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ORIGINAL PAPER

Enhanced biomass production of *Synechocystis* sp. PCC 6803 by two associated bacteria *Paenibacillus camelliae* and *Curtobacterium ammoniigenes*

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Abstract
Synechocystis sp. PCC 6803 grown continuously in a 5-L photo-bioreactor for 20 months was found to have associated consortia with heterotrophic microorganisms. Two strains of bacteria were isolated from the long-term cultures of cyanobacteria with the aim to test whether their presence affects cyanobacterial growth and metabolism. The two strains were phylogenetically identified as *Paenibacillus camelliae* and *Curtobacterium ammoniigenes*, respectively. Co-culturing the *Synechocystis* sp. with either of the isolates under photoautotrophic and photoheterotrophic conditions exerted a statistically significant growth enhancement effect on cyanobacteria. Under co-culture experiments, the addition of *P. camelliae* resulted in a four-fold higher biomass yield with a considerable decrease in the stationary period. The growth was more pronounced on the addition of acetate to the culture media. Growth-enhancing factors like indole acetic acid (IAA) and siderophores were detected in the co-culture conditions which proved to be the main driving force in boosting cyanobacterial growth. Thus, the cyanobacteria–bacteria consortia can be very useful for augmenting biomass production by circumventing the time factor which can be further exploited for various biotechnological applications.

Keywords Biomass yield · Co-culture · *Curtobacterium ammoniigenes* · *Paenibacillus camelliae* · *Synechocystis* sp. PCC 6803

Introduction

Phytoplankton and bacteria numerically are the dictators of the aquatic community. The evolution of these organisms dates back to 2150 million years ago when anoxygenic and later oxygenic organisms evolved (Ramanan et al. 2016). Phytoplanktons mainly cyanobacteria are the cosmopolitan photosynthetic prokaryotes, directly responsible for the oxygenic environment and indirectly for the evolution of aerobic organisms. These ubiquitous organisms are considered to be the most attractive bio-prospecting resources of recent decades due to their high photosynthetic efficiency, low nutritional requirement as well as ability to synthesize a variety of organic biochemicals (Issa et al. 2020). The phytoplanktons along with the bacteria are the key biological aspects that constitute the pillars of the ecosystem. These two groups exemplify all possible modes of interaction ranging from mutualism to parasitism (Fuentes et al. 2016). Under natural conditions, most of the cyanobacteria are found to be in association with various aerobic and anaerobic bacteria. One of the most interesting sites of interaction is the 'phycosphere' where, the heterotrophic bacteria inhabit the surface of alga, stimulated by its extracellular products (Variem and Kizhakkedath 2021). Certain long-term laboratory microalgal cultures have also been found to maintain symbiotic association with certain bacteria (Park et al. 2008). The presence of bacteria in the axenic cultures of alga might be due to nutrient enrichment and the presence of desirable carbon sources (Kumar et al. 2014). Co-inoculation of such symbiotic bacteria is reported to have improved algal growth than that of the mono-algal culture (Subashchandrabose et al. 2011). Bacteria are normally considered to be a mere contaminant in algal cultures; but recent studies advocate their synergistic effect on each other's physiology

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and metabolism. Gonzalez and Bashan (2021) presented a list of bacterial partners which enhances the algal growth under artificial microalgae–bacteria consortia by supplying CO_2 for photosynthesis while in return utilizing photosynthetic O_2 and some fixed organic carbon reserves (Gonzalez and Bashan 2021). Moreover, this interaction is useful for flocculation which is a major challenge in algal biotechnology. The chemistry between the algal bacterial interactions includes the exchange of molecular signals, secretion of metabolites, transporters, and other molecules (Fuentes et al. 2016). Besides carbon dioxide–oxygen exchange, bacteria establish beneficial interaction with the microalga by secreting various growth-promoting substances like indole acetic acid (Dao et al. 2018), vitamin B_{12} (Tandon et al. 2017), and siderophores (Rajapitamahuni et al. 2018) to the environment.

