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Comparative Study of Microalgae Samples from Nepal for bio-fuel potentials

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Abstract

This paper is on a comparative study on growth rate and lipid content of various microalgae samples collected from biologically and geographically diversified areas on Nepal. Comparisons were also made on samples with same genus isolated from geographically diversified areas (i.e. high altitude (>4000 m) and mid hill (>1000 m)), and effect of seasonal variation (with respect to temperature) on similar species of microalgae. *Euglena sp.*, *Chlorella vulgaris*, and *Scenedesmus sp.* with growth rate of 0.401 day^{-1}, 0.377 day^{-1}, and 0.342 day^{-1} and lipid content above 27 % were taken for the later studies. Mountain varieties showed slightly higher growth rate with difference in their lipid contents up to 0.5 % as compared to mid-hill varieties. In case of seasonal effect, there was increase in the growth rate of all three species during summer temperature of 27 ± 2 ^{o}C; whereas, due to this temperature negative affect was seen in the lipid content of these species.

Keywords- Altitude, Biodiesel, Growth Rate, Lipid, Microalgae, Temperature.

Introduction

Microalgae could be considered as one of the most versatile microorganism as it could be used in various fields including food, feed, fuel, waste water treatment, and air quality improvement (CO_2 sequestration) (Priyadarshani and Rath, 2012; Li *et. al.*, 2008). These are unicellular and simple multicellular photosynthetic micro-organisms, either prokaryotes (cyanobacteria) or eukaryotes (also called microalgae in a more narrow sense). They reproduce quickly and capable of all year round production. Microalgae could produce more biomass than any other terrestrial plants; estimation has been made that they could produce 50 times more biomass compared to switchgrass (fastest growing terrestrial plant) (Nakamura, 2006).

Commercial culture of microalgae dates back to 1960s in Japan (Borowitzka, 1999). Initial researches on microalgae were mostly based on feed and nutrition aspect, for human and aquaculture. Correlating excessive use of fossil fuel and climate change, these days much interest is been shown on fuels from microalgae. Even though literatures reports on microalgae being capable of producing various fuel types including biodiesel, bio-hydrogen, ethanol, and butanol; much focus has been given towards biodiesel (Radakovits *et. al.*, 2010; Dragone *et. al.*, 2010). This is because of its fast growth rate and high oil content (up to 50% of its dry weight) (Priyadarshani and Rath, 2012; Li *et. al.*, 2008; Pienkos, 2007). Biomass from microalgae could be produced throughout the year; with oil yield up to 1,5000 l/ha, which exceeds the yield of the best oilseed crops (Pienkos, 2007). Along with fuel, algae could address problem related to global warming as it is good atmospheric CO_2 sequester, i.e. 1 kg of dry algal biomass utilizes about 1.83 kg of CO_2 (Chisti, 2007).

Nepal's dependency upon petroleum product is increasing yearly. Out of total 12.1% commercial fuel consumed, 8.2% is comprised with petroleum products (WECS, 2010). Among all petroleum production, consumption of diesel is very high and increasing (average increment rate from Fiscal Year 2007/08 to 2012/13 is 83,598 kL/yr) with population increment and nation's development (NOC, 2014). With high reproductive rate and good lipid content, fuel from microalgae could act as an alternative source to support country energy demand. Geographical diversity, biodiversity, water resources, and pleasant solar irradiance of the nation could be advantages for commercial microalgae culture for biofuel production. Even though there has been less effort in R&D work on microalgae culture for commercial purpose, taking glance over the country's geographical diversity and its climatic condition, an appropriate environment could be obtained for the culture of various species of microalgae.

This paper is based on comparative study on growth rate and lipid content of various microalgae samples collected from biologically and geographically diversified areas on Nepal. Comparisons were also done on samples with same genus isolated from geographically diversified areas (i.e. high altitude (>4000 m) and mid hill (>1000 m)), and effect of seasonal variation (with respect to temperature) on similar species of microalgae. *Euglena* sp., *Chlorella vulgaris*, and *Scenedesmus* sp. with growth rate of 0.401 day^{-1}, 0.377 day^{-1}, and 0.342

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day^{-1} and lipid content above 27 % were taken for the later studies. Mountain varieties showed slightly higher growth rate with difference in their lipid contents up to 0.5 % as compared to mid-hill varieties. In case of seasonal effect, there was increase in the growth rate of all three species during summer temperature of 27 ± 2 ^{o}C; whereas, due to this temperature negative affect was seen in the lipid content of these species.

