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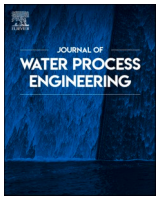

Unlocking the potential of microalgae: Cultivation in algae recycled effluent with domestic wastewater for enhancing biomass, bioenergy production and CO_2 sequestration

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ABSTRACT

The current study aimed to enhance microalgal biomass productivity by re-using microalgae supernatant obtained from an algae cultivation system. In addition, raw and final effluent from a domestic wastewater treatment plant (WWTP) were integrated with the recycled effluent (supernatant). In order to accomplish the aim, the performance of various dilutions of raw wastewater (RW), recycled effluent (RE) and wastewater final effluent (FE) was assessed for resource recovery and biomass production. The 10 different dilutions were 100RW, 25RW + 75RE, 50RW + 50RE, 75RW + 25RE, 100RE, 100FE, 25FE + 75RE, 50FE + 50RE, 75FE + 25RE and RW + RE + FE. Results showed that the maximum biomass production (1.59 g L^{-1}) was observed in 75RW + 25RE followed by RW + RE + FE (1.13 g L^{-1}) diluted wastewater and lowest in 100FE (0.53 g L^{-1}). Similarly, the highest lipid content was in 75RW + 25RE (32.16 %) and lowest in BG11. The highest protein was obtained in BG11 (48.65 %) followed by 33.32 % in 75RW + 25RE. Furthermore, maximum CO_2 fixation rate (0.19 gCO_2 L^{-1} d^{-1}), theoretical biochemical methane potential (471.54 mL CH4 g^{-1} VS) and high heating value (21.52 Kg J^{-1}) were observed in 75RW + 25RE. These findings indicated that 75RW + 25RE is a suitable combination that could be used as a potential ratio to achieve optimum biomass production. This may be due to the presence of phytohormones within RE in combination with other nutrients from RW. Moreover, 75RW + 25RE showed high metabolites yield, high CO_2 fixation rate, nutrient removal efficiency and high heating value. The mixture of RW and HE may be a sustainable and feasible strategy for acquiring fast microalgal growth while reducing dependency on nutrient and freshwater.

1. Introduction

Microalgal biomass is used to produce an array of sustainable and environmentally friendly bioproducts such as feed, biofuels, biofertilizers etc. However, the growth of microalgae requires huge quantities of water and nutrients, hence, microalgae cultivation in various types of wastewater (WW) has been extensively explored to reduce the need for freshwater as well as costly nutrients [1]. Microalgae cultivation in synthetic media is expensive, e.g., BG11, Fogg's and CHU 12 mediums were ~0.328, 0.04 and 1.89 $/L, respectively, compared to cultivation in WW streams, which have little to no cost [2]. This significantly affects the overall production costs of microalgae biomass.

Yang et al. [3] reported that ~0.33 kg and 0.71 kg of nutrients nitrogen (N) and phosphorous (P), respectively, are required for production of 1 kg of microalgae biomass. Approximately 1000 kg of water is required to produce 1 kg of microalgae biomass [4] and producing 1 kg of microalgal biodiesel requires 3726 kg of water [5]. Microalgae can utilize the nutrients (e.g., nitrates, nitrites, phosphates etc.) in WW streams as a source for their growth. The use of WW for microalgal cultivation reduces reliance on clean water resources, decreases carbon emissions associated with water treatment, aids in phycoremediation processes, reduces costs (no requirement for fertilizers/ nutrient supplementation) and promotes a circular economy by resource recycling [1].

WW streams present a viable (economical and environmentally friendly) option as a water source/ culture medium for the cultivation of microalgae [1]. There are various WW streams suitable for microalgal cultivation such as domestic (sewage), industrial, agricultural (livestock

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and aquaculture industry) etc. The different types of WW's are unique in their physical properties and chemical compositions and contain varying nutrient profiles. Municipal WW is commonly used for microalgal cultivation since it is readily available and is a cost-effective substrate. Municipal WW may be divided into three groups based on the stage of the process in WW treatment: (i) raw sewage/ influent (WW prior to primary settling), (ii) secondary effluent (WW after activated sludge treatment) and (iii) centrate (a by-product of sludge dewatering process) Many studies have been conducted using different WW effluents at various stages along the WW treatment system for algal growth, often at a variety of dilutions since nutrient loads differ greatly at the different process points ([1,6–9] and references therein).

There have been many investigations into diluting and/or mixing different WW effluents at different levels/ratios in order to decrease nutrient concentrations since it can strongly impact nutrient removal ability by microalgae as well as biomass production ([8,10,11]; and references therein). The use of secondary effluent WW is generally preferred, especially at a commercial scale, since the microbial load in the post-chlorinated WW is decreased which reduces the risk of bacterial/viral contamination; however, N and P are deficient in this type of water and may require nutrient supplementation to improve biomass productivity.

After harvesting the biomass, the supernatant may be reused for subsequent microalgal growth cycles since it contains residual nutrients [12]. Reusing the supernatant/recycled effluent (RE) can reduce water requirements by 84 % and nutrient intake by 55 % [3]. Studies have reported a positive, negative, or neutral influence of harvested media on microalgal growth depending on factors such as strain, culture conditions, harvesting methods, water reuse regimen, etc. [12]. Morocho-Jácome et al. [13] observed an increase (13.5 %) in the growth of *A. platensis* when grown in harvested effluent combined with flocculants and activated carbon (powdered) compared to its growth in standard media. Yang et al. [14] found that reused harvested water had stimulatory effects on *Chlorella* sp. growth by 5–11 %. A more recent study by Sha et al. [15] showed that biomass production in phycoremediated (harvested) water supplemented with activated carbon (granular) was almost the same compared to BG11 media. These findings show that it is possible to enhance biomass yield and foster microalgal growth in recycled water. This is advantageous for long-term, widespread microalgae cultivation. A potential approach to improve microalgal growth performance could involve supplementing nutrient depleted final effluent or high nutrient concentration raw WW with harvested effluent, which contains residual nutrients and possibly phytohormones. To the best of our knowledge, there are no studies reported thus far using RE mixed with FE or RW to ascertain a suitable combination for improving biomass production.

In this study, three different WW streams (RW, RE and FE) were used for the cultivation of *Tetradesmus obliquus*. The aim of this work was to determine the most appropriate WW dilution ratio for optimal microalgal growth, physiology, biochemical compositions, nutrient removal capacity, CHNS, theoretical biochemical methane potential, CO_2 sequestration potential, and cost-saving potential.

2. Materials and methods

2.1. Microalgae cultivation

Tetradesmus obliquus was isolated from a maturation pond at the Kingsburgh wastewater treatment plant (WWTP) in Durban, South Africa. The strain was purified through sub-culturing using the streak plate method. *T. obliquus* was inoculated in 1 L flasks containing BG11 medium to initiate the seed culture. These cultures were maintained in a controlled environment, with a temperature of 22 ± 2 °C, under Sylvania Gro-Lux lamps that provided 80 μmol·photons·m^{-2} s^{-1} irradiance using a 16/8 h light/dark cycle. The cultures were agitated at 100 rpm on a shaker to prevent algal clumping.

2.2. Experimental plan

Raw wastewater (RW), and final effluent (FE) (post-chlorinated water) were collected from a domestic WW treatment in Durban, South Africa. Recycled effluent (RE) (supernatant) was collected from a continuous microalgal biomass decanter (TOMOE Engineering Co., Ltd.; Japan) harvesting 5000 L hr^{-1} from a 300,000 L raceway pond located at the same WWTP. The raceway contained FE supplemented with 250 mg L^{-1} of $NaNO_3$ and 40 mg L^{-1} of K_2HPO_4 under natural sunlight (18–30 °C) with light intensity (400–1200 $\mu m^2 s^{-1}$). RW, RE, and FE were mixed in varying ratios for the growth and analysis of microalgae.

The various dilutions of RW, RE, and FE used for the cultivation of *T. obliquus* are shown in Table 1. The culture was also grown in BG11 using freshwater, as a control. Experimental sets were inoculated with 10 % of the stock culture of *T. obliquus* (optical density 2.2 @ 680 nm). All experiments were performed in triplicate in 1 L flasks with 500 mL working volume. The study was conducted for 16 days.

