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Synechococcus elongatus BDU 130192, an Attractive Cyanobacterium for Feedstock Applications: Response to Culture Conditions

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Abstract

Marine cyanobacteria are attractive organisms as feedstock for bioethanol and biotechnological applications. Previously, a marine cyanobacterial strain *Synechococcus elongatus* BDU 130192 was identified which can accumulate high polyglucan levels without the need to resort to N-deprivation. In this study, among the three different temperatures (30, 34, and 38 °C) tested, the highest biomass accumulation and total carbohydrate occur at 34 °C. The biomass and total carbohydrate increased with increasing light intensities, while protein, chlorophyll, and carotenoid contents were reduced. The best growth occurred at 1.8% NaCl concentration, even though the strain could grow well until 5.8% NaCl. Among the different concentrations of CO_2 tested, the best growth occurred at 5% CO_2 in the air. $NaNO_3$ gave the best growth, though the cells were able to grow well on urea and NH_4Cl too. Tripling the concentration of either N or P in the medium led to a significant increase in growth rate. The strain does not require the addition of vitamin B_{12} for growth, and about 90% flocculation could be achieved in an hour with the addition of chitosan. High productivities of 0.5 g/L/day of biomass and 0.23 g/L/day of carbohydrates could be reached in a 3-L photobioreactor (PBR) bubbled with air. This work shows many unique properties of this strain such as high carbohydrate productivity, ability to tolerate high light intensities and high concentrations of salt and CO_2, and efficient flocculation. These properties make *Synechococcus elongatus* BDU 130192 an attractive candidate as a feedstock for bioethanol and biotechnological applications.

Keywords Feedstock · Marine cyanobacteria · Photosynthesis · Flocculation · Productivity · Carbohydrate

Introduction

Photosynthetic biomanufacturing is promising for the sustainable and economical production of fuels, chemicals, pharmaceuticals, and feedstock [1, 2]. Cyanobacteria are oxygenic photoautotrophs, belonging to a morphologically and geographically diverse group of photosynthetic prokaryotes responsible for the production of one-third to one-half of total global biomass and oxygen in the atmosphere [3, 4]. Cyanobacteria and algae may be better photosynthetic platforms than plants due to their higher biomass production rates, photosynthetic efficiencies (3–9% in cyanobacteria and 1–3% in plants) [5], and ability to grow in fresh, sea, and wastewater which provides additional benefits of whole year cultivation in non-arable land [6]. Marine photosynthetic microorganisms have an additional advantage as they can be cultivated in seawater, which is not used for human consumption and agricultural use [7, 8]. Another quality of marine cyanobacteria is their potential to withstand higher salt concentrations which reduces the possibility of contamination in open ponds [9], though the higher ash content may pose a challenge while converting the biomass into energy in processes such as gasification. In addition to potentially high biomass productivity, cyanobacteria, being photoautotrophic, can help fix atmospheric CO_2 and hence may help regulate the levels of the greenhouse gas [10]. Cyanobacteria have highly effective carbon-concentrating mechanisms (CCM), such as active HCO_3^- and CO_2 transporters, to facilitate CO_2 fixation [11,

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12]. Glycogen is the primary carbon storage polymer in cyanobacteria and the organisms may accumulate more than 50% of their biomass depending on strain and growth conditions [13]. Glycogen is potentially useful as a substrate for bioethanol production [14]. Another advantage is that the biomass hydrolysate will also have peptides and amino acids that will support the growth of the organism and may require little additional nitrogen to be added [15].

The usefulness of the microorganism for industrial purposes strongly depends on their biological characteristics such as their optimal pH, temperature, and light intensity [16]. Other parameters that affect productivity are the choice of nitrogen, phosphorus sources [17], and the concentration of CO_2 used. Optimal physical parameters help to improve growth conditions, biomass productivity, and ultimately in the accumulation of useful products. Cyanobacterial biomass grows best under saturating light intensities [18], with 100–800 μmol/m^2/s and temperatures of 30–38 °C used in most previous studies with *Synechococcus* species [19]. An important consideration while developing processes based on unicellular photosynthetic organisms is how to harvest them efficiently as it may contribute to about 20–30% of the total biomass production costs [20]. Thus, cells that grow rapidly, accumulate high amounts of glycogen/carbohydrates, can tolerate a variety of temperature and light intensities, are salt-tolerant, and easy to harvest would be useful for further development as a potential feedstock for fermentation and bioethanol production [21, 22].

A marine cyanobacterial strain isolated from Indian salt pans with good growth and high cellular carbohydrate content was previously reported. Previous works include the publication of its genome sequence [23] and reconstruction of its genome-scale metabolic model as well as some biochemical aspects [24]. These works suggested general robustness of the organism, e.g., the existence of alternate methionine synthesis pathways suggested that this strain should not be auxotrophic to vitamin B_{12} while the scanning electron microscopic (SEM) analysis indicated many surface polymers suggesting rapid flocculation for harvesting biomass. In this work, experimental validations of some of these predictions and responses to various process parameters are presented. The overall schematic diagram of the experiments done is shown in Fig. 1. The results indicate the suitability of this organism for feedstock application due to its several advantageous characteristics.

