrophic	0.16	0.065 ± 0.01	21.9
Mixotrophic in bioreactor	0.20	0.069 ± 0.01	22.51

were 3.39 ± 0.03 g/L, 0.069 ± 0.01 g/L d, and 22.51%, respectively (Table 2). *Scenedesmus* sp. grown in a bioreactor exhibited a specific growth rate of 0.2 d^{-1}. During the growth, the Chl-a content increased from 0.56 ± 0.01 mg/L to 6.4 ± 0.6 mg/L over four days. The highest Chl-a content (9.90 ± 0.06 mg/L) was achieved on day 10 of incubation. This revealed that *Scenedesmus* sp. DDVG I consumed organic matter in the PMWW efficiently in a mixotrophic mode. In agreement with this study, Engin et al. (2018) reported mixotrophic cultivation of *Micractinium* sp. *ME05* with vinasse in a 5-L bioreactor, achieving a higher biomass concentration of 1.95 ± 0.2 g/L of and 0.32 ± 0.2 mg/L d of biomass productivity when compared to a 500 mL flask study. Similarly, mixotrophic growth of *Auxenochlorella protothecoides* UMN280 with concentrated municipal wastewater in a 25-L BIOCOIL reactor achieved high biomass concentration and biomass productivity of 1.30–1.78 g/L and 0.92 mg/L d, respectively. Mitra et al. (2012) reported that mixotrophically grown *Chlorella vulgaris* in a 6L bioreactor could increase cell densities from 8.0 to 9.8 g/L and biomass productivities from 2.0 to 2.5 g/L d by scaling up from 250 ml flask cultivation. The augmentation in the cell growth and biomass productivity with increased working volume signified a correlation between the working volume and biomass productivity. This might be due to more space for light exposure to microalgal cells and proper aeration in the bioreactor than in smaller flasks (Engin et al., 2018).

3.4. Nutrient removal by Scenedesmus sp. DDVG I cell from PMWW

The patterns of nutrient removal by *Scenedesmus* sp. from the culture medium (PMWW) in heterotrophic and mixotrophic mode over 10 days cultivation period are illustrated in Fig. 2. As shown in Fig. 2a, the concentration of COD decreased gradually in mixotrophic mode when compared to heterotrophic over 10 days of culture duration. In mixotrophic, the COD decreased from 484.8 ± 8.5 mg/L to 225 ± 5.5 mg/L when the cells were in the exponential phase (day 4) of the growth. The lowest residual COD concentration in the culture medium after 10 days was 120 mg/L, achieving an overall COD removal efficiency of 75.2 ± 0.1%. In a study by Chaudhary et al. (2018), *Scenedesmus obliquus* ACHB 417 exhibited a COD removal efficiency of 75.9% from municipal wastewater. Likewise, the higher concentration of COD (3429.33 mg/L) in the slaughterhouse wastewater was reduced to 855.6 mg/L by *Chlorella pyrenoidosa*, achieving a 75.4% removal efficiency (Azam et al., 2020). These values were comparable with this present study. However, Wang et al. (2010) stated that the centrate sludge obtained after centrifugation of municipal wastewater generally had 20,180 mg/L of COD. The study further reported the effective reduction of COD to 6065 mg/L by *Chlorella* sp. with a 70% removal efficiency which was relatively lower than the value of this present study.

Results in Fig. 2b and c indicated that the initial concentrations of NH_3–N (40.5 mg/L) and TN (44.9 mg/L) in the medium declined abruptly, resulting in 19.92 mg/L and 7.7 mg/L with removal efficiencies of 50.8% and 82.8 ± 1.2%, respectively after day 4, which corresponded to the exponential phase of *Scenedesmus* sp. DDVG I growth. At the end of the experiment (day 10), the lowest TN concentration remained in the culture medium was 0.05 mg/L, resulting in a nutrient removal efficiency of 99.8%. Meanwhile, the NH_3–N removal

