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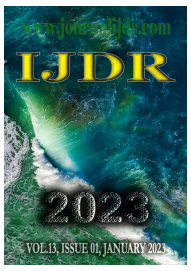

RESEARCH ARTICLE OPEN ACCESS

GROWTH PERFORMANCE OF FIVE SPECIES OF MARINE MICROALGAE

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ABSTRACT

Growth performance of five marine microalgae *Isochrysis galbana, Chaetoceros calcitrans, Tetraselmis suecica, Nannochloropsis oculata*, *Aphanocapsa* species was studied under different culture conditions i.e. Conway and f/2 media at 20, 30 and 40ppt salinities (‰). The microalgae culture was undertaken in the indoor facility with 24±1° C temperature, P^H 8.7±1 under 24hour photoperiod. Overall highest cell density (56.23million cells/ml) was recorded in *Aphanocapsa* sp. in Conway medium at 30‰ followed by *N. oculata* (32.78million cell/ml) in Conway medium at 20‰, whereas lowest value (0.6million cell/ml) was recorded in *T. suecica*in f/2 medium at 40‰. Overall highest specific growth rate (0.23%) was recorded in *T. suecica* cultured in Conway medium at 20‰ and the lowest value (0.01%) also recorded in *T. suecica* in f/2 medium at 40‰. It is further observed that the Conway medium was the best medium for the attainment of maximum cell growth and cell density of the five species studied.

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INTRODUCTION

Microalgae are microscopic photosynthetic organisms that can be found in both freshwater and marine environments.They contribute significantly to the ocean's productivity and serve as prime producers in the marine food chain by synthesizing the organic material from carbon dioxide and water through sunlight. Apart from that, they correspondingly produce the necessary oxygen which is required for the metabolism of the consumer organisms (Chacón-Lee and González-Mariño, 2010). Microalgae cultures are regularly fed to fish larvae, crustacean larvae and molluscs in all phases of their development (Meireles *et al*., 2003). Microalgae have long been produced at a large scale successfully by utilizing either domestic or bio-industrial wastes or inorganic chemicals (Shelef & Soeder, 1980; Fabregas *et al*., 1985a). The medium for culture must be non-hazardous to larvae, so the utilization of waste-supported microalgal biomass in larval rearing is often doubtful. Therefore, hygienic nutrient sources often be used, and these media are usually prepared in research laboratory (Stein, 1973; Soeder, 1980; Fabregas *et al*., 1984a, 1985a). The pure strain culture of microalgae requires a more rigorous nutrient composition in their growth media.

Distinct media formulations like Conway (Walne, 1970) and f/2 (Guillard & Ryther, 1962) media are commonly employed to yield microalgae monocultures on a laboratory scale. Microalgal biomass production decreases greenhouse gas emissions and produces bio-fuel as a substitute for fossil fuels. They have been used for the production of food, health supplements, fodder, aquaculture, biofuel, fine chemicals and a variety of biotechnological applications (Raja *et al*., 2014). Microalgae have been considered as one of the biofuel feedstocks to resolve the global energy predicament due to their high photosynthetic rate of more than 6.9 x 10^4 cells m/L/h (Suali & Sarbatly, 2012). Cyanobacteria, correspondingly called blue- green algae, are often treated as part of the same group because of their physiological and photo fermentation features (Hakobyan *et al*., 2019). They can be grown as single cells or associated in chains or tiny colonies (Postma *et al*., 2016) and also play an essential role in aquatic ecosystems because of their photosynthetic competence (Malcata *et al*., 2018). On the other hand, the rapid growth of the world's population is expected to make attaining adequate nourishment a rising worldwide concern in the next decades, which makes microalgae farming a potential alternative food crop as food and feed (Sun *et al*., 2019). According to the earlier studies, most of the authors are cultured microalgae in fixed ambient conditions. Due

to the paucity of information in this study area on the culture of microalgae at different salinities in different culture media, the present study designed to determine the growth performance of five species *Isochrysis galbana* Parke 1949, *Chaetoceros calcitrans* (Paulsen) Takano 1968, *Tetraselmis suecica* (Kylin) Butcher 1959, *Nannochloropsis oculata* (Droop) Hibberd 1981 and *Aphanocapsa* sp. Nägeli 1849 (cyanobacteria) in different culture media, more or less similar to Conway (Walne, 1970) & f/2 (Guillard & Ryther 1962) at three different salinities (20 ppt, 30 ppt & 40 ppt). This study was undertaken in algal laboratory in Dept. of Marine Living Resources, Andhra University, Visakhapatnam, India during 2016-2017.

