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Research Article

Characterization and Growth Evaluation of Marine *Chlorella* sp. for Biomass Production

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Graphical Abstract

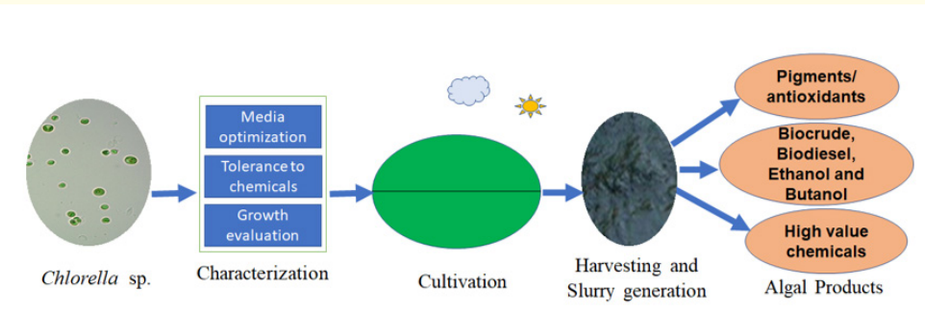

Figure a

Abstract

The fresh water and nutrients are costly inputs in algal cultivation. Nowadays, marine algae are in focus since they can be cultivated in seawater with minimum freshwater addition. In this study, marine *Chlorella* sp. was characterized in lab and open ponds ($1m^2$) of greenhouse to evaluate the potential of biomass production. In lab studies, the growth performances (OD and biomass production) of algae in industrial grade nutrient sources were found similar to lab grade sources. The growth at 12:12 h. of light: dark cycle was at par with 24 h. of continuous light illumination. The minimum inhibitory concentrations for hydrogen peroxide, benzalkonium chloride, and sodium hypochlorite were determined to be 2.5, 5, and 5 mgL^{-1} respectively. In pond studies, the strain was found to tolerate salinity up to 5.5%. Highest aerial, volumetric productivities and nutrient removal were observed at 10 cm as compared to 15 and 20 cm depths. The aerial productivities and biomass composition in semiturbidostat mode cultivation at 0.5 and 0.7 OD's of harvest were found comparable and semiturbidostat mode was found more productive than batch mode. Overall study showed that the marine *Chlorella* sp. is a robust strain and can be cultivated in open ponds using seawater.

Keywords: Characterization; *Chlorella*; Growth Optimization; Open Ponds; Semiturbidostat

Abbreviations

AFDW: Ash Free Dry Weight; BAC: Benzalkonium Chloride; H: Hydrogen; H_2O_2: Hydrogen Peroxide; MIC: Minimum Inhibitory Concentration; NaOCl: Sodium Hypochlorite; N: Nitrogen; OD: Optical Density; TN: Total Nitrogen; TP: Total Phosphorus.

Introduction

Microalgae have the potential of serving as one of the major sources of renewable energy in the world [1,2]. Algae can be grown on any marginal land without competing with agriculture lands, have high growth rate [3], and are very attractive because of high

lipid content [4]. *Chlorella* strains are known for their robustness and can grow as photoautotrophs, heterotrophs, or mixotrophs [5]. Amongst the widely studied algal strains, *Chlorella* is already mass produced at industrial scale and tested for biofuel production. Because of its robustness and lipid (14-30%) content [1,6], it's one of the most cultivated microalgae for commercial biodiesel production worldwide [5,7].

The economic feasibility of biofuel production from microalgae primarily depends on higher biomass productivity, lipid yield, and low-cost downstream processes [8]. Media cost is one of the major contributive factors to the economics of biofuel production [2]. There are several media's available for cultivation of *Chlorella*, and most of these consists of lab grade chemicals. The lab grade chemicals are very expensive hence, relatively cheaper media would make algae business more economically feasible. The urea and phosphoric acid were used as N and P sources in large scale algal cultivation [9-11]. The industrial grades of these sources need to be evaluated. The quality of water is another expensive input in algal cultivation. Due to scarcity of fresh water, the focus has been shifted to seawater. The prime requirement for seawater based algal cultivation is higher tolerance of algae to fluctuating salinity levels. The marine algae have the capability to grow in wide range of salinity and could be a viable option. The raceway ponds are considered as cheaper than photobioreactors [12,13]. The light penetration in raceway pond is affected by culture depth and cell density [14,15]. Generally, the water depth used for mass cultivation varies from 10-50 cm [16] and it should be optimized to minimize the expense on nutrient addition and to achieve higher biomass productivity [17].

The control of biotic stresses with chemical agents depends on tolerance level of algae and non-target organism to any given chemical [18]. For better control, the tolerance level of algae to particular chemical should be always higher than the organisms [19]. The chemicals such as benzalkonium chloride, sodium hypochlorite, and copper sulfate were evaluated for control of biotic stresses in algal cultivation without affecting algal productivities [18,20].

In this study, attempts were made to evaluate the impact of industrial grade urea and phosphoric acid, different photoperiods on growth of *Chlorella* sp. and characterized algae for its tolerance to various crop protection chemicals such as benzalkonium chloride (BAC), sodium hypochlorite (NaOCl), and hydrogen peroxide (H_2O_2) under controlled lab conditions. The strain was also evaluated in 1m^2 ponds under greenhouse conditions for its salinity tolerance, verified the biomass production, and nutrient consumption at different depths. The biomass productivities in batch and semi-continuous mode cultivation at optimum depths were compared, while offering information to reduce the cost with respect to cultivation of microalgal strain.