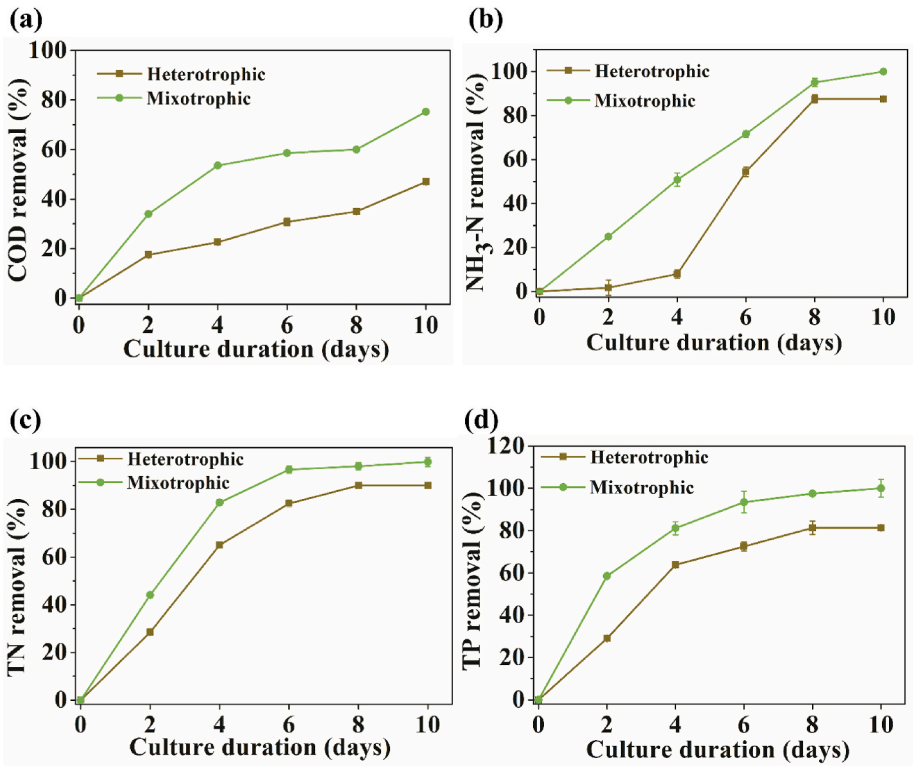

Fig. 2. Nutrient concentrations and their corresponding removal efficiencies by *Scenedesmus* sp. during 10 days culture period in the PMWW: (a) COD concentration, (b) COD removal efficiency, (c) NH_3–N concentration, (d) NH_3–N removal efficiency, (e) TN concentration, (f) TN removal efficiency, (g) TP concentration, and (h) TP removal efficiency.

efficiency achieved by *Scenedesmus* sp. was 100%. A study conducted by Daneshvar et al. (2019) reported that the removal efficiency of TN from dairy wastewater by *Scenedesmus quadricauda* was 87.6%. Chaudhary et al. (2018) found out 91.5% of NH_3–N removal by *Scenedesmus obliquus* ACHB 417 from municipal wastewater. Likewise, *Chlorella* sp. showed the highest removal of NH_3–N (91.3%) from the centrate sludge (Wang et al., 2010). As shown in Fig. 2d, the initial TP concentration (7.9 mg/L) in PMWW was reduced to 0.52 mg/L with a removal efficiency of 93.4% after day 6. After day 10, the TP was removed efficiently by *Scenedesmus* sp., resulting in a 100% removal efficiency. Daneshvar et al. (2019) reported that TP from the dairy wastewater was completely removed (100% removal efficiency) by *Scenedesmus quadricauda*. Similarly, complete removal of TP (100%) from dairy wastewater by *Acutodesmus dimorphus* was reported (Chokshi et al., 2016). Results in Fig. 2 showed that in heterotrophic mode, the nutrient removal efficiency achieved by *Scenedesmus* sp. was relatively lower when compared to the mixotrophic condition. The COD removal efficiency was 46.9 ± 1.1% over 10 days of cultivation. While the removal of TN, NH_3–N, and TP over 10 days of cultivation were 89.9 ± 1.1%, 87.5%, and 81.2 ± 1.1%, respectively. The high nutrient removal efficiency even after the lesser cell densities of *Scenedesmus* sp. might be contributed by the growth of other microbes in heterotrophic as mentioned in section 3.2. In such dark conditions, bacteria tend to assimilate nitrogen and phosphorus efficiently as instant energy that are required for cell division and growth (Cid et al., 1992).