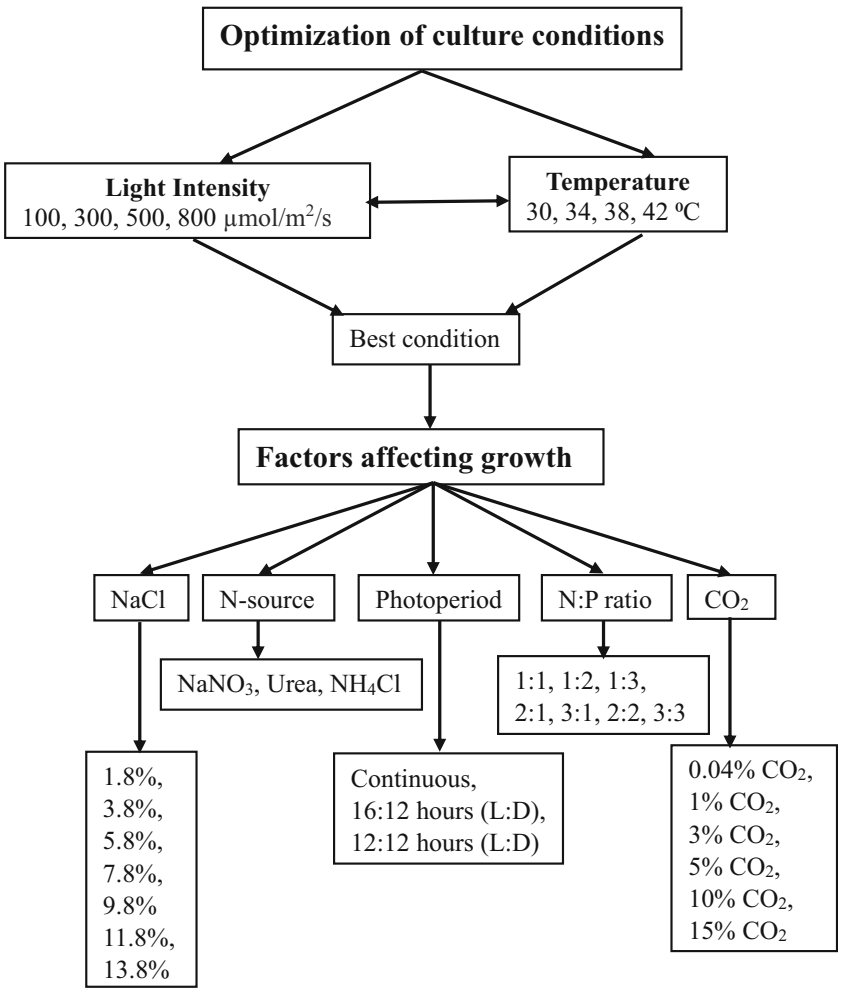

Fig. 1 Schematic overview of the experimental work

Material and Methods

Chemicals and Materials

NaCl and Tris base were purchased from Fisher Scientific (Mumbai, India), KCl was from Amresco (Solon, OH, USA), and $MgSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, Na_2EDTA, H_3BO_3, $CaCl_2 \cdot 2H_2O$, KH_2PO_4, $NaNO_3$, vitamin B_{12}, $FeCl_3 \cdot 6H_2O$, $MnCl_2 \cdot 4H_2O$, $ZnCl_2$, and $CoCl_2 \cdot 6H_2O$ were from Sigma-Aldrich (St. Louis, MO, USA). All chemicals were of analytical reagent (AR) grade.

Cyanobacterial Strain and Regular Culture Maintenance

The cyanobacterium *Synechococcus elongatus* BDU 130192 was obtained from the National Facility for Marine Cyanobacteria (NFMC), Bharathidasan University (BDU), Thiruchirapalli, India, and grown in A^+ medium containing 4 μg/L vitamin B_{12} [25]. Strain purity was checked by an absence of growth in LB medium when incubated in the dark at 37 °C. For regular culture, the cells were grown in 250-mL Erlenmeyer flasks containing 100-mL culture in an incubator shaker (Innova 44, New Brunswick) maintained at 30 °C, 150 rpm agitation, and light intensity of 150 μmol/m^2/s with 16:8 h light/dark cycle, illuminated by LED lamps (Design Innova, India).

Variation of Medium Components

To evaluate the effect of nitrogen sources, $NaNO_3$ in A^+ medium (11.8 mM) was replaced with either 5.9 mM urea or 11.8 mM NH_4Cl [26]. To test the effect of different concentrations of $NaNO_3$ (N), KH_2PO_4 (P), or their combination, their concentrations were kept the same as in A^+ medium (1×) [27] or increased to 3 times the concentration in A^+ medium (3×). Salt concentration was varied by adding more NaCl to A^+ medium as desired. To evaluate the necessity of vitamin B_{12}, vitamin B_{12} was omitted from the medium.