2.3. Biomass and nutrient analysis

The biomass concentration was measured using a spectrophotometer (DR6000, Hach) at a wavelength of 680 nm. To determine the dry cell weight (DCW), the algal samples were filtered using pre-dried and pre-weighed 25 mm Whatman No. 1 filter paper (11 μm) and then dried in an oven at 105 °C overnight and placed in a desiccator until cool and weighed to determine the DCW. The biomass productivity was calculated using Eq. (1).

$$\text{Biomass productivity } \left(\text{mg L}^{-1}\text{d}^{-1}\right) = \frac{\textit{Biomass yield } (\textit{mg L} - 1)}{\textit{Number of days}} \quad (1)$$

Nutrients including nitrate, nitrite, ammonia, and phosphate concentration of microalgae culture were determined using calorimetric with Gallery TM (Thermo Scientific™ Gallery™ Automated Photometric Analyzer, Germany).

2.4. Physiology of microalgae and chlorophyll analysis

Before analysis of microalgae, culture was kept in dark conditions for 20–30 min. The maximum quantum efficiency of photosynthesis II charge separation (F_v/F_m) was calculated according to the following equation.

$$F_v/F_m = F_m - F_o/F_m \quad (2)$$

Chlorophyll-*a* determination was carried out according to method of Porra et al. [16]. Briefly, 1 mL algal sample was centrifuged and the pellets were collected. The pellet was then resuspended in 1 mL methanol and incubated in a water bath at 60 °C, after which the sample was removed to cool down. The optical density (OD) of the algal sample was measured at 652 nm, 665.2 nm, 670 nm, and 750 nm. Finally, the quantity of chlorophyll-*a* was estimated using Eq. (3).

Table 1
Different dilutions of RW, RE and FE used for cultivation of *T. obliquus*.

No. of combinations	RW (%)	RE (%)	FE (%)
1	100	–	–
2	25	75	–
3	50	50	–
4	75	25	–
5	–	100	–
6	–	–	100
7	–	75	25
8	–	50	50
9	–	25	75
10	33.3	33.3	33.3
Control (BG11)	–	–	–

$$Chlorophyll-a\ (\mu g\ mL-1) = 16.29\ (A_{665.2}–A_{750})–8.54\ (A_{665.2}–A_{670}) \quad (3)$$

2.5. Biomass harvesting, elemental and biochemical composition analysis

After 16 days of cultivation, the biomass was harvested by centrifuging at 3000 rpm for 5 min. The obtained wet algae biomass was subjected to a freeze dryer (Mini Lyotrap, LTE Scientific Ltd., United Kingdom) where wet biomass was used to lyophilize after overnight freezing at −84 °C in a biofreezer (Glaciar NU9668E, Nuaire, Japan).

The elemental (CHNS) analysis of algal biomass was quantified using a CHNS analyzer (PerkinElmer 2400, USA), and oxygen (O) content was determined on an ash ash-free basis [100 – (C + H + N + S)]. The higher heating value (HHV) microalga biomass sample were calculated by following the formula Eq. (4) [17]. Moisture and ash content were determined according to the standard method [18].

$$\mathrm{HHV}\ \left(\mathrm{Kg\ J^{-1}}\right) = 5.22C^2 - 319C - 1647H + 38.6C \times H + 133\ N + 21,028 \quad (4)$$

The lipid extraction process utilized a microwave-assisted technique, where 50 mg of dried biomass was mixed with 20 mL of a solvent mixture (chloroform and methanol in a 2:1 v/v ratio). The mixture was then heated at 100 °C for 10 min at 1000 W in a microwave digester. The lipids yield was determined gravimetrically, and lipid productivity was calculated using Eq. (5).

$$\text{Lipid productivity}\ \left(\mathrm{mg\ L^{-1}d^{-1}}\right) = \text{Biomass productivity}\ \left(\mathrm{mg\ L^{-1}d^{-1}}\right) \times \frac{Lipid\ content\ (\%)}{100} \quad (5)$$

The microalgal biomass was analyzed for protein content by following the protocol of López et al. [19]. The absorbance at 750 nm was measured using a spectrophotometer (DR6000, Hach). Bovine serum albumin (BSA) was used to prepare standards for calibration and protein quantification [19]. The protein productivity was calculated using Eq. (6).

$$\text{Protein productivity} = \text{Biomass productivity}\ \left(\mathrm{mg\ L^{-1}d^{-1}}\right) \times \frac{Protein\ (\%)}{100} \quad (6)$$

The total carbohydrates in algae biomass were determined using the phenol-sulfuric acid method [20]. In brief, dried biomass was mixed with 2 % (v/v) sulfuric acid and then autoclaved for 30 min at 121 °C for hydrolysis. The resulting solution was then measured for absorbance at 490 nm using a spectrophotometer (DR6000, Hach). The total carbohydrates were determined by referring to a calibration curve prepared using glucose as a standard. Carbohydrate productivity was calculated using Eq. (7).

$$\text{Carbohydrate productivity}\ \left(\mathrm{mg\ L^{-1}d^{-1}}\right) = \text{Biomass productivity}\ \left(\mathrm{mg\ L^{-1}d^{-1}}\right) \times \frac{Carbohydrate\ (\%)}{100} \quad (7)$$

2.6. Theoretical biochemical methane potential (TBMP)

Theoretical biomethane potential (TBMP) was calculated as per Eq. (8) [21].

$$TBMP\ \left(mL\ CH4\ g^{-1}VS\right) = \frac{22.4 \times \left(\frac{a}{2} + \frac{b}{8} - \frac{c}{4} - \frac{3d}{8} - \frac{e}{4}\right)}{12.017a + 1.0079b + 15.999c + 14.0067d + 32.065e} \quad (8)$$

where,

$$a = \frac{aultimas}{mmC} = \frac{aultimass}{12.0107}$$

$$b = \frac{bultimas}{mmH} = \frac{bultimass}{1.0079}$$

$$c = \frac{cultimas}{mmO} = \frac{cultimass}{15.999}$$

$$d = \frac{dultimas}{mmN} = \frac{dultimass}{14.0067}$$

$$e = \frac{eultimas}{mmS} = \frac{eultimass}{32.065}$$

The equation has the following assumptions:

(a) Constant temperature
(b) Ideal bacterial conditions (full digestion)
(c) Substrate consists of C, H, N, O and S
(d) Products of reaction include only CH_4, CO_2, NH_3, and H_2S
(e) No accumulation of ashes

2.7. Statistical analysis

The experiment was carried out in triplicate, and the data are reported as mean values with standard deviations (mean ± SD, n ≥ 3) or with error bars in all graphs. One-way ANOVA was carried out to investigate significant differences between each dilution.

3. Results and discussions

3.1. Wastewater characterization

The physicochemical parameters for collected WW are shown in Table 2. The ranges of nitrate, nitrite, ammonia and phosphate were 10.07–28.17, 0.01–1.7, 0.34–5.37 and 1.5–4.5 mg L^{-1}, respectively. The nutrient concentration was similar to reported research papers. For example, Wang et al. [9] used untreated WW which contained 33.4 mg L^{-1} ammonia-nitrogen, and 5.56 mg L^{-1} orthophosphates to cultivate *Chlorella* sp. Satheesh et al. [22] cultivated *S. obliquus*, *C. sorokiniana* and

Table 2
Characterization of RW, RE, FE and BG11 used for the cultivation of *T. obliquus*.

Characteristics	Unit	Raw wastewater (RW)	Final effluent (FE)	Recycled effluent (RE)	BG11
Colour	–	Grey	Clear	Light yellow	Clear
Odor	–	Unpleasant	Odorless	Fishy	Odorless
Temp	°C	24.5	23.5	22.2	23.3
Conductivity	μS cm^{-1}	894	635	2701	2322
TDS	mg L^{-1}	0.58	0.413	1.75	–
Salinity	mg L^{-1}	0.43	0.31	1.38	1.2
DO	mg L^{-1}	0.5	1.65	1.51	7.41
pH	–	7.63	7	9.43	8.11
COD	mg L^{-1}	454	26	238	–
Nitrate	mg L^{-1}	18.38	10.07	28.17	240
Ammonia	mg L^{-1}	5.37	0.34	0.39	0.4
Phosphate	mg L^{-1}	4.5	1.5	1.5	16.8
Bacterial density	cfu mL^{-1}	1.2×10^5	6.8×10^4	6.2×10^5	–

C. pyrenoidosa in domestic WW containing 25.65 mg L^{-1} of nitrate, 7.81 mg L^{-1} of phosphates and 1.23 mg L^{-1} of nitrite. The WW utilized also contained a high concentration of bacteria which was 1.2×10^5, 6.8×10^4, and 6.2×10^5, respectively in RW, FE, and RE.