Further, scaling up of mixotrophic growth in 3 L bioreactor showed the maximum removal efficiency of COD (75.6 ± 1.5%), NH_3–N (100%), TN (99.8%), and TP (100%) which were comparable with the literature report as shown in Table 3. But, the incomplete removal of COD might be due to the presence of intractable organic compounds in the wastewater, which cannot be easily degraded by microalgae. Nevertheless, the present study exhibited higher COD removal efficiency when compared to literature reports (Eladel et al., 2019; Leong et al., 2019). From all these analyses and results, we observed that when the cell densities increased, the extent of pollutants present in the culture medium gradually decreased which is represented in Fig. S3(a) and Fig. S3(b) of ESM. The nutrients were consumed by the microalgal cells, resulting in enhanced cell densities and the lowering of pollutants. Highly polluted municipal wastewater discharges are detrimental to the environment (Kothari et al., 2012; Wang et al., 2010). The mixotrophic cultivation of *Scenedesmus* sp. DDVG I could be an alternative method for the treatment of wastewater. Further studies are still required to improve the overall nutrient removal and biomass production. Some studies suggested that efficient removal of COD could be achieved via either introduction of cyanobacteria to the microalgal growth (Tsolcha et al., 2017, 2018b, 2021, 2018b) or a mixed microbial consortium (Patrinou et al., 2020; Tsolcha et al., 2018a). Besides, an optimal N/P ratio in the medium would enhance the performance of bioremediation and microalgal growth (Choi and Lee, 2015). Hence, future research will focus on the microalgal-based consortium in balanced N/P medium to get efficient removal of the overall nutrient and enhanced biomass productivity.

3.5. *Effect of mixotrophic cultivation on biochemical composition of Scenedesmus sp. DDVG I*

3.5.1. *Amino acid profile*

The amino acid profile (in mg/g DCW) of whole dried algal cells obtained from *Scenedesmus* sp. DDVG I is summarized in Table 4. We observed that the biomass comprised nine EAA and eight NEAA. The EAAR value of the *Scenedesmus* sp. grown in the PMWW was 0.45, which was slightly higher than the EAAR value (0.43–0.44) of soybean (Serretti et al., 1994). Miao et al. (2016) reported the EAAR value achieved by mixotrophically grown *Chlorella vulgaris* in domestic wastewater was 0.37. Furthermore, the EAA content (159.8 mg/g DCW) of this study was comparable to other reference proteins such as soybean (EAA content, ranging from 160 to 220.4 mg/g DCW) (Serretti et al., 1994), *Spirulina*

Table 3
Comparison of biomass concentration and nutrient removal efficiency by *Scenedesmus* sp. DDVG I under 3L mixotrophic growth in primary municipal wastewater with previously reported bioremediation studies.