Further, microalgae are known to inhibit the growth of bacteria by secreting some antibacterial substances or by altering the pH and temperature of the surrounding (DellaGreca et al. 2010; Munoz and Guieysse 2006). Complex relationships between autotrophic algae and heterotrophic bacteria can be used for nutrient removal from waste water and in subsequent bioremediation processes (Kim et al. 2014). Negative interactions in the form of parasitism also occur in the environment where bacterial cells secrete certain substances like phenol, cellulose, alkaloid, glycosidase, etc. which pose an inhibitory effect to the micro-algal growth (Luo et al. 2013). However, this mode of interaction in the environment is helpful for controlling algal blooms (Cirri and Pohnert 2019), degradation of hydrocarbons, pollutant removal (Subashchandrabose et al. 2013), and maintenance of aquaculture (Han et al. 2019). Hence, both synergistically and antagonistically, the interactions between the two groups of photoautotrophs and heterotrophs are areas of recent research interest on a global scale (Amin et al. 2015). Thus, the algal bacterial consortium can find innumerable applications in the field of biotechnology and environmental technology.

There are reports available elucidating the algal bacterial interactions; however, in this study, attempts were made to understand the interaction between cyanobacteria and bacteria where one partner is photoautotrophic like algae and another one is heterotrophic. Despite differences in food sources, both partners share similar cellular characteristics. *Synechocystis* sp. PCC 6803, unicellular non-nitrogen-fixing cyanobacteria are widely been used as a model organism for cyanobacterial research. The distinctive feature of short generation time, fast growth without any specific nutritional demands, and easy transformation ability has made it popular for various biotechnological processes. In this study, efforts have been made to isolate and identify the associated bacteria from 20-month-old photo-bioreactor culture of *Synechocystis* sp. PCC 6803. The associated bacteria were then co-inoculated under photoautotrophic and photoheterotrophic conditions to examine the interaction between the test cyanobacterium and bacteria. We detected their interactions and elucidated the mechanism involved in the growth-promoting effect of bacteria on cyanobacteria under co-cultured conditions.

Materials and methods

Culture conditions of *Synechocystis* sp. PCC 6803

Synechocystis sp. PCC 6803 (source: Pasteur Culture Collection of Cyanobacteria, Pasteur Institute, Paris, France) was grown axenically in 250 ml Erlenmeyer flasks containing 100 ml of BG-11 medium (Rippka et al. 1971). The test organism was kept in a temperature-controlled incubator at 27 ± 2 °C under illumination with 18 W fluorescent light at an intensity of 75 µmol photon m^{-2} s^{-1} PAR and photoperiod of 14:10 h. Tris–HCl (4 Mm) buffer was used to maintain the pH of the media at 8.5. For continuous long-term culture, *Synechocystis* sp. PCC 6803 was inoculated in a 5-L photo-bioreactor equipped with a magnetic stirrer and thermostatic jacket to maintain the temperature. Two-third of the culture was replaced by fresh BG-11 medium after every 7 days. Illumination was provided with the help of four white lights. The total amount of air sparging the culture medium was 1 L min^{-1}.

Isolation of associated bacteria from *Synechocystis* sp. PCC 6803

50 ml of 20-month-old photobioreactor cultures of *Synechocystis* sp. was transferred to a centrifuge tube. After centrifugation (7500×*g* for 5 min at 25 °C), a white layer was seen on the surface of cyanobacterial pellet. The white layer was scraped down using a sterile inoculating loop and re-suspended in 2 ml sterilized distilled water. The re-suspended bacteria in distilled water were inoculated on Nutrient agar (Himedia) plates and incubated at 37 °C for 24 h in an incubator under dark. The colonies thus developed were further subjected to single-colony isolation by repetitive culture in the fresh nutrient agar plates. Single bacterial colonies of the isolates were inoculated in Nutrient Broth at 37 °C for 48 h in a rotary shaker to obtain sufficient bacterial cells for further experiments.

Identification of cyanobacterial associated bacterial isolates

The bacterial strains isolated from the old cultures of *Synechocystis* sp. were further characterized morphologically and genetically. The bacterial colonies were tested

morphologically for colony characteristics and Gram staining.

