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A novel, low cost, sterilizable and disposable in-house culture vessel for laboratory study of microalgae cultures

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Abstract

Algal industry is expanding as a source of energy, food, feed, recombinant proteins and high value chemicals. As the research need is increasing, many laboratories are getting involved. Photobioreactors are used to achieve uniform light illumination and CO_2 supply to achieve faster algal growth. Use of commercially available PBR in algal study is limited to few highly funded laboratories due to large initial capital requirement and high operational cost. To address the issue of uniform light illumination and CO_2 supply at low cost, an In-house Culture Vessel (ICV) has been designed and the algal growth was compared between ICV vs. conventional 20L carboys which are generally used in lab for relatively larger quantity of inoculum development. An in-built air-sparger has been provided to achieve both air lift mixing of culture and CO_2 supply. The well mixed culture with high surface area allows uniform light illumination. Picochlorum sp. was grown in the designed culture vessel and in conventional carboys. Growth rate in ICV was 16 times more as compared to carboy. This enhanced growth rate indicates more and/or even availability of light to algal cells in ICV compared to carboy as the starting nutrient level was same in both. Growth rate of 0.16 OD/day even at 35 OD in ICV indicates that the culture of Picochlorum sp is still in growing phase in ICV whereas the same culture reached stationary phase in the 20L carboy at 3.73 OD with negligible growth rate of 0.02 OD/day. The proven fact of chlorophyll a/b ratio adjustment in response to light was observed as well with maintained Nitrogen level for Picochlorum sp. The In-House Culture Vessel was demonstrated to be a promising and inexpensive tool which could be used as a closed system for developing actively growing high density microalgae cultures at laboratory scale for further studies.

Keywords: In-house Culture Vessel, Photobioreactor, microalgae, Algae cultivation, Picochlorum sp., CO_2.

Introduction

Microalgae are considered to be a great production system for many valuable products such as pigments, polyunsaturated fatty acids (PFA), and lipids[1]. Recently Microalgae are even used as a production system for recombinant proteins as well[2]. In lab scale, Microalgae can be conventionally studied in either conical flasks or glass bottles. Growth in conical flasks and glass bottles can be limited by inadequate supply of photosynthetically active radiation (PAR) and dissolved carbon dioxide[3,4]. To overcome these challenges in the conventional culture vessels, a varied series of automated lab-scale PBRs including flat plate-based PBRs[5], cylindrical bubble column PBRs[6], and flow tube PBRs[7] have been developed. To achieve uniform light illumination, sophisticated and internally illuminated photobioreactors (IIPBR), equipped with optical fiber's to increase uniform light availability were also developed[8,9]. However, these above mentioned PBRs involve high construction costs[10-12] and all are not easily sterilizable.

Microalgae also have the ability to adjust their pigment ratio on exposure to the light regime to which they are exposed. Microalgae were observed to adjust their chlorophyll content as well as chl a/b ratio during photo acclimation[13]. More pigmentation is expected to be beneficial to the microalgal cells exposed to low light but causes extensive photosystem (PS) oversaturation of the cells exposed to high light. The excess amount of light energy in highly pigmented cells is dissipated as heat and fluorescence[14]. In the culture conditions where light is limiting, microalgae increase the accessory pigment number (i.e. antenna size) to absorb more light energy[5]. Microalgae has natural tendency to decrease in pigmentation to maximize productivity when irradiated with high light because low pigment content would reduce extra light energy consumption and improved performance at the high light[5]. The Chl *a/b* ratio is supposed to respond to light availability and nitrogen availability in two ways[15];1) Chl *a/b* ratios increases with increasing light exposure at a given nitrogen concentration in the media or 2) Chl *a/b* ratios increases with decreasing nitrogen availability under high light conditions. The relationship between Chl *a/b* ratio and light irradiance has been supported by many studies in plants[16,17]. Adjustment of the Chl *a/b* ratio was found to be an integral feature of acclimation to high light conditions in stable nitrogen availability in plants[18].

In the present study a small scale, In-house Culture Vessel (ICV) has been designed and studied for microalgal growth.