Experimental Setup and Cultivation Conditions

All the experiments were performed in a Multi-cultivator system (MC 1000-OD, Photo System Instruments, Czech Republic), unless otherwise indicated. The Multi-cultivator system allows culture in 8 tubes at chosen light intensities in parallel at a given temperature [28]. The initial optical density (OD) of inoculum was set to 0.1 with a culture volume of 60 mL and each tube was bubbled with sterile filtered ambient air at an inlet flow rate of 0.8 mL/min. Light intensities were set at 100, 300, 500, and 800 μmol/m^2/s in parallel tubes while the temperatures were set sequentially at 30 °C, 34 °C, 38 °C, or 42 °C, thereby covering the full factorial design of light intensity and temperature.

When testing the effect of photoperiod, the cultures were illuminated for only 12 or 16 h with 300 μmol/m^2/s light intensity.

For normal cultures, the tubes were bubbled with air. For experiments where the effect of CO_2 concentration in the air was tested, CO_2 at the desired concentration was bubbled using specially ordered gas cylinders (Laser gases, India).

Analytical Methods

Growth of the cells in MC was monitored by measuring the OD_{730} (spectrophotometrically, UVmini-1240, Shimadzu Co.) after every 3 h for 2 days while examining the effect of light and temperature. When required, the samples were diluted to obtain the OD within linear range and the obtained value was multiplied by the corresponding dilution factor. Dry cell weight (DCW) was measured as per an earlier published protocol [29]. Thirty milliliters of culture was centrifuged at 5000*g* for 10 min, the pellet was washed twice with distilled water, and the cells were transferred to a pre-weighed 2-mL microcentrifuge tube, dried in a dry bath at 65 °C for about 24 h until the weight of the tube became constant. The weight of a blank microcentrifuge tube was then subtracted from the filled microcentrifuge tube to obtain the biomass weight in 30-mL culture and then calculated accordingly for weight per liter of culture.

The total carbohydrate (TC) was measured by a concentrated sulfuric acid method [30]. The total protein (TP) was extracted in 1 N NaOH [31] and estimated by the bicinchoninic acid (BCA) method [32]. Photosynthetic pigments were extracted in methanol and analyzed as per previously published protocols [24].

For estimating N and P utilization, 1-mL culture was taken every day. The cells were pelleted and the supernatant was filtered through a 0.45-μm syringe filter. Nitrate was estimated in the filtrate by the salicylic acid method [33]. $NaNO_3$ was used as a standard in the concentration range of 0–23.8 mM. The estimation of phosphate was carried out by the ammonium molybdate method [34]. KH_2PO_4 was used as a standard in the concentration range of 0–0.72 mM.

Flocculation of Cells with Chitosan

The cyanobacterial strains *Synechococcus* sp. PCC 7002 and *Synechococcus elongatus* BDU 130192 were grown in A^+ medium until OD_{730} reached ~ 6. To test flocculation in the presence of chitosan [20], 16 mg/L chitosan (from shrimp shells, Sisco Research Laboratory Pvt. Ltd., India) was added to the cultures, the contents were mixed, and the tubes were placed at room temperature on a laboratory bench. The cultures were allowed to settle for up to 60 min. A supernatant

sample was taken at 0, 1, 2, 5, 10, 20, and 60 min using a pipette and its OD_{730} nm was measured. The flocculation efficiency was calculated as the percentage reduction of the OD_{730} value in the supernatant after treatment with chitosan in comparison with the initial concentration.

Cell Culture in a Bioreactor

Cells were cultivated in a 3-L automated bioreactor (Applikon Biotechnology, Holland) [35], with a working volume of 1.5 L. The temperature was set at 34 °C and the stirring at 300 rpm. Air was bubbled at 2 mL/min. The bioreactor was continuously illuminated from the outside by LED lights (Design Innova, Delhi, India). The light intensity was kept initially at 100 μmol/m^2/s and was increased by 100 μmol/m^2/s every 8 h up to 1000 μmol/m^2/s. Seeding OD was 0.1 and the subsequent ODs were taken at 730 nm spectrophotometrically after every 12 h for 4 days.

Statistical Analysis

All the experiments were performed in biological triplicates and the data are expressed as mean ± standard deviation. All plots were statistically analyzed and plotted in Sigma Plot version 12.5, Systat Software Inc. Statistical significance was calculated using the paired Student's *t* test or two-way analysis of variance (ANOVA) followed by Tukey's honest significant difference (Tukey's HSD) post hoc tests. The ANOVA was conducted in R.

Results

Effect of Different Light Intensities, Temperature, and Photoperiod on Growth

The initial experiments had indicated that the cells grow better in A^+ as compared to ASN-III medium as shown in Supplementary Fig. S1. So all further experiments were conducted in the A^+ medium.

