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High yield cultivation of marine macroalga *Ulva lactuca* in a multi-tubular airlift photobioreactor: A scalable model for quality feedstock

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ABSTRACT

Conventional off-shore and on-shore cultivation methods for marine macroalgae are both inadequate to pitch macroalgae as scalable renewable feedstock that can be grown across all coastal locations. With on-shore cultivation likely to be sustainable and preferred over eco-damaging open seas cultivation, new reactor systems need to be developed for on-shore cultivation of seaweeds at scale. The present work is an attempt to use the indigenously designed vertical multi-tubular air-lift photobioreactor system to grow *Ulva lactuca* through the entire year under natural conditions. Optimized operation of the 1000 L photobioreactor assembly demonstrated a year-round averaged productivity of 0.87 kg $m^{-2}.d^{-1}$ (fresh weight) implying 1800 ton.$ha^{-1}.y^{-1}$ feedstock production. Carbon dioxide supplementation (5%), optimized circulation velocity (0.25–0.35 m/s), and managing nitrogen supply (17 ppm), under natural light intensities (500–1400 μmol $m^{-2}.s^{-1}$) provided a year-round sustained and continuous production of *Ulva lactuca* biomass. The photobioreactor system designed as a modular, linearly scalable, and resilient system operates with low land and water footprints, and gives a multi-fold increase in renewable feedstock production compared to the conventional sea-based and other on-shore tank-based practices.

For the video summary of this article, see the file in the supplemental data.

1. Introduction

Terrestrial farming provides almost all of the food to the planet, while petroleum and coal provide a major share of energy and carbon-based products. Serious concerns on climate change have however forced mankind to start development of renewable carbon sources for both energy and materials. As a result, technologies based on renewable agricultural and forest produce are being scaled up. However, the twin matters of degradation of arable lands through persistent tilling and use of chemical fertilizers, and increasing shortage of freshwater reserves in major agricultural regions, are both likely to change the way agriculture will be practiced in coming decades. Fast growing ocean phytoplanktons, long known as significant sinks for anthropogenic carbon dioxide, provide an alternative renewable carbon option. Controlled farming of select seaweeds can potentially be useful for a wide range of applications stretching over '*food on the plate to fuel in the plane*' (Tiwari and Troy, 2015). Indeed, the seaweed industry has been growing at rapid pace as the number of potential applications have increased (Pandey et al., 2020).

Seaweeds on an average grow at a magnitude faster rate than terrestrial plants and thus can form an attractive option for providing food as well as raw material for a range of products otherwise derived from petroleum or agricultural resources. However, for seaweeds to become a significant supplement to terrestrial farming, ocean farming in its current form shall need to be practiced at far bigger scales than being done today. Typical open ocean farming gives an annual seaweed yield of about 4 tonnes dry weight per hectare with current practices. This compares poorly with any terrestrial biomass yield despite the far higher photosynthesis rates in seaweeds. Besides, all seas are not suitable for growing seaweeds. Seaweed is cultivated today in open seas at only select locations using traditional techniques that suffer from several disadvantages. The location specific challenges include limited sea

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accessibility, steep sloping of photic zones, monitoring and maintenance of deeper farms, labour-intensive activities, increased seawater temperatures, ocean acidification, wave actions, natural calamities, adverse effects on benthic habitat, and seasonal variations.

At the present-day productivities, the desired scales of off-shore seaweed production shall require large usage of open seas that is certain to adversely affect the delicate ocean ecologies. One sustainable alternative is the use of coastal non-agricultural lands for growing seaweeds. For example, the authors' country India has a coastline of about 7000 km of which nearly 40% comprises of arid and semi-arid regions. This coastal zone can provide more than 2 million hectares for on-shore seaweed farming. On-shore seaweed farming is however very different from open sea cultivation and fraught with different challenges. It is today in its infancy with initial attempts made with on-shore tanks and ponds. The present work is aimed at providing a step up in large-scale on-shore seaweed farming.

The combined constraints of delicate ocean ecologies and current inefficient open sea farming practices necessitate that new technologies be developed for on-shore seaweed farming that should lead to a precision phyconomy to not only allow large-scale cultivations but also bring in rewards of continuous and consistent round-the-year production. Onshore seaweed farming using tanks and raceway ponds has shown the possibility of overcoming the open-sea cultivation challenges (Msuya and Neori, 2008). However, shallow raceway ponds are not the best bioreactors for seaweed production even though deployed at large scales for microalgae cultivations. Simple deeper tank-based cultivations do provide higher volumetric productivities but are still limited by limited light exposure due to larger depths used. Praeger et al. (2019) reported vertical stacking of attached seaweed species in deep tanks as an effective strategy to increase the areal productivity of land-based seaweed cultivation wherein productivities more than 40 ton dw ha^{-1} y^{-1} could be achieved. Multiple layers of seeded ropes of *Ulva tepida* were stacked into tanks in layers to a culture depth of 50–350 mm below the water surface. However, with larger stackings, algal biomass deep in the water column inevitably has lower light exposure. In another attempt, a detailed analysis of physiological plasticity parameters conducted during land-based cultivation in 40 m^3 depth ponds established that increase in seaweed density was directly related to the drop in light availability and dissolved inorganic carbon (Revilla-Lovano et al., 2021). Land-based production of red and green macroalgae for human consumption in the Pacific Northwest with an estimated annual production of 50–70 ton dw ha^{-1} y^{-1} has been reported by Gadberry et al. (2018), which is significantly higher than conventional terrestrial crops. However, the cultivation cycle was reported to be seriously affected by seasonal variations with the lowest being around winter when the growth was negative with sea lettuce.

In order to overcome the issues of limited light exposure in depth-tanks and mechanical limitations of shallow raceway ponds, a wide variety of photobioreactors (PBRs) have been designed and tested. Well-designed PBRs are better equipped for controlling abiotic and biotic stresses during cultivation and have been well explored for microalgae cultivation. Cultivation of seaweed in PBRs was first reported using 15 L airlift PBR for micro-propagation of red seaweed *Kappaphycus alvarezii* by Yeong et al. (2014) who also demonstrated development of clonal propagules in different types of airlift PBRs. Chemodanov et al. (2017) experimented with a polythene bag based PBR system for indoor small-scale *Ulva rigida* production. A ring-shaped cultivation system with algae moving in a circular way simulating the movement pattern in a standard tank cultivation vessel was also evaluated by Stefan Sebök et al. (2019). In all the mentioned studies, seaweed cultivation in PBRs was primarily performed for propagating seed material or done as laboratory-scale exploratory studies carried out indoor and/or outdoor in low-volume PBRs over a limited time span without accounting for seasonal variations. For developing and proposing any PBR design, it is necessary to study the effects of abiotic factors such as variations in temperature, illumination, nutrients, and pCO_2. These factors are all known to challenge consistent biomass composition and yield, the two parameters identified as key challenges for any industrial-scale seaweed production. Thus, PBRs have been relatively unexplored for scale-up of on-shore seaweed cultivation until the recent work published by Mhatre et al. (2018), wherein attempts were made to cultivate *Ulva lactuca* using a flat panel PBR. A 1000 L flat-panel photobioreactor system was successfully used for the cultivation of *U. lactuca* and was reported to achieve biomass productivity up to 300 g fw.m^{-2}.d^{-1} which extrapolates to 900 ton fw ha^{-1}.y^{-1} corresponding to approximately 90 ton dw ha^{-1} y^{-1}. Even though the proposed PBR system could surpass the biomass productivities reported by other reported land-based cultivation systems, the flat panel geometry can be imagined to impose structural as well as operational limitations at scale. Therefore, in the current study, an attempt has been made to address the limitations of the PBR and land-based cultivation systems reported so far.