Material and Methods

Strain maintenance and culturing methods

Marine *Chlorella* sp. used in this study was obtained from germplasm collection of Reliance Industries Ltd. *Chlorella* sp. was maintained on ASN III agar slants. The monoalgal inoculum was developed in ASN III liquid medium under standard conditions in Kuhner shaker such as 26 ± 2 ºC, pH 7.0-7.5, rpm 120, 3.5% salinity, 2% CO_2 and 250 µmol m^{-2} s^{-1} of light intensity at 12:12 light: dark cycle.

Media preparation

Industrial and lab grade media composition

To prepare the industrial media, industrial grade urea (80%) and phosphoric acid (75%) were sourced locally. For lab media preparation, lab grade urea (99.9% pure) and phosphoric acid (85% pure) were procured from Sigma-Aldrich. Using these sources, 100 mgL^{-1} nitrogen and 6.25 mgL^{-1} phosphorous concentrations were ensured in both the media and maintained 16:1 N/P ratio.

F/2 trace metals composition

Trace metals are required in very smaller quantity; hence, lab grade chemicals were used in preparing the stock (Table 1). From sub stock solution, 1 mL F/2 solution was added per liter of final media.

Compound	Main stock Solution	Quantity of Main stock added to prepare 1 L Sub stock
$FeCl_3 \cdot 6H_2O$	-	3.15 gm
$Na_2EDTA \cdot 2H_2O$	-	4.36 gm
$CuSO_4 \cdot 5H_2O$	9.8 gm/L	1 mL
$Na_2MoO_4 \cdot 2H_2O$	6.3 gm/L	1 mL
$ZnSO_4 \cdot 7H_2O$	22.0 gm/L	1 mL
$CoCl_2 \cdot 6H_2O$	10.0 gm/L	1 mL
$MnCl_2 \cdot 4H_2O$	180.0 gm/L	1 mL

Table 1: F/2 trace metal composition.

Evaluation of industrial and lab grade N and P sources for cultivation of *Chlorella* sp.

The growth of *Chlorella* sp. was studied in industrial media and results were compared with lab media. The mid log phase inoculum grown in lab media was used for inoculation. The inoculum was centrifuged, cell pellet was washed and re-suspended in distilled water to remove the residual media effect. The industrial and lab grade media (250 mL media/500 mL flask; n=3) were inoculated at an initial cell density of 0.5 optical density (OD). All the flasks were incubated for eight days in Kuhner shaker under standard conditions. The samples were withdrawn on daily basis for analysis of OD. The culture morphology was observed for any variations under microscope.

Evaluation of different photoperiods on growth of *Chlorella* sp.

Effect of photoperiod on growth of *Chlorella* sp. was evaluated by exposing inoculated flasks (250 mL industrial media/500 mL flask; n=3) to 250 µmol m^{-2} s^{-1} light intensity at 12:12 hour light: dark cycle and 24-hours for 8 days. Samples were withdrawn on daily basis for analysis of OD.

Determination of minimum inhibitory concentration (MIC) of various chemicals on *Chlorella* sp.

The minimum inhibitory concentration of chemicals, which inhibits the growth of *Chlorella* sp. by ≥50% (MIC50) was studied. The experiment was conducted in three biological replicates (250 mL industrial media in 500 mL conical flask). The effect of chemical on culture growth was studied by adding each chemical separately into culture medium at cell density of 0.7 OD. BAC and NaOCl were tested at 1, 5, 10, and 15 mgL^{-1}, whereas the H_2O_2 was tested at 1, 2.5, 5, 10 and 15 mgL^{-1}concentrations. All the inoculated flasks were incubated under standard conditions and MIC50 value was calculated using measured OD.

Greenhouse studies

All the greenhouse studies were conducted in $1m^2$ ponds (100 cm length X 100 cm width) in industrial media in three biological replicates. The culture in the pond were mixed by paddle wheel at 0.21 m s^{-1}. In case of batch mode evaluation, nutrients were added initially only once, while in case of semiturbidostat mode cultivation the lost nutrients through harvested culture was replaced with fresh sea water media and maintained 16:1 N/P ratio. The pH of the culture media was adjusted to 7.0-7.5 by sparging CO_2 at every one-hour interval. The evaporation losses from the ponds were corrected by adding fresh water at the end of the day before taking samples for analysis and salinity was maintained as per the experimental design.

Characterization of *Chlorella* sp. for salinity tolerance in greenhouse open ponds

The salinity tolerance of the strain was tested by growing the *Chlorella* sp. in sea water media adjusted to various salinity levels (3.5-4.5%, 4.5-5.5%, 5.5-6.5%, and 6.5-7.5%). The experiment was started at an initial OD of 0.5 and conducted for 10 days in three biological replicates. The natural seawater comes with 3.5-4.0% salinity and to achieve the desired salinity NaCl was added. The effect of salinity on growth of algae was measured in terms of OD and ash free dry weight (AFDW). The photosynthetic response of algae to increased salinity stress was measured in terms of Fv/Fm.

