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THE EVALUATION OF THE GROWTH PERFORMANCE OF A CYANOBACTERIAL ISOLATE *PHORMIDIUM LUCIDUM* (KÜTZİNG EX GOMONT 1892) GROWN UNDER DIFFERENT ENVIRONMENTAL CONDITIONS AND ITS USE AS A DIETARY SOURCE FOR *DAPHNIA MAGNA* (STRAUS 1820)

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ABSTRACT. This study presents the effect of cyanobacterium isolated from Bolluk Lake (Konya, Türkiye) which is a saline lake on the growth performance of *Daphnia magna*. Isolated cyanobacteria species were identified as *Phormidium lucidum* according to its 16S rDNA sequences. The effects of different growth conditions including pH (7.18, 8.15, 9.17 and 10.26), light intensity (1200, 2400, 3600 and 4800 lux), temperature (10, 20, 25 and 30°C) and nitrogen concentrations (0.25, 0.5, 1.0 and 1.5 g/L) on *P. lucidum* was studied. Effects of each environmental factor on biochemical composition (total protein, total lipid and chlorophyll-a concentration) of *P. lucidum* were also studied. The optimum growth conditions were found as pH 7.18, ambient temperature 20°C, nitrogen 0.25 g/L and light intensity 3600 lux, after a 2-week incubation period. The effects of various mixtures of the cyanobacteria and *Chlorella vulgaris* which is a common feed for Daphniids were also evaluated for their effects on the growth rates of *D. magna*. The best growth rate for *D. magna* was obtained in the medium containing 100% *P. lucidum* at the end of the 13thday.

1. INTRODUCTION

Members of the cyanobacteria are called blue-green algae due to the pigments they contain. These prokaryotic organisms are known as the first photosynthetic organisms of the earth. They are mainly found in the oceans, thermal waters, freshwaters including lakes, rivers, streams, marshes, wetlands, permanent or temporary water bodies and saline ponds. They can also survive in very harsh environmental conditions such as temperatures up to 74°C, deserts, polar regions, rock interiors, terrestrial environments exposed to UV radiation [1-4].

Biomass increase in cyanobacterial cultures depends on various environmental factors such as pH, temperature, N and P content, salinity and light intensity [5-11]. Several studies indicated the changes in the concentration of metabolic

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products including total lipid, total protein and fatty acid produced when they are exposed to different environmental conditions such as pH, temperature, N and P amount, salinity and light intensity. It is also known that such parameters are species specific [11-13]. pH is considered one of the most important factors affecting the species diversity, growth and development of cyanobacteria [14-18]. Light intensity may lead to remarkable changes in the chemical composition, pigment content and photosynthetic activities of cyanobacteria [11,19-22]. Temperature may affect the growth and chemical composition of cyanobacteria through alterations in the metabolic rate [9]. Nitrogenous compounds are also known to effect cynabobacteria growth and chemical composition particularly lipid content [23-24].

Daphnia spp. is one of the most important and common food sources for freshwater fish both in aquaculture facilities and natural aquatic habitats. They are favored due to their rapid growth rate, high reproductive potential and short life cycles [25]. Daphniids tend to grow rapidly in environments where food items such as bacteria, yeast and microalgae are sufficient [26-27]. Several zooplanktonic species including Daphniids can not synthesize enough essential fatty acids and lipid. Although, several well known microalgal species are used widely in the culture of various zooplankton species including Daphniids [26-31], it is vital to determine lipid and fatty acid composition of new candidate microalgal and cyanobacterial species for Daphniid cultures. Extreme habitats such as alkaline lakes or saline lakes have a potential for isolation of new algal strains which could be used as an alternative dietary source for zooplankton cultures. Therefore, in this study we focused on a cyanobacteria species isolated from a saline lake, Bolluk Lake located in the Tuz Lake Basin which is home to several endemic taxa [32]. The strain was identified using 16S rDNA gene sequences and grown under several environmental conditions to determine optimum growth conditions. We compared chlorophyll-a, total protein and total lipid concentrations of *Phormidium lucidum* in order to assess its growth rates. Then, the growth rates of *Daphnia magna* fed with various mixtures of the isolate and a common green microalga, *Chlorella vulgaris* were tested to determine and compare the dietary potential of the isolate for *Daphnia magn*a culture. We choose *Chlorella vulgaris* as a control feed type since it is one of the most common feed types for Daphniids and thus enabling us to compare the nutritional value of the new isolate.

