Trivedi *et al. Phycology Journal* (2026) 2:1
https://doi.org/10.1186/s44377-026-00006-0

Phycology Journal

RESEARCH Open Access

Process development for scaled-up land-based cultivation of *Ulva ohnoi* (Hiraoka & S.Shimada) using spores-derived seedlings

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Abstract

Ulva is edible green seaweed with higher rate of productivity, and considerable nutritional benefits. However, its seasonal nature and reproductive behaviour hinder opportunities for continuous cultivation in land-based systems. In the present study, a scaled-up process was developed for the controlled production of spore-based seedlings of *Ulva ohnoi*. In a Phase I, production of ~ 100,000 seedlings (in 45 days) was achieved and while Phase II yielded two kg fresh biomass (in next 45 days) in systematic land-based cultivation. In phase I, various abiotic stresses, namely, different stocking densities of mother tissue as well as seedlings were standardized to ensure obtaining uniform and the highest number of seedlings available for farming. Fragmentation (~0.1 cm) induced 100% sporulation by Day 2, while 0.3 cm disks showed 37.2% sporulation by Day 2. Freshwater exposure (10 min) triggered 81.7% sporulation on Day 2, and desiccation (1 h) induced over 90% by Day 2. Salinity and temperature had negligible effects on sporulation induction. A stocking density of 0.1 g fragments per 100 ml seawater produced 5200 ± 200 seedlings by day 15. Through this method, mass production of ~ 100,000 seedlings was attempted and successful. The subsequent stepwise culturing protocol ensured production of seedlings of 0.97 ± 0.35 cm by Day 45. In Phase II, further stocking size, stocking densities of seedlings and light intensities for the land-based cultivation system were also standardized to scale-up the entire process to 250-L reactor. This yielded the production of 2 kg contaminant free biomass derived from ~ 100,000 seedlings. This scaled-up protocol recorded a growth rate of 14.26 ± 0.29% day^{-1}, and biomass yield of 33.54 ± 0.97 g ww m^2 day^{-1}. The nutritional compositions such as protein (35.25 ± 0.81% of dw), and carbohydrate (38.98 ± 0.55% of dw) were significantly improved during scaled-up production. This is the first attempt to produce spores-derived seedlings of *U. ohnoi* [at the scale of ~ 100,000]

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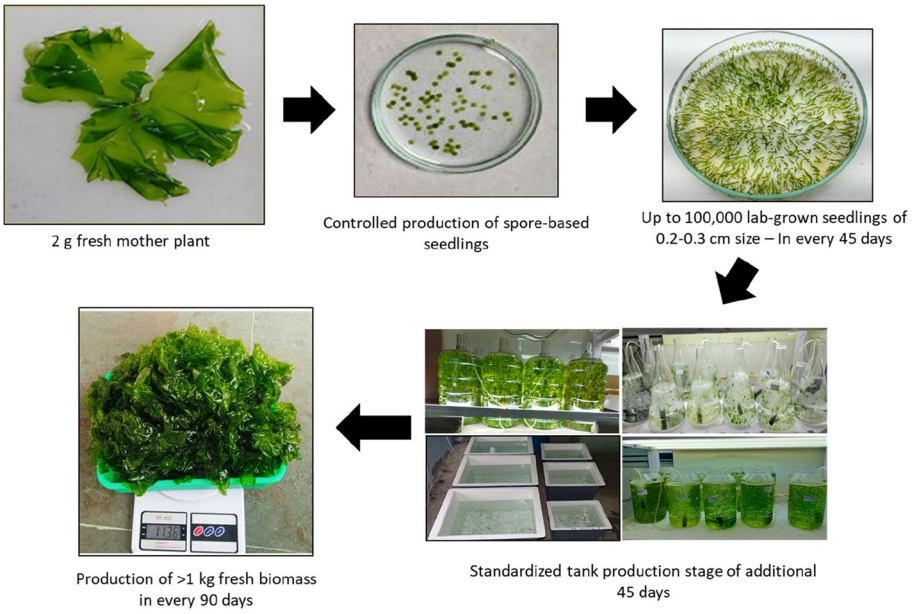
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followed by biomass production in land-based cultivation system. This study would undoubtedly be helpful in food industries, seaweed farmers, stakeholders and *Ulva* biomass production industries for further scale-up.

Keywords *Ulva*, Functional food, Spores, Seaweed, Land-based cultivation

Graphical Abstract



Background

The global seaweed aquaculture has experienced rapid expansion, surpassing other marine production sectors with an annual growth rate of 7.7% since 1970 [6]. The future of seaweed aquaculture is debated, with Hafting et al. [21] that emphasizes on the development of high-value markets for functional foods, cosmeceuticals, nutraceuticals, and pharmaceuticals. Despite 47% of global primary production being marine, only 1.4% of human food is sourced from the sea [48]. Hafting et al. [21] foresee a shift towards consuming products from lower trophic levels, such as algae, mussels and herbivorous fishes.

Among the various macroalgae, *Ulva* is positioned as a versatile solution as it has health benefits for consumers, that makes it valuable for both the functional food market and addressing global food needs [48]. It has been proposed as a model organism for land-based aquaculture [29]. This is essentially due to a cosmopolitan distribution and high biomass productivity (up to 16%). It is rich in protein (4–26% dw basis), carbohydrates (16–65% dw basis), dietary fibers, and vitamins, and also a suitable source for biogas, bioplastics, alcohol, and biocrude production [2, 8, 25]. It is also rich in potential biopolymers, such as Ulvan, which has bio-medical applications [9]. Due to its high capacity for nutrient uptake, it also has bioremediation potential, particularly in removing nutrients waste from animal aquaculture system [19] and can be used as a biofilter of marine ecosystems [55].

Ulva is typically cultivated using the vegetative method which has several limitations including, it solely releases spores once reaching reproductive maturity. The main challenge in vegetative propagation lies in selecting non-reproductive strains, which may lead to reduced genetic diversity and increased production risks over time. To achieve profitable cultivation coupled with enhanced productivity, it is necessary to establish an advanced cultivation protocol through artificial seed production that ensure a sustainable and consistent supply of high-quality biomass [43].

Traditionally, the production of *Ulva* seedlings involved entrapment of zoospores through placing nets under natural beds [47]. However, the success rate of this technique heavily relies on the availability of sexually mature plants in the wild population that limits its practical feasibility throughout the year. Moreover, contamination of intertidal coastal waters, where seaweed beds are located, by sewage and other effluents increases the vulnerability of field-collected spores to microbial attack and raises questions about the quality of developed seedlings. Considering these challenges, other viable methods have been explored for seedling production in the laboratory, including protoplast isolation [50], germling clusters [27], and dark light treatment [40] for artificial seeding. Mantri

et al. [43] also attempted seedling production through reciprocation of values of salinity and temperature for *U. fasciata*, yielding considerable results. However, none of these methods has scaled-up further that can prove their commercial viability. In the present study, spore-based seedling production of *U. ohnoi* has been scaled up to a level of ~ 100,000 viable seedlings through a standardized method which can be practised throughout the year. Furthermore, these seedlings were successfully cultivated in customized land-based tanks (henceforth referred to as reactors) of various sizes to achieve a targeted biomass of 2 kg (fresh). The whole process has been standardized to restrict the cultivation cycle in a total of 90 days, that created an opportunity to undertake four cycles in a year. In addition, another *Ulva* species was also included to evaluate whether the treatments are effective in other *Ulva* spp.

Material and methods

Phase I: Induction of sporulation and spore-based seedling production

Collection of mother plant

Fresh and healthy thalli of *Ulva ohnoi* Hiraoka & S.Shimada and another *Ulva* spp. were collected from single populations found in the intertidal zones of the sea coast at Mithi Virdi village, Bhavnagar district (21.47°N, 72.24°E), and Veraval, Gir Somnath district (20.91°N, 70.36°E), respectively, in Gujarat state of India. Fronds were immediately transported to the laboratory in sufficient seawater under cool conditions. Every thallus was thoroughly washed several times with filtered seawater to remove adhering epiphytes and dirt. Clean algal fronds were cut into 4–5 cm size and placed for acclimatization in an autoclaved seawater for one week at 25 °C, 30 psu salinity under white fluorescent LED light of irradiance intensity 150 µmol photons m^{-2} s^{-1} with 12:12 h light: dark photoperiod. Seawater was replenished every alternate day during the acclimatisation period. As natural seawater has 25 °C temperature and 30 psu salinity, the cultures were further exposed to similar controlled culture conditions. Species identification was done by using rbcL molecular marker as a part of another study in case of *U. ohnoi* Hiraoka & S.Shimada [5], whereas molecular identification was not performed for another species. That species was hereafter mentioned as *Ulva* spp.