Extraction of bacterial genomic DNA

Genomic DNA was extracted from a loop full of overnight grown bacterial cells on nutrient agar using a QIAmp DNA mini kit (Qiagen, Duesseldorf, Germany) as per manufacturers' protocol. The purity and concentration of DNA were examined by electrophoresis on agarose gel (1%) and quantified with a NanoDrop 2000c spectrophotometer (Thermofischer Scientific Inc, Walthum, MA, USA). The genomic DNA was stored at − 20 °C until PCR amplification.

PCR amplification and 16S rRNA gene sequencing

Two universal primers: 27f (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 907r (5′-CCC CGT CAATTC ATT TGA GTT T-3′) were used for PCR amplification of 16S rRNA gene to generate a PCR product equivalent to 918 bp long nucleotide of the *Escherichia coli* 16S rRNA sequence (Weisburg et al. 1991).

A 25 µl reaction mixture was prepared in a 0.2 ml PCR tube using 2 µl template DNA, 0.5 µl of each primer (forward and reverse), 0.2 µl Taq-DNA polymerase, 2 µl dNTP and 2.5 µl Taq buffer. The reaction was performed in a thermocycler (Bio-rad T100, USA) with an initial 5 min denaturation at 95 °C, followed by 35 cycles of 1-min denaturation at 95 °C, 1 min annealing at 55 °C, 2 min extension at 72 °C, and a final extension step of 10 min at 72 °C. Then the PCR products were resolved in agarose gel (1.2%) with ethidium bromide (1 µg/ml) staining and visualized by a gel documentation system (Bio-rad Gel Doc XR +, USA) after electrophoresis. The PCR products were purified using a DNA purification kit (illustra™ GFX™ PCR DNA and Gel Band purification kit, GE Healthcare, UK) as described in the manufacturer's protocol. Purified amplicons were then sequenced using ABI Genetic Analyzer (ABI 3500, Applied Biosystem, India). The DNA sequences thus obtained were compared with available sequences of bacterial lineages in National Centre for Biotechnology Information Gene Bank using nucleotide BLAST (http://www.ncbi.nlm.nih.gov/blast). A similarity score of 99% and above with the available sequences in the Gene Bank was used to determine the identity of the bacterial strains. Multiple sequence alignment and Neighbor-Joining phylogenetic tree (according to the Jukes–Cantor model) were constructed using MEGA X software (Tamura et al. 2011). The topology of the phylogenetic tree was estimated by the bootstrap resampling method with 1000 replicates (Felsenstein 1985).

Purification of *Synechocystis* culture

Synechocystis sp. PCC 6803 was purified and axenic cultures were prepared from the consortium by addition of antibiotics (single or in combination). Triple-antibiotic solution was prepared by mixing penicillin, streptomycin, and chloramphenicol at a concentration of 100 mg, 50 mg and 10 mg in 10 ml distilled water. Chloramphenicol was however, mixed in 1 ml of 95% ethanol before mixing with the penicillin–streptomycin solution. The filtered antibiotic solution was supplemented to the cyanobacterial consortium medium at different concentrations following Singh et al. (2002) and incubated for 2 weeks. After the incubation, the culture broth was observed by microscopy and inoculated on Nutrient agar plate to confirm the purity of *Synechocystis* sp. Bacteria-free clones were selected and maintained on agar slants for further experiments.

Co-culture of the isolated bacteria with *Synechocystis* sp. PCC 6803

Co-culture experiments were established with the axenic cultures of isolated bacteria and test cyanobacteria in the BG-11 medium by taking the combination of one bacterial strain with the cyanobacteria at a time. Each bacterial isolate was harvested by centrifugation (7500×*g* for 10 min), re-suspended in BG-11 media, and diluted serially to attain the required cell density before the experimentation. The inoculum density of *Synechocystis* sp was adjusted to 7.3×10^5 cells ml^{-1} based on the absorbance value at 750 nm. The prepared bacterial dilution was inoculated with *Synechocystis* sp. aseptically in the ratio 1:3 (bacteria: cyanobacteria) in 250 ml flasks containing 100 ml BG-11 media each (Guo and Tong 2014). The experimental setup was subjected to photoautotrophic and photoheterotrophic (supplementation of 0.4% sodium acetate) conditions for 12 days (Guo and Tong 2014). The cyanobacterial cell concentration was assessed by counting the number of cells under a microscope using a haemocytometer. The bacterial cell density was also determined by spreading 100 µl of serially diluted cells on nutrient agar plates. The CFU count was performed at a regular interval of 2 days.

