



# Growth Performance and Biochemical Composition of *Desmodesmus* sp. Green Alga Grown on Agricultural Industries Waste (Cheese Whey)

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**Abstract** Agricultural industries produce vast amounts of liquid waste, which contains a significant concentration of nutrients. In the context of emphasizing the huge increase in population, climate changes, and pollution which results in depletion of fresh water resources, a sustainable solution for a greener future is needed. Wastewater treatment by the use of microalgae can mitigate a part of the problem by restoring water for irrigating agricultural crops. Little studies give insights on the physiological responses and ultrastructure of the Chlorophyta alga *Desmodesmus* sp. as it grows in cheese whey (CW). The algal strain was mixotrophically grown in a growth medium composed of CW only and CW supported with Bold's basal medium (BBM) for 14 days. The potent response was observed with algal cultures fed by 15% CW enriched with 50% BBM. Fifteen percent CW in combination with 50% BBM

significantly improved *Desmodesmus* sp. growth (303%), productivity (325%), and accumulation of cell metabolites, mainly lipids (3.89%), and carbohydrates (1.95%). On the contrary, protein and photosynthetic pigment (chlorophyll *a*, chlorophyll *b*, and carotenoids) contents were higher in BBM than in all treatments. Fatty acid composition demonstrated that the predominantly accumulated fatty acids were palmitic (25.86%), oleic (35.31%), and linoleic acid (13.22%). In conclusion, *Desmodesmus* sp. can be a good candidate for phycoremediation when cultivated on CW, whereas it can reduce the nutrition costs and water demand of algal cultivation by 50% and 15%, respectively. Therefore, it may be an effective strategy for algal mass production in sustainable agricultural systems.

**Keyword** Algal biomass · Biochemical composition · Cheese whey · *Desmodesmus* sp. · Phycoremediation · Water scarcity

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## 1 Introduction

Fresh water availability has a crucial role in sustaining agricultural production. Many countries are suffering from severe water pollution and shortage resulting from the rapid increase in population (Sofy et al., 2022). It is well known that huge amounts of water are consumed to meet household, industrial, and agricultural needs which affects the quantity and

quality of available water (El-Mahdy et al., 2021). The planet contains approximately 1400 million km<sup>3</sup> of water resources (F.A.O., 2017), of which only 3% is fresh, usable water (Kılıç, 2020).

Contaminated industrial wastewater, overloaded with excessive nitrogen and phosphorus, known as eutrophication, jeopardizes marine ecosystems (Dawood et al., 2023). Cyanobacterial algal blooms degrade water quality in aquatic ecosystems (Bali & Gueddari, 2019; Sonarghare et al., 2020; Su et al., 2022). Low levels of dissolved oxygen resulting from cyanobacterial algal bloom lead to fewer number of fish, which is a significant loss of important food sources for some mammals and birds, leading to the death of some marine animals (Kim et al., 2020; Sonarghare et al., 2020). Furthermore, eutrophication limits sunlight penetration required by plants for photosynthesis, thus resulting in an eventual destruction of sea grass bed (Almanassra et al., 2021; Kim et al., 2020). In addition, excessive growth of algae clogs irrigation and water pipes, thereby affecting transportation and power generation. Also, the increased rate of eutrophication of aquatic ecosystems has been associated with the rise of new diseases (Matei & Racoviteanu, 2021). Moreover, the existence of nitrates in drinking water has been reported to form carcinogenic compounds (Matei & Racoviteanu, 2021).

Nowadays, conserving water resources and reducing wastewater pollutants require highly effective methods that are cost-effective and eco-friendly to maintain climate change and ecological stability (Bhatt et al., 2021). The United Nations Water (UN Water) estimates that the number of those who will suffer from water scarcity will increase to about 1.8 billion people by 2025 (Awad et al., 2019; Baskar et al., 2022). Taking early precautions, including wastewater treatment, may aid in providing clean and safe water for drinking, agriculture, or industrial use. Some of the common wastewater treatment techniques include chemical treatment, physical treatment, the use of biological organisms, and sludge treatment (Kamali et al., 2019).

Recently, global demand and consumption of dairy products is elevating, which led to an enormous growth of dairy industries with the repercussion of dairy waste production including whey, dairy sludges, and wastewater. They are characterized by their high nutrient concentration, biological oxygen demand

(BOD), chemical oxygen demand (COD), and organic and inorganic contents (Ahmad et al., 2019).

Dairy wastewaters can be treated by physio-chemical, biological, and biotechnological methods. The physio-chemical treatment (coagulation or flocculation) is used for destruction of milk fat and protein colloids in dairy wastewater, requiring considerable amount of chemicals, energy, and operating costs. Biological treatments including the use of microalgal cultivation in dairy wastewater have been proposed in recent years. Several algal species including *Chlorella pyrenoidosa*, *Anabaena ambigua*, *Scenedesmus abundans*, *Chlorella vulgaris*, *Chlamydomonas polypyrenoides*, and *Acutodesmus dimorphus* have been successfully cultivated in dairy wastewater (Brar et al., 2019). Biological treatments are considered a more eco-friendly approach than physio-chemical methods. Moreover, aerobic along with anaerobic process treatments can be applied in order to reach the effluents' discharge limits for dairy wastewater. Biotechnological processes are implemented recently and can result in the formation of significant products to the industries, such as whey-derived products, bioplastics, biofuels, bioenergy, organic acids, bioactive peptides, and enzymes (Ahmad et al., 2019).

Microalgae are photosynthetic microorganisms that can grow in wastewater as an alternative culturing medium adding to their ability in sequestering carbon from flue gases (Muhammad et al., 2020). When exposed to sunlight, they can carry out photosynthetic processes that turn carbon dioxide into biomass. Microalgae have long been used by humans in the industrial sector; one such use is wastewater treatment, which is effective and energy-efficient in eliminating pollutants (Tan et al., 2023). Wastewaters are typically contaminated with nitrogen, phosphorus, and other trace elements, which microalgae require for their cell growth (Goyal et al., 2023). Additionally, they have high affinity for absorbing heavy metals (Abd El-Hameed et al., 2018; El-Beltagi et al., 2022). Using this strategy, a dual benefit can be obtained by removing pollutants and accumulating algae biomass (Rodríguez-Rángel et al., 2022). Besides bioremediation, the resulting algal biomass can be used to produce biofuel, biofertilizers, and economical bioproducts (Silva et al., 2020), along with fish feed (Ansari et al., 2021). Several microalgal species, such as *Desmodesmus*, *Chlorella*, *Scenedesmus*, *Neochloris*, *Chlamydomonas*, *Nitzschia*, and

*Cosmarium*, have been applied to various types of wastewater treatments coupled with biofuel production under sterilized or non-sterilized conditions (Madakka et al., 2019). More specifically, the green microalga *Desmodesmus* sp. is adaptable to different growth conditions. It can grow under autotrophic, mixotrophic, and heterotrophic conditions (Ríos et al., 2018). Also, it can survive and grow efficiently under a wide range of temperatures, light intensity, CO<sub>2</sub>, and pH (Chiu et al., 2016; Ferreira et al., 2021).

There is no doubt that large-scale algal production cultivation requires a huge amount of fresh water for cultivation. Large water consumption is necessary, as microalgae are aquatic species that require a substantial amount of water for survival and cell proliferation (Farooq et al., 2015). According to Nogueira Junior et al. (2018), the production of 1 kg of algae requires 1564 l of water under pond conditions and 372 l when photobioreactors are used. Therefore, recent studies are focusing on cultivating algae using agricultural and industrial wastewater (Abdelfattah et al., 2023). In this regard, agro-industrial wastewater effluents of apple vinegar (Giovanardi et al., 2013), cheese whey (Ribeiro et al., 2017), vinasse (Santana et al., 2017), palm oil mill (Kamyab et al., 2019), and molasses (Yew et al., 2020) were used for cultivating algae. For example, Wang, Wang, Miao, and Tian (2018) evaluated the ability to use tofu whey as an alternative medium for *Chlorella pyrenoidosa* under mixotrophic and heterotrophic conditions.