Induction of sporulation

Induction of sporulation was tested by applying five different abiotic stresses (fragmentation, salinity, fresh water, desiccation and temperature) separately in *U. ohnoi*. Briefly, fragmentation stress was applied by cutting marginal portion of 4–5 cm large fragment into 0.1, 0.3, 0.4, 0.5, and 1.0 cm size disks, using Biopsy Punch apparatus (Acumen Schimatle, India), following three-time washing for 15 min with sterile seawater [36]. A separate biopsy punch apparatus was used for each disk size. About 1 cm size disk was considered as a whole thallus (control). Hypo- (10 psu) and hyper- (50 psu) salinity stresses were tested as per the procedure described by Mantri et al. [43] with slight modifications. *Ulva* disks, approximately 1 cm in size, were excised from the margins and incubated under salinity conditions of 10 psu, 30 psu, and 50 psu. The salinity of 30 psu was considered as a control. To give freshwater treatment, 1 cm size disks were treated with freshwater for 10, 30, and 60 min respectively [7]. Desiccation stress was applied by dehydrating the disks of 1 cm size for up to 1, 2, and 3 h, respectively. Lastly, temperature stress was tested by keeping 1 cm size disks into 15, 20, 25, 30, and 35 °C [43]. In this experiment a disks kept at 25 °C were considered as a control. All experiments were conducted in triplicate ($n = 3$). Each replicate comprised nine disks of each size, arranged in three plates (three disks per plate to accommodate larger disk sizes).

Sporulation was also tested by applying combination of fragmentation, salinity, desiccation and temperature stresses on *Ulva* spp. For combined stress, 0.3 cm disks prepared from *Ulva* spp. and were incubated with variable salinity (15, 20, 25, 30, and 35 psu), desiccation time (0.5, 1, 2, and 3 h.) and temperature (15, 20, 25, 30, and 35 °C) and the induction of spore release percentage were recorded. The detailed treatments are provided in Supplementary Table 2. As 0.3 cm size disk gave better response (produced sporangia in 24–30 h time, Fig. 3) for *U. ohnoi*, and found easy to handle, it was the only disk size which was tested in the combined stress treatments for *Ulva* spp. The experiment was performed in triplicates ($n = 3$). Each replicate comprised ten disks of 0.3 cm size.

U. ohnoi seedlings were further chosen to standardize the rest of the process. In each set of experiments, disks were kept in a 10 cm size petri plate filled with 25 ml autoclaved seawater at a ratio of 10 disk petri $plate^{-1}$ (standardized in other experiments described below). Every experiment was carried out in three replicates and each replicate contains ten disks of similar sizes. Before applying stress, microscopic observations were taken using Zeiss Axio Imager 1 microscope to make sure all the cells were in vegetative stage. After inducing sporulation at 11 AM in the morning, disks were kept in the dark till the next morning at 8 AM. Microscopic observations were taken every 6, 24 (day 1), 30, 48 (day 2), 54, 72 (day 3), 78, and 96 h (up to four days). Induction of sporulation percentage was recorded by observing the number of oriented cells with respect to disoriented cells. Sporulation was considered induced when ≥ 50% of the cells in a disk showed chlorophyll orientation. Three random microscopic fields from each disk were observed and an

average was taken to display the final readings. Water was replenished every 24 h (up to four days) to ascertain removal of the sporulation inhibitors (such as cross-linked glycoprotein found in the blade cell wall, which is secreted in large amounts to prevent blade cell differentiation Wichard and Oertel [67]). On the fifth day, all the disks were removed, and the released spores were cultured for 10 more days under the controlled conditions as described before.

Standardizing the stocking density

Stocking density of disks The disks 1, 10, 30, 50, and 100 of 0.3 cm size were cultured in petri-plates [10 cm diameter] to standardize the stocking density of disks per petri-plate for achieving maximum output in terms of viable spore-based seedlings. The experiment was performed in triplicates ($n = 3$). The disks were treated with fragmentation stress as described above and cultured for 15 days. On 15th day, the grown seedlings were counted and expressed as the total number of viable seedlings per petri plate and the number of viable seedlings per 0.3 cm disk. After the fourth day, the water was replenished at every five days intervals up to 15 days.

Stocking density of early spore-based seedlings Small seedlings (0.02 ± 0.002 cm) were grown in petri plates for 15 days. They were then transferred to flasks and distributed at stocking densities of 500, 1000, 5000, and 10,000 seedlings L^{-1}, and cultured for an additional 15 days in flask. Thus, the total culture period was of 30 days. Four replicates ($n = 4$) were prepared for each stocking density. Seedling growth was assessed by measuring their length at five-day intervals under a light microscope. For each replicate, five seedlings were randomly selected, and their average length was recorded as the average value was considered. The water was replenished every five days.

Mass production of spore-based seedlings

For mass production of ~ 100,000 seedlings, a new method was developed. Considering that 10 disks (0.3 cm each) produce average of ~ 1067 ± 176 seedlings per petri plate (Fig. 3 in the results) to achieve the target of ~ 1,00,000 seedlings, 100 such petri plates would be required. To reduce the need for glassware, space, and the effort involved in preparing 0.3-cm-sized disks, a less time-consuming novel method was tested by crushing *Ulva* thalli in a handheld mixer grinder. In brief, *U. ohnoi* thalli were crushed in a handheld mixer grinder up to achieve the fragments of ca. 0.2–0.3 cm. The homogenate was repeatedly passed through a fine strainer to remove fragments < 0.2–0.3 cm, while the retained material was spread onto petri plates, and larger fragments were manually removed to select the fragments of 0.2–0.3 cm size. Produced fragments were exposed to fresh water for 10 min followed by desiccation stress for 60 min to achieve maximum sporulation. Variable weight of such fragments, 0.1 g (~ 50–55 fragments), 0.3 g (~ 150–160 fragments), 0.5 g (~ 240–260 fragments), and 1 g (~ 480–520 fragments), were incubated in 150 ml conical flasks containing 100 ml of autoclaved seawater (30 psu salinity) for 15 days. At the end of the initial 15 days culture, produced seedlings were further grown at a density of 10,000 seedlings L^{-1} for another 15 days (total 30 days). The incubation period extended for 15 more days (in total 45 days) to foster the growth of seedlings to the targeted size of approx. 1.0 cm. At the end of 30 days, seedlings were further re-distributed with a lower stocking density of 50, 100, 500, and 1000 seedlings L^{-1} for faster growth. Effect of lower stocking densities on seedling length was checked at the end of 45 days.

Phase II: Systematic land-based cultivation of spore-based seedlings

Process for acclimatization of translocated seedlings

To ensure the successful acclimatization of transported *U. ohnoi* seedlings, they were packed in moist, small size containers (50 mL) which controls temperature (ice cubes and air conditioner facility not required). The unhealthy seedlings were removed after inspection once the seedling consignment reached specific destination (in this study seedlings transported from CSMCRI, Bhavnagar, Gujarat to Marine Algal Research Station, Mandapam camp, Tamil Nadu). The road distance is approximately 2,000 km, and transportation of the seedlings takes about five days. Temperature (23 ± 2 °C) and light conditions (150 µ mol m^{-2} s^{-1}) were maintained during the acclimatization period. The seedlings were transferred from the Falcon tube to a petri plate containing the same water on day 46 (here the days required for transportation are excluded). The seedlings were placed in a 250 ml conical flask with 100 ml of seawater (50 ml existing water + 50 ml new seawater) on day 47. Water exchanges along with ESS media [14] (Supplementary Table 1) was conducted and the seedlings were maintained in the 250 ml round-bottom conical flask with 200 ml of fresh seawater on day 48 and the same method was followed on day 51. The further cultivation protocol explained in Table 1. The irradiance level is ~ 30 µ mol m^{-2} s^{-1} up to 5 lit capacity, 50 µ mol m^{-2} s^{-1} for 20 lit capacity, 150 µ mol m^{-2} s^{-1} for 250 lit capacity.

