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RESEARCH ARTICLE - BIOLOGICAL SCIENCES

A Dual Role of Marine Microalga *Chlorella* sp. (PSDK01) in Aquaculture Effluent with Emphasis on Initial Population Density

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Abstract The marine microalga *Chlorella* sp. (PSDK01) was cultured in shrimp-cultured effluent under different initial population densities (IPDs) ranging from 0.25 to 12.0 g L^{-1} for dual purpose (excess nutrient consumption and biomass productivity). It was found that the IPD had affected the nutrient consumption and biomass significantly ($P < 0.001$). A higher biomass productivity compared to that of other IPDs was achieved in 0.5 g L^{-1} IPD, and the concentration had reached 0.78 g L^{-1} d^{-1}. In the same time, higher IPD derived higher biomass concentration (up to 5.5 g L^{-1}) in 6 days growth, but at the end of the experiment (9th day), the biomass was slightly decreased (2 %). In the current observation, while starting IPDs 0.25–0.5 g L^{-1}, with the increase in IPD, the biomass productivity also increased, when IPD exceed the 0.5 g L^{-1}, the biomass productivity reversely decreased. The maximum nutrients consumption was recorded in 0.5 g L^{-1} IPD at the end of the experiment (9th day) as 96, 69, and 67 % for phosphate, nitrate, and nitrite, respectively. However, the highest NH_3^- consumption (63 %) was observed in 0.25 g L^{-1} on 9th day. Maximum ammonia consumption in other IPDs was resulted at 6th day, after that ammonia concentration was slightly increased from the previous concentration due to the decay of microalgae. Based on these results, to obtain the maximum nutrient consumption and biomass productivity of *Chlorella* sp. (PSDK01) in diverse wastewater on large-scale level, it is necessary to select a suitable IPD at around 0.5 g L^{-1}.

Keywords *Chlorella* sp. · Initial population density · Wastewater bioremediation · Marine microalgae · Biomass productivity

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1 Introduction

Aquaculture industry is one of the major concerns of pollution along the coastal water. Various environmental rules have been implementing strictly in order to reduce water pollution. So, issues have been arised in terms of water quality and other biological organisms, the fight against pollution has become a major issue. Wastewater from aquaculture is one of the eutrophic sources that could cause some problems like eutrophication and contamination when excessively released due to its high contents of nitrogen and phosphorus [1–5]. It would be necessary and beneficial to treat the aquaculture wastewater before it is being released into larger water bodies. Microalga is one of the nature's gifts; the use of microalgae for the removal of nutrients is attractive because they are able to serve in multiple functions such as bioremediation as well as generating biomass for biofuel production with concomitant carbon sequestration [6–8]. In addition, wastewater remediation by microalgae is also an eco-friendly method since it does not discharge any secondary pollutant as long as the biomass produced is continuously reused and thus an efficient nutrient recycling is maintained [7,9–11]. Culturing algae in the wastewater using immobilized biosorption techniques is commonly used for bioremediation. On the other hand, commercially available artificial culture media (ACM) and live feed are widely utilized for cultivating algae; ACM

plays a major role in microalgal culture. There are a number of predictable media, such as Walne's or Conway, F/2 media, Miquel's, and Scheiber's, being used for the culture and maintenance of microalgae in research laboratories as well as in fish and shrimp hatcheries [12,13]. Usually, the artificial media contains varieties of inorganic recipes. The production of ACM is tedious and often too costly. Moreover, the microalgae can effectively remove the surfeit nutrients from the water and fix the carbon from the atmosphere by photosynthesis activity [14,15]. They consume nitrogen and phosphorous as well as the carbon sources from the wastewater. Therefore, culture of marine microalgae in the aquaculture wastewater may serve as a potential and sustainable approach toward the wastewater treatment [16,17] compared to other treatment methods [18]. Solely, limited studies were carried out on the marine microalgae culture using wastewater from aquaculture farm for a dual purpose like nutrient removal and biomass productivity. The microalgal species such as *Skeletonema costatum, Chaetoceros coarctatus* [19], *Chlorella* sp. [20], and *Chlorella marina* [11] were used for the above purpose. In this work, *Chlorella* sp. (PSDK01) was used to treat the shrimp-cultured water by culturing. The influence of microalga inoculation density on the growth rate, nutrients removal efficiency, and biomass productivity was investigated.

