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Morphological Characterization, Pigments and Biochemical Composition of Isolated Microalgae from South Eastern Freshwater Habitat of Bangladesh

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ABSTRACT

Identification and characterization of the microalgae is prerequisite before using them for any kind of commercial use. So, the study was designed to determine the growth, pigments and biochemical composition of four different freshwater microalgae (*Nephrocytium* sp., *Nannochloropsis* sp., *Protococcus* sp., and *Pectinodesmus* sp.). Growth performance was evaluated by collecting the data of cell density and optical density. Isolated microalgae were mass cultured in Bold Basal Media and harvested at their early stationary phase. Result showed that, *Nephrocytium* sp. showed significantly ($p < 0.05$) highest chlorophyll a and chlorophyll b and *Protococcus* sp. resulted highest carotenoid. Protein and lipids were significantly ($p < 0.05$) highest in *Nannochloropsis* sp. Moreover, *Protococcus* sp. resulted higher amount of saturated fatty acids, monounsaturated fatty acids, and omega 3 polyunsaturated fatty acids. Current results will aid to choose microalgal strains that possess fast growth, suitable pigments and biochemical composition to utilize them in several industrial applications.

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Introduction

Microalgae are unicellular and multi-cellular microscopic autotrophs/heterotrophs where above forty thousand microalgae species have been identified, which are considered as natural producers of protein, lipid, carotenoids, and fatty acids. Microalgae are important not only in human and animal nutrition but also in medicines [1-3]. Protein content of microalgae is considered as one of the crucial components influencing their nutritional value [4]. Microalgae lipid content may vary from 20% to 50% of dry weight and the high oil containing microalgae species has been utilized in biofuel production. Microalgal fatty acids that have carbon chains between C14 and C20 are generally exploited for biodiesel production [5,6]. As well as microalgal lipids that have chains longer than C20 are usually PUFAs which include ω-6 and ω-3 fatty acids like DHA and EPA, not only important nutrients for humans but also act as food supplements [7]. Compared to other microalgae compounds, carbohydrates have a lesser energy value, but they are the preliminary raw component for the synthesis of biofuels via biotechnological conversion [8]. Pigments are considered as one of the most essential products from microalgae and chlorophylls, carotenoids, and phycobilins are the significant pigment group found in microalgae [9]. Chlorophyll is one of the significant bioactive materials that can be extracted from microalgae and have antioxidant property, utilized as a natural food coloring agent and used widely in pharmaceutical products [10,11]. Moreover, carotenoid, a natural, fat-soluble, yellow to red pigments, is principally dominant in plants, where they play an important role in photosynthesis of algae and photosynthetic bacteria [12]. Along with this, phycobiliproteins are used as natural dyes and extensively applied as nutraceuticals and in other biotechnological applications like food, cosmetics, diagnostics, and pharmaceutical industries [13].

Microalgae which have valuable characteristics like lipid, carbohydrate and protein, are applied in aquaculture, and have economic potential [14]. A deep knowledge of the behavior of a specific microalgal strain in response to various culture conditions, like nutrient supply, is important for the optimization of mass microalgal production [15]. Hence, to develop a reliable and commercially viable process for the mass production of microalgae, selection of algal species and strain can be considered as first and most important step. Selection of the best performing tropical strains should be carried out to utilize the nutritional properties as they are well adapted to similar environment, exhibit better performance and robustness than those from a strain bank collection [16,17]. But microalgal research has not been so developed in Bangladesh.

Therefore, the aim of this study was to isolate microalgae from different south-eastern freshwater habitats of Bangladesh (Kaptai Lake, Halda River and Karnaphuli River) and to compare the growth, pigments, proximate composition and fatty acids of isolated microalgae.

Materials and Method

Water Sampling and Isolation of Microalgae

The freshwater microalgae samples were collected from March to May from three different sites in Chattogram, Bangladesh including Kaptai Lake, Rangamati (22o64′ N, 92o19′ E), Halda River, Chattogram (22o51′ N, 91o84′ E) and Karnaphuli River (22°50' N 92°14' E). The water temperature, pH, DO and salinity were measured from the surface water by using a glass thermometer, a handheld pH meter (pHep-HI98107, HANNA, Romania), a dissolved oxygen meter (DO-5509, Lutron), and a handheld ATC refractometer (YEGREN), respectively. All the instruments were calibrated before use. As well as, TAN, NO2-N, and SRP were determined by Parsons et al. analytical methods [18].

For the water samples, 40-50L freshwater was filtered through the plankton net (60 µm mesh size). The filtered samples were collected in 300 ml sample bottles and maintained at refrigerated conditions while transferring them to the laboratory for isolation. The filtered raw freshwater samples were concentrated by centrifuging at 4000 rpm for 5 minutes in the laboratory, and then isolated by streak plating on 1.5% BBM agar in Petri dishes [19].

The filtered freshwater samples were also preserved by adding a few drops of Lugol's iodine to determine the species composition of microalgal community. Morphological identification of microalgae was conducted under a microscope at 40X magnification based on the morphological characteristics [20,21].

Determination of Growth Curve

Cultures were grown at 24 ± 1°C in 500 mL triplicate sterile borosilicate Erlenmeyer flask containing 350 mL of BBM with 2% microalgae stock. Cultures were grown at 150 µE m-2 s-1 for 24 h light condition with 4.53 ± 0.53 mgL-1 aeration rate. Based on the cell density (cells ml-1) and optical density growth curve was determined for each species until the death phase. Cell density and optical density both were analyzed in this study to reduce the experimental errors.