Standardization of cultivation conditions during land-based cultivation of spore-based seedlings under controlled conditions

To optimize factors influencing the enhancement and scaling up of *U. ohnoi* biomass production, the growth rate and length was evaluated at various cultivation parameters like stocking size, stocking density, and light

Table 1 Overall acclimatization and cultivation process of geographically translocated *U. ohnoi* seedlings for successful land-based seaweed farming. The days required for seedling translocation were excluded from the calculation of the total cultivation period

Period	Process
Day 46	Transfer from falcon tube to Petri dish with same water
Day 47	250 ml round bottom conical flask with 100 ml of seawater (50 ml existing water + 50 ml new seawater)
Day 48	Water exchange along with media (ESS media) and maintain in 250 ml round bottom conical flask with 200 ml of seawater
Day 51	Water exchange along with media (ESS media) and maintain in 250 ml round bottom conical flask with 200 ml of seawater
Day 54 & 57	Water exchange along with media which contains ($NaNO_3$) and phosphate ($Na_2HPO_4 \cdot 12H_2O$) in a ratio of 30,000:2,000 mg dissolved in 1 L + *KA* extract 1.0 ml L^{-1} and maintain in 1000 ml round bottom conical flask with 750 ml of seawater with aeration
Day 60 & 63	Water exchange along with media which contains ($NaNO_3$) and phosphate ($Na_2HPO_4 \cdot 12H_2O$) in a ratio of 30,000:2,000 mg dissolved in 1 L + *KA* extract 1.0 ml L^{-1} and maintain in 6000 ml round bottom plastic container with 5000 ml of seawater with aeration
Day 66 & 71	Water exchange along with media which contains ($NaNO_3$) and phosphate ($Na_2HPO_4 \cdot 12H_2O$) in a ratio of 30,000:2,000 mg dissolved in 1 L + *KA* extract 1.0 ml L^{-1} and maintain in 20 L round bottom plastic culture tub, further maintain in 19 L of seawater with aeration
Day 76, 79, 82, 85 & 88	Water with media which contains ($NaNO_3$) and phosphate ($Na_2HPO_4 \cdot 12H_2O$) in a ratio of 30,000:2,000 mg dissolved in 1 L + *KA* extract 1.0 ml L^{-1} and maintained in 290 L of plastic bags with 250-L seawater with aeration
Day 90	Harvest (production for 90 days and harvest is starting of 91 st day)

intensity during cultivation process and these experiments were done in the jars (310 mm height, 180 mm outer diameter, 11.5 mm mouth diameter, 6 lit capacity with 5 lit of water). To ensure higher survival rates the experiments on standardizing stocking length sizes (large: 1.65 cm; medium: 0.75 cm; small: 0.5 cm) for 21 days, and stocking densities (0.5, 1.0, 1.5 and 2.0 g L^{-1}), light intensities (18.0, 32.0, 42.0 and 74.0 µ mol m^{-2} s^{-1}) experiment for 15 days in 0.006 m^3 round bottom oval-shaped plastic jars were conducted. The experiments were carried out under controlled conditions in triplicates ($n = 3$), maintaining a temperature of 23 ± 2 °C, 30 PSU of salinity, 12:12 h L:D of photoperiod, and a light intensity of 47–50 µ mol m^{-2} s^{-1} (except light intensity experiment).

Scaling-up at 250-L capacity

For scaling up of *U. ohnoi* cultivation in large scale level, the standardized parameters were implemented in the 250-L filtered seawater in the capacity of 290 L reactor under controlled culture conditions (temperature-23 ± 2 °C; pH-7.8; light intensity-150 µ mol m^{-2} s^{-1}). Between 1.0 to 1.5 cm sized *U. ohnoi* seedlings with the initial stocking density of 1.67 g L^{-1} were inoculated and experimental cultivation lasted for 15 days in triplicates ($n = 3$). Every three days, partial water exchange (50%) was conducted, along with media (3N:P + 1.0 mL^{-1} of K-sap; K-sap is a extract of *Kappaphycus alvarezii*) [14] and full water exchange along with cleaning of bag was carried out if the water contamination is high. After 15 days of cultivation, the grown *U. ohnoi* biomass was harvested and the biomass was subjected to DGR, length, nutritional compositions analysis.

Analysis

Growth rate

During a specific time interval (5 days once), the algal fronds were collected from reactor and subjected to the DGR measurement by given formula; daily growth rate (% day^{-1}) = In (FW/IW)/t × 100, where FW denotes final weight and IW denotes preliminary weight of *U. ohnoi,* and t is denoting the cultivation days [12]. Algal fronds' length was measured to evaluate length gain on a scale. The biomass yield was evaluated as per the procedure of Evans [18] with slight modifications and calculation of biomass yield (g wet wt m^2 day^{-1}) = [(W_t-W_0/t]/SA, where W_0 and W_t are initial and final wet weights (wt) in grams, t is time of cultivation days and SA is the side area of the cultivation reactor. The calculation of side area calculation was followed as per following formula (Side Area = π × D × H); D and H are the diameter and height of cultivation reactor in cm.

Photosynthetic pigments

Pigment (chlorophyll a, chlorophyll b, and total chlorophyll) analysis was performed using the 80% ice-cold acetone method described by Arnon [1]. The detailed methodology given in the supplementary file.

Evaluation of nutritional compositions

The nutritional compositions like protein, and carbohydrate content were estimated as per the methods described by Lowry et al. [38], and Dubois et al. [16], respectively. The detailed methodology given in the supplementary file.

Amino acid profiling

Amino acid profiling was performed according to the method mentioned by Kwanyuen and Burton [32]. Briefly, 100 mg dried *U. ohnoi* powder was subjected to hydrolysis using 750µL of 6 N HCl at 110 °C for 24 h in a glass vial. After hydrolysis, both the sample and 10µL of the amino acid standard (AAS18, Sigma-Aldrich, USA) were dried under vacuum. The dried sample was then neutralized using 100µL of an ethanol–water–triethylamine

(TEA) mixture in a 2:2:1 ratio (v/v/v), vortexed thoroughly, and dried again under vacuum. The resulting residue was derivatized by adding 100µL of a mixture containing ethanol, water, TEA, and Phenyl isothiocyanate (PITC) in a 7:1:1:1 ratio (v/v/v/v) and incubated at room temperature for 20 min. Following derivatization, the sample was vacuum dried, and the residue was reconstituted in 2.5 ml of 5 mM sodium phosphate buffer (pH 7.4) containing 5% acetonitrile (v/v). Finally, the solution was filtered through a 0.45 µm membrane filter and analyzed using HPLC (Shimadzu HPLC system, Japan), equipped with a 2695 separation module, a 2996 photodiode array detector, and an auto-sampler. Amino acid separation was performed using a mobile phase composed of two eluents: Eluent A, which consisted of 150 mM sodium acetate with 6% (v/v) acetonitrile and 0.05% (v/v) TEA, adjusted to pH 6.4; and Eluent B, a mixture of acetonitrile and water in a 6:4 (v/v) ratio. Chromatographic separation was carried out on a C18 column (Shim-pack, Shimadzu, Japan) with specifications of 5 µm particle size, 100 Å pore size, and dimensions of 4.6 × 150 mm. The injection volume was set to 20µL, and the flow rate was maintained at 1.5 mL/min. Elution followed a linear gradient: 100% A at the start (0 min), transitioning to 80% and 20% B at 5.5 min, 54% A and 46% B at 10 min, reaching 100% B at 12.5 min, and returning to 100% A by 30 min. Detection of amino acids was performed at 254 nm, with identification based on the comparison of retention times to those of known standards. Relative quantification was achieved by normalizing the peak areas of the amino acids to those of the standards and expressed in mg 100 g-1 of dry *Ulva* biomass.

Statistical analyses

The acquired data were analysed using one-way ANOVA in OriginPro 2024, followed by Tukey's and Fisher's post hoc tests to identify statistically significant differences ($P < 0.05$). All analyses were performed in triplicate, and results are presented as mean ± standard deviation.