The cells were grown in different combinations of light intensities and temperature. The temperature and light intensity range were chosen based on previous reports on *Synechococcus* sp. strains [19, 36]. The growth rate of the cells increased with increasing light intensity at all the temperatures tested (Fig. 2a) with a maximum of 0.067 ± 0.001 h^{-1} at 34 °C and 800 μmol/m^2/s. The growth rate was significantly affected by light intensity and temperature as well as their interaction as per two-way ANOVA.

The growth of the strain was abruptly affected upon increasing the temperature to 42 °C and the cells started to die (not shown). In agreement with the growth rate data, light intensity, temperature, and their interaction also affected the biomass concentration after 2 days of growth (Fig. 2b). However, at the extreme light intensities tested (100 and 800 μmol/m^2/s), there was no significant effect of temperature on biomass accumulation. The culture achieved a dry cell weight of 586 ± 11 mg/L ($n = 3$) at the maximum light intensity and at 34 °C (Fig. 2b).

The cells grow best under constant illumination; 8 h of darkness a day reduced the growth by about 40% while 12 h darkness reduced the growth by about 75% (Fig. 2c).

Thus, the results show that among temperature and light intensity, the latter has a greater effect on biomass accumulation of the cells and constant illumination significantly increases biomass accumulation compared to dark: light cycles.

Effect of Light and Temperature on Biochemical Composition

Similar to the effect seen on biomass concentration of light and temperature, light intensity had a significant effect on TC content while temperature had a lesser, but significant, effect in the range tested (Fig. 3a). The TC content increased with light intensity though the increase was significant when the intensity was increased from 100 to 300, but lesser afterward. At all the light intensities tested, the TC content of the cells was highest at 34 °C. It was found that the maximum carbohydrate of approximately 55–60% DCW was accumulated at 34 °C and 800 μmol/m^2/s.

In contrast to the trend observed for biomass and TC, the TP content showed a decreasing trend with increasing light intensities (Fig. 3b). Here too, light was a major factor while temperature was a minor factor that affected TP. Interestingly, at all light intensities, the cells grown at 34 °C had a significantly lower protein content.

Similar to the trend observed for TP, the photosynthetic pigments (chlorophyll *a* and carotenoid content) showed a decreasing trend with increasing light intensity (Fig. 3c and Fig. 3d). There was no significant difference in pigment content between 30 and 34 °C. However, incubation at 38 °C significantly reduced the pigments at all light intensities tested, in agreement with the paler appearance of the cells at this temperature (not shown).

Therefore, the optimal temperature chosen for this strain is 34 °C (based on the growth rate, DCW, and TC content).

Effect of Different Concentrations of Salt and CO_2 on Growth

Synechococcus elongatus BDU 130192 was found to be capable of tolerating high salt (NaCl) concentrations although the growth decreases with increasing salt concentrations (Fig. 4a). There was a drastic reduction in growth when going from salt concentration of 7.8 to 9.8%. Thus, ~ 8% salt concentration seems like an upper limit of tolerance of this strain.

