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A comparative study on the productivity of selected tropical freshwater microalgae

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Abstract. The wide spread use of microalgae in various fields of human activity including fishery generated a need of screening of highly productive microalgae. Therefore, in this study, growth rate and productivity of selected freshwater microalgae species (*Nephrocytium* sp., *Nannochloropsis* sp., *Selenastrum* sp., *Sphaerocystis* sp., *Ankistrodesmus* sp., *Monoraphidium* sp., *Pectinodesmus* sp. and *Scenedesmus* sp.) were studied. Selected microalgae were cultured in Bold Basal Medium and cell density was determined to analyze specific growth rate (SGR), cell duplication time and cell doublings per day. In addition, biomass data and lipid data were recorded to calculate volumetric, areal and lipid productivity. Results showed that *Monoraphidium* sp. had significantly highest ($p<0.05$) SGR in lag phase (0.861 ± 0.017/day) and exponential phase (0.437 ± 0.016/day) where *Ankistrodesmus* sp. showed significantly highest ($p<0.05$) SGR in stationary phase (0.221 ± 0.009/day) compared to others. *Selenastrum* sp. and *Scenedesmus* sp. showed significantly ($p \leq 0.05$) highest and lowest cell duplication time and cell doublings per day was highest in *Monoraphidium* sp. Volumetric (47.738 ± 0.576mg/L/Day) and areal (4.774 ± 0.058mg/cm^2/day) productivity were significantly ($p\leq0.05$) highest in *Scenedesmus* sp. but lipid productivity was in *Monoraphidium* sp. (8.513 ± 0.258 mg/L/Day). This study concluded that *Monoraphidium* sp. performed best and accumulated much higher lipid in a day in BBM which will contribute to produce biomass that can be utilized for different commercial application.

Keywords: Freshwater microalgae, SGR, Cell duplication time, Cell doublings per day

Introduction

Microalgae are prokaryotic or eukaryotic unicellular autotrophic-heterotrophic photosynthetic microorganisms (Mata *et al.* 2010) that use solar energy to combine water with CO_2 to create biomass and found in all ecosystems both aquatic and terrestrial like marine, fresh, brackish, and residual waters under a wide range of temperatures, pH, and nutrients (Koller *et al.* 2014). Although, there are over thousands or even millions of microalgae species existing in nature (Hannon *et al.* 2010), 30,000 species have been studied; however, so far not fully exploited (Mata *et al.* 2010) and only a few of them have been successfully produced commercially for the production of high value products (Saha and Murray 2018). The high oil production of various microalgae species has been utilized in biofuel production (Hussain *et al.* 2017) due to their advantages over other conventional biofuel sources which is based on its non-seasonality, biodegradability, non-toxic nature and renewability. Moreover, microalgae have the potential to reduce the dependence on conventional raw materials in aquafeed that could potentially replace or reduce common feed stuff because of their nutritional quality and positive effect on the rate of growth of aquatic species due to increased triglyceride and protein deposition in muscle, improved resistance to disease, decreased nitrogen output into the environment, omega-3 fatty acid content, physiological activity, and carcass quality (Becker 2004). Along with this,

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microalgae presents excellent advantages, like high growth rates, high productivity, no requirement of agricultural land for their cultivation, short harvest cycles, ease of cultivation, high lipid content, and high photosynthetic efficiency (Nascimento *et al.* 2015). The biomass and product yields are well known to depend on the cultivation conditions, where theoretical biomass yield of microalgae was reported as 100–200 g dry weight m^{-2} day^{-1} and the practical productivity rate was 15–30 g dry weight m^{-2} day^{-1} (Subramanian *et al.* 2013). In microalgae, the metabolism of reserving materials and energy is prior to steady growth and division of microalgae (Chen 1996). In addition, under stress conditions they resulted between 20% and 50% lipids in terms of the dry weight of the biomass (Wang *et al.* 2016) that is assumed to be between 15 and 25 t ha^{-1} $year^{-1}$, which would correspond to 4.5 and 7.5 t ha^{-1} $year^{-1}$ of lipid production (Choong *et al.* 2020). Along with this, several nutritional factors such as nitrogen, phosphorus, carbon and iron are recognized as one of the most important factors influencing the yield of biomass and the lipid accumulation (White *et al.* 2013). Growth and productivity of microalgae also differ between species to species. Chemical composition and productivity of potentially important indigenous species should be analyzed to assess the nutritional properties of them as they generate a less environmental impact where the system is operating, due to more ecological suitability and high level of adaptability to local environment. However, very little research has been done on the characterization of microalgae in Bangladesh.