Consequently, agro-industrial carbon-rich wastes as an alternative, sustainable, and low-cost source of nutrients are an attractive approach for raising algae production, with a particular interest in high-value-added co-products such as vitamins, proteins, and essential amino acids, monounsaturated and polyunsaturated fatty acids, polysaccharides, minerals, and pigments, to improve the economics (Barone et al., 2019; de Medeiros et al., 2020; El-Sayed et al., 2020; El-Sayed et al., 2022; Fetyan et al., 2022; Kamal et al., 2017). For example, Yuan et al. (2021) cultured eight microalgal species of *Scenedesmus* and *Desmodesmus* on potato wastewater to purify and produce valuable by-products.

The dairy industry, as an example of the agricultural industry, is usually considered the largest industrial food wastewater source, especially in Europe (Karadag et al., 2015). The main important by-product in milk processing is whey, which accounts

for about 85–95% of milk volume and 55% of milk components. Carbohydrates, mostly lactose, represent about 4.8–5% of whey (Carvalho et al., 2013; Pires et al., 2021). Endogenous  $\beta$ -galactosidase activity was exhibited in many species of *Chlorophyceae*, so it could be a potential candidate for cultivation in a lactose-containing medium (Li et al., 2023). In Egypt, the production of Domiati cheese was 326,000 tons in 2015, and its production increases annually by 2.4% (El Soda & Awad, 2022). According to de Almeida Pires et al. (2022), *C. vulgaris* can grow on cheese whey and efficiently consume carbon, nitrogen, and phosphorous from this medium. Moreover, Pereira et al. (2019) studied the mixotrophic cultivation of *Spirulina platensis* in Zarrouk's medium diluted with different concentrations of mozzarella cheese whey. They found that biomass production, biochemical composition, and antioxidant capacity were positively affected.

In the context of emphasizing the huge increase in population, climate changes, and pollution which results in depletion of fresh water resources, a sustainable solution for a greener future is needed. Wastewater treatment by the use of microalgae can mitigate a part of the problem by restoring water for irrigating agricultural crops. The present study aims for a dual benefit in terms of wastewater treatment and a green biofuel production using an eco-friendly candidate. Using agro-industrial wastewater, specifically cheese whey, as an alternative culturing medium, a green microalga *Desmodesmus* sp. was cultivated. The physiological performance of *Desmodesmus* sp. and its ability to accumulate biomass with value-added products were evaluated to confirm the vital role of microalgae in the bioremediation of wastewaters and its potential to produce biofuel as an alternative source of energy.

## 2 Materials and Methods

### 2.1 Microalga Isolate and Growth Conditions

The green microalga *Desmodesmus* sp. was obtained from the Algal Biotechnology Unit, National Research Centre, Dokki, Giza, Egypt. An actively growing culture was maintained in Bold's basal medium (BBM) according to Bischoff (1963) at pH 7.1, temperature  $25 \pm 2$  °C, under continuous

illumination of about 5000 lux provided by white fluorescent lamps, along the incubation period (7 days). Using a batch-mode culturing system for *Desmodesmus* sp., both experiments were performed using 1-l flasks. Aeration was performed through a sterilized air system using an air pump (power 5 W and max flow 3.5 l min<sup>-1</sup>) and a disposable syringe filter (0.45 µm).

## 2.2 Experimental Design

Domiaty cheese is one of the most popular soft white pickled cheeses in Egypt. Its production was 326,000 tons in 2015 and increases annually by 2.4% (El Soda & Awad, 2022). Whey is the main by-product of the dairy industry which is produced during cheese manufacturing. Through two experimental stages, Domiaty cheese whey was used as an alternative culturing medium in comparison to BBM growth medium as a control. CW was directly collected from the Dairy Technology Unit, Faculty of Agriculture, Cairo University, Egypt, and stored at -20 °C until use. The composition of cheese whey (CW) was determined by measuring the following parameters: color, pH, biological oxygen demand (BOD), chemical oxygen demand (COD), moisture, lactose, protein, fat, nitrogen, sodium, potassium, calcium, iron, magnesium, and phosphate (Table 1).

For stage I, four different concentrations of CW (i.e., 5, 10, 15, and 20 % v/v BBM medium) with a working volume of 700 ml were autoclaved at 121°C, 1.5 bar, for 20 min. Under this condition, all treatments were inoculated with 0.1 g l<sup>-1</sup> of pre-washed *Desmodesmus* sp. then inoculated flasks were incubated for 14 days under optimum growth conditions

**Table 1** Composition of the used cheese whey

Parameter	value	Parameter	value
Color	Yellowish	Nitrogen*	0.47
pH	6.10	Sodium*	8.22
BOD*	2.09	Potassium*	1.284
COD*	35.22	Calcium*	0.42
Moisture %	89.45	Iron**	3.74
Lactose*	65.91	Magnesium**	98.03
Protein*	3.80	Phosphate**	55.97
Fat*	2.41		

\*Concentration unit g l<sup>-1</sup>, \*\*concentration unit mg l<sup>-1</sup>

as described above. At this stage, the best concentration of CW for *Desmodesmus* sp. growth will be selected.

Stage II: For enhancing *Desmodesmus* sp. growth, the best growth-promoting concentration of CW for *Desmodesmus* sp. was 15%, as resulted from stage I. In stage II, a combination of 15% CW and three different concentrations of BBM (25, 50, and 75 % v/v) were examined compared to the control (100% BBM). Autoclaving, inoculation with *Desmodesmus* sp., and incubation were carried out as previously described.

At a 2-day interval, using 3 replicates for each treatment, dry weight, photosynthetic pigments (Chl *a*, Chl *b*, and carotenoids), and pH were determined. Also, total protein, carbohydrates, and lipids were measured at 14 days of the incubation period. The trophic mode, in addition to the initial concentration for both nutrients (N, P, K, Na, Mg, and Fe) and lactose, is listed for all treatments in Table 2.

## 2.3 Analytical Method

### 2.3.1 Microalga Growth Determination and Media Reaction Measurement (pH)

The growth of microalga was determined through dry weight (DW) measurement, specific growth rate ( $\mu$ ), and biomass productivity. For dry weight, 10 ml of homogenized *Desmodesmus* sp. culture was filtered over a pre-weighed (w1) Sartorius cellulose nitrate sterile membrane filter (pore size 0.45 µm), and filters were dried at 105°C in a circulated oven (until getting constant weight). Dried filters were kept over anhydrous calcium chloride until room temperature and re-weighed (w2). The difference between weights (w1 and w2) indicated the net dry weight of the grown alga within a defined sampling time. At the exponential growth phase, *Desmodesmus* sp.-specific growth rate and biomass productivity were calculated according to Liang et al. (2013) as follows:

$$\text{Specific growth rate } \mu \text{ (day}^{-1}\text{)} = \text{Ln} (N_2 - N_1) / (T_2 - T_1) \quad (1)$$

$$\text{Biomass productivity (g l}^{-1}\text{day}^{-1}\text{)} = (N_2 - N_1) / (T_2 - T_1) \quad (2)$$

where  $N_1$  and  $N_2$  are the biomass at the initial time ( $T_1$ ) and final time ( $T_2$ ), respectively.