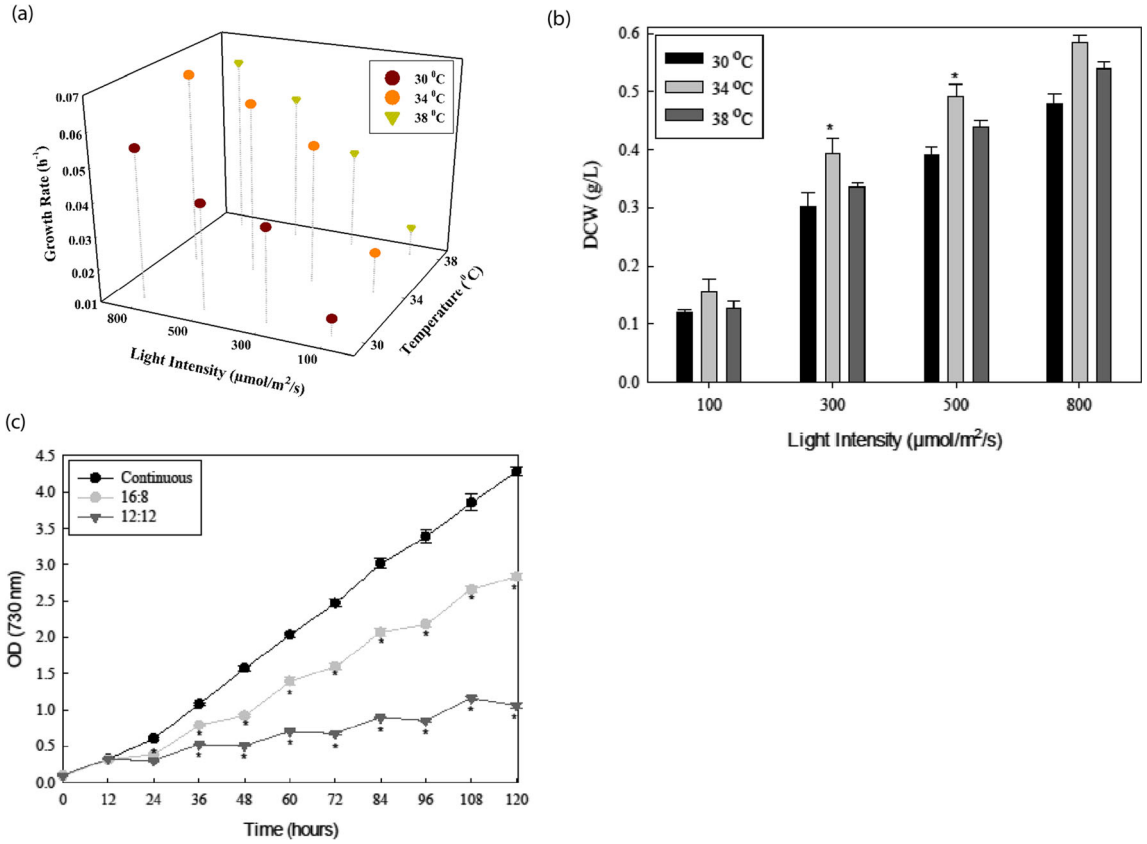

Fig. 2 Effect of light intensity and temperature, and photoperiod on the growth of *Synechococcus elongatus* BDU 130192 cells. The cells were grown in a multicultivator with air bubbling. **a** A 3D plot showing change in growth rate with varying light and temperature. **b** Biomass concentration (as DCW in g/L) after 48 h of growth at the different temperature and light intensities. For a and b, the cells were grown under continuous illumination. **c** Effect of photoperiod on the growth of cells when cultivated at 34 °C and 300 μmol/m^2/s. The asterisk represents statistically different values compared to the cells grown under continuous illumination, $p < 0.05$, $n = 3$

This strain was also grown under varying CO_2 concentrations and 5% CO_2 in the air gave the highest growth (Fig. 4b). Concentrations higher than 5% CO_2 had an inhibitory effect on growth and the growth was even lesser than that on compressed air.

Growth in a Medium Without Vitamin B_{12}, in Presence of Different Nitrogen Sources, Consumption of N and P with the Growth Curve, and the Variation of N and P Levels

In a previous study, this strain was identified as a close homolog of *Synechococcus* sp. PCC 73109 [24] which can grow well without the addition of vitamin B_{12}. Additionally, the metabolic network of these cells has alternative pathways for the synthesis of methionine [24]. Therefore, the requirement of vitamin B_{12} addition was tested and the results showed that these cells do not require vitamin B_{12} addition for growth (Fig. 5a).

Among the different N sources tested, $NaNO_3$ was found to be the best for growth as shown in Fig. 5b. The cells could also grow reasonably well on urea and NH_4Cl, though the growth was slightly lower than that with $NaNO_3$.

The utilization profile of the substrates on normal A^+ medium showed that 60–70% of nitrate was consumed while 40–50% of phosphate was consumed after 5 days of growth (Fig. 5c).

As nitrogen and phosphorus are the major components of cyanobacterial media, the strain was further studied under different nitrate and phosphate concentrations (modified A^+ media). Figure 5 d illustrates that increasing the N or P concentration in the medium increased the growth of the cells. However, increasing both N and P did not increase the growth any more than that observed with a higher level of N or P alone.

Growth in a PBR with Increasing Light Intensity

A significant increase in growth was observed when the cells were grown in a PBR with increasing light intensity and the OD_{730} reached ~ 7 in 4 days as shown in Supplementary Fig. S2. The TC was in the range of 45–50% DCW and the dry cell weight to OD_{730} conversion was 291 mg/L/OD_{730}. Thus, biomass productivity of 0.5 g/L/day and carbohydrate productivity of approximately 0.23 g/L/day could be reached in a PBR with air bubbling.