2. MATERIALS AND METHODS

2.1. Isolation of cyanobacteria *Phormidium lucidum*

Bolluk lake (38°32′25″N 32°56′34″E) is a salina lake located in Konya Closed Basin in Türkiye. This lake is chracterised by its high sodium sulphate content.

Water samples were taken with sterile dark colored glass bottles from 20 cm below the surface in duplicates. The samples were transferred to laboratory in ice boxes within 6 hours and were immediately inoculated in sterile flasks containing liquid BG-11 media. Samples were left to grow under a 2400 lux light intensity with white fluorescent bulbs, with a pH of 7.18 at ambient temperature (23 ± 2°C). Isolated algal cells were transferred to petri dishes and purified under aseptic conditions. Isolated algal culture under sterile conditions were added to 200 ml sterile flasks containing 100 ml of BG-11 medium for stress conditions experiment [33-34].

2.2. Phylogenetic analysis of isolated strain

Isolated cyanobacteria strain was identified according to its 16S rDNA gene sequences. EurX GeneMATRIX Bacterial & Yeast DNA isolation kit (Poland) was used for isolation of DNA from the isolated samples. In the PCR study (Kyratec thermocycler), gene regions targeted for species identification were amplified with 27F (5' AGAGTTTGATCMTGGCTCAG 3') – 1492R (5' TACGGYTACCTTGTTACGACTT) primers as universal primers. PCR steps were adjusted to 95°C for 5 min, 30 cycles of 95°C for 45 sec., 57°C for 45 sec. and 72°C for 60 sec. The amplification results obtained by PCR were carried out in 1.5% agarose gel prepared with 1x TAE buffer at 100 volts for 90 min and their image was taken in UV light using ethidium bromide dye.

2.3. Microalgae *Chlorella vulgaris* culture

Microalgae *Chlorella vulgaris* (CCAP-211/12) were provided by CCAP (Culture Collection of Algae and Protozoa, Scotland, UK). Freshwater microalgae *C. vulgaris* were cultured in 3N-BBM+V medium ($NaNO_3$+ $CaCl_2xH_2O$+ $MgSO_4x7H_2O$+ $K_2HPO_4x3H_2O$+ KH_2PO_4+ NaCl+ trace minerals+ Vitamin B1 and Vitamin B12) [35]. Cultured microalgae then harvested at stationary phase. In order to calculate growth numbers, microalgae were counted daily by Neubauer Hemocytometer. The microalgae culture was grown in liquid BG-11 medium at pH 7.18 for 14 days and freshly harvested *C. vulgaris* culture was used in the experiments.

2.4. Effects of experimental conditions (pH, light, nitrogen and temperature) on total protein, total lipid and chlorophyll-a concentrations in *P. lucidum*

In order to determine the optimum growth conditions for *P. lucidum* isolated from Bolluk Lake, we tested the growth performance of the cyanobacterium grown in BG-11 medium at different pH levels (7.18, 8.15, 9.17 and 10.26), temperature (10, 20, 25 and 30°C), light intensities (1200, 2400, 3600 and 4800 lux) and nitrogen concentrations (0.25, 0.5, 1.0 and 1.5 g/L ($NaNO_3$, Sigma

≥99.0%)). Experimental conditions tested were based on preliminary experiments and relevant studies [10-11,19,36]. Experiments were carried out in 200 ml flasks containing 100 ml of BG-11 at pH 7.18 for 14 days in triplicate. The results are given as the average of 3 repetitions.

2.5. Maintainace of *Daphnia magna* stock culture

Daphnia magna stock culture was maintained according to the procedure described by OECD guidline 211 (OECD, 2012) Stock cultures were kept in 100 lt aquaria under a photoperiod of 8 hours dark:16 hours light. One third of the water was changed every second day with dechlorinated tap water. The temperature for the stock cultures was kept constant at 20.2±1.3°C.