Estimation of photosynthetic pigments

Chlorophyll and carotenoid estimation

The estimation of chlorophyll was carried out using methanol following Ritchie (2008). 25 ml of cultures was drawn aseptically and harvested by centrifugation (7500×*g* for 5 min). Pellets obtained after discarding the supernatant were washed with distilled water and suspended in methanol overnight at 4 °C. The cells were again centrifuged and

absorbance of the supernatant was measured at 470, 665, and 720 nm. The amount of chlorophyll and carotenoid was determined using the following equation (Wellburn 1994):

$$\text{Chlorophyll } a = 12.9447\left(A_{665} - A_{720}\right), \quad (1)$$

$$\text{Carotenoid} = \frac{1000\left(A_{470} - A_{720}\right) - 2.86(\text{chl}a)}{221}. \quad (2)$$

Phycocyanin estimation

After chlorophyll extraction, 10 ml of distilled water was added to the pellets and kept in a temperature-controlled water bath at 50 °C for 30 min. Then, the suspension was centrifuged (7500×*g* for 5 min) to get the supernatant and the absorbance was measured at 650 and 620 nm, respectively. Phycocyanin content was estimated using the following equation (Bennett and Bogorad, 1973):

$$\text{Phycocyanin} = \frac{A_{620} - 0.47 \times A_{650}}{5.34}. \quad (3)$$

Determination of dry cell weight

Dry cell weight was determined gravimetrically. Cyanobacterial cells were centrifuged and the pellets were washed with distilled water and dried (60 °C) in an oven to constant weight (Rai et al. 1991).

Measurement of pH and dissolved oxygen

pH of both the monoculture and co-culture was measured using a pH meter at an interval of 2 days. Dissolved oxygen (DO) concentration in the medium was also measured by a DO meter (Hansatech, UK) on every alternative day.

Detection and estimation of indole acetic acid (IAA)

IAA production in *Synechocystis* sp. was estimated by without and with the supplementation of tryptophan (500 µg/ml) with BG-11 media for 12 days. Cells were harvested by centrifugation (7500×*g* for 10 min at 4 °C) at an interval of 2 days and the supernatant was tested for the presence of IAA. The occurrence of IAA was determined using Salkowski colorimetric technique (Ahmed et al. 2010). Salkowski reagent (1 ml of 0.05 M $FeCl_3$ mixed in 50 ml of 35% perchloric acid) was added to the supernatant in 1:2 (*v*/*v*) ratios and kept in dark for 30 min. The presence of IAA was noted by the development of pink color which was further confirmed spectrophotometrically at 535 nm. The values were then calculated using a calibration curve of standard IAA. In another set of experiment, exogenous IAA (100 µg/ml) was added to the culture media of the axenic cyanobacteria as well as co-cultures while inoculation, and the presence of IAA in the medium along with biomass accumulation was estimated after the incubation period.

IAA production was further confirmed by HPLC analysis (Sergeeva et al. 2002). The supernatant obtained from the cultures with tryptophan supplementation was acidified to pH 2.8 with 1.0 M HCl. The solution was then extracted thrice with ethyl acetate (1:3 *v*/*v*). Extracts were then evaporated using a rotary evaporator (IKA RV 10, USA) at 37 °C. The residual aqueous fraction was adjusted to pH 7.0 with 1 N NaOH and again extracted thrice with water-saturated *n*-butanol (1:3 *v*/*v*) followed by drying in a vacuum using a rotary evaporator. The collected extract was dissolved with 80% methanol and filtered through Whatman filter paper (0.45 µm) (Ahmed et al. 2010, Varalakshmi and Malliga 2012). 20 ml of the methanol extracts was injected into the HPLC system (Merck, Germany) using a C18 column (150×2.1 mm; 5 mm). The mobile phase used was methanol/water/acetic acid (36:64:1) at a flow rate of 1 ml/min. Elutes were detected using a detector (Shimadzu SPD-M20A PDA, Japan) at 220 nm and IAA was quantified by integrating the areas under the peak with an authentic IAA standard (Sigma-Aldrich, U.S.A.).