Therefore, the present approach was to compare the growth rate and productivity of eight different freshwater microalgae (*Nephrocytium* sp., *Nannochloropsis* sp., *Selenestrum* sp., *Sphaerocystis* sp., *Ankistrodesmus* sp., *Monoraphidium* sp., *Pectinodesmus* sp. and *Scenedesmus* sp.) which may contribute to select potential strains that possess fast growth and high lipid productivity.

Materials and Methods

Collection of freshwater isolates: Eight different freshwater tropical microalgae pure stock were obtained from the previously isolated and preserved samples at Live Feed Research Corner, Department of Aquaculture, Chattogram Veterinary and Animal Sciences University (CVASU), Bangladesh.

Determination of microalgae growth: The microalgae were cultivated using BBM (average pH 7.72 ± 0.17) (table.1). Cultures were grown at 24.0 ± 1.0°C temperature in a in sterile 500 mL borosilicate Erlenmeyer flasks each having 350 ml of culture volume for each species with three replicates where 2% pure culture stocks were added. Microalgae cultures were subjected to a 24 h light condition at 150 μ E m^{-2} s^{-1} intensity with continuous gentle aeration (24 hrs) at a rate of 4.53 ± 0.53 mg/L. The experiment was continued until the death phase.

Determination of cell density: Microalgae cell count was carried out every day by using a Neubauer hemacytometer (0.0025 mm^2, 0.1 mm deep chambers, Hecht Assistent, Germany) under the magnification of 40X. Cells were counted by using the based on the formula of Lavens and Sorgeloos (1996).

$$\text{Cell density (cell/ml) for 5 squares} = \frac{\text{Total number of cells counted}}{10\times4} \times 10^6$$

$$\text{Cell density (cell/ml) for 25 squares} = \frac{\text{Total number of cells counted}}{50\times4} \times 10^6$$

Where 10 and 50 = the squares of the 2 hemacytometer chambers and 4 x 10^{-6} = the volume of samples over the small square areas, that were equivalent to 0.004 mm^3 (0.2 mm x 0.2 mm x 0.1 mm), expressed in cm^3 (ml).

Determination of biomass: Biomass determination is prerequisite for productivity analysis. Biomass were determined by filtering of 1ml microalgae sample from each replication of individual microalgae through a pre-weighted (after marking of filter paper rinsed with 10ml distill water and dried at 100°C for 4hours in hot air oven) glass microfiber filter paper, which was further rinsed with 10ml distill water for three times. Then the filter paper with biomass was oven dried at 100°C for 4hours. After that, final weight of filter paper was taken followed by 15min of desiccation and dry biomass was calculated according to Ratha *et al.* (2016).

***Lipid extraction**:* Lipid was determined according to Bligh and Dyer (1959) and Folch *et al.* (1957). In a centrifuge tube, 50 mg of each sample was taken and diluted into 5x volume using distilled water. Then, 3 mL methanol: chloroform (2:1, v/v) solution was added and mixed properly with tissue homogenizer and centrifuged at 1000 rpm for 4 min at 4°C. After centrifugation, the supernatants were transferred into clean tubes and placed them in ice. In the sample tubes, again 3 mL of methanol: chloroform (2:1, v/v) solution was added and mixed with tissue homogenizer properly and centrifuged again at the same conditions, and the supernatants were transferred to the previous supernatants tubes. In the combined supernatants, 1.5 mL of 0.9% NaCl was poured and mixed well by using vortex mixture and kept in the refrigerator for 1 hr at 4°C temperature. Then the tubes were centrifuged at 1000 rpm for 10 min at 4°C temperature and the lower layer was transferred in pre-weighted aluminum dish. The aluminum dish was weighed to get the final weight after the evaporation of solvent at 60°C using hot air oven and lipid weight in the samples was determined by subtracting initial weight from the final weight.