A multi-tubular vertical airlift photobioreactor was designed and used for optimization of seaweed cultivation. An attempt was made to demonstrate pilot scale cultivation of *Ulva lactuca* in the in-house designed 1000 L multi-tubular airlift photobioreactor system operated under natural light conditions. Optimization studies included effects of reactor hydrodynamics, irradiance characteristics, nutrient management, stocking biomass density, and CO_2 supply. Importantly, the studies were conducted over different seasons of the year to establish if the cultivation system could accommodate abiotic and biotic stresses encountered due to seasonal variations and provide a consistent supply of biomass.

2. Materials and methods

2.1. Airlift photobioreactor (A-PBR): geometry, arrangement, and instrumentation

The vertical A-PBR erected and installed on the open terrace of the building housing the DBT-ICT Centre for Energy Biosciences in Mumbai, India, comprised a set of 20 interconnected parallel vertical tubes, with ten tubes acting as risers and other ten as downcomers alternatively. The tubes were made from UV-resistant transparent polycarbonate tubes (150 mm diameter; 2500 mm height) interconnected with high-density polyethylene (HDPE) bottom C-connectors and top H-connectors (Fig. 1). The alternating riser and downcomer tubes completed ten vertical loops with the vertical tubes spaced 150 mm apart occupying a total land footprint of 2.7 m^2. The total working volume of the reactor was 1000 L. The reactor was equipped with stainless steel (SS 316) cooling fingers dipped in vertical tubes from the top and connected to a central temperature controlled chilling unit in order to maintain the cultivation temperature in the A-PBR. The A-PBR was connected to the seawater supply tank for initial filling and replenishment as and when required. A special seaweed harvesting trap made from nylon mesh was deployed that could harvest the entire biomass when dipped into the top of any of the downcomer tubes. CO_2 enriched air (up to 5% v/v) could be used, if desired, through a CO_2 cylinder and a flow meter connected to the compressed air supply line. This also helped maintain the pH of the system at desired levels.

2.2. Growth studies of U. lactuca in A-PBR

The seed macroalga *U. lactuca* was collected from Veraval beach (Latitude 20°.90′ N, Longitude 70°.35′ E) in the state of Gujarat, India. Experiments in the A-PBR were carried out between April 2019 and June 2019 (summer) and between November 2019 and January 2020 (winter) under natural diurnal light. Each growth cycle included biomass inoculation, cultivation, harvesting, and growth analysis.

The A-PBR system was provided with continuous aeration in the range of 20–50 LPM via the ten riser tubes under the operating pressure of 0.2 MPa (gauge) resulting in the circulation of the macroalgae and the medium through the 20 tubes. The 10 tube spargers were placed at the

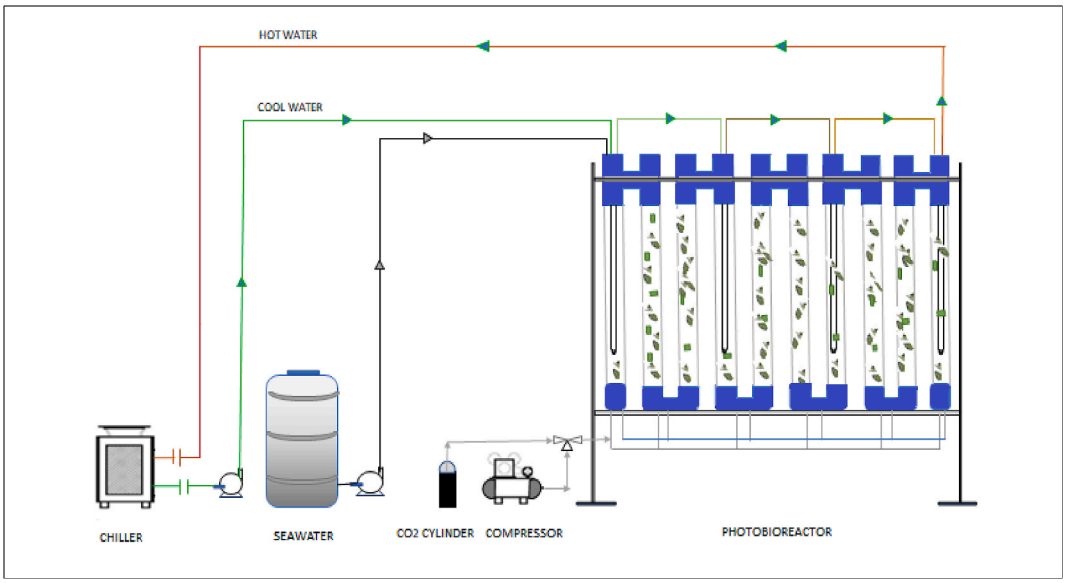

Fig. 1. The schematic of process flow and instrumentation of A-PBR.

bottom C-connectors and centrally to the riser tube. Two types of tube spargers were studied; the length of both spargers was 60 mm while hole diameters were 80 μm and 10 μm. Selected healthy fronds were inoculated in the A-PBR filled with filtered natural seawater (pH 8.35 and salinity of 30 psμ) supplemented with nutrient-enriched medium (MP1) (Suto, 1959). Cultivation was carried out at different initial stocking densities (SD) of 1 g/L, 3 g/L, 5 g/L, and 7 g/L. At stocking densities of 5 g/L and 7 g/L, biomass was harvested daily to restore the initial stocking density. However, experiments with SD 7 g/L had to be discontinued due to biomass over-crowding that resulted in irregular circulation of macroalgae in the A-PBR. The daily growth rate (DGR, %), biomass productivity (BP) (g fw.L^{-1}.d^{-1}), and areal biomass productivity (g fw. m^{-2}.d^{-1}) were recorded. The biomass collected was analyzed for proximate and ultimate composition. Protein content of the algal biomass was calculated by multiplying the nitrogen content measured by CHNS/O Analyzer (Thermo Scientific TM FLASH, 2000 CHNS/O Analyzers) by a factor of 5. (Angell et al., 2016).

Nitrogen uptake rate of the macroalgae was calculated by measuring nitrate present in the cultivation media on a daily basis during the cultivation cycle. The nitrate measurement was done spectrophotometrically using Angell et al., 2016 Section 4500 NO_3–B method and the nitrate uptake rate was calculated using the following equation:

$$\text{Nitrate uptake rate} = \frac{NO_3\ t_0 - NO_3\ t}{t - t_0}$$

where $NO_3\ t_0$ = nitrate supplemented on the first day of the cultivation cycle.