Siderophore production

The isolated bacterial strains were assayed for siderophore production following Dave et al. (2006). The isolates were incubated in TYES media and incubated at 37 °C. Cultures (24-h-old) were centrifuged at 7500 rpm for 5 min and the supernatant was examined for extracellular siderophore production. The production of reddish–orange color on the addition of 1–5 ml of 2% $FeCl_3$ indicated the presence of siderophore.

Statistical analysis

All the experiments were carried out in triplicates and results were expressed as mean of triplicates. Differences between the groups were compared by correlation coefficient and the means were separated using one way ANOVA and Duncan's multiple range test ($P = 0.05$).

Result

Identification and characterization of associated bacteria

Two different bacterial strains were isolated from the old cultures of cyanobacteria and characterized morphologically

from their colony characteristics. Both the bacteria were found to be Gram-positive and rod-shaped under a light microscope. The colony characteristics showed distinct variations for both the bacteria as shown in Table 1. Bacteria A appeared to be transparent white colonies, whereas bacteria B were yellow opaque colonies on Nutrient agar plates. Bacteria A displayed dull appearance while the other one appeared sticky.

Further, the bacterial isolates were identified at the genetic level by comparing the 16S rRNA sequences with the data available in Gene Bank using BLAST homology search. Of the two isolates, one displayed 99% similarity with *Paenibacillus camelliae*, whereas the other bacterium showed 95% similarity with *Curtobacterium ammoniigenes* (Fig. 1). The strains belonged to Firmicutes and Actinobacteria based on the phylogenetic reconstruction of gene sequences. Both the sequences were submitted to the NCBI gene bank via accession no. A: MW298678 and B: MW298679.

Table 1 Morphological characterization of bacterial isolates from old *Synechocystis* culture

Characters	Colony form	Appearance	Elevation	Margin	Color	Optical density	Gram stain	Cell shape
Bacteria A	Irregular	Dull	Flat	Wavy	White	Transparent	+	Rod
Bacteria B	Circular	Sticky	Raised	Entire	Yellow	Opaque	+	Rod