***Determination of productivity**:* Biomass productivity, areal productivity and lipid productivity were calculated according to Mercado *et al.* (2020) and Sukkrom *et al.* (2016). Productivity data was calculated at the end of the exponential phase. Specific growth rates and Cell doublings per day were calculated according to Daniel and Srivastava (2016) and cell duplication time was calculated according to Chiu *et al.* (2009).

***Biomass productivity**:* Following equation was used to calculate biomass productivity:

Biomass productivity/Pb (mg L^{-1} day^{-1}) = $\frac{(X_2 - X_1)}{(t_2 - t_1)}$

Where X_1 and X_2 were the biomass concentrations (mg L^{-1}) on days t_1 (start of study) and t_2 (end of the study).

Areal productivity: Following equation was used to calculate areal productivity:

AP (mg cm^{-2} day^{-1}) = $\frac{VP \times V}{A}$

Where, VP = Volumetric Productivity, V = Total Volume of the culture, A = surface area occupied ground.

***Lipid productivity analysis**:* Lipid productivity was calculated by using the following equation:

Lipid Productivity (mg L^{-1} day^{-1}) = $\frac{\text{Biomass Productivity (mg } L^{-1} \text{ day}^{-1}) \text{ x (\% lipid)}}{100}$

Specific growth rates: Specific growth rate was calculated using the formula as follows:

$$\text{SGR (r)} = \frac{\ln Nt - \ln No}{\Delta t}$$

Where Nt is the final cell count and No is the initial cell count; t is the number of days.

Cell duplication time: Cell duplication time was calculated using the following formula:

Cell duplication time $td = 0.693/\mu$

Cell doublings per day: Cell doublings per day was calculated using the following formula:

$$\text{Cell doublings per day (K)} = \frac{\ln Nn - \ln Ni}{ln2(tn - ti)}$$

Where Nn is the final cell count and Ni is the initial cell count; tn is the final time in days and ti is the initial time in days.

Statistical analysis*:* Mean and standard error of mean were calculated using MS excel. When assumptions were met, all statistical analyses regarding the SGR, cell duplication time, cell doublings per day, volumetric productivity, areal productivity and lipid productivity were performed using the IBM SPSS (v. 26.0). The collected data were analyzed using a one-way analysis of variance (ANOVA) by using Tukey's multiple comparison tests at 95% confidence interval level. Post-hoc test was utilized to discern differences between groups.

Results

Specific growth rate of selected freshwater microalgae*:* In lag phase, exponential phase and stationary phase, SGR differ among the eight microalgae (Fig.1). Significantly highest ($p<0.05$) SGR was detected in lag phase from all of the microalgae which was declined gradually in exponential and stationary phase. Among the eight microalgae, *Monoraphidium* sp. showed significantly highest ($p<0.05$) SGR in lag phase (0.861 ± 0.017/day) and exponential phase (0.437 ± 0.016/day) where *Ankistrodesmus* sp. resulted significantly highest ($p<0.05$) SGR in stationary phase (0.221 ± 0.009/day). But in lag phase, no significant difference was detected among *Sphaerocystis* sp., *Selenastrum* sp., *Pectinodesmus* sp., *Ankistrodesmus* sp. and *Scenesdesmus* sp. Moreover, in exponential phase, *Nephrocytium* sp., *Sphaerocystis* sp., *Pectinodesmus* sp. and *Scenesdesmus* sp. showed no significant difference in SGR. Along with this, *Nephrocytium* sp., *Nannochloropsis* sp., *Monoraphidium* sp., *Sphaerocystis* sp., *Selenastrum* sp. and *Scenesdesmus* sp. showed almost similar SGR in stationary phase.

