

## **Production of quality biomass of *Gelidium micropterum* Kützing through optimization of nutrients and salinity for sustainable land-based cultivation**

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### **Abstract**

In this study, the seedling density (w/v), Provasoli Enriched Seawater (PES) medium strength, and salinity of the culture medium were optimized to obtain higher growth rates and superior biomass quality of *Gelidium micropterum* Kützing. Among the tested seeding densities, 30 mg of biomass per 100 mL of seawater was optimal. The samples cultured in 0 to 100% PES medium strengths at 35 PSU salinity exhibited a daily growth rate (DGR) between 2.73 and 5.79%. A considerably higher DGR% of  $5.79 \pm 0.15$  was obtained by utilizing a 75% PES medium strength. In the salinity experiment, samples cultured with optimized PES medium (75%) at 28 to 36 PSU salinity, showed DGR ranging from 1.84 to 6.20%. The combination of optimized PES medium (75%) and salinity (34 PSU) gave the highest DGR of  $6.20 \pm 0.04\%$  over other tested combinations. The sample exhibited enhanced levels of total phenols ( $2.37 \pm 0.16$  mg GAE g<sup>-1</sup> FW), total proteins ( $20.75 \pm 0.27\%$  DW), and total carbohydrates ( $43.05 \pm 0.18\%$  DW) when cultured under optimized culture conditions. The results of this study could offer valuable primary insights for expanding the cultivation of this important alga in tubular photobioreactors and tank systems on land.

**Keywords:** Carbohydrates, Growth rate, Pigments, Protein, Red seaweed, TPC

## Introduction

In the 21<sup>st</sup> century, seaweed resources have received unprecedented attention worldwide and emerged as one of the -sustainable mariculture systems for the production of food, hydrocolloids climate-friendly feed, chemicals, bio-stimulants, functional ingredients, nutritional supplements, biofuels, biodegradable alternatives to fossil-based plastics, etc. (Yong et al., 2024). While transforming the entrepreneurial opportunities for coastal communities, the innovations in seaweed processing technologies have further expanded the sustainability of the seaweed industry on a global scale (World Bank, 2023). The majority of seaweed harvested globally is primarily utilized in the food industry, followed by the extraction of commercially important hydrocolloids (agar, carrageenan, and alginates) and other commercial applications (Buschmann et al. 2017). Certain seaweed species possess a diverse array of chemical components and nutritional qualities rendering them suitable for both food and hydrocolloid applications. For instance, *Gelidium* and *Laminaria* are used for both edible and hydrocolloidal applications. Agar, a polysaccharide found in the cell walls of red seaweed species, is one of the most valuable hydrocolloids. Agar, which is derived from *Gelidium*, *Gelidiella*, and *Gracilaria* spp., is widely used as an ingredient in a wide range of industries, including biotechnology, food, pharmaceuticals, beverages, healthcare, personal care, textiles, cosmetics, paper coating, printing, etc. (Baghel et al. 2014, Wijayanto et al. 2020). The total trade value of agar globally is estimated at USD 275.36 million in 2023 and is projected to reach USD 375.23 million by the end of 2030 with a CAGR of 4.52% (MMR report, 2024).

Ltd[https://www.maximizemarketresearch.com/market-report/agar-market/199693/#:~:text=Agar%20Market%20was%20valued%20at,4.52%25%20from%202024%20to%202030.](https://www.maximizemarketresearch.com/market-report/agar-market/199693/#:~:text=Agar%20Market%20was%20valued%20at,4.52%25%20from%202024%20to%202030.,), assessed on August 13, 2024). The rising global demand for agar from the food and beverage industries has fueled price rises. Expanding the production of seaweeds, especially agarophytes, is crucial to bridge the growing gap between supply and demand. Besides edible applications, *Gelidium* biomass has also been studied for the production of natural pigments (Mittal et al. 2019), bioethanol (Kim et al. 2015, Park et al. 2012), microfibrillated cellulose (Istianah et al. 2024), and fine chemicals (Qiu et al. 2024, Zhang et al. 2021). Previously, *Gelidium* cultivation has been attempted in the sea at California using a range of designs including rigid, damped, and tensioned test farms in calmer and more turbulent environments and varying depths with/without chemical fertilization and reported specific growth rates of 2% per day in ideal conditions (Melo. et al., 1991). The reattachment process in *Gelidium sesquipedale* (Clem.) Born. et Thur. was studied and reported the ideal temperature (16 and 18 °C) and photoperiod (14:10, 16:8) for maximizing the process of attachment (Salinas and Valdés, 1993). Also, *Gelidium rex* sporulation and the spores' settlement on

mollusk shells has been studied (Rojas et al., 1996). Boulus et al. (2007) studied the effect of outdoor conditions on the growth rate and chemical composition of *Gelidium crinale* in culture and concluded that temperature is the major limiting seasonal factor that positively affects growth rate, dry weight, and gel strength while negatively affecting the agar percentage. Similarly, Li et al. (2019) reported that 22 °C to 26 °C are the optimal temperatures for healthy photosynthesis in *G. amansii*. Recently, an attempt has been made to cultivate *Gelidium pusillum* on the Southeast coast of India and reported a daily growth rate of 0.56–1.05% (Veeragurunathan et al., 2018).

Traditional seaweed cultivation methods rely on either vegetative fragments or spores for their propagation. The seaweeds which are used for carrageenans and agar phycocolloids extraction are farmed using vegetative propagules as their thallus architecture allows the tying of propagules to the ropes. On the contrary, those seaweeds which are either small in size (*G. micropterum*, *G. pusillum*) and/or sheet like thallus (*Ulva*, *Monostroma*, *Porphyra*, etc.) have been farmed using spores and occasionally farmed vegetatively as a tumbling/suspension cultures in tanks and ponds onshore. Also, the open sea cultivation is not feasible throughout the year due to high wave action and variations in seawater composition and temperature in some seasons, and thus exerts a negative impact on the growth of seaweeds and their overall annual yield. In contrast, onshore cultivation offers a promising and reliable option. Though some attempts have been made to cultivate *Gelidium* (Santelices, 1988; Santos and Duarte, 1991, Melo, 1998, and McHugh, 2003) earlier, no one succeed in demonstrating a successful technology for the commercial farming (Friedlander, 2008). The *Gelidium* processed commercially at present comes from wild harvest and some from aquaculture. There has also been an attempt to cultivate *G. amansii* on a small scale in ponds in Taiwan (Santos & Melo, 2018). The development of onshore cultivation systems provides a controlled environment for growing seaweeds year round with consistent quality, yield, and traceability etc. (Suthar et al. 2019, Hafting et al. 2012).

The daily growth rate (DGR) of algal species is one of the important parameters that determines the economic success of seaweed cultivation. The growth rate and chemical composition of seaweeds in general are significantly influenced by salinity and nutrient concentrations in the culture medium. Salinity plays a crucial role in seaweed growth due to osmotic stress (Reis et al. 2011, Macler et al. 1988). Nutrients concentration also be a limiting factor in seaweed growth (Wijayanto et al. 2020). The availability of data for species-specific culture conditions is a must for setting up an onshore culture system. *G. micropterum*, is commonly found as dense clumps (growing up to 5 cm in length) on rocks/ hard surfaces in the intertidal zone (Jha et al., 2009) was selected for this study. The effect of various factors on the growth and chemical composition of *G. micropetrum* remains a critical knowledge gap that requires scientific investigation. Thus, the present study aims at optimizing the

seeding density, PES medium strength, and salinity under a controlled environment for maximising the production of *G. micropterum*. Furthermore, the biochemical composition of biomass grown in different nutrient and salinity regimes has also been evaluated for pigments (R-PE and R-PC), total phenols, protein, and carbohydrates. The present work, for the first time, provide important insights for the establishing onshore tubular bioreactor and tank system intended for the large scale production of *G. micropterum*, an important red seaweed.

## **Material and Methods**

### **Sample collection**

The red alga *Gelidium micropterum* Kützinger was collected from the intertidal rocky shore facing strong water motion at Hawaii Beach (15.4548 N, 73.8063 E) in Dona Paula, Goa, India in January 2023. Samples were brought to the laboratory in Ziploc bags filled with seawater in cool conditions. The contaminants, such as sand, mud, and epiphyte, were cleaned gently with a brush in filtered seawater. The cleaned samples were subjected to acclimatization in the laboratory for 2 weeks in filtered autoclaved seawater (35 PSU salinity) supplemented with 20 mL / L of PES stock (Provasoli, 1968) nutrient enrichment (Supplementary Table 1) at  $25 \pm 1$  °C under daylight white fluorescent lamps at  $15 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  with a 12:12 h light-dark photoperiod, under continuous aeration. The culture medium changed every alternate day.