Results and discussion

Effect of various abiotic stresses on induction of sporulation

Fragmentation, salinity, fresh water, desiccation and temperature stresses were individually tested on *U. ohnoi* cells while the combined effect of these stresses (except fresh water) was tested on another *Ulva* spp. Experimental results indicate that cells derived from the smallest size disks (0.1 cm) exhibited the highest percentage of induced sporulation, reaching 100% on Day 2 (Figs. 1A and 2). None of the salinity was found to be able to induce sporulation in more than 50% of cells. However compared to control (30 psu) both low and high salinity led to higher sporulation (Fig. 1B). Exposure to freshwater for a mere 10 min can elicit sporulation in 81.7% of cells on Day 2, that is in line with the results obtained on Day 3 when mother tissue fragmented to 0.3 cm size disks (Fig. 1C). The induction of sporulation percentage was investigated by subjecting thalli to desiccation for various time duration, followed by re-suspension in seawater with a salinity of 30 psu. Desiccating *Ulva* thalli for 1 h and subsequently re-suspending them in 30 psu salinity can stimulate sporulation in over 90% of cells by Day 2 (Fig. 1D). The lowest temperature tested (15 °C) demonstrated the highest induction of sporulation percentage

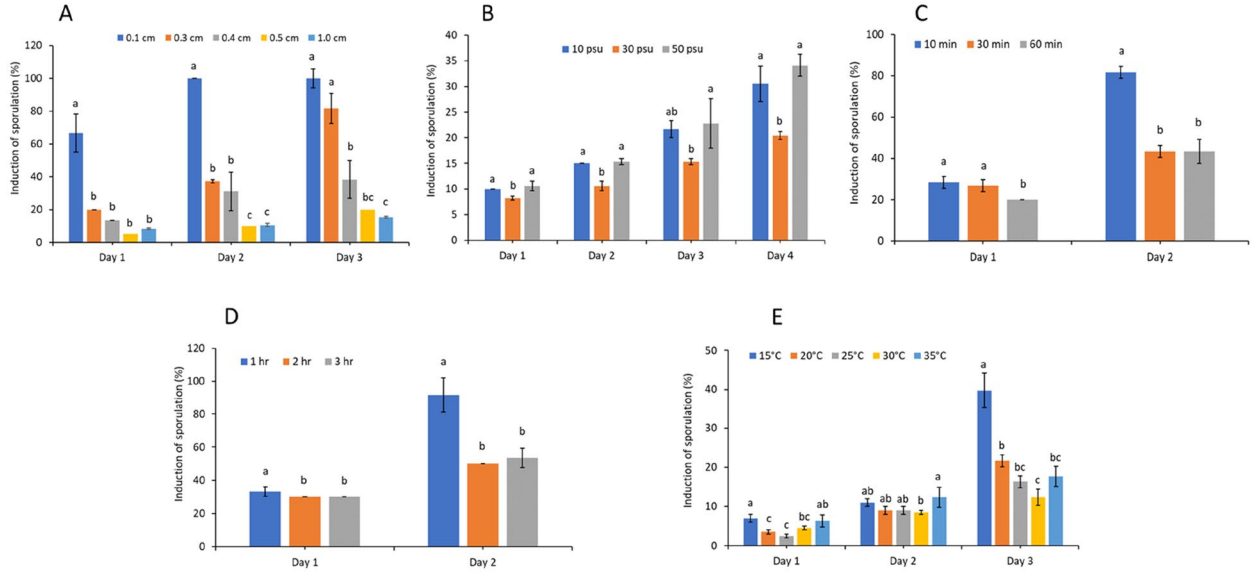


Fig. 1 Effect of various abiotic stresses on induction of sporulation %. **A** Fragmentation **B** Salinity **C** Fresh water **D** Desiccation **E** Temperature. Values are mean of three replicates. Bars followed by different alphabets within the treatment are significantly different at $P < 0.05$ using Tukey's HSD

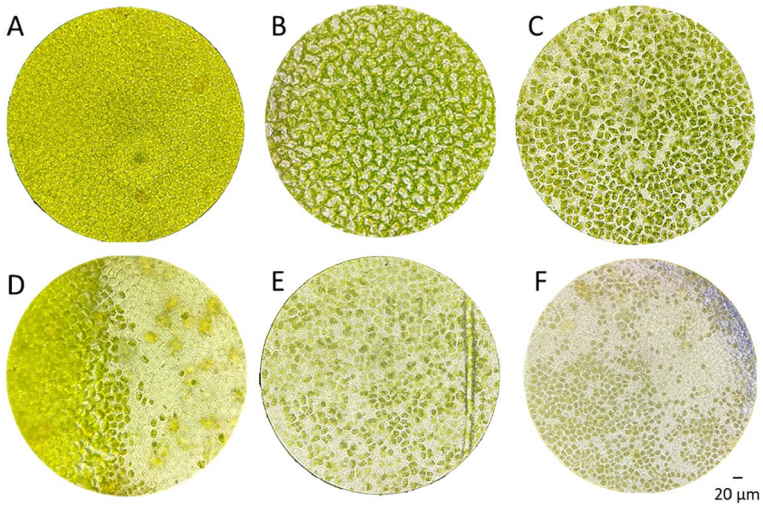


Fig. 2 Light micrographs of *U. ohnoi* cells in the process of sporulation under fragmentation (0.1 cm) stress (**A** Vegetative cells, at 0 h (11 AM); **B** Reproductive cells with oriented chlorophyll, 6 h after induction (5 PM); **C** Reproductive cells before discharging gametes, 24 h after induction (next day at 11 AM);, Reproductive cells discharging gametes, 30 h after induction (next day at 5 PM); **E** & **F** Reproductive cells after discharged gametes, 48 (second day at 11 AM) and 54 h (second day at 5 PM) after induction, respectively. The scale bar represents 20 µm in **A**-**F**

(39.7% by Day 3) compared to all other temperature conditions tested (Fig. 1E).

Experimental results indicate that 0.3 cm size disks of another *Ulva* spp. when kept in standard laboratory conditions (at 30 psu salinity and 25 °C temperature), induction of sporulation was reported up to 70% of its cells. Further, when they were kept in 15 psu salinity or dehydrated for minimum 60 min, sporulation can be induced in 96.7 or 100% of its cells, respectively (Supplementary Fig. 1). 0.3 cm size disks can able to induced sporulation in 70% of *Ulva* spp. cell on day 2 and 81.7% of *U. ohnoi* cells on day 3 (Fig. 1A), that suggests that the fragmentation treatment can be applied for various tropical *Ulva* species, adapted to the same local environment, to produce higher number of spores.

The study enhances our understanding of the effects of various abiotic factors on inducing sporulation in tropical *Ulva* species. Fragmentation, exposure to freshwater, and desiccation stress were found to be effective in inducing sporulation, while variations in salinity and temperature had negligible effects. These findings are consistent with previous studies in other *Ulva* species. Hiraoka and Enomoto [26] reported that 90% of cells exhibited sporulation induction when *U. pertusa* thalli were fragmented into 0.9 mm-sized disks. Similarly, *U. fasciata* Delile achieved 100% zoospore formation within two days when 5 mm size disks were incubated at 15 psu salinity and temperatures of 25–30 °C [43]. *U. mutabilis* Foyn also demonstrated nearly 100% sporulation following fragmentation of thalli into ~ 1,000-cell fragments [46]. *U. prolifera* also exhibited the highest sporulation in 0.75 mm disk fragments, though specific percentages were not mentioned [66].

Although the exact mechanism underlying the differential sporulation response to various abiotic conditions is not clearly understood, some studies provide useful clues. For instance, fragmentation is known to cause the leakage of sporulation inhibitors, prompting cells to enter the reproductive phase more rapidly than non-fragmented cells [26]. Accordingly, in our study, smaller disk sizes led to greater sporulation induction, likely due to more efficient leakage of sporulation inhibitors (such as cross-linked glycoprotein) into the medium [67]. Additionally, the daily replacement of the medium may have facilitated further removal of these inhibitors, thereby supporting faster release of spores compared to non-fragmented cells subjected to other stress treatments. Further, transcriptomic studies have also identified the transcription factor containing RWP-RK domain as a key regulator linking fragmentation stress to the initiation of gametogenesis [36]. Additionally, salinity reduction due to fresh water exposure and desiccation also led to many physiological changes such as alteration in osmotic pressure and cell turgor, which may act as a cue for zoospore formation by stimulating membrane stress signaling, which results in higher sporulation. For instance, Bastos et al. [3] observed higher induction of reproduction when salinity reduced from 35 to 25. Together these studies highlight that sporulation in *Ulva* is regulated through multiple physiological and molecular mechanisms.