2 Materials and Methods

2.1 Stock Culture

The water sample was collected from the Palk Bay region of Muthukuda coast (Lat. 9°51′48″ N; Long. 79°7′15″ E), Tamil Nadu, southeast coast of India. Isolation and identification of microalgae was done by agar plating technique [21]. Indoor cultivation of microalgae was done under controlled conditions. *Chlorella* sp. (PSDK01) was inoculated in 2,000-mL Erlenmeyer flask containing 800 mL of sterilized seawater by adding Walne medium [22]. Chemical composition of the Walne medium is as follows: 1.3 g of $FeCl_3 \cdot 6H_20$, 0.36 g of $MnCl_2 \cdot 4H_20$, 33.6 g of H_3BO_3, 45.0 g of EDTA (Disodium salt), 20.0 g of $NaH_2PO_4.2H_2O$, 100 g of $NaNO_3$ 100.0 g and 1 mL of trace metal solution (2.1 g of $ZnCl_2$, 2.0 g of $CoCl_2\ 6H_2O$, 0.9 g of $(NH_4)6Mo_7O_{24}{\cdot}4H_2O$, 2.0 g of $CuSO_4 \cdot 5H_2O$ in 1 l of distilled water), 10.0 mg of vitamin B_{12} (cyanocobalamin), 10.0 mg of vitamin B_1 (thiamine. HCl) and 200.0 µg of vitamin H (biotin) in to the 100 mL of distilled water. Microalgae inoculum of 10 % was added to the culture flask provided with 12:12-h light (5,000 lux) and dark cycle. After 5–8 days, the maximum exponential phase was obtained. Temperature in the range of 23 and 25 °C was furnished with a salinity of 30 ppt maintained for the entire culturing period.

2.2 Microalgae Inoculation and Experimental Conditions

Indoor-cultured *Chlorella* sp. (PSDK01) was harvested after 6 days, and the microalgal cells in exponential growth phase were obtained by centrifuging at 5,000 × *g* for 10 min. Then, the pellets were resuspended in different volumes of sterilized seawater to prepare seven levels of cell density: 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, and 12 g L^{-1}. The resuspended cultures were transferred to a round-shaped 3 l plastic container filled with wastewater and provided with vigorous aeration using fish aquarium motor for avoiding settlement. Two fluorescence bulbs with 5,000 lux capacity were furnished to enable 12:12-h light and dark cycle. The wastewater sample (100 mL) was collected from the chamber for every 3 days for the analysis of nutrients.

2.3 Evaluation of Nutrients Removal and Biomass Productivity

During the sampling period, the nutrients (PO_4, NO_3, NO_2, and NH_4) were estimated according to Strickland and Parsons [23] and Jenkins and Medsken [24]. The algal biomass was estimated by the gravimetric method according to Richmond et al. [25] with minor modifications. A 10-mL aliquot of the culture was vacuum filtered onto a pre-weighed (IW) glass fiber (GF/C) filter paper (pore size 0.45 µm), and washed twice with 10 mL distilled water to remove adhering inorganic salts. After drying overnight in an oven at 105 °C, the weight of algal cells along with the filter paper was measured (FW). The biomass concentration was calculated as:

$$\text{Biomass}\ \left(\text{g L}^{-1}\right) = \frac{\text{FW} - \text{IW}}{10/1{,}000}$$

2.4 Stastical Analyses

The statistical relationship between primary microalgal cell density (0.25, 0.5, 1.0, 2.0, 4.0, 8.0, and 12.0 g L^{-1}) and experimental duration (initial, 3rd day, 6th day, and 9th day) was assessed using two-way analysis of variance (ANOVA) using by GraphPad Prism (version 5.0) software.