**Table 2** The initial concentration of nutrients and lactose ( $\text{mg l}^{-1}$ ) in control and different concentrations of CW, and trophic mode in stages I and II

Stages	Treat.*	Trophic mode	N	PO <sub>4</sub>	K	Na	Mg	Fe	Lactose
Stage I	T <sub>1</sub>	Auto	41.11	163.2	72.32	76.829	12	1.12	0
	T <sub>2</sub>	Mixo	23.33	2.70	69.11	411.64	0.50	0.187	3295
	T <sub>3</sub>	Mixo	46.66	5.39	138.21	823.27	0.99	0.374	6590
	T <sub>4</sub>	Mixo	69.99	8.09	207.32	1234.91	1.48	0.561	9885
	T <sub>5</sub>	Mixo	93.32	10.78	276.43	1646.54	1.98	0.748	13,180
Stage II	T <sub>6</sub>	Mixo	80.27	48.89	225.40	1254.12	4.48	0.841	9885
	T <sub>7</sub>	Mixo	90.54	89.69	243.48	1273.32	7.48	1.121	9885
	T <sub>8</sub>	Mixo	100.82	130.49	261.56	1292.53	10.48	3.921	9885

T<sub>1</sub>, BBM (control); T<sub>2</sub>, 5% CW; T<sub>3</sub>, 10% CW; T<sub>4</sub>, 15% CW; T<sub>5</sub>, 20% CW; T<sub>6</sub>, 15% CW + 25% BBM; T<sub>7</sub>, 15% CW + 50% BBM; T<sub>8</sub>, 15% CW + 75% BBM

For further biochemical analysis, *Desmodesmus* sp. biomass was harvested by sedimentation and further centrifuged at 4000 rpm for 10 min. The obtained algal slurry was washed with distilled water to remove salts and then dried in an oven at 70°C overnight.

The growth media reaction (pH) of *Desmodesmus* sp. cultures was measured against BBM as a blank using a pH meter (HANNA Instruments pH 211).

### 2.3.2 Determination of Photosynthetic Pigments, Carbohydrates, Lipids, and Protein

Photosynthetic pigment content of *Desmodesmus* sp. was photometrically determined for chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), and total carotenoids. The pigments were extracted using dimethyl sulfoxide (DMOS) according to Wellburn (1994) and calculated ( $\mu\text{g ml}^{-1}$ ) as the following equations:

$$\text{Chl } a = 12.47 \times A_{665} - 3.62 \times A_{649} \quad (3)$$

$$\text{Chl } b = 25.06 \times A_{649} - 6.5 \times A_{665} \quad (4)$$

$$\text{Carotenoids} = (1000A_{480} - 1.29 \times \text{Chl } a - 53.78 \times \text{Chl } b) / 220 \quad (5)$$

For carbohydrate extraction, 0.1 g of dry biomass was hydrolyzed by 20 ml HCl (2.5M) at 100 °C for 30 min then neutralized to pH 7 by using Na<sub>2</sub>CO<sub>3</sub>. The volume was adjusted to 100 ml by distilled water and then filtered (Miao et al., 2004). Total carbohydrates were quantified using the phenol-sulfuric acid method (Dubois et al., 1956), using glucose as a standard.

The spectrophotometric readings were performed at 490 nm. The extraction of lipids from dried biomass was performed according to the procedure of Bligh and Dyer (1959). The nitrogen (N) content was measured by the elemental CHNS analyzer of dried biomass equipped with a thermal conductivity detector (TCD). Protein contents were calculated from the N content using the conversion factor (4.78), which was used as the nitrogen-to-protein conversion factor instead of 6.25 according to Lourenço et al. (2004).

### 2.3.3 Fatty Acids Profile and Prediction of Biodiesel Properties

Algal lipids were converted to fatty acid methyl ester (FAME) using the methyl ester boron trifluoride method (AOAC, 1998). This method was implemented by adding sodium methoxide, boron trifluoride solution, and a boiling chip to the produced algal oil and boiled under reflux until the oil droplets disappear (5–10 min) then heptane was added to the mixture. Boiling was continued for 1 min to extract the FAME and then analyzed by using Shimadzu GC-2010, equipped with a flame ionization detector (FID). Calibration curves between peak area and concentration were established by injecting reference FAME samples of known concentrations into the GC-FID. The analysis occurred at the Regional Center for Food and Feed (RCFF), Agricultural Research Center, Giza, Egypt. By using computer software, predicted biodiesel properties based on a fatty acid profile were calculated (Biodiesel analyzer © ver.2.2.) (Talebi et al., 2014).

### 2.3.4 Ultrastructure Analysis of *Desmodesmus* sp. Using TEM

The ultrastructure of microalgae (*Desmodesmus* sp.) was studied using a transmission electron microscope (TEM) to know the effect of CW compared to control (BBM). Cells were harvested after 14 days and then fixed in a 2% (w/w) glutaraldehyde solution in 0.1 M sodium cacodylate buffer at room temperature for 0.5 h, and then post-fixed for 4 h in 1% (w/v) OsO<sub>4</sub> in the same buffer. After dehydration through a graded ethanol series, the samples were embedded in an epoxy resin. Ultrathin sections were made with a Leica Ultracut UCT Ultramicrotome, stained with lead citrate and uranyl acetate according to Reynolds (1963), and examined under a Jeol JEM 1400 (JEOL, Tokyo, Japan) microscope in Cairo University Research Park (CURP).

### 2.4 Statistical Analysis

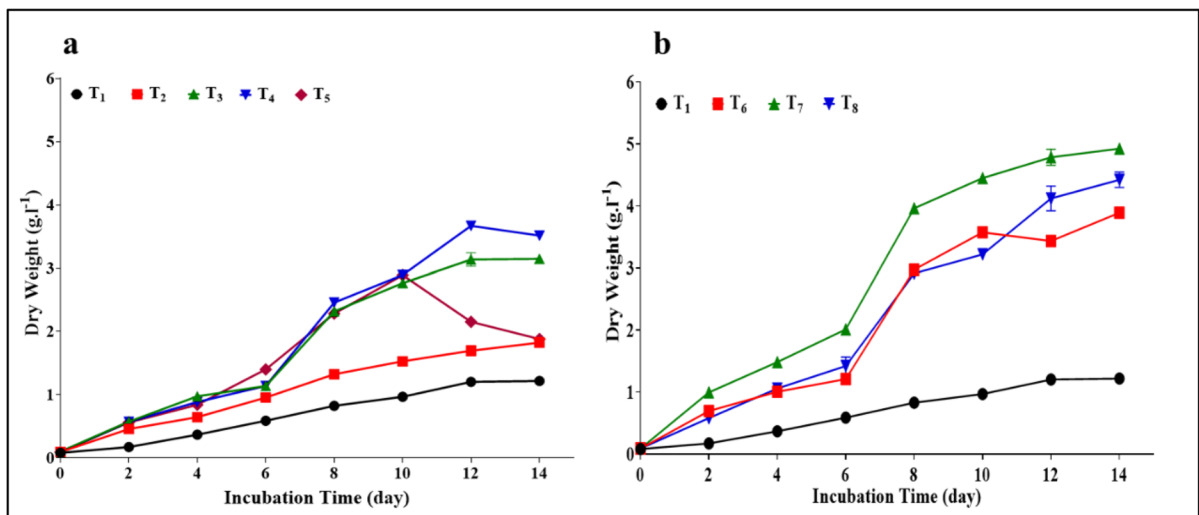
The mean values of this study, together with the standard deviation (SD) of three replicates, are reported in tables and figures. A two-way ANOVA was used to analyze the effect of CW on growth and photosynthetic pigments of *Desmodesmus* sp. and the pH of cultures, as well as the effect on biomass productivity, specific growth rate, and biochemical

composition, which was analyzed by one-way ANOVA. The differences among means were compared by Duncan's multiple range test as given by Snedecor and Cochran (1994) by using the Assistat program (Silva & Azevedo, 2009), and figures were generated using Prism version 8.0.2 (GraphPad Software, USA).