### **Growth experiments**

To achieve high growth rates and high-quality biomass, seeding density, PES medium strength, and salinity were optimized in triplicate experiments. In the first experiment, the biomass to water make ratio (seeding density) was optimized in autoclaved seawater. After the identification of the optimum biomass to water volume ratio, the PES medium strength was optimized while keeping the salinity of the culture medium at 35 PSU. Following the optimization of PES medium strength and seeding density, the salinity concentration was optimized. All experiments were conducted in flat-bottom borosilicate culture flasks, and randomized in the culture racks in the laboratory. Racks were equipped with white light, and aeration was provided with a Hi Blow HP-80 air pump using an aeration tube and air stones. Aeration speed was controlled by airflow control valves. Hanna Instruments Marine Salinity Tester (HI98319) was used to measure the salinity.

### **Optimization of seeding density**

To optimize the biomass-to-water volume ratio, 10, 20, 30, 40, and 50 mg/100 mL of blotted wet biomass were inoculated in each flask containing 400 mL of filtered autoclaved seawater at 35 PSU salinity and  $\text{pH } 8.27 \pm 0.01$ . The culture flasks were randomized in the culture rack

(Supplementary Fig. 1) and allowed to grow for 2 days at  $25 \pm 1$  °C under a 12:12 h light-dark photoperiod with continuous aeration. The culture period was set to 2 days because in all further experiments, it was planned to change seawater and nutrients on an alternate day (48 h). The salinity changes that occurred due to the evaporation of water was adjusted by making up the water volume using filtered and sterile fresh water after 24 hours of culture setup. After a 2-day culture period, final fresh weights were measured, and daily growth rates (DGR%) were calculated using the following formula (Gupta et al. 2018):

$$\text{DGR \%} = [(W2/W1)^{1/t} - 1] \times 100$$

where *W1* represents the initial fresh weights in milligrams, *W2* represents the final fresh weight in milligrams, and *t* is the total culture days.

### **Optimization of PES nutrient enrichment dosage**

For the optimization of PES nutrient enrichment dosage, an optimized biomass of 120 mg (30 mg biomass/ 100 mL) of blotted wet samples was added to each flask containing 400 mL autoclaved seawater (35 PSU salinity) supplemented with 25%, 50%, 75% and 100% PES enrichment (5, 10, 15 and 20 mL of PES enrichment per L of seawater) in three replicates, randomized in a culture rack (Supplementary Fig. 2) and grown for 12 days at  $25 \pm 1$  °C under a 12:12 h light-dark photoperiod with continuous aeration. The sample cultured in normal autoclaved seawater without PES medium enrichment was taken as a control. The pH in the culture flasks ranged from  $8.16 \pm 0.01$  to  $8.27 \pm 0.01$  (Supplementary Table 2). The salinity of the culture medium was adjusted as described above. The culture medium was changed every alternate day and biomass weights were recorded. Salinity of adjusted every day by making up the water volume using filtered and sterile fresh water. After a 12-day culture period, final fresh weights were measured, and daily growth rates (DGR%) were calculated using the formula mentioned in the seed density optimization section.

### **Optimization of salinity concentration**

To optimize the salinity concentration, the samples were grown for 12 days in a PES medium with different salinities ranging from 28, 30, 32, 34, and 36 PSU in triplicates with an optimized dosage of PES medium strength (75%) as determined from the previous experiment. The PES medium of different salinities was prepared by diluting the filtered sterile seawater using filtered and sterile fresh water and salinities were tested with a Marine Salinity Tester. The average pH in the culture flasks was  $8.18 \pm 0.01$  (Supplementary Table 2). All culture conditions (biomass density, culture medium volume, and photoperiod) were the same as in the PES medium strength optimization experiment (Supplementary Fig. 3).

## Validation of optimized nutrient concentration and culture conditions at the 4 L scale

*G. micropteron* was cultured on a 4 L scale to test the reproducibility of growth results under optimized biomass and culture conditions for 18 days in triplicates. A 1.2 gm (30 mg/100 mL optimized seed density) of blotted wet sample was added to 5 L of flat-bottom culture flasks containing 4000 mL 75% strength of PES medium (optimized strength) prepared in filtered autoclaved seawater at 34 PSU salinity randomized in a culture rack. The rest of the culture conditions, such as photoperiod, aeration and salinity adjustment were remained the same as in other growth experiments (Supplementary Fig. 4). The PES medium was changed on an alternate day along with the biomass weights measurement.

## Biochemical analysis of the harvested biomass

Following the completion of the PES and salinity optimization experiments, biomass was harvested from each treatment, blotted with paper to remove surface water, and then weighed. A  $50 \pm 2$  mg of wet biomass from each sample was dried at 60 °C for 24 h, powdered, and stored for estimation of total carbohydrates and protein. Another  $50 \pm 2$  mg of wet biomass from each sample was dried at 105 °C for 6 h to estimate dry mass. The percentage of change in biomass in comparison to wet biomass was then used for calculating the dry mass. The remaining wet biomass for each of the samples was stored separately at -80 °C for pigments and TPC analysis.

Aqueous extracts were prepared from culture-grown samples collected from both experiments for determining phycobiliproteins (PBPs) and total phenolic content (TPC). In brief, 50 mg of -80 °C wet frozen samples were powdered using liquid nitrogen and added to 1 mL of Milli-Q water. Further, samples were vortexed and incubated for 12 h in the dark at 4 °C. Following the incubation, samples were centrifuged for 10 min at 10,000 rpm, and supernatants were collected and used for pigment and TPC estimations. Phycobiliprotein pigments R-Phycoerythrin (R-PE) and R-Phycocyanin (R-PC) were quantified by recording the absorbances at 564 nm for the R-PE-specific peak, 618 nm for the R-PC-specific peak, and 730 nm for baseline correction using a UV-1800 spectrophotometer (Shimadzu, Japan). The contents of R-PE and R-PC were calculated according to the formula of Sampath-Wiley (Sampath-Wiley et al. 2007):

$$\text{R-PC} = 0.154(A_{618} - A_{730})$$

$$\text{R-PE} = 0.1247((A_{564} - A_{730}) - 0.4583(A_{618} - A_{730}))$$

The total phenolic content (TPC) of the samples was analyzed using the Folin-Ciocalteu method (Baghel et al. 2021). Briefly, 50 µL of the aqueous extract of the samples was added to the 50 µL of Milli-Q water and mixed with 500 µL of Folin-Ciocalteu reagent (1:10 diluted with water) in a glass

test tube and incubated at 25 °C for 5 min. Following the reaction, 400 mL of 7.5% Na<sub>2</sub>CO<sub>3</sub> (w/v in water) was added to each sample and incubated for 2 h at 25 °C in the dark. The UV visible spectrophotometer (UV-1800, Shimadzu, Japan) was used to read absorption spectra at 760 nm. TPC contents were calculated with the help of a gallic acid standard curve, and values were presented in mg g<sup>-1</sup> gallic acid equivalents (GAE) TPC on a fresh weight basis.

To quantify total protein content, 5 mg of 60 °C dried powdered samples were added to 800 µL of Milli-Q water vortexed and incubated at 4 °C for 12 h. After incubation, 200 µL of a 7.5 M NaOH solution was added to make a final molarity of 1.5 and autoclaved for 90 min at 100 °C. Autoclaved samples were neutralized using 6 N HCl and centrifuged at 10000 rpm for 10 min. The estimation of protein content was done following the Lowry method (Lowry et al. 1951). Total protein contents were calculated with the help of a BSA standard curve, and values were presented on a dry weight basis. To analyze the total carbohydrates, 10 mg of powdered samples and 100 µL of Milli-Q water were taken in 10 mL of the test tube, mixed by vortexing, and incubated at 4 °C for 12 h. Following the incubation, 193 µL of 98% H<sub>2</sub>SO<sub>4</sub> (w/w) was added to make a final H<sub>2</sub>SO<sub>4</sub> concentration of 72% and vortexed. Further, samples were hydrolyzed in two steps; in the first step, samples were incubated at 37 °C for 1 hour. After incubation, the H<sub>2</sub>SO<sub>4</sub> concentration was decreased to 4% (w/w) by diluting with Milli-Q water, and it was further hydrolyzed for 2 h at 120 °C. The pellets were removed from the hydrolyzed samples by centrifugation at 5000 rpm for 5 min. Carbohydrate estimation was carried out following the phenol-sulphuric acid colorimetric assay (Dubois et al. 1956). The calculation was done with the D-glucose standard curve, and values were presented in percentages of dry weight.

