Evaluating the growth performance of *Synechocystis pevalekii* and *Tetradesmus obliquus* under different culture conditions

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ABSTRACT: In recent decades, much attention has been directed toward algae-based biotechnology, encompassing wastewater treatment, biofuel generation, medicines, nutraceuticals, and environmental remediation. Optimizing algal growth conditions is crucial to maximizing their potential and improving their efficacy in many biotechnological applications. This study examined the growth performance of *Tetradesmus obliquus* (Chlorophyta) and *Synechocystis pevalekii* (Cyanobacteria) under various culture media (Bold's Basal medium, BG-11 medium, Khul's medium, and Zarrouk's medium), nitrogen sources ($NaNO_3$, KNO_3, NH_4Cl, and peptone), and pH levels (7, 9, 11, and 13). Our research indicated that both species achieved optimal growth performance utilizing Bold's Basal medium. Of these conditions, total chlorophyll concentrations of *S. pevalekii* and *T. obliquus* were significantly elevated by 27.98 and 22.28 times, respectively. The findings indicated that sodium nitrate was the most efficacious nitrogen source, markedly augmenting the optical density and chlorophyll content of both species, with OD increasing by 15.74 and 8.27 times for *S. pevalekii* and *T. obliquus*, respectively, in relation to the beginning value. Concerning pH niches, pH 7 was ideal for *S. pevalekii*, but pH 13 was most conducive for *T. obliquus*.

Keywords: *Synechocystis pevalekii, Tetradesmus obliquus*, growth, chlorophyll content, biomass, nitrogen sources

INTRODUCTION

Algae are a diverse group of micro- and macroorganisms encompassing both unicellular and multicellular forms thriving in all aquatic and terrestrial environments. The algae biorefinery concept represents a sustainable approach to producing biofuels and high value biochemicals (Salami *et al*., 2021). Microalgae, being photosynthetic entities, have a growth speed that surpasses that of terrestrial plants. They inhabit a wide range of aquatic systems globally, including nutrient-rich environments. By utilizing light and carbon dioxide (CO_2) via photosynthesis, microalgae could synthesize key bioactive substances like proteins, carbohydrates, lipids, pigments, and vitamins which can be refined into bioproducts for diverse biotechnological applications and health sectors (Ho *et al*., 2014; Tang *et al.,* 2020). Cyanoprokaryotes and microalgae account for 32% of global photosynthesis, playing a vital role in supporting various ecosystems (Rath, 2012). The valuable contents of metabolites in microalgae and cyanobacteria have been the focus of biotechnological studies, with the possibility to produce a large scope of uses in many fields such as medicine, food, energy, and agriculture (Martínez-Ruiz *et al*., 2022). The capacity of microalgae to consume heavy metals and absorb nutrients, like phosphate and nitrogen, makes them useful for wastewater treatment (Morsi *et al.,* 2021).

It also has been found that abiotic stress factors can enhance or decrease the production of these valuable beneficial compounds. Consequently, much more research investigations are still required to better understand the best cultivation growth conditions for cyanobacteria and microalgae to optimize their industrial outputs (Patel *et al*., 2019). Algal growth is closely regulated by its complicated microenvironment including parameters such as temperature, phosphorus, nitrogen levels, and light intensity (Liu *et al.,* 2024). Nitrogen is a pivotal macronutrient for microalgal growth and produces proteins, lipids, and carbohydrates (Yodsuwan *et al*., 2017; Zarrinmehr *et al*., 2020). Overall, nitrogen content greatly has a significant impact on microalgal biochemical makeup. For instance, when nitrogen levels in the cultivation medium are depleted, growth diminishes, but lipid production tends to increase (Vooren *et al.,* 2012). pH also has a direct impact on numerous biochemical and physiological processes in microalgae, keeping the pH within an optimal extent is crucial for enzyme functions, cell growth, and nutrient absorption (Abinandan *et al*., 2021). Although numerous studies have focused on optimizing the growth conditions of various microalgae species, there is still a gap in understanding the specific growth requirements and performance of *Synechocystis pevalekii* and *Tetradesmus obliquus* under different culture conditions.