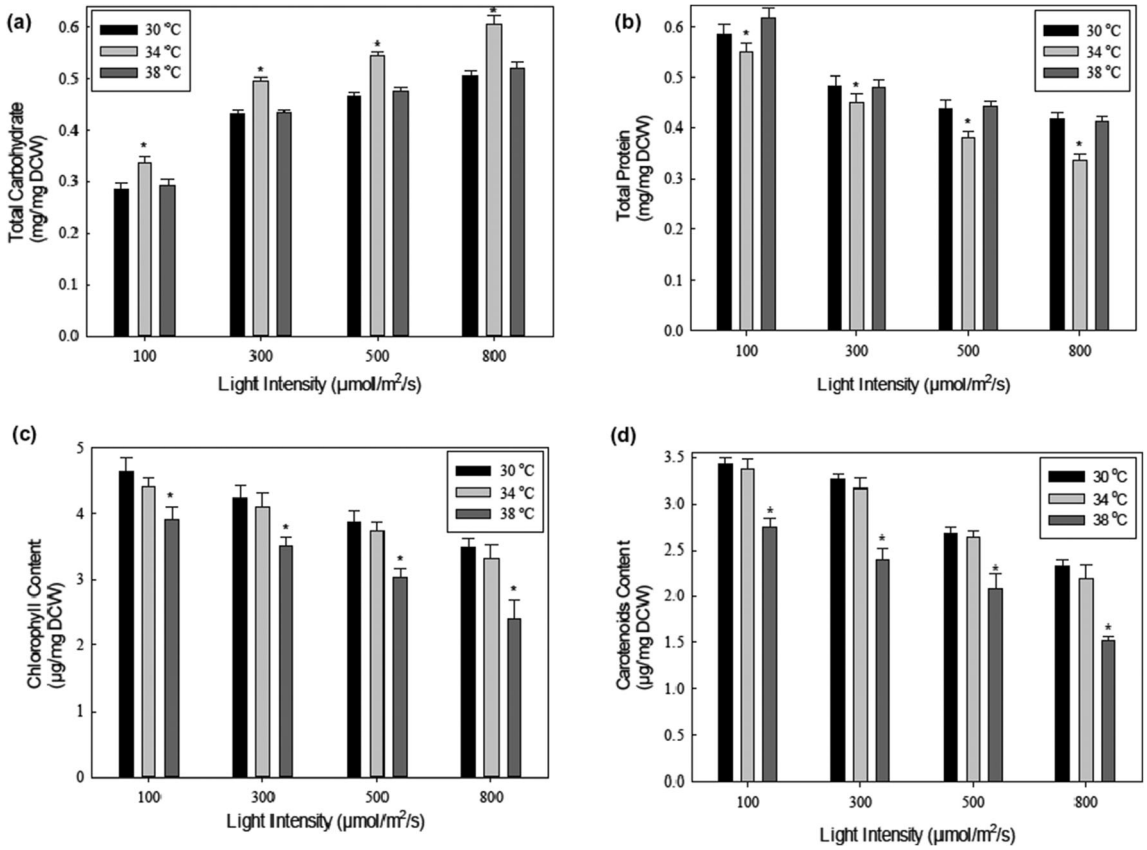

Fig. 3 Response of some biochemical parameters to variation in light intensity and temperature. The cells were grown at the indicated light intensities and temperatures and the biochemical parameters were measured. **a** Total carbohydrates (TC) in mg/mg DCW. **b** Total protein (TP) content in mg/mg DCW. **c** Content of photosynthetic pigments. **d** Carotenoids content. The asterisk represents statistically different values, $p < 0.05$, $n = 3$

Flocculation of *Synechococcus elongatus* BDU 130192 and *Synechococcus* sp. PCC 7002

The preliminary experiments showed that in the range of 0–30 mg/L chitosan, the lowest concentration that provided efficient settling of *Synechococcus elongatus* BDU 130192 was 16 mg/L. Because *Synechococcus elongatus* BDU 130192 was previously shown to have more surface polymers than *Synechococcus* sp. PCC 7002 [24], the flocculation of these two strains in presence of 16 mg/L chitosan was compared. Indeed, *Synechococcus elongatus* BDU 130192 flocculated significantly more (~ 95% flocculation) in an hour as

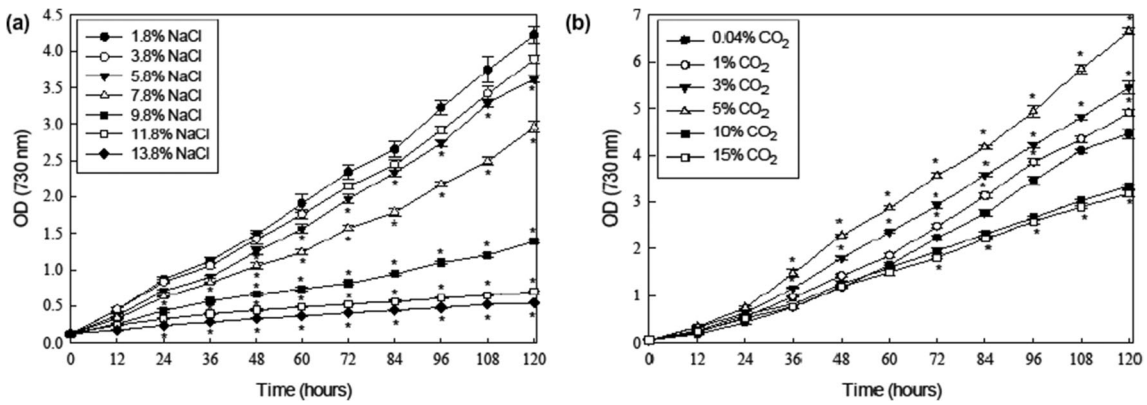

Fig. 4 Effect of salinity of the medium and CO_2 concentration on the growth of *Synechococcus elongatus* BDU 130192. **a** Effect of salinity of the medium. **b** Effect of CO_2 concentration. For this experiment, the cells were grown in Dreschel (gas-washing) bottles as indicated in the methods section. The asterisk represents significantly different values compared to cells grown at 1.8% NaCl concentration (for **a**) or 0.04% CO_2 (for **b**), $p < 0.05$, $n = 3$