Stocking density of disks

The highest total number of viable seedlings (1067 ± 176) in a single petri-plate was obtained when 10 disks were incubated together, compared to other stocking densities. However, the number of seedlings per disk was higher

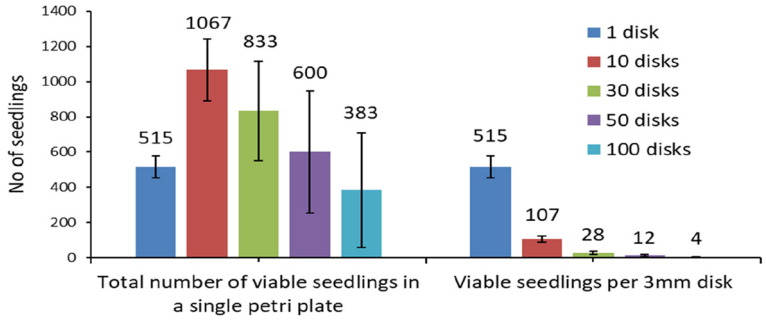


Fig. 3 Effect of stocking density of disks on number of viable seedlings produced per petri-plate

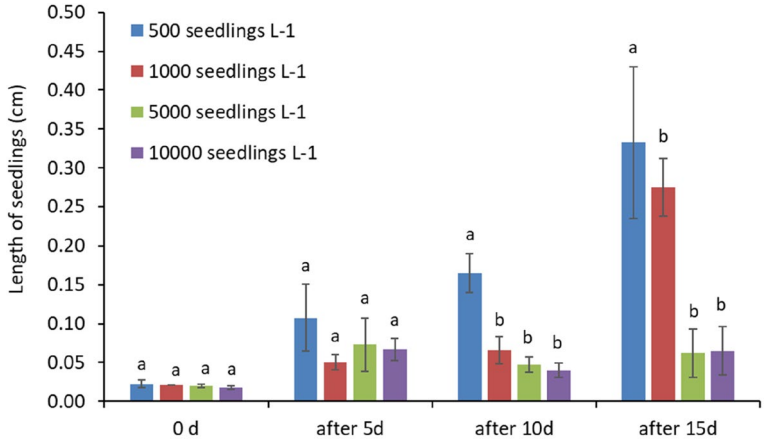


Fig. 4 Effect of different stocking densities on seedling length, where seedlings were cultured for 15 days (days 16–30). Values are mean of four replicates. Bars followed by different alphabets within the days are significantly different at $p < 0.05$ using Tukey's HSD

when a single disk was incubated alone (515 ± 59) (Fig. 3). The number of seedlings decreased as the number of disks incubated together increased. To get the maximum number of seedlings per plate while utilizing lesser resources, a stocking density of ten disks per petri-plate can be utilized.

Increasing the number of disks per unit volume may have elevated the concentration of sporulation inhibitors in the medium, thereby reducing the number of seedlings per plate. In contrast, a single disk, having the largest volume of surrounding medium relative to its own inhibitor release, likely experienced the greatest dilution of sporulation inhibitors, resulting in the highest number of seedlings. This observation aligns with previous findings that the accumulation of sporulation inhibitors can suppress reproductive induction in *Ulva* [26].

Stocking density of seedlings

Effect of various stocking densities (500, 1000, 5000, and 10000 seedlings L^{-1}) on seedling length were further estimated. At the end of the 15-day incubation period (in total 30 days), seedlings cultured with a stocking density of 500 and 1000 seedlings L^{-1} performed better reaching up to 0.33 and 0.27 cm, respectively compared to others (Fig. 4).

Mass production of spore-based seedlings

From the tested variable weight of fragments, the minimum number of fragments (0.1 g $flask^{-1}$) produced the highest number of viable seedlings (5200 ± 200). Based on these results, only 20 such flasks will be required to produce ~ 100,000 seedlings, compared to the 100 petri plates needed if the spore induction step was performed using petri-plates (Table 2). In the first 15 days, 5200 ± 200 seedlings were produced from a stocking density of 0.1 g fragments per 100 ml of seawater.

Approximately 100,000 seedlings of 20 such flasks were then redistributed at a density of 10000 seedlings L^{-1} of seawater and cultured for an additional 15 days (up to 30 days in total), during which each seedling reached a size of 0.065 ± 0.031 cm (Fig. 4). During next step, the seedlings grown with a stocking density of 1000 seedlings L^{-1} were able to gain the target size of 1 cm (an average length of 0.97 ± 0.35 cm) (Fig. 5). To manage with minimum number of glassware, two hundred flasks (500 ml each) were stocked with 500 seedlings per flask (with a stocking density of 1000 seedlings L^{-1}) and cultured for 45 days, yielding an average seedling length of 0.97 cm, from which they were taken forward to Phase II of land-based cultivation.

Table 2 Effect of stocking density of variable fragment weight on number of viable seedlings produced per 150 ml conical flask and the comparative requirement of 150 ml flasks and petri plates in the first stage (at initial 15 days)

Variable weight of 0.3 cm size fragments (g)	Number of fragments	No. of viable seedlings achieved per flask of 100 ml seawater	No. of flasks (150 ml capacity) required to achieve ~ 100,000 seedlings	No. of petri plates required considering ~ 1000 seedlings per petri-plate
0.1	50–55	5200 ± 200	**20**	**100**
0.3	150–160	3967 ± 153	25	
0.5	240–260	3033 ± 153	33	
1.0	480–520	2633 ± 153	38	

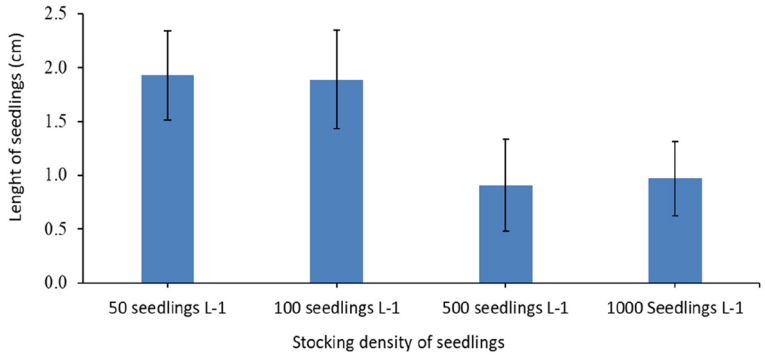


Fig. 5 Length of seedlings after 45 days of culture with different stocking densities applied during the last 15 days (days 31–45). Values are the mean of three replicates

Process for acclimatization of translocated seedlings

The deliberate transportation of planting material between different countries, regions, and locations has directly contributed to the exponential growth of the seaweed industry in Asia over the past decade [10]. Hence, the acclimatization of transported seedlings is considered an important factor that mostly deals with survival rates, and it directly correlates with *Ulva* growth, especially in tanks [14]. From the day of shifting from the seedling facility to the land-based cultivation facility on day 51. As a result of this meticulous acclimatization protocol (Table 1), the survival rate of the seedlings reached 93 ± 0.89% by day 51, marking the sixth day of the acclimatization period. This high survival rate demonstrates the effectiveness of the process in preparing *U. ohnoi* seedlings for successful growth in new environments. Acclimatizing *Ulva* seedlings is crucial for their survival and healthy growth in a new environment. This process gradually helps the seedlings adapt to new temperature, light, and water quality conditions. By doing so, it reduces transplant shock and minimizes stress, ultimately preventing high mortality rates that can result from sudden environmental changes. In addition to improvement in survival rates, acclimatization also increases the likelihood of robust growth and better biomass production.

This process plays a crucial role in stabilizing photosynthetic activity, allowing seedlings to gradually adjust to new light and temperature conditions. Furthermore, acclimatization helps seedlings adapt to differences in water quality, such as salinity, pH, and nutrient levels through prevention in osmotic stress and nutrient imbalances. Reducing stress also enhances the seedlings' resistance to diseases and pests, that ensures strong immune responses. Effective acclimatization protocols are essential for both research and commercial applications, as they ensure reliable and productive seaweed cultivation practices. To adopt and survive various environmental conditions, synthesis of ion and organic osmolytes to maintain the osmotic pressure internally [30]. The changing of light and saline conditions during seaweed cultivation or relocation of, made significant changes in photosynthetic efficiency stated by Marambio et al. [44] who investigated *Desmarestia aculeata* and observed that the changing of maximal quantum yield of PSII. The chlorophyll 'a', and β-Carotene also get influenced by the acclimatization process which will positively enhance the light absorption rate at different irradiance levels after translocation [23]. However, the advantages like cells may protect from oxidative damage when the phlorotannin levels are increased due to lower salinity metabolic reaction stress [44].

Growth improvement by standardizing cultivation conditions

The growth improvement through standardizing cultivation conditions were carried out in 5 lit jars with reference to seedling size, stocking density, and light intensity. The high growth rate (7.53 ± 0.32% day^{-1}) recorded

in large size of seedling, 4.29 ± 0.18% day^{-1} in 1.5 g/5 L stocking density, and 15.60 ± 1.76% day^{-1} in 74.0 μ mol m^{-2} s^{-1} light intensity (Fig. 6). *Ulva* reproduces naturally in a way that seems to be connected to the moon's cycle. Many researchers have extensively studied this natural process, but haven't carried out much research on how to make *Ulva* reproduce artificially [14, 43]. The artificial induction spores may enhance many alternative methods like stress, stimulators and other possible ways [13]. However, there is no report available on the growth improvement of spore-based *Ulva* seedlings in land-based system except studies from Salvi et al. [54] and da Silva et al. [11] who made attempt on through germling cluster method and bench scale Seeded Algal Turf Scrubber system (60 cm L × 13 cm W). Our group previous attempt on 0.005 m^3 plastic containers, 0.1 m^3 and 0.5 m^3 FRP tanks at controlled and semi-controlled conditions on *U. fasciata* [14] and *U. ohnoi* [13].