This study aimed to uncover the optimal growth conditions for these algae by testing multiple growth media, nitrogen sources, and pH niches. By doing so, it provides valuable insights into enhancing their growth and biomass production, particularly for large-scale biotechnological applications such as local wastewater treatment. The findings of this study could be pivotal in developing sustainable and efficient algal-based treatment systems tailored to specific environmental conditions.

MATERIAL AND METHODS

Algal isolates

Synechocystis pevalekii and *Tetradesmus obliquus* were isolated in June 2022 from agricultural wastewater in Tala province, Menoufia governorate, Egypt, located at approximately 30° 40' 54" N and 30° 56' 51" E, with an altitude of 9.40 m (30.83 ft) above sea level. After the samples were collected, they were enriched with Bold's Basal medium, and the algae were purified by streaking on agar plates. The colonies were re-streaked several times to ensure purity before identification. The two species were morphologically identified using a BEL® photonics biological microscope (BEL® Engineering Co., Monza, Italy) at Prof. Abd El-Salam M. Shaaban's Lab (the Phycology Lab No. 341), the Botany Department, Faculty of Science, Ain Shams University, Cairo, Egypt, following the relevant taxonomic literatures of Komárek and Anagnostidis (1999) and Komárek and Fott (1983).

Algae growth media

The two species were cultured under various nutrient media, including BG-11 medium (Ilavarasi *et al*., 2011), Bold's Basal medium (Bischoff and Bold, 1963), Kuhl's medium (Kuhl and Lorenzen, 1964), and Zarrouk's medium (Zarrouk, 1966). 1000 ml conical flasks were set up, each with 500 ml of the corresponding medium. Using 0.1M sodium hydroxide (NaOH) and 0.1M hydrochloric acid (HCl), the pH of each medium was adjusted with a pH meter (HANNA HI 991301). Before the experiment, the media underwent sterilization in an autoclave at 121 °C and 1.5 atm for 20 min. After sterilizing the medium, 10% (v/v) microalgal growth was added. The flasks were equipped with air pumps and kept at 28 ±2 °C with 60 µmol photons m^{-2} s^{-1} of constant light. To prevent bacterial contamination, a sterile syringe filter with a pore size of 0.45 µm was used. Triplicate runs of the experiment were conducted, with growth tracked over thirty days by measuring optical density (OD) and total chlorophyll content.

Influence of different sources of nitrogen and pH

After characterizing the optimal growth medium, several nitrogen sources such as ammonium chloride (NH_4Cl), potassium nitrate (KNO_3), sodium nitrate ($NaNO_3$), and peptone were separately tested in BBM for both species. Additionally, the impact of pH on algal growth was considered using BBM with $NaNO_3$ as the nitrogen source. pH levels were adjusted to 7, 9, 11, and 13 in 1000 ml conical flasks, each containing 500 ml of the nutritional medium. As previously mentioned, all flasks were autoclaved for 20 min at 121 °C, and then they were inoculated and allowed to be incubated.

Algal growth measurements

Optical density (OD)

For growth analysis, each algal species was assessed by measuring optical density, i.e. 560 nm for *T. obliquus* (El-Sheekh *et al*., 2014) and 750 nm for *S. pevalekii* (El-Sheekh *et al*., 2005) using a UV-Vis spectrophotometer (Spectronic Unicam, Rochester, USA) at the five-day interval.

Estimation of photosynthetic pigment (total chlorophyll)

To extract and estimate photosynthetic pigments (total chlorophyll), a 5 ml volume of each algal sample was centrifuged at 1788.8 g for 10 min. The pellet was rinsed twice by distilled water, then treated with 5 ml of 95 % methanol, and heated in a water bath at 60 °C for 30-60 min. Thereafter, the sample was centrifuged again at 1788.8 g for 10 min. This extraction process was repeated to ensure completeness of the extraction process (Mackinney, 1941). Total chlorophyll content was determined by UV-Vis spectroscopy at 665 and 650 nm, with total chlorophyll concentration calculated by applying the following formula (Bajwa *et al.*, 2018). Total chlorophyll (mg/ml) = $((0.4 \times 10^{-2}\ E665) + (2.55 \times 10^{-2}\ E650)) \times 10^3$.

Statistical examination

SPSS (version 19) was used to analyze the results, with data presented as means ± standard deviation (SD). There were three (n = 3) repetitions of each measurement. ANOVA (one-way analysis of variance) was utilized to determine statistical significance at the probability level of 5% ($P \leq 0.05$).