Species	Wastewater	Cultivation period (days)	Biomass content	Nutrient content in untreated wastewater	Nutrient content in treated wastewater	Nutrient removal efficiency (%)	Reference
			(g/L)	(mg/L)	(mg/L)		
Scenedesmus sp. DDVG I	Municipal wastewater	10	3.4	COD (484.8 ± 8.5)	COD (118.0 ± 1.5)	COD (75.6 ± 1.1)	**This study**
				NH_3–N (40.2 ± 1.1)	NH_3–N (ND)	NH_3 (100)	
				TN (44.9 ± 1.9)	TN (0.052)	TN (99.8)	
				TP (7.9 ± 0.5)	TP (NR)	TP (100)	
Scenedesmus sp. LX1	Municipal wastewater	15	0.11	TN (15.5)	TN (0.24)	TN (98.45)	Xin et al. (2010)
				TP (0.5)	TP (0.01)	TP (98)	
Chlorella sp.	Centrate from sludge centrifuge	9	1.5	COD (20180)	COD (6054)	COD (70)	Wang et al. (2010)
				NH_3–N (1434.3)	NH_3–N (124.7)	NH_3 (91.3)	
Scenedesmus obliquus ACHB 417	Municipal Wastewater	10	0.8	COD (293 ± 3.3)	COD (70.5)	COD (75.9)	Chaudhary et al. (2018)
				NH_3–N (43.67 ± 0.72)	NH_3–N (3.7)	NH_3 (91.5)	
				PO_4^{3-} (18.53 ± 0.05)	PO_4^{3-} (1.6)	PO_4^{3-} (91.3)	
Scenedesmus obliquus	Municipal wastewater	7	0.31	TN (8.7 ± 0.5)	TN (ND)	TN (100)	Ji et al. (2013)
				TP (1.71 ± 0.3)	TP (ND)	TP (100)	
Parachlorella kessleri sp.	Municipal wastewater	12	1.01 ± 0.01	NH_3–N (100)	NH_3–N (ND)	NH_3–N (100)	Aketo et al. (2020)
				TN (130)	TN (2.6)	TN (98)	
				PO_4^{3-} (65)	PO_4^{3-}(0.65)	PO_4^{3-}(99)	
Chlorella vulgaris	Municipal wastewater	14	1.0 ± 0.03	COD (160–200)	COD (64–80)	COD (55.5–64.45)	Leong et al. (2019)
				NH_3–N (43–46)	NH_3–N (ND)	NH_3–N (100)	
Chlorella sorokiniana	Municipal wastewater	10	0.07	COD (44)	COD (16.9)	COD (61.5)	Eladel et al. (2019)
				NH_3–N (0.5)	NH3–N (0.12)	NH3–N (76)	
				TP (1.9)	TP (0.32)	TP (83.16)	
Scenedesmus quadricauda	Dairy wastewater	12	0.47	TN (85.99)	TN (10.66)	TN (87.6)	Daneshvar et al. (2019)
				PO_4^{3-} (8.7)	PO_4^{3-} (0.87)	PO_4^{3-} (90.0)	
Chlorella pyrenoidosa	Livestock slaughterhouse wastewater	15	0.4	COD (3429.33)	COD (855.6)	COD (75.05)	Azam et al. (2020)
				BOD (1798.33)	BOD (547.6)	BOD (69.5)	
				NO_3–N (331.66)	NO_3–N (48.0)	NO_3–N (85.5)	
				PO_4^{3-} (34.13)	PO_4^{3-} (4.4)	PO_4^{3-} (87.1)	

ND, not detected.

(EAA content = 226.4 mg/g DCW) (Dewi et al., 2016), *Nannochloropsis granulate* (EAA content = 172.6 mg/g DCW) (Tibbetts et al., 2015), and *Scenedesmus acuminatus* (149.4 mg/g DCW of EAA) (Zhang et al., 2019). This indicated that PMWW could induce the accumulation of EAA in *Scenedesmus* sp. DDVG I, showing the feasibility for use in animal and aquaculture feed.

Despite its high nutritional values, precautions should be taken before the establishment and commercialization of microalga as feed supplements. Some algal species could accumulate heavy metals at higher concentrations higher from the environment and produce biogenic toxins including purines that cause neurodegenerative disorders (Jin et al., 2006; Lum et al., 2013). Therefore, future research is still required to understand the toxicological property of *Scenedesmus* sp. DDVG I for consuming as feed supplements.