Likewise, the present study was aimed to improve the growth rate with reference to stocking size, stocking density, and light intensity during cultivation of *U. ohnoi*. The photosynthetic rate, dark respiration, and light usage was mostly depending upon the algal density when it comes to tanks [14], and maintaining proper stocking density plays a crucial role to achieve higher growth rate and biomass yield [51].

Similarly, *Ulva* spp. cultivation at a stocking density of 1–1.5 kg/m^2 in an oxygenated medium resulted in faster growth rates and larger densities, up to 4 kg/m^2, that resulted in no reduction in growth rates [63]. Also, algal stocking densities in tanks may be directly correlated with solar radiation absorption and it should be

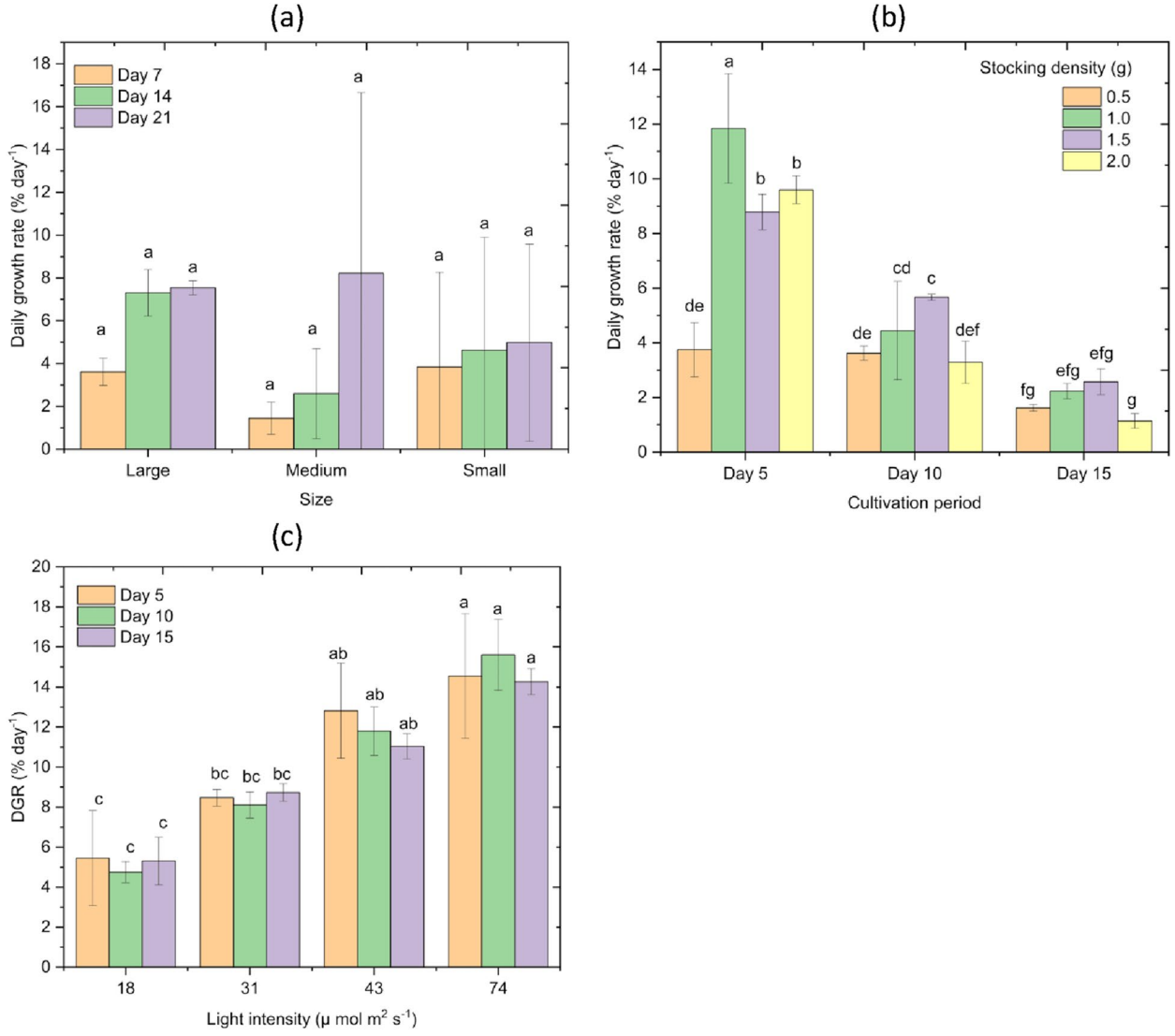


Fig. 6 Daily growth rate of *U. ohnoi* during standardization stocking size (**a**), stocking density (**b**), and (**c**), light intensity. values given as means with SD ($n = 3$). Values in same sampling period different letters differ significantly from each other (ANOVA, $p < 0.05$)

moderate (5–6 kg/m^2) stated by Bidwell et al. [4]. However, the optimal stocking density in tank cultivation may vary with species, culture technique, and climate. But, the higher densities (like 4 kg/m^2) might only provide limited benefits due to competition for resources in tanks [65]. In *Ulva* cultivation, the seedling settlement density in tanks is directly propounded with the size and biomass of the seaweeds, and it also influences the biomass yield and growth rate [58]. The algal frond settlement density is mostly dependent on the seeding density stated by Carl et al. [7], who made an experiment on filamentous *Ulva* cultivation in polypropylene ropes for continuous biomass production under controlled conditions. Steen and Scrosati [58] stated that size and density of seedlings may play an important role in the growth and survival of algal fronds with relation to shading effects and intraspecific competitions, additionally there is a possibility of higher epiphyte accumulations density because of not maintaining proper stocking size and density, especially lower stocking [58]. However, to control biofouling on cultured seaweeds, higher stocking density may help indirectly [39]. The results showed that the highest length observed in large size seedlings was 3.71 ± 0.18 cm, which was statistically significant ($p < 0.05$). In terms of stocking density, the maximum length of 4.51 ± 0.13 ($p < 0.05$) cm was recorded at a stocking density of 1.5 g/L on day 5. Regarding light intensity, the highest length of 3.78 ± 0.03 cm ($p < 0.05$) was achieved under a light intensity of 74 µmol m^{-2} s^{-1} (Fig. 7. The initial energy for photosynthesis in seaweeds is triggered by light, and light illumination enhancement may boost the photosynthesis activity up to a certain threshold; however, excessively high or low light levels can inhibit this process [45, 68] and standardization of light intensity is essential for seaweed cultivation especially when it comes to green seaweed in tanks [14]. The present study results show that higher light intensity (74 µ mol m^{-2} s^{-1}) exhibit better growth than the other tested light intensities (18, 31, 43 µ mol m^{-2} s^{-1}). Makarov [42] stated that the algae have the ability to adapt under various light levels by modification of the ratio of light-harvesting through photo-protective pigments. However, after a certain limit of high light, the light-harvesting pigments and photo-protective pigments are negatively correlated [52]. Hence, the ratio of pigment is considered as a key indicator for algal photosynthesis when light intensity is an influencing factor [60].

Scaling-up production in 250-L capacity

Controlled cultivation and harvesting will be a merit advantage which is available in land-based seaweed cultivation systems and *Ulva* cultivation being on top in that case [22] for selecting tank-based/land-based systems. However, only limited commercial tank-based seaweed cultivation systems (Nordic Seafarm, Algalplus, Monterey Bay Seaweed, and Blue Evolution) is in the operating conditions due to high operational and maintenance cost with low commercial interests by investors [21, 22, 61]. In this study we have demonstrated land-based *Ulva* cultivation at 250-L reactor scale. This offers a viable alternative for large-scale, land-based cultivation of *Ulva*. It represents a low-cost and easily deployable cultivation system that can be established at diverse locations. When adequate light is provided, the system supports higher biomass yields while minimizing the risk of contamination. Minimizing water usage, and energy footprints, managing contamination, standardizing nutrient and carbon provisions, and maintaining optimum temperatures are considered as important key factors for scaling up. The growth rate significantly ($p < 0.05$) increased from day 0 to day 15 and the value was 14.26 ± 0.29% day^{-1}, similarly the biomass yield (33.54 ± 0.97 g ww m^2 day^{-1}) was high on day 10 ($p < 0.05$), and comparatively lesser yield (33.53 ± 0.21 g ww m^2 day^{-1}) was observed on day 15 ($p > 0.05$) (Fig. 8a).