Cell duplication time,cell doublings per day and cell density on harvest of selected freshwater microalgae: Cell duplication time, Cell doublings per day (K) and cell density on harvest varied among different microalgae (Table I) where *Selenastrum* sp. and *Scenedesmus* sp. showed significantly ($p \leq 0.05$) highest and lowest cell duplication time. Along with this, *Nannochloropsis* sp. and *Sphaerocystis* sp. showed almost similar cell duplication time. On the other hand, significantly highest ($p \leq 0.05$) amount of cell doublings was detected from *Monoraphidium* sp. while *Selenastrum* sp. resulted the lowest. At the end of the exponential phase, significantly highest and lowest ($p \leq 0.05$) amount of cell density was detected from *Nannochloropsis* sp. and *Scenedesmus* sp., respectively. In terms of cell density, no significant difference in cell density was detected among *Monoraphidium* sp., *Selenastrum* sp., *Sphaerocystis* sp., and *Ankistrodesmus* sp.

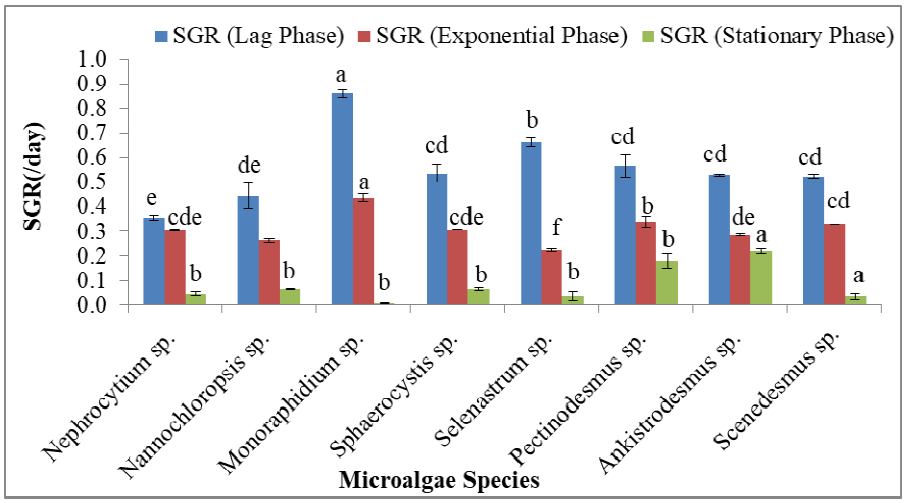


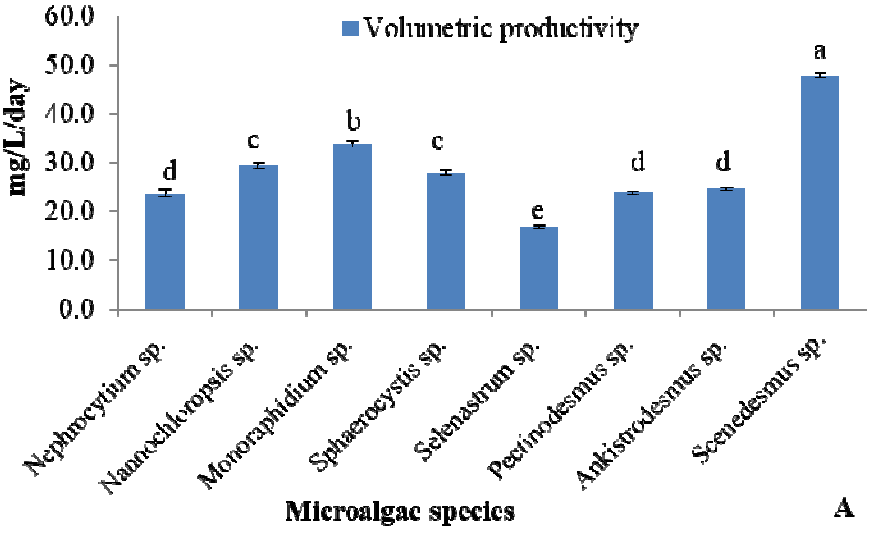
Fig 1. Specific growth rate (mean ± SE) of selected tropical freshwater microalgae cultured in Bold Basal medium.