Material and methods

Design and Assembly of In-House Culture Vessel: Polypropylene (PP) bags were chosen as these are readily available and low cost (INR 140.00perKg. which is approximately INR 2.00perbag). Sparger's were made using 6.00mm diameter pipes which are used in water filtration systems, as these pipes remain straight on heat straightening and the holes made for sparging remains stable during water bath sterilization. Silicone tubing was used for air inlet as it is autoclavable. The In-House Culture Vessel was developed in two main components such as complete polypropylene sheet structure and sparger tubing. PP material was chosen as it is autoclavable. PP bags were used for making the body of the In-House Culture Vessel. The culture vessel can accommodate up to 1L of culture and can be used for minimum of 200ml. Operational thickness that can be adjusted between 6mm to 15 mm. The body of the culture vessel is divided in to two halves to facilitate bubbles movement from bottom to top which allows uniform mixing. In-House Culture Vessels were inoculated from the air sparger tube by removing air filter and connecting the tube with inoculum flask. Culture movement for inoculation was performed with the help of peristaltic pump. Tube light arrangements from top and bottom were provided to achieving 280 to 300µM/M2/s irradiation from both the side.

Inoculum Preparation: Inoculum of *Picochlorum* sp. was prepared in 200 ml flask in Kuhner shaker at Light: dark regime of 12:12 hours, 1% CO_2 during light period and 26^0C temperature. 40% light was used which is around 20000 - 22000lux from top.

Growth Medium: Urea as a nitrogen source, Phosphoric Acid as a phosphorus source[19] and f/2 micronutrients[20,21] N:P ratio of 16:1[22,23] was used in media with 150 to 200ppm Nitrogen maintained throughout the experiment and f/2 trace elements[20] were added 1ml per liter of medium from 1000X stock solution.

Experimental setup: Culture was inoculated through sparging tube with the help of peristaltic pump in aseptic conditions. Light:dark regime of 15:09 hours was manually operated. Temperature was maintained 27-28^{0}C by maintaining room temperature. 2% CO_2 with 98% atmospheric air was sparged during light period with flow rate of 4 l/m, while during dark period only atmospheric air was sparged with same flow rate. White tube lights were used to provide light from top and bottom. Four white fluorescent tube lights from each side were used achieving illumination around 280 -300µM/m^2/s from each side.

All the algal growth experiments were carried out in triplicates in ICV vs. carboys and mean of data points was taken to plot graphs with standard deviation. First experiment was initiated with high initial density (3.14OD), considering high illumination may affect the culture growth and adaptation in ICV. Second batch of experiment was conducted with low initial density (0.49OD), to test the culture growth at higher illumination in ICV.

Analytical Methods: Various methods were used to measure productivity. Spectrophotometric absorbance was used as a primary method to quantify algal biomass, due to its reliability and as it correlates cell density to light absorbance at 750nm wavelength from earlier recommendations[24,25] at an interval of 24hrs.

The quantitative analysis of chlorophyll-*a* (Chl-*a*) and chlorophyll-*b* (Chl-*b*) in whole pigment extract of microalgae in methanol extracts was quantified by UV-VIS spectroscopy. 1ml of 1OD (750nm) normalized samples were centrifuged and pallets were homogenized in 100% methanol and incubated at 60^{o}C for 15min to completely extract pigments in methanol. The extract was then centrifuged and supernatant was used for spectrophotometric analysis. Reading were subjected to calculations with Šesták and Lichtenthaler method to get the concentrations of individual pigments[26,27].

Calculations: Growth rate: Growth rate per day (expressed in µ) for two OD values, OD_1 (initial) and OD_2 (final), for corresponding time points t1 and t2 in days was calculated with below formula[28,29,30].

$$\mu = (\ln OD_2 - \ln OD_1) / (t_2 - t_1)$$

Determination of Doubling Time: Fundamentally, doubling time is the time required for all the cells to divide in to two. More the doubling time, slower is the growth and vice versa. Doubling time was calculated with the given formula-1 below[28-30].

$$T_g = \ln(2) / \mu \quad (1)$$

As ln(2) is equal to 0.6931, generation time can be calculated in days with the below formula-2:

$$T_g = 0.6931 / \mu \quad (2)$$

Chlorophyll content: Pigment content using methanol as a solvent were calculated by below formulae-3, 4 and 5 after taking absorbance readings of chlorophyll extract at 652nm and 665nm[26,27].

Chla (µg/ml): 16.72 X (A665nm) – 9.16 X (A652nm) (3)
Chlb (µg/ml): 34.09 X (A652nm) – 15.28 X (A665nm) (4)
Chla+b (µg/ml): 1.44 X (A665nm) – 24.93 X (A652nm) (5)

Results and discussion

The given study was conducted with two experiments, one with higher initial OD (3.14OD) and another with lower initial OD (0.49OD). Algal growth in terms of OD was followed for five days and total chlorophyll content was estimated at second day.