3.2. Growth and physiology of *T. obliquus* cultivated in different wastewaters

Fig. 1 shows that *T. obliquus* grew well initially for 3 days in all types of WWs with different dilutions which indicates that they are appropriate mediums for algal adaptation and growth. For the first three days of cultivation, biomass production was similar in all dilutions. Due to limited nutrient concentration in 100FE, it can be observed that it had lower biomass production compared to other dilutions. The lag phase was not observed when *T. obliquus* was grown in different WW, suggesting that this algae strain exhibited good adaptability in selected WW dilution without further acclimatization. Xue et al. [23], observed a similar trend when *C. sorokiniana* was cultivated in silk industry WW using a bubble column bioreactor with different initial cell densities and aeration rates. The maximum biomass production (1.59 g L^{-1}) was obtained in 75RW + 25RE, followed by 1.13 g L^{-1} (RW + RE + FE), 1.07 g L^{-1} (100RE), 1.0 g L^{-1} (100RW), and lowest in 0.53 g L^{-1} (100FE). Whereas corresponding biomass productivities were 99.37, 70.9, 67.18, 72.87, and 33.43 mg L^{-1} d^{-1}. In comparison to 75RW + 25RE there were slight increases (5.37 %) in biomass production in BG11 media, where the biomass concentration was 1.68 g L^{-1} and productivity was 105.08 mg L^{-1} d^{-1} (Table 3). The biomass obtained from 75RW + 25RE was 2.97, 2.11, 1.89, 1.8 and 1.58 folds higher than those of 100FE, 75FE + 25RE, 25FE + 75RE, 50FE + 50RE and 100RW. Biomass production in 75RW + 25RE WW dilution medium was shown similar to BG11 medium (1.68 g L^{-1}). The suitability of 75RW + 25RE could be readily observed since the required nutrient was already present in it. At all different dilutions, reasonable growth for *T. obliquus* was observed except for 100FE. Furthermore, when the proportion of FE to RE increased, biomass production decreased (Fig. 1). For example, in 100FE growth of *T. obliquus* was inhibited due to the presence of limited nutrient concentration. These results are in accordance with those observed for continuous culture of *C. vulgaris* grown in the recycled medium using photobioreactor for 56 and 63 days and observed <1.5 g L^{-1} of biomass production [24,25]. Castrillo et al. [26] studied the effect of different harvesting techniques and the reuse of recycled water for *S. obliquus* growth. They observed slightly higher biomass production of 1.6 and 1.7 g L^{-1} in the supernatant of centrifugation and flocculation, respectively.

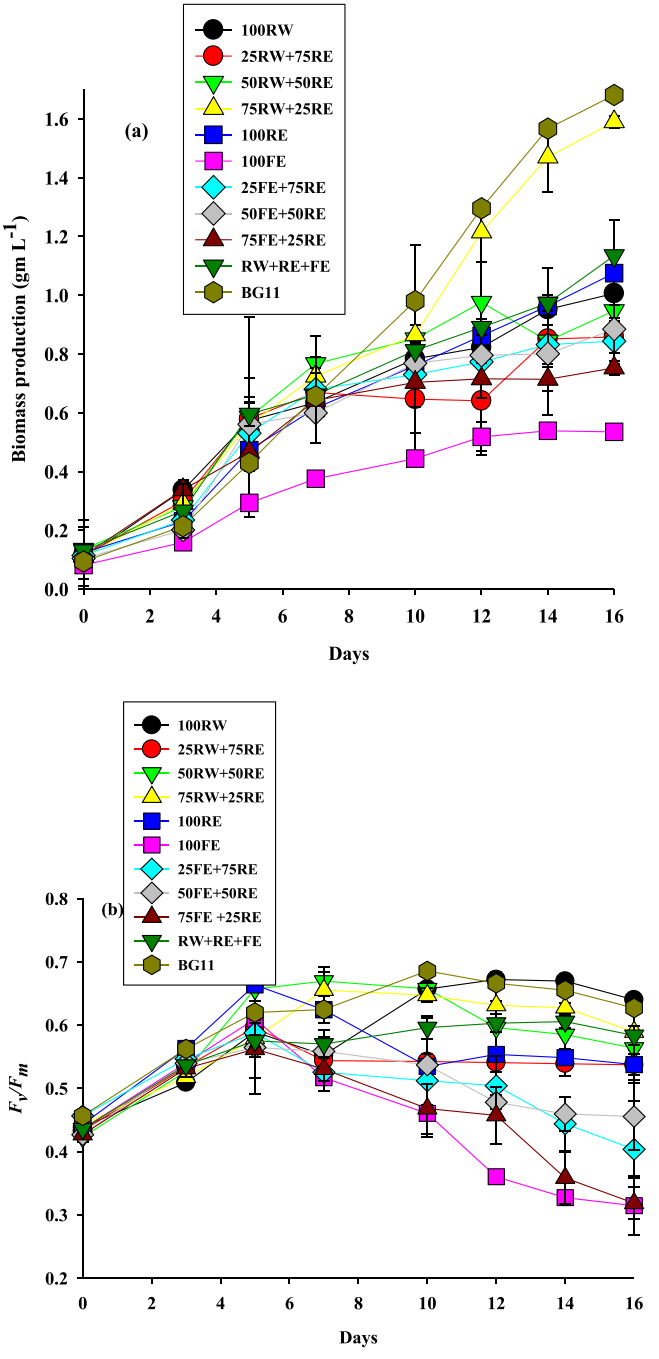

Fig. 1. Biomass production (a) and physiology (b) of *T. obliquus* grown in different dilutions of wastewater.

Mata et al. [27] reported 0.9 g L^{-1} biomass production and 64.28 mg L^{-1} d^{-1} productivity, respectively, when cultivating *S. obliquus* in brewery WW. Ansari et al. [28], cultivated *S. obliquus* in domestic WW and found that 0.89 g L^{-1} and 55 mg L^{-1} d^{-1}, production, and productivity respectively. Gupta et al. [1], observed that 50RW + 50FE was optimal for *S. obliquus* for the overall experimental duration while 75RW + 25FE provided marginally sub-optimal conditions. Apandi et al. [29] cultivated *Scenedesmus* sp. in meat market WW and they observed biomass productivity of 98.5 mg L^{-1} d^{-1}. Khalid et al. [30], observed significantly higher biomass productivity (107.5 mg L^{-1} d^{-1}) when cultivated *C. sorokiniana* in palm oil effluent. Findings indicate that types of algal strains and cultivation conditions have a major impact on microalga growth. It has also been reported that different phytohormones are released during microalgae cultivation by cell lysis that accumulate in harvested effluent [5]. Studies have shown supplementation of phytohormones in growth media improved biomass production and productivity [31–33]. Therefore, it is reasonable to assume that due to the presence of phytohormones in RE, biomass production was improved.

Fig. 1b shows the physiological (F_v/F_m) condition of *T. obliquus* grown in different diluted WW. The value of F_v/F_m in each dilution was between >0.5, which means there was no inhibitory effect on the photosynthetic system of *T. obliquus* except 100FE. Nevertheless, the value of F_v/F_m on the first day of dilution was around 0.43 and this was probably due to the new growth environment. As dilution increases with FE, the F_v/F_m values decrease. Changes in F_v/F_m values may be due to inappropriate nutrient profiles of the RW, RE, and FE as well as different ratios of P/N and COD/N. Under normal cultivation conditions, the values of F_v/F_m can be maintained however, in algae culture subjected to stress, the F_v/F_m value exhibits a decrease [34]. The 75RW + 25RE and 100RW remained healthy similar to the BG11 culture but started decreasing F_v/F_m value after 14 days as the nutrient started to become a limiting factor. Similar results were obtained by Satheesh et al. [22], when *C. sorokiniana* (0.57) *C. pyrenoidosa* (0.58) and *S. obliquus* (0.57) were cultivated in domestic WW.

Table 3
Biomass composition, production, and productivity of *T. obliquus* cultivated in different dilutions of wastewater.