3 Results

3.1 Consumption of Inorganic Nutrients by Microalga

Phosphate consumption was significantly influenced by initial population cell density ($P < 0.01$) and the experimental duration ($P < 0.001$) (Table 1; Fig. 1). In the experiment, the maximum consumption (96.6 %) of PO_4 resulted in 0.5 g L^{-1} IPD ($P < 0.001$) on the final day of the experiment, and second IPD (62.6 %) was obtained in 1.0 g L^{-1}

Table 1 Nutrients concentration (PO_4^{3-}, NO_3^-, NO_2^-, and NH_3^-) under different IPDs in shrimp-cultured effluent

IPD (g L^{-1})	0.25	0.5	1.0	2.0	4.0	8.0	12.0
Phosphate (μ mol L^{-1})							
Initial	0.30^{a}	0.30^{b}	$0.30^{b,c}$	0.30^{a}	0.30^{a}	0.30^{a}	0.30^{a}
3rd day	0.24^{a}	$0.19^{b,c}$	$0.24^{a,c}$	0.27^{a}	0.27^{a}	0.28^{a}	0.28^{a}
6th day	0.21^{a}	$0.07^{a,c}$	$0.19^{a,c}$	0.22^{a}	0.23^{a}	0.24^{a}	0.25^{a}
9th day	0.20^{a}	0.01^{a}	0.11^{a}	0.17^{a}	0.21^{a}	0.23^{a}	0.27^{a}
Nitrate (μ mol L^{-1})							
Initial	$3.00^{b,c}$	3.00^{b}	$3.00^{b,c}$	$3.00^{b,c}$	3.00^{a}	3.00^{a}	3.00^{a}
3rd day	$1.90^{a,c}$	1.20^{a}	$2.87^{b,c}$	$2.82^{a,c}$	2.75^{a}	2.90^{a}	2.97^{a}
6th day	1.27^{a}	1.02^{a}	$1.97^{a,c}$	$1.99^{a,c}$	1.90^{a}	2.26^{a}	2.68^{a}
9th day	1.05^{a}	0.93^{a}	1.24^{a}	1.35^{a}	1.47^{a}	2.20^{a}	2.54^{a}
Nitrite (μ mol L^{-1})							
Initial	$3.80^{b,c}$	3.80^{b}	$3.80^{b,c}$	3.80^{a}	3.80^{a}	3.80^{a}	3.80^{a}
3rd day	$3.35^{a,c}$	$3.10^{b,c}$	$3.00^{b,c}$	2.80^{a}	2.79^{a}	2.90^{a}	3.00^{a}
6th day	$2.65^{a,c}$	$2.40^{a,c}$	$2.60^{a,c}$	2.65^{a}	2.54^{a}	2.79^{a}	2.91^{a}
9th day	2.12^{a}	1.25^{a}	1.48^{a}	2.49^{a}	2.50^{a}	2.70^{a}	2.85^{a}
Ammonia (μ mol L^{-1})							
Initial	11.9^{b}	$11.90^{b,c}$	$11.90^{b,c}$	$11.90^{b,c}$	11.9^{a}	11.90^{a}	11.90^{a}
3rd day	$9.30^{b,c}$	$10.20^{a,c}$	$10.50^{a,c}$	$10.90^{a,c}$	10.4^{a}	10.60^{a}	10.70^{a}
6th day	$7.50^{a,c}$	8.10^{a}	$8.60^{a,c}$	$8.92^{a,c}$	8.90^{a}	9.20^{a}	9.50^{a}
9th day	4.30^{a}	6.80^{a}	6.98^{a}	7.30^{a}	9.10^{a}	9.50^{a}	9.90^{a}

a, b, c Values within a column with different superscripts are significantly different ($P < 0.05$)