Microalgal cells were counted by hemacytometer (Assistent, Germany) and determined according to the formula of Lavens & Sorgeloos, [22]. Maximum absorbance was determined by scanning the microalgae sample between 300 to 700 nm, using a Nano Drop spectrophotometer (Nano Plus, Wave Analytics, Germany). Optical density was observed at the wavelength of 430 nm for *Nephrocytium* sp., 450 nm for *Nannochloropsis* sp. 426 nm for *Protococcus* sp., and 362 nm for *Pectinodesmus* sp. The optical density (OD) at those wavelengths was used to determine the growth curve of each species.

Experimental Design for Pigment and Proximate Composition Determination

Further experiments were conducted to determine the pigment, proximate composition, and fatty acids. In large sterile 2 L borosilicate Erlenmeyer flasks, 1.7 L pure BBM were used for this experiment. Each of the microalgae species was cultured as described above until the stationary phase. From the fresh cultured sample, chlorophylls and carotenoids were analyzed at the end of their exponential phase. As phycobiliprotein and proximate composition analysis required dry biomass, all the cultures were harvested at the end of their exponential phase by centrifugation (himac CR 21g-II, Hitachi, Japan), and dried at 40°C and preserved at refrigerated condition (4°C) for further use.

Determination of SGR

Biomass was determined and calculated according to the procedure of Ratha et al., [23].

SGR (mgday-1) of the cultured microalga was calculated according to the following formula:

$$SGR\ (mg/day) = \ln (X1 - X2)/(t1 - t2) \quad (1)$$

where X1 and X2 are biomass concentration of the end and beginning of selected time interval, respectively, and t1 – t2 is time elapsed between the selected time in the day [24].

Determination of Chlorophylls, Carotenoids, and Phycobiliproteins

For the determination of chlorophylls, microalgae were extracted according to the procedure of Dixit et al., [25]. Concentrations (mg/L) of chlorophyll a and chlorophyll b were determined with the absorbance at 664, 647, and 630 nm [26,27].

Carotenoid from microalgae was extracted according to the procedure reported by Khatoon et al., [28]. Carotenoid concentration (mg/L) was calculated from the absorbance at a wavelength of 450 nm.

To determine the phycobiliproteins, extraction of microalgae was done by following the procedure reported by Siegelman et al., [29]. The amount (mgmL-1) of PC, APC, and PE in the sample was calculated from the absorbance at the wavelength of 562, 615, 652, and 720 nm [29-31].

Determination of Proximate Composition and Fatty Acids

Protein, carbohydrate and lipid were determined by following the methods of [32-34]. Where fatty acids were analyzed by "Two steps transesterification (2TE)" method followed by [35].

Statistical Analysis

All data (growth, biochemical composition, pigments content) were analyzed statistically by using the IBM SPSS (v. 26.0). For each of the parameters of each microalgae descriptive statistics was done and a test for homogeneity of variance was performed. One-way analysis of variance (ANOVA) was used to examine all the data, and Tukey's multiple comparison tests were used to determine whether there were any significant differences across microalgal species at the 95% confidence interval level. A post-hoc test was used to identify group differences.

Results

Water Quality Parameters and Species Composition of Microalgal Community of The Sampling Sites

The physicochemical parameter of collected water from different sites was shown in Table 1 where differences in physical and chemical parameters were observed among different sites. Temperature and pH were highest in Kaptai lake and DO was higher in Karnaphuli River and sampling was done in midafternoon in Karnaphuli River. Together with this, total ammonia nitrogen was almost similar in three sampling site; both soluble reactive phosphate and nitrite-nitrogen were highest in Halda River. Species composition of the collected water from different sampling sites was shown in Table 2 and Figure 1.

Table 1: Water Quality Parameters of the Sample Water Collected from Different Freshwater Sites:

Parameters	Halda river	Karnaphuli river	Kaptai lake
Temperature (˚C)	26.4	28.2	30
DO (mgL^{-1})	4.2	7.7	6.33
pH	8.9	8.0	8.4
TAN (mgL^{-1})	0.03	0.02	0.03
SRP (mgL^{-1})	0.16	0.04	0.09
NO2-N (mgL^{-1})	0.12	0.06	0.04

Table 2: Species Composition in Different Sampling Sites Where "*" Represents the Presence of Species in the Sampling Site.

Microalgae species	Halda river	Kaptai lake	Karnaphuli river
Nannochloropsis sp.	*		
Pinnularia sp.	*		
Cryptomonas sp.	*		
Navicula sp.	*		*
Thalassiosira sp.	*	*	*
Cyclotella sp.	*	*	*
Synedra sp.	*		
Nitzschia sp.	*		*
Oedogonium sp.		*	
Coelastrum sp.		*	
Staurastrum sp.		*	*
Protococcus sp.		*	
Cosmarium sp.		*	
Ceratium sp.		*	*
Anabaena sp.		*	*
Tribonema sp.		*	
Pediastrum sp.		*	*
Microcystis sp.		*	*
Dinobryon sp.		*	*
Tetraedron sp.		*	*
Chroococcus sp.		*	*
Guinardia sp.			*
Spirogyra sp.			*
Epithemia sp.			*
Gomphonema sp.			*
Nephrocytium sp.			*
Pectinodesmus sp.			*