### **Statistical analysis of data**

All the experiments were performed in triplicate. With JMP software (JMP®, Version 18. SAS Institute Inc., Cary, NC, 1989–2024), data was analyzed for one-way ANOVA followed by statistical significance using Tukey's HSD post hoc test. The values displayed are the three biological replicates' averages ± standard deviation. According to Tukey's post hoc test, different letters (a, b, and c) denote significant differences between means (P < 0.05).

## **Results and discussion**

### **Growth rates**

For the seaweed to water volume optimization, the daily growth rates ranged from 0.69 ± 0.13 to 3.40 ± 0.26%. The culture inoculated with 10 mg/100 mL to 30 mg/100 mL wet biomass was recorded with a significantly higher growth rate (3.31 to 3.40%) in comparison to growths (0.69–1.46%) for 40 mg and 50 mg/100 seeding (Fig. 1 and Supplementary Table 3). However, there was no significant

difference recorded for 10–30 mg/100 mL seeding, so the 30 mg/100 mL seed density was considered optimal for further growth experiments.

The PES nutrient enrichment is a mix of macronutrients, micronutrients, trace metals, and vitamins that are required for the growth of a wide range of seaweeds. However, the optimal strength of the PES medium for higher growth may vary from species to species (Yuniarti et al., 2018). The PES medium strength 0, 25, 50, 75, and 100% at 35 PSU salinity showed a wide range of DGR% from  $2.73 \pm 0.39$  to  $5.79 \pm 0.15$  % (Fig. 2A). The lowest DGR% was recorded for the control (0% PES strength) and the highest with the 75% PES strength which was significantly higher than 0%, 25% and 50% studied (Supplementary Table 3). The PES medium strength in culture medium showed a linear relationship with DGR up to 75% and thereafter reduced, although it was marginal. This could be due to nutrient saturation with 100% PES medium strength, and precipitation was also witnessed in the medium. The 75% PES medium strength was found optimal, and this concentration was used for further salinity optimization experiments.

Among the investigated salinities (28, 30, 32, 34, and 36 PSU), the DGR ranged from  $1.84 \pm 0.49$  to  $6.20 \pm 0.61$  % (Fig. 2B). The highest DGR was recorded for 34 PSU, which was significantly higher than the DGR obtained for other ranges investigated in this study (Supplementary Table 3). The DGR (6.20%) recorded in the present study was higher than those previously reported: 1.9% for *Gelidium corneum* (formerly *G. sesquipedale*) (Carmona et al. 1996), 1-3.6% for *Gelidium robustum* (Pacheco-Ruiz et al. 1995), and comparable with 7% for *Gelidium sclerophyllum* (Rodríguez et al. 1996), 6.5% for *G. crinale* (Boulus et al. 2007), and 6.7% for *G. amansii* (Shunzo, 1971) cultured in the laboratory, in situ, or ex-situ (in tanks). Further, the DGR% obtained in this study was also superior than those reported for field cultivated *Gelidium pussilum* 0.56 to 1.05% (Veeragurunathan et al. 2018) and *Gelidiella acerosa* (1.11–1.31), from the Indian sea coast (Ganesan et al. 2011). In this study, the supplementation of culture medium with 75% PES nutrient enrichment and 34 PSU salinity yielded the highest DGR (6.20%).

### **Reproducibility of growth results at 4 L culture scale and culture scalability**

The primary experimental data generated from optimisation studies has also been further further validated by culturing *G. micropterum* in 4 L scale for 18 days. The growth rate recorded for the 0-6 days was  $5.36 \pm 0.14\%$  d<sup>-1</sup>, for 6-12 days  $6.49 \pm 0.82$  and for 12-18 days was  $4.50 \pm 0.42$  d<sup>-1</sup> (Fig. 3). The DGR% recorded for the 12-18 days was significantly lower as compared to those recorded for the 6-12 days culture period (Supplementary Table 3). The DGR% reduction could be due to the cultures reaching growth saturation. To have the alga in a continuous growth state even after a 12-day, there is



a need to either increase the culture medium volume proportionately, spike the nutrient content, or lower the seeding density in the culture. However, harvesting the grown biomass by leaving behind earlier determined seed material once DGR% shows declining trend would be a better strategy than the other alternatives. DGR for a 0-12 days period ( $5.85 \pm 0.47\% \text{ d}^{-1}$ ) was comparable to those obtained with a 400-mL culture scale. The culture medium used for culturing this alga in the lab was filtered and autoclaved to make it free from contaminants. Therefore, there was no visible epiphytic growth observed in the culture system during the experimental period. The reproducibility of growth results on a 4 L scale indicates the scope for further expansion of cultivation of this alga in a tubular airlift photobioreactor under controlled conditions in the laboratory. Nevertheless, cultivation in open-air tanks needs further investigation to understand how the temperature impacts the growth, epiphytes, abiotic stress arising from changes in the salinity due to seawater evaporation, etc. According to earlier reports, one of the main concerns with *Gelidium* cultivation in open tanks or ponds was epiphytic contamination (Friedlander, 2008).

Three types of methods can be used to manage epiphytic contamination in the culture system (Sahu et al., 2020): 1) physical methods, 2) chemical methods, and 3) biological methods. Among the physical methods, a) using contamination-free planting material, b) filtering seawater using diatomaceous earth or filter cartridges or sand filters, and c) frequent changes of seawater in the tanks may be a possible solution to minimize the incidence of epiphytic growths in the culture system. Further, this alga grows naturally on an intertidal rocky shore facing strong wave action showing its natural tolerance to slamming waves. Thus, providing strong water motion in the culture system may also help to minimize the epiphytic attachment on the thalli.

In chemical methods, certain chemical treatments, such as rinsing seaweed in specific concentrations of chlorine and copper, were found effective in reducing epiphytic growths (Sahu et al., 2020). The pre-treatment of the seawater, tanks, and equipment has mostly involved the use of sodium hypochlorite, found effective in avoiding epiphyte growths (Ugarte and Santelices 1992). According to several studies, epiphytes can be suppressed by regulating pH levels (Friedlander 1991), especially when pH levels are high (Haglund and Pedersén 1992).

In the biological methods, grazers such as *Gammarus lawrencianus* and *Idotea baltica* were found effective in eliminating epiphytic growth of *Enteromorpha* spp (now grouped in *Ulva*). and *Ectocarpus* spp. from the culture system (Shacklock and Dolye 1983). Other than this, growing seaweeds in high densities in tanks may be helpful to control the epiphytes (Bidwell et al. 1985). However, as the efficacy of these techniques varies from species to species, appropriate strategies to prevent epiphytic contamination for this alga may be chosen after systematic experimentation.

## Dry Mass

The dry mass for the samples grown in 0-100% PES medium strength at 35 PSU ranged from  $40.24 \pm 0.53$  to  $44.92 \pm 0.48\%$  (Fig. 4A). Dry mass for the control and 100% PES medium strength were higher than the samples grown at the rest of the PES strength. Samples grown in 25–75% PES medium strength had marginally high water content, which may be due to the tendency of seaweeds to retain high water content in optimal culture conditions. At the same time, dry masses for samples grown in 75% PES medium strength in 28–36 PSU salinity ranged between  $40.24 \pm 0.09$  and  $42.04 \pm 1.21\%$ , respectively, without any significant differences (Fig. 4B and Supplementary Table 3). The dry mass obtained in this study for *G. micropterum* was higher than those reported for tank cultivated *Gelidium crinale* 25% (Boulus et al., 2007), *Gelidium robustum* 15-26% (Sousa-Pinto et al., 1986), and for other red seaweeds 13.4–30.5% (Olsson et al. 2020). Low biomass density to water volume is one of the major concerns when seaweeds are cultivated in photobioreactors, tanks, and ponds. This can be addressed by selecting the species forming higher dry mass. For example, *Gracilaria dura*, a source of microbiology-grade agar, showed a  $12.24 \pm 0.09\%$  dry mass and  $25.15 \pm 0.78\%$  agar yield (Baghel et al., 2015), while *G. micropterum* exhibited  $\geq 40\%$  dry mass and  $23.54 \pm 0.52\%$  agar content (Baghel et al., 2014). Although the agar content of *G. micropterum* is comparatively lower than that of *G. dura*, the processing of an equal quantity of fresh biomass of both species, *G. micropterum* could provide more agar than *G. dura* due to its high dry mass formation quality. The high organic content in total dry biomass is advantageous in value chain development studies and leads to the recovery of higher product yield over those with less organic content in dry biomass.