Materials and methods

Samplings were done from various freshwater resources situated at different geographical location of Nepal as shown in Table 1. Water samples containing microalgae were collected from these resources in a 15 ml centrifuge tube with 10 ml of Bold Basal Medium (BBM) Bischoff and Bold, 1963). On laboratory, all samples were filtered, and washed with distilled water and BBM respectively. Samples were examined microscopically for the presence of algal species prior to isolation. Isolation were done using 2% (wt/vol.) bacteriological agar (Hi-Media) in BBM and incubated at 25 ± 2^{0}C with irradiance of 28 µmol/m^{2}/s for 2-3 weeks. Cool daylight florescence tubes of 6500 ^{0}K from Bajaj Electrical Limited were used to provide irradiance to these cultures. Light intensity was altered by maintaining culture distance from the light source. Isolated samples were identified on the basis of their morphological features and dimensions following Prescott (1951), Philipose (1967), and Bellinger and Sigee (2010), at Department of Botany, Post Graduate Campus, TU, Biratnagar, Nepal.

For all comparative growth studies, fully matured isolated samples were culture in 250 ml borosilicate conical flask (Borosil). Initial volumes of all cultures were maintained to 250 ml with BBM at pH 6.8. All cultures were subjected to continuous aeration of 0.3 l/min using aquarium air pump. Rotameter from Flowstar (FSA 100) was used to determine aeration rate to the cultures. Cultures were maintained at 12:12 light: dark photoperiod and irradiance of 56 µmol/m^{2}/s All studies, expect effect of temperature with respect to seasonal variation, were done at temperature of 25 ± 2 ^{o}C. Later study was done on the month of December (with average room temperature of 18 ± 2 ^{o}C) and on the month of May (with average room temperature of 27 ± 2 ^{o}C). *Chlorella vulgaris*, *Euglena* sp. and *Scenedesmus* sp. were taken for later two experiments. All experiments were conducted at Department of Biotechnology, Kathmandu University, Dhulikhel, Kavre, Nepal.

To estimate dry weight of biomass, cultures were harvested and washed for two times using distilled water by centrifuging at 4500 rpm for 10 mins. Concentrated samples were dried in hot air oven at 60°C till constant weight appeared. Lipid contents were extracted following Bligh and Dyer (1959) method, with slight modification as described by Lee et. al. (2010) and Ryckebosch et. al. (2011). Extraction was done by using mixture of chloroform: methanol: water at 1:1:0.8 ratio (v/v), chloroform layer containing lipids was separated and dried at 40 ^{o}C. Lipids were measured gravimetrically and reported as percentage of dry their weight.

Analytical Method: Cultures growths were monitored spectrophotometrically at 750 nm, using Chromtech, CT-1500 spectrophotometer, on daily basis. Optical Density (OD) reading was plotted on semi logarithmic scale for the calculation of growth rate (μ) as described by Friedrich Widdel (2007). Growth rates for initial study of 8 samples were calculated for growth phase of 0-3 days (rate increasing period of exponential phase) culture and 4-10 days (rate decreasing period of exponential phase). Averages of these two phases were also calculated. For later studies growth rate was taken from the initial log phase to final log phase from the semi-log plot. Following equation was used for growth rate measurement:

$$\mu = \frac{\ln(OD2 - OD1)}{T2 - T1}$$

Where, *OD2* and *OD1* are the optical densities of culture at time *T2* and *T1* respectively.

For reproducibility of process, all culture experiments and lipid extraction steps were performed in triplicate. Standard errors of the mean of the experiments were calculated and shown either in Fig. or by plotting in illustrations.

Result and discussion

Species isolation and identification

List of the isolated species are given in Table 1. This includes two samples of *Chlorella vulgaris*, *Chlamydomonas* species and *Euglena* sp.; three samples of *Scenedesmus* sp.; a sample of *Tetraedron caudatum (Corda) Hansg*, and *Chlorella* sp.

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Table 1: Isolated single species according to their location.