Effect of culture depth on growth and biomass production of *Chlorella* sp.

The effect of operating depth on growth and nutrient removal by algae was studied by cultivating culture at 10, 15, and 20 cm (100 L, 150 L, and 200 L culture volume respectively) in $1m^2$ pond. The experiment was started at an initial OD of 0.5 and carried out for 10 days in three biological replicates. Daily samples were withdrawn for analysis of OD, AFDW, and nutrient consumption.

Semiturbidostat mode cultivation of *Chlorella* sp.

The growth and biomass production of *Chlorella* sp. was studied at 0.5 and 0.7 set ODs of harvest in semiturbidostat mode. The experiment was conducted in $1m^2$ ponds at 10 cm depth (100 L, culture volume) in three biological replicates. The semiturbidostat mode was operated for 20 days and culture was harvested at the end of every day. The AFDW concentration was measured from daily harvested culture and the aerial and volumetric productivities were calculated using measured AFDW.

Aerial productivity ($g \cdot m^{-2} \cdot d^{-1}$) = (A x B)/C

Where:

A is harvested culture volume (L), B is AFDW (g/L), C is total pond area (m^2)/Day.

Volumetric Productivity ($mg \cdot L^{-1} \cdot d^{-1}$) = (E x F)/G

Where:

E is aerial productivity ($g \cdot m^{-2} \cdot d^{-1}$), F is pond Area ($m^2$), and G is total volume of culture in the pond (L).

Harvest (%) = (A-B)/A x 100

A is final OD and B is set OD of harvest.

Analytical measurements

Optical Density measurement

The growth of algae was measured in terms of OD. OD measurement was done at 750 nm wavelength using Shimadzu make UV-visible spectrophotometer (Model-UV-1800). In the measurements, media was used as a blank.

Growth rate and doubling time measurement

The growth rate and doubling time of algae were calculated using measured OD [21].

Growth rate (µ) = ln $(X_2-X_1)/(T_2-T_1)$

Where

X_2: Final OD

X_1: Initial OD

T_2: Final Time (Days)

T_1: Initial Time (Days)

Doubling Time (Td) = 0.6931/ growth rate (µ)

Ash free dry weight measurement (AFDW)

The dry weight and ash free dry weight of algal samples were measured by gravimetric method [22]. To measure the ash free dry weight, 10 mL of algal culture was filtered using glass fiber filters (Whatman 45 mm, GF/C 1.2 µm filters) and washed with 4% ammonium bicarbonate (1:2). The filter paper containing washed biomass was dried to a constant weight at 105 °C in an oven for 2 hour. The dry weight (DW) was calculated as below.

$$\text{Dry weight } (gL^{-1}) = \frac{(A-B) \times 1000}{\text{Sample Volume (mL)}}$$

Where:

A: Weight of filter paper + dried biomass (g)

B: Weight of filter paper (g)

To determine the ash free dry weight, dried biomass was burnt in a muffle furnace at 550 °C for 1 hour and cooled to a room temperature in a desiccator and reweighed.

$$\text{AFDW } (gL^{-1}) = \frac{(C-D) \times 1000}{\text{Sample volume (mL)}}$$

Where:

C: Weight of filter paper (g) + Dry weight of algae before ashing (g)

D: Weight of filter paper (g) + Ash weight (g).

Total nitrogen (TN) and total phosphorus (TP) estimation

Approximately 10 mL of algal culture was centrifuged at 10000 rpm for 10 min and supernatant was used for estimation of TN and TP. The TOC and TN analyzer was used for estimation of TN [23]. The total phosphorous was estimated according to APHA method [24].

Carbon (C), hydrogen (H), and nitrogen (N) elemental analysis

The C, H, and N content from biomass of *Chlorella* sp. was estimated using CHNS analyzer (Elementar vario MACRO cube). For estimating C, H, and N content, one-liter of culture was centrifuged at 10000 rpm for 10 min. The supernatant was discarded, and cell pellet was washed with water to remove the residual nitrogen and the adsorbed salts. The pellet was dried at 105 °C overnight and used for estimation of elements [25].

Fv/Fm measurement

Mini PAMII (Walz Germany) was used to measure the Fv/Fm values. The dense algal culture OD was normalized to 0.5 and dark adapted for 15 min before estimation.

Microscopic observation

The cell morphology was observed using a Nikon ECLIPSE Ci-E microscope with DS-Ri2 camera and images were captured under 40 X magnification.

Results

Evaluation of industrial and lab grade nutrient (N and P) sources for cultivation of *Chlorella* sp.

The *Chlorella* sp. grown in industrial and lab grade media have shown similar growth rate, doubling time (0.27 and 2 days, respectively) and OD (Figure 1A). Morphologically the cells grown in lab (Figure 1C) and industrial (Figure 1D) grade media sources were found similar in shape and size. This indicate that, the *Chlorella* sp. can be grown in industrial grade nutrient sources without compromising on growth parameters (growth rate, morphology, and division pattern).