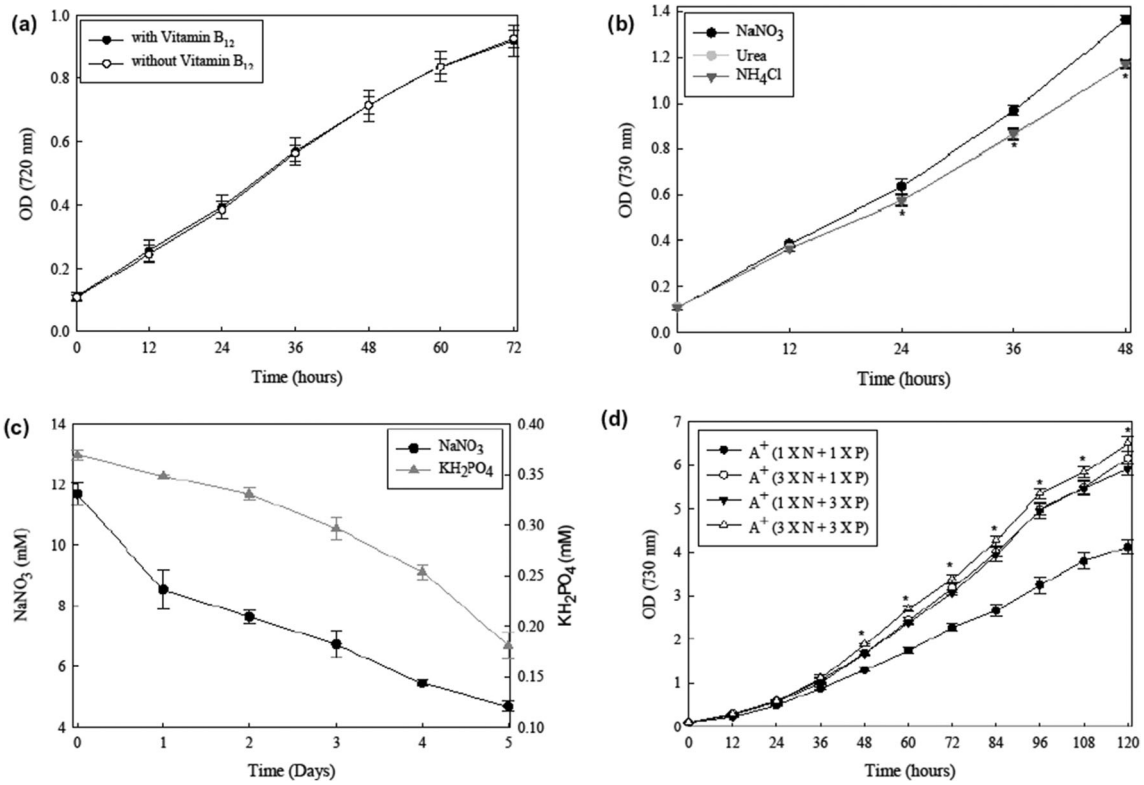

Fig. 5 Effect of variation of medium components on the growth of *Synechococcus elongatus* BDU 130192 cells. The cells were grown with air bubbling. **a** Growth of cells in A^+ medium with and without vitamin B_{12}. **b** Different nitrogen sources. The asterisk indicates significantly higher than other nitrogen sources, $p < 0.05$, $n = 3$. **c** Substrate utilization profiles. **d** Effect of 3 times higher concentrations of N and/ or P in the medium on the growth of the cells. The asterisk represents significantly higher value compared to control medium (A^+ (1× N + 1× P)), and is applicable to all other curves $p < 0.05$, $n = 3$

compared to *Synechococcus* sp. PCC 7002 (~ 40% flocculation) at this chitosan concentration (Fig. 6).

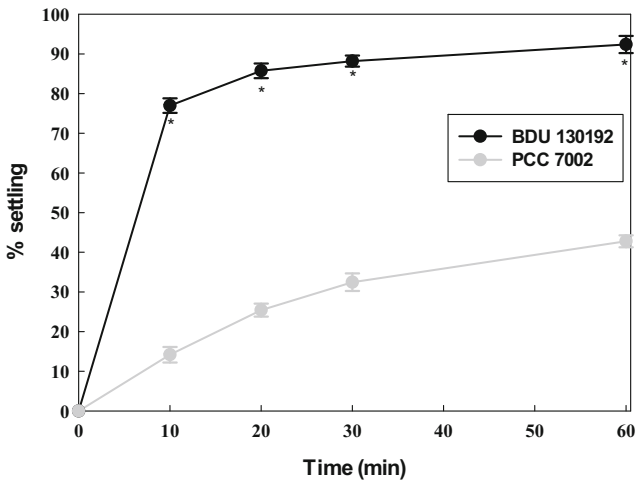

Fig. 6 Kinetics of chitosan-mediated flocculation of cells. Settling of *Synechococcus* sp. PCC 7002 and *Synechococcus elongatus* BDU 130192 cells was measured after the addition of 16 mg/L chitosan to the cultures. The asterisk represents statistically different values $p < 0.05$, $n = 3$