SN	Sample Code	Location	Description of Location	Isolated Species	Altitude (meter)	Coordinates
1	BNP i	Banepa, Kavre	Fish Pond	*Chlorella vulgaris*	1450	27°37'55"N 85°31'15"E
2	DM v	Gahana Pokharai, Ktm	Pond	*Chlorella* sp.	1400	27°43'0"N 85°19'58"E
3	GW vi	Godavari, Lalitpur	Pond	*Chlamydomonas* sp.		27.59°N 85.39°E
4	DM xi	Gahana Pokharai, Ktm	Pond	*Euglena* sp.	1400	27°43'0"N 85°19'58"E
5	SMKU xii	Semlar VDC, Rupandehi	Stream	*Tetraedron caudatum (Corda) Hansg.*	< 300	27°40'00.6"N 83°23'26. 5"E
6	BM xiii	Banglamukhi, Lalitpur	Drainage of natural water tap	*Scenedesmus* sp.	1400	27°40'35"N 85°19'34"E
7	DM xv	Gahana Pokharai, Ktm	Pond	*Scenedesmus opoliensis Richter*	1400	27°43'0"N 85°19'58"E
8	KU xvii	Dhulikhel, Kavre	KU's water collection site	*Chlamydomonas* sp.	1600	27°37'8"N 85°32'16"E
9	GOSCV	Gosainkunda, Rasuwa	Pond	*Chlorella vulgaris*	4400	28°4'56"N 85°24'48"E
10	GOSSCN	Gosainkunda, Rasuwa	Pond	*Scenedesmus* sp.	4400	28°4'56"N 85°24'48"E
11	GNKEUG	Ganeshkunda, Rasuwa	Pond	*Euglena* sp.	4600	28°4'33"N 85°25'29"E

Culture growth and lipid content

During preliminary screening of eight different samples, higher growth rates (on average basis) were seen in *Euglena* sp. (DM xi), *Chlorella vulgaris* (BNP i) and both *Chlamydomonas* sp. (KU xvii & GW vi) which were 0.401 day^{-1}, 0.377 day^{-1}, 0.365 day^{-1} and 0.367 day^{-1} respectively (Fig. 2:(a)). All species showed higher growth rates on initial 3 days of the culture and growth rate decreased along with the culture time. For DM xi both growth rates i.e., from 0-3 days and from 4-10 days were comparatively higher than other samples. Even though growth rate from 0-3 days was higher for *Chlamydomonas* sp. as compared to BNP i, its growth rate from 4-10 days was lower. Thus, BNP i had better overall growth than KU xvii & GW vi.

Although, growth rate for *Chlamydomonas* sp. and *Euglena* sp. were higher, these samples tends to attain early stationary phase thus producing biomass less than 0.50 g/l of dry weight, at end of exponential phase (Fig. 1 & 2(a)). On other hand, *Scenedesmus opoliensis Richter* (DM xv) and *Scenedesmus* sp. (BM xiii) had average growth of 0.237 day^{-1} and 0.342 day^{-1} still they produced comparatively

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higher biomass which was 0.56 g/l and 0.55 g/l on dry weight basis respectively. Among all eight samples of microalgae BNP i sample seems most promising with high growth rate, highest dry mass production capability of 0.71 g/l and average lipid content of 28.3 % (Fig. 2: (a) & (b)).

Lipid content was almost similar in all the eight microalgae stains with standard error ±1.3% (on average) among the samples with expectation for *Tetraedron* sp. (SMKU xii) as seen in Fig. 2:(b). Growth rate and lipid content in SMKU xii sample was found to be comparatively low, i.e., 0.220 day^{-1} and 20.8 % respectively. Highest lipid content of 30.1 % was seen in DM xv. Our study report, lipid percentage of the given microalgae species lies between 20 % to 30 % and this was comparatively similar to other research findings (Ryckebosch *et. al.*, 2011; Mahapatra *et. al.*, 2013; Abdo *et. al.*, 2013). During these experiments, lipids from all above cultures were extracted at time period of 12 to 13 days. As some species attained stationary phases faster compared to other, further trails are required on harvesting and extraction at similar growth phases for all these samples to obtain more optimum result for dry biomass and lipid content.