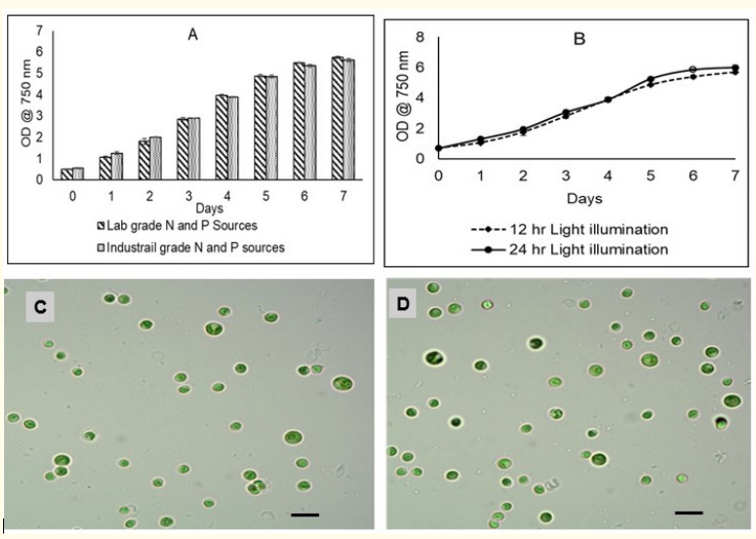

Figure 1: Growth of culture in lab grade and industrial grade media (A), different photoperiods (B), microscopic images of *Chlorella* sp. grown in lab (C), and industrial grade (D) N and P sources. Scale bar: C-D, 4µm.

Evaluation of the effect of photoperiods on growth of *Chlorella* sp.

The growth of *Chlorella* sp. was found similar at both the illuminations and comparable results were observed between 24 hours of continuous illumination and 12: 12 hours of light dark cycle (Figure 1B).

Determination of MIC of various chemicals on the *Chlorella* sp.

The MIC50 values for BAC (Figure 2A), NaOCl (Figure 2B), and H_2O_2 (Figure 2C) were determined as 5 mgL^{-1}, 5 mgL^{-1}, and 2.5 mgL^{-1} respectively.

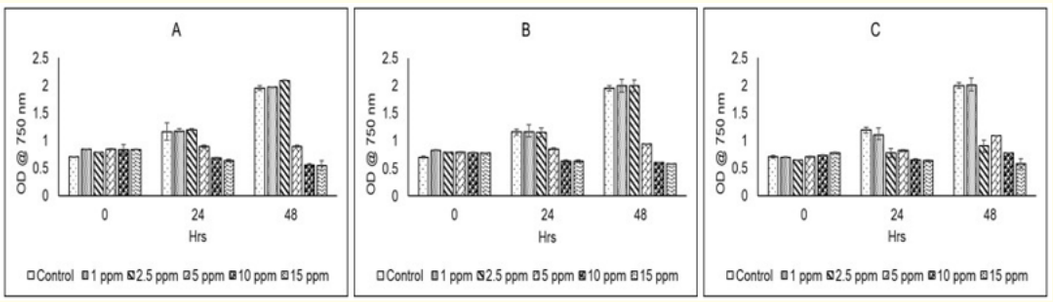

Figure 2: MIC50 of benzalkonium chloride (A), sodium hypochlorite (B), and hydrogen peroxide (C) on *Chlorella* sp.

Characterization of *Chlorella* sp. for higher salinity tolerance

The highest OD (2.80 ± 0.148), OD jump per day (0.22), AFDW (0.679 ± 0.024 gL^{-1}), aerial (5.3 ± 0.263 $g{\cdot}m^{-2}{\cdot}d^{-1}$), and volumetric (53 ± 2.63 $mg{\cdot}L^{-1}{\cdot}d^{-1}$) productivities and least doubling time (4.51 days) were observed at 3.5-4.5% salinity (Figure 3). The increasing salinity levels > 5.5%, were found to affect the OD (Figure 3A) and AFDW (Figure 3B and Table 2). The culture grown in 3.5-4.5% salinity recorded the higher Fv/Fm (0.57 ± 0.05) ratio followed by 4.5-5.5% salinity (0.538 ± 0.007) and it was decreased drastically with increasing salinity levels (Figure 3C).

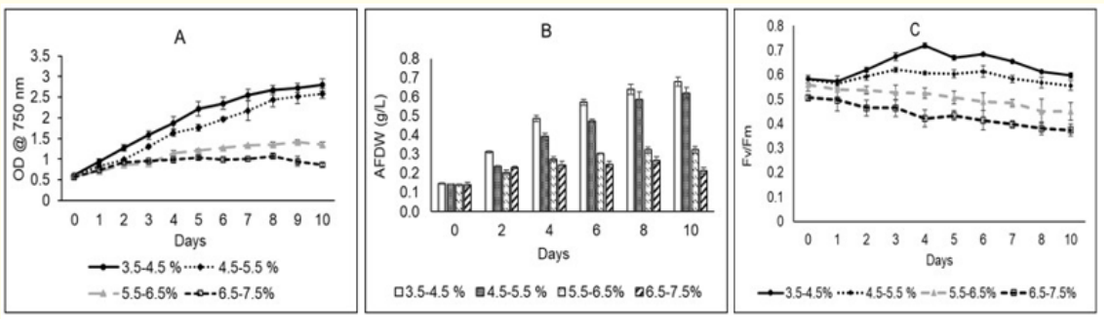

Figure 3: OD (A), AFDW (B), and Fv/Fm (C) profile of *Chlorella* sp. cultivated at various salinity levels.