3.5.2. Fatty acid profile, and biodiesel assessment

The fatty acid profile (together with C_{14}–C_{24} content) of *Scenedesmus* sp. DDVG I obtained over the 10 days of cultivation is presented in Table 5. The FAME were consisting of saturated fatty acid (SFA), unsaturated fatty acid (MUFA), and polyunsaturated fatty acid (PUFA). The total FAME content of the mixotrophically grown *Scenedesmus* sp. DDVG I in wastewater was 22.04% in DCW. The MUFA content (65.8%) in total FAME was predominant, followed by PUFA (19.1%), and SFA (15.1%). A higher concentration of MUFA in the FAME indicated a better quality of the biodiesel, owing to the oxidative stability and low-temperature fluidity (Cao et al., 2014). Furthermore, *Scenedesmus* sp. DDVG I was rich in C18:2 (linoleic acid) or also known as omega 6 fatty acid (18.9%). This FA is generally deficient in the human diet and animal feed (Mitra et al., 2012; Sinclair, 1990). The remarkable content of C18:2 in the present study showed a promising solution for a dietary source of human, aquaculture, and animal feed.

Based on the FAME profile, biodiesel parameters (including, DU, SV, IV, CN, LCSF, CFPP, CP, PP, OSI, HHV, FP, ρi, and η) were determined and summarized in Table 6. The results were represented with correspondence to the characteristics of the biodiesel standards EN 14214 (Europe) and ASTM D6751 (United States). We observed that all the tested parameters were comparable to the corresponding standard values. The IV (89.3 g I_2/100 g) of *Scenedesmus* sp. meets the current standard limit of the IV (it should be < 120). Thus, the biodiesel found from the lipid of this strain showed high oxidative stability. As shown in Table 6, the CN value (54.8) exceeded the minimum standard value of CN (it should exceed >51) which suggested that the combustibility of the fuel obtained from this biomass might be appropriate.

According to the analysis, the HHV value of *Scenedesmus* sp. (39.9 MJ/kg) attained the minimum standard value (>35 MJ/kg). This parameter allowed estimation of the energy potential and economic efficacy. Furthermore, density (0.87 g/cm^3) and viscosity (4.4 mm^2/s) met their corresponding standard values (Table 6). This demonstrated superior biodiesel, allowing transfer of a small mass of fuel to the engine, effective atomization, energy efficiency, as well as less deposition of fuel

Table 4
Amino acid (mg/g DCW) composition of whole biomass produced from *Scenedesmus* sp. DDVG I grew in the 3-L bioreactor under a mixotrophic condition.

Amino acids	*Scenedesmus* sp. DDVG 1 [a]	*Scenedesmus acuminatus* [b]	*Nannochloropsis granulate* [c]	*Spirulina* [d]	*Chlorella vulgaris* [e]	Soybean [f]
Non-essential amino acid (NEAA)						
Alanine	34.6 ± 0.0	23.31	23.0	45.9	11.2 ± 0.1	19.1–24.4
Aspartic acid	28.2 ± 0.1	25.58	35.5	59.9	13.7 ± 0.3	50.8–67.4
Cystine	2.9 ± 0.0	2.85	2.7	5.9	2.4 ± 0.2	5.3–6.5
Glutamic acid	39.7 ± 0.0	43.76	40.4	91.3	15.7 ± 0.5	75.4–102.5
Glycine	23.2 ± 0.0	14.8	18.3	31.3	8.6 ± 0.1	18–23.4
Hydroxyproline	34.8 ± 0.3	12.74	0.3	NR	0.7 ± 0.0	NR
Serine	17.3 ± 0.0	11.27	14.8	27.6	7.5 ± 0.1	22.7–29.4
Tyrosine	11.7 ± 0.1	9.31	14.1	25	6.4 ± 0.2	15.5–19.5
Essential amino acid (EAA)						
Arginine	21.3 ± 0.0	38.74	25.4	43.1	9.8 ± 0.1	29.3–44.4
Histidine	6.7 ± 0.0	9.01	7.5	10	3.1 ± 0.2	12.3–16.4
Isoleucine	14.3 ± 0.0	11.08	17.4	35	6.5 ± 0.0	12.3–15.4
Leucine	33.7 ± 0.0	24.96	32.4	53.8	13.4 ± 0.1	28.9–37.6
Lysine	19.1 ± 0.0	15.88	24.1	4.8	9.2 ± 0.3	24.2–37.6
Methionine	6.3 ± 0.0	4.71	8.7	11.7	3.5 ± 0.1	5.0–6.2
Phenylalanine	20.2 ± 0.0	16.21	19.1	NR	8.2 ± 0.1	19.8–25.2
Threonine	18.9 ± 0.1	14.02	16.5	28.6	6.4 ± 0.2	15.5–21.2
Valine	19.3 ± 0.09	14.78	21.5	39.4	9 ± 0.0	12.7–16.4
Total EAA	159.8	149.4	172.6	226.4	69.1	160–220.4
Total EAAR	0.45	0.50	0.53	0.44	0.51	0.43–0.44