The growth rate significantly decreased when the cultivation days extended and this was similar in *Ulva* in tank cultivation and this finding is in line with the findings of Dinesh Kumar et al. [14] and Majumder et al. [41]. The extending cultivation period more than 15 days could not provide significant enhancement in the biomass yield and growth rate. Extending the cultivation period for seaweed often does not yield significant benefits due to several factors [70] especially when it comes to tank-based cultivation. As seaweed matures, its growth rate tends to plateau, making further cultivation less effective in increasing biomass [37]. Additionally, the other conditions such as nutrient availability in culture container can become less favourable over time, potentially leading to reduced growth or even degradation of the seaweed [31] and also there will be possibility of releasing molecular iodine, and volatile short-lived organo-iodines which is playing major role on biogeochemical cycle [35]. Economically, the extended cultivation period may not be cost-effective, as the additional resources required often outweigh the marginal gains in yield [31]. Moreover, prolonged cultivation can increase the risk of issues like disease and biofouling, further diminishing the quality and market value of the seaweed [24].

Photosynthetic pigments and nutritional characteristics

The photosynthetic pigments constituents were found to vary significantly and the values were 44.52 ± 0.95 mg g^{-1} fw (chlororophyll 'a'), 39.85 ± 1.13 mg g^{-1} fw (Chlorophyll 'b'), and 102.25 ± 1.91 mg g^{-1} fw (total chlorophyll) during end of the cultivation period (Fig. 8b). The pigments level of in *U. ohnoi* are significantly inclined by culture conditions like environmental factors (pH, salinity, temperature) and media along with bio-effectors (level of

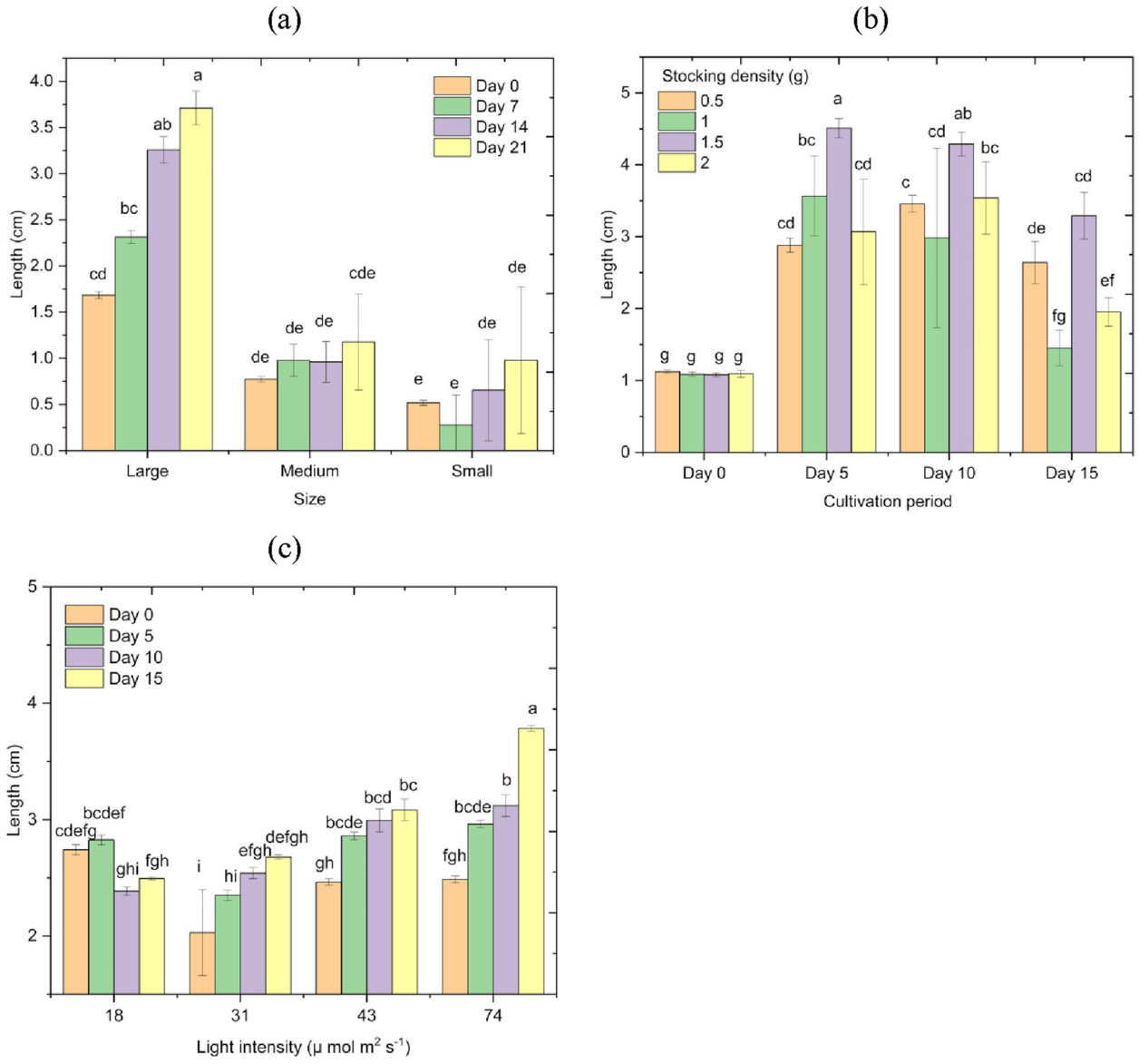


Fig. 7 Length variation of *U. ohnoi* during standardization stocking size (**a**), stocking density (**b**), and (**c**), light intensity. values given as means with SD ($n=3$). Values in same sampling period different letters differ significantly from each other (ANOVA, $p<0.05$)

nitrogen, bio-stimulants, and other bio-elicitors) and this would consider as crucial factor during scaling-up of cultivation especially *Ulva* species which play a crucial role in scaling up its cultivation [14]. Similarly, the biostimulant, AMPEP, has delivered capable outcome by pre-treating *U. ohnoi* with the concentration of 0.01% for 30 min significantly lifts chlorophyll 'a' content to 144.8 μg g^{-1} and total chlorophyll to 235.3 μg g^{-1} during lab level studies [20]. The reported values in lab level (144.8 μg g^{-1}) and during scaling-up (44.52 ± 0.95 mg g^{-1} fw) are comparatively higher which is highlighting potential of AMPEP's to improvement of pigments contents in large-scale systems. The chlorophyll dynamics are fully depending on provision of lights with quality and quantity which will be considered as equally important [33].

The blue and fluorescent lighting may help maintain more pigments accumulation in *U. pertusa* while red LED may reduce the chlorophyll 'a' level up to 40% [34] and the present study we engaged with white LED to retain the chlorophyll content in *U. ohnoi*. The well-controlled and balanced cultivation set-up may help to achieve successful tank-based *Ulva* cultivation along with maximized level of chlorophyll content [17] and white or blue LED combined with AMPEP pre-treatment strategy may provide excellent outcome on tank-based cultivation of *U. ohnoi*.

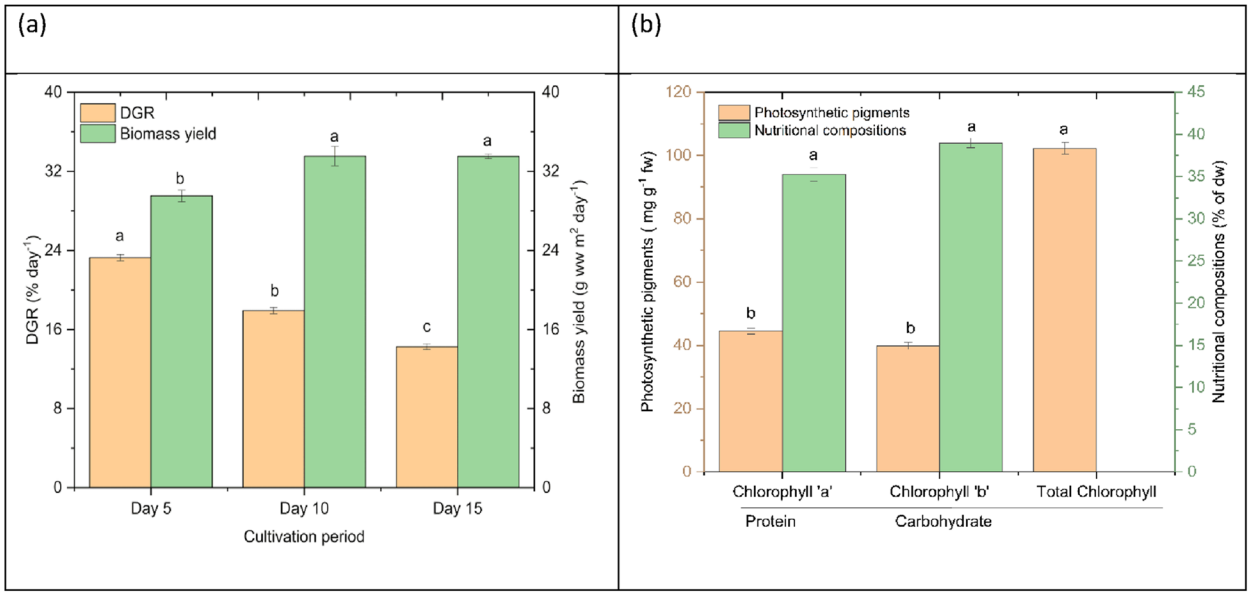


Fig. 8 Growth rate and biomass yield (**a**), photosynthetic pigments and nutritional compositions (**b**) of *U. ohnoi* in 250 L reactor; values given as means with SD ($n = 3$). Values in same sampling period different letters differ significantly from each other (ANOVA, $p < 0.05$)