Salinity levels	Volumetric productivity ($mg{\cdot}L^{-1}{\cdot}d^{-1}$)	Aerial productivity ($g{\cdot}m^{-2}{\cdot}d^{-1}$)	OD jump per day	Growth rate (μ)	Doubling time (Days)
Control (3.5-4.5 %)	53 ± 2.63	5.3 ± 0.263	0.22	0.153	4.51
4.5-5.5 %	47 ± 2.63	4.7 ± 0.263	0.198	0.146	4.74
5.5-6.5 %	18 ± 1.2	1.8 ± 0.12	0.076	0.082	8.45
6.5-7.5 %	7.5 ± 0.07	0.75 ± 0.08	0.029	0.042	16.50

Table 2: Growth kinetic details of *Chlorella* sp. at various salinity levels.

Effect of culture depth on growth and biomass production of *Chlorella* sp.

The highest OD (2.9 ± 0.05), AFDW (0.696 ± 0.02 g L^{-1}) (Figure 4A and 4B), aerial (5.5 ± 0.15 g·m^{-2}·d^{-1}), and volumetric (54.98 ± 0.1 mg·L^{-1}·d^{-1}) productivities were reported for 10 cm depth as compared to other depths. The nutrient consumption by algae was found directly proportional to the growth of algae. The highest nitrogen (42%) and phosphorous (72.7%) consumption were observed at 10 cm depth as compared to other depths (Figure 4C and 4D). However, per OD consumption of N (20.62 ± 2.31 g) and P (2.0 ± 0.078 g) were found similar at various depths (Table 3). Overall, the OD, AFDW, biomass productivities, and nutrient consumption were found decreased with increasing depths (>10 cm) (Figure 4; Table 3).

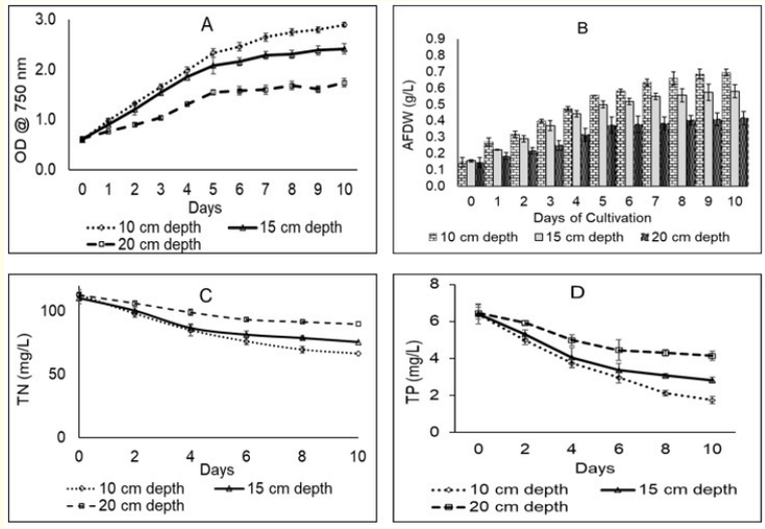

Figure 4: OD (A), AFDW (B), residual nitrogen (C) and residual phosphate (D) profile at various depths in batch mode.

Culture depth (cm)	Aerial productivity (g·m^{-2}·d^{-1})	Volumetric productivity (mg·L^{-1}·d^{-1})	OD jump Per day	Growth rate (μ)	Doubling time (Days)	Total nitrogen consumption on per OD basis (g)	Total phosphorous consumption per OD basis (g)
10	5.5 ± 0.15	54.98 ± 0.1	0.23	0.157	4.41	20.62 ± 2.31	2.0 ± 0.078
15	4.3 ± 0.17	42.58 ± 0.13	0.18	0.139	4.98	19.72 ± 1.02	1.98 ± 0.1
20	2.7 ± 0.16	27.2 ± 0.15	0.11	0.104	6.66	21.2 ± 3.19	2.08 ±0.08

Table 3: Summary of *Chlorella* sp. cultivated at different depths.

Semi-continuous mode cultivation of *Chlorella* sp.

During the semiturbidostat mode cultivation, ~600-800 μmol m^{-2} s^{-1} light intensity was recorded (Figure 5A). At 0.5 and 0.7 set ODs of harvest significant difference were not reported for OD jump per day (Table 4), aerial (Figure 5A), and volumetric productivities (Table 4). However, the highest growth rate (0.442 ± 0.03) (Figure 5B) and higher% harvest (35.96% ± 0.07) of culture were observed at 0.5 set OD as compared to 0.7 set OD of harvest (Table 4).

Set OD for harvest	Culture depth (cm)	Aerial productivity (g·m^{-2}·d^{-1})	Volumetric productivity (mg·L^{-1}·d^{-1})	OD jump per day	% Harvest	AFDW (g/L)	AFDW/OD
0.5	10	8.25 ± 0.7	84.6 ± 1.24	0.292 ± 0.028	35.96 ± 0.07	0.230 ± 0.02	0.290
0.7	10	8.23 ± 0.6	82.5 ± 0.729	0.278 ± 0.002	28.6 ± 0.60	0.288 ± 0.04	0.297

Table 4: Kinetics of semi-continuous mode cultivation of *Chlorella* sp. at 0.5 and 0.7 set OD of harvest in 1m^2 pond at 10 cm culture depth.