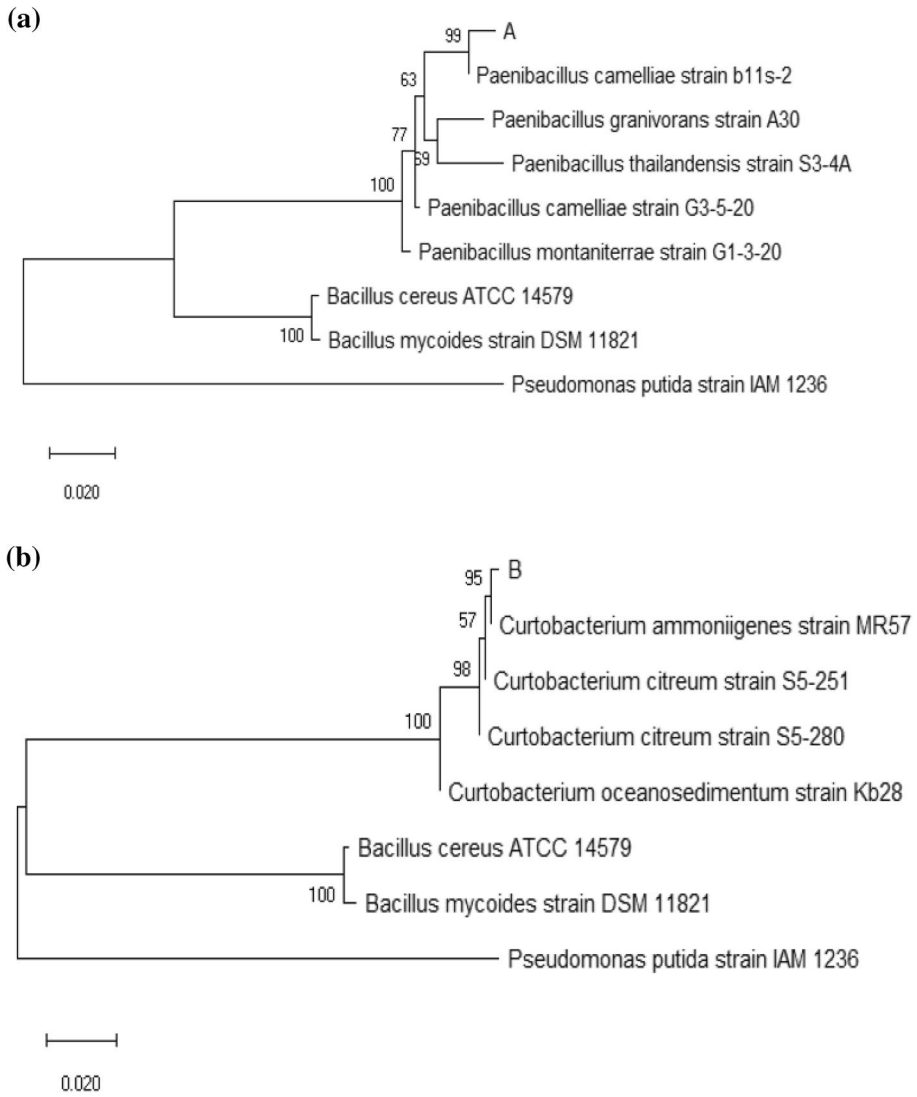

Fig. 1 Phylogenetic tree showing the relationship among the bacterial isolates with representatives of other related taxa **a** *Paenibacillus camelliae*, **b** *Curtobacterium ammoniigenes*. The tree was constructed using the software MEGA-X by the neighbor-joining method using Jukes–Cantor model. The number at the branching points indicates the levels of bootstrap support based on data for 1000 replicates. The scale bar indicates 0.02 substitutions per nucleotide position

Growth of *Synechocystis* sp. with associated bacteria under photoautotrophic and photoheterotrophic condition

Estimation of dry weight

Growth of *Synechocystis* was measured in terms of dry weight. The axenic culture of *Synechocystis* reached the stationary phase of growth on 20th day of incubation with the maximum biomass of 125.88 mg/L under photoautotrophic conditions. When *Synechocystis* was co-cultivated with *P. camelliae*, the cyanobacterial cells reached the stationary phase on 8th day of incubation with a biomass of 124.58 mg/L. However, co-culture with *C. ammoniigenes* was found to have 88.91 mg/L biomass accumulation on 6th day of incubation thereby reaching the stationary phase (Fig. 2a).

A similar trend of biomass accumulation was also observed under photoheterotrophic conditions. The axenic culture of *Synechocystis* sp. under the supplementation of 0.4% acetate showed a maximum boost in biomass (142.08 mg/L) on 16th day (stationary phase) in comparison to 20th day under photoautotrophic condition (Fig. 2b). The co-culture with *P. camelliae* and *C. ammoniigenes* along with acetate (0.4%) demonstrated a shift in cyanobacterial biomass accumulation of 141.22 mg/L and 114.35 mg/L on 6th and 5th day, respectively. Under the photoheterotrophic condition, the stationary phase in both the consortium shifted from 8 and 6th day to 6th and 5th day, respectively. The decrease in time to reach the stationary phases of the test cyanobacteria with increased biomass (specific growth rate (μ) of 0.81 day^{-1} for co-culture with *P. camelliae* and 0.73 day^{-1} for co-culture with *C. ammoniigenes)* was evident under acetate supplementation.