Daphnids used in the experiments were selected among individuals i. reproducing by parthenogenesis, and ii. were at least third generation and iii. age <24 hours. A total of 50 mature individuals were taken into the aquarium and the females were transferred to a new tank every week, and during this period the individuals ready to spawn were transferred to separate tanks and the newly hatched individuals were collected within 24 hours [37]. As a starting point, 10 individuals ready to lay eggs were used [38-40]. The experimental period lasted 21-days; a time in which *Daphnia magna* is reported to reach maxiumum output [26,41].

2.6. Growth rates of *Daphnia magna*

The nutritional value of *P. lucidium* were compared to *C. vulgaris* which is one of the most common feed types for Daphniid stock cultures. The mixtures tested were selected based on the approach reported by Lürling [42]. The mixtures including cynabocateria and *C. vulgaris* were mixed in a volume-to-volume ratio. To use cyanobacteria and microalgae cell density at approximate values, filamentous algae cells were transformed into smaller forms by morter. After obtaining the approximate cell density for both algae species, experimental feeding groups were created. Experimental feeding groups were 100% *Chlorella vulgaris* (100CV), 50% *Chlorella vulgaris* + 50% *Phormidium lucidum* (50CV+50PL), 25% *Chlorella vulgaris* + 75% *Phormidium lucidum* (25CV+75PL), 75% *Chlorella vulgaris* + 25% *Phormidium lucidum* (75CV+25PL) and 100% *Phormidium lucidum* (100PL) with 5 different food combinations (100 ml 10%, per 1 liter) have been evaluated. The use cell densities equally for all algae the filamentous cells were divided into small pieces in a mortar and the approximate cell number was obtained for both algae. The mixtures including cyanobacteria and microalgae were mixed in a volume-to-volume ratio.

The total number of daphniids were counted in each experimental food group every two days during the 21-day trial period to estimate the growth performance of *D. magna*. The formulae [43] given below was used for calculations (2.1).

$$r = (In\ Nt - In\ No)/t \quad (2.1)$$

No= Number of daphniids at the beginning
Nt= Number of daphniids at the end of each trial
t= Time (days) to reach the maximum number of individuals per unit volume (ml)
% r = Growth rate

2.7. Chlorophyll-a analysis

The chlorophyll-a concentration in the media for each cyanobacterium group was determined in aqueous 90% acetone (≥99.5%, Isolab) solution. The concentration of chlorophyll-a was found with optical absorption at 630, 645, 665 ve 750 nm, respectively [44]. Calculation is made and the amount of chlorophyll-a is calculated by writing the resulting value into the formula below (2.2).

Chlorophyll-a = (11.85 × OD665) – (1.54 × OD645) – (0.08 × OD630) × dilution rate (2.2)

2.8. Total protein (TP) and total lipid (TL) analysis

The protein concentration in the samples *P. lucidum* were measured spectrophotometrically [45] after homogenization within in a mortar. Total lipid concentration in each cyanobacterium group grown at different conditions was calculated using a modified method of Bligh and Dyer [46] with the following formulae (2.3).

Lipid% = [amount of lipid extracted (g)/ weight of microalgae sample (g)] × 100 (2.3)

2.9. Statistical analysis

The data are given as mean ± standard deviation (SD) of the 3 replicates for each group. The data were tested for goodness of fit to a normal distribution prior to the analyzes using Shapiro Wilk-W test. One Way ANOVA and Duncan multiple comparison tests were performed to analyze significant differences among groups. Results were considered significant where $P<0.05$. All statistical analyzes were performed using the statistical package program, SPPS (v23.0).

3. RESULTS

3.1. The physicochemical parameters of Bolluk Lake (Konya, Türkiye)

The physicochemical parameters of the lake water measured simultenaously a WTW portable multi meter device were as follows: temperature 28.9°C, pH 10.7, dissolved oxygen 5.37 mg/L and electrical conductivity 129 µs/cm.