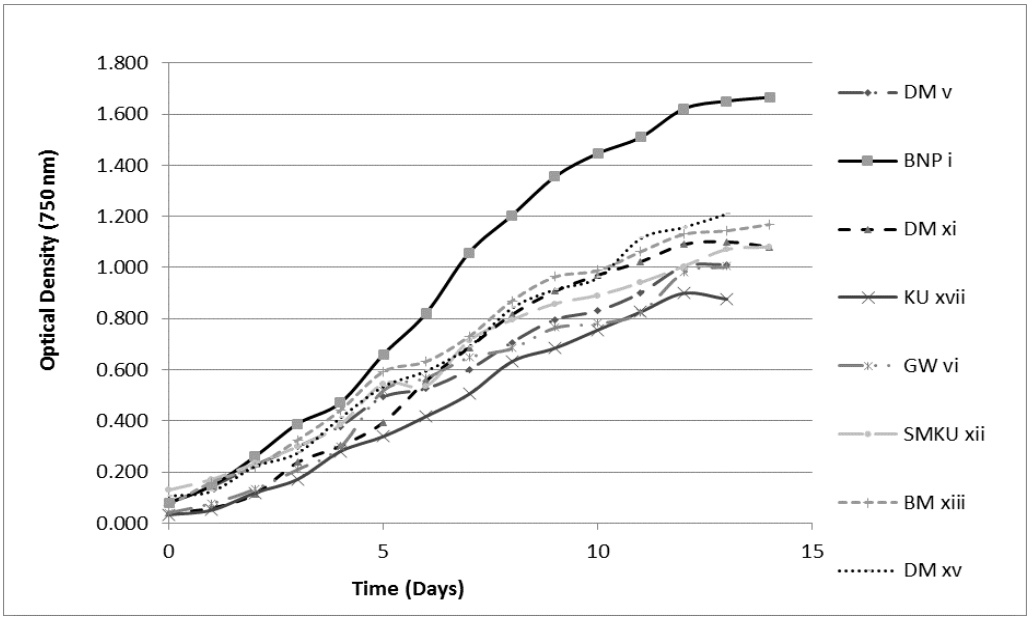

Fig. 1: Comparative growth study of eight samples of microalgae with continuous aeration of 0.3 l/min, and culture condition of 25 ± 2 ^{0}C, 12:12 light: dark photoperiod and irradiance of µmol/m^2/s. (Average of Standard Error of the Mean: DM v= 0.056; BNP i= 0.049; DM xi= 0.1; KU xvii= 0.052; GW vi= 0.094; SMKU= 0.053; BM xiii= 0.091; and DM xv= 0.020)

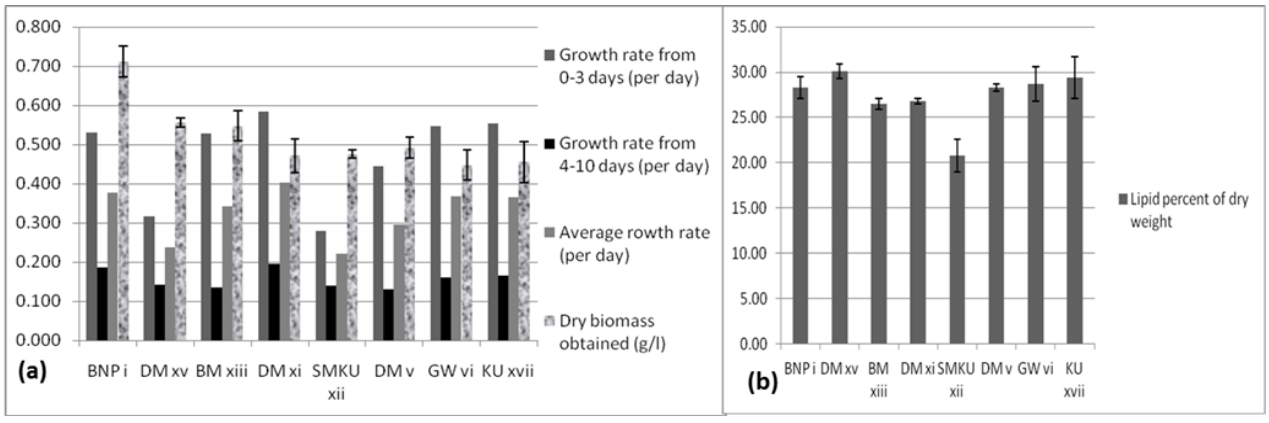

Fig. 2: (a) Growth rates and dry biomass content of eight microalgae samples with respect to Fig. 1. (b) Lipid percentage on the basis of dry biomass of microalgae samples.