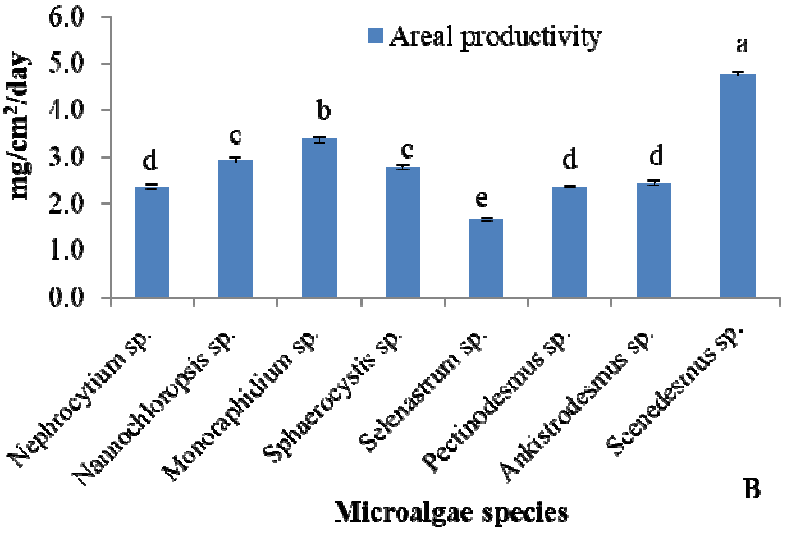
Table I. Cell duplication time (Day), cell doublings per day (K) and cell density on harvest (cells/ml) of freshwater microalgae, cultured in Bold Basal Medium

Microalgal species	Cell duplication time (Day)	Cell doublings per day (K)	Cell Density on harvest (cells/ml)
Nephrocytium sp.	1.156 ± 0.004^{c}	0.493 ± 0.008^{d}	$4.037 \times 10^{7} \pm 0.157^{c}$
Nannochloropsis sp.	1.042 ± 0.003^{e}	0.485 ± 0.020^{d}	$6.374 \times 10^{7} \pm 0.144^{a}$
Monoraphidium sp.	1.022 ± 0.003^{f}	0.910 ± 0.013^{a}	$5.604 \times 10^{7} \pm 0.061^{b}$
Sphaerocystis sp.	1.049 ± 0.002^{e}	0.574 ± 0.017^{c}	$2.711 \times 10^{7} \pm 0.042^{d}$
Selenastrum sp.	1.555 ± 0.005^{a}	0.482 ± 0.008^{d}	$5.658 \times 10^{7} \pm 0.083^{b}$
Pectinodesmus sp.	1.073 ± 0.002^{d}	0.625 ± 0.014^{bc}	$1.521 \times 10^{7} \pm 0.081^{e}$
Ankistrodesmus sp.	1.395 ± 0.003^{b}	0.581 ± 0.005^{c}	$2.923 \times 10^{7} \pm 0.029^{d}$
Scenedesmus sp.	0.820 ± 0.001^{g}	0.644 ± 0.001^{b}	$0.936 \times 10^{7} \pm 0.004^{f}$

Volumetric, areal and lipid productivity of different freshwater microalgae: In this study, different freshwater tropical microalgae species were cultured in BBM providing uniform parameters to determine the difference in their productivity. Fig. 2A showed the variation in volumetric productivity for the eight microalgae species where significantly highest and lowest ($p \leq 0.05$) amount of volumetric productivity was detected from *Scenedesmus* sp. (47.738±0.576 mg/L/Day) and *Selenastrum* sp. (16.680±0.393 mg/L/Day), respectively. Moreover, *Nephrocytium* sp., *Pectinodesmus* sp. and *Ankistrodesmus* sp. resulted almost similar amount of volumetric productivity (23.628±0.648, 23.656±0.318 and 24.459±0.423 mg/L/Day, respectively). The results showed that areal productivity varied among the species (Fig. 2B). The areal productivity varied as 2.363±0.065, 2.922±0.061, 3.366±0.066, 2.774±0.044, 1.668±0.039, 2.366±0.032, 2.446±0.042 and 4.774±0.058mg/cm^2/day among *Nephrocytium* sp., *Nannochloropsis* sp., *Monoraphidium* sp., *Sphaerocystis* sp., *Selenastrum* sp., *Pectinodesmus* sp. *Ankistrodesmus* sp. and *Scenesdesmus* sp., respectively. Areal productivity was significantly ($p < 0.05$) highest in *Scenesdesmus* sp. and lowest in *Selenastrum*