NR: not reported;.
[a] present study.
[b] (Zhang et al., 2019).
[c] (Tibbetts et al., 2015).
[d] (Dewi et al., 2016).
[e] (Tibbetts et al., 2015).
[f] (Serretti et al., 1994).

Table 5
FAME compositions of *Scenedesmus* sp. DDVG I in mixotrophic growth using 3-L bioreactor in the PMWW for a culture duration of 10 days.

FAME	%FAME in DCW	% FAME in total FAME
C14:0 (myristic)	0.53	2.4
C16:0 (palmitic)	0.37	1.7
C16:1 (palmitoleic)	5.89	26.7
C18:0 (stearic)	2.07	9.4
C18:1 (oleic)	8.38	38.0
C18:2 (linoleic)	4.17	18.9
C18:3 (linolenic)	0.02	0.2
C20:0 (arachidic)	0.35	1.6
C20:1 (gadoleic)	0.09	0.4
C22:1 (erucic)	0.07	0.3
C24:1 (nervonic)	0.10	0.4
SFA	3.32	15.1
MUFA	14.53	65.8
PUFA	4.19	19.1
Total FAME	22.04	100

Table 6
Properties of *Scenedesmus* sp. DDVG I-derived biodiesel grown mixotrophically in the primary municipal wastewater (PMWW).

Properties	*Scenedesmus* sp. DDVG I	EN 14214	ASTM D6751
DU	103.6	NR	NR
SV (mg KOH/g oil)	191.1	NR	NR
IV (g I_2/100 g)	89.3	<120	NR
CN	54.8	>51	>47
LCSF	8.4	NR	NR
CFPP (°C)	9.9	NR	NR
CP (°C)	−3.0	NR	NR
PP (°C)	−10.2	NR	NR
OSI	4.4	NR	>3h
HHV (MJ/kg)	39.9	NR	>35
FP (°C)	421.1	>120	>93
ρ_i at 15 °C (g/cm^3)	0.87	0.86–0.90	0.82–0.90
η at 40 °C (mm^2/s)	4.4	3.5–5.0	1.9–6.0

NR: not reported.

in the engine. Thus, *Scenedesmus* sp. DDVG I-derived biodiesel showed the viability of use in automobiles.

4. Conclusions

The novel strain *Scenedesmus* sp. DDVG I isolated from a fresh water-logged area was proven to be a robust candidate for wastewater bioremediation. Depending on the high growth rate and biomass yield, municipal wastewater can be an alternative low-cost medium for mixotrophic cultivation of *Scenedesmus* sp. The operation of a 3-L bubble photobioreactor for 10 days provided net biomass productivity of 0.069 g/L. d of dried algae, a lipid yield of 22.5% DCW, and a FAME content of 22.04% in DCW. This study identified that *Scenedesmus* sp. DDVG I had valuable fatty acids and amino acid profile (EAA of 159.8 mg/g DCW) that can be converted to biodiesel as well as animal feed, respectively.

CRediT authorship contribution statement

Nongmaithem Debeni Devi: Conceptualization, Investigation, Methodology, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Xiao Sun:** Formal analysis, Writing – review & editing. **Lingkan Ding:** Formal analysis, Writing – review & editing. **Vaibhav V. Goud:** Supervision, Conceptualization, Data curation, Writing – review & editing, Visualization, Validation. **Bo Hu:** Supervision, Funding acquisition, Conceptualization, Data curation, Writing – review & editing, Visualization, Validation, All authors have approved the final version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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