$NO_3\ t$ = residual nitrate on day t.

Subsequently, the amount of N was calculated using the following equation.

$$\text{N uptake rate} = 0.22 \times \text{nitrate uptake rate}$$

2.3. CO_2 enrichment studies for improved biomass productivity

The pH of the A-PBR was maintained between 7.5 and 9.5 by mixing 5% v/v CO_2 into the airline. The CO_2 dosing period was taken to be the interval between the upper limit pH of 9.5 when CO_2 supplementation was started to when pH hit the lower limit of 7.5, as denoted by Equation (1):

$$T[CO_2]_D = \Delta t_{[CO_2]}\ (pH\ A - pH\ B) \quad (1)$$

where, $T[CO_2]_D$ is the time interval in hours of CO_2 dosing, while $pH\ A$ and $pH\ B$ are the upper (9.5) and lower (7.5) limits of pH, respectively, for use in the macroalgal cultivation, Δt is the time interval per unit pH change.

2.4. Effect of bubble size and flow rate on circulation velocity of U. lactuca fronds

Porous spargers with pore sizes of 80 μm and 10 μm were used to provide variation in bubble size at any given air flow rate. Bubble sizes were measured by capturing images of the riser column in a high-resolution camera (Canon 1200D). The recorded images were analyzed using an image analysis software (WebPlotDigitizer) to determine the average bubble size (Hendre et al., 2018). To determine the circulation velocity for *U. lactuca* fronds, five thalli of random size with a mean diameter of 3–7 cm (referred to as vehicles) were dropped in the riser tube, and their travel velocity was monitored visually. Circulation velocity was determined by the linear axial displacement of the vehicle per unit time ($V = \Delta S/_{\Delta t}$).

2.5. Solar irradiance model for A-PBR

For A-PBR, the distance (P_{direct}) travelled by a direct ray of light from the tube's surface to the point within the culture was calculated using solar position and polar coordinates. The solar position was determined as the point of direct light incidence on the reactor's vertical surface. For polar coordinates, the position of the point (r_i, φ) along the cross-section of the tube is considered. For a vertical tube in the A-PBR, the distance (P_{direct}) to the point (r_i, φ) was calculated as described by Camacho et al. (1999).

$$P_{direct} = \frac{a_i\ cos\ \omega}{\cos\left(\frac{\pi}{2} - \theta'_z\right)} = \frac{R\ sin\ \varepsilon - r_i\ sin\varphi}{\cos\left(\frac{\pi}{2} - \theta'_z\right)} \quad (2)$$

where the parameter a_i is,

$$a_i \frac{r_i\ cos\ \varphi - R\ cos\ \varepsilon}{sin\ \omega} = \frac{R\ sin\ \varepsilon - r_i\ sin\ \varphi}{cos\ \omega} \quad (3)$$

The different lengths and angles relevant to eq. (2) and (3) are provided in the supplementary material for reference. The angle θ'_z is the zenith angle modified by the light refraction in the culture using Snell's law. Lamberts-Beer's law was applied to obtain the local irradiance

$I_{Bt}(r_i, \varphi)$ at the point (P_{direct}) as

$$I_{Bt}(r_i, \varphi) = I_{Bt}\, exp(-K_a.C_b.P_{direct}) \quad (4)$$

where $C_b$is the concentration of biomass and $K_a.C_b$is the apparent net absorption coefficient for *Ulva lactuca* at C_b and was determined as

$$K_a.C_b = C_b .\frac{2.303A}{t} \quad (5)$$

where A = absorbance, and t = thickness of thalli, considering thalli at given concentration acting as a homogeneous medium. The attenuation coefficient for *U. lactuca* was considered as 0.84 which corresponds to the 55% absorption of incoming irradiance (Brush and Nixon, 2003).

In addition to direct radiation (P_{direct}), the algal thalli also experience dispersed radiation due to light attenuation from the reactor wall and other thalli. This can be estimated from equation (4) as disperse irradiance $I_{Dt}(r_i, \varphi)$ for the same points. Since the A-PBR is mounted on an elevated skid, ground reflectance was considered negligible and thus ignored.

According to Fernández (Acién Fernández et al., 1997) integration of local values namely $I_{Bt}(r_i, \varphi)$, $I_{Dt}(r_i, \varphi)$ over the length and radius of the tube yields the average solar irradiance (I_{av}) on the culture;

$$I_{av} = \frac{1}{\pi R^2}\left\{\left(\int_R\int_\varphi I_{Bt}(r_i, \varphi)\, r\, dr\, d\varphi\right) + \left(\frac{1}{2\pi}\int_R\int_\varphi\int_\varepsilon I_{Dt}(r_i, \varphi)\, r\, dr\, d\varphi\, d\varepsilon\right)\right\} \quad (6)$$

2.6. Effect of stocking densities on solar energy conversion efficiency

The photon flux density (PFD) incident on the culture depends on the following factors: External irradiance due to solar position during the day; the photobioreactor geometry; orientation and inclination of a photobioreactor; culture density; and morphology of algae. The available fraction of irradiance on the reactor surface plays a critical role in determining photosynthetic active radiation (PAR). For this purpose, irradiances were measured using two types of devices. PAR on culture surface was measured with a Li-Cor Spherical Quantum Sensor (LI 193SA, Li-Cor, NE), while the instantaneous solar irradiance reaching the longitudinal tube surface was estimated using a lux meter (Onset_ HOBO_ sensor UA-002-08 by Onset Inc. MA), and these were subsequently converted into photon flux density. The instantaneous solar irradiance values were also measured at the surface of the tube at the top, middle, and bottom points of the central tubes on the A-PBR rack. Photon flux density was measured inside the tubes of the A-PBR in the central tubes (6th and 15th) and the end tubes (1st and 20th, 10thand 11th), where measurements were carried out at a 250 mm distance inside from the top of the respective tubes in presence of the culture medium. The positions for the measurement points have been provided in the supplementary material.

The daily solar average irradiance (kWh.m^{-2}) was estimated as the irradiance from 6 a.m. to 6 p.m. on each day of the cultivation experiment and verified by Equation (6). The total solar input in W/m^2 (E_{solar}) was evaluated for the A-PBR's total footprint area of 2.7 m^2, and the maximum PAR to the chemical energy conversion efficiency of *U. lactuca* biomass in the A-PBR was calculated from Equation (7) (Chemodanov et al., 2017).

$$\%\eta_{max} = \frac{\Delta(fw_f - fw_i)_{max} \cdot fw/_{dw} \cdot e \cdot N}{E_{solar} \cdot \Delta t} \cdot 100\% \quad (7)$$

where, $\Delta(fw_f - fw_i)_{max}$ in (g_{fw}) is the maximum biomass produced; *fw/dw* is wet to dry biomass weight ratio measured by drying biomass at 105 °C for 24 h; e (MJ/kg) is the specific energy in biomass according to ASTM D5865-13; N is the number of cultivation reactors; and Δt (d) is the number of the days of cultivation. Chemical energy conversion from PAR in the A-PBR was calculated for the selected stocking densities of 1, 3, and 5 g/L.