3.2. Identification of isolated cyanobacteria

The 16S rDNA sequences of the isolated cyanobacterium indicated that the strain had a >99% similarity to *Phormidium lucidum* according to the NCBI Gen-Bank.

3.3. The effects of different growth conditions biochemical content in *P. lucidum*

3.3.1. pH

The total protein and lipid concentrations of the cyanobacterium *P. lucidum* grown at different pH levels (7.18, 8.15, 9.17 and 10.26) are given Figure 1a. The highest (26.7±0.02 mg/L) and lowest (13.4±0.07 mg/L) protein concentrations were observed in groups grown at 7.18 and 10.26, respectively. There was gradual decrease in the TP concentration with increasing pH levels. An opposite pattern was observed for TL concentration (Figure 1a); with the highest (11.5±0.07%) content observed at pH 10.26 and lowest (3.83±0.09%) when grown at pH 7.18. Chl-a concentrations of *P. lucidum* showed a decreasing pattern with increasing pH levels (Figure 1a). The highest and lowest chl-a concentrations were 1.88±0.41 µg/L and 0.41±0.26 µg/L when grown at pH levels of 7.18 and 10.26, respectively.

3.3.2. Light intensity

Biochemical responses of *P. lucidum* grown under various light intensities are summarized in Figure 1b. The highest TP (18.9±0.03 mg/L) and TL (7.96±0.39%) concentrations were observed in cyanobacterium grown under 3600 lux. Chl-a concentrations were found 0.661±0.034 µg/L, 0.537±0.013 µg/L, 0.459±0.016 µg/L and 0.343±0.032 µg/L when cultivated in media under 1200 lux, 2400 lux, 3600 lux and 4800 lux different light illumination, respectively.

3.3.3. Nitrogen concentrations

The effects of different nitrogen concentrations on the growth of *P. lucidum* was shown in Figure 1c. The TP concentrations showed a gradual increase with decreasing N concentration in the growth media. The highest TP concentration

23.74±0.01 mg/L was recorded in cyanobacteria grown in the media containing 0.25N g/L. Lipid concentrations showed a slight variation depending on the N content in the growth medium. There was gradual increase in the chl-a concentration with decreasing N concentraion. The highest (2.265±0.068 µg/L) and lowest (0.807±0.112 µg/L) chl-a concentrations were observed in groups grown at 1.5N g/L and 0.25N g/L, respectively.