## 3 Results and Discussion

### 3.1 Effect of CW on Dry Weight, Specific Growth Rate, and Biomass Productivity

The results obtained from the growth curve of *Desmodesmus* sp., based on dry weight (Fig. 1), revealed that the mixotrophic mode (CW and CW+BBM) enhanced *Desmodesmus* sp. growth compared to the autotrophic mode. As shown in Fig. 1a, the increasing concentration of CW as an alternative culturing medium in stage I markedly induced algal growth along the incubation period. The maximum dry weight indicated in 15% CW (3.51g l<sup>-1</sup>) was 1.87 times higher than the control (1.22g l<sup>-1</sup>) after 14 days of incubation. Similarly, the specific growth rate ( $\mu$ ) and biomass productivity of *Desmodesmus* sp. in 15% CW ( $T_4$ ) were higher than in the control ( $T_1$ ) by 38.11% and 201.234%, respectively. Also, it is



**Fig. 1** Growth curve of *Desmodesmus* sp. during **a** stage (I) and **b** stage (II).  $T_1$ , BBM (control);  $T_2$ , 5% CW;  $T_3$ , 10% CW;  $T_4$ , 15% CW;  $T_5$ , 20% CW;  $T_6$ , 15% CW + 25% BBM;  $T_7$ , 15%

CW + 50% BBM;  $T_8$ , 15% CW + 75% BBM. Data are presented as means  $\pm$  SD ( $n = 3$ )

notable that increasing CW up to 20% caused a slight decrease in growth as compared to 10 and 15% CW, which might return to the high concentration of  $\text{Na}^+$  (Table 2). Salinity stress might cause growth inhibition because of high salt levels that cause ion toxicity, hyperosmotic stress, and secondary stresses such as oxidative damage (Fal et al., 2022; Qiao et al., 2021). Furthermore, high turbidity limits the amount of light that penetrates the growth medium, which in turn inhibits the growth of the microalgae (Chang et al., 2016).

Regarding the combination between CW and BBM, stage II, it was found that enriching the alternative media (CW) with BBM significantly induced *Desmodesmus* sp. growth as compared to the control (Fig. 1b). The best growth was achieved using 15% CW + 50% BBM ( $4.92 \text{ g l}^{-1}$ ), which was three times higher than the control. Adding BBM to CW with more than 50% started to decrease algal growth significantly as compared to 15% CW + 50% BBM treatment.

It could be concluded that supplying *Desmodesmus* sp. with lactose as an external carbon source derived from CW positively affected metabolic pathways in such a way as to lead to more biomass accumulation. In this regard, a lot of studies found that the mixotrophic growth of microalgae on wastewater triggered biomass accumulation rather than autotrophic growth (Bonett et al., 2020; Gumbi et al., 2021). In mixotrophic growth, photosynthesis and respiration work together in green microalgae, which avoid the negative effect of oxidative stress resulting from  $\text{O}_2$  accumulation (Patel et al., 2019), and reuse C, N, P,

and Fe, which are produced from respiration (Beardall & Raven, 2016). Girard et al. (2014) reported similar results when adding whey protein to BBM media for cultivating *S. obliquus*, resulting in  $3.6 \pm 0.4 \text{ g l}^{-1}$  of dry weight after 13 days.

Calculating the specific growth rate ( $\mu$ ) of *Desmodesmus* sp. at stages I and II (Table 3) reflected that cheese whey could positively affect the rate of cell divisions and growth phases along the incubation period. Treatment with 15% CW and 50% BBM at stage II recorded the highest growth rate of *Desmodesmus* sp. In turn, the biomass productivity of *Desmodesmus* sp. significantly increased (Table 3), as compared to the control, due to the presence of CW in the culturing media. The high content of N, K, and lactose in CW (Table 1) could be a significant factor in inducing *Desmodesmus* sp. growth and productivity. So, combining CW with BBM, specifically 15% CW plus 50% BBM, causes a synergistic effect leading to vigor algal growth. The synergism arises from the high nutritional load of CW, along with the initial content of P, Mg, and Fe in BBM (Table 2).

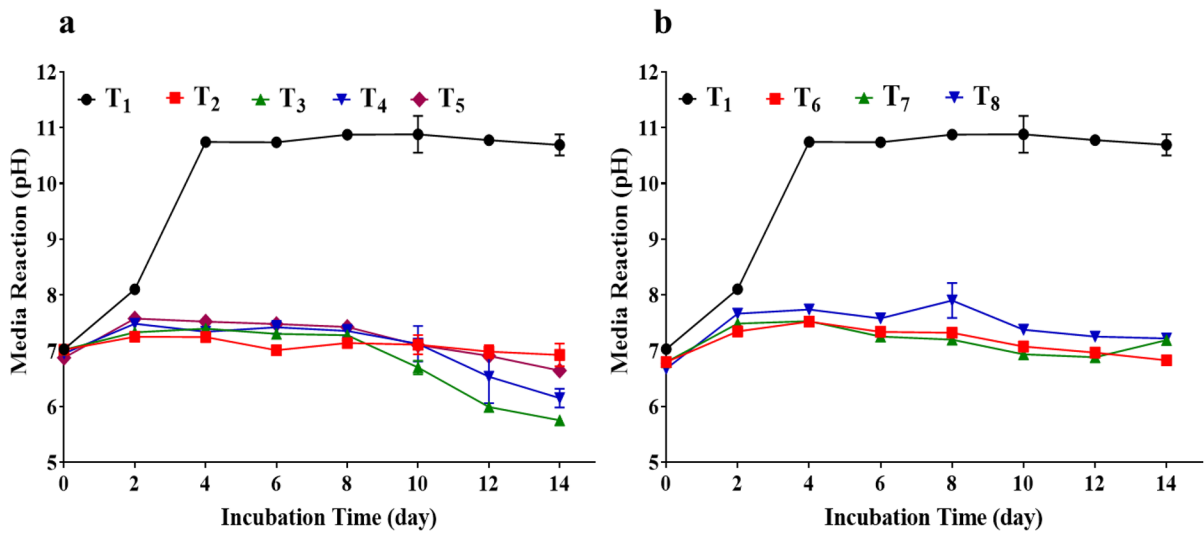
### 3.2 Impact of CW on Media Reaction (pH)

In this study, the initial pH of the media for all treatments was set to 7.0 before inoculation with *Desmodesmus* sp. and kept uncontrolled during the 14 days of incubation. In autotrophic cultures (control), the pH increased to 10.74, by the fourth day. Then, it gradually levelled off until the 10th day, followed by a slight decrease from the 12th to the 14th day (Fig. 2a).