2.7. Water footprint of macroalgae cultivation in the photobioreactor

The water footprint (WF) is the ratio of freshwater used to the biomass (*dw*) produced (Béchet et al., 2017). The use of seawater or wastewater as a culture medium for algal cultivation can reduce freshwater usage in the cultivation cycle by nearly 90% since freshwater needs to be used irrespective of culture media for maintaining salinity against evaporative losses, nutrient preparations, and cleaning of the biomass (Yang et al., 2011).

The annual water footprint of algal cultivation WF_{year} (m^3.kg^{-1}) in the A-PBR was calculated as the ratio of areal water demand (WD_{year}) upon the areal productivity (P_{year}) as described by Bechet et al. (Béchet et al., 2017)

$$WF_{year} = \frac{WD_{year}}{P_{year}} \quad (8)$$

The more realistic annual water demand associated with any algal cultivation in a photobioreactor, WD_{year} (m^3.m^{-2}.yr^{-1}), is also the function of the sum of the water demands incurred between each culture change during each batch operation.

2.8. Use of alternate N-source for sustainable cultivation of U. lactuca

Mhatre et al. (2018) demonstrated that poultry litter extract (PLE) and urea could successfully be used as an alternative N-source for *Ulva* cultivation. PLE and urea media were prepared as described by Mhatre et al. (2018). The effect of PLE and urea media was studied in A-PBR independently, and the biomass growth along with the proximate and ultimate analysis of biomass produced.

2.9. Statistical analysis

All data is presented as mean ± standard deviation. One-way analysis of variance (ANOVA) was performed to confirm significant differences in results. A multiple comparison test by Tukey's honest significance difference (HSD) was carried out to find significant differences at P = 0.05 in response from controls.

3. Results and discussion

3.1. Circulation velocity of U. lactuca in A-PBR

The performance of an airlift photobioreactor is strongly influenced by the rate of aeration and air bubble size (Guieysse et al., 2011). The bubbles seen in the A-PBR experiments were large and non-spherical, and were approximated as oblate spheroids and measured in 2D-*XY* plots. The effect of variation in bubble sizes at different airflow rates was

Table 1
Effect of airflow rate and bubble size on circulation velocity of *U. lactuca* in A-PBR.

Airflow rate (LPM)	Bubble size (mm)	Circulation velocity (m/s)	Gas hold up (ε_g)	Time to complete loop (s)
20	4.0 ± 0.5	0.251 ± 0.010	0.007	220 ± 10
20	1.7 ± 0.3	0.261 ± 0.012	0.012	240 ± 10
30	4.0 ± 0.6	0.301 ± 0.011	0.0126	190 ± 12
30	1.7 ± 0.4	0.310 ± 0.010	0.0204	210 ± 12
40	4.2 ± 0.7	0.334 ± 0.013	0.0168	166 ± 11
40	1.7 ± 0.5	0.341 ± 0.009	0.0249	180 ± 9
50	4.2 ± 0.7	0.342 ± 0.013	0.0248	160 ± 12
50	1.8 ± 0.5	0.350 ± 0.010	0.0299	160 ± 8

investigated for its effect on the circulation velocity of *U. lactuca* fronds suspended in the seawater medium. Table 1 shows that two bubble sizes, with Sauter mean diameters of 4.0 ± 0.7 mm and 1.7 ± 0.5 mm, were observed with the two spargers used in the entire aeration rate range of 20–50 LPM. The corresponding range of circulation velocity was 0.25–0.35 m/s. Table 1 also indicates the effect of airflow rate and bubble size on circulation velocity and gas hold-up capacity of *U. lactuca* in A-PBR. The gas hold up (ε_g) of A-PBR was calculated as a relative increase in liquid height (in the riser tube) when aerated compared to static liquid height. The gas hold up at different airflow rates was estimated for the biphasic system (gas & liquid) and was found to be in the range of 0.007–0.029. There were clearly no significant changes in the liquid circulation velocity ($p > 0.05$) with bubble size in the range of airflow rates used on account of low gas hold-ups (1–3%).

In the presence of the solid phase (*U. lactuca*), the overall gas hold-up increased due to flow resistance and bubble breakup in the presence of macroalgae fronds in the tri-phasic (air + media + *U.lactuca*) system. The presence of algal fronds is known to result in the breakup of the large bubbles thereby increasing gas hold-up and increase in gas-liquid-solid interfacial area (Reyna-Velarde et al., 2010). Presence of fronds also resulted in significant reduction in the biomass circulation velocity by almost 20% at stocking densities of 3 g/L and 5 g/L with both mean bubble sizes (see Supplementary material). This can be attributed to the drag force generated by increased culture density causing the reduction of circulation velocity of macroalgal fronds. It is reported that high stocking density results in increased energy dissipation due to higher flow resistance, the random collision between macroalgal fronds, trapping of air bubbles, and friction at the reactor walls (Sebök et al., 2019). Hence to maintain the required circulation velocity between 0.25 and 0.35 m/s, about 10% increase in air flow rate was needed with high stocking densities of 3 g/L and 5 g/L. However, volumetric airflow rate beyond 30 LPM was observed to adversely affect culture growth indicated also by a reduced nutrient uptake. At stocking densities of 1 g/L, 3 g/L, and 5 g/L, small bubbles (1.7 ± 0.5 mm) with the finer sparger were seen to get trapped with the *U. lactuca* fronds. This resulted in reduction of frond bulk density and accumulation of algal biomass at the top of the tubes which resulted in disruption of the circulating flow of the through the A-PBR. Small bubble size was also seen to result in mechanical damage and shear stress to the culture. Hence, the use of the finer sparger and small bubble sizes was discontinued for further experiments. The larger mean bubble size of 4 ± 0.7 mm, on the other hand, was seen to favor the growth of *U. lactuca* even at higher stocking densities. Hence, all further studies were conducted using sparger that gave mean bubble size of 4 ± 0.7 mm at airflow rates in the range of 20–30 LPM.

3.2. Distribution of PAR and solar irradiance

Incident sunlight changes with weather, diurnal cycle, and seasons. Under any given condition, the photobioreactor and its geometry also affect the amount of light reaching the culture. As algal biomass grows, the changing culture morphology and density both dictate the light distribution inside the photobioreactor. Therefore, in addition to logging the changes in the external light incident on A-PBR, it is also important to understand and control the light reaching inside the reactor system. As shown in Fig. 2, the distribution of the solar irradiance and PAR both varied along with the height of the reactor at any time of the day. Light available to algal biomass through the light and dark cycles is also greatly influenced by other factors including light transmission of tube material (which was ~92%); inter-distance between the tubes (which in this was 0.16 m); circulation velocity of thalli; and cumulative shading effect caused by shading effect of the reactor and *U. lactuca* fronds. On the other hand, continuous movement of the thalli brings them to the surface and back for more uniform direct or diffused irradiance in their passage along the length of the vertical reactor tubes.