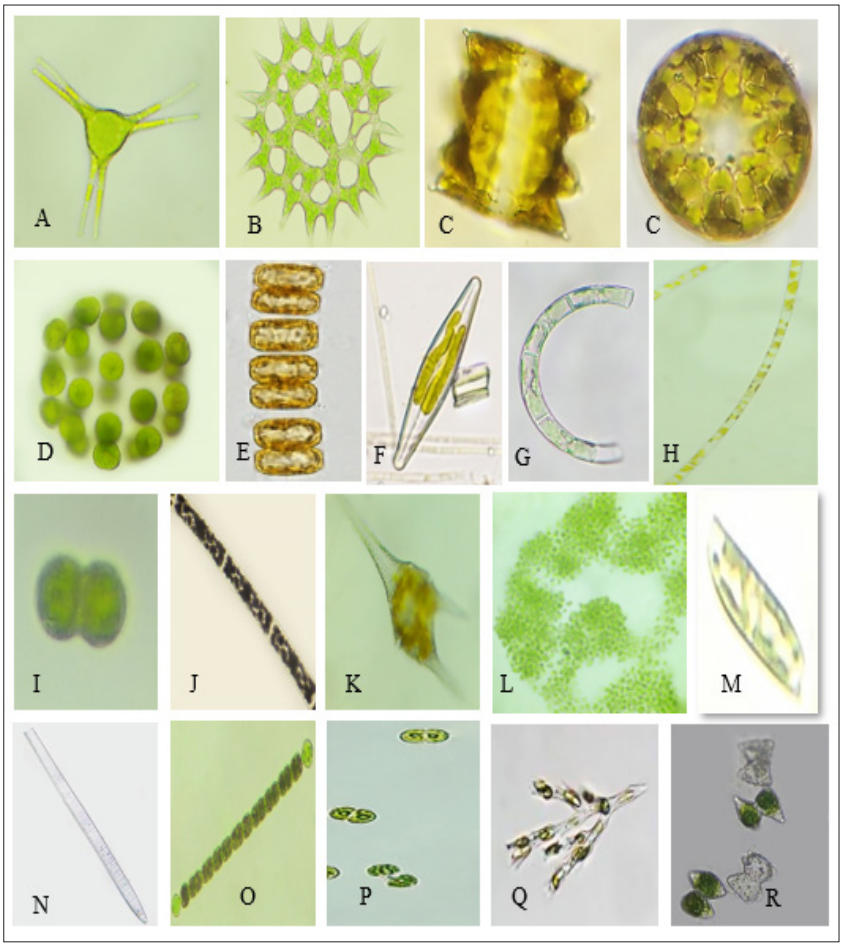


Figure 1: Light microscopic pictures of commonly found microalgae in three different sampling stations, *Staurastrum* sp. (A), *Pediastrum* sp. (B), *Cyclotella* sp. (C), *Coelastrum* sp. (D), *Thalassiosira* sp. (E), *Navicula* sp. (F), *Guinardia* sp. (G), *Oedogonium* sp. (H), *Chroococcus* sp. (I), *Spirogyra* sp. (J), *Ceratium* sp. (K), *Microcystis* sp. (L), *Pinnularia* sp. (M), *Synedra* sp. (N), *Anabaena* sp. (O), *Cosmarium* sp. (P), *Dinobryon* sp. (Q) and *Tetraedron* sp. (R)

Characterization of Isolated Microalgae

Four species of microalgae were isolated in this study (Figure 2). Isolated microalgae were *Nephrocytium* sp. (A), *Nannochloropsis* sp. (B), *Protococcus* sp. (C) and *Pectinodesmus* sp. (D) of which the largest one is *Pectinodesmus* sp. (32 µm) and the smallest one is *Nannochloropsis* sp. (3.3 µm). Among the four microalgae, *Protococcus* sp. was isolated from the Kaptai lake, where *Nephrocytium* sp. and *Pectinodesmus* sp. from Karnaphuli river and *Nannochloropsis* sp. from Halda river.

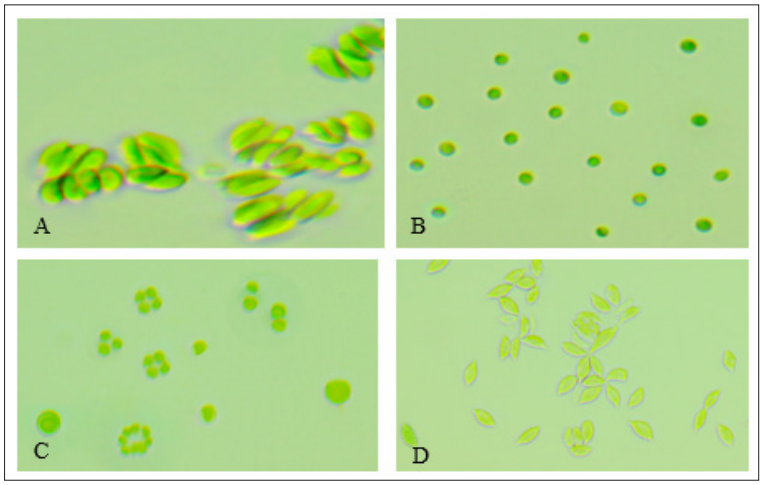
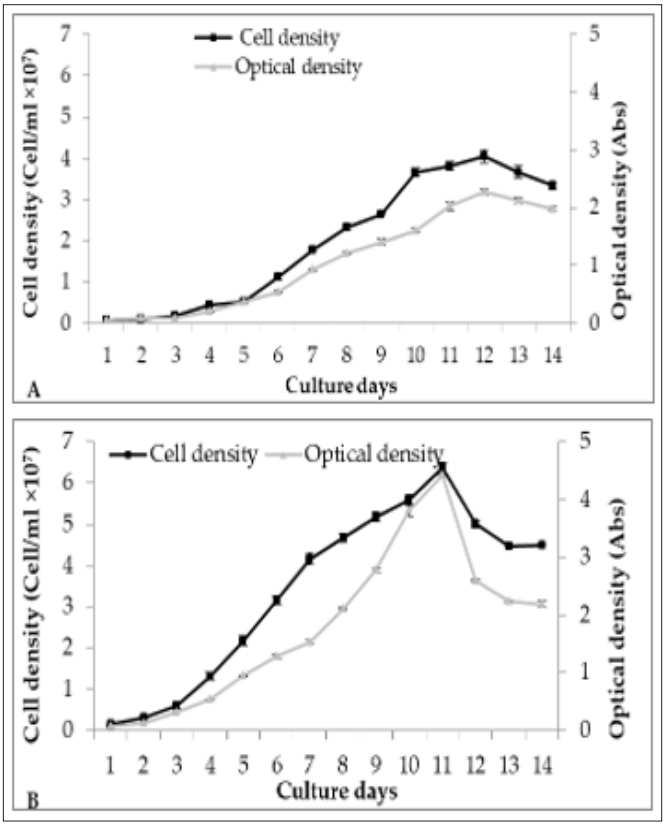
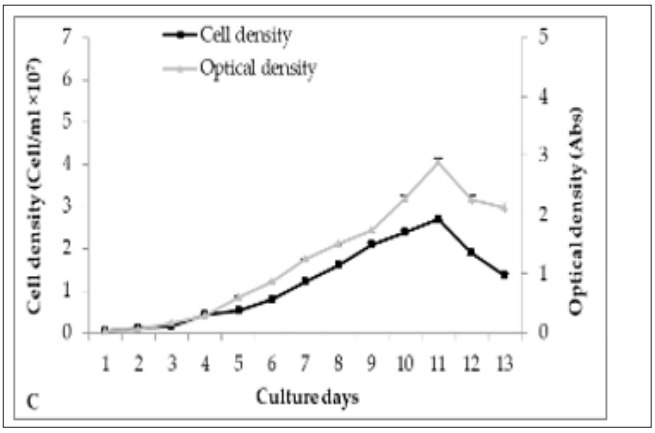