**Table 3** Biomass productivity and specific growth rate ( $\mu$ ) of *Desmodesmus* sp. during stages I and II

Stages	Treatment	Biomass productivity $\text{g l}^{-1} \text{ day}^{-1}$	$\mu \text{ day}^{-1}$
Stage I	$T_1$	BBM	$0.081 \pm 0.005^d$
	$T_2$	5% CW	$0.124 \pm 0.002^c$
	$T_3$	10% CW	$0.218 \pm 0.001^b$
	$T_4$	15% CW	$0.244 \pm 0.003^a$
	$T_5$	20% CW	$0.127 \pm 0.003^c$
Stage II	$T_1$	BBM	$0.081 \pm 0.005^d$
	$T_6$	15% CW + 25% BBM	$0.271 \pm 0.003^c$
	$T_7$	15% CW + 50% BBM	$0.345 \pm 0.001^a$
	$T_8$	15% CW + 75% BBM	$0.309 \pm 0.009^b$

Data are presented as means  $\pm$  SD ( $n = 3$ ) in each column and for each part means with different letters (a, b, c, d, e) are significantly different according to Duncan's multiple range test at  $P < 0.05$



**Fig. 2** pH changes in *Desmodesmus* sp. culturing media during stage I (a) and stage II (b) along the incubation period. T<sub>1</sub>, BBM (control); T<sub>2</sub>, 5% CW; T<sub>3</sub>, 10% CW; T<sub>4</sub>, 15% CW;

T<sub>5</sub>, 20% CW; T<sub>6</sub>, 15% CW + 25% BBM; T<sub>7</sub>, 15% CW + 50% BBM; T<sub>8</sub>, 15% CW + 75% BBM. Data are presented as means  $\pm$  SD ( $n = 3$ )

This trend is expected, because of the fixation of inorganic carbon during algal photosynthesis, which increased pH due to the formation of OH ions resulting from the release of oxygen molecules as a by-product of photosynthesis (Sarwer et al., 2022). In contrast, in mixotrophic cultures of stage I, the pH slightly increased up to 8 days of incubation, while the pH decrement occurred from day 10 until the end of the incubation period, as shown in Fig. 2a. In this regard, we attribute the reduction of pH in mixotrophic cultures to the degradation of soluble organic monomers into organic acids through the processes of acidogenesis and acetogenesis (Deng et al., 2019). In stage II of the experiment, the pH of mixotrophic cultures was increased gradually until the 8th day, followed by a slight decrement until the end of the incubation period (Fig. 2b). A concluding remark is that adding BBM to cheese whey cultures made the pH more stable than cheese whey alone.

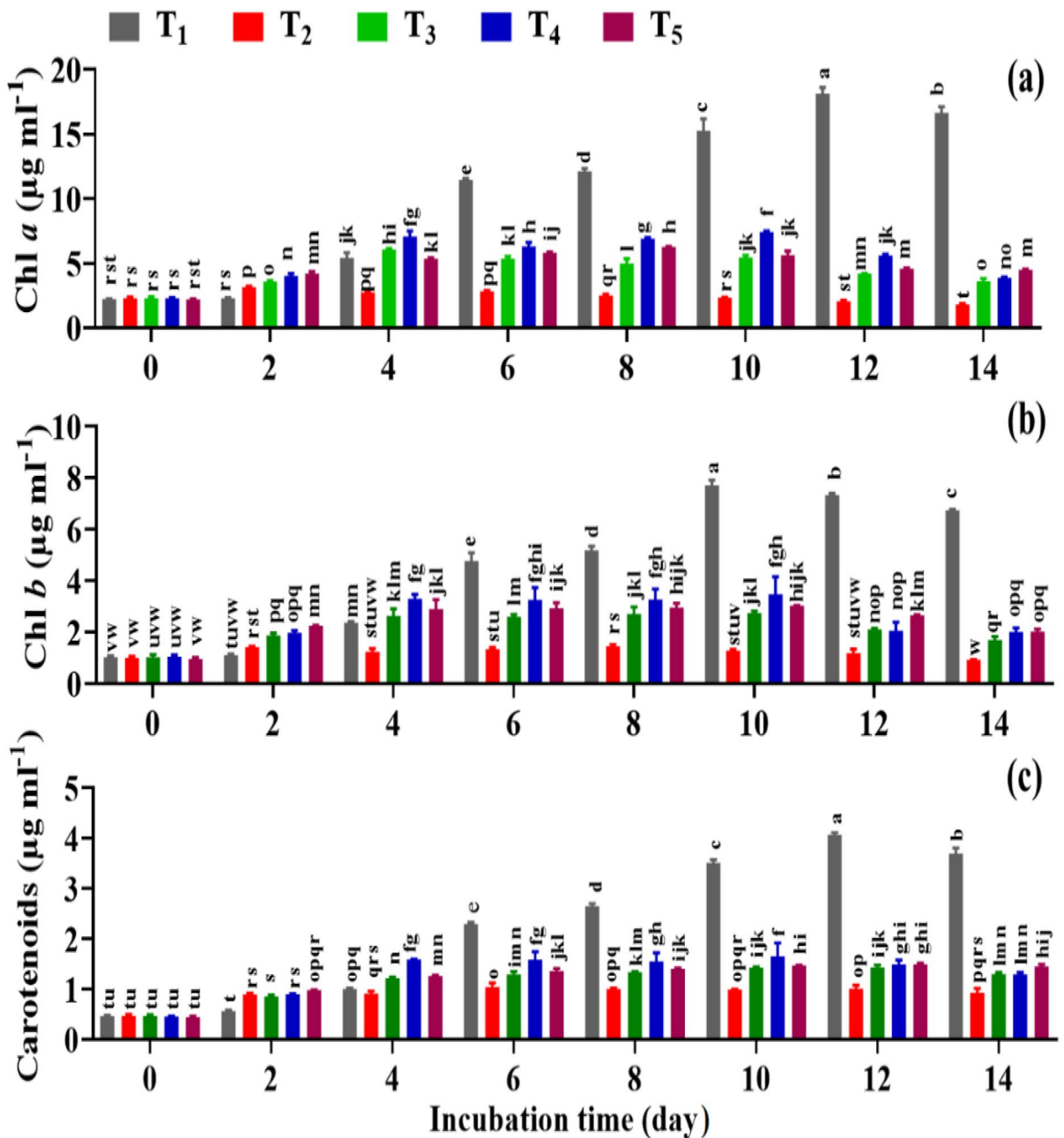
### 3.3 Photosynthetic Pigments

Algal growth requires a healthy chloroplast structure and function; hence, the photosynthetic pigments are significant physiological indicators through which we can examine *Desmodesmus* sp. adaptation in different treatments of cheese whey as an alternative growth media. The photosynthetic

pigment (Chl *a*, Chl *b*, and carotenoid) concentrations of *Desmodesmus* sp. at a 2-day interval during 14 days of growth in different concentrations of cheese whey was illustrated in Fig. 3. The pigment concentrations of all mixotrophic cultures were lower than the photoautotrophic control (BBM) Chl *a* (18.14  $\mu\text{g ml}^{-1}$ ), Chl *b* (7.72  $\mu\text{g ml}^{-1}$ ), and carotenoids (4.07  $\mu\text{g ml}^{-1}$ ). Because Chl *a* is the major pigment for light harvesting (converting light energy to chemical energy) and Chl *b* is an accessory pigment, the values of Chl *a* in microalgae were greater than that of Chl *b* (Tang et al., 2022). Several studies found that acetone was found to be excellent in extracting chlorophyll from microalgae (Tang et al., 2023).

These results indicate that the addition of cheese whey inhibited photosynthesis. Whereas under mixotrophic conditions, algal cells are able to hydrolyze lactose into glucose and galactose by producing extracellular  $\beta$ -galactosidase (Li et al., 2023). Deng et al. (2019) reported that adding glucose to algal culture caused a great reduction in Chl *a* content which was attributed to its impact on Chl *a* biosynthesis through inhibition of coproporphyrinogen III-oxidase, which forms protoporphyrinogen IX from coproporphyrinogen III (Stadnichuk et al., 1998). Kong et al. (2020) studied the effects of various carbon sources on *Chlorella vulgaris* 31 and reported that organic carbon