The mean PAR values inside the reactors were 67.0, 56.2, and 38.0 μmol $m^{-2}.s^{-1}$ for the stocking densities of 1, 3, and 5 g/L, respectively. These PAR values have been validated with values calculated using

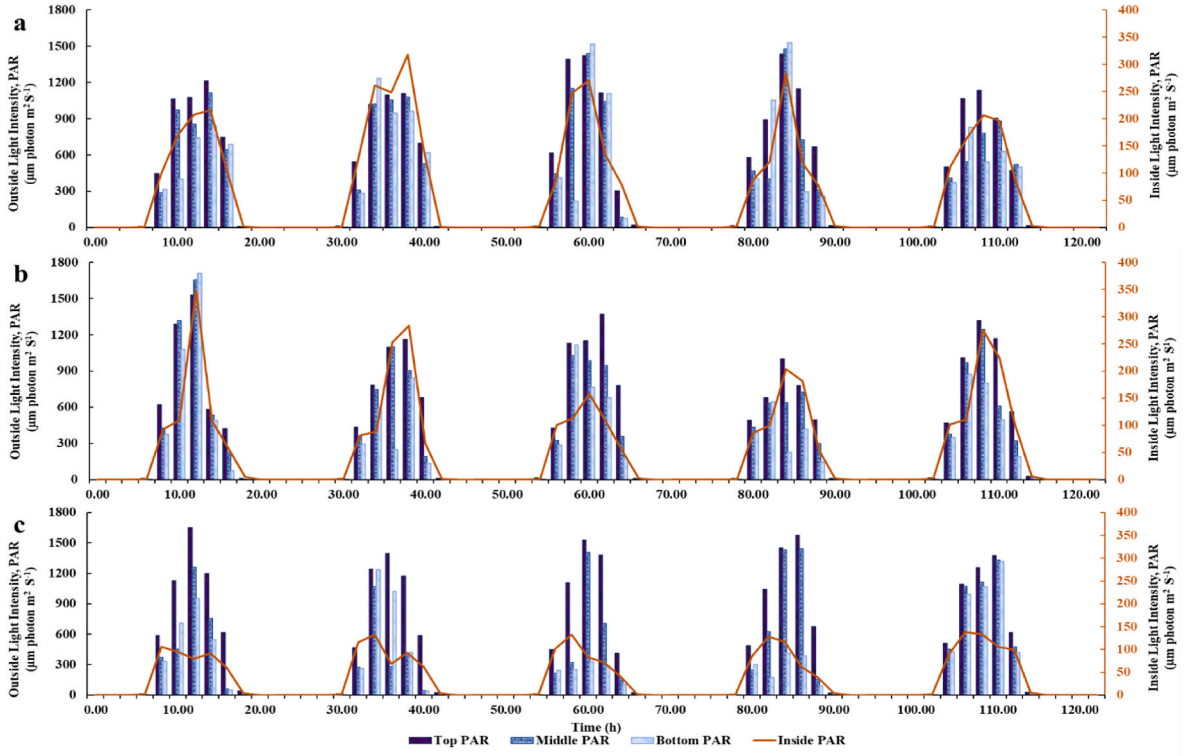

Fig. 2. Incident light distribution pattern along the length of the photobioreactor (top, middle, and bottom) and corresponding actual PAR reaching the U. lactuca culture. Both measurements were carried out at different time points (with 2 h interval for 5 days) as measured at the axis of the vertical PBR tube. The experiment was carried out at three different stocking densities: (a) 1 g/L, (b) 3 g/L, and (c) 5 g/L.

Equation (6) within ±5%. Insufficient irradiance or light attenuation can impair photosynthesis and the subsequent growth of seaweed. However, it was noteworthy that the overall productivities in this work were not significantly affected at high culture densities of 3 g/L and 5 g/L as further discussed in Section 3.3, indicating unhindered photosynthesis.

3.3. Effect of stocking density on growth rate and biomass productivity in summer and winter seasons

Initial biomass stocking density affects both biomass productivity and composition. Low densities may limit productivities when light exceeds the photosynthesis demand, while high densities may limit light availability thereby decreasing biomass productivities (Mata et al., 2016; Bruhn et al., 2011). Effect of different stocking densities on biomass productivity was monitored during different average light conditions i.e. winter and summer seasons while the temperature of culture media was maintained at 27 ± 2 °C at all times (Table 2). The daily growth rate (DGR, % d^{-1}) ranged from 31.3% d^{-1} to 15.7% d^{-1}, with the trend of DGR inversely related to stocking density in both seasons. The highest DGR observed in summer was 30.8% d^{-1} at the stocking density of 1 g/L. Similarly, the highest DGR of 31.3% d^{-1} was recorded in winter at the same stocking density. During both seasons, DGR obtained at a stocking density of 1 g/L was double the DGR obtained at a stocking density of 5 g/L (15.7% d^{-1} in summer, 16.4% d^{-1} in winter). Thus, the DGR at the stocking density of 1 g/L (30.8% d^{-1} in summer and 31.3% d^{-1} in winter) was significantly higher than other stocking densities irrespective of the seasonal variations and was confirmed with $p < 0.01$ (Tukey's HSD Test).

In contrast to DGR, the trend of biomass productivity was expected in direct proportion to the stocking density. The highest productivity of 608.2 g $fw.m^{-2}.d^{-1}$ was achieved at a stocking density of 5 g/L (Table 2) in winter ($p > 0.05$; Tukey's HSD test). The highest productivity of 522.3 g $fw.m^{-2}.d^{-1}$ in summer was also recorded at the density of 5 g/L (Tukey's HSD test $p > 0.05$). Further, changing stocking densities significantly influenced biomass productivities in both seasons ($p > 0.05$). Thus, the increase in average productivity was 100% d^{-1} for winter and 105% d^{-1} for summer for stocking density going from 1 g/L to 3 g/L; while the increase was only 25% d^{-1} for both seasons when stocking density was increased from 3 g/L to 5 g/L. In a study with red seaweed *Porphyra dioica*, a 13% $week^{-1}$ increase in biomass production was obtained at the highest stocking density of 1.5 g $fw.L^{-1}$, compared to the lowest stocking density of 0.2 g $fw.L^{-1}$ at a light intensity 150 µmol $m^{-2}.s^{-1}$ (Pereira et al., 2006).