Figure 2: Light Microscopic Pictures of Isolated Microalgae, *Nephrocytium* sp. (A), *Nannochloropsis* sp. (B), *Protococcus* sp. (C), *Pectinodesmus* sp. (D)

Growth Phases of Microalgae Species

Growth showed that onset of stationary phase (9-14 days) varied among the four species. Based on the growth curve it is possible to determine the growth phases of those four microalgae. The growth phases are almost same for *Nannochloropsis* sp. (Fig. 3. B), *Protococcus* sp. (Fig. 3. C) and *Pectinodesmus* sp. (Fig. 3. D) that showed the lag phase on days 1 to 3, the exponential phase on days 3 to 11, the stationary phase on days 10 to 11, and finally the phase of death after 11 days. Similarly, *Nephrocytium* sp. showed the lag phase on 1 to 3 days, the exponential phase on 3 to 12 days, the stationary phase on 11 to 13 days and the death phase from 13 days (Fig. 3. A). In the stationary phase, cell density was significantly higher ($p < 0.05$) in *Nannochloropsis* sp. (6.374×107 cellsml-1) compared to the other microalgae.





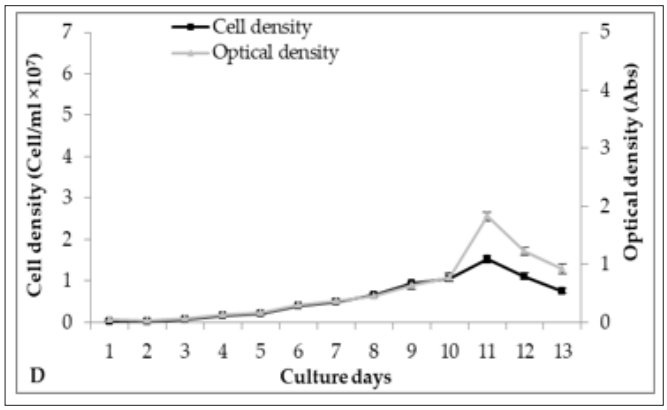


Figure 3: Growth Curves of Isolated Microalgae *Nephrocytium* sp. (A), *Nannochloropsis* sp. (B), *Protococcus* sp. (C), and *Pectinodesmus* sp. (D) in terms of cell density (cells/ml×107) and optical density. Values are means ± standard error

SGR of Microalgae Species

Different microalgae showed a significant variation in SGR (Fig. 4). The SGR of *Nannochloropsis* sp., *Nephrocytium* sp., *Pectinodesmus* sp., and *Protococcus* sp. was 0.665±0.002, 0.60±0.002, 0.646±0.001, and 0.661±0.001 mgday-1, respectively. Significantly highest ($p < 0.05$) SGR were observed in *Nannochloropsis* sp. and *Protococcus* sp. while lowest ($p < 0.05$) SGR were in *Nephrocytium* sp.

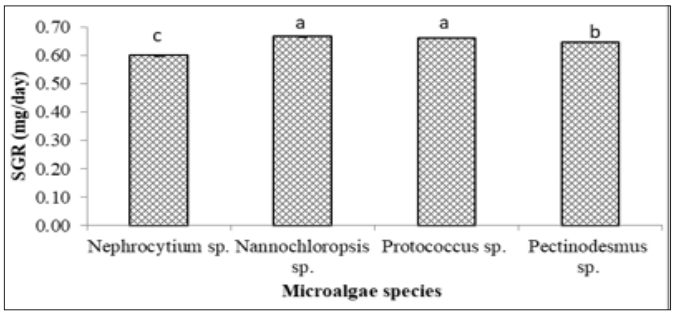


Figure 4: SGR (mean ± SE) of isolated microalgae. Values with the different letters within each series indicate significant differences ($p < 0.05$) among the four species

Chlorophyll, Carotenoid and Phycobilipreoteins Content of Isolated Microalgae

Chlorophyll, carotenoid, and phycoboliproteins content were investigated in this study. Different microalgae showed a considerable variation in chlorophyll, carotenoid (Fig. 5) and phycobiliproteins (Table 4) content. Chlorophyll and carotenoid were recorded in µg/ml as they were determined from wet samples and phycobiliproteins was recorded in mg/g as it was determined from dry microalgal biomass.