sp. Lipid productivity in various microalgae also differs from species to species which is represented in Fig. 2C. *Nephrocytium* sp., *Nannochloropsis* sp., *Monoraphidium* sp., *Sphaerocystis* sp., *Selenastrum* sp., *Pectinodesmus* sp., *Ankistrodesmus* sp. and *Scenesdesmus* sp. resulted in 4.810±0.303, 5.935±0.093, 8.513±0.258, 4.068±0.132, 2.915±0.075, 2.790±0.102, 4.006±0.070 and 6.623±0.260 mg/L/Day of lipid productivity, respectively. Significantly ($P < 0.05$) maximum and minimum amount of lipid productivity were detected from *Monoraphidium* sp. and *Pectinodesmus* sp. Together with this, similar kind of lipid productivity was detected from *Nephrocytium* sp., *Sphaerocystis* sp. and *Ankistrodesmus* sp.





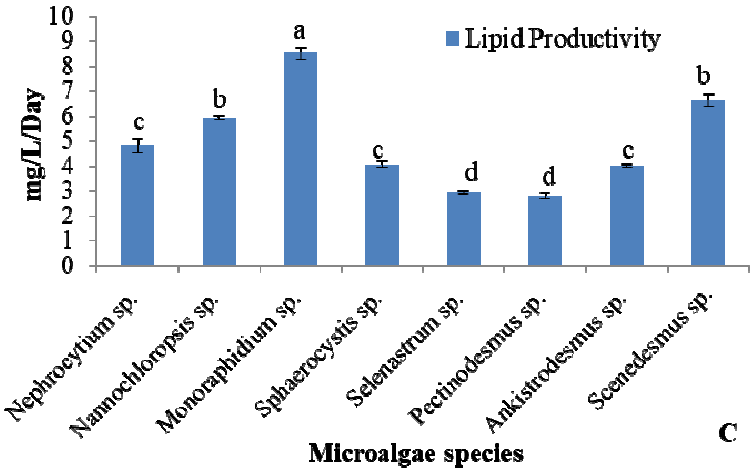


Fig 2. Volumetric productivity (A), Areal productivity (B) and Lipid productivity (C) (mean±SE) of tropical freshwater microalgae, cultured in Bold Basal medium.

Discussion

***Specific growth rate of selected freshwater microalgae*:** In Bangladesh, a very limited attention has been paid to the indigenous microalgae and its potentialities. No previous study has been reported in our country on the growth and productivity analysis of *Nephrocytium* sp., *Nannochloropsis* sp., *Monoraphidium* sp., *Sphaerocystis* sp., *Selenastrum* sp., *Pectinodesmus* sp. *Ankistrodesmus* sp. and *Scenesdesmus* sp. In this study, SGR of microalgae was highest in lag phase, and gradually decline up to stationary phase. Similar to this result, Zarrinmehr et al. (2020) reported that SGR of *Isochrysis galbana* in different nitrogen concentrations gradually declined during cultivation time. Some world wide data are available regarding the SGR of *Nannochloropsis* sp., *Monoraphidium* sp. *Selenastrum* sp. *Ankistrodesmus* sp. and *Scenesdesmus* sp. Yustinadiar *et al.* (2020) reported almost similar growth rate in case of *Nannochloropsis* sp. isolated from marine habitat and resulted about a growth rate of 0.25/day in stationary phase and 0.55/day on day 1 at 0.75:0.25 hr flashing light treatment in Walne medium. Moreover, *Scenedesmus* sp. for 11 days of culture duration resulted 0.51 ± 0.06 day^{-1} and 0.54 ± 0.14 day^{-1} SGR in BG11medium and 100% dairy wastewater medium, respectively (Mercado *et al.* 2020). Along with this, Dhup and Dhawan (2014) observed that growth rates of *Monoraphidium* sp. affected by different nitrate concentrations in BG11medium for a period of 15 days culture where highest SGR was 0.089/day at 0.072 mg/lNO_3^-concentrations. Okomoda *et al.* (2021) reported about 0.443 ± 0.001/ day of specific growth rate in *Ankistrodesmus falcatus* in BBM. Previous study done by Maa *et al.* (2012) reported about 0.25 ± 0.02/ day of growth rate from *Selenastrum* sp. with 10 days of cultivation time. Therefore, compared with the circumstances stated above, it was found that, locally isolated *Nephrocytium* sp., *Nannochloropsis* sp., *Monoraphidium* sp., *Sphaerocystis* sp., *Selenastrum* sp., *Pectinodesmus* sp. *Ankistrodesmus* sp. and *Scenesdesmus* sp. performed differently in BBM which can be justified as, microalgae growth characteristics vary from species to species and impacted by

multiple factors, like reactor feature, culture conditions (light, nutrients, temperature, pH, aeration) and the physiological need of the microalgae species (Guedes and Malcata 2012).