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Comparisons of microalgae samples from two different altitudes

In our study we compare similar genus of microalgae from mountain range (above 4000 m) and mid hill range (above 1000 m) of Nepal. Growth comparison was done on, i.e. *C. vulgaris* (BNP i Vs GOSCV), *Euglena* sp. (DM xi Vs GNKEUG) and *Scenedesmus* sp. (BM xiii Vs GOSSCN) on the basis of their growth curve, as seen in Fig. 3:(a), (b) & (c). It was observed that growth rate of all three genus from high altitude samples were higher than of mid hill region (Fig. 4:(a)). For *C. vulgaris* difference in growth rates among GOSCV and BNP i was 0.02 day^{-1}, whereas growth rates *Euglena* sp.(GNKEUG and DM xi) and *Scenedesmus* sp. (GOSSCN and BM xii) were differed by 0.04 day^{-1}.

There was minimal difference seen in lipid percentage of dry weight among two altitudes samples. In case *C. vulgaris* difference was only 0.2%, whereas in *Euglena* sp. and *Scenedesmus* sp. difference was of 0.4% and 0.5% respectively (Fig. 4:(b)). *C. vulgaris* and *Scenedesmus* sp. showed higher growth and lower lipid accumulation but the difference in each of these properties were very minimum. There was negligible effect seen on lipid percentage of algal sample according to their origin (sampling altitude) and growth rate. Even though GNKEUG had comparatively high growth rate to DM xi, its lipid content was also higher. Species of *C. vulgaris* was confirmed on the basis of their morphological features and dimensions, whereas for *Euglena* sp. and *Scenedesmus* sp. it is yet to be confirmed. Identification of all these samples on their species and genetic level is still required to determine their biological and genetic variation. This could ensure compared samples belongs to same species with or without variation, thus our result on above comparisons would be more précised.

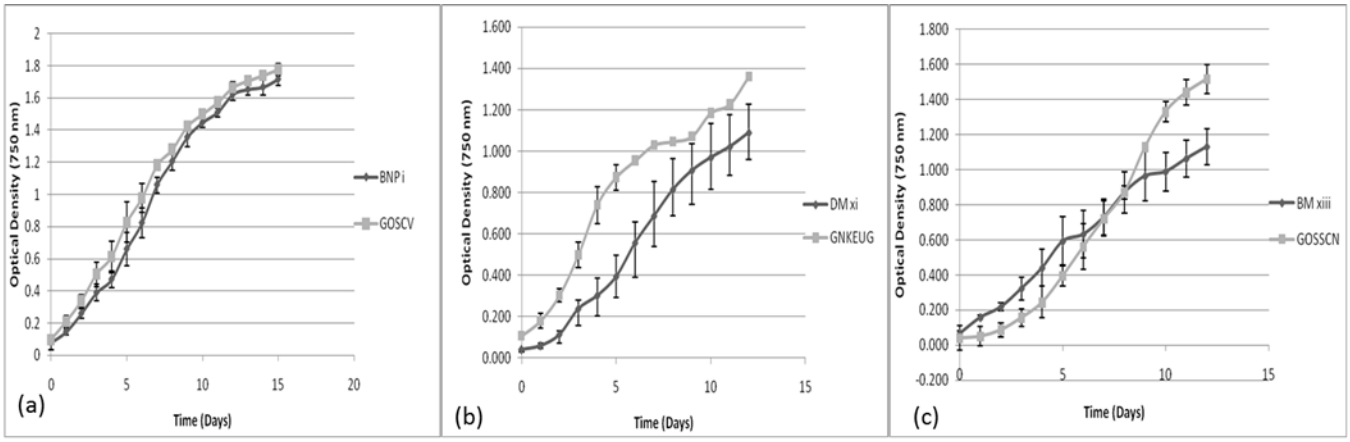

Fig. 3: Growth curve of microalgae samples from mountain range (>4000 m) and mid hill range (>800 m) cultured with continuous aeration of 0.3 l/min and culture condition of 25 ± 2 ^{0}C, 12:12 light: dark photoperiod and irradiance of 56 µmol/m^2/s. (a) *C. vulgaris;* (b) *Euglena* sp.; and (c) *Scenedesmus* sp.