Significantly highest ($p < 0.05$) amount of chlorophyll a (7.98±0.02 µgmL-1) and chlorophyll b (1.28±0.05 µgmL-1) were observed in *Nephrocytium* sp. on days 11. On the contrary, *Pectinodesmus* sp. showed significantly lowest ($p < 0.05$) amount of chlorophyll a (2.51±0.03 µgmL-1) and chlorophyll b (0.54±0.03 µgmL-1) on days 10. Moreover, *Nannochloropsis* sp. and *Protococcus* sp. resulted about 5.557 ± 0.03 µgmL-1 and 5.87±0.02 µgmL-1 of chlorophyll a whereas *Protococcus* sp. showed 1.09±0.03 µgmL-1 of chlorophyll b but no chlorophyll-b content was recorded from *Nannochloropsis* sp. On the other hand, total carotenoid content

was also varied among those microalgae. Significantly ($p < 0.05$) highest quantity of carotenoid accumulations was observed in *Protococcus* sp. on days 10 (6.80±0.12 µgmL-1) whereas those of *Pectinodesmus* sp. was lowest on days 10 (2.07±0.07 µgmL-1) among the four strains.

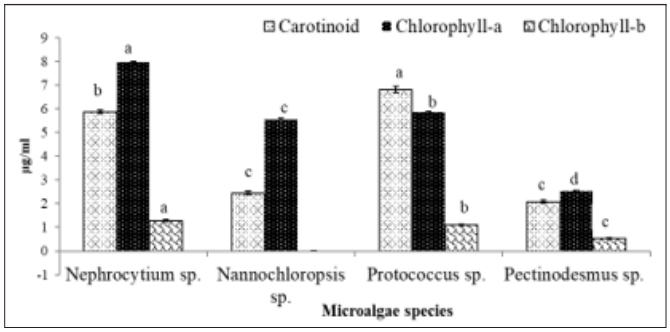


Figure 5: Carotenoid, chlorophyll a, and chlorophyll b content of isolated microalgae. Values are means ± SE. Values with the different letters within each pigment indicate significant differences ($p < 0.05$) among the four species

Phycobiliproteins analysis found that PC, APC, PE, and total phycobiliprotein content were significantly ($p < 0.05$) highest in *Nephrocytium* sp. on days 11 and lowest in *Pectinodesmus* sp. (Table 3). *Protococcus* sp. and *Pectinodesmus* sp. did not show any significant difference in total phycobiliprotein content.

Table 3: Phycobiliproteins content (mg/g) of isolated microalgae cultured in BBM. Values are means ± SE. Values with the different letters within each series indicate significant differences ($p < 0.05$) among the four species.

Species	PC	APC	PE	Total phycobiliproteins
Nephrocytium sp.	0.68±0.01[a]	2.27±0.02[a]	0.55±0.01[a]	3.497±0.023[a]
Nannochloropsis sp.	0.50±0.00[b]	1.67±0.03[b]	0.48±0.01[b]	2.627±0.041[b]
Protococcus sp.	0.23±0.01[c]	0.90±0.02[c]	0.17±0.00[f]	1.296±0.007[c]
Pectinodesmus sp.	0.21±0.01[c]	0.66±0.01[d]	0.29±0.01[d]	1.152±0.012[d]

Proximate Composition of Isolated Microalgae

Protein, carbohydrate, and lipid contents were also determined for the four microalgae species (Fig. 6). *Nannochloropsis* sp. showed significantly ($p < 0.05$) higher amount of protein (34.34±1.22% DW) and lipid content (20.33±0.55 % DW) whereas significantly ($p < 0.05$) highest amount of carbohydrate content was observed in *Pectinodesmus* sp. (25.23±0.61 %DW), *Nephrocytium* sp. (22.79±1.16 %DW), and *Protococcus* sp. (24.78±1.25 %DW). *Nephrocytium* sp. (30.20±1.13%DW, *Protococcus* sp. (30.46±1.18%DW) and *Pectinodesmus* sp. (29.47±0.64%DW) showed almost equivalent amount of protein. Along with this, *Nephrocytium* sp., *Protococcus* sp. and *Pectinodesmus* sp. showed significant difference ($p < 0.05$) in lipid content which were about 20.32±0.78, 14.69±0.70 and 11.79±0.27 % DW, respectively.