In the experiment initiated with 3.14OD culture, Carboy culture reached an average of 3.73OD at 5 days of inoculation whereas culture in In-House Culture Vessel reached 35.17OD (Figure-1). In the second experiment started at 0.49 initial OD, cultures in carboys reached 1.53OD whereas cultures in In-House Culture Vessels reached 25.74OD at fifth day of inoculation (Figure-1).

In the experiment started with 3.14OD, maximum OD jump per day was observed 8.07 in ICV whereas it was 0.22 in carboys. Average OD jump for five days in ICV was observed 6.4 and 0.12 in carboys (Figure-2). In the experiment started with 0.49OD, maximum OD jump per day was observed 8.26 in ICV whereas 0.28 in carboys. Average OD jump for five days in ICV was observed 5.1 whereas it was 0.21 in carboys (Figure-2).

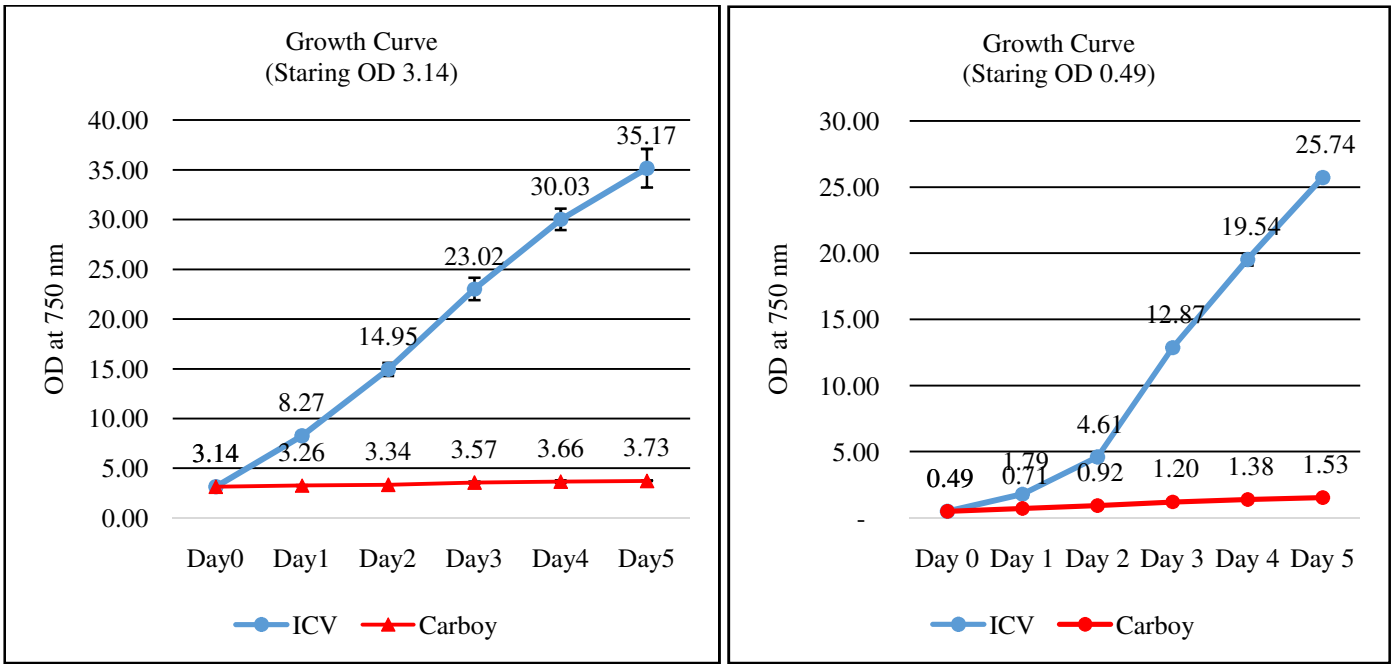

Figure-1: Growth comparison between In-House Culture Vessel and Carboy in term of OD at 750nm under similar conditions with different initial densities.