## Pigments and TPC contents

Natural pigments come in a variety of hues and have long been extensively used in everyday life. A few sectors deserving of attention are food production, the textile and paper industries, water science and technology, and agricultural practice and research (Cserháti et al. 2006). Pigments are of great value in the industrial perspective due to their important features and their favorable biological activities as antioxidants and anticancer agents. As a result, they have a huge potential to meet present economic demands, which have been increasingly directed toward the biotechnology and health sectors in search of natural compounds and goods with proven positive effects on humans (Cserháti et al. 2006). Seaweed contains a variety of pigments, including phycobiliproteins, fucoxanthin, violaxanthin, antheraxanthin, zeaxanthin, lutein, and neoxanthin. In red seaweeds, the phycobiliproteins are the predominant pigments. The R-PE and R-PC content varied with PES medium strength in the culture medium and ranged from 0.58 to  $3.1 \text{ mg g}^{-1}$  and 0.20 to  $0.76 \text{ mg g}^{-1}$  respectively on a fresh weight basis (Fig. 5A). The samples grown in culture medium supplemented

with PES enrichment showed significantly higher R-PE and R-PC content than the control (Supplementary Table 3). However, there was no significant difference recorded among the different dosages of PES enrichment. Whereas the samples grown at 28 PSU had the lowest R-PE and R-PC concentrations without significant differences compared to those grown at other salinities (Fig. 5B). Among the samples grown at 30-36 PSU salinities, there was no significant variations observed. The sample with the highest growth rate (75% PES strength and 34 PSU salinity) contained R-PE of 2.05 mg/g fresh weight and R-PC of 0.5 mg/g fresh weight basis. The R-PE and R-PC contents were higher than those of *G. micropterum* (1.03 and 0.26 mg) and *G. pusillum* (0.88 and 0.44 mg) reported for wild-harvested samples (Baghel et al. 2014).

The samples grown at 35 PSU salinity with different PES enrichment dosages (0–100% PES strength), TPC content ranged from 1.76 to 2.81 mg GAE g<sup>-1</sup> fresh weights (Fig. 6A). The highest TPC content was recorded for the sample cultured in 75% and 100% PES medium strength, which were significantly higher than other PES strengths (0 to 50%) (Supplementary Table 3). All PES-enrichment-supplemented samples showed significantly higher TPC content than the control. With increasing dosages of PES enrichment, it showed an increasing content of TPC. The TPC content was found to remain stable and did not show any variations with changing salinities and it ranged between 2.37 and 2.72 mg/g GAE g<sup>-1</sup> fresh weights (Fig. 6B). The samples with the highest growth rate (75% PES strength and 34 PSU salinity) contained 2.37 ± 0.16 mg GAE g<sup>-1</sup> FW of TPC on a fresh weight basis (or 5.7 mg g<sup>-1</sup> for corresponding dry weight). The estimated TPC content is higher than those reported for for *Pyropia vietnamensis* (*Phycocalidia vietnamensis*, Rhodophyta) with 0.563 mg GAE g<sup>-1</sup> FW (Baghel et al. 2023), 2.04 mg GAE g<sup>-1</sup> dry weight for the edible green alga *Caulerpa lentillifera* (Chlorophyta) (Nguyen et al. 2011), and 4-5 mg g<sup>-1</sup> DW for several red seaweeds (Tibbetts et al. 2016) and comparable to 6.82 ± 0.26 GAE g<sup>-1</sup> reported for *Gelidium corneum* (Cavaco et al. 2021). Phenolic compounds, or phenolics, are metabolites that have drawn significant attention recently due to their bioactive properties with several health benefiting applications advantages. Phenolics, which comprise tannins, chalcones, coumarins, and flavonoids, are known antioxidant metabolites, and their presence enhances nutraceutical values of foods.

### **Total Protein content**

One of the most common macromolecules in a cell is a protein, which is essential for many metabolic activities as well as cell growth. The need for protein has grown worldwide due increasing world population, and plant based proteins, particularly seaweed-based protein is gaining traction as an alternative to animal protein (Gajaria et al. 2017). Seaweed proteins have been the subject of research in recent times due to their bioactive properties such as anticancer, anticoagulant,

antibacterial, antiviral, and antioxidants (Pliego-Cortés et al., 2020). The protein content in samples grown in 0-100% PES medium strengths ranged from  $7.73 \pm 0.47$  to  $20.31 \pm 0.12\%$  on dry weight basis (Fig. 7A). The highest total protein content was recorded for the samples grown in 75% PES medium strength (20.31%), which was significantly higher than the control and the 25% PES medium strength but not statistically different from those grown in medium with 100% (18.80%) and 50% (18.45%) PES medium (Supplementary Table 3). When compared to the control, all samples grown in the PES medium had significantly higher protein content indicating the positive effect of nitrogen content in the PES medium. Further, in the salinity optimization (28–36) experiment, the protein content ranged from  $17.99 \pm 0.26$  to  $20.75 \pm 0.27\%$  on dry weights (Fig. 7B). The samples that showed the highest growth rate when grown in medium within 75% PES strength and 34 PSU salinity showed the highest protein content (20.75%), which was significantly higher than the samples grown in 28 and 36 PSU salinities; however, the samples grown in 30, 32, and 34 PSU salinities did not show significant differences in their protein contents (Supplementary Table 3). The protein content recorded with 75% PES medium strength and 34 PSU salinity was higher than the 6.25% for *G. corneum* (Cavaco et al. 2021),  $13.23 \pm 1.06\%$  for *Gelidium* sp. (Ashoush et al., 2017), 19.31% for *G. pusillum*, 17.56% for *P. vietnamensis* (Baghel et al. 2023) and lower than 23.40% for *G. microdon* (Paiva et al. 2017). In this study, it was confirmed that the PES enrichment dosage plays a significant role in increasing total protein contents. *Gelidium* biomass with good protein content could be used for direct consumption or could be utilized for protein extraction for nutraceutical applications.

### **Total carbohydrates**

The total carbohydrate contents of samples grown in medium with 0-100% PES strength ranged from  $37.69 \pm 0.32$  to  $58 \pm 0.44\%$  on a dry weight basis (Fig. 8A). The highest total carbohydrate content was recorded for control ( $58 \pm 0.44\%$ ), followed by 75% PES medium strength (43.61%). The high carbohydrate content in control may be attributed to the reduction of other components, such as protein and pigments, due to nutrient deficiency. It was comparable to the carbohydrate content  $53.66 \pm 1.21\%$  reported for the *Gelidium* sp. collected from the Egypt (Ashoush et al., 2017) and (56.73%) reported for the moderately bleached red alga *P. vietnamensis* (Baghel et al. 2023). Further, in the salinity optimization (28–36) experiment, the carbohydrate contents ranged from  $39.81 \pm 0.43$  to  $43.05 \pm 0.18\%$  on dry weights (Fig. 8B). The total carbohydrate contents recorded for 34 and 36 PSU salinity-grown samples were significantly higher than those grown at 28 and 30 PSU salinity. The samples grown with the highest growth rate (75% PES strength and 34 PSU salinity) recorded a carbohydrate content of  $43.05 \pm 0.18\%$ , which was higher than the 39.5% for *G. corneum* (Cavaco et al. 2021),

40.29% for healthy *P. vietnamensis* (Baghel et al. 2023) and 17.62% for *G. microdon* (Paiva et al. 2017).

## **Conclusion**

The present investigation revealed that the growth rates and biochemical composition of *G. micropterum* are significantly influenced by the PES medium strength and salinity. The findings of this study suggest 75% PES medium strength with 34 PSU salinity could be used to achieve higher growth rates as well as high-quality biomass for *G. micropterum*. The biomass derived from optimized culture conditions can be used for edible applications or valorised for the production of a stream of products in a biorefinery model, improving the overall sustainability. The results of this study may offer a fundamental understanding for expanding *G. micropterum* farming onshore.

## **Acknowledgments**

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## **Author's contribution**

We declare that all Authors listed on the title page have contributed significantly to work. R.S.B. designed the experiments; R.S.B., A.AK.S. and ADV performed the experiments; R.S.B. and CRK.R. analyzed and interpreted the data. R.S.B., A.AK.S. and CRK.R. wrote the article and all authors approved the final manuscript.

## **Declarations**

**Ethical Approval** Not applicable.

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**Availability of data and materials** The authors assert that the data underpinning the findings of this study are accessible within both the article and its supplementary information files.