Loss in biomass yields is often encountered on account of seasonal variations resulting from severe stress due to temperature fluctuations, changes in irradiance and other abiotic conditions. In a study by Hung et al. (2009), all morphotypes of *Kappaphycus* were reported to exhibit seasonal variation in their growth rates with the brown macrophyte showing a higher growth rate (3.5–4.6% d^{-1}) from September to February, and a lower growth rate (1.6–2.8% d^{-1}) from March to August. High growth rates for the red (3.6–4.4% d^{-1}) and green (3.7–4.2% d^{-1}) morphotypes were obtained from September to February (Hung et al., 2009). Hernandez and co-workers reported seasonal changes in DGR and biomass productivity for *Ulva* spp. In sea-based cultivation. In the study, positive daily growth rates were observed in spring and autumn (0.25% d^{-1}), and were negative in winter and summer (Niell, 1997). Altamiranol et al. also reported seasonal changes in growth rates in sea-based cultivation of *UIva olivascens* wherein the highest growth rate was observed in March (10.9 ± 1.6% d^{-1}), which declined by 68% in summer (3.5 ± 0.6% d^{-1}) (Altamiranol et al., 2000). Contrary to all these reports *U. lactuca* in the present study did not suffer seasonal aberrations and its yields were not influenced by seasons. This was possible thanks to maintaining constant temperatures throughout the cultivations. With no significant changes in the biomass productivities between summer and winter seasons ($P > 0.05$, Tukey's HSD test), the performance of A-PBRs in this study becomes unique for consistent round-the-year macroalgae cultivation. Also, this work has bettered the earlier reported work by Mhatre et al. who demonstrated the cultivation of *U. lactuca* in 1000 L Flat-panel PBR (F-PBR) wherein biomass productivity of 45 g $dw.m^{-2}.d^{-1}$ was achieved and was highest among the studies reported then for *U. lactuca* (Mhatre et al., 2018).

Table 2
Effect of stocking density on growth and productivity yields in summer and winter with and without (w/o) CO_2 supplementation.

Season	SD (g/L)	DGR (%) (w/o CO_2)	Productivity (g $fw.m^{-2}.d^{-1}$) (w/o CO_2)	DGR (%) (With CO_2)	Productivity (g $fw.m^{-2}.d^{-1}$) (With CO_2)
Summer	1	30.8 ± 4.5	202.8 ± 14.61	45.8 ± 1.8	415.6 ± 15.9
	3	21.4 ± 2.5	416.4 ± 69.5	29.1 ± 2.0	636.4 ± 45.5
	5	15.7 ± 5.4	522.3 ± 90.4	22.5 ± 5.5	855.3 ± 47.7
Winter	1	31.3 ± 3.2	242.4 ± 48.9	42.8 ± 4.9	473.5 ± 64.4
	3	25.5 ± 7.4	484.8 ± 46.7	32.4 ± 2.7	684.2 ± 65.0
	5	16.4 ± 4.9	608.2 ± 67.5	25.5 ± 2.9	871.6 ± 110.9

3.4. Effect of CO_2 enrichment on growth and productivity

Cultivation of high-density cultures of 3 g/L and 5 g/L was seen to show increase in the pH of the growth media from 9 to 12 which can adversely affect the growth. Supplementing air with CO_2 was found to decrease alkalinity. To study the effect of CO_2 enrichment on growth and biomass productivity, *U. lactuca* was grown at stocking densities of 1, 3, and 5 g/L with air containing 5% v/v CO_2. Mass transfer of CO_2 from air to liquid medium is known to be the rate-limiting factor for algal growth (Liu et al., 2020). Low CO_2 concentrations (less than 400 ppm) coupled with a relatively low CO_2 transfer rate can contribute to CO_2 limitation (Putt et al., 2011). Besides, it is reported that pH of the culture can be controlled by periodic CO_2 dosing (Guo et al., 2015). In the current study with the stocking density of 5 g/L, the pH of the media was found to shoot up to 12 during the peak hours of solar irradiance (the active photosynthetic hours), irrespective of the season.

There have been reports on different methods of CO_2 supplementation to algal reactors; the most commonly suggested method is still the direct injection of CO_2 enriched air into the growth medium (Pegallapati and Nirmalakhandan, 2013; Israel et al., 1999). In the present study, it was found that pH rise in the peak irradiance hours could be controlled by sparging 5% CO_2 supplemented air over 5 h in summer and 4 h in winter. As described in Section 2.3, upper and lower pH was set as 9.5 and 7.5, respectively, and the pH was manually controlled at 8.5 ± 1 at all times. This significantly increased productivity at all the three stocking densities for summer and winter (Table 2). Liu et al. also commented on the pH maintenance via CO_2 supplementation as accountable for the joint operation of the C4 pathway and a CA-supported HCO_3^- mechanism in *U. prolifera* and which is responsible for improved biomass production (Liu et al., 2020)

In the current study, the highest productivity observed was 871.6 g $fw.m^{-2}.d^{-1}$ at a stocking density of 5 g/L, which was significantly higher than 602.8 g $fw.m^{-2}.d^{-1}$ obtained without CO_2 supplementation ($p > 0.05$, TSD test). A similar trend was also observed in DGR values of cultures grown with and without CO_2 for all stocking densities in both seasons. Growth rate and biomass productivity were enhanced with CO_2 supplementation for *Cladophora coelothrix* by 26% and 24% for *Chaetomorpha linum* (de Paula Silva et al., 2013). A comparison of CO_2 supplementation with the present study is given in Table 3. In the current study, the best biomass productivities obtained for the selected stocking densities of 1, 3, and 5 g/L were 473.5 ± 17 g $fw.m^{-2}.d^{-1}$, 684.2 g $fw.m^{-2}.d^{-1}$, and 871.6 g $fw.m^{-2}.d^{-1}$, respectively during winter with CO_2

Table 3
The comparison of macroalgal species for their growth performance with and without CO_2 supplementation.

Sr no.	Species	Growth without CO_2 supply	Growth with CO_2 supply	Reactor volume	CO_2%; dosing time	Stocking Density	Study
1	*Oedogonium* spp.	8.3 g dw.m^{-2}.d^{-1}	3.37 g dw.m^{-2}.d^{-1}	15,000 L	99.9%; 11 h	0.5 **g/L**	Cole et al., 2014
2	*Cladophora coelothrix*	12.5 g dw.m^{-2}.d^{-1}	16.8 g dw.m^{-2}.d^{-1}	5 L	99.9%; 24 h	3 **g/L**	Sathakit et al., 2020
3	*Chaetomorpha linum*	9.5 g dw.m^{-2}.d^{-1}	12 g dw.m^{-2}.d^{-1}	5 L	99.9%; 24 h	3 **g/L**	Sathakit et al., 2020
4	*Gracilaria tikwahiea*	6% DGR	12% DGR	2.5 L	5%; 24 h	–	Young and Gobler, 2017
5	*Ulva lactuca*	17% DGR	24% DGR	2.5 L	5%; 24 h	–	Young and Gobler, 2017
6	*Ulva intestinalis*	2.19% DGR	2.31% DGR	10 L	1%; 24 h	0.05 **g/L**	Sathakit et al., 2020
7	***Ulva lactuca***	**90.7 ± 10 g dw.m^{-2}.d^{-1},16.4% DGR**	**130.1 ± 16 g dw.m^{-2}.d^{-1}, 25.5% DGR**	**1000 L**	**5%; 4h (winter)**	**5 g/L**	**Present study**
8	***Ulva lactuca***	**77.9 ± 13 g dw.m^{-2}.d^{-1}, 15.7% DGR**	**127.6 ± 7 g dw.m^{-2}.d^{-1}, 22.5% DGR**	**1000 L**	**5%; 5h (Summer)**	**5 g/L**	**Present study**

DGR- Daily growth rate.

supplementation. The measured specific energy content for *Ulva lactuca* was 14.67 MJ/kg. This translates to PAR to chemical energy conversion efficiency of 2.3% for a stocking density of 1 g/L; 1.75% for a stocking density of 3 g/L; and 3.47% for a stocking density of 5 g/L. Bruhn et al. have reported photosynthetic efficiency of 1.6% with a stocking density of 4 g/L for *U. lactuca* (Bruhn et al., 2011). In another work, the energy conversion efficiency in the range of 0.87–1.3% was observed for *U. compressa* at an initial cultivation density of 2.5 g/L (Chemodanov et al., 2017).