Sample	Biomass production (g L^{-1})	Biomass productivity (mg L^{-1} d^{-1})	Lipid (%)	Lipid productivity (mg L^{-1} d^{-1})	Protein (%)	Protein productivity (mg L^{-1} d^{-1})	Carbohydrate (%)	Carbohydrate productivity (mg L^{-1} d^{-1})
100RW	1.01	62.88	27.85	17.51	30.50	19.18	27.97	17.59
25RW + 75RE	0.86	53.63	24.10	12.92	29.43	15.78	25.36	13.6
50RW + 50RE	0.95	59.22	19.22	11.39	30.64	18.14	25.6	15.16
75RW + 25RE	1.59	99.38	32.16	31.97	33.32	33.11	24.56	24.4
100RE	1.07	67.19	21.41	14.39	28.86	19.39	28.98	19.48
100FE	0.54	33.44	20.86	6.98	15.65	5.23	17.46	5.83
25FE + 75RE	0.84	52.72	24.08	12.69	29.98	15.81	23.61	12.45
50FE + 50RE	0.88	55.28	20.51	11.34	25.87	14.30	23.76	13.14
75FE + 25RE	0.75	47.03	25.87	15.4	20.89	12.44	24.22	14.42
RW + RE + FE	1.13	70.90	24.18	17.15	31.53	22.36	28.44	20.17
BG11	1.68	105.09	16.17	17	48.65	51.13	16.49	17.33

3.3. Effect of different growth media on Chlorophyll-a content and CHNS of T. obliquus

To evaluate the effect of the various proportions of RW, RE, and FE on the photosynthetic activity of microalgae, the contents of chlorophyll- *a* of *T. obliquus* were measured. The chlorophyll-*a* content of *T. obliquus* was in line with nutrient as well as biomass production (Table 2 and Fig. 1a). The highest chlorophyll-*a* content (14.49 μg mL^{-1}) was observed at 12 days of cultivation in BG11 media followed by 75RW + 25RE (14.07 μg $mL^{-1)}$. Cultures growing in 100FE, 75FE + 25RE, and 50FE + 50RE showed 3.19, 4.52, and 9 μg mL^{-1}, respectively. The chlorophyll-*a* content in 75RW + 25RE was 9.4 % and 77.32 % higher than microalgae grown in 100RW and 100FE, respectively. Similar chlorophyll content was observed in 75RW + 25RE (14.07 μg mL^{-1}) and BG11 (14.49 μg mL^{-1}). Satheesh et al. [22] found 9.34, 10.12 and 8.47 μg mL^{-1} chlorophyll-*a* content for *C. pyrenoidosa*, *S. obliquus* and *C. sorokiniana* when cultivated in domestic WW. As shown in Fig. 2, chlorophyll-*a* contents of microalgae gradually increased with increasing RW concentration. When the proportion of RW and RE reached 75RW + 25RE, the chlorophyll-*a* content of microalgae reached 14.073 μg mL^{-1} similar to BG11. These findings indicated that cultivating *T. obliquus* in` RW, and RE promoted chlorophyll synthesis (75RW + 25RE). This demonstrates an adaptive mechanism of microalgae to various dilutions of RW, RE, and FE environments. Thus, promoting the growth of microalgae in WW with a suitable dilution was required to improve productivity.

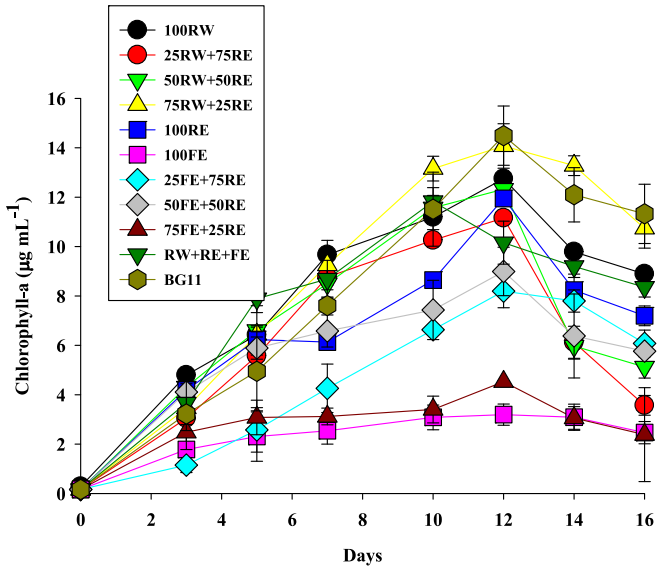

Fig. 2. Chlorophyll-*a* content (μg mL^{-1}) of *T. obliquus* grown in different dilutions of WW.

Table 4 shows the elemental composition of *T. obliquus*. The highest C content was observed in 75RW + 25RE (52.4 %), followed by RW + RE + FE (49.23 %), BG11 (48.72 %), 100RE (48.72 %), 50RW + 50RE (46.13 %), and lowest was 100FE (31.57 %). The N of *T. obliquus* cultivated in different diluted medium ranges from 5.7 to 8.49 % which was similar to the nitrogen content (6.64–7.72 %) reported by Pandey et al. [35]. High N content is attributed to the presence of a high concentration of total nitrogen in the culture medium. The ranges of H and N contents were (4.4–5.93 %) and (4.79–8.49 %), respectively. The N content directly depends on the presence of nutrients in the growth medium, where BG11 shows a higher N content than other growth conditions. Higher dilutions with FE resulted in a more remarkable decrease in N content in algal biomass since the nitrogen concentration in the diluted growth medium has a direct impact on the algal biomass. The range of O content was 35.27–53.17 % while S content in all types of biomasses was <1 % except BG11 (1.59 %) (Table 4). The findings suggest that the elemental composition of microalgae biomass varies from growth medium. The biomass shows high C and H content which makes them a suitable feedstock candidate for biofuel production as compared to other feedstock such as coal or lignocellulosic biomass.

The higher heating values of *T. obliquus* cultivated in different diluted WW are listed in Table 4. The highest HHV varied from 15.82 to 21.52Kg J^{-1}, where the highest HHV was observed in 75RW + 25RE and the lowest in 100FE. The lower oxygen content (34.74 %) in 75RW + 25RE growth medium resulted in a higher HHV of 21.52Kg J^{-1} which was similar to the previously reported study. Ansari et al. [28] observed 19.97Kg J^{-1} and 17.96Kg J^{-1} of HHV when they cultivated *S. obliquus* in WW and BG11, respectively. HHV is strongly dependent on biomass composition, mainly on oxygen-to-carbon (O/C) and hydrogen-to-carbon (H/C) ratios. It increased with decreasing O/C or increasing H/C ratio. Current findings suggest that because of the high C content, high HHV value and low N and S content in 75RW + 25RE-grown algae, the biomass can potentially be used as feedstock for biofuel production.

3.4. Nutrient and COD removal efficiency of T. obliquus cultivated in different wastewater

The nutrient removal efficiencies of *T. obliquus* in different diluted

Table 4
CHNSO, moisture and ash content (%) of *T. obliquus* cultivated in different dilutions of wastewater.

Sample	C (%)	H (%)	N (%)	S (%)	O (%)[a]	HHV (Kg J^{-1})[b]	Moisture (%)	Ash (%)
100RW	46.89	4.4	6.09	0.78	41.84	19.07	6.62	7.44
25RW + 75RE	44.27	4.88	6.02	0.98	43.85	18.24	6.21	7.87
50RW + 50RE	46.13	4.75	6.14	0.8	42.18	18.87	6.43	6.76
75RW + 25RE	52.4	5.27	6.71	0.88	34.74	21.52	6.65	5.34
100RE	48.72	5.42	6.02	0.69	39.15	19.94	6.54	8.82
100FE	36.57	4.93	4.79	0.54	53.17	15.82	7.12	12.34
25FE + 75RE	43.66	4.93	5.7	0.6	45.11	18.00	6.78	7.98
50FE + 50RE	43.36	4.91	6.26	0.79	44.68	17.97	6.34	8.9
75FE + 25RE	39.46	4.65	6.07	0.37	49.45	16.80	7.16	10.33
RW + RE + FE	49.23	5.23	6.72	0.82	38	20.19	6.12	5.89
BG11	48.72	5.93	8.49	1.59	35.27	20.39	6.21	5.54

[a] Calculated by differences.
[b] Calculated by HHV = $5.22C^2 - 319C - 1647H + 38.6C \times H + 133N + 21{,}028$ [17].

WW are shown in Fig. 3a–d. Fig. 3a depicts the nitrate removal efficiency of *T. obliquus* from different diluted WW. *T. obliquus* showed the highest nitrate removal efficiency in 100FE (93.09 %), which was due to low levels of initial nitrate concertation (10.07 mgL^{-1}) in FE followed by 75FE + 25RE (90.8 %), 50FE + 50RE (78.6 %) and 25RW + 75RE (78.44 %). Due to low initial concentration, nitrate removal efficiency in 100FE was significantly ($p < 0.001$) higher than other WW growth medium. Similarly, 75FE + 25RE showed significantly ($p < 0.001$) higher nitrate removal efficiency. Nitrate considerably decreased from 18.98 to 4.06 mg L^{-1}, 24.33 to 5.24 mg L^{-1}, 20.56 to 5.63 mgL^{-1} and 28.17 to 7.52 mg L^{-1} in 50FE + 50RE, 25RW + 75RE, 100RE and 75RW + 25RE, respectively after 16 days of cultivation. It was also observed that nitrate removal efficiency in all WW diluted samples was >70 % (Fig. 3a). Similar results were found by other researchers. For example, He et al. [36], cultivated *S. obliquus* and *C. pyrenoidosa* in swine water diluted (8 times) with domestic WW. They found 87.87 % and 100 % nitrate removal efficiency respectively, for *S. obliquus* and *C. pyrenoidosa*. Pandey et al. [37] observed 95.1 % nitrate removal efficiency when cultivated *Scenedesmus* sp. in outdoor conditions using nutrient-supplemented dairy effluent. It has been reported that low nutrient-containing growth medium, both algae and bacteria compete for nutrients which suppresses the growth of both species. However, in a nutrient-rich medium, algae and bacteria co-exist and support the growth of each other [1].