RESULTS AND DISCUSSION

Synechocystis pevalekii has the following diagnostic characteristics: Cells blue-green, spherical, solitary or in pairs a short time after division, 1.95–2.6 µm in diameter. Cell content homogeneous (Komárek and Anagnostidis, 1999). *Tetradesmus obliquus* (Figure 2) is colonies usually of 4 erect cells arranged in a linear series, fusiform with acute apices, inner cells with straight sides and sometimes irregularly alternating, outer sides of the terminal cells concave to slightly convex, cell walls smooth, without teeth or spines, 14–16 µm long and 5–6 µm wide. Chloroplast single parietal with a prominent pyrenoid (Komárek and Fott, 1983).

S. pevalekii and *T. obliquus* reached their highest growth performance in BBM, indicating that the nutrient composition of BBM is highly suitable for both species, promoting efficient nutrient absorption and metabolic activity. The findings demonstrated that a notable growth of *S. pevalekii* was revealed at BBM on the 20th day.

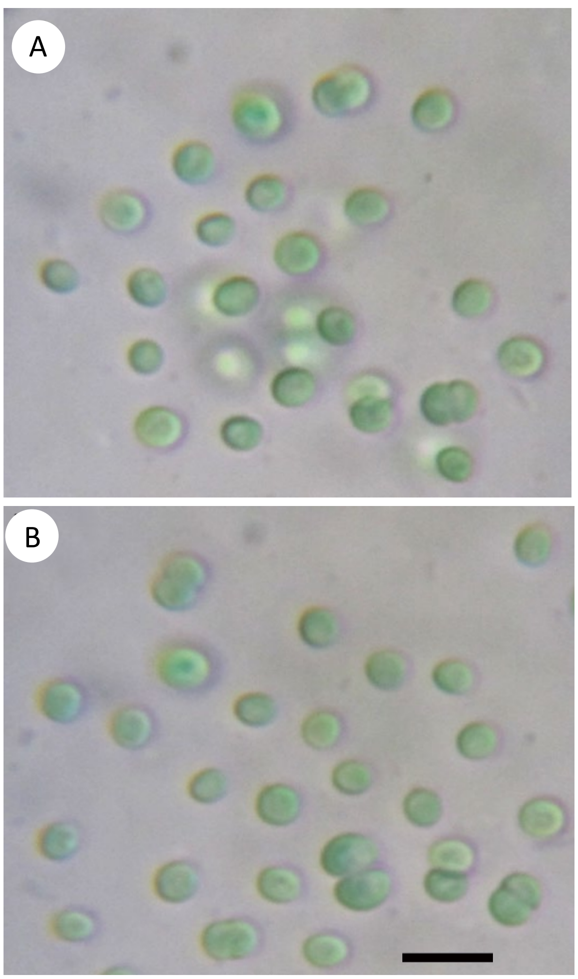

Figure 1. Light micrographs of *Synechocystis pevalekii* showing the different morphotypes of this species under the culture conditions of the study. Scale bar: 5 µm.