3.5. Effect of seasonal variation on the nutrient uptake rates and biomass composition

It thus emerges that with the temperature-controlled A-PBR system deployed in this work there was no significant seasonal variation seen in biomass productivity which can otherwise be expected in the typical tropical climates. It is, however, important to also study the effects on biomass quality. Seasonal changes can stimulate or inhibit biosynthesis of seaweed components that depend on nutrient uptake rates (Marinho-Soriano et al., 2006). The nutritional composition of seaweeds and their seasonal oscillation is inadequately known and primarily evaluated based on chemical compositions.

In the present study, important biomass constituents were measured and the results are provided in Table 4. A significant difference in the averaged total chlorophyll content was observed between biomass grown in summer (0.69 ± 0.12 mg g^{-1} fw) compared to that grown in winter (1.43 ± 0.007 mg g^{-1} fw) ($p < 0.05$, Tukey's HSD). The average carotenoid content in summer (0.4 ± 0.04 mg g^{-1} fw) was also significantly lower than the carotenoid content in the winter (0.21 ± 0.04 mg g^{-1} fw). Fully pigmented cells under high light exhibit reduced efficiency of light-to-biomass conversion because their large antenna size results in a high photon energy transmission rate (Polle et al., 2002). As a result, more than 80% of the absorbed photons are wasted (Melis, 2012). Furthermore, at high light intensities the available photo-protective mechanisms become insufficient to completely quench the absorbed energy, thus causing permanent damage to photosystems. Therefore, in order to avoid damage to the photosystem in the high light of summer, there is a reduction in cellular pigment production.

Table 4
Average values (mean ± SD) of N-uptake and biomass composition of *U. lactuca* in summer and winter.

Parameters	Summer period	Winter period
Averaged irradiance (μmol.m^{-2}.s^{-1})	1399	524
N-uptake (mM. g^{-1} dw. d^{-1})	5705 ± 90.7	305 ± 5.3
Carbohydrate content (% dw)	50.21 ± 0.127	56 ± 0.3
Lipid content (% dw)	3.02 ± 0.10	1.4 ± 0.05
Protein content (% dw)	13.05 ± 0.07	16 ± 0.1
Ash (% dw)	21.35 ± 0.4	15.7 ± 0.5
Moisture (% dw)	11.16 ± 0.25	10.3 ± 0.2
Chl a (mg.g^{-1} fw)	0.58 ± 0.12	1.10 ± 0.1
Chl b (mg.g^{-1} fw)	0.10 ± 0.007	0.27 ± 0.01
Total Chl (mg.g^{-1} fw)	0.69 ± 0.12	1.43 ± 0.007
Carotenoids (mg.g^{-1} fw)	0.21 ± 0.04	0.40 ± 0.04

Nitrogen (N) uptake rate was also found to vary significantly with season ($p < 0.05$, Tukey's HSD). A high N-uptake rate of 705 ± 90.7 mM g^{-1} dw.d^{-1} was observed in the summer compared to the N-uptake (305 ± 5.3 mM g^{-1} dw.d^{-1}) in winter. High light conditions induce higher N-uptake and photosynthetic protein catabolism, leading to decreased total protein content in algae and higher plants (Yang et al., 2007). The protein content was high (16 ± 0.1% dw) in the biomass produced under winter irradiance, although Tukey's test defined no significant difference (p value > 0.05). Reduction in protein content through high biomass accumulation is speculated as physiology in green algae to dilute the functional photosynthetic proteins such as antennae molecules. Thus overall, although carotenoid contents and N-uptake rates varied significantly between the seasons, there was no significant variation in carbohydrate, protein, and lipid content of biomass ($p > 0.05$) (Table 4).

Seasonal variation in productivity and biomass composition is a major concern in open sea-based cultivation and has led many to attempt seaweed cultivation in on-shore tanks. Mata et al. studied green seaweeds *Derbesia tenuissima* and *Ulva ohnoi* in a 10,000 L tank reactor over six months; they found significant variation in monthly biomass productivity for both seaweeds, possibly on account of temperature changes, while in the case of *U. ohnoi*, carbohydrate content (ulvan) remained consistent while lipid and protein contents varied throughout the cultivation (Mata et al., 2016). Monteiro et al. reported significant differences in the harvesting period recorded for carbon, hydrogen, and sulfur content in kelp cultivation (Monteiro et al., 2021). Consistency in biomass productivities and bio-composition is often highlighted as an imperative feature of industrial seaweed cultivation. The A-PBR system used in the present work gave consistent biomass production in both quality and quantity. This may be attributed to the A-PBR assembly's controlled environment with uniform light distribution, temperature, nutrient inputs, salinity, and CO_2 supplementation.

3.6. Alternative N supplementation for U. lactuca growth in A-PBR

Since nitrogen supplementation is a crucial part of the algae cultivation and a significant cost contributing factor, alternative and cheap N-sources for A-PBR-cultivation of *U. lactuca* were explored. Two nitrogen sources, namely, PLE and urea, were used as alternatives. Growth characteristics of *U. lactuca* in PLE and urea-containing media were compared to the MP1 medium with respect to biomass productivity; and

carbohydrate, protein, lipid, and pigment contents. Results are presented in Table 5. Biomass productivities of 833 g fw.m^{-2}.d^{-1} and 848 g fw.m^{-2}.d^{-1} were observed in urea and PLE media, respectively, and these were not significantly lower than the productivity obtained in the MP1 media ($p > 0.05$). It has been reported that seaweeds do uptake urea for growth in urea enriched conditions (Han et al., 2017). Although the N-uptake rates varied significantly between the media, there was no apparent variation in biomass carbohydrate, protein, and lipid content ($p > 0.05$).