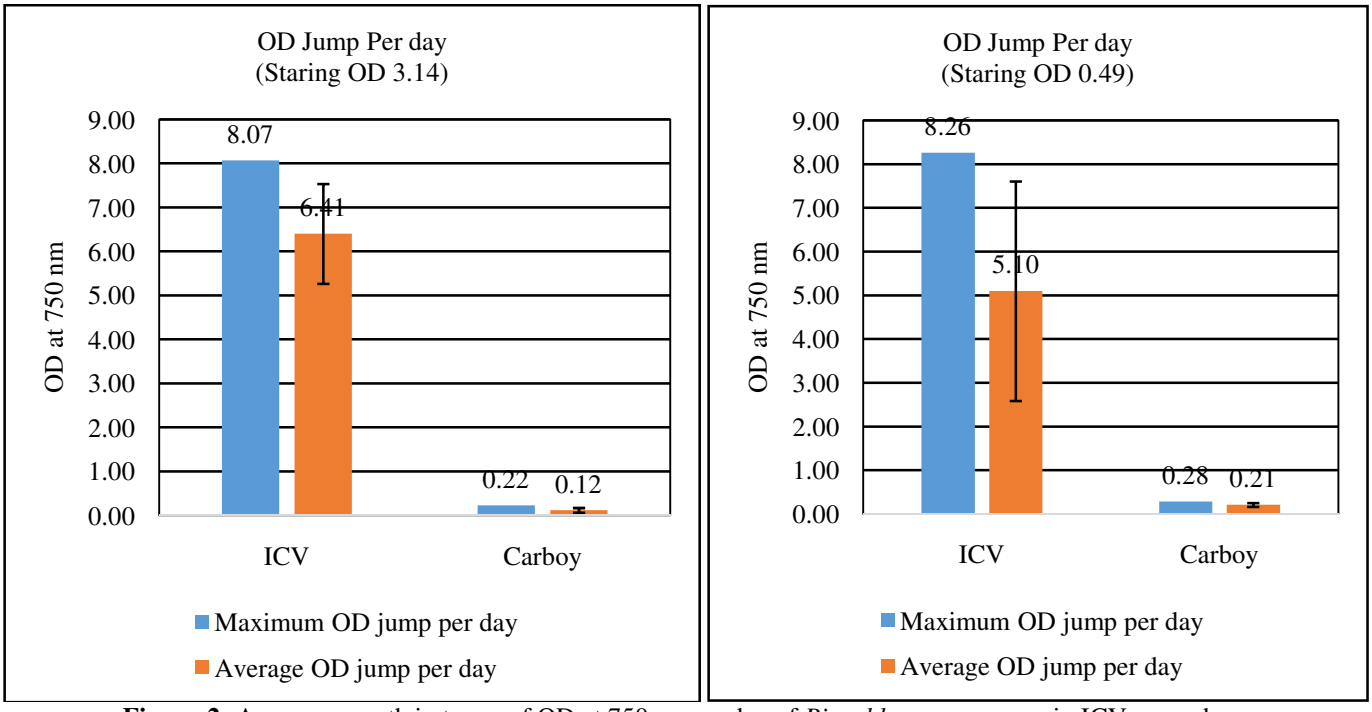

Figure-2: Average growth in terms of OD at 750nm per day of *Picochlorum* sp.grown in ICV vs. carboy.

In the experiment started at 3.14OD inoculation, average growth rate in ICV was found 0.48 per day whereas 0.03 per day in carboys. Growth rate in carboy was negligible resulting 16 times more growth rate in ICV as compared to carboy. This huge difference in growth rate may indicate more and/or even availability of resources (CO_2 and Light) in ICV compared to carboy as nutrient level was same in both the cases. In the second experiment started with 0.49OD inoculation, average growth rate was found 0.79 per day in ICV whereas in carboys it was 0.23 per day. Growth rate in ICV was 3.4 times more as compared to carboy. Difference in growth rate between ICV and carboy has reduced to 3.4 times in the experiment inoculated at 0.49 starting OD as compared to 16 times when inoculated at 3.14 starting OD.

In the experiment started at 3.14OD inoculation, doubling time in day was increase in ICV from 0.72 at 1st day to 4.39 at 5th day whereas in carboys it was increased from 17.99 at 1st day to 34.94 at 5th day. Average doubling time in ICV was 1.43 whereas in carboys it was 20.02 day. In the experiment started at 0.49OD inoculation, average doubling time in ICV was found 0.87 day whereas in carboys it was found 3.04 day. As the OD increased, in ICV doubling time was increase from 0.49 day to 2.52 whereas in carboys it has increased from 1.85 to 6.43 (Table-1).