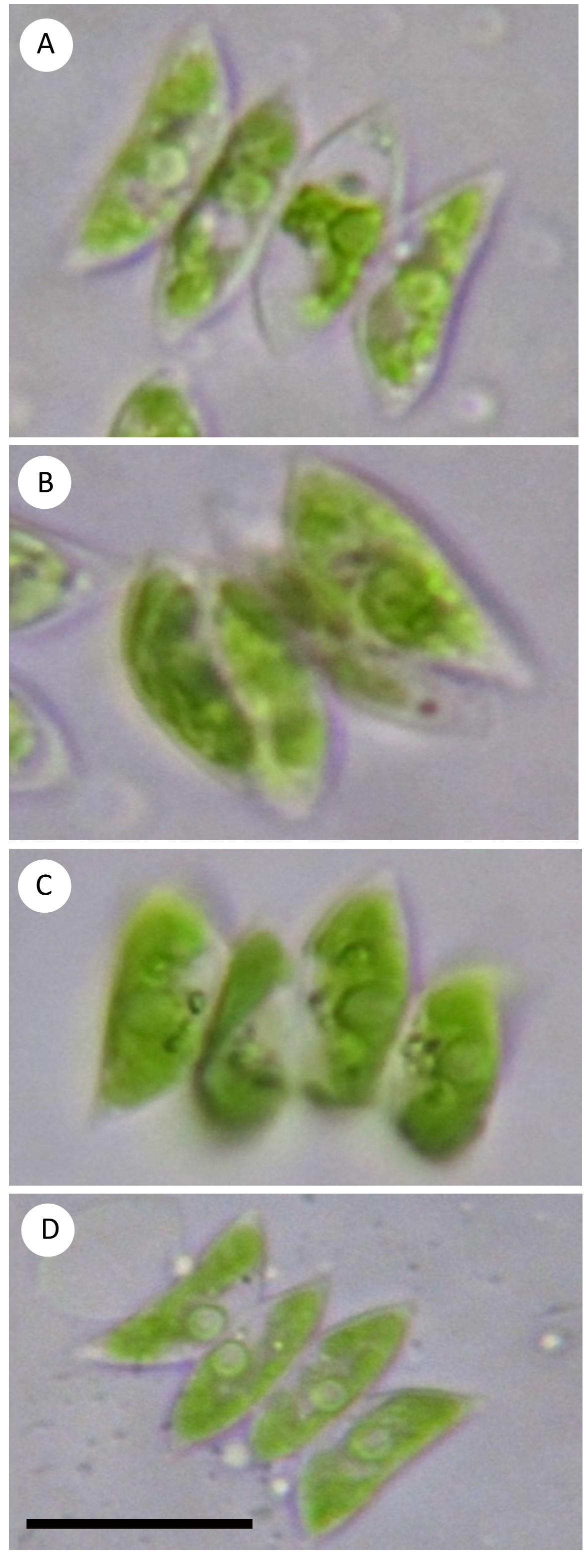

Figure 2. Light micrographs of *Tetradesmus obliquus* showing the different morphotypes of this species under the culture conditions of the study. Note the slight variations in cell outline and arrangement of internal cells. Scale bar: 10 µm.

As shown in Figures 3 and 4, the optical density (OD) and total chlorophyll contents were raised-by 25.95 and 27.98 times in comparison to the initial record, respectively. Khul's medium displayed a shift of 12.78 in OD and 10.4 times in total chlorophyll. On day 25, *S. pevalekii* showed limited growth in BG-11 and Zarrouk's media compared to the pronounced growth in the bold medium. *T. obliquus* data proved that BBM also supported the highest growth performance for both species. The optical density (OD) of the algal culture increased from an initial value of 0.017 to 1.983 on the 25-day, also chlorophyll content showed an increase from its initial value of 1.6625 mg/ml to 37.046 (mg/ml) on the 25-day, demonstrating a significant growth over the 25 days, followed by BG-11 medium as OD recorded 0.957 on the 25-day and total chlorophyll recorded 17.057 on day 25 compared to the initial value. Zarrouk's medium showed the lowest growth on day 20 in all growth parameters. In previous studies, *T. obliquus* was cultivated using various growth media. Kata et al., (2024) utilized Bold's Basal Medium (BBM) with a modified triple nitrate formulation (3N-BBM) at a triple concentration (3×3N-BBM) to prevent nutrient shortages in high-density bioreactors, thereby enhancing growth and biomass production.

Another study on *T. obliquus* UJEA_AD from Emmarentia Dam, Johannesburg, South Africa, used BG11 medium and reported substantial biomass accumulation of 2.78 g L^{-1}, a specific growth rate of 0.13 day^{-1}, and a CO_2 bio fixation rate of 0.265 g CO_2 L^{-1} day^{-1}. This strain also exhibited a favorable biochemical composition with 27.17% carbohydrates, 38.00% proteins, and 31.2% lipids, including fatty acids suitable for biodiesel production (Ahiahonu et al., 2022). In a study on *Synechocystis* sp. PCC 6803, researchers discovered that the wild-type strain could thrive in an artificial seawater (ASW) medium enriched with nitrogen and phosphorus sources. While adding HEPES buffer enhanced cell growth, the growth achieved in ASW remained lower than that in synthetic BG-11 medium (Iijima *et al*., 2015). Furthermore, another investigation showed that by optimizing factors like sulfate and iron levels in BG-11 medium, the growth rate of *Synechocystis* sp. was significantly improved under controlled conditions (Van Alphen *et al*., 2018). This confirms the importance of selecting and optimizing the appropriate growth medium to enhance algal growth and productivity, and it highlights the species-specific requirements necessary to achieve maximum output.