Fig. 3b shows the ammonia removal efficiency from different diluted WW. The initial concentration of ammonia ranges from 0.29 to 5.44 mg L^{-1} where the highest was in 100RW and the lowest in 100FE and 75FE + 25RE. In terms of removal efficiency, the highest ammonia removal

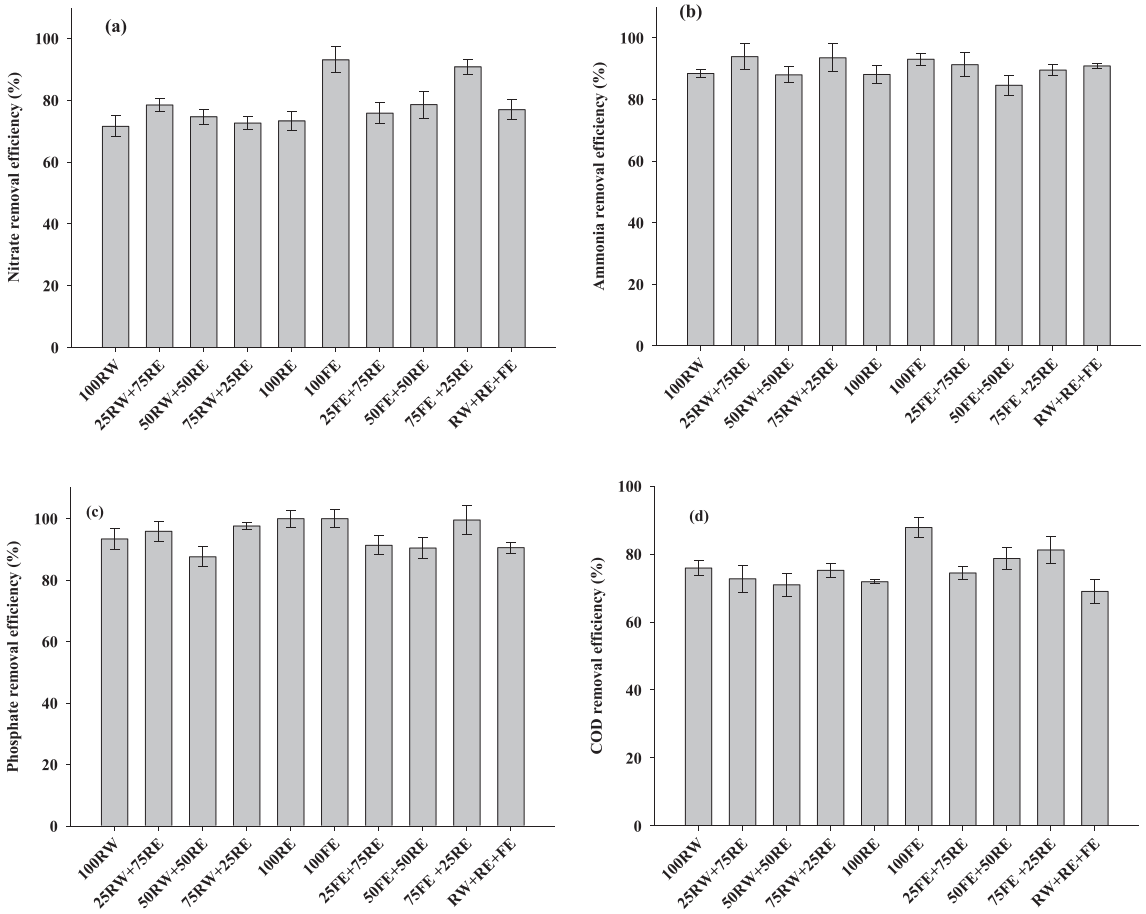

Fig. 3. Nutrients (a) nitrate, (b) ammonia, (c) phosphate, and (d) COD removal efficiencies of *T. obliquus* cultivated in different dilutions of wastewater.

efficiency was observed in 25RW + 75RE (93.89 %) and the lowest in 50FE + 50RE (84.59 %). In addition, the ammonia removal efficiency was higher in all WW growth media after 16 days of cultivation. There was no significant difference in ammonia removal efficiency in the selected growth medium except 25RW + 75RE and 50FE + 50RE ($p = 0.03$). The current finding is in agreement with Kong et al. [38], who found 91.6 % ammonia removal efficiency when they cultivated *Scenedesmus* in municipal WW. Similarly, Gupta et al. [39], cultivated *S. acutus* and *Tetradesmus* sp. at a ratio of 3:1 (v/v) using anaerobically digested dairy WW and untreated dairy WW in fed-batch mode. They observed 92 and 75 % of ammonia removal efficiency respectively. Ammonia in the growth medium was removed >80 %, mainly because ammonium serves as the preferred nitrogen source for microalgae growth which requires less energetic cost during its assimilation, followed by nitrate, nitrite and urea [39]. In algae culture, pH increases as growth progresses and changes in pH values are related to the photosynthesis of microalgae. Microalgae assimilate CO_2 and HCO_3^- through photosynthesis, which affects the inorganic carbon balance among CO_2, H_2CO_3, HCO_3^-, and CO_3^{2-}, resulting in an elevation of pH [40]. In addition, an increase in pH is responsible for some amount of ammonia removal by volatilization. Almomani et al. [41], found that the amount of ammonia removed by volatilization was 6 % and 2 % at working pH values of 9.6 and 7.5, respectively.

The changes in phosphate removal efficiency are depicted in Fig. 3c. The initial concentration of phosphate ranged from 1.5 to 4.53 mg L^{-1}, where 100RW contained the highest phosphate concentration while 100FE and 100RE were the lowest. Among the tested raw and different diluted WW, 100FE, 100RE and 75FE + 25RE, achieved the maximum phosphate removal efficiency of ~100 %, ~100 % and 99.55 %, respectively. Furthermore, *T. obliquus* removed >87.57 % phosphate in all tested dilutions. The phosphate removal efficiency increases when FE or RE is diluted with RW (Fig. 3c). The phosphorus removal efficiency is in line with previously reported research works. For example, Sivakumar and Kumar [42] found 90.7 % phosphate removal efficiency, when they cultivated *Scenedesmus* in municipal WW using a photobioreactor. Similarly, Miyawaki et al. [43] used *T. obliquus* for phytoremediation, and they observed that phosphorus (99.2 %) can be effectively removed from the biodigested swine manure medium, which is in agreement with the present study. The higher phosphorus removal could also be due to higher biomass production. However, the correlation between biomass production and phosphate removal in the current study was low. For example, 100FE, and 75FE + 25RE considerably showed the highest phosphorus removal but low biomass production. It can be suggested that factors such as pH other than assimilation could be taking place to remove phosphorus [44]. However, in the current study as the pH of the medium was not controlled, removal of phosphorous cannot be entirely attributed to microalgal uptake. The precipitation of phosphorus normally takes place in the ranges of pH values 8.9–9.5. Since the pH of most algae culture systems is usually higher than 8.5, it can be reasonably speculated that the precipitation of some number of phosphates with actions including Ca^{2+}, Fe^{2+} and $Mg^{2}+$ contributes toward the removal [45]. The other possible reason for the higher removal efficiency of ammonia is the presence of phytohormone in RE. It has been reported that phytohormone (e.g., IAA) promotes nitrogen metabolism in microalgae by increasing the activity of enzymes involved in nitrogen metabolism [34].