**Competing interests** The authors declare no competing interests.

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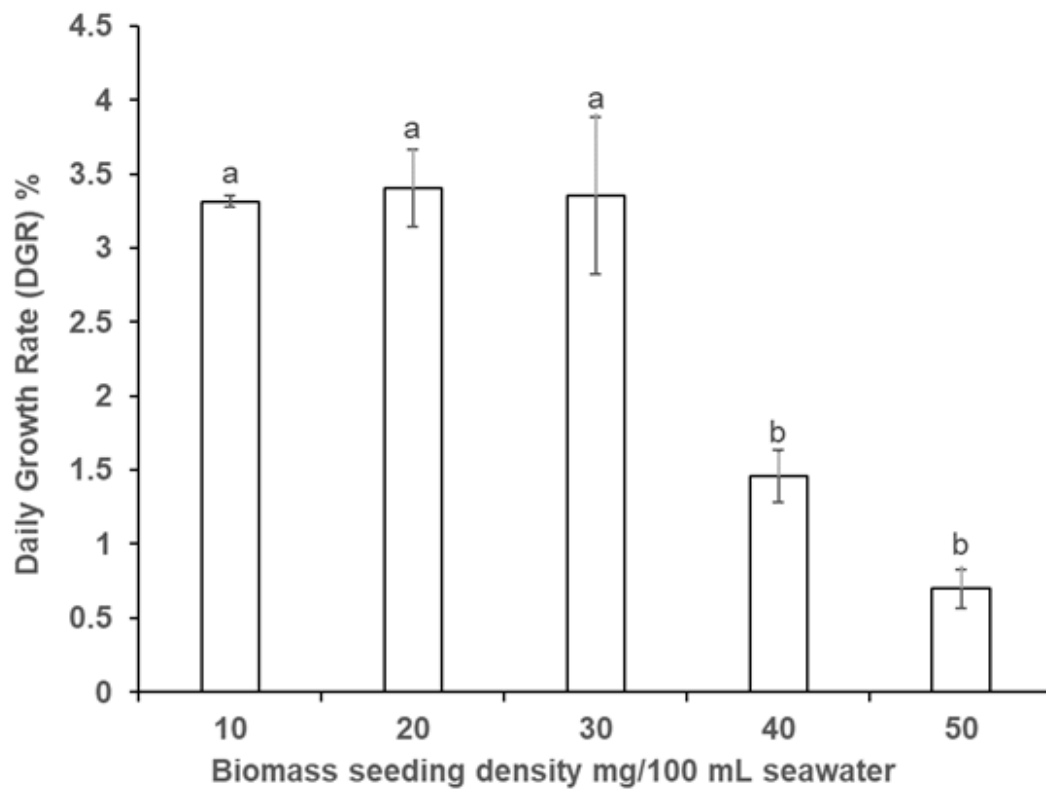
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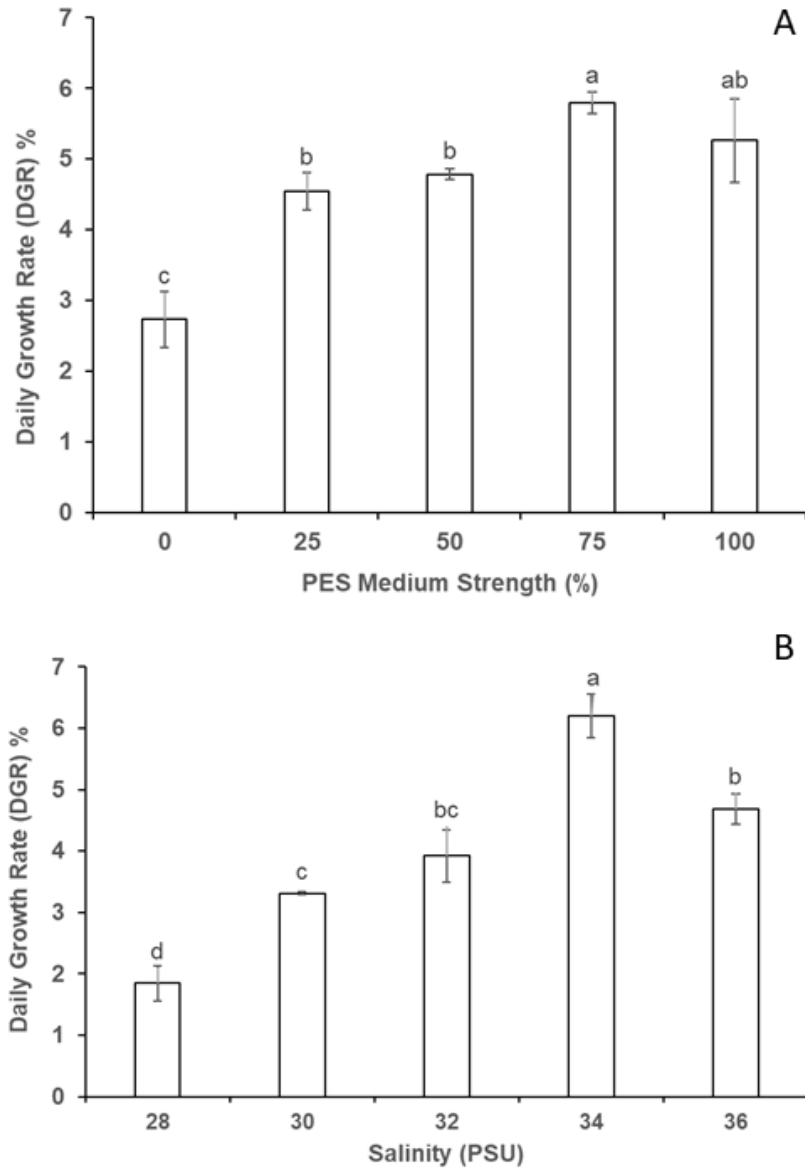


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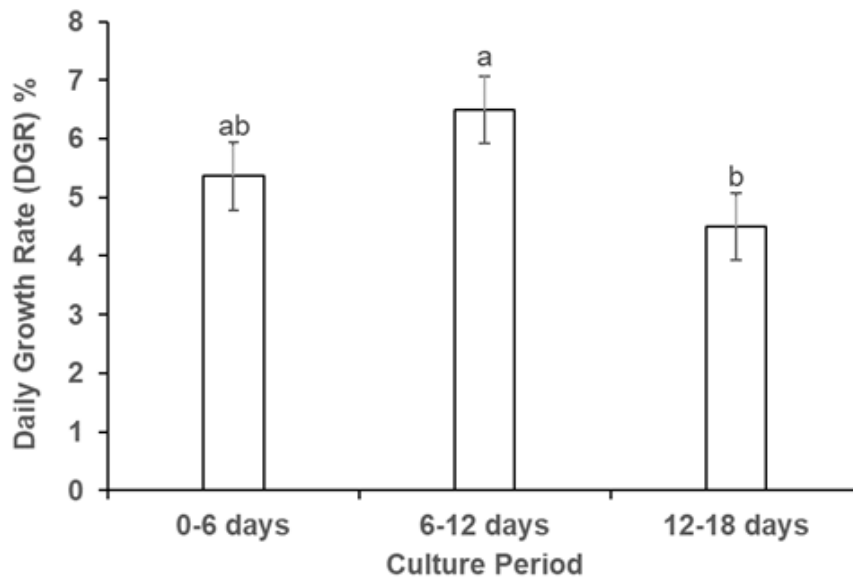
## Figures