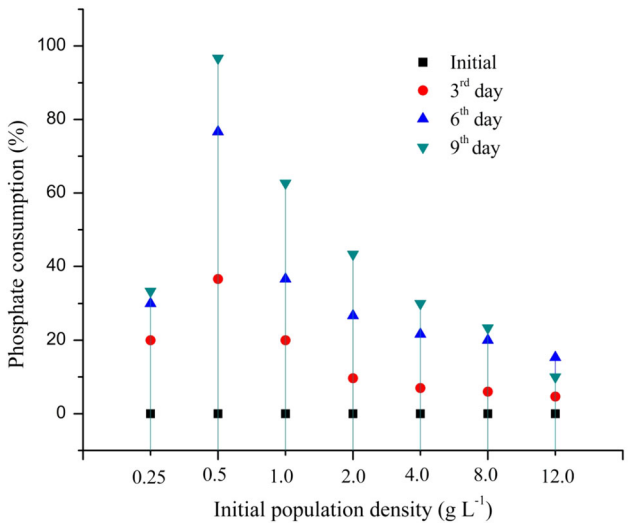

Fig. 1 Effect of IPD on phosphate consumption from the aquaculture effluent

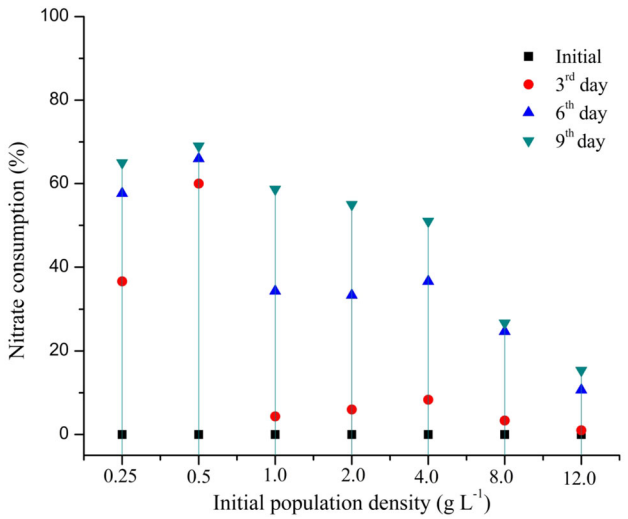

Fig. 2 Effect of IPD on nitrate consumption from the aquaculture effluent

($P < 0.01$) at the end of the experiment (9th day). The concentration of phosphate significantly reduced from 0.3 to 0.01 and 0.3 to 0.11 μmol L^{-1}, respectively, while using 0.5 and 1.0 g L^{-1} IPDs. The other IPD's setups (0.25, 2.0, 4.0, 8.0, and 12.0 g L^{-1}) did not show any significant variation in nutrient consumption during the study period.

Consumption of NO_3^- (69 %) was quiet lower than that of the PO_4 (96 %) during the experiment. The nitrate consumption was significantly varied (sampling periods—$P < 0.001$; IPD—$P < 0.01$) during the experiment. The maximum nitrate consumption (69 %) was found in 0.5 g L^{-1} IPD, and the concentration of nitrate was reduced from 3.0 to 0.93 μmol L^{-1} (Fig. 2). The IPDs 0.25, 1, and 2 showed significant variation on the consumption of nitrate ($P < 0.01$). The percentage of nitrate consumption at 0.25, 1, and 2 IPDs were 65, 58, and 55 % respectively. First, two sampling (3rd