The COD removal efficiency (%) by *T. obliquus* grown in different diluted WW is shown in Fig. 3d. The initial concentration of COD ranges from 26 to 454 mgL^{-1}, where the highest was in 100RW followed by 382.3 mg L^{-1} (75RW + 25RE), 343.65 mg L^{-1} (50RW + 50RE), 275. 89 mg L^{-1} (25RW + 75RE) and lowest in 100FE. In terms of removal efficiency, highest COD removal efficiency was observed in 100FE (87.78 %), followed by 75FE + 25RE (81.17 %), 50FE + 50RE (78.66 %), RW (75.85 %) and lowest in RW + RE + FE (69 %). Due to low initial concentration (26 mg L^{-1}) of COD in FE, significantly ($p < 0.001$) higher COD removal efficiency compared to all growth medium (except 75FE + 25RE). This might be due to nutrient variation in the growth medium. The significant COD removal efficiency implies that *T. obliquus* was able to utilize organic carbon present in WW. *S. obliquus* can undertake mixotrophic growth when organic carbon and light are available. Mata et al. [27], observed 57.5 % of COD removal efficiency when cultivating *S. obliquus* in brewery WW. This finding was in accordance with the study by Katiyar et al. [46] they cultivated *Chlorella minutissima* and *Chlorella sorokiniana* to treat municipal WW, and removed 61.69 % and 72.17 % of COD, respectively. The COD removal efficiency was higher than the value reported by Ansari et al. [47] where 42 % of COD removal efficiency was observed when they cultivated *S. obliquus* in aquaculture WW. For instance, Nicodemou et al. [48], achieved 100 % COD removal efficiency when they adopted a two-stage strategy to cultivate *S. obliquus* in olive processing WW. It is evident from several studies that *T. obliquus* has promising potential to cultivate in different types of WW and reduce the COD, simultaneously producing valuable biomass (Table 5). The COD removal efficiency by different microalgae strains grown in different growth media is variable, due to the microalgae characteristics in metabolic pathway and enzyme activity.

3.5. Effect of different growth media on biomass composition and productivity

3.5.1. Lipid

Fig. 4a–c shows the lipid, protein and carbohydrate yield in algal biomass cultivated in different diluted WW. The different dilutions of WW within RW, RE and FE, reconstruct the concentration of the nutrient which implies the reallocation of carbon flux in the metabolic pathways of microalgae. Therefore, the biochemical composition of *T. obliquus* was investigated to unravel the effect of different dilutions of RW, RE and FE as shown in Fig. 4a–c. The lipid yield of *T. obliquus* was analyzed and the lipid productivities were calculated in combination with the biomass productivities of microalgae (Table 3). The maximum lipid yield (32.16 %) was observed in 75RW + 25RE and lowest in BG11 (16.17 %). Lipid yield (%) in 75RW + 25RE was significantly ($p < 0.001$) higher than other growth medium except for 100RW ($p = 0.063$) (Fig. 4a). Microalgae *T. obliquus* cultivated in BG11 medium showed ~50 % lower lipid yield compared to 75RW + 25RE.

As shown in Table 2, nutrient concentrations such as nitrogen and phosphorus were insufficient in the WW relative to those in the BG11 medium, even FE further diluted the nutrient concentration. Different abiotic stress such as temperature variation, light conditions, nutrient stress (especially nitrogen) metal ions, and high salt concentration are commonly used to enhance lipids yield [51]. Stress conditions promote oxidative stress in microalgae cells and thus accumulate ROS (reactive oxygen species) such as oxygen free radicals, leading to oxidative stress of microalgae cells and changes in cell composition. Previous studies have postulated that nitrogen stressed growth conditions might lead to improved lipid accumulation [28,51]. Apandi et al. [29] observed lipid yield of 23.2 %, when they cultivated *Scenedesmus* sp. in meat market WW. Kumar et al. [52] also observed 33.4 % of lipid yield in *Ascochloris* sp. when cultivated in raw dairy WW. Ganeshkumar et al. [53], observed significantly higher lipid content (51 %) when the cultivated *Chlorella* sp. MM3 diluted winery and piggery WW (80:20). Castrillo et al. [26], observed ~50 less lipid to the current finding when they cultivated *S. obliquus* in supernatant obtained after centrifugation and flocculation.

In terms of productivity, the maximum lipid productivity (31.97 mg L^{-1} d^{-1}) was observed in 75RW + 25RE (17.51 mg L^{-1} d^{-1}) followed by 100RW and RW + RE + FE (17.15 mg L^{-1} d^{-1}) while lowest was in 100FE (6.98 mg L^{-1} d^{-1}) (Table 3). Similar lipid productivity was observed by Han et al. [54] when *S. obliquus* was cultivated in WW obtained from primary (38 mg L^{-1} d^{-1}) and secondary tanks (33 mg $^{-1}$L d^{-1}). J. Li et al. [55] and Y. Li et al. [56] also observed similar lipid productivity of 31.2 mg L^{-1} d^{-1}, when they cultivated *Chlorella* sp. in municipal WW. Castrillo et al. [26], observed 26 mg L^{-1} d^{-1} of lipid productivity when they cultivated *S. obliquus* in supernatant obtained

Table 5
Microalgae cultivation in different growth biomass production, biochemical composition and nutrient removal efficiency.

Algae	Medium	Biomass production (g L^{-1})	Biomass productivity (mg L^{-1} d^{-1}),	Biochemical composition (%) L = Lipid P=Protein C=Carbohydrate	Lipid, protein, and carbohydrate productivity (mg L^{-1} d^{-1}) LP = Lipid pro PP=Protein Pro CP=Carb. pro	Nutrient removal (%)			COD removal efficiency (%)	References
						Nitrate	Ammonia	Phosphate		
S. obliquus	Domestic wastewater	0.90	–	L = 25.67 P = 35.76 C = 30.32	–	81.34	–	–	70–75	[22]
C. pyrenoidosa	Domestic wastewater	0.71	–	L = 25.34 P = 34.43 C = 33.21	–	78.3	–	–	70–75	[22]
C. sorokiniana	Domestic wastewater	0.76	–	L = 26.23 P = 33.35 C = 32.32	–	85.54	–	–	70.75	[22]
C. vulgaris	Aquaculture and pulp	–	187	L = 9.07 P = 47.5 C = 19.09		TN = 76.5		TP = 92.7	75.5	[49]
S. obliquus	Brewery	0.9	64.28	–	–	TN = 20.8	–	–	57.5	[27]
S. obliquus	Aquaculture Wastewater	1.25	89.28	L = 30.85 P = 19.52 C = 35.05	LP = 27.55 PP = 17.50 CP = 31.41	77.7	88.7	~100	42-	[47]
S. obliquus	Domestic Wastewater	0.89	55	L = 26.5 P = 28.3 C = 27.5	LP = 14.58 PP = 15.56 CP = 15.12	~100	81.9	94.1	71.2	[28]
S. obliquus	Domestic Wastewater	–	–	L = 28.36	–	TN = 98.54	–	97.9	76.3	[1]
Scenedesmus sp	Meat market		98.5	L = 23.2 P = 41.2	–	TN = 90	–	TP = 85	–	[29]
S. obliquus	Brewery effluent	–	–	L = 64 (bio-oil) P = 31.4 C = 0.2-1mgmL^{-1}(phenol)	–	TN = 88	–	TP = 30	71	[50]
T. obliquus	75RW + 25RE	1.59	99.38	L = 32.16 P = 33.32 C = 24.56	LP = 31.38 PP= CP	72.63	93.52	97.59	75.18	This study
T. obliquus	100RE	1.07	67.19	L = 21.41 P = 28.86 C = 28.98	19.39	73.3	88.12	83.29	71.84	This study
T. obliquus	100RW	1.006	62.875	L = 27.85 P = 30.5 C = 27.97	29.17	71.54	88.42	93.38	65.85	This study
T. obliquus	RW + RE + FE	1.13	70.90	L = 24.18 P = 31.53 C = 28.44	22.36	76.96	90.89	90.55	69	This study

L = Lipid (%), P = Protein (%), C = Carbohydrate (%), LP = Lipid productivity, PP = Protein productivity, CP = Carbohydrate productivity, TN = Total nitrogen, TP = Total phosphate, COD = Chemical oxygen demand.

after centrifugation and flocculation. Wang et al. [57] cultivated *Chlorella* sp. in digested dairy manure (20× dilution) and digested manure (no dilution) and they found 11 and 7 mg L^{-1} d^{-1} of lipid productivity respectively, which was significantly lower than the current finding.