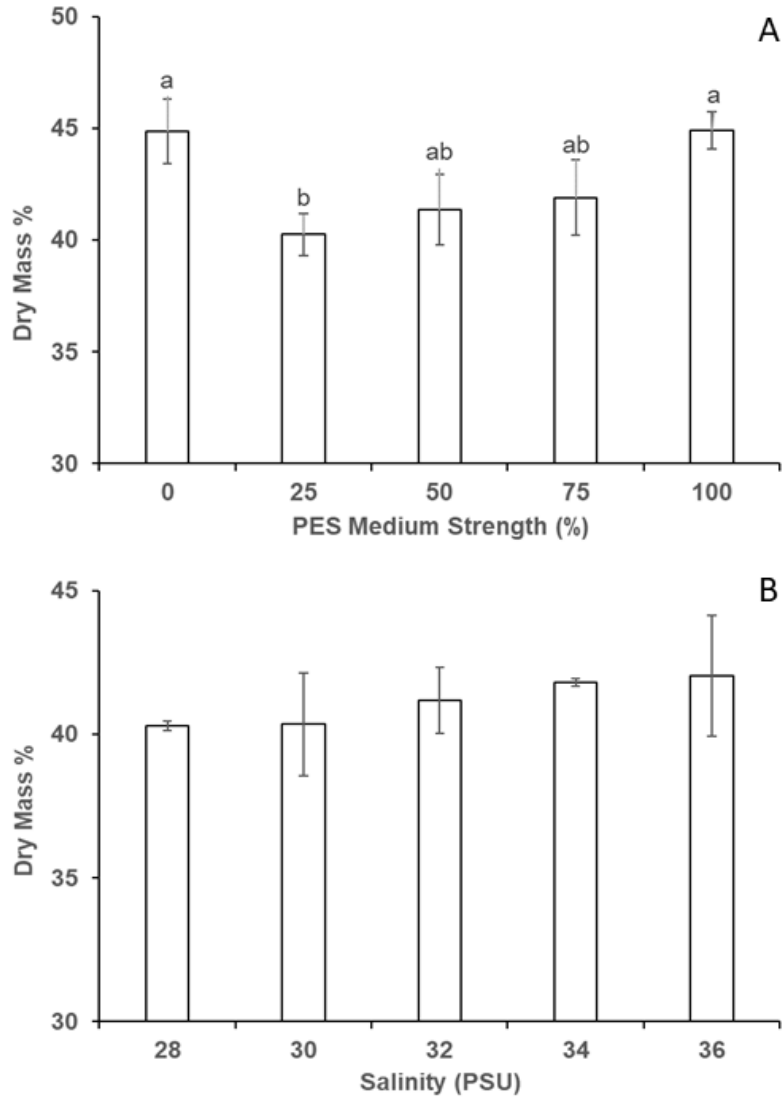
**Figure 1** Growth rates for *G. micropterum* obtained with different seeding densities. Values are averages of three biological replicates ( $n = 3$ )  $\pm$  SD. Different letters indicate significant differences according to the one-way analysis of variance and Tukey's test ( $p \leq 0.05$ ).



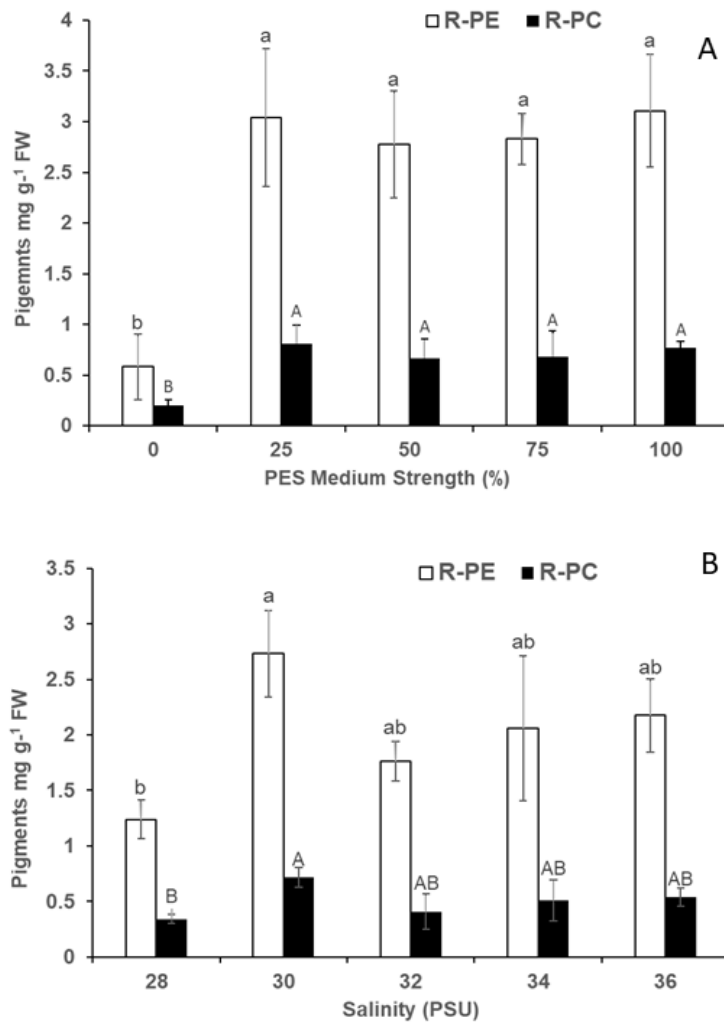
**Figure 2** Growth rates for *G. micropteryum*: A, *G. micropteryum* grown at different PES medium strengths with 35 PSU salinity, B, *G. micropteryum* grown at different salinities with 75% PES medium strength. Values are averages of three biological replicates ( $n = 3$ )  $\pm$  SD. Different letters indicate significant differences according to the one-way analysis of variance and Tukey's test ( $p \leq 0.05$ ).



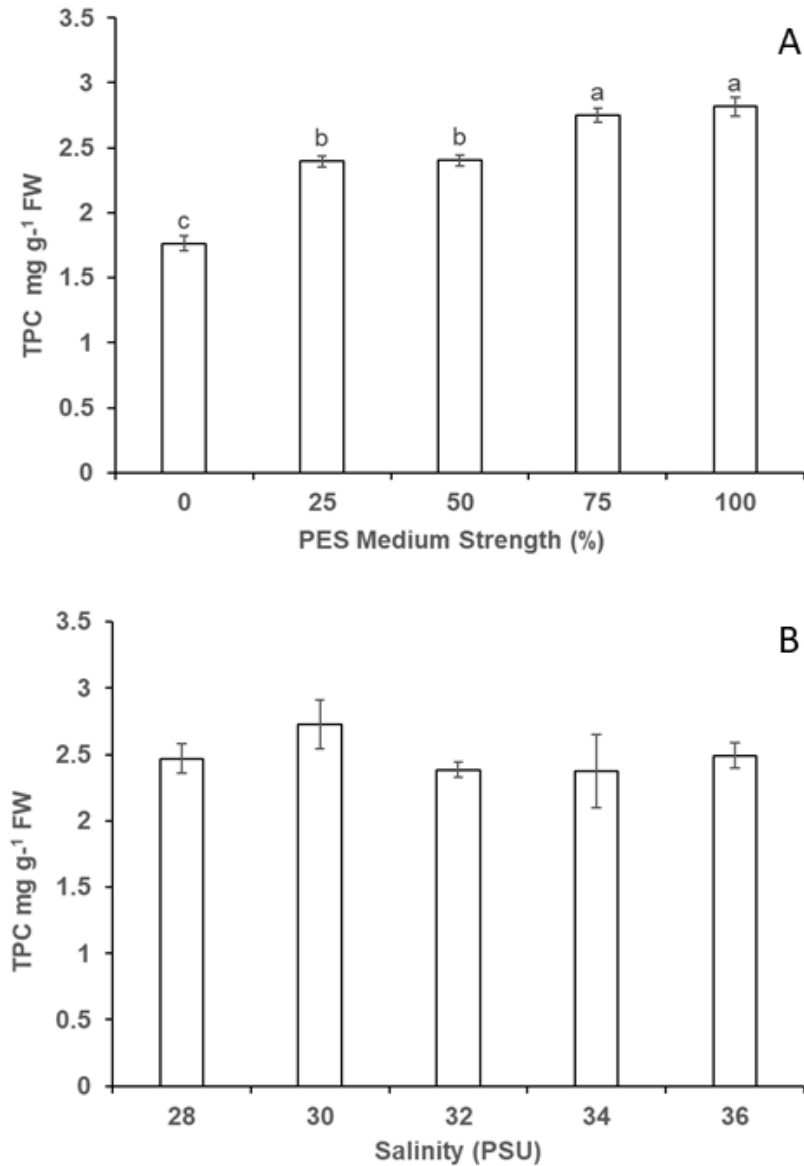
**Figure 3** Growth rate for *G. micropterum* cultured for 18 days at 4 liters' scale with optimized PES medium strength (75%) and salinity (34 PSU). Values are averages of three biological replicates ( $n = 3$ )  $\pm$  SD. Different letters indicate significant differences according to the one-way analysis of variance and Tukey's test ( $p \leq 0.05$ ).