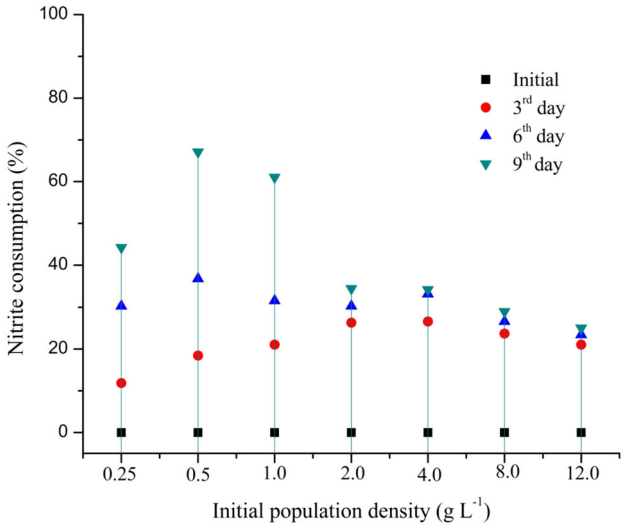

Fig. 3 Effect of IPD on nitrite consumption from the aquaculture effluent

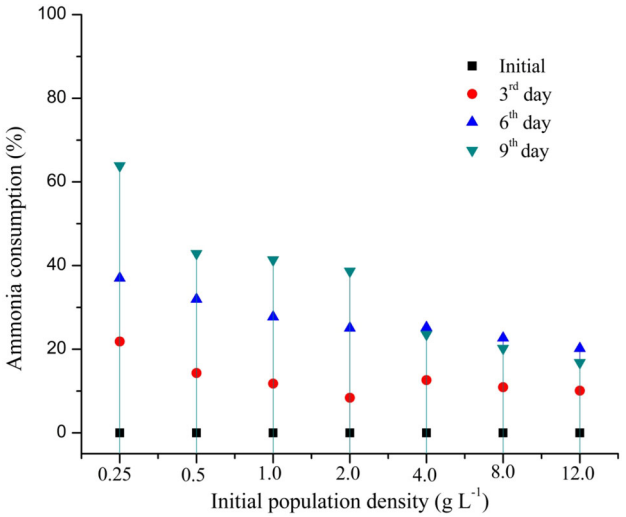

Fig. 4 Effect of IPD on ammonia consumption from the aquaculture effluent

and 6th) periods showed the 40 and 15 % of NO_3^- consumption, which was higher when compared to that in 9th day (8 %). The rate of nitrate consumption by microalgae quickly decreased after 6th day of the experiment.

The consumption of nitrite shows significant variation ($P < 0.001$) with respect to cultivation periods, but did not show any significant variation with the IPDs. Among the experiment with seven IPDs, the most efficient nitrite consumption (67.1 %) was recorded in 0.5 g L^{-1} IPD on 9th day ($P < 0.01$) (Fig. 3). The second (61 %) was obtained in 1.0 g L^{-1} IPD on 9th day ($P < 0.05$). The other IPDs did not show any significant nitrite consumption.

Ammonia consumption was significantly influenced by both IPD and cultivation periods. The maximum (64 %) ammonia consumption was recorded in 0.25 g L^{-1} IPD on 9th day ($P < 0.001$), where the concentration was reduced from 11.9 to 4.3 µmol L^{-1}. Compared to other organic nutrients consumption, ammonia showed reverse result in case of maximum consumption noticed at 0.25 g L^{-1} IPD. The other IPDs (0.5, 1.0, and 2.0 g L^{-1}) consumed 42 % ($P < 0.01$), 41 % ($P < 0.01$), and 38 % ($P < 0.05$), respectively (Fig. 4). At 4.0, 8.0, and 12.0 g L^{-1} IPDs, the ammonia slightly increased after 6th day of the experiment which was reported as 2, 3, and 3.3 % respectively.

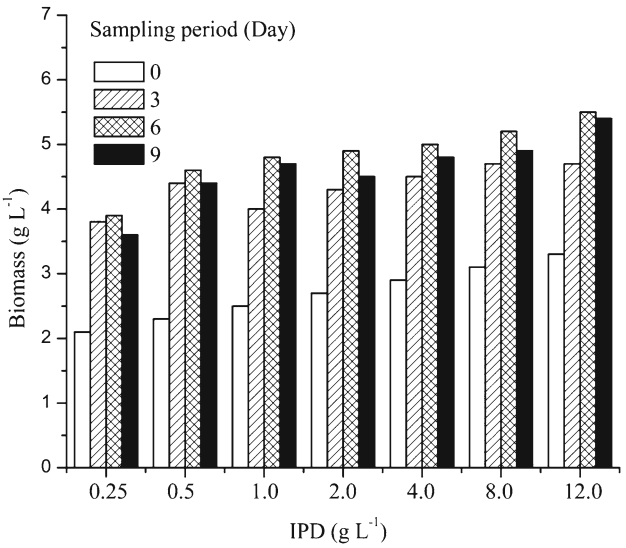

Fig. 5 Effect of IPD on algal biomass using aquaculture effluent

3.2 Effect of IPD on the Algal Growth

Figure 5 shows the algal growth curve under different IPDs. The biomass of *Chlorella* sp. (PSDK01) increased with increasing PDs till 6th day followed by a slight decrease. Higher IPDs could get higher biomass concentration, but the daily biomass productivity was decreased (Fig. 6). For example, when IPD was 0.5 g L^{-1}, the cell growth reached 4.6 g L^{-1} on 6th day, the highest biomass productivity was only about 0.78 g L^{-1} d^{-1} (Fig. 7). However, when IPD was 12.0 g L^{-1}, the cell growth reached 5.5 g L^{-1} on 6th day, but the daily productivity decreased to 0.35 g L^{-1} d^{-1}. Furthermore, when IPD was about 0.25 g L^{-1} or lower, there was a longer lag time of about 3 days. Nutrients concentrations were found to be decreased for all the seven IPD treatments during the experiment. The fastest consumption of nutrients was occurred at IPD of 0.5 g L^{-1}, and on day 9, at which more than 60 % nutrients (PO_4^{3+}—96 %; NO_3^-—69 % and NO_2^-—67 %) had been utilized by the *Chlorella* cells except ammonia (40 %).