Cell duplication time, cell doublings per day and cell density on harvest of selected freshwater microalgae*:* The microalga *Scenedesmus* sp. showed lower cell duplication time compared to the other microalgae strains. Microalgae growth characteristics vary from species to species and impacted by multiple factors, like reactor feature, culture conditions (light, nutrients, temperature, pH, aeration) and the physiological need of the microalgae species (Guedes and Malcata 2012). Moreover cell doublings per day was also varied among the species because of the variation in lag phase, log phase and stationary phase of individual species and due to variation in growth rate which depend not only microalgae species but also culture environments. In different growth phases, microalgae growth rate differ due to the variation in several activity like in lag phase it adapts to the culture condition such as medium, pH, temperature and lighting (Krishnan *et al.* 2015). Then start to undergo rapid cell division and the cell of the culture will increase gradually in exponential order, as enzymes and metabolites needed for cell division are available here (Prayitno 2016). After which, stationary phase taken place when the equal rate of the cell division and cell death occur due to depletion of nutrients in the medium (Krishnan *et al.* 2015). Along with this, *Nannochloropsis*sp. resulted highest cell number on harvest because smaller size species grow rapidly than the larger ones because of their large surface or volume ratio of smaller sized cells which simplify assimilation of nutrients at comparatively faster rate (Phatarpekar *et al.* 2000).

Volumetric, areal and lipid productivity of selected freshwater microalgae: The biomass production of microalgae is a mere function of the instantaneous growth rate and algal cell concentration in culture (Daniel and Srivastava 2016). In the present study, different microalgae showed variation in biomass productivity which can be justified as productivity of microalgae is also influenced by the microalgal strain used and the characteristics of the environment, where it grows (Mercado *et al.* 2020). Moreover, lipid productivity of microalgae also varied among species as it depends on biochemical composition of microalgae, nutrient content of culture media and different stress factors. Microalgae cells accumulated a larger quantity of lipid when they were subject to unfavorable culture conditions (Lucas-Salas *et al.* 2013). Though no previous study reported on *Nannochloropsis* sp. culture in freshwater media, but in case of marine *Nannochloropsis* sp. strains, Daniel and Srivastava (2016) detected about1088.44 g m^{-3} d $^{-1}$ mean volumetric productivity, 10.10 g m^{-2} d $^{-1}$ mean areal productivity from 30 mm thickness of thick tubular photobioreactor. Dhup and Dhawan (2014) detected 0.19g/l/day of lipid productivity from *Monoraphidium* sp. at 0.36mg/l NO_3 concentration. The maximum lipid productivity of 38.32 mg/L/day was recorded from*Ankistrodesmus* sp. in modified BG11 medium at the dilution rate of 0.16 day (Sukkrom *et al.* 2016). *Scenedesmus* sp. resulted about 350.81 $\pm$ 33.05 mg g^{-1} of lipid productivity in standard BG11 culture medium (Mercado *et al.* 2020). Based on the outcomes of the current study, *Nannochloropsis* sp., *Monoraphidium* sp., and *Scenesdesmus* sp. can be utilized a potential source of biofuel production as they showed higher lipid productivity.

Considering the results achieved from the current study it can be concluded that SGR of microalgae varied among different growth phases. Interestingly, *Scenedesmus* sp. showed lowest cell duplication time and *Monoraphidium* sp. resulted highest cell doublings per day which can be utilized for mass production of microalgae. Moreover, *Monoraphidium*sp. accumulated much

higher lipid in a day and can be as a potential species for biodiesel production. Further study will require on fatty acid analysis of those microalgae, to boost up the biodiesel production.

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