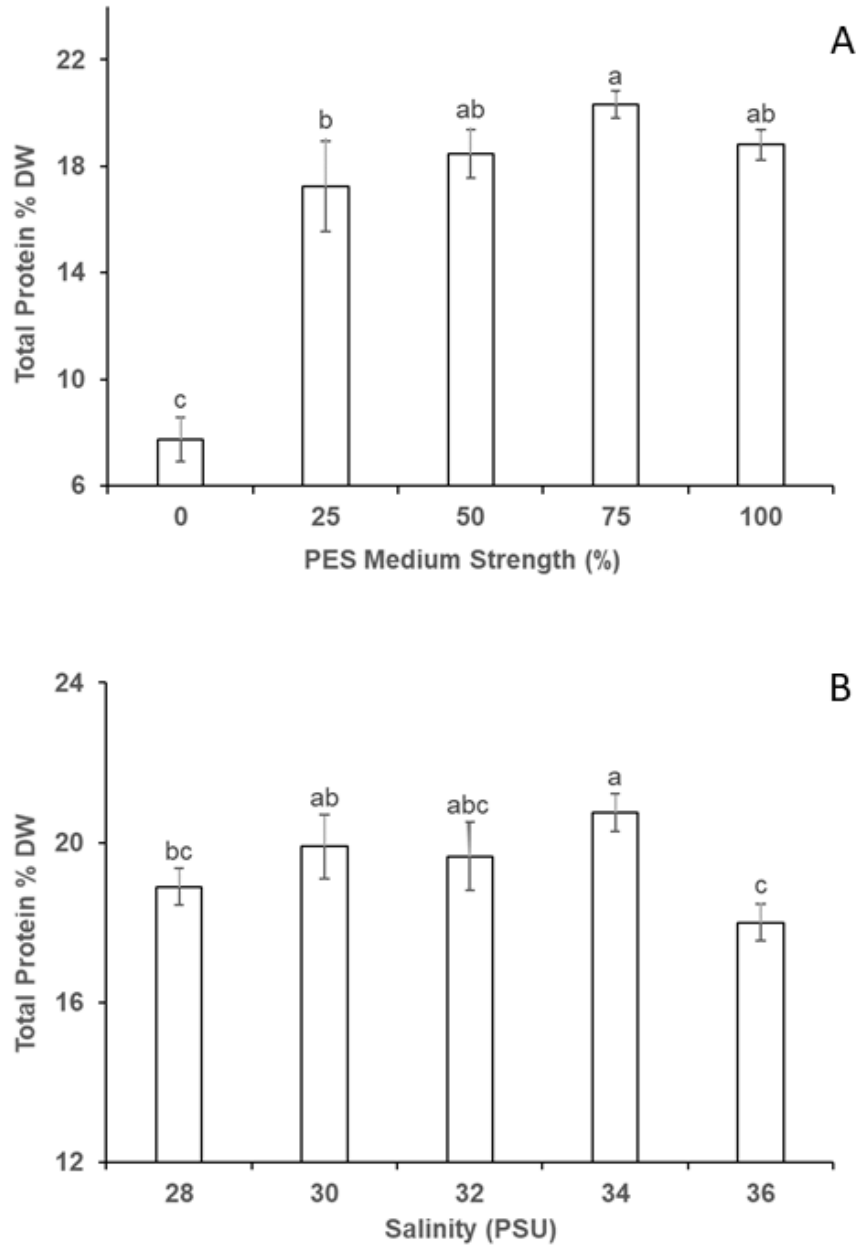
**Figure 4** Effect of PES medium strengths and salinity concentration in dry mass: A, *G. micropterum* grown at different PES medium strengths with 35 PSU salinity, B, *G. micropterum* grown at different salinities with 75% PES medium strength. Values are averages of three biological replicates ( $n = 3$ )  $\pm$  SD. Different letters indicate significant differences according to the one-way analysis of variance and Tukey's test ( $p \leq 0.05$ ).



**Figure 5** Effect of PES medium strengths and salinity concentration in R-PE and R-PC contents: A, *G. micropteron* grown at different PES medium strengths enrichment with 35 PSU salinity, B, *G. micropteron* grown at different salinities with 75% PES medium strength. Values are averages of three biological replicates ( $n = 3$ )  $\pm$  SD. Different letters indicate significant differences according to the one-way analysis of variance and Tukey's test ( $p \leq 0.05$ ).

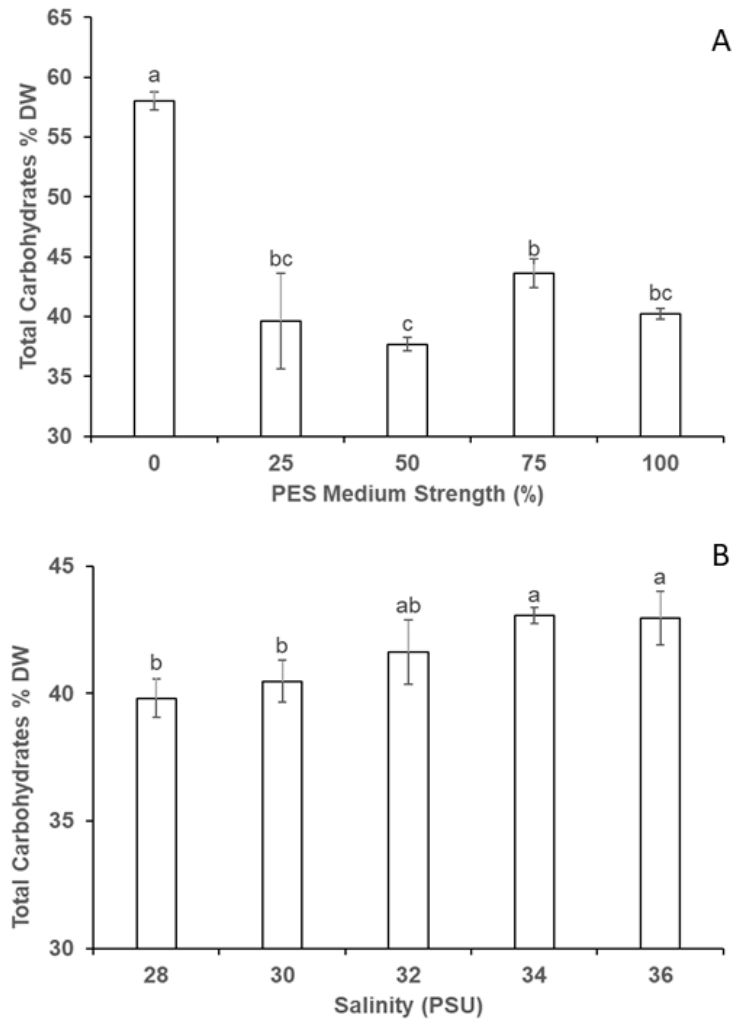


**Figure 6** Effect of PES medium strengths and salinity concentration in total phenolic content (TPC): A, *G. micropterum* grown at different PES medium strengths with 35 PSU salinity, B, *G. micropterum* grown at different salinities with 75% PES medium strength. Values are averages of three biological replicates ( $n = 3$ )  $\pm$  SD. Different letters indicate significant differences according to the one-way analysis of variance and Tukey's test ( $p \leq 0.05$ ).



**Figure 7** Effect of PES medium strengths and salinity concentration on total protein content: A, *G. micropterum* grown at different PES medium strengths with 35 PSU salinity, B, *G. micropterum* grown at different salinities with 75% PES medium strength. Values are averages of three biological replicates ( $n = 3$ )  $\pm$  SD. Different letters indicate significant differences according to the one-way analysis of variance and Tukey's test ( $p \leq 0.05$ ).





**Figure 8** Effect of PES medium strengths and salinity concentration in total carbohydrates: A, *G. micropterum* grown at different PES medium strengths with 35 PSU salinity, B, *G. micropterum* grown at different salinities with 75% PES medium strength. Values are averages of three biological replicates ( $n = 3$ )  $\pm$  SD. Different letters indicate significant differences according to the one-way analysis of variance and Tukey's test ( $p \leq 0.05$ ).