3.5.2. Protein

As depicted in Fig. 4b, the protein yield of *T. obliquus* was measured in different diluted WW. The results show that protein content is considerably affected by the nutrient concentration in the growth medium. Due to optimal nutrient concentration in BG11, the highest protein (48.65 %) was observed which was significantly ($p < 0.001$) higher than other growth medium. Among other growth medium, *T. obliquus* cultivated in 75RW + 25RE yielded (33.23 %) higher protein content whereas the lowest was observed in 100FE (15.65 %). Protein yield in 75RW + 25RE was significantly ($p < 0.001$) higher than 100FE, 50FE + 50RE and 75FE + 25RE. Due to low initial concentration of nitrogen as FE dilution increases in RW or RE protein content decreases. In comparison to the BG11 medium 31.51–67.83 % reduction in protein yield was observed when *T. obliquus* was cultivated in raw and different diluted WW which clearly resembled the variation of nutrient concentration, especially nitrogen. A similar protein content (35.76, 34.43 and 33.35 %) was observed by Satheesh et al. [22] when they cultivated *S. obliquus, C. pyrenoidosa* and *C. sorokiniana* respectively, in domestic WW. Ferreira et al. [50], found 31.4 % of protein yield when they cultivated *S. obliquus* in brewery effluent. Renuka et al. [31], cultivated *Acutodesmus obliquus* in a modified BG11 medium supplemented with kinetin (1 mg L^{-1}) and they found 30.14 % of protein content. In contrast, Apandi et al. [29], obtained a higher (41.2 %) protein content when they cultivated *Scenedesmus* sp. in meat market WW. Findings show that protein content was highly depends on growth medium specially nitrogen concentrations.

In terms of productivity, highest protein productivity was observed in BG11 (51.13 mg L^{-1} d^{-1}) and lowest in 100FE (5.83 mg L^{-1} d^{-1}) (Table 3). 100FE demonstrated the lowest protein productivity due to lower biomass production and productivity. Additionally, the drop in cellular nitrogen reduces cellular nitrogen-rich components such as chlorophyll, amino acids, proteins, and nucleotides. Under nitrogen stress conditions, these cellular components get degraded to form ammonium to perform other metabolic biosynthesis processes.

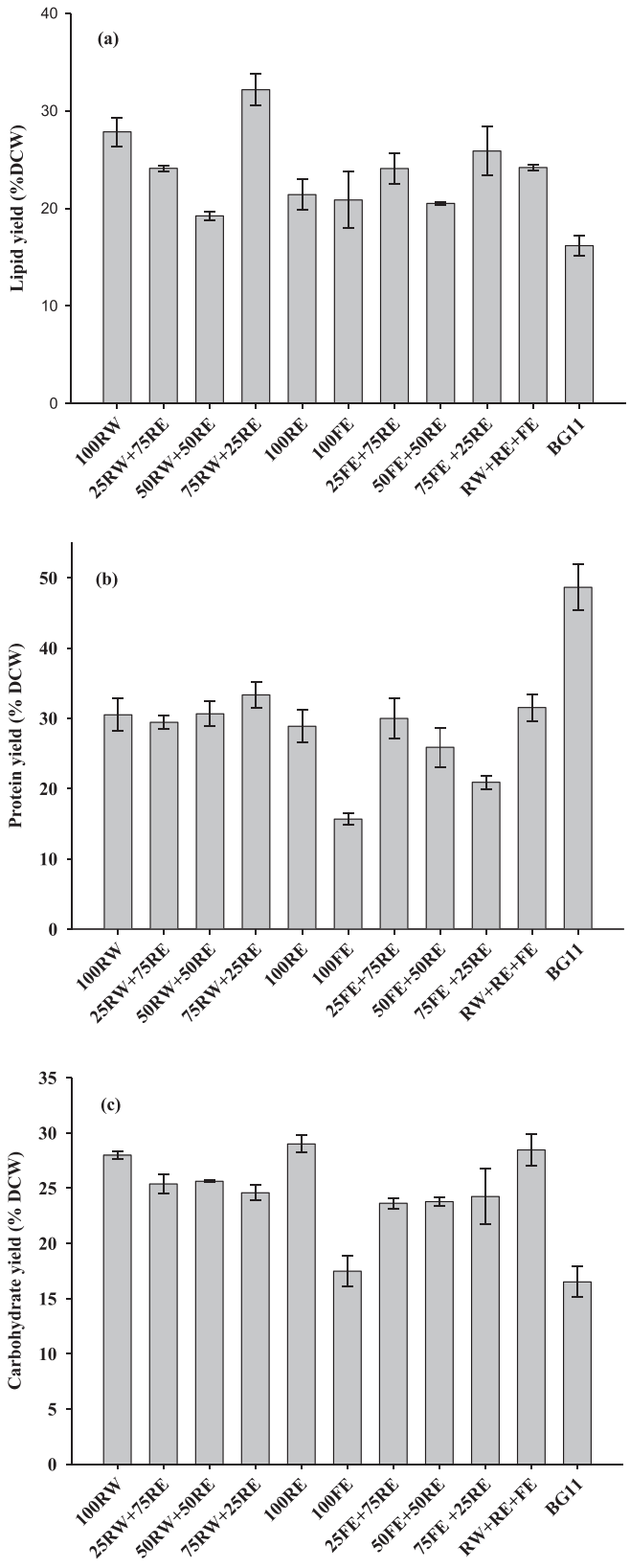

Fig. 4. Biochemical compositions of lipid (a) protein (b) and carbohydrate (c) of *T. obliquus* grown in different dilutions of wastewater.

Ramsundar et al. [58], observed similar protein productivity (23.88 mg L^{-1} d^{-1}) when cultivated *C. sorokiniana* mixotrophically using a combination of waste activate sludge and final effluent of domestic WW.

3.5.3. Carbohydrate

Carbohydrates are another important component of microalgae biomass. The carbohydrate-rich biomass could provide a carbon source for animal feeds. It can also be used to further convert value-added products through fermentation. The carbohydrate content of *T. obliquus* grown in different diluted WW is shown in Fig. 4c. Similar to lipid yield, it was found that the *T. obliquus* cultivated in raw and different diluted WW attained higher carbohydrate compared to BG11 (16.49 %). The ranges of carbohydrate yield in different diluted WW were 17.46–28.98 %. For example, carbohydrate yield in 100RE, RW + RE + FE, 100RW, 50RW + 50RE were 28.98, 28.44, 27.97 and 25.6 %, respectively. The corresponding carbohydrate productivity achieved were 19.48, 20.17 and 15.16 mg L^{-1} d^{-1}, respectively. Interestingly, even lower carbohydrate yield (24.56 %) in 75RW + 25RE the carbohydrate productivity (24.4 mg L^{-1} d^{-1}) was higher than the 100RE, RW + RE + FE, 100RW, 50RW + 50RE (Table 3). Carbohydrate yield in all WW grown medium (except 100FE) was significantly ($p \leq 0.001$) higher than BG11, while there was no significant difference ($p = 0.990$) observed between BG11 (16.49 %) and 100FE (17.46 %). Moreover, in continuation to the earlier observations related to biomass, chlorophyll-*a*, lipid and protein yield in 100FE were lowest, possibly due to limited nutrient availability to support growth and metabolic process.

Most microalgae tend to accumulate carbohydrate and lipid under nutrient stress, especially nitrogen. Nutrient stress conditions could lead to stimulate the storage of energy substances as a self-protective strategy under adverse conditions. Daneshvar et al. [49] cultivated *C. vulgaris* in aquaculture and pulp WW and found 19.09 % carbohydrate content. Similarly, Gao et al. [59], cultivated *C. vulgaris* in vinegar WW and observed 20.3 % carbohydrate content. Satheesh et al. [22] cultivated *S. obliquus, C. pyrenoidosa* and *C. sorokiniana* in domestic WW and found 30.32 % 33.21 % and 32.32 % respectively of carbohydrate yield.

Our current study was in agreement with this observation, in which WW contained limited nutrient concentrations compared with BG11 medium, which indicates a stress condition. Algae cultivated in a limited nitrogen medium modified their metabolic pathway to survive in unfavorable conditions where they start producing more carbohydrates. Among this great diversity of potential products, the direct application of biomass as fertilizer has attracted much interest due to their high N and P content [60]. This is especially true for the microalgae cultivated in WW where the assimilation of nutrients into biomass is the major uptake mechanism. In the present study, biomass production, biochemical composition of biomass and elemental analysis of *T. obliquus* biomass was higher when cultured in 75RW + 25RE. Therefore, this carbohydrate and lipid rich biomass could be used for biofuel production and residual biomass subsequently be reapplied as fertilizer.