3.7. Quantification of water footprint

Water footprint (WF) has perhaps the most serious impact on the sustainability of any on-land algal cultivation. Large-scale freshwater algal cultivation massively impacts the local water resources (Guieysse et al., 2013). In the present study, seawater was used as the culture medium and thus lowers the WF. Water footprint in such a case can be quantified based on the freshwater demand (WD) of the end-to-end process of algal cultivation. The following aspects were considered while estimating the WD in *U. lactuca* cultivation in the present work with A-PBR: (1) Freshwater is required to maintain salinity owing to the media evaporation; (2) Water is required for reactor system cleaning and crop cleaning; and (3) Freshwater is required for controlling the temperature of A-PBR using chiller and cooling coils circuits. Precipitation water was not considered since the reactor system is a closed system, and seawater can be also be used in place of fresh water on the two of the above three counts especially when operated at larger scales, except for crop cleaning.

Typically, WD largely stems from the volumes of fresh water required on account of evaporation that results in rising salinity, and fresh water required to clean the A-PBR system, and also clean the crop free from seawater constituents. It was observed that average evaporative losses of 0.173 m^3 m^{-2}.yr^{-1} in winter and 0.281 m^3 m^{-2}.yr^{-1} in summer were measured on the A-PBR in the tropical climate of Mumbai, India. These numbers are low compared to typical losses seen from raceway ponds in similar climates. Martins et al. also reported negligible evaporative losses while studying a closed pilot-scale 1.5 m^3 multi-tubular photobioreactor (Martins et al., 2018). In the present study, the hydraulic retention time (HRT) of culture media (seawater) in the A-PBR was for four cultivation cycles, which was followed by freshwater use for A-PBR cleaning, while the harvested crop was also washed with fresh water. After considering the cultivation cycles and reactor maintenance cycles, the WD for the cultivation of *U. lactuca* was estimated to be 1.78–1.95 m^3 m^{-2}.yr^{-1} which corresponds to a WF of 0.05 m^3/kg macroalgal biomass for 5 g/L of culture density.

To the best of our knowledge, there are no reports that discuss water footprint for macroalgae cultivation while a few assessments are available for microalgae cultivation. Most of the reports discussing WD for microalgae cultivation are based on empirical data or empirically derived formulas that are too site-specific to be universally applicable. Guieysse et al. (2013) compared the WF of microalgae cultivation in open ponds in different geographical locations evidencing the significant variability and uncertainty in the WF quantification. As per this study, the water evaporation rate varies from 0.476 to 2.275 m^3 m^{-2}.yr^{-1} in the open raceway ponds with the total WD being in the range of 5.49–6.39 m^3 m^{-2}.yr^{-1} (Guieysse et al., 2013). Martins et al. estimated a water footprint of 2.4–6.8 m^3/kg dry microalgae biomass in a closed pilot-scale PBR of volume 1.5 m^3 though 90% of the water was reused after each harvesting and about 10% of makeup water was added (Martins et al., 2018).

The present study cannot be directly compared with reported literature due to differences in algal species and cultivation media. Overall, with the related assumptions and system boundaries, the present study demonstrates that use of seawater and closed photobioreactors has a combined beneficial effect on reduction in WF significantly and thus improves the overall sustainability of the land-based cultivation of seaweeds. Recycle of freshwater used for reactor and biomass cleaning can further reduce the water footprint undoubtedly.

Table 5
Average values of growth yields and proximate composition (means ± SD) of *U. lactuca* in MP1 media, urea media, and PLE media different media grown in A-PBR. (Data represented is the average of samples collected in triplicate at every month).

Parameters	Urea	PLE	MP1
Biomass productivity (g fw.m^{-2}.d^{-1})	833 ± 93	848 ± 105	871 ± 110
Carbohydrate content (% dw)	55.3 ± 0.54	55 ± 0.3	57 ± 0.2
Lipid content (% dw)	1.5 ± 0.4	1.4 ± 0.01	1.36 ± 0.01
Protein content (% dw)	16.3 ± 0.44	16.2 ± 0.2	18 ± 0.5
Chl a (mg.g^{-1} fw)	1 ± 0.12	0.85 ± 0.01	1.16 ± 0.13
Chl b (mg.g^{-1} fw)	0.21 ± 0.03	0.21 ± 0.01	0.27 ± 0.13
Total Chl (mg.g^{-1} fw)	1.20 ± 0.05	1.069 ± 0.5	1.43 ± 0.008
Carotenoids (mg.g^{-1} fw)	0.33 ± 0.01	0.29 ± 0.01	0.43 ± 0.54
Total N-uptake (mM.g^{-1} dw.d^{-1})	146 ± 3.02	93.84 ± 2.1	134.5 ± 5.2

3.8. Salient features of cultivation of Ulva lactuca in A-PBR

The multi-tubular photobioreactor system presented in the work is a modular, resilient and scalable system and offers ease of operation, control, cultivation and biomass harvesting. The system can be scaled up wherein multiples of 1000 L assemblies can be deployed and operated standalone, or in tandem. Changes in stocking densities and seasonal variations normally have significant influence on biomass productivities. However, the multi-tubular photobioreactor system with appropriate hydrodynamics presented in this work provides precise control over temperature, nutrients management, CO_2 supply, efficient light transmission, and thus can ensure year-round supply of consistent biomass. Thus, the prototype model supports an enhanced phyconomy and can enable seaweed cultivation in non-conducive weather on uncultivable sea-shores.

4. Conclusion

The presented work was aimed at developing a photobioreactor technology for scalable round-the-year high yield cultivation of *Ulva lactuca* under natural conditions. A 1000 L vertical multi-tubular airlift photobioreactor system was designed and optimized against several operating parameters. The system was operated under optimized conditions in both summer and winter, and gave an average productivity of 871.6 g fw.m^{-2}.d^{-1} with a starting stocking density of 5 g/L in winter when supplemented with CO_2. This number transforms to 1800 ton fw ha^{-1}.y^{-1} or 270 ton dw ha^{-1}.y^{-1} of feedstock production. This result presents immense potential and can play a significant role in transforming the emerging on-shore cultivation of seaweeds in all regions blessed with abundant sunlight, seawater, and arid coasts that should become centres of a novel 'green revolution' with a boosted 'blue economy' despite geographical impediments like steep shores, arid climate, and rough seas. The designed A-PBR system is linearly scalable in numbers to cover several hectares of land, is easy to operate and maintain, consumes low energy and fresh water, and provides clean, controlled, and consistent seaweed production.

CRediT authorship contribution statement

Prashant Savvashe: Conceptualization, Formal analysis, Methodology, Photobioreactor management, Validation, Investigation, Writing – original draft, Resources, Software. **Akanksha Mhatre-Naik:** Conceptualization, Methodology, Validation, Investigation, Writing – original draft, Data curation. **Gayatri Pillai:** Validation, Formal analysis, Visualization, Writing – original draft, **Juilee Palkar:** Conceptualization, Visualization, **Mayur Sathe:** Conceptualization,

Photobioreactor management. **Reena Pandit:** Data curation, Project administration, Supervision, Writing – review & editing, C. R. K. Reddy: Resources, writing-review. **Arvind M. Lali:** Project administration, Supervision, Writing – review and editing, Funding acquisition. All authors provided critical feedback and helped shape the research analysis and manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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