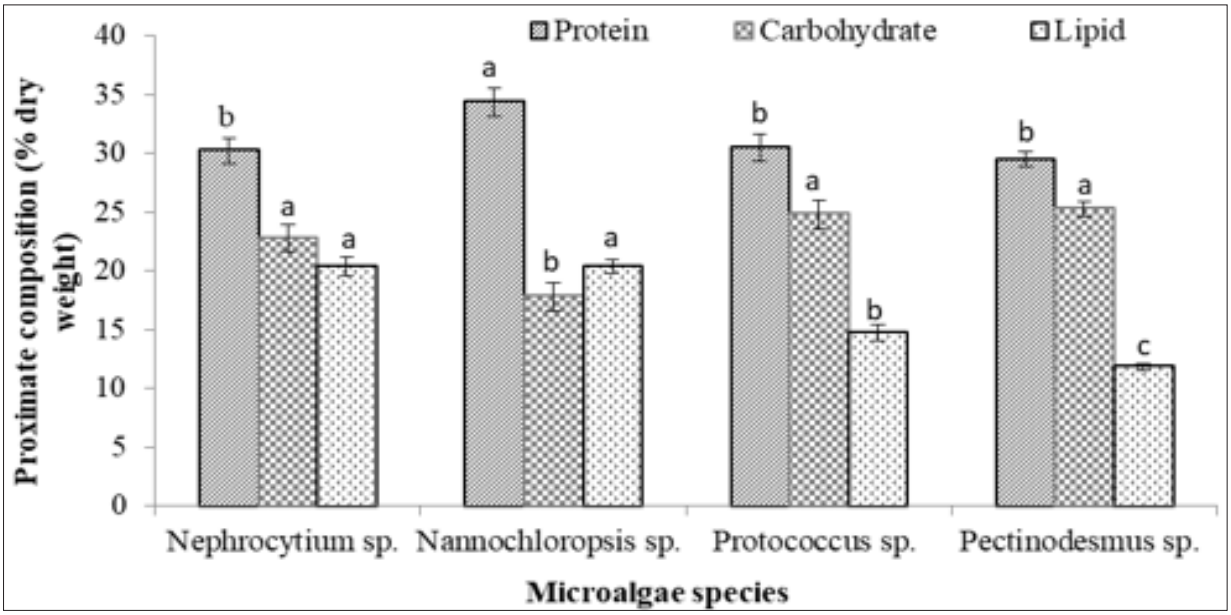


Figure 6: Proximate Composition (% dry weight) (mean ± SE) of Isolated Microalgae. Values with the Different Letters within Each Series Indicate Significant Differences ($p < 0.05$) Among the Four Species

Fatty Acids Content of Isolated Microalgae

The results of fatty acids analysis by GCMS are represented in Table 4. There were significant ($p < 0.05$) differences in SFA, MUFA, PUFA, HUFA, omega 3 fatty acids (n-3) and omega 6 fatty acids (n-6) contents among the four species (Fig. 7). In case of SAF, *Protococcus* sp. (18.44 ± 0.23 %) and *Nannochloropsis* sp. (2.09 ± 0.03%) showed maximum ($p < 0.05$) and minimum ($p < 0.05$) value where *Nephrocytium* sp. and *Pectinodesmus* sp. showed about 16.14±0.6 and 5.59± 0.29% of total fatty acids. Whereas, *Protococcus* sp. showed significantly ($p < 0.05$) higher amount of MUFA with 12.31± 0.76% of total fatty acids and significantly ($p < 0.05$) lower in *Nephrocytium* sp. with 0.80± 0.2% of total fatty acids. On the other hand, *Nannochloropsis* sp. (94.43 ± 0.03%) and *Pectinodesmus* sp. (90.98 ± 0.59%) did not show any significant difference in PUFA content, and *Nephrocytium* sp. and *Protococcus* sp. showed 83.05± 0.8% and 69.25 ± 0.53% of total fatty acids. Together with this, HUFA content is comparatively low in the four species where it was highest ($p < 0.05$) in *Protococcus* sp. with 5.00± 0.27% and lowest ($p < 0.05$) in *Nephrocytium* sp. with 0.89± 0.17% of the total fatty acids. Moreover, *Protococcus* sp. (29.8 ± 0.62%) showed significantly highest amount of n-3 fatty acids where, *Pectinodesmus* sp. (1.46 ± 0.12%) and *Nephrocytium* sp. (0.67 ± 0.12%) showed no statistical difference in n-3 fatty acids content. *Pectinodesmus* sp. resulted significantly ($p < 0.05$) maximum amount of n-6 fatty acids with 89.2 ± 0.75 of the total fatty acids where *Nephrocytium* sp. (82.16 ± 1.11%) and *Nannochloropsis* sp. (83.25 ± 0.04%) showed no statistical difference and *Protococcus* sp. showed the lowest content with 38.59 ± 0.20% of the total fatty acids. In case of EPA and DHA content, *Nannochloropsis* sp. and *Protococcus* sp. showed greater amount of EPA but all of the four species showed no detectable amount of DHA.

Table 4: Fatty Acids Content (% of the total fatty acids) of Different Tropical Microalgae Species. Values are mean± SE.

Carbon	Fatty acids	*Nephrocytium* sp.	*Nannochloropsis* sp.	*Protococcus* sp.	*Pectinodesmus* sp.
SFA					
C8:00	Methyl Octanoate	0.53±0.01	-	-	-
C10:00	Methyl Decanoate	-	-	-	-
C12:00	Methyl Laurate	0.12±0.01	-	0.15±0.02	-
C13:00	Methyl Tridecanoate	1.16±0.05	-	-	-
C14:00	Methyl Myristate	-	-	0.11±0.01	-
C16:00	Methyl Palmitate	0.31±0.01	0.44±0.15	3.24±0.28	1.67±0.5
C18:00	Methyl Stearate	-	0.85±0.04	1.8±0.23	0.159±0.005
C20:00	Methyl Arachidate	1.32±0.006	0.28±0.07	1.28±0.16	-
C17:00	Methyl Heptadecanoate	-	-	-	-
C21:00	Methyl Heneicosanoate	12.57±0.47	0.16±0.03	11.54±0.94	3.48±0.2
C22:00	Methyl Hehenate	-	0.25±0.12	0.15±0.002	-
MUFA					
C16:01	Methyl Palmitpleate	0.44±0.14	3.52±0.13	7.16±0.07	2.03±0.23
C18:01	Methyl Oleate	-	0.13±0.001	2.9±0.24	0.2±0.01
C22:01	Methyl Eirocate	0.18±0.03	-	1.84±0.16	1.19±0.07
C20:01	Methyl 11-Eicosenponoate	-	-	0.41±0.29	-
C24:01:0	Methyl Nervonate	0.1±0.007	-	-	-
PUFA					
C18:02	Methyl Linoleate	81.93±1.17	82±0.01	36.65±0.23	88.59±0.78
C18:03	Methyl Linolenate	0.11±0.001	7.64±0.15	26.75±0.38	0.6±0.05
C20:04	Methyl Arachidonate	0.11±0.03	0.6±0.03	0.97±0.01	0.32±0.02
C20:05	Methyl Eicosapaennoate	0.55±0.07	2.75±0.1	3.04±0.24	0.85±0.08
C20:03	Methyl 11-14-17-Eicosatrienoate	0.33±0.22	1.39±0.06	1.82±0.12	0.62±0.05
C22:06	Methyl Docosahexanoate	0.03±0.01	-	-	-
C22:05	Methyl Docosapentaenoate	0.09±0.02	-	-	-