MATERIALS AND METHODS

***Collection of marine microalgal species*:** The stocks of five marine microalgal species *I. galbana, C. calcitrans, T. suecica, N. oculata* and *Aphanocapsa* sp. were used in this study procured from the Central Marine Fisheries Research Institute (CMFRI), Visakhapatnam, Andhra Pradesh, India. Stocks of each culture were maintained in Walne medium (Walne, 1970) at salinity 34 ppt and at 23±2° C temperature under 12:12 h light: dark cycle with a light intensity of 80 µmol photons.$m^{-2}.s^{-1}$.

Microalgal culture

Cleaning and sterilization: Preceding to the microalgae culture, all new glassware was soaked in 10% 1M hydrochloric acid for overnight to remove the chemical residues. The glassware and also the other culturing equipment were then washed with leboline (Decon-90), a phosphate-free detergent, then rinsed few times with tap water and finally with distilled water. Used glassware was bleached with 10% NaOCl then washed thoroughly. All culture equipment was autoclaved to maintain the sterile conditions. Inoculation, sub culturing, sampling and transfer of medium were carried out near a spirit lamp inside the laminar air flow chamberby cleaning the surface with 70% ethanol.

Seawater uptake and purification: Raw seawater, pumped from the seashore, was filled in a storage tank through sand filtration; the stocked water was chlorinated using commercially available chlorine (5 ppm) and dechlorinated by giving aeration for 24 hours. After dechlorination, ethylenediaminetetraacetic acid (EDTA-5ppm) treatment was done. Then the treated seawater was transferred to another tank for storage.

Preparation of nutrient media: Distilled water (Merck) was used for the preparation of nutrient stock medium and purified seawater was (filtered through 0.45 µm nitrocellulose membrane) used for culture media. The seawater's salinity was adjusted to the required concentration by adding the filtered fresh water, then final salinity was checked with a portable hand Refractometer model E-2. The filtered seawater was autoclaved at 121° C for 15 min and left to cool to room temperature prior to the addition of nutrients and culture inoculation. The detailed nutrient composition was mentioned in Table 1.

Maintenance of stock culture: The Samples were cultured in appropriate medium and subjected to consecutive rounds of serial dilution and streaked on agar plates and test tube slants. The isolates then maintained on agar plates and in cotton plugged test tube slants at 24 ± 1° C, 12 h light: 12h dark photoperiod with an intensity of 60-80 µmol photons.$m^{-2}.s^{-1}$ light. The stock cultures of microalgae were maintained in 50ml conical flasks stoppered with cotton plugs and were sub-cultured in fresh medium for every 2 weeks. Stock culture flasks were manually shaken three to five times a day to prevent the cells from settling. The cultures were maintained in a sterile room at 24 ± 1° C temperature equipped with cool-white fluorescent lights with an irradiance of 60-80 µmol photons.$m^{-2}.s^{-1}$ for 16:8 h light: dark cycle.

Indoor Mass culture: The selected microalgal species were cultured in Walne & f/2 medium at 20, 30 & 40 ppt salinities from initial culture volume of 100 ml, 250 ml, 2 L, and up to 20 L under 24 h light period with an intensity of 80 µmol photons.$m^{-2}.s^{-1}$ irradiance. Lighting was provided with a cool-fluorescent tube (Anaga & Abu, 1996) along with constant aeration by an aerator, with 150 bubbles per minute through a drip set fitted with a controller (Anaga & Abu, 1996) at 24±1° C temperature and P^H 8.7±1. The cultures were swirled manually three to five times a day to maintain the vigour of cells. Indoor mass culture was carried out in 200 L round vertical Fiberglass Reinforced Plastic (FRP) transparent tank. Prior to that, the internal and external walls of the vertical tanks were thoroughly scrubbed with a dilute solution of laboline. The tanks were then rinsed and filled up to the rim with tap water to which chlorine pellets [2mg/l at 600 g/kg chlorine as $Ca(OCl)_2$] were added. The tanks were then left overnight with the chlorinated water. Then the chlorinated water was discarded and the tanks were scrubbed thoroughly with tap water prior to the inoculation of culture. The exponential phased 20 L (transparent PET jars) algal culture was used as inoculumand added into 180 L culture media in 220L capacity of the vertical fibre glass tank (Figure 1). The mass culture of five microalgae was performed in Conway and f/2 medium at three salinities separately to obtain maximum cell density without bacterial contamination. All experiments were performed with three replications. The average values were used for the preparation of graphs and presentation of data. This work was undertaken from June 2016- May 2017.