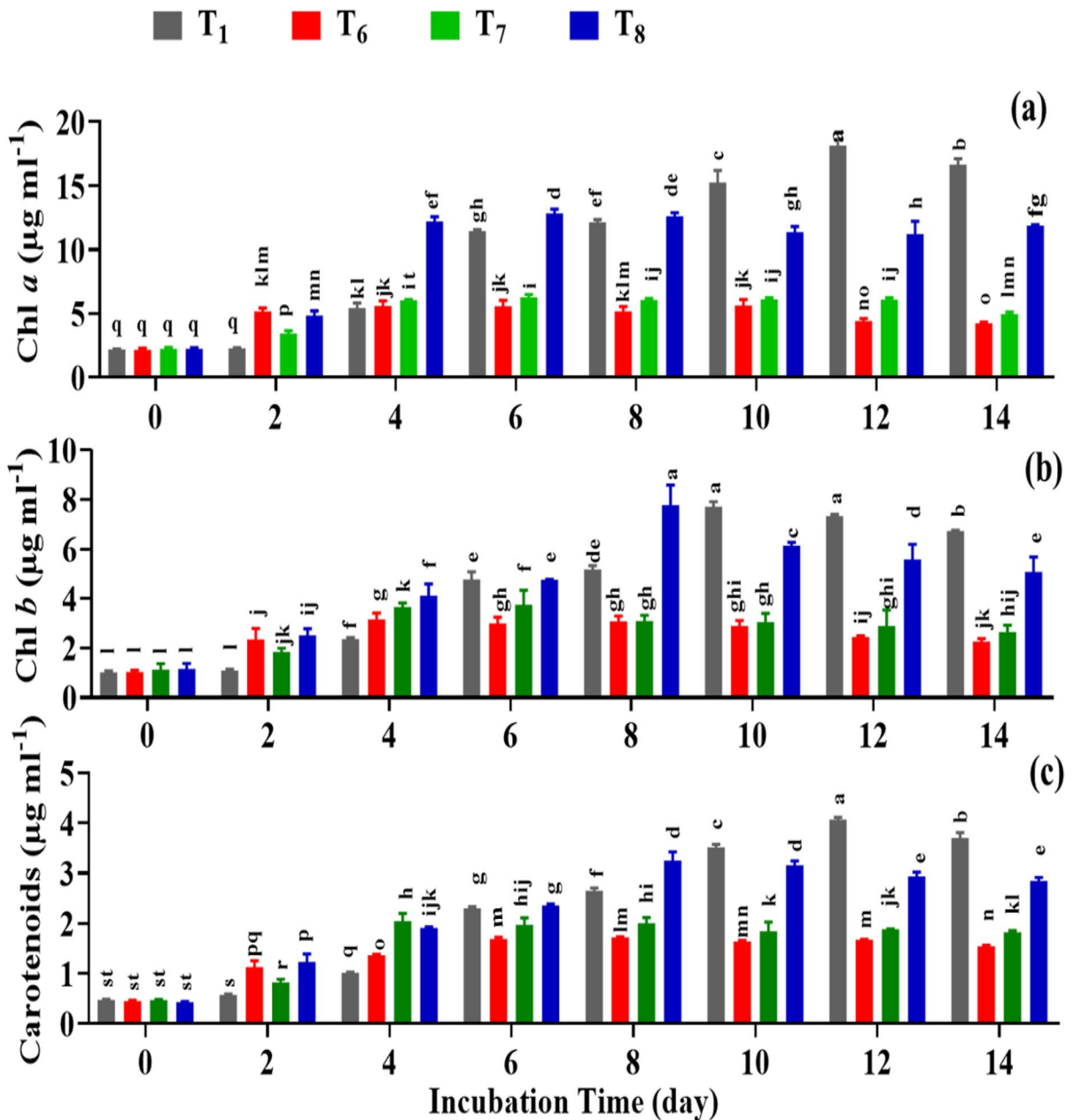
**Fig. 3** Effect of different concentrations of CW (5, 10, 15, and 20%) as well as control (stage I) on chlorophyll a (a), chlorophyll b (b), and carotenoids (c) of *Desmodium* sp. incubated for different intervals (2 days).  $T_1$ , BBM (control);  $T_6$ , 15% CW

+ 25% BBM;  $T_7$ , 15% CW + 50% BBM;  $T_8$ , 15% CW + 75% BBM. Data are presented as means  $\pm$  SD ( $n = 3$ ) with significantly different letters (a-t) according to Duncan's multiple range at  $P < 0.05$

sources inhibited photosynthesis and promoted respiration. de Almeida Pires et al. (2022) found that the cultivation of *C. vulgaris* on BBM with 1% CW increased chlorophyll value by 100–132% compared

to BBM, but high concentrations of CW harm the pigment accumulation.

Not only lactose but also nutrient limitations, especially N,  $\text{PO}_4$ , Mg, and Fe, affect photosynthetic



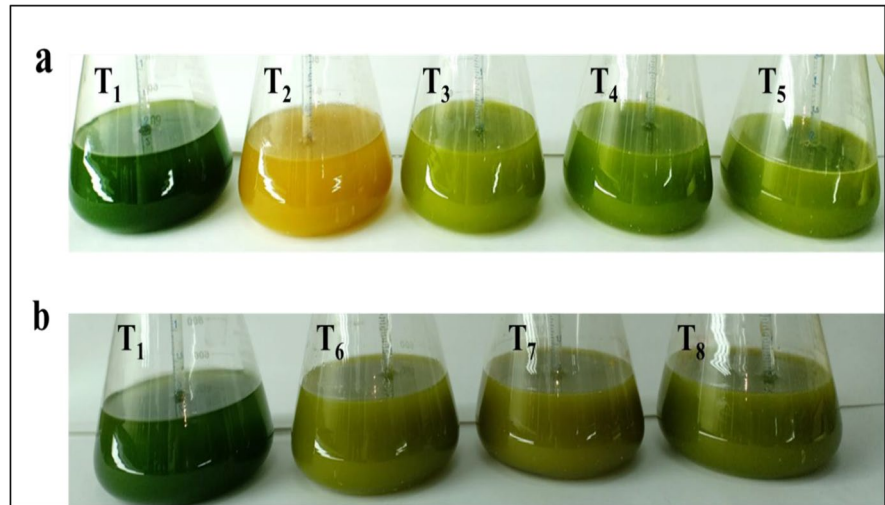
**Fig. 4** Effect of 15% CW supplemented with different concentrations of BBM (25, 50, and 75 %) as well as control (stage II) on chlorophyll a (a), chlorophyll b (b) and carotenoids (c) of *Desmodium* sp. incubated for different intervals (2 days).

T<sub>1</sub>, BBM (control); T<sub>6</sub>, 15% CW + 25% BBM; T<sub>7</sub>, 15% CW + 50% BBM; T<sub>8</sub>, 15% CW + 75% BBM. Data are presented as means  $\pm$  SD ( $n = 3$ ) with significantly different letters (a-q) according to Duncan's multiple range at  $P < 0.05$

pigment synthesis, according to the data in Table 2. The treatment of 5% CW contains the lowest level of nutrients, negatively affecting chlorophyll synthesis so T<sub>2</sub> obtained a yellow culture after 14 days of

incubation (Fig. 5). Iron and magnesium are considered essential elements for the photosynthesis pigments in algae (Ermis et al., 2020). In this regard, Xing et al. (2007) reported that Fe<sup>+2</sup> limitation causes

**Fig. 5** Visual changes in *Desmodesmus* sp. cultures as affected by cheese whey concentrations and Bold's basal growth medium during stage (I) (a) and stage (II) (b).  $T_1$ , BBM (control);  $T_2$ , 5% CW;  $T_3$ , 10% CW;  $T_4$ , 15% CW;  $T_5$ , 20% CW;  $T_6$ , 15% CW + 25% BBM;  $T_7$ , 15% CW + 50% BBM;  $T_8$ , 15% CW + 75% BBM



a simultaneous loss of chlorophyll and degeneration of chlorophyll structure because iron is associated with proteins and enzymes, including photosynthesis (e.g., cytochrome, ferredoxins, NADP<sup>+</sup> reductase), cellular respiration (e.g., Aconitase), nitrogen assimilation, and vitamin synthesis (Marchetti & Maldonado, 2016), where magnesium is a vital metal for algal growth because it has a critical position in the porphyrin ring (chlorophyll molecule) and controls the activity of several photosynthetic enzymes (Li et al., 2023).