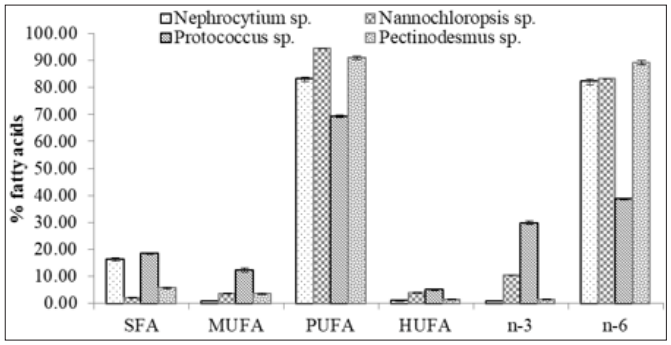


Figure 7: Fatty Acids Content (% of total fatty acids) of Isolated Microalgae Species Cultured in BBM. Values are mean± SE.

Discussion

Water Quality Parameters of the Sampling Sites

Sufficient amounts of nutrients mainly nitrogen, phosphorus is mandatory to achieve optimum growth rates in microalgal cells [36]. Some other factors like temperature, light, salinity, pH etc. also play a major role in growth and biochemical compositions of microalgae [37]. According to Santhosh & Singh pH should be between 6.5 and 9.0, which were observed from all sampling sites. On the other hand, DO >5 mgL^{-1} is essential to support good fish production, and DO of Karnaphuli River and Kaptai Lake were in optimal range [38]. DO in Halda River was below the optimum range as sampling was done in the early morning in Halda River, and DO is maximum in mid-afternoon due to photosynthesis and minimum in the early morning due to highest respiration and decomposition than photosynthesis [39]. The temperature of the sampling sites was in ideal level where the optimum growth temperature is mostly between 20 and 30˚C for most marine microalgae [40]. The optimum phosphorus concentration for microalgae is between 0.001 gL^{-1} to 0.179 gL^{-1}, where TAN concentration must be less than 0.5 mgL-1 and the desirable range of nitrite-nitrogen is 0-1 mgL^{-1} [41,42]. In the entire sampling site, nitrogen and phosphorus concentration was in the ideal range that is required for plankton growth.

Growth Phases of Microalgae Species

In the present study, different microalgae showed different cell concentration, pigment concentration and proximate composition as those can vary from species to species. No previous study has been reported over *Nephrocytium* sp. But according to the present study, it can be concluded that *Nephrocytium* sp. can easily be cultured by BBM as it showed a good cell density in BBM. Moreover, no previous studies found over the freshwater strain of *Nannochloropsis* sp. in BBM. But in case of marine water strain of *Nannochloropsis* sp., Ermavitalini et al., reported almost similar growth pattern (lag, exponential and stationary phase) in the combined treatment media of Indole 3-acetic acid (IAA) and 6-Benzyl Amino Purine (BAP). But cell density of *Nannochloropsis* sp. was higher in the present study than the earlier study reported by Khatoon et al., who found 4.877 × 107 cells mL-1 in Conway media [43,44]. The differences in the cell density observed herein can be linked to different growth media used in *Nannochloropsis* sp. growth and different strains from different environment. In the current study, *Protococcus* sp. and *Pectinodesmus* sp. represented their maximum cell density and optical density on day 10 with a high cell density which concluded that, this species can easily be cultured by using BBM in a commercial scale. Among the four species cell density of *Nannochloropsis* sp. was the highest because the growth of smaller size species multiply rapidly than the larger ones due to their large surface or volume ratio which simplify absorption of nutrients at comparatively faster rate [45].

Specific Growth Rate (SGR) of Microalgae Species

Yustinadiar et al., reported that, marine strains of *Nannochloropsis* sp. showed 0.35 day-1 of SGR in Walne medium at 35+1 ppt salinity [46]. But present study reported higher SGR of freshwater strains of *Nannochloropsis* sp. in BBM. In the current study, all of the microalgal strains show variation in SGR in BBM as SGR is also influenced by the microalgal strain used and the characteristics of the environment where it grows. According to the findings, it can be said that all of the four species can be utilized for higher microalgal biomass production for different commercial application as it required large amount of biomass for any commercial application of microalgae.

Chlorophyll, Carotenoid and Phycobilipreoteins Content of Isolated Microalgae

Present study showed higher amounts of chlorphylls a and b in *Nephrocytium* sp., which are almost similar with the values reported by Singh et al. from *Chlorella vulgaris* cultured in urban wastewater medium [47]. In the case of freshwater *Nannochloropsis* sp. it showed considerable variation in chlorophylls a and b content from marine water *Nannochloropsis* sp. cultured in Walne medium reported by Fakhri et al. [48]. This difference can be justified as nutrient composition of the culture medium may also influence the content of chlorophyll [49]. On the other hand, chlorophylls a and b of *Protococcus* sp. in this study were almost similar amounts with *Ankistrodesmus falcatus* in BBM at 702 lux light intensity in a 12-day culture period [50]. Moreover, chlorophyll content in *Pectinodesmus* sp. in the current study was almost similar with the previous study on *Dunaliella tertiolecta* in the urea containing Tk medium [51]. From this study, it was found that *Nephrocytium* sp., *Nannochloropsis* sp., and *Protococcus* sp. can extensively be used as a great source of chlorophyll for pharmaceutical industry and also for food color preparation, as chlorophyll utilized as a natural food coloring agent [10] and also used widely in pharmaceutical products [11].