Cell density and growth: The growth rate of algal cultures was observed according to the cell counts using Neubauer haemocytometer under a bright-field contrast microscope (Olympus CH20*i* magnification 400-1000X).

The total cells were counted under the microscope and calculated using the formula as specified by Schoen [1988].

$$\text{Total cell count } (\times 10^6 \text{ cell/ml}) = \frac{\text{Number of cells counted}}{\text{Number of squares counted}} \times \text{Total number of squares} \times 10^4$$

The specific growth rate of algal cultures was calculated from the exponential regression of the logarithmic portion of the growth curve, as mentioned by Wood *et al*. [2005].

$$\text{SGR\% } (\mu) = \frac{\ln (N_t) - \ln (N_0)}{t - t_0}$$

Where ln is log, N_t is cell density at time (t), and N_0 is cell density at time t_0.

Statistical analysis: ANOVA was carried out for the growth performance of microalgae using Microsoft Excel.

RESULTS AND DISCUSSION

Cell Density: The growth of microalgae is generally decided by the quality of the culture medium which is used for their cultivation (Lam & Lee 2012; Li et al., 2012; Prathima Devi et al., 2012). Adequate supplement of nutrients is a key factor to the performance of the microalgae growth (Xin et al., 2010). According to the results of the present study highest cell count (56.23±7.69 million cell/ml) was recorded in cyanobacteria *Aphanocapsa* sp. cultured in Conway medium at 30 ppt salinity, whereas in other microalgae N. oculata attained maximum cell count (32.78±2.01 million cell/ml), when cultured in Conway medium at 20ppt salinity, but the lowest cell count (0.60±0.15 million cell/ml) was reported in T. suecica cultured in f/2 medium at 40ppt salinity A fairly moderate cell count was recorded in other species *I. galbana and C. calcitrans* in all the conditions. These variations may be attributed to cell size and culture media and salinity. The variations of cell density in five microalgae cultured in different media and salinity showed in Table 2. Similar findings were also noticed in various microalgal species. Lourenço *et al*., (1997) reported that culturing *T. gracilis* in Conway medium found the highest cellular density value and he suggested that the high

Table 1. Composition of the nutrient media

Conway or Walne's Medium		f/2 medium	
Nutrient Solution	*Quantity*	*Nutrient Solutions*	*Quantity*
KNO_3	100.0g	$NaNO_3$	75g
$NaH_2PO_4.2H_2O$	20.0g	$NaH_2PO_4H_2O$	5g
EDTA di-sodium salt	45.0g	Dissolved in Distilled water	Make up to 1L
H_3BO_3	33.6g	$Na_2SiO_3\,9H_2O$	35g/L
$MnCl_2.4H_2O$	0.36g	*Trace Metals solution*	
$FeCl_3.6H_2O$	1.3g	Na_2EDTA	4.36g
Dissolved in Distilled water	Make up to 1 L	$FeCl_3.6H_2O$ (Ferric Chloride)	3.15g
		Dissolved in Distilled water	Make up to 1 L
Trace metal solution		*Primary Metals stocks*	
		(Each solution should be prepared separately)	
$ZnCl_2$	2.1g	$CusO_4.5H_2O$	1.0g/100ml
$CoCl_2.6H_2O$	2.0g	$ZnSO_4.7H_2O$	2.2g/100ml
$(NH_4)6Mo_7O_{24}.4H_2O$	0.9g	$CoCl_2.6H_2O$	1.0g/100ml
$CuSO_4.5H_2O$	2.0g	$MnCl_2.4H_2O$	1.8g/100ml
Conc.Hcl	Few drops	$Na_2MoO_4.2H_2O$	0.63g/100ml
Dissolved in Distilled water	Make up to 100ml	Add 1ml of each of 5 to the above Trace Metals solution	
Vitamin solution		*Vitamin Stock Solution*	
Vitamin B_{12} (Cyanocobalamin)	0.1g	Vitamin B_{12}	0.1g
Vitamin B1(Thiamine.HCL)	2g	Vitamin B_1	0.2g
		Biotin	0.1g
Dissolved in Distilled water	Make up to 1 L	Dissolved in Distilled water	Make up to 1 L
Making Final Medium		Making Final Medium	
Nutrient solution	*1ml*	$NaNO_3$+ NaH_2PO_4 Stock solution	*1.0 ml*
Trace metal solution	*0.5ml*	$Na_2SiO_3\,9H_2O$	*1.0 ml*
Vitamin solution	*0.1 ml*	Trace Metals Stock solution	*1.0 ml*
		Vitamin Stock Solution	*1.0 ml*
Sterilized seawater	1L	Sterilized seawater	1 L