The biomass generated from 0.5 and 0.7 set ODs of harvest did not differ significantly in elemental composition. The biomass harvested from 0.5 (Figure 5C) and 0.7 (Figure 5D) set OD of harvest reported ~ 51% C, ~8.3-8.63% N, and 6.7-6.8% H. The microscopic observations revealed that the culture harvested at 0.5 (Figure 6A) and 0.7 (Figure 6B) set ODs of harvests did not show any marked differences in cell morphology and shape.

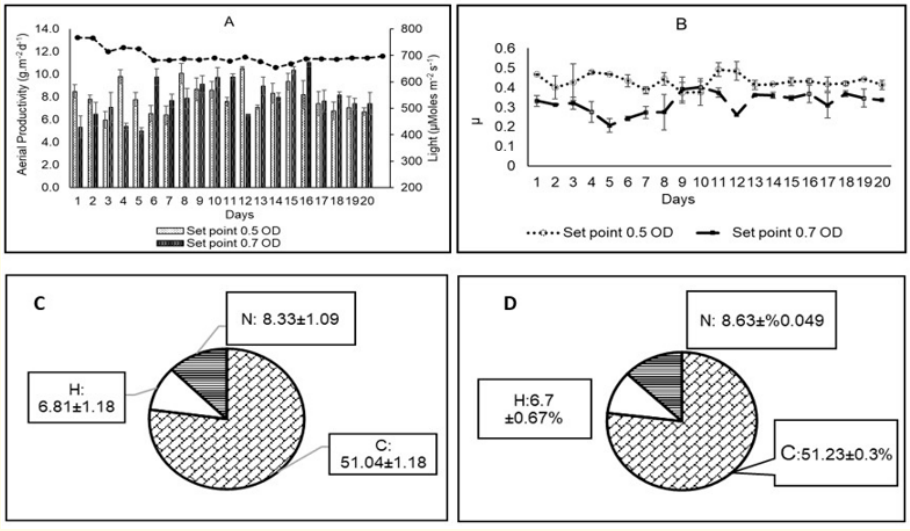

Figure 5: Aerial productivity (A), growth rate (B), elemental composition of *Chlorella* sp. cultivated in semiturbidostat mode at 0.5 (C) and 0.7 (D) set OD of harvest in 10 cm depth under greenhouse conditions.

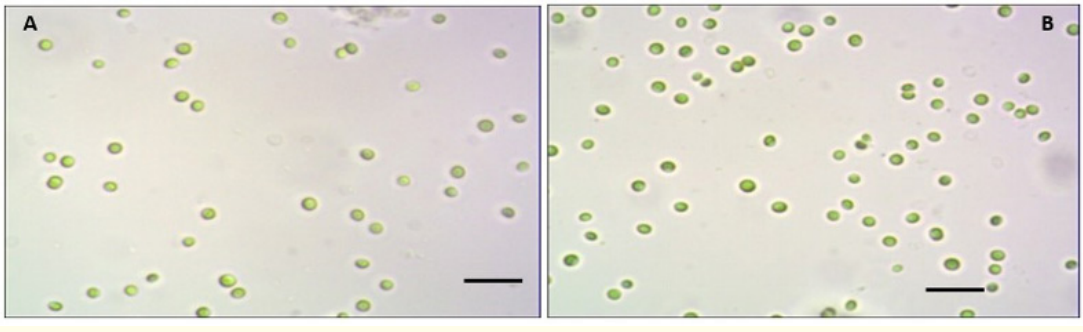

Figure 6: Microscopic (40 X magnification) images of *Chlorella* sp. cultivated in semiturbidostat mode at 0.5 (A) and 0.7 (B) set OD of harvest. Scale bars: A-B, 4 µm.

Discussion

The study with industrial grade N and P sources suggests that, the industrial grade nutrient sources have not affected culture growth, growth rate, morphology, and cell division pattern of algae. Therefore, these sources could be used to replace costly lab grade nutrients in algal cultivation. Tania., *et al*. [26] observed the similar results when they grown *Chlorella* sp. in media prepared using industrial grade urea, single super phosphate, and murate of potash as N, P, and K sources respectively. In their study, the cell number/mL and growth performance of the algae grown in industrial media were found comparable to standard lab grade media.

In our light illumination study, neither growth promotion nor growth inhibition was observed at 24 hours of continuous light illumination and results were at par with the 12:12 hours light: dark

photoperiod. These results indicate that the culture was tolerant for prolonged light exposure and further growth advantages were not observed at 24 hours of light illumination. Therefore, 12:12 hours light: dark cycle with 250 µmol m^{-2} s^{-1} is enough to attain the highest growth. Similarly, Iriarte., *et al*. [27] studied the effect of 24 hours continuous light illumination and 12:12 hours of light: dark cycles on *Pycnococcus provasolii*. An increased chlorophyll- b/a ratio and reduced photorespiration rate were observed under 12:12 light: dark cycle as compared to 24 hours continuous light illumination. They have concluded that 24 hours of light illumination was not harmful to algae, however light energy was used less efficiently as compared to 12:12 hours of light: dark cycle.