The axenic culture of bacteria *P. camelliae* and *C. ammoniigenes* in BG-11 medium attained stationary phase of growth on 6th and 5th day of incubation with a biomass yield of 2.95 mg/L and 2.39 mg/L, respectively, (Fig. 2c) under photoautotrophic condition. Addition of acetate to both the axenic bacterial culture significantly enhanced the biomass without affecting the stationary phase.

Estimation of photosynthetic pigments

The photosynthetic pigments of *Synechocystis* sp. viz. chlorophyll, carotenoid, and phycocyanin were estimated to study the growth of axenic and xenic cultures of cyanobacteria under photoautotrophic conditions.

Maximum chlorophyll content of 3.32% dry weight (dw) was found in co-culture of *Synechocystis* sp. with *P. camelliae* as compared to the axenic culture of *Synechocystis* sp. (1.03% dw) on 8th day of incubation (Fig. 3a). Co-culturing the test organism with *C. ammoniigenes* also increased the

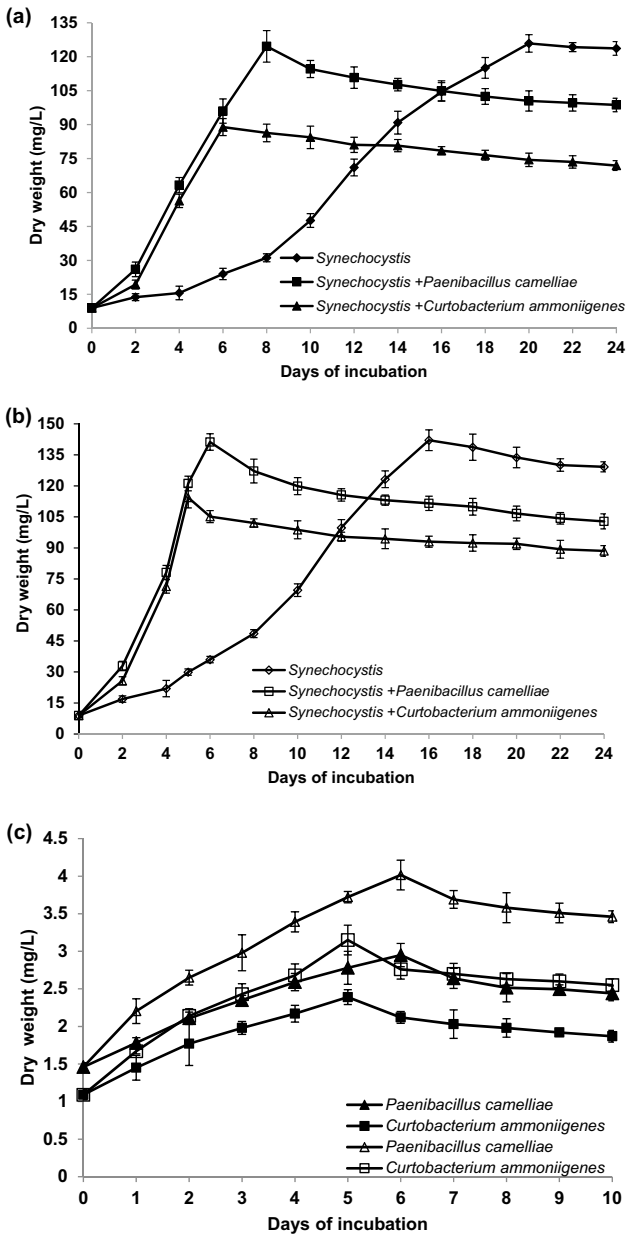

Fig. 2 Dry weight of *Synechocystis* sp. in axenic and co-culture with two associated bacterial strains under photoautotrophic condition. **a** Axenic *Synechocystis (filled diamond)*, co-culture with *P. camelliae (filled square)*, co-culture with *C. ammoniigenes (filled triangle)*, $F_{(2,6)} = 108.92$ on 8th day, $F_{(2,6)} = 123.03$ on 6th day $P < 0.05$ and under photoheterotrophic condition. **b** Axenic *Synechocystis (empty diamond)*, co-culture with *P. camelliae (empty square)*, co-culture with *C. ammoniigenes (empty triangle)* $F_{(2,6)} = 116.75$ on 8th day, $F_{(2,6)} = 132.49$ on 6th day $P < 0.05$. **c** axenic *P. camelliae (filled triangle),* axenic *C. ammoniigenes (filled square)* without acetate and axenic *P. camelliae (empty triangle);* axenic *C. ammoniigenes (empty square)* with acetate (0.4%) in BG-11 medium. All values are mean ± SE, $n = 3$