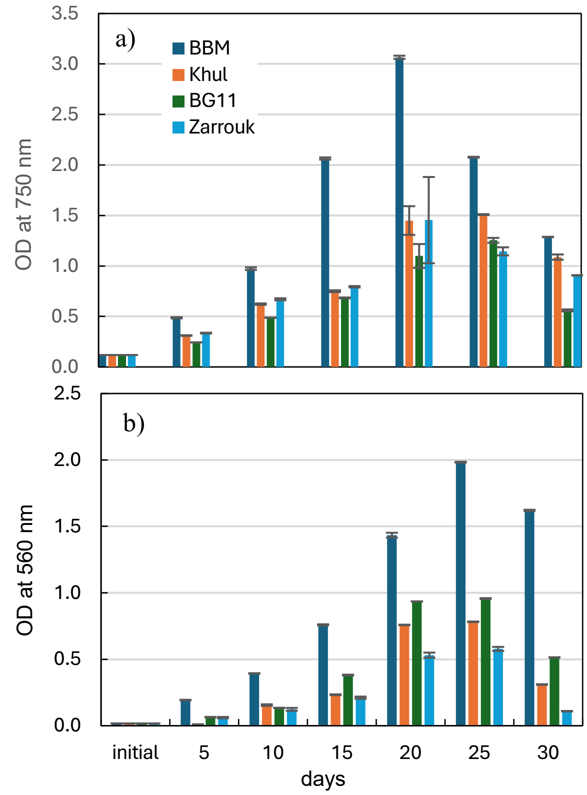

Figure 3 Effects of different growth media on optical density (OD) readings in *S. pevalekii* (a) and *T. obliquus* (b).

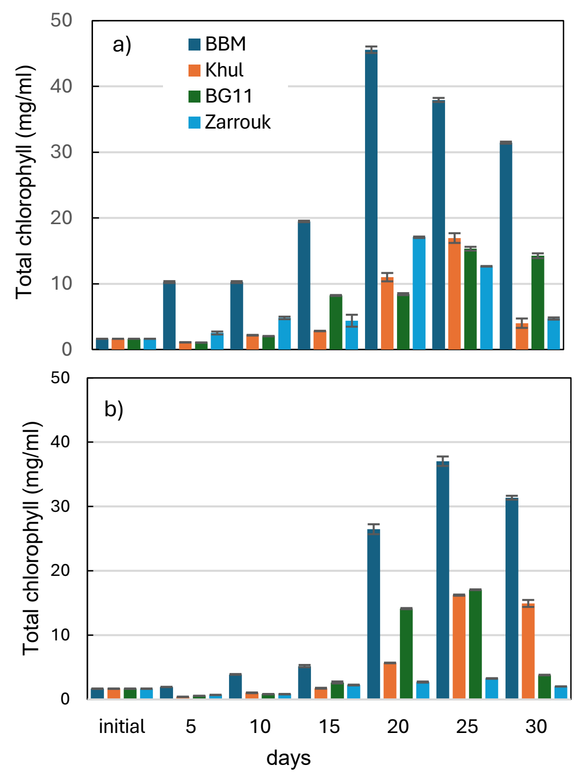

Figure 4. Effect of different growth media on total chlorophyll content in *S. pevalekii* (a) and *T. obliquus* (b).