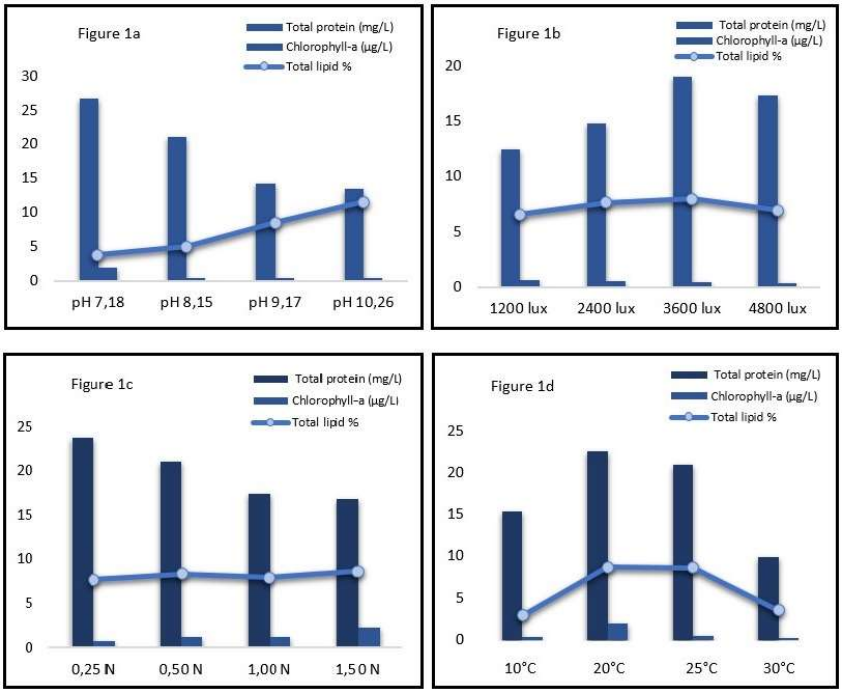

FIGURE 1. The other parameters applied while determining TP, TL and Chl-a amount in the stress conditions experiment, respectively; a: 2400 lux, 1N, 23 ± 2°C, b: pH 7.18, 1N, 23 ± 2°C, c: pH 7.18, 3600 lux, 23 ± 2°C, d: pH 7.18, 0.25 N, 3600 lux.

3.3.4. Temperature

In order to test the effects of increasing temperature on *P. lucidum*, the ambient temperature range was set to four different temperatures ranging from 10 to 30°C. Both TP and TL concentrations showed a gradual increase up to 30°C, but decreased at 25°C and reached to min levels at 30°C (Figure 1d). A similar trend was observed for chl-a levels with a highest level at 20°C (1.921±0.130 µg/L) and lowest at 30°C (0.171±0.072 µg/L).

According to the results mentioned above the optimum growth conditions for *P. lucidum* was set to 7.18 for pH, 3600 lux for light intensity, 0.25 g/L for N and 20°C for temperature. Bioexperiments for *D. magna* were carried out with *P. lucidum* grown in optimum growth conditions.

3.3.5. Evaluation of diet composition on the growth rates of *Daphnia magna*

Daphnia magna were fed with five different nutrient groups indlucing various concentrations of microalgae *C. vulgaris* and cyanobacteria *P. lucidum* to determine the optimum growth rates for *D. magna*.

4. DISCUSSION

The number of adult females and juveniles were calculated separately and it was found that there was no recruitment of juveniles up to the first 5 days of the experiments (Table 1). Similarly, there was no changes in the number of adult females during this initial phase. It was observed that the number of juveniles increased with the addition of *P. lucidum* to the as diet, while for adult females it increased when *C. vulgaris* was added to the as diet. In this study, it was seen that the use of PL as a diet supported the increase in the number of new individuals depending on the algae rate and it was seen that it gave weight to development and maturation depending on the adult female CV rate. However, a sharp increase was noted on the 13th day in all diet groups. The highest recruitment was observed in daphniids fed with 100% *P. lucidum* (Figure 2). An identical pattern was also observed for the number of adult females on the 13th day.

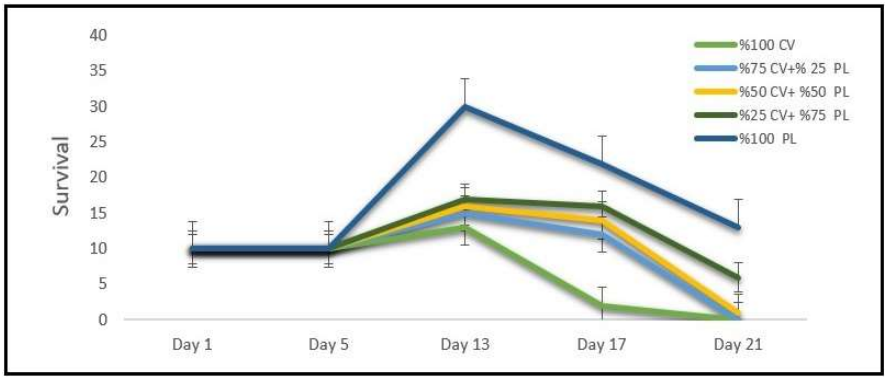

FIGURE 2. Number of individuals surviving different diets for 21 days experimental period

Bolluk Lake is an extreme habitat due to its high sodium sulphate and located in Tuz Lake Basin which is home to several endemic species, such as *Silene salsuginea* and *Saponaria halophila* [47-48]. Thus, have a potential to be home to new isolates of microalgae and cyanobacteria which might have a nutritional value for zooplankton cultures. Therefore, in this study we aimed to determine i. the optimum growth conditions (pH, light intensity, nitrogen and temperature) for the cyanobacteria, *Phormidium lucidum*, isolated from Bolluk Lake and ii. its

potential to be used as an alternative food source for *Daphnia magna*. Several studies indicated that the optimum growth rates and thus the biomass of various algal species depend on several environmental parameters including light intensity, temperature, nutrient composition, salinity and pH [5-11].