Growth rate in ICV as well as in carboys was decreased gradually as the density increased. Doubling time increased in both the experiments as the density increased.

An interesting observation to note here is that, growth rate of *Picochlorum* sp. in ICV even at 35OD (μ = 0.16 at day 5) was higher than growth rate in carboy at 1.38OD (μ = 0.13 at day 4). Growth rate of 0.16 at day 5 with 35OD in ICV indicates that the culture *Picochlorum* Sp is still in growing phase in ICV whereas the same culture was almost at stationary phase in the carboy at 3.73OD with negligible growth rate of 0.02 at day 5. Along with fast growth, the culture in the ICV also remained uni-algal devoid of any contamination after five days of growth as in the carboy. There was no microscopic difference in the culture health observed in the ICV grown culture and carboy grown cultures.

Total chlorophyll content and Chlorophyll a/b ratio from experiment inoculated at 3.14OD: The proven fact of chlorophyll-a content and chl a/b ratio adjustment in response to light with maintained N level availability in media[5,13,14] was observed in the experiment with *Picochlorum* sp.

Total chlorophyll content analysis was done at second day of growth. Total chlorophyll content in the ICV grown culture was only 43.53% of the carboy grown culture (56.47 % reduction). It has been reported earlier that when light is limiting, microalgae has natural tendency to decrease pigmentation to maximize productivity when irradiated with high light[5,13] as lower amount of pigment content would reduce extra energy consumption and improve performance[5]. In comparison *chlb* content reduction (ie. 66.85%) was more than *chla* (i.e. 52.79), resulted in increased *chla/b* ratio in ICV (i.e. 4.0) than in carboy (i.e. 2.8), which is supported with earlier references[14]. These results indicates that there is more light available in the ICV as compared to carboy which resulted in reduced pigmentation (i.e antenna size) and increased a/b ratio[13,14].

Table-1: Growth rate and doubling time calculated from OD values at 750nm in ICV and carboy inoculated with Picochlorum sp at similar conditions.

Experiment Starting at 3.14 initial OD					Experiment Starting at 0.49 initial OD				
	Growth Rate		Doubling Time			Growth Rate		Doubling Time	
Day	ICV	Carboy	ICV	Carboy	Day	ICV	Carboy	ICV	Carboy
1	0.97	0.04	0.72	17.99	1	1.42	0.38	0.49	1.85
2	0.59	0.02	1.17	29.85	2	0.82	0.25	0.85	2.72
3	0.43	0.07	1.61	10.34	3	1.03	0.27	0.68	2.58
4	0.27	0.02	2.61	28.37	4	0.42	0.13	1.66	5.15
5	0.16	0.02	4.39	34.94	5	0.28	0.11	2.52	6.43
Average	0.48	0.03	1.43	20.02	Average	0.79	0.23	0.87	3.04

Table-2: Total chlorophyll content analysis normalized to 1OD (750nm). All the numbers are mean of experiments performed in triplicates.

Chlorophyll	Chlorophy content mg/L			Comparative percentage of chlorophyll			Ratio
	a	b	a + b	a	b	a + b	a / b
Carboy	7.37	2.61	9.98	100	100	100	2.8
ICV	3.48	0.87	4.35	47.21	33.15	43.53	4.0

Conclusion

Growth rate of *Picochlorum* sp. in ICV was found multiple times higher than the growth in conventional carboys in two different starting OD. This could be the result of uniform light availability to algal culture in ICV than in carboy as the CO_2 supply and nutrient levels were maintained same in both ICV and carboy. The theory of light as the concluding factor for faster growth in ICV was proven with chlorophyll content analysis as well. Less chlorophyll content and more chlorophyll a/b ratio in ICV grown culture was observed as compared to carboy grown culture. The results are in line with the proven fact, when irradiated with high light, microalgae tend to reduce pigmentation and increase Chl a/b ratio to improve performance[5] with maintained Nitrogen level availability.

The In-House Culture Vessel is significantly cost effective alternative to a photobioreactor which could be developed In-house in any research or academic institute and can accommodate in simple racks. This In-house Culture Vessel model could be used as a closed system for high density microalgae cultivation at laboratory scale. This In-house culture vessel can be used at lab level for many experiments such as screening fast growing algae, to study effect of light quality and quantity on growth of various algal strains and for fast inoculum development.

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