Carotenoid concentrations are higher in photoautotrophic cultures than in mixotrophic cultures (Figs. 4 and 5c). These findings are consistent with those of Abreu et al. (2012), who also cultivated *Chlorella vulgaris* using hydrolyzed cheese whey powder solution

as mixotrophic cultures in comparison to photoautotrophic ones. The light-harvesting complex of photosynthesis is linked to carotenoids in photosynthetic organisms, which are known for their photoprotective properties (Castillo et al., 2021). The high ratio of carotenoids to total chlorophyll indicates nutritional stress (nutrient limitation) and photo-oxidation, as reviewed by Wong et al. (2017).

Our findings showed that by increasing CW concentrations (stage I) the pigment concentrations were increased significantly ( $p < 0.05$ ) with increasing CW concentrations to 15% and combining BBM with 15% CW decreases the negative effect of nutrient limitation in CW and enhances algal growth and pigment synthesis, as shown in Figs. 4 and 5.

**Table 4** Biochemical composition of *Desmodesmus* sp. cultivated in different concentrations of CW (stage I), 15% CW supplemented with different concentrations of BBM as well as control for 14 days of incubation period (stage II)

Stages	Treatment		Carbohydrate (%)	Protein (%)	Lipid (%)
Stage I	$T_1$	BBM	$31.93 \pm 0.44^{ab}$	$23.96 \pm 0.24^a$	$31.60 \pm 1.04^c$
	$T_2$	5% CW	$30.68 \pm 0.74^b$	$16.11 \pm 0.68^{bc}$	$36.18 \pm 0.07^b$
	$T_3$	10% CW	$32.21 \pm 0.06^a$	$15.65 \pm 0.51^c$	$35.69 \pm 0.36^b$
	$T_4$	15% CW	$32.05 \pm 0.29^{ab}$	$16.86 \pm 0.61^b$	$35.70 \pm 0.76^b$
	$T_5$	20% CW	$29.07 \pm 1.25^c$	$15.33 \pm 0.24^c$	$43.04 \pm 2.18^a$
Stage II	$T_1$	BBM	$31.27 \pm 0.13^{ab}$	$23.96 \pm 0.24^a$	$31.60 \pm 1.04^b$
	$T_6$	15% CW + 25% BBM	$30.43 \pm 0.72^c$	$16.23 \pm 0.30^b$	$34.94 \pm 0.67^a$
	$T_7$	15% CW + 50% BBM	$31.88 \pm 0.47^a$	$17.43 \pm 1.38^b$	$32.83 \pm 0.27^b$
	$T_8$	15% CW + 75% BBM	$30.55 \pm 0.10^{bc}$	$22.45 \pm 1.65^a$	$31.76 \pm 1.49^b$

Data are presented as means  $\pm$  SD ( $n = 3$ ) in each column and for each part means with different letters (a, b, c) are significantly different according to Duncan's multiple range test at  $P < 0.05$

### 3.4 Biochemical Composition

Carbohydrate, protein, and lipid concentrations of a stationary phase–harvested algal biomass after 14 days of incubation, as affected by different treatments compared to control, are summarized in Table 4. At stage I, the different concentrations of CW, as compared to control, significantly affected the carbohydrates, proteins, and lipids of *Desmodesmus* sp., and based on dry weight, in a different manner.

The highest carbohydrates and lipid concentrations were respectively obtained upon cultivating *Desmodesmus* sp. with 10 and 20% CW, as compared to control (Table 4). On the other hand, protein concentration significantly decreased with all CW concentrations as compared to control. Accordingly, it seems that autotrophic culturing mode enhances protein assimilation in *Desmodesmus* sp. as compared to mixotrophic mode, which suppresses it. In this regard, Wang, Wang, Tao, et al. (2018) reported that adding an organic carbon source to the culturing medium decreased protein content in *Chlorella* spp. as compared to autotrophic cultures.

Additionally, *Desmodesmus* sp. accumulated more lipids at 20% CW, and this could be due to the high content of  $\text{Na}^+$  in CW as well as protein biosynthesis retardation (Table 2). This result is consistent with Gour et al. (2020) who stated that increasing salinity enhances lipid content and productivity. Furthermore, Wang et al. (2016) reported that the high C/N

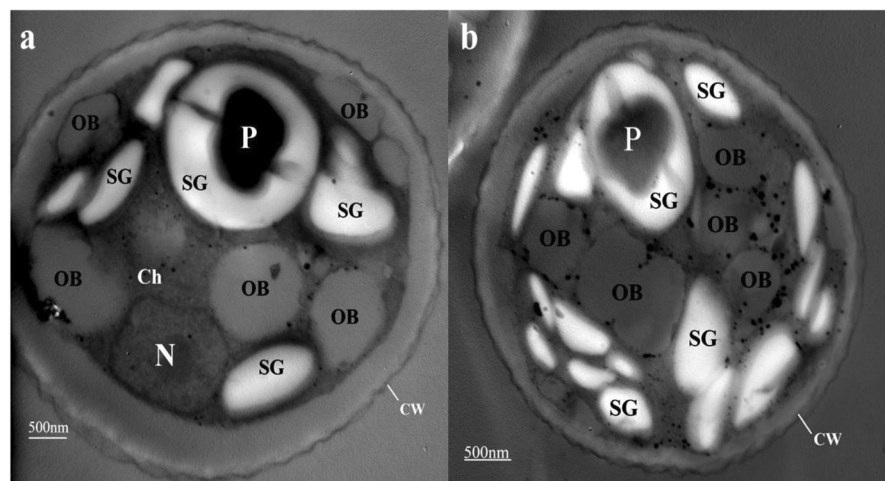
ratio in growth media increases the lipid content in microalgae.

Since microalgae accumulate more lipids upon exposure to environmental stress such as salinity and nutrient limitation (Sun et al., 2018), therefore, the highest concentration of CW in this study caused the highest lipid accumulation in *Desmodesmus* sp. This could be explained by a synergistic effect between high  $\text{Na}^+$  concentrations in CW and nutrient limitation at the harvesting phase (Khatoun et al., 2017).

What can be concluded is that starting nutrient limitation at the stationary phase of *Desmodesmus* sp. growth led to limited assimilation of protein, although carbohydrates and lipid accumulation increased. In consonance, Zhu et al. (1997) reported that the assimilation of carbohydrates and neutral lipids increased at the early stationary phase of *Isochrysis galbana* while protein, phospholipids, and glycolipids decreased.

Fortifying CW with different concentrations of BBM in stage II, as indicated in Table 4, revealed that increasing BBM concentration by 25% and 50% significantly increased lipids and carbohydrates in *Desmodesmus* sp., respectively, as compared to control. On the other hand, 75% BBM significantly decreased carbohydrate concentration and non-significantly affected protein and lipids, all compared to control. In general, protein concentrations in *Desmodesmus* sp. gradually increased with increasing BBM concentrations. In contrast, lipid concentration gradually decreased by increasing BBM concentration, both as compared to control.