In the MIC study with various chemicals, H_2O_2 was found more toxic to algae at a lower concentration (2.5 mgL^{-1}) than BAC (5 mgL^{-1}) and NaOCl (5 mgL^{-1}). However, all these chemicals could be used to control biotic stresses (below the MIC concentrations) without affecting the algae. Park., *et al*. [20] controlled *Brachionus calyciflorus* invasion in *Chlorella kessleri* cultivation without affecting algal health using NaOCl (>0.4 mg Cl L^{-1}) and observed 100% control within 24-hours of treatment. Karuppasamy., *et al*. [18] tested the effect of BAC on *Chlorella vulgaris* and biotic stress caused by *Euplotes* sp. and *Oxyrrhis* sp. In this lab studies, 10 mgL^{-1}, 1 mgL^{-1} and 2 mgL^{-1} of BAC was determined as MIC50 values for *C. vulgaris*, *Oxyrrhis* sp., and *Euplotes* sp. respectively. Complete control of all these non-target organisms (*Oxyrrhis* sp. and *Euplotes* sp.) was observed at 2.5 mgL^{-1} of BAC in 24 hours of treatment. From the results it was found that *C. vulgaris* was more tolerant to BAC than biotic stress causing agents. Our study suggests that *Chlorella* sp. has wide tolerance to various chemicals. Any of these tested chemicals could be used to control biotic stresses and BAC would be an ideal chemical owing to its cost and availability.

In our study, 3.5-5.5% salinity was observed as optimum for cultivation of *Chlorella* sp. and above which the growth parameters were affected. The tolerance of strain to 3.5-5.5% salinity could be due to the production of osmoprotectants (spermine and spermidine) [28,29]. Similarly, Pandit., *et al*. [30] reported the decreased growth rate of *C. vulgaris* from 0.127 day to 0.093 day with increase in salinity from 0.06 M to 0.4 M. In another study Monika., *et al*. [31] reported, the effect of salinity on biomass production of *Chlorella* sp. In their study, the maximum biomass production (1.021 ± 0.070 gL^{-1}) and the lowest biomass production (0.016 ± 0.021 gL^{-1}) were observed at 0.2 M and 1.1 M NaCl respectively. In our study at 3.5-4.5% salinity, the Fv/Fm values were near to physiological maxima (0.7). The decreased Fv/Fm with increased salinity could be due to the osmotic stress and deleterious effects of reactive oxygen species on photosynthetic machinery [32]. Similarly, Matthias., *et al*. [33] studied the effect of salt stress on photosynthetic efficiency (Fv/Fm) of *Micrasterias* cells exposed to 200 mM KCl, 200 mM NaCl or 339 mM sorbitol for 0.5, 1, 3, 6, 12, and 24 hours. In their study until 6 hours of treatment, the control and treated cells have shown Fv/Fm values near to 0.67-0.77. After 24 hours of exposure, Fv/Fm values were decreased in all the treatments, the lowest was observed in KCl (Fv/Fm: 0.39) followed by NaCl (Fv/Fm: 0.51), and sorbitol (Fv/Fm: 0.66) treated cells. The poor growth, biomass production and lower Fv/Fm values observed at higher salinity could be due to the osmotic stress and lower energy availability for algal growth. According to Kirst [34], the energy required for osmotic regulation (e.g., osmolyte synthesis, ion transport, and morphological changes) exceeds the energy needed for cell division and thereby it affects the growth of algae. Water evaporation is a common phenomenon in open ponds and is associated with the salinity increase. Our study suggests that, salinity is an important parameter to be considered while cultivating algae in open ponds to achieve higher productivity. The optimum salinity required for the strain must be maintained by adding fresh water. The higher salinity tolerant strain is an added advantage because it can tolerate the salinity fluctuations and reduces quantity of fresh water required for salinity correction.

In our study, the increased culture depth was found to affect the algal growth and nutrient consumption. The decreased growth of culture with increased depth of pond (>10 cm) could be attributed to the low light availability for algal growth. Many studies have revealed that, the depth of ponds must be as shallow as possible to achieve the maximum light penetration [35,36]. The biomass productivity depends on photosynthesis whereas, photosynthesis depends on the light availability in growth medium. Therefore, the effective light regulation is a key parameter for economical algal biomass production [37]. Light availability in pond is not only affected by depth but also influenced by cell density [38]. It's evident from our depth study, where the OD, biomass, and nutrient consumption were found higher in the initial period of batch and reduced as the cell density increases irrespective of depth. This increased cell density caused self-shading and had affected the light penetration. Kim., *et al*. [39], in their study with mixed algal cultures of *Chlorella* sp. *Scenedesmus* sp. and *Stigeoclonium* sp. observed the aerial productivities of 6.06 ± 0.32 $g{\cdot}m^{-2}{\cdot}d^{-1}$, 4.67 ± 0.26 $g{\cdot}m^{-2}{\cdot}d^{-1}$, and 4.18 ± 0.39 $g{\cdot}m^{-2}{\cdot}d^{-1}$ at 20, 30, and 40 cm depths respectively. The volumetric productivities of 30.28 ± 1.60 $mg{\cdot}L^{-1}{\cdot}d^{-1}$, 15.55 ± 0.86 $mg{\cdot}L^{-1}{\cdot}d^{-1}$, and 10.45 ± 0.98 $mg{\cdot}L^{-1}{\cdot}d^{-1}$ were reported for these corresponding depths. The nutrient consumption was also found higher at lower culture depth (20 cm). At 20, 30, and 40 cm cul-