## Supplementary Materail

### Supplementary Tables

**Supplementary Table 1.** Composition of PES medium and its preparation (Provasoli,1968)

<b>Composition of PES stock (100 mL)</b>		<b>*PII metals stock (1000 mL stock)</b>	
NaNO <sub>3</sub>	350 mg	NA <sub>2</sub> EDTA	1 g
Glycerine phosphate Na	50 mg	FeCl <sub>3</sub>	49 mg
Fe EDTA (2Na)	18.8 mg	H <sub>3</sub> BO <sub>3</sub>	1.14 g
*PII metals	25 mL	MnCl <sub>2</sub> .4H <sub>2</sub> O	143 mg
*Vitamin mixture	1 mL	ZnCl <sub>2</sub>	10 mg
Tris	500 mg	CoCl <sub>2</sub> .6H <sub>2</sub> O	4 mg
pH	7.8		
Distilled water	100 mL	Distilled water	1000 mL
<b>Vitamin composition</b>		<b>*Primary stock of Vitamins</b>	
Required concentration		Biotin	50 mg/100 mL
Biotin	5 µg/litre		(Dissolved in 0.1N NaOH)
Cyanocobalamin	10 µg/litre	Cyanocobalamin	10 mg/100 mL
Thiamine HCl	0.5 mg/litre	Thiamine HCl	500 mg/100 mL
<ul style="list-style-type: none"> <li>• Add 1 mL of primary stock of biotin, 10 mL of cyanocobalamin and 10 mL of thiamine HCl and finally make up to 100 mL.</li> <li>• Add 20 mL of above PES stock to make 1 liter of seawater culture medium of full strength (100%).</li> </ul>			

**Supplementary Table 2.** pH of culture medium in the flasks in growth experiments

<b>PES optimization experiment</b>	
PES strengths	pH
Control (0%)	8.27±0.017
25 %	8.26±0.01
50%	8.23±0.01
75%	8.18±0.006
100%	8.16±0.015

<b>Salinity optimization Experiment</b>	
Salinity concentrations	pH
28 PSU	8.17±0.005
30 PSU	8.17±0.01
32 PSU	8.18±0.015
34 PSU	8.19±0.015
36 PSU	8.18±0.01

**Supplementary Table 3.** One-way analysis of variance followed by statistical significance using the Tukey's HSD post hoc test

<b>Source</b>	<b>DF</b>	<b>Sum of Squares</b>	<b>Means Square</b>	<b>F Rtaio</b>	<b>Prob &gt; F</b>
Analysis of variance for daily growth rates (DGR) % at different seed density (Fig. 1)					
Between groups	4	19.591445	4.89786	60.4202	<.0001
Within groups	10	0.810633	0.08106		
C. total	14	20.402077			
Analysis of variance for daily growth rates (DGR) % at different PES strengths (Fig. 2a)					
Between groups	4	15.854540	3.96364	32.6250	<.0001
Within groups	10	1.214906	0.12149		
C. total	14	17.069447			
Analysis of variance for daily growth rates (DGR)% at different salinity concentrations (Fig. 2b)					
Between groups	4	30.080372	7.52009	38.8238	<.0001
Within groups	10	1.936981	0.19370		
C. total	14	32.017353			
Analysis of variance for daily growth rates (DGR) % at 4 liters' scale culture (Fig. 3)					
Between groups	2	5.9944021	2.99720	10.1874	0.0118
Within groups	6	1.7652440	0.29421		
C. total	8	7.7596461			
Analysis of variance for dry weight contents at different PES strengths (Fig. 4a)					
Between groups	4	54.458201	13.6146	7.6149	0.0044
Within groups	10	17.878758	1.7879		
C. total	14	72.336959			
Analysis of variance for dry weight content at different salinity concentrations (Fig. 4b)					
Between groups	4	7.780704	1.94518	1.0756	0.4184
Within groups	10	18.084861	1.80849		
C. total	14	25.865564			
Analysis of variance for R-PE contents in different PES strengths (Fig. 5a)					
Between groups	4	13.542388	3.38560	13.9155	0.0004
Within groups	10	2.432974	0.24330		
C. total	14	15.975361			
Analysis of variance for R-PC contents in different PES strengths (Fig. 5a)					
Between groups	4	0.71223747	0.178059	9.1809	0.0022
Within groups	10	0.19394645	0.019395		

C. total	14	0.90618392			
<hr/>					
Analysis of variance for R-PE contents in different salinity concentrations (Fig. 5b)					
Between groups	4	3.8998720	0.974968	5.2944	0.0149
Within groups	10	1.8415077	0.184151		
C. total	14	5.7413797			
<hr/>					
Analysis of variance for R-PC contents in different salinity concentrations (Fig. 5b)					
Between groups	4	0.22373811	0.055935	5.0663	0.0171
Within groups	10	0.11040553	0.011041		
C. total	14	0.33414364			
<hr/>					
Analysis of variance for TPC contents at different PES strengths (Fig. 6a)					
Between groups	4	2.0951889	0.523797	171.6234	<.0001
Within groups	10	0.0305201	0.003052		
C. total	14	2.1257091			
<hr/>					
Analysis of variance for TPC contents at different salinity concentrations (Fig. 6b)					
Between groups	4	0.24572739	0.061432	2.2547	0.1355
Within groups	10	0.27245656	0.027246		
C. total	14	0.51818394			
<hr/>					
Analysis of variance for total protein contents at different PES strengths (Fig. 7a)					
Between groups	4	303.24702	75.8118	79.2189	<.0001
Within groups	10	9.56990	0.9570		
C. total	14	312.81692			
<hr/>					
Analysis of variance for total protein contents at different salinity concentrations (Fig. 7b)					
Between groups	4	13.128128	3.28203	8.1989	0.0034
Within groups	10	4.003031	0.40030		
C. total	14	17.131160			
<hr/>					
Analysis of variance for total carbohydrate contents at different PES strengths (Fig. 8a)					
Between groups	4	808.09150	202.023	55.0586	<.0001
Within groups	10	36.69236	3.669		
C. total	14	844.78386			
<hr/>					
Analysis of variance for total carbohydrate contents at different salinity concentrations (Fig. 8b)					
Between groups	4	25.105867	6.27647	7.7677	0.0041
Within groups	10	8.080198	0.80802		
C. total	14	33.186065			

## Supplementary figures



**Supplementary Figure 1.** Culture flasks are randomized in the culture racks for seed density optimization experiment.

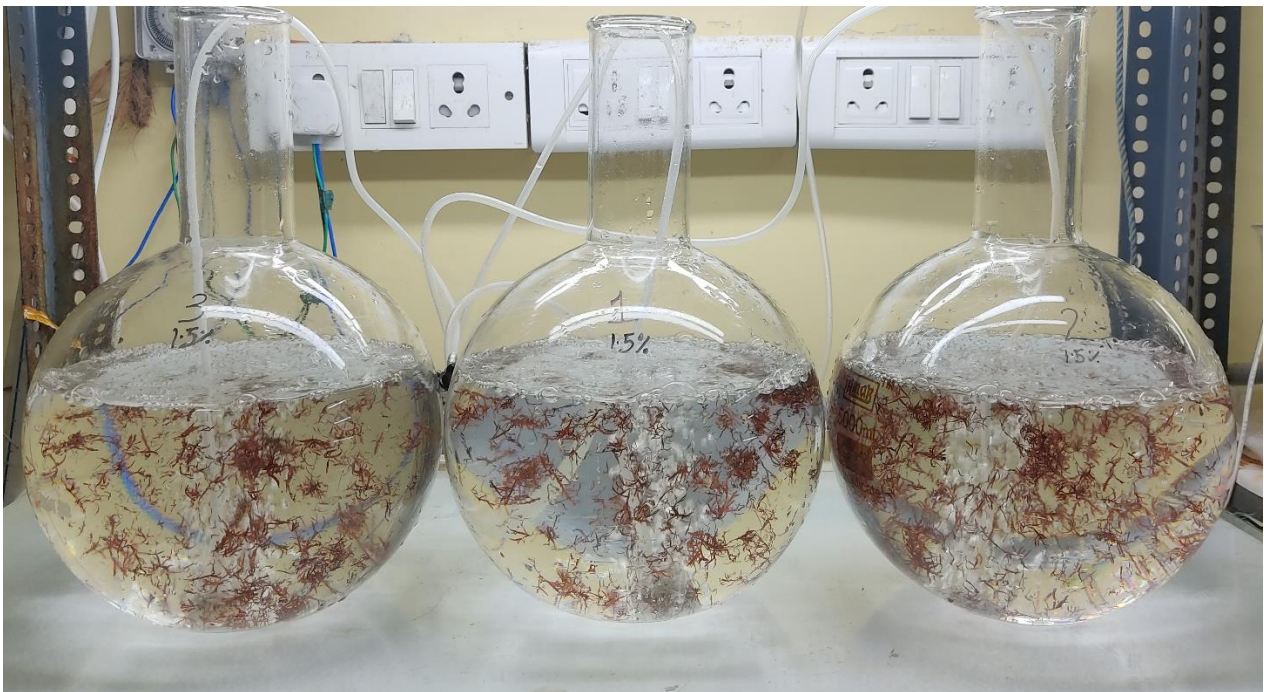


**Supplementary Figure 2.** Culture flasks are randomized in the culture racks for PES strength optimization experiment.





**Supplementary Figure 3.** Culture flasks are randomized in the culture racks for salinity concentration optimization experiment.



**Supplementary Figure 4.** Culture flasks are randomized in the culture racks for culture scale up at 4 liters' scale contains 75% PES medium strength at 34 PSU salinity, grown for 18 days.