3.6. CO_2 sequestration of *T. obliquus* cultivated in different wastewater

Effects of raw and different diluted WW on CO_2 fixation of *T. obliquus* are depicted in Table 6. The highest CO_2 fixation rate was observed in 75RW + 25RE (0.19 gCO_2 L^{-1} d^{-1}) followed by, BG11 (0.19 gCO_2 L^{-1} d^{-1}). However, the CO_2 fixation rate was approximately 79 % higher in 75RW + 25RE and BG11 than in 100FE (Table 6). The finding shows that CO_2 fixation depends on biomass productivity, carbohydrate yield and C (%) content of biomass. Due to low nutrients in 100FE lower biomass productivity, carbohydrate yield carbon content and CO_2 fixation were obtained. The CO_2 fixation rate is significantly affected by the growth conditions provided. CO_2 fixation rate of *T. obliquus* cultivated in 75RW + 25RE was relatively higher than the values reported during *Scenedesmus acutus* LB0414 (0.08 gCO_2 L^{-1} d^{-1}) and *Graesiella* sp. WBG-1 (0.01 gCO_2 L^{-1} d^{-1}) cultivation in modified BG11 (raceway pond) and BG11 (flat-plate), respectively [61,62]. Ho et al. [63] cultivated *S. obliquus* CNW-N using a modified Detmer's medium in tubular PBR to see the effect of seasonal variation on CO_2 fixation rate. They found that during summer (0.34 gCO_2 L^{-1} d^{-1}) *S. obliquus* showed a higher CO_2

Table 6
CO_2 fixation rate and TBMP of *T. obliquus* grown in different dilute wastewater.

Growth medium	CO_2 fixation rate ($gCO_2\ L^{-1}\ d^{-1}$)	TBMP ($mLCH_4\ g^{-1}$ VS)
100RW	0.11	375.05
25RW + 75RE	0.09	412.04
50RW + 50RE	0.10	375.41
75RW + 25RE	0.19	471.54
100RE	0.12	430.22
100FE	0.04	262.27
25FE + 75RE	0.08	350.69
50FE + 50RE	0.09	345.13
75FE + 25RE	0.09	287.65
RW + RE + FE	0.13	428.88
BG11	0.19	441.85

fixation rate than in winter (0.20 $gCO_2\ L^{-1}\ d^{-1}$). Xu et al. [64] carried out a detailed review of current advances in CO_2 fixation by microalgae with various cultivation modes, including a high-rate algal pond, and observed that algae manage to fix CO_2 from 0.02 g L^{-1} day^{-1} to 2.16 g L^{-1} day^{-1}. The current study demonstrates that the range established in the present experiment's mixtures is consistent with those discovered in published literature.

3.7. TBMP of T. obliquus grown in different diluted wastewater

Theoretical biochemical methane potential (TBMP) represents the theoretical maximum volume of biomethane that would be yielded if the biomass was transferred directly to fermentation and fermented completely [65]. TBMP was calculated of *T. obliquus* biomass cultivated in raw and diluted WW (Table 6). The ranges of TBMP were 262.27–471.54$mLCH_4\ g^{-1}$ VS where maximum TBMP was observed in 75RW + 25RE and minimum in 100FE. The results show that with a higher dilution of RE with FE, the TBMP decreased considerably. The TBMP obtained in 75RW + 25RE was 6.29 %, 9.04 %, 8.76 %, 12.61 %, higher than BG11, RW + RE + FE, 100RE and 25RW + 75RE, respectively. While TBMP in 75RW + 25RE (471.54$mLCH_4\ g^{-1}$ VS) was 44.38 % higher than 100FE (262.27$mLCH_4\ g^{-1}$ VS). Klin et al. [65], cultivated *Monoraphidium contortum* BA-5, *Monoraphidium* sp.BA-165, and *Chlorella vulgaris* BA-2 in modified F/2 for TBMP and they found 267, 255, and 244$mLCH_4\ g^{-1}$ VS, respectively which was lower than the current finding. Parimi et al. [66], compared the theoretical and experimental CH_4 production in the original biomass of *S. platensis*, disrupted biomass and protein extracted biomass. The theoretical CH_4 production of original biomass, disrupted biomass and protein extracted biomass were 558.4, 558.4 and 374 CH_4 yield (mL g^{-1} VS), respectively. However, the experimental CH_4 yield was considerably lower than the theoretical such as 181.1, 245.5 and 236.1 CH_4 yield (mL g^{-1} VS) respectively, for original, cell disrupted, and protein extracted biomass. Due to several assumptions (e.g., constant temperature, ideal bacterial conditions, no accumulation of ashes, etc.) higher CH_4 production was achieved [21]. The biochemical methane potential of microalgae biomass is significantly influenced by substrate to inoculum ratio, biomass concentration and pretreatment method.

3.8. Economic feasibility assessment of commercialization

Industrial scale manufacture of microalgal products is restricted to but a few items, primarily due to the high costs of production. An analysis by J. Li et al. [55] and Y. Li et al. [56] extrapolated in detail the costs involved in the hypothetical large-scale production of astaxanthin from *Haematococcus pluvialis* compared to current synthetic astaxanthin. The study showed that commercial astaxanthin prices were reported at 2000-3000USD kg^{-1} whereas final costings for natural astaxanthin were estimated at 718USD kg^{-1} and 14USD kg^{-1} for biomass. The study found that the production of natural astaxanthin was theoretically feasible due to the low cost of equipment technology and manpower (e.g., Chinese and American labour costs at ~120USD kg^{-1} and ~600USD kg^{-1}, respectively). Bhandari et al. [11] estimated that for the production of 100 kg d^{-1} of *S. obliquus* biomass (DCW), the freshwater and nutrient (BG11) cultivation costs were 56.06 and 18,097.51 USD, respectively.

In the present work, the use of WW limits biomass application toward bioenergy and agriculture. Cultivation of *T. obliquus* using 75RW + 25RE shows a similar pattern of biomass production and CO_2 fixation rate to BG11, while lipid, carbohydrate, and TBMP were higher than BG11. Therefore, this work suggests that 75RW + 25RE is more suitable for the growth of *T. obliquus* for biomass, biochemical components and TBMP, where no additional nutrients are required for cultivation.

Based on 75RW + 25RE as the ideal culture medium, the cost for nutrients and water would be fully eliminated compared to artificial medium (BG11) costing ~338USD L^{-1} [67]. A lab-scale economic analysis by Colusse et al. [68] demonstrated that nutrients N, P and K (potassium) had the highest costs, with approximate media costs for BG11, CHU and WC at 18.36, 11.55 and 2.61USD 10^{-3} L, respectively. While NPK required for cultivation are expensive, they are vital for cell growth and metabolism. This further emphasizes the importance of nutrient recycling in culture media, especially on a large scale.

Several case studies have reported the projected economics of microalgal biomass and the final product for scaled-up scenarios. For example, Branco-Vieira et al. [69] reported the cost of biomass around 2.18USD kg^{-1} and 0.33USD L^{-1} for biodiesel. Yang et al. [3], investigated the life cycle assessment for water and nutrients usage of microalgae-based biodiesel production and reported 3726, 0.33 and 0.71 kg of water, N and P respectively, would be required to produce 1 kg of biodiesel. Fernández et al. [70] reported that operational expenditure for microalgal biomass production ranged from 108.88 to 10.89USD kg^{-1} using closed bioreactors and raceways, respectively, where this value could be decreased to 1.09USD kg^{-1} if effluents are utilized.

It can be concluded that microalgal growth in 75RW + 25RE is appropriate for selected applications, producing rich biomass. Furthermore, the proposed methodology (75RW + 25RE) will help in attaining economic sustainability on a commercial basis owing to the lack of nutrient demand. Therefore, the combination 75RW + 25RE could be considered as a suitable algae growth media for industrial scale cultivation for relevant applications. Overall, this strategy has potential to reduce biomass production costs by translating same technologies on a larger scale.

4. Conclusion

In this study, the biomass production, biochemical composition, CO_2 fixation rate and TBMP of microalgae were successfully improved by *T. obliquus* in WW and containing algae-harvested effluent. The highest productivities of microalgae, lipid, protein, carbohydrate and CO_2 sequestration were obtained in 75RW + 25RE. Moreover, the addition of 25RE in RW effectively improved the nutrient requirements which was conducive to efficient biomass production and CO_2 sequestration from the environment. The biochemical composition of *T. obliquus* was significantly affected by the different dilutions and could be used as a high impact feedstock for value added products such as aquaculture feed, animal feed, food additive, or as feedstock for bioenergy production. The process of 75RW + 25RE improving biomass production mainly included suitable nutrient concentration and possibly the presence of phytohormones in RE. The study showed that combined WW treatment and algal cultivation is an effective way for resource recovery, biomass production and reducing environmental pollution. This provided a basis for further research on how to utilize the RE and further scale up for improving biomass production for various applications.

CRediT authorship contribution statement

Humeira Hassan: Writing – review & editing, Writing – original

draft, Investigation, Formal analysis, Data curation. **Faiz Ahmad Ansari:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ismail Rawat:** Writing – review & editing, Writing – original draft, Conceptualization. **Faizal Bux:** Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare no conflict of competing or financial interests.

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Data availability

Data will be made available on request.

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