TABLE 1. *Daphnia magna* growth rate, adult females and juveniles individuals

Days /diets	**100% CV**	**75% CV + 25% PL**	**50% CV+ 50% PL**	**25% CV + 75% PL**	**100% PL**
Juveniles					
Day 1	0	0	0	0	0
Day 5	0	0	0	0	0
Day 13	5.333±1.528	8.333±3.055	9.667±2.082	9.667±2.309	21.333±4.163
Day 17	2±2	7.333±2.082	8.333±4.726	10.667±2.517	15± 6.083
Day 21	0	0	0.667±0.577	4.333±2.517	8.333±1.528
Adult females					
Day 1	10	10	10	10	10
Day 5	10	10	10	10	10
Day 13	12.524±0.577	6.667±0.577	6.333±1.528	7.333±1.528	9.333±0.577
Day 17	0.333±0.57	4.333±0.577	5.333±2.082	5.667±1.155	6.667±1.528
Day 21	0	0	0.333±0.577	1.667±0.577	4.667±2.517
Growth rate %					
Day 1	0	0	0	0	0
Day 5	0	0	0	0	0
Day 13	12.231	14.231	15.231	16.231	30.231
Day 17	1.411	11.411	13.411	15.411	21.411
Day 21	0	0	0.524	5.524	12.524

pH is considered as one of the most important factors affecting the growth rates, distribution and diversity of cyanobacteria species [14-18]. We found that TP and chl-a concentrations showed a gradual increase with decreasing pH levels with being highest at pH 7.18. An opposite pattern was observed for TL concentraions reaching maximum levels at pH 10.26. Chandra and Rajashekhar [18] reported that the optimum pH level ranges between 5.5-10 for *P. lucidum* and they observed highest growth rate at pH 7.5 with a maximum chl-a concentration. Other reports are also available; indicating optimum pH was 9 for *Spirulina platensis* with a considerably higher TP and chl-a concentration or demonstrating favorable pH levels were approximately 8 for *Halomicronema hongdechloris* [49]. Yadav et al. [11], found no differences for TL concentrations and biomass production among two different *Phormidium* sp. grown under different pH levels.

Several studies are available indicating the effect of light intensity on the growth of cyanobacteria species [11,19-22]. In this study we found that the highest TP and TL concentrations are observed in *P. lucidum* grown under 3600 lux. Yadav et al. [11], reported a similar pattern demonstrating that the increasing light intensity had a positive effect on lipid content in *Phormidium* sp. However, an opposite pattern was observed for chl-a concentrations with increasing light intensity, being highest at 1200 lux in our study. Similar results are available for

Phormidium sp. with a positive effect on biomass production and a decrease in chl-a concentrations with increasing light intensity had [21] and for *Spirulina platensis* with a decrease in chl-a concentration and an increase in TP concentrations with increasing light intensity [49] and for *Phormidium* sp. with the highest chl-a concentration at lowest light intensity [10]. Several studies report that higher light intensity is associated with lower chl-a concentrations, higher lipid content in algal cells [10,19], there are also studies showing increasing light intensity did not lead to significant alterations in *Nostoc spongiarforme* and *Phormidium corium* [50]. Furthermore, it is also known that color and photoperiod may affect the production of fatty acids in cyanobacteria [10,17,20-21,51-53]. It has been shown that optimum growth rates, chl-a, TP and TL concentrations vary depending on the species-specific light intensity and color requirements of the algal species including *Phormidium* sp., *Spirulina platensis, Nostoc sphaeroides, Scenedesmus abundans, Chlorella sorokiniana, Pseudanabaena galeata, Microcystis aeruginosa, Synechococcus* sp., *Cyanobium* sp., *Oscillatoria* sp. [10-11,19,49,54-58].