Discussion

For feedstock applications for bioethanol production and other biotechnological processes, a strain should be fast-growing, robust, have good carbohydrate productivity, and easy to harvest. Though cyanobacterial biomass itself is not a feedstock for lipid production, the sugar and protein produced could, in principle, be used to cultivate oleogenic yeast strains to produce lipids and biodiesel. Here, the response of *Synechococcus elongatus* BDU 130192 to various process parameters such as a wide range of light intensity and different photoperiods, temperature, salinity, and flocculation ability is shown. The strain has a high level of tolerance to different environmental stresses (CO_2, light, and salts). These results, along with the high biomass and carbohydrate productivities obtained, suggest that the strain may be an encouraging organism for the production of carbohydrates and protein. Good growth on urea makes it possible to use this strain for treating human and animal liquid wastes while producing biomass rich in sugars and protein. No requirement of vitamin B_{12} would help to make the medium cheaper.

The salinity tolerance of this strain is higher than that reported for *Synechococcus* PCC 7002, which shows a reduction in growth when cultured in 1 M NaCl [37] and mutated

Synechocystis PCC 6803 cells that grow well in 3.5% salt [38]. Cultivation in a high-salt medium could not only protect against contamination but may also increase autofermentation rates and the product yields through increased "sodium stress cycling" [39]. Additionally, given that the cells were originally isolated from salt pans, it is likely that adaptive laboratory evolution could be used to further increase salinity tolerance of the cells.

It has been suggested that light intensity affects cyanobacterial biomass [40]. Indeed, light intensity had a greater effect on cellular responses compared to temperature in the range tested. While biomass and carbohydrate content increased with increasing light intensity, total protein and pigments reduced. This trend of increased biomass and reduced chlorophyll is in agreement with previous studies [41]. Few previous studies have looked into the effect of light intensity on cellular protein content. Interestingly, there was an inverse relation between the biomass and the cellular protein and pigment content. This result is in agreement with the thesis that cyanobacterial growth is affected by resource allocation [42].

An important decision in the culture of photosynthetic organisms is whether to utilize an open-pond or a photobioreactor system. Open-pond systems require significantly reduced CAPEX but have issues of reduced productivity and greater chances of contamination. Additionally, in open-pond systems, the cells are exposed to daylight cycles and poor control of important process variables such as light intensity, pH, and temperature. Here, it is shown that while the strain may be amenable for open-pond cultivation due to tolerance of high light intensities, ability to withstand high salinity, and good growth at different temperatures tested, the growth is significantly improved when the cells are cultivated under constant illumination and under high light intensities. Therefore, it may be worthwhile to consider a "hybrid" open-pond system with continuous illumination to achieve good productivity while keeping the costs low.

The cells grew best when cultured with bubbling of 5% CO_2 in the air. There was a significant increase in growth at 5% CO_2 compared to 3% CO_2 bubbling. The OD with 5% CO_2 bubbling was about 1.5-fold than that with 1% air bubbling. Thus, the better growth at 5% compared to 1% CO_2 is different from that observed for *Synechococcus* sp. PCC 7002, which shows only a slight increase in biomass when grown at 2% CO_2 compared to 1% CO_2 [43]. The ability to grow at higher CO_2 concentrations may be beneficial for applications involving the use of exhaust gases to grow cyanobacterial biomass.

The net carbohydrate productivity in the fermenter was approximately 0.23 g/L/day. While this is slightly lesser than the value reported in a previous study [24], this productivity was achieved with air bubbling and at a larger scale (1.5-L culture vs. 60-mL tube cultures in the previous study). It is likely that higher, comparable productivity can be achieved by bubbling in air-CO_2 mixtures as in other studies. Additionally, the experiments with higher nitrate or phosphate concentrations show another way of increasing biomass accumulation rates.

Harvesting unicellular cultures contribute significantly to total production costs. The efficient harvesting of *Synechococcus elongatus* BDU 130192 cells with a low concentration of chitosan could be very beneficial in reducing the harvesting costs as well. The difference in kinetics of settling as compared with another popular marine cyanobacterial strain suggests a difference in membrane structure and composition.

Conclusion

Synechococcus elongatus BDU 130192 exhibits several properties such as very high carbohydrate productivity; ability to tolerate high light intensities, salt, and CO_2 concentrations; not needing vitamin B_{12} for growth; and rapid and efficient flocculation with chitosan. These properties make it an attractive feedstock candidate for bioethanol generation and other biotechnological applications. The results with increased biomass and TC through an increase in light intensity and nitrate or phosphate concentrations in medium provide ways to increase cyanobacterial biomass and carbohydrate productivity.

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Data Availability Data will be made available to all interested researchers upon request.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethics Approval/Consent to Participate Not applicable to the article

Consent for Publication All the authors have read the manuscript and agree to its publication.

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