4 Discussion

Commonly, shrimp pond effluent has been a very high load of solid particles in suspension, which contributes to increased turbidity [26,27]. This high concentration of solid particles in the marine environment limits the light absorption by the primary food producers affecting the photosynthetic capacity of phytoplankton and their growth. To reduce the pressure of solid material in suspension on microalgae growth, the shrimp effluent used in the experiment was subjected to two days of sedimentation, which reduced turbidity by around 90 % since natural sedimentation effectively reduced the turbidity from the effluent. This result was confirmed by the previous reports [27–29].

During the culture of microalgae in wastewater, their initial population density (IPD) played a significant role in nutrients and other pollutant removal. Some researchers had intensively studied the relationships between the IPD and growth of different species of microalgae [30–32]. All the work has demonstrated that IPD plays a major role in the wastewater treatment using microalgae. But, there are no reports on IPD of microalgae to remediate wastewater, especially for marine microalgae. Chen et al. [33] have reported the influence of IPD on the growth, biomass, and lipid production by using *Nannochloropsis* sp. in artificial seawater. Another work was carried out by Courtois de Viçose et al. [34] for the effect of initial inoculums cell density (IICD) on the growth and biochemical variation.

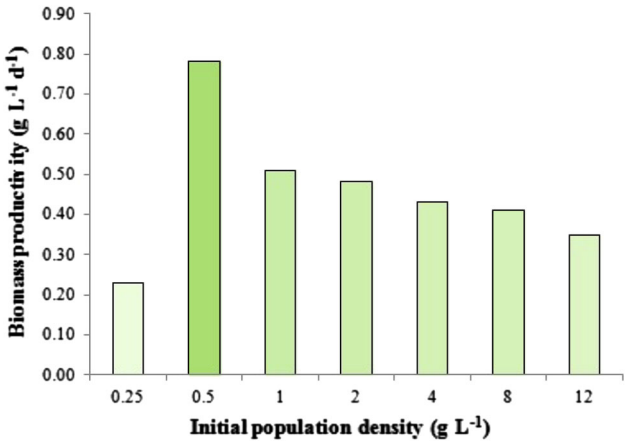

Fig. 6 Effect of IPD on daily biomass productivity of *Chlorella* sp. (PSDK01) using aquaculture effluent

Among the seven IPDs (0.25, 0.5, 1.0, 2.0, 4.0, 8.0, and 12.0 g L^{-1}) tested, the maximum biomass productivity (0.78 g L^{-1} d^{-1}) was obtained at 0.5 g L^{-1} IPD while lowest IPD (0.25 g L^{-1}) results lower biomass productivity and low nutrient consumption. IPD of 0.5 g L^{-1} resulted higher biomass productivity leading to a faster nutrient consumption due to the increased algal cell growth compared to other IPDs. The maximum nutrients (PO_4^{3+}, NO_3^- and NO_2^-) consumption were noticed in 0.5 g L^{-1} IPD except ammonia. The consumption of ammonia was found to be high in 0.25 g L^{-1} IPD. Higher IPDs (1.0–12.0 g L^{-1}) resulted low biomass productivity and nutrient consumption. Although a higher biomass concentration could be obtained at the end of the experiment. These different responses of the algal culture to be affected by IPD might be due to the lighting conditions under which the cells grow. Under moderate IPDs, the light source captured by microalgal cells was "oversaturated," which led to down-regulated photosynthesis rate, while under high IPD treatments, the light energy was insufficient to support continuous fast growth as agreed by early workers [33,34]. Chen et al. [33] have proved that lower IPDs (0.98 g L^{-1}) can produce higher biomass productivity and lipid production compared to higher IPDs (2.63–9.09 g L^{-1}). Courtois de Viçose