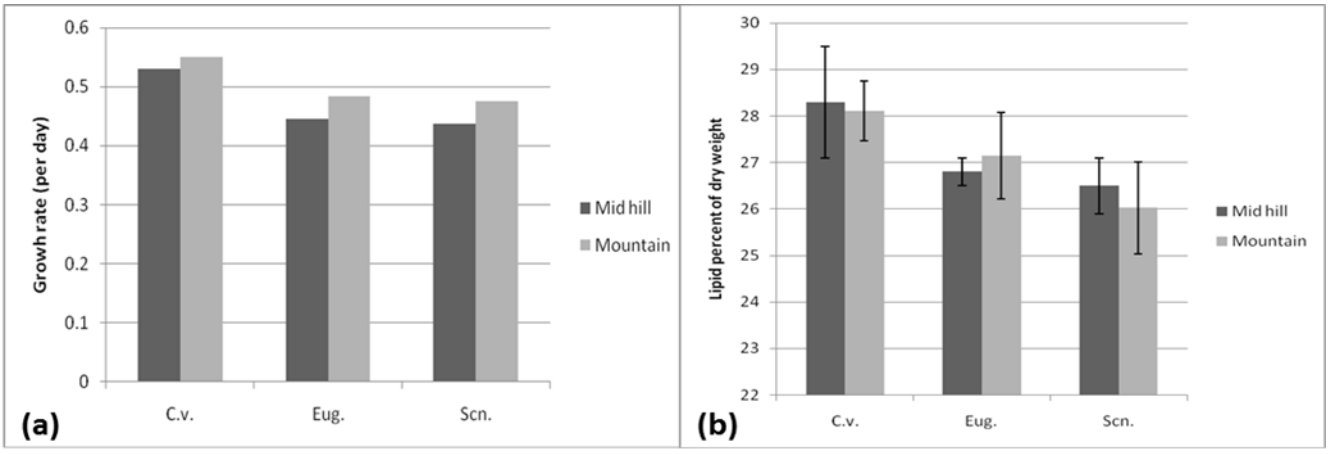

Fig. 4: (a) Comparative growth rate of six microalgae samples with respect to Fig. 3:(a), (b) & (c); and (b) comparison of lipid percentage on the basis of their dry weight among six microalgae samples from mountain (>4000 m) and mid hill (>800 m) range.

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Comparison of microalgae samples at summer and winter season temperature

Further, we experimented on the effect of seasonal temperature on growth rate and lipid content of *C. vulgaris*, *Euglena* sp. and *Scenedesmus* sp. Growth of BNP i, DM xi and BM xiii were studied on two different month of the year i.e., December and May (Fig. 5:(a), (b) & (c)). We find out difference on growth rate and lipid content on these species with respect to the two extreme climates (Fig. 6: (a) & (b)). With increase in temperature by 10 ^{o}C growth rate of BNP i, DM xi and BM xiii increased by 0.05 day^{-1}, 0.12 day^{-1} and 0.08 day^{-1}, whereas lipid content decreased by 10 %, 14 % and 13 % respectively. This reveals that there is an inverse relationship between lipid percentage and growth rate depending to the temperature. Rukminasari (2013) reported increase in lipid percentage with decrease of growth at low temperature (18 ^{o}C) for freshwater *Scenedesmus* sp. and vice versa at 25 ^{o}C. On the other hand, marine algae, *Dunaliella tertiolecta* had shown better result at 18 ^{o}C, with increased growth rate and lipid content. In our study we observed, during summer season biomass productivity is higher compared to winter with lower lipid productivity and vice versa during winter for all three freshwater species.