It is well known that microalgae can assimilate nitrogen in a variety of forms such as urea, ammonium, nitrate, and nitrite (Xu *et al*., 2001). In the present study, BBM was the best growth medium for both algal species. Accordingly, the same medium was utilized to test four different nitrogen sources ($NaNO_3$, KNO_3, NH_4Cl, and peptone) to identify the most effective one for enhancing algal growth. Considering our findings, *S. pevalekii* reached its maximum growth using $NaNO_3$ as a nitrogen source on the 20th day. The optical density (OD) and total chlorophyll concentrations were 15.47 and 12.16 times, respectively, compared to the initial day (Figures 5 and 6). On the other hand, KNO_3 showed an increase of 4.72 times in total chlorophyll and 3.35 times in OD. Importantly, both peptone and NH_4Cl gave the lowest growth rates for *S. pevalekii*. As regards *T. obliquus*, sodium nitrate was also the best N-source, where the OD increased from 0.144 on the initial day to 0.943 on the day 25th. Additionally, there was a distinct increase in the level of total chlorophyll from 2.0845 mg/ml on the initial day to 8.769 mg/ml on the day 25th. NH_4Cl was the second-best nitrogen source with an OD of 0.867 and a total chlorophyll content of 8.079 mg/ml on the day 25th. Contrarily, peptone and potassium nitrate weakly supported *T. obliquus* growth rates (Figures 5 and 6). Sodium nitrate was found to be the best source of nitrogen in the current study for both *S. pevalekii* and *T. obliquus*. For *S. pevalekii*, the optical density (OD) increased from 0.153 on the first day to 2.367 on the day 25th, and the chlorophyll content rose from 2.33 mg/ml to 28.36 mg/L. Similarly, *T. obliquus* exhibited significant growth with sodium nitrate, confirming its effectiveness as a nitrogen source. On contrarily, growth rates were notably lower when peptone was used, most likely due to the complexity of organic nitrogen sources which require additional energy for utilization and consequently slow down the algal growth. These findings coincide with former studies affirming that inorganic nitrogen sources, such as nitrate, are more easily absorbed and metabolized by algae. Accordingly, Yaakob *et al*. (2021) found that nitrate is most often preferred in the cultivation of microalgae due to its stability and reduced potential for pH fluctuations compared to other ammonium salts. However, Khazi *et al.* (2018) tested the same nitrogen sources on different cyanobacterial species, including *Arthrospira platensis*, *Phormidium* sp., and *Pseudoscillatoria* sp., and found that the latter two species were successfully used all N-sources and NH_4Cl achieved the maximum growth rates, while *A. platensis* grew well in $NaNO_3$.

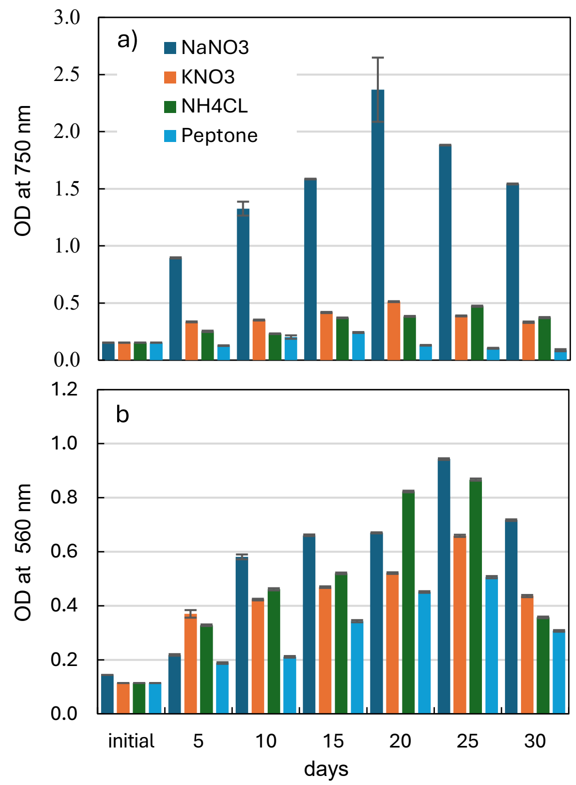

Figure 5. Effect of different nitrogen sources on optical density (OD) readings in *S. pevalekii* (a) and *T. obliquus* (b).