ture depths 82.5%, 43.4%, and 18.6%, total nitrogen and ~89.7%, 36.0%, and 32.3%, total phosphate consumption was observed respectively. In their study, it was found that 20 cm depth as the most optimum, where the highest aerial and volumetric productivities were observed. The amount of nutrient addition depends on the culture depth. Nutrients are costly input materials in algal cultivation, unproductive ponds with higher depth ultimately increases the cost of cultivation. Hence, the depth should be considered as one of the most important parameters to be optimized for effective algal cultivation and to achieve higher biomass productivities [40].

The semiturbidostat mode cultivation at different set OD's of harvest (0.5 and 0.7) shown similar aerial and volumetric productivities. However, the operation of semiturbidostat at 0.5 set OD of harvest shown 22% higher growth rate and 7.36% higher harvest volume as compared to 0.7 set OD. These higher values at 0.5 set OD of harvest could be due to the maintenance of lower cell density (0.5 OD) during the cultivation. The lower cell density resulted in reduced self-shading and improved light penetration. However, the harvest obtained at 0.5 set OD was found to contain AFDW concentration (0.230 ± 0.02 gL^{-1}) lower than 0.7 set OD of harvest (0.288 ± 0.04 gL^{-1}). Since the cultivation at 0.5 set OD generates huge culture volume and lower biomass concentration, the processing of such huge volume to recover the biomass ultimately increase the harvesting cost. The downstream harvesting cost can be significantly reduced by operating the semi-continuous mode at higher set OD of harvest (0.7) which generates the low volume high dense culture. Hence, operating semi-continuous mode at 0.7 or higher set OD of harvest is more economical than operating at lower set OD (0.5) of harvest. The semiturbidostat mode cultivation (Table 4) at 10 cm depth shown, ~33% higher biomass productivity (aerial / volumetric) as compared to batch mode cultivation (Table 3). The reason for higher productivities in semiturbidostat mode was due to maintenance of culture in log phase throughout the cultivation period of 20 days, this was achieved by daily harvest and daily dilution, and it was evident from our batch study. Initially a very high growth rate (0.487) was observed in batch mode cultivation at 10 cm depth and as the cultivation time increases the growth rate was decreased and an average growth rate of 0.157 was observed at the end (Table 3). Whereas, in case of semiturbidostat mode (0.7 set OD of harvest) the growth rate was observed between 0.3-0.4 from start to end of cultivation period (Figure 5B). The biomass generated from 0.5 and 0.7 set OD of harvest did not differ in the percent composition of C, H, and N content. The chemical composition of *Chlorella* sp. reported in our study was found similar to various other reports and species of *Chlorella*. Phukan., *et al*. [41] characterized the biomass of indigenously isolated *Chlorella* sp. by CHNS analyzer and reported 47.54%, 7.1%, and 6.73% of C, H, and N respectively. Chen., *et al.* [42] reported 47.84%, 6.41%, and 9.01% of C, H, and N in *C. vulgaris*. Kumar., *et al*. [43] reported 49.74%, 8.25%, and 7.60% of C, H, and N in *Chlorella sorokiniana*. Overall study confirms that the set OD of harvest does not affect the elemental composition of algae. Microscopic observation of cells cultivated at 0.5 and 0.7 set OD of harvest were found healthy, green and did not differ in morphology. Application of 2.5 mgL^{-1} BAC was effective for combating the growth of any non-target organism, and was necessary to achieve the sustainable biomass by *Chlorella* sp.

Conclusion

The industrial grade urea and phosphoric acid were found to support the growth of *Chlorella* sp. as equal to the lab grade sources and can be used as low cost nutrients in large scale cultivation. The culture growth at 12:12 hours light: dark photo-period was comparable to 24 hours continuous light illumination and this showed the strain adaptability to varying light conditions. The wider biocide (BAC: 5 mgL^{-1}, NaOCl: 5 mgL^{-1}, and H_2O_2: 2.5 mgL^{-1}) and salinity (3.5-5.5%) tolerant properties, makes this strain as one of the best candidate for outdoor cultivation. Amongst all the tested depths, maximum growth and biomass productivities were observed at 10 cm depth under greenhouse conditions. However, the optimum depth for cultivating *Chlorella* sp. must be optimized with respect to light availability and cultivation systems. In semiturbidostat mode, the set ODs of harvest (0.5 and 0.7) did not affect the productivities (8.23-8.25 $g{\cdot}m^{-2}{\cdot}d^{-1}$) and biomass composition of *Chlorella* sp. From downstream point of view, operating semiturbidostat mode at higher cell density (0.7 set OD of harvest) would be more economical than lower (0.5 set OD of harvest) cell density. As compared to batch mode, the semiturbidostat mode with a robust biotic stress management practice was found more productive for long-term cultivation. Overall study showed that the, marine *Chlorella* sp. is a robust strain which can be used for sustainable biomass production in outdoor.

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Conflict of Interest

All authors have read and given their final approval and do not have any conflict of interest.

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