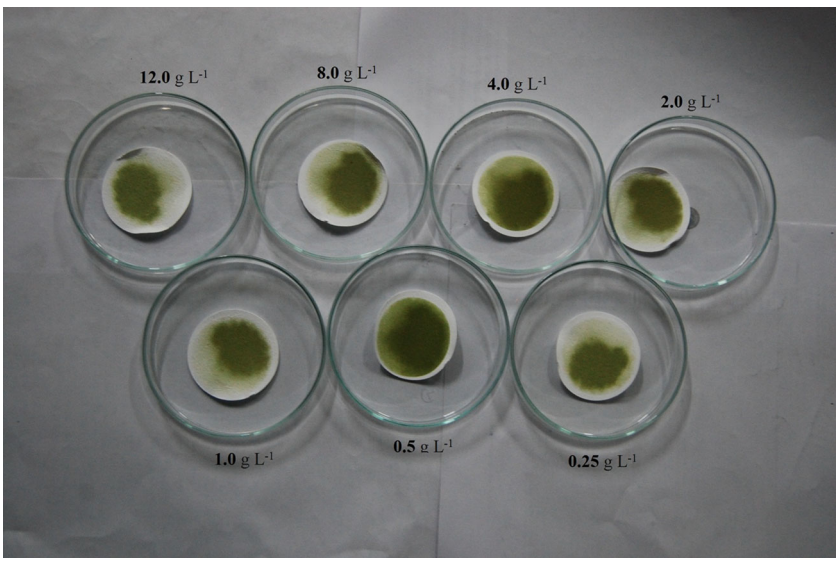

Fig. 7 Harvested biomass with reference to different IPDs

et al. [34] have found the higher biochemical compositions in the low (0.10×10^{-6} cells/mL) initial inoculums cell density (IICD) compared to higher (0.25×10^{-6} cells/mL) IICD.

A strong relationship was found between nutrients consumption and biomass concentration in the present experiment. The maximum consumption of PO_4^{3+} (92 %), NO_3^- (69 %), and NO_2^- (67 %) was recorded at the end of the treatment when biomass reached nearly 50 % from the initial. Our previous work [11] found a strong relationship between the nutrients removal and biomass growth in diverse wastewater using marine microalga *Chlorella marina*. While seeing ammonia consumption in the wastewater after 6th day, the consumption stopped and concentration of ammonia increased from the previous concentration in some IPDs (4.0, 8.0, and 12.0 g L^{-1}) except 0.25, 0.5, 1.0, and 2.0 g L^{-1}. For example, in 12.0 g L^{-1} IPD, the maximum ammonia consumption (3 %) was found on the 6th day, where the concentration varied from 11.9 to 9.5 μmol L^{-1}. After 6th day, ammonia concentration was found to be increased from previous concentration (9.5–9.9 μmol L^{-1}), the percentage of increase was nearly 4 %. This might be due to the decomposition of algae during the experiment; while by using algae or other biological sources for nutrients removal, decay of organisms might increase ammonia concentration [11].

Our results inferred that IPD is an important parameter for the wastewater treatment when using microalgal cells. But the selection of suitable IPD is still not easy to formulate. While selecting microalgal cultivation method for nutrient consumption, we have to consider IPD as an important factor. On the other hand, microalgal cultivation is not only practiced for excessive nutrients reduction, but also used for other purposes like biomass production for biodiesel and feed formulation means. It is wondering to determine the appropriate IPD when we want to obtain more biomass and remove more nutrients simultaneously. This study reveals that the moderate IPDs showed higher nutrient consumption and biomass productivity compared to both higher and lower IPDs. Therefore, it leads to high biomass yield, which could be used as feedstock for biodiesel, pigments, and other value-added products [33,35–37]. At the same time, for large-scale wastewater treatment, using suitable IPDs could reduce the cost of operation.

5 Conclusions

In the summary, *Chlorella* sp. (PSDK01) was cultured in shrimp-cultured effluent for dual purposes (nutrient consumption and biomass production) from low IPD to high population density (0.25–12.0 g L^{-1}). Maximum nutrients consumption was noticed in moderate IPD (0.5 g L^{-1}) except ammonia (0.25 g L^{-1}). The higher biomass was obtained at higher IPD (12.0 g L^{-1}), but maximum productivity was achieved in moderate IPD (0.5 g L^{-1} d^{-1}). On the other hand, moderate IPD resulted maximum nutrients consumption while produced limited biomass. Based on this study, microalgae cultivation technique with different IPDs in diverse wastewater would contribute to an increase in the nutrient consumption rate and biomass productivity efficiency developing into a large-scale treatment system.

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