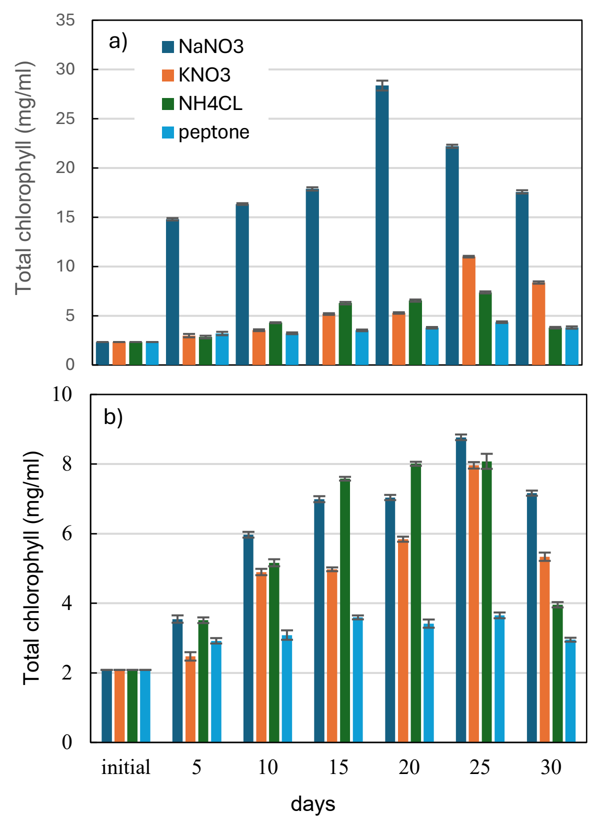

Figure 6. Effect of different nitrogen sources on total chlorophyll content in *S. pevalekii* (a) and *T. obliquus* (b).

pH has a dynamic role in the growth of microalgae, with most species thriving in a neutral pH range (Razzak *et al*., 2023). The typical pH for each species of algae differs based on how the microalgae physiologically respond to changes in environmental pH, the cell ability to endure these pH shifts depends on how effectively it can adjust its physiology to counterbalance the pH gradient, a process that may require energy (Gerloff-Elias *et al.,* 2005). pH fluctuations may interfere with enzymatic activities and, similarly, impact microalgae's overall metabolism (Filali *et al.,*2021). The best pH value for *S. pevalekii* was detected at pH 7 on the 20th day, which recorded 18.41 folds OD and 19.42 folds total chlorophyll from the initial record (Figures 7 and *8*). At pH 11 recorded an increase of 16.05 folds OD, and 17.15 folds total chlorophyll compared with the initial record. The pH 9 and pH 13 increased by 12.74 and 12.87 folds OD; 16.56 and 14.63 folds total chlorophyll, respectively.

The ideal pH for *T. obliquus* was 13, where the OD increased from 0.144 on the initial day to 2.145 on day 25. A significant increase was illustrated through chlorophyll content, from 2.0845 mg/ml on the initial day to 27.228 mg/ml on day 25. Then pH 11, with an OD of 1.808 and chlorophyll content of 22.928 mg/ml on day 25, pH 7 and 9, OD increased from its initial value to 1.25 and 1.534, and chlorophyll content reached (7.401 and 14.876) mg/ml on day 25th, respectively (Figure 7 and Figure 8). In former studies, *Synechocystis* cultures grown in BG-11 medium at pH levels between 7.5 and 10 exhibited near-optimal productivity, with only a 5% reduction at pH 10. However, when the pH was increased to 11, productivity dropped by over 30% (Touloupakis *et al*., 2016). In our study, *S. pevalekii* reached the maximum growth at pH 7, using BBM with the contribution of sodium nitrate as a source of nitrogen. Accordingly, Bano and Siddiqui (2004) isolated six cyanobacterial species, including *Katagnymene accurata*, *Lyngbya contorta*, *Pseudoanabaena lonchoides*, *Spirulina major*, and *Synechocystis aquatilis*. Their findings revealed that all the isolates preferred a pH range close to neutral or slightly alkaline, specifically between 7.4 and 8. *T. obliquus* demonstrated the highest growth levels at pH 13 when cultured in BBM with sodium nitrate. In line with our observations, Nadzir *et al*. (2019) observed that *T. obliquus* achieved the maximum biomass yield of 115±1.4 mg/L/day in BG-11 medium with sodium nitrate at pH 9.8.