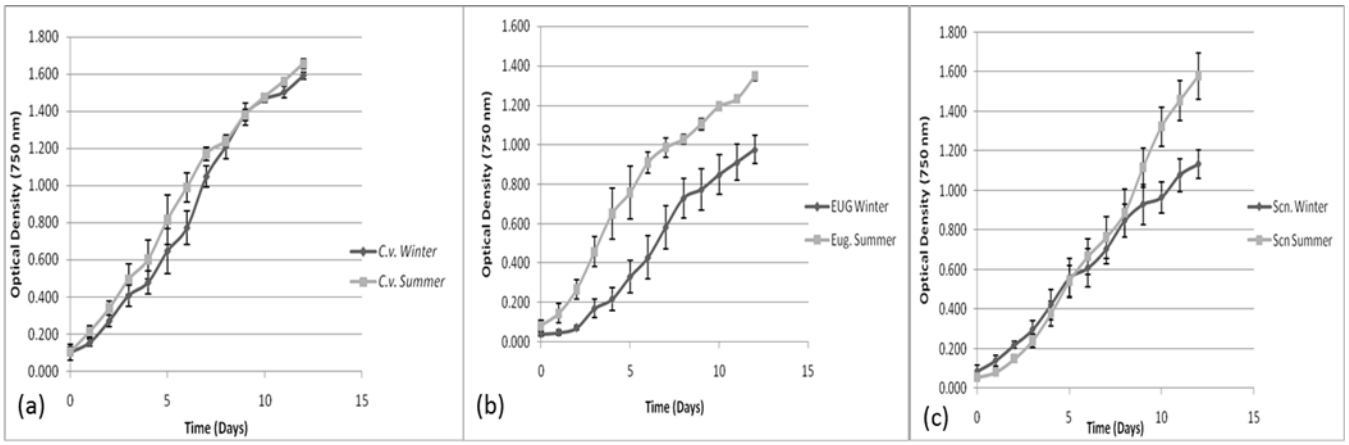

Fig. 5: Growth curve of microalgae samples with seasonal variation according to temperature. Samples culture in winter season (18 ±2 ^{0}C) and summer season (27 ±2 ^{0}C) with continuous aeration of 0.3 l/min, 12:12 light: dark photoperiod and irradiance of 56 µmol/m^{2}/s. (a) *C. vulgaris* (BNP i); (b) *Euglena* sp. (DM xi); and (c) *Scenedesmus* sp. (BM xiii).

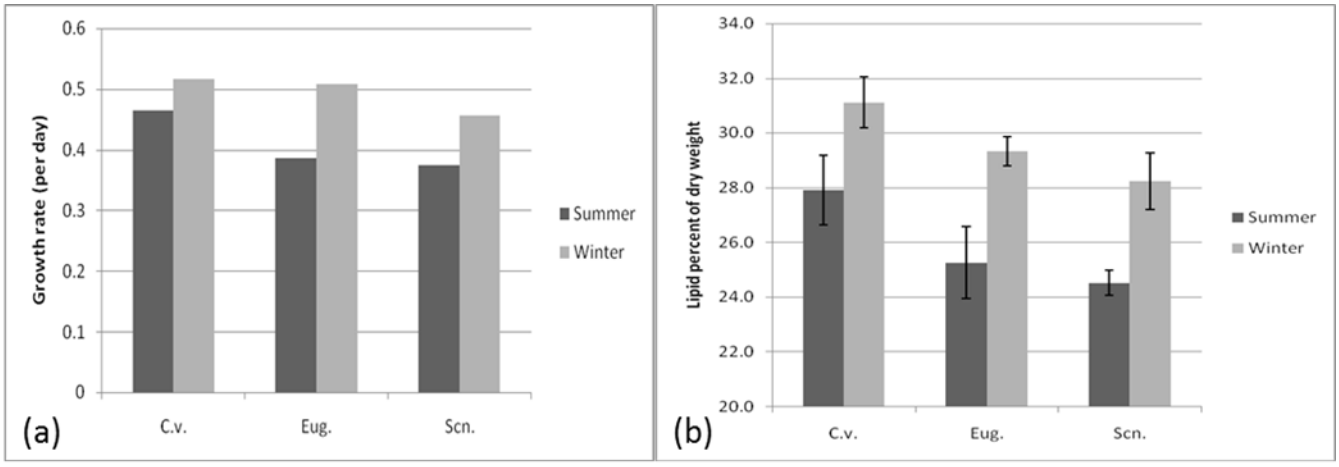

Fig. 6: (a) Comparative growth rate of three microalgae samples with respect to Fig. 5:(a), (b) & (c); and comparison of lipid percentage on the basis of dry weight among three microalgae species during summer (27 ±2 ^{0}C) and winter (18 ±2 ^{0}C) season.

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Conclusion:

Growth rate and lipid content varied according to the selected samples and their culture temperature. *C. vulgaris*, *Eugelena* sp., and *Scenedesmus* sp. were found to have better species on basis of their growth rate and lipid content. These species could be possible used in scale up culture and analyze their productivity for large scale culture as feedstock for biodiesel production. Moreover, summer temperature of 27 ±2 ^{0}C could be considered as better condition to obtain improved yeild in biomass as this had positive effect on the growth rate, along with this, negative impact of temperature on lipid content should also be taken into account. Species from mountain range of the country were comparatively better. Study on the mountain and mid-hill ranged samples is necessary, based upon identification of their species at genetic level, to confirm above comparision on species and it's genetic strain basis.

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