**Fig. 6** Transmission electron micrographs of *Desmodesmus* sp. cultivated in BBM (a) and 15% CW + 50% BBM (b). Ch, chloroplast; CW, cell wall; N, nucleus; OB, oil body; P, pyrenoid; SG, starch granule



**Table 5** Fatty acids profile of *Desmodium* sp. cultivated in 15% CW+ 50% BBM ( $T_7$ ) for 14 days

Fatty acid	Amounts %
<b>Saturated fatty acid (SFA)</b>	
Tridecanoic acid (C13:0)	1.10 ± 0.12
Myristic acid (C14:0)	1.72 ± 0.33
Palmitic acid (C16:0)	25.86 ± 0.04
Heptadecanoic acid (C17:0)	3.12 ± 1.01
Stearic acid (C18:0)	7.23 ± 0.22
Sum of SFA	<b>39.01 ± 1.20</b>
<b>Monounsaturated fatty acid (MUFA)</b>	
Palmitoleic acid (C16:1 $\omega$ 7)	1.73 ± 0.12
Oleic acid (C18:1 $\omega$ 9)	35.31 ± 0.33
Vaccenic acid (C18:1 $\omega$ 7)	2.20 ± 0.18
Sum of MUFA	<b>39.23 ± 0.03</b>
<b>Polyunsaturated fatty acids (PUFA)</b>	
Hexgonic acid (C16:3 $\omega$ 4)	2.59 ± 0.15
Linoleic acid (C18:2 $\omega$ 6)	13.22 ± 1.78
Linolenic acid (C18:3 $\omega$ 3)	5.95 ± 0.44
Sum of PUFA	<b>21.76 ± 1.20</b>

These obtained results were consistent with the transmission electron micrographs, as indicated in Fig. 6. Supplying 15% CW with 50% BBM affected the ultrastructure of *Desmodium* sp. in terms of starch granules and oil bodies. It is obvious that starch granules occupied most of the *Desmodium* sp. cells as compared to the control. Also, oil bodies were larger and denser as compared to controls. Peng et al.

(2019) reported that mixotrophic cultivation by adding glucose and sodium acetate to wastewater enhanced the accumulation of lipids and carbohydrates in *C. vulgaris*. As shown in Fig. 6, the pyrenoid of *Desmodium* sp. grown on BBM is larger than that grown on 15% CW + 50% BBM ( $T_7$ ). This finding is similar to that of Yang et al. (2018), who found that *Scenedesmus* sp. pyrenoid became smaller under phosphorus stress, which improved lipid accumulation.

### 3.5 Fatty Acids Profiling and Biodiesel Parameters Calculation

The fatty acid composition of *Desmodium* sp. grown in 15% CW + 50% BBM ( $T_7$ ) for 14 days is listed in Table 5, and the data were expressed as relative percentages. The obtained results showed that *Desmodium* sp. mainly produced high amounts of palmitic acid (25.86%), oleic acid (35.31%), and linoleic acid (13.22%), as saturated, monounsaturated, and polyunsaturated fatty acids, respectively. The highest fatty acid was oleic acid (C18:1 $\omega$ 9) which agrees with the results of Chen et al. (2020) and Hu et al. (2013). Gogna et al. (2020) correlated the increase of oleic acid and reduction of linoleic acid to the negative effect of toxic ions ( $\text{Na}^+$  and  $\text{Cl}^-$ ) on  $\Delta^{12}$  desaturase (oleate desaturase).  $\Delta^{12}$  desaturase is an enzyme that synthesizes linoleic acid from oleic acid by adding a double bond at position 12 (Ghassemi-Golezani & Farhang-Abriz, 2018; Gogna et al., 2020).

**Table 6** Biodiesel parameter calculated from the fatty acids composition of *Desmodium* sp. cultivated 15% CW + 50% BBM for 14 days

Biodiesel properties	Biodiesel standard		
	<i>Desmodium</i> sp.	ASTM-D6751	EN 14214
Degree of unsaturation (DU)	79.49	-	-
Saponification value (SV) mg/g	200.738	-	-
Iodine value (IV) g/100 g	77.122	ND	≤120
Cetane number (CN)	56.137	>47	≥51
Long-chain saturated factor (LCSF)	6.279	-	-
Cold filter plugging point (CFPP)	3.25	-	-
Cloud point (CP) °C	8.626	-15.0 to 10.0	ND
Pour point (PP)	2.543	-3.0 to 12.0	ND
Allylic position equivalent (APE)	77.85	-	-
Bis-allylic position equivalent (BAPE)	25.76	-	-
Oxidation stability (OS)	8.452	3	≥ 6
Higher heating value (HHV)	38.44	NA	NA
Viscosity ( $\nu$ ) mm <sup>2</sup> /s	3.688	1.9–6.0	3.5–5.0
Density ( $\rho$ ) g/cm <sup>3</sup>	0.853	0.84–0.90	0.86–0.9

Fatty acid compositions highly affect biodiesel properties (Khethiwe et al., 2020; Salah et al., 2023). As shown in Table 5, *Desmodesmus* sp. contents of saturated, monounsaturated, and polyunsaturated were 39.01, 39.23, and 21.76%, respectively. The majority of fatty acids in *Desmodesmus* sp. are short-chain fatty acids (C14–C18), which are the dominant components of biodiesel (Choi, 2016). Data in Table 6 shows the predicted biodiesel properties resulting from *Desmodesmus* sp. grown in 15% CW + 50% BBM ( $T_7$ ) as culture media, with comparison to the American Society of Testing and Materials (ASTM) D6751 and the European (EN) 14214 standards. The characterization of biodiesel produced from *Desmodesmus* sp. was in the normal range of the ASTM-D6751 and EN 14214 standards, which revealed excellent suitability for biodiesel production.

#### 4 Conclusion

The growth curve of *Desmodesmus* sp. based on dry weight during 14 days of incubation revealed that the mixotrophic mode (CW and CW+BBM) enhanced algal growth compared to the autotrophic mode (BBM). The best growth was achieved using 15% CW + 50% BBM and was three times higher than BBM (control). In mixotrophic growth, photosynthesis and respiration function together in green microalgae which avoid the negative effect of oxidative stress resulting from  $O_2$  accumulation and reuse of C, N, P, and Fe that are produced from respiration. Microalgal cultures grown on CW and CW+BBM accumulated fewer concentrations of chlorophyll a, chlorophyll b, and carotenoids compared to cultures grown on BBM. The highest carbohydrate and lipid concentrations were respectively obtained upon cultivating *Desmodesmus* sp. with 10 and 20% CW, as compared to BBM (control). On the other hand, protein concentration significantly decreased with all CW concentrations as compared to BBM (control). Protein concentrations in *Desmodesmus* sp. gradually increased with increasing BBM concentrations. In contrast, lipid concentration gradually decreased by increasing BBM concentration compared to BBM (control). Transmission electron micrographs of *Desmodesmus* sp. show that cultivation of alga on 15% CW + 50% BBM enhanced the accumulation of starch granules and oil bodies in algal cells more than control. Based on fatty acid composition of *Desmodesmus* sp. grown on 15%

CW combined with 50% BBM, palmitic (25.86%), oleic (35.31%), and linoleic acid (13.22%) were the most abundant fatty acids. So, the produced algae have a significant amount of short-chain fatty acids (C14–C18) which make up the majority of biodiesel. In conclusion, the highly accumulated biomass of *Desmodesmus* sp., along with the high contents of carbohydrates and lipids, makes this strategy a pioneer for phycoremediation and biofuel production and also, the potential application of CW can help reduce the dairy industry's negative impacts on the environment.

**Author Contributions** The authors confirm their contribution to the paper as follows: study conception and design: A.S., H.S., A.B.E., R.M.E., and A.A.; data collection: A.S.; analysis and interpretation of results: A.S., H.S., A.B.E., R.M.E., H.I.M., and A.A.; draft manuscript preparation: A.S., H.S., A.B.E., R.M.E., H.I.M., and A.A. All authors reviewed the results and approved the final version of the manuscript.

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**Data Availability** All data generated or analyzed during this study are included in this published article.

#### Declarations

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

**Consent to Participate** Not applicable

**Consent for Publication** The manuscript is original. It has not been published previously by any of the authors and even not under the consideration in any other journal at the time of submission.

**Competing Interests** The authors declare no competing interests.

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