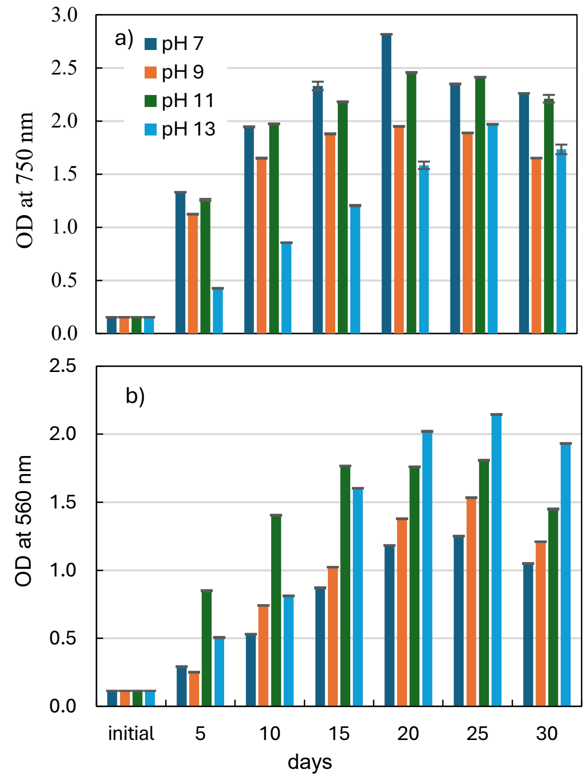

Figure 7. Effect of different pH niches on optical density (OD) readings in *S. pevalekii* (a) and *T. obliquus* (b).

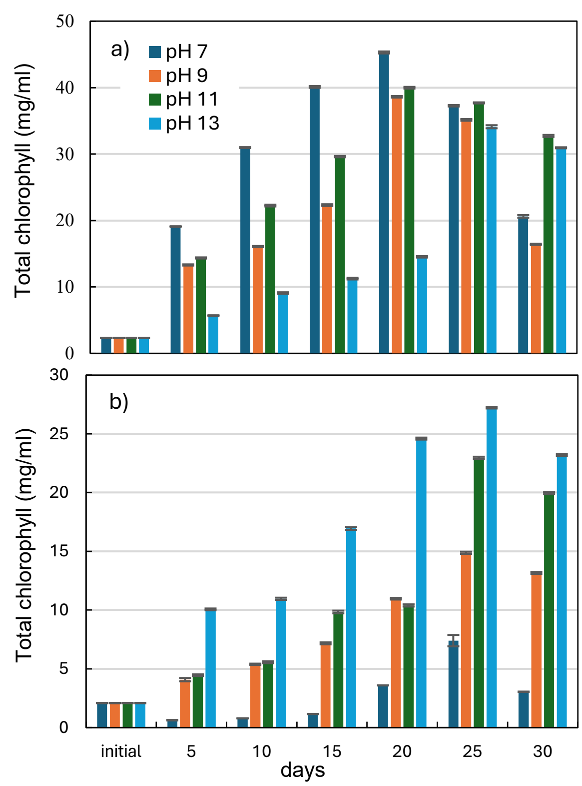

Figure 8. Effect of different pH gradients on total chlorophyll content in *S. pevalekii* (a) and *T. obliquus* (b).

Another study by Bai *et al.* (2024) found that *Tetradesmus* cf. *obliquus* ZYY1 exhibited vigorous heterotrophic growth in a BG-11 medium enriched with glucose, with the pH maintained between 7 and 8.5. Recently, Akgül (2024) pinpointed that *T. obliquus* could reach its maximum growth via cultivation in BG-11 medium at pH 7.5 and 1.5 g L^{-1} of sodium nitrate.

CONCLUSION

The study demonstrated that Bold's Basal medium was the most suitable growth medium for both *S. pevalekii* and *T. obliquus*, providing optimal conditions for maximizing algal biomass. Among the different nitrogen sources tested, sodium nitrate was identified as the most effective one for both species, significantly enhancing their growth rates and total chlorophyll contents. *S. pevalekii* reached the maximum growth at pH 7, while *T. obliquus* achieved the highest growth at pH 13. These findings emphasize the importance of optimizing culture media, nitrogen sources, and pH niches to improve the algal growth which can be beneficial for further biotechnological applications. Furthermore, this work contributes to achieving several Sustainable Development Goals (SDGs). Specifically, it supports SDG 6 (Clean Water and Sanitation): By optimizing algal growth conditions for wastewater treatment, the study helps improve water quality and promotes sustainable management of water resources. SDG 7 (Affordable and Clean Energy): The study also aligns with this goal through the potential use of algae in biofuel production. These findings highlight the broader impact of our research in promoting sustainable and environmentally friendly solutions to global challenges.

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