In case of carotenoid content of *Nephrocytium* sp., Khatoon et al. reported almost equivalent amount of carotenoid in 0.7M salt concentration in Conway medium from *Dunaliella salina*. Carotenoid contents of *Nannochloropsis* sp. varied from that of the marine habitat species in Walne medium reported by Fakhri et al. [28,48]. May be the differences in temperature, light, culture media and different strain from different habitat were the principal reasons for observed differences herein. *Micractinium* sp. (CCAP IPOME-2) a chlorophyta microalgae like *Protococcus* sp. showed almost similar amount of carotenoid concentrations (6.04 ± 0.03 µgmL-1) in BBM that has been detected from *Protococcus* sp. in the current study. Eze et al., (2022) earlier found that, *Desmodesmus subspicatus* resulted about 2.3 ± 0.1 mgg-1 carotenoid by urea supplementation using BG11 medium in the flask culture [52]. *Pectinodesmus* sp. as a species of Scenedesmaceae family, findings of this study were almost equivalent with the finding of Eze et al. in flask condition. Present study also concluded that, P*rotococcus* sp., and *Nephrocytium* sp. has high carotenoid content and huge potentiality to contribute in human, animal food industry as well as in aquaculture [53].

In an earlier study Zuorro et al.opined that, *Oscillatoria* sp. grown at 28 °C temperature, 12:12 h of light: dark cycle with 100 µmol m–2 s–1 light intensity in optimized BG-11 media for

15 days resulted with values of 15.21, 3.95, and 1.89 (% w/w), PC, APC and PE, respectively. However, very lower amount of total phycobilipreotain was recorded from *Nephrocytium* sp., *Nannochloropsis* sp., *Protococcus* sp., and *Pectinodesmus* sp. compared to Cyanobacterial strains, as the light harvesting pigments phycobiliprotein commonly found in cyanophyceae and cryptophyceae [54,55]. On top of that all the microalgae in the present study belong to the Chlorophyceae class, and Chlorophyta (Green microalgae) reported to contain mostly chlorophylls and carotenoid [56].

Proximate Composition of Isolated Microalgae

Present study showed that, protein, lipid, and carbohydrate content in *Nephrocytium* sp. was significantly higher among the four species and unanimous with the previous study by Renaud et al. indicated that microalgae hold approximately 30-40 % (w/w) protein, 10-20 % (w/w) lipids and 5-15 % (w/w) carbohydrates in the late-exponential growth phase [57]. The protein and lipid content in *Nannochloropsis* sp. differ from Khatoon et al. reported earlier at 30ppt salinity in Conway medium [44]. As biochemical composition of microalgae varies according to the culture medium compositions and culture conditions, hence variation in salinity and different strains from different habitat could be the reason for the differences. On the other hand, *Protococcus* sp. and *Nannochloropsis* sp. showing higher contents of protein and lipid can be a potential species in feed industry for fish and other animals [58,59]. Protein and carbohydrate contents of *Pectinodesmus* sp. in this study were much greater than that cultured in BG-11 medium reported by Samadhiya et al., however, they showed lower lipid content. Lipid content may vary due to changes in the growth condition or nutrient concentration [60,61]. According to the finding of the current study, all of the four species can be utilized as a good protein source for aquaculture or other commercial application. As well as, *Nannochloropsis* sp. and *Nephrocytium* sp. can also be used as a potential raw material for crude lipid production.

Fatty Acids Content of Isolated Microalgae

The experimental species showed variation in fatty acids production, this is because of the variation in genetic material of each species, which is unique for each species [60]. Among the four species, *Nephrocytium* sp. and *Protococcus* sp. showed considerably higher amount of SFA, thus it can be exploited in biodiesel industry. As good amount of saturated fatty acid and lower quantity of unsaturated fatty acids content indicate the good quality biodiesel production [62]. All the four species showed higher amount of PUFA in controlled commercial BBM because unsaturated fatty acids tend to be synthesized when their present optimal growth condition whereas saturated fatty acid is likely to be synthesized in unfavorable condition [63,64]. Moreover, the experimental species can be highly utilized in pharmaceuticals industry as PUFAs have demonstrated protective and curative activities against chronic inflammatory diseases [65].

Conclusions

Considering the results achieved from the current study, *Nephrocytium* sp., *Nannochloropsis* sp., *Protococcus* sp. grow well in BBM with elevated quantity of protein and lipid content and shows their importance in fish or animal feed industry and fuels production. Interestingly, *Nephrocytium* sp., *Protococcus* sp. showed higher chlorophyll and carotenoid content and confirms their potentiality in pigment production. *Nephrocytium* sp. and *Protococcus* sp. can be utilized in biodiesel production due to their higher amount of SFA. Because of the good amount of PUFA, all of the four species can act as a raw material for pharmaceuticals and feed industry. Further study will require on amino acid analysis of those microalgae, to boost up the feed industry.

CRediT Authorship Contribution Statement

Zannatul Nayma: Methodology; Data collection; data curation; statistical analysis; original draft. Helena Khatoon: Conceptualization; project administration; and submission. Mohammad Redwanur Rahman and Tashrif Mahmud Minhaz: Review and editing. Fardous Ara Mukta and Razia Sultan: Formatting manuscript.

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Competing Interest

The authors declare that the research has no conflict of interest.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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