Table 2. Average cell density of five microalgae under different culture conditions (million cells/ml)

Species	Conway			f/2		
	20ppt	30ppt	40ppt	20ppt	30ppt	40ppt
I. galbana	13.27±2.06	3.20±0.73	10.90±0.9	13.30±1.83	3.00±0.81	4.20±0.32
C. calcitrans	9.10±1.15	2.96±0.51	2.05±0.20	7.57±2.38	2.85±0.40	0.85±0.10
T. suecica	4.70±1.25	1.90±0.13	1.25±0.18	3.37±1.89	1.77±0.97	0.60±0.15
N. oculata	32.78±2.01	32.37±1.00	13.8±1.52	25.51±5.69	23.45±1.90	5.50±1.20
Aphanocapsa sp.	43.50±6.49	56.23±7.69	27.55±2.26	37.60±2.85	30.70±2.16	26.20±2.30

Growth performance of five species of marine microalgae

a. Algal culture laboratory *b. Flask culture*

c. Carboys culture *d. Vertical cylinder culture*

Figure 1. Microalgal culture

densities of cultures were possibly because Conway medium was more nutritive than f/2 Guillard's medium. Lananan *et al*. (2013)also reported that the *Isochrysis* sp. cultured in Conway medium showed maximum cell density, whereas *Dunaliella* sp. in f/2 medium showed the lowest value of cell density. He also suggested that the larger cells required more nutrients for their growth and reproduction. The studies of Phatarpekar *et al*. (2000); Huerlimann *et al*. (2010) were also in agreement with the present study. The response of microalgae on the utilization of the culture media was also based on their specific biological requirements and thus it varied between different genera (Collet *et al*., 2011).

Table 3. Specific growth rate in five microalgae under different culture conditions (%)

Species	Conway			f/2		
	20ppt	30ppt	40ppt	20ppt	30ppt	40ppt
I. galbana	0.19	0.11	0.09	0.15	0.10	0.08
C. calcitrans	0.22	0.17	0.10	0.22	0.16	0.02
T. suecica	0.23	0.11	0.08	0.13	0.11	0.01
N. oculata	0.18	0.16	0.10	0.15	0.14	0.04
Aphanocapsa sp.	0.17	0.21	0.10	0.16	0.11	0.11

Specific growth rate: In the present study, all the species showed a high specific growth rate (0.17 to 0.23%) in Conway medium at 20ppt salinity except *Aphanocapsa* sp. where it showed a high specific growth rate (0.21%) in Conway medium at 30ppt salinity. The second fastest specific growth rate (0.13 to 0.22%) was noticed in all species cultured in f/2 medium at 20ppt salinity, whereas the lowest specific growth rate (0.01 to 0.11%) was recorded in all species cultured in Conway and f/2 media at 40ppt salinity. The variations of specific growth rates in five microalgae cultured in different media and salinity showed in Table 3. These findings agreed with the earlier studies on the culture of various microalgal species (Glover *et al*., 1987; Phatarpekar *et al*., 2000; Chisti, 2007; Huerlimann *et al*., 2010; Gour*et al*., 2014). Differences in growth rate could be the reason for differences in culturing method/system (Borowitzka, 1999; Wen & Chen, 2000), strain (Illman *et al*., 2000) and culture conditions (Yongmanitchai & Ward, 1991). ANOVA for Cell density in five microalgal species cultured under different conditions showed statistical significance ($P<0.05$), whereas specific growth rate showed no statistical significance ($P>0.05$) between species, but showed significance ($P<0.05$) within the species cultured at different culture conditions.

CONCLUSION

The result of the present study indicates that the five microalgal species have the potential to yield in large scale culture. It is further observed that the Conway medium was the best medium for the attainment of maximum cell growth and cell density of the five species studied. But the production cost needs to be reduced. Such enhancements can be attained potentially by discovering alternative sources for nutrients of the culture medium. Conducting further research is necessary to find out a solution for reducing the nutrient cost because the production cost is higher than the vending cost due to the expensive growth medium.

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