Nitrogen content is one of the cheapest and easiest methods used to increase lipid content in algal cells [59]. We found that the increase in nitrogen amount in the growth medium led to an increase on chl-a concentration of *P. lucidum*. On the other hand, we observed an opposite pattern for TP concentrations. Essential nutrients such as nitrogen are vital for growth and synthesis of proteins, nucleic acids and celular components in algal cells [60]. Cyanobacteria can use a variety of nitrogen sources, including nitrate (NO_3^-), nitrite (NO_2^-) and ammonium (NH_4^+) [23-24]. However, any changes in the concentrations of nitrogenous compounds or the sources used for nitrogen may lead to changes in the growth rate, development, biomass production, N_2 fixation rates, presence of heterocysts, chl-a, polysaccharide composition, secondary metabolite levels, TP and TL concentrations in cyanobacterial strains [56-57,60-64].

The changes in the ambient temperature affects the rate of cell metabolism and biochemical composition of cells [9]. Several studies have shown the effects of temperature are species speficic [9,16,65-66] and may lead to changes in protein production, biomass, growth rate and lipid content in algal cells [11,19,22]. In our experiments, we found that the highest growth rates and TP, TL and chl-a concentrations were observed in *P. lucidum* grown at 20°C. Yadav et al. [11], reported that TL content in *Phormidium* sp. increased with increasing ambient temperature. We also observed that P. lucidum was also able to tolerate temperatures up to 25°C. Similar findings were also reported by Hotos [10] who stated that optimum range is between 21 and 22.5 °C for *Phormidium* sp.

Each zooplankton species has its own specific requirements for suitable food type and quantity in order to obtain a high growth rate [67-69]. Several natural or commercially produced nutrient types are available for daphniids. *Ankistrodesmus* sp., *Botrycoccus* sp., *Chlorella* sp., *Cylindrospermopsis* sp., *Nannochloropsis* sp., *Scenedesmus* sp. and *Stephanodiscus* sp. are among the

most used natural food sources for *Daphnia* spp. [26-31,68]. However, no information exists on the use of *P. lucidum* as a food source for *D. magna*. Choi et al. [30], found that *C. vulgaris* was not effective to obtain a larger Dapniid size when used alone. However, they reported a larger size when *D. magna* was fed with *Stephanodiscus hantzschii-Chlorella vulgaris* or *Stephanodiscus hantzschii* alone. *Ölmez et al*. [29], found that highest growth rates for *D. magna* were recorded when they are fed with a containing *Scenedesmus acuminatus*. On the other hand, in another study where the mixtures of *Clamydomonas* sp. and *Chlorella* sp. were tested to feed *D. magna*, highest growth rates were observed when they are fed with single *Chlorella* sp. cell cultures [70]. Bednarska et al. [71], in their study with *C. raciborskii* and *S. obliquus*, stated that the first reproductive age increased in clones fed with *C. raciborskii* due to temperature and *D. magna* clones would gain durability in adapting to the environment. They found that feeding green algae promoted early maturation, increase in body size and egg size.

4. CONCLUSIONS

Daphnia magna is a valuable zooplankton species which is widely used in fish production. Although, several microrganisms including *Scenedesmus* spp., *Chlorella* spp. and yeast generally have been used for Daphniid culture [26-31,68-69] testing new microalgal strains for their nutritional value is vital for aquaculture development. In this study, the effects of the mixtures of *C. vulgaris* and the cyanobacterial isolate *P. lucidum*, on the growth rates of *D. magna* were investigated. We found that *Phormidium lucidum* is a productive species in terms of biomass under optimum growth conditions and has a potential to be used as an alternative feed for *Daphnia magna* cultures.

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Author Contribution Statements SA-supervising, resources, conceptualization, writing–review. TA-conceptualization, investigation. MBE-data curation, formal analysis, manuscript editing. KME-project development, manuscript editing. KK-data analysis, manuscript writing and manuscript editing. All authors have read and approved the manuscript.

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

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