Table 3 Amino acid profiling of *U. ohnoi* biomass produced by spore-based seedlings. Values given as means with SD ($n = 3$). ND, Not Detected

Amino acids	Quantity (g 100 g protein^{-1})
Non-essential amino acids	
Aspartate	0.090 ± 0.003
Glutamate	0.041 ± 0.002
Serine	0.021 ± 0.003
Glycine	0.061 ± 0.012
Arginine	0.058 ± 0.016
Alanine	0.118 ± 0.025
Proline	0.027 ± 0.006
Tyrosine	0.053 ± 0.005
Cysteine	0.012 ± 0.001
Essential amino acids	
Histidine	0.037 ± 0.004
Valine	0.032 ± 0.002
Isoluceine	0.031 ± 0.001
Leuceine	0.071 ± 0.012
Phenylalanine	0.036 ± 0.002
Lysine	0.022 ± 0.001
Methionine	0.016 ± 0.004
Threonine	ND

These standardized conditions not only enhance the biomass yield and also improve the downstream applications like aquaculture feeds from *Ulva* and pigments (chlorophyll) rich *Ulva* can positively influence pigmentation which taken as feed, and would be profitable commercial feed business. The nutritional component such as protein (35.25 ± 0.81% of dw), was lower than the carbohydrate (38.98 ± 0.55% of dw). Usually in the *U. ohnoi*, the crude protein content reported between 19.2% to 69.4% and it will vary based on the culture conditions and nutrients supplements and present study finding were in line the reported values. This protein source could be utilized for multiple applications, including human and animal consumption as fodder [53]. Usual range of carbohydrate (35% of dw) in *U. ohnoi* and in extreme conditions it will reach 74% of dw [28] and the present study values are little bit less than compared to early reported values. Lobban and Harrison [37] stated that low carbohydrates levels in *Ulva* species might be attributed due to their active role on the formation of the cell wall. Wherever the low carbohydrates reported in *Ulva*, there is possibility of high content of rhamnose (Ulvan) which is accumulated in the cell wall of *Ulva* as uronic acids especially *U. ohnoi* [15]. The accumulated primarily polysaccharides in *Ulva* have multiple health-promoting assets and it can be utilized in food and pharma applications [49].

The amino acid profile of dried *U. ohnoi* powder is presented in Table 3. Biomass produced from spore-based seedlings showed relatively higher concentrations of alanine, aspartate, and leucine, and lower concentrations of serine, methionine, and cysteine. The total amino acid content ranged from 0.012 ± 0.001 to 0.118 ± 0.025 g per 100 g of protein. These values are lower than previously reported ranges for various *Ulva* species (1.87–19.11 g/100 g protein) and *Ulva* spp. specifically (0.4–14.2 g/100 g protein) as reported by Uribe et al. [62] and Shuuluka et al. [56]. Variability in amino acid composition can be attributed to several factors, including the drying method employed. In this study, solar drying was used, which may have contributed to the relatively lower amino acid concentrations observed,

consistent with findings that thermal processing can alter protein composition and reduce amino acid content [62]. Notably, aspartate emerged as one of the predominant amino acids in the present study, aligning with previous observations in *Ulva* species [56]. Additionally, essential amino acids accounted for 33.7% of the total amino acid content, comparable to values reported in *U. capensis*, *U. lactuca*, and *U. rigida*, which ranged from 30.8% to 33.6% [56]. These findings indicate that *U. ohnoi* biomass derived from spore-based seedlings offers a nutritionally relevant amino acid profile with potential for functional food and feed applications.

Industrial prospects, constraints in scaling-up, pathways for betterment

The mixing factors of biological, environmental and engineering are challenging for a land-based scaling-up production of *Ulva*. Indoor tank system consisting tight control of temperature, light, salinity and nutrients while outdoor conditions does not guarantee due to seasonal and site-specific variability (storms, temperature swings, turbidity) that reflect on production yield and nutritional compositions change [69]. Consistent reproduction, and seedling supply are the bottlenecks in large-scale cultivation due to sporulation patterns of *Ulva* and their environmental dependency. Hence, attaining uniform and high-density seedling production at large-scale remains technically challenge [13, 59]. Increasing culture volume or area leads to mass transfer limitations and hydrodynamic which is directly connects with optimal flow, light penetration, and exchange of nutrients along with possibility of self-shading due to higher density, nutrient depletion or anoxic micro-zones that reduce the growth [71]. Outdoor cultivation systems are at higher risk with biofouling, grazers, disease, and inconsistent product quality. Their capital and operational costs including harvesting, and post-harvest handling—along with regulatory constraints, can hinder economic scalability unless carefully engineered and matched to the site [64]. The various industrial applications from Ulva biomass, including functional food ingredients, biofilters, bioplastics (such as ulvan and PHAs), biochar, and bio-oil, due to its high content of proteins, carbohydrates, polysaccharides, minerals, and bioactives [57]. Yield and nutritional composition variations by unpredicted environmental and seasonal fluctuations may not guaranteed sustainable biomass [69]. Infrastructure development requires high investment for reactors, tanks, culture room, temperature control facility, energy requirements (mixing and light control) and post-harvest processing. Improvement with technical understanding is necessary for making *Ulva* production at an industrial scale economically viable and reliable by selecting more vigorous strains, improving reactor designs, managing sporulation, developing continuous cultivation systems, and proper post-harvest processing to sustain product quality.

Conclusion

The controlled production of spore-based *U. ohnoi* seedlings and their scaled-up cultivation in land-based reactor systems are feasible once standardized with proper protocol which is scalable and repeatable. The factors like abiotic stressors, stocking densities, and growth circumstances were all optimized, and the findings of the present study would be considered to increase the yield of high-quality biomass with improved nutritional composition. Other than the standardized and developed process, eliminating contaminants, maintenance of controlled temperature are considered as critical factors. However, these entire processes offer a solid baseline data for the commercial production of spore-based *U. ohnoi* and generate exciting prospects for use in the biomass-based and functional food sectors.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s44377-026-00006-0.

Supplementary Material 1.

Acknowledgements

Mr. Mujeer Habsi thank Dr. Sachin Rathod for sharing his experience and expertise on spore induction techniques in the initial stage of the project.The authors would like to express their gratitude to the Director of CSIR-CSMCRI, Bhavnagar, for support, guidance, mentorship. This contribution has CSIR-CSMCRI PRIS registration number (CSIR-CSMCRI-96/2025).

Authors' contributions

Khanjan Trivedi, conceptualization, resources, conducting experiments, writing original draft; S. Dinesh Kumar, conceptualization, resources, conducting experiments, writing original draft, review and editing, funding acquisition; Abantika Majumder, conducting experiments; and writing review and editing. Mujeer Habsi, conducting experiments; R. Suhail Haq, conducting experiments; literature review; M. Hariprakash, conducting experiments; literature review; K. Logesh, conducting experiments; literature review; Jibananand Nayak, conducting experiments; V. Veeragurunathan, project administration, supervision; Arup Ghosh, project administration, supervision; Vaibhav A Mantri, conceptualization, resources, project administration, supervision, literature review and editing. All authors provided critical feedback and helped shape the review analysis and manuscript.

Funding

SDK would like to express his gratitude to the ANRF (formerly SERB-DST), Govt. of India, support through the EEQ scheme (File No. EEQ/2023/000216, dated 20.12.2023) – GAP2181.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Competing interests

The authors declare no competing interests.

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Received: 18 June 2025 / Revised: 6 January 2026 / Accepted: 6 January 2026
Published online: 26 January 2026

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