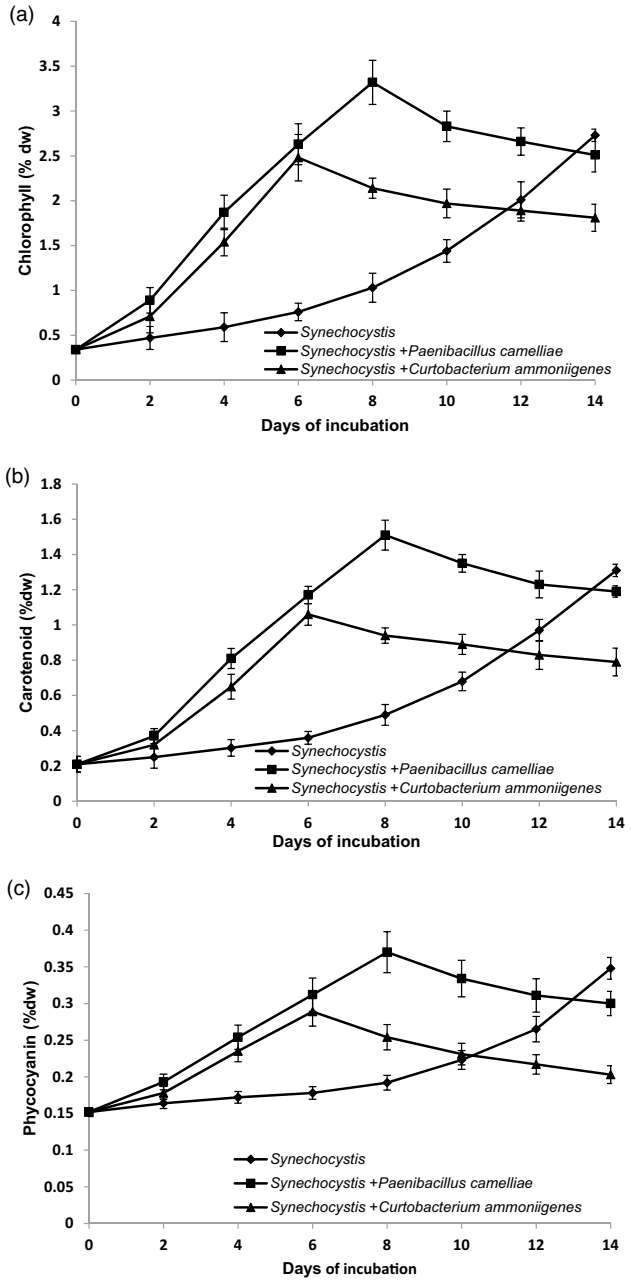

Fig. 3 **a** Chlorophyll content, **b** Carotenoid content, **c** Phycocyanin content of *Synechocystis* in axenic and co-culture with two associated bacterial strains under photoautotrophic condition. Axenic *Synechocystis (filled diamond)*, co-culture with *Paenibacillus camelliae (filled square)*, co-culture with *Curtobacterium ammoniigenes (filled triangle)*. All values are mean ± SE, $n = 3$

chlorophyll content significantly (2.48% dw, $P < 0.05$) in comparison to axenic culture on 6th day of incubation.

Nevertheless, under photoautotrophic conditions, the carotenoid content also illustrated an increasing trend. Co-cultivation experiments with *P. camelliae* showed a maximum carotenoid content of 1.51% dw as compared to the axenic culture which depicted 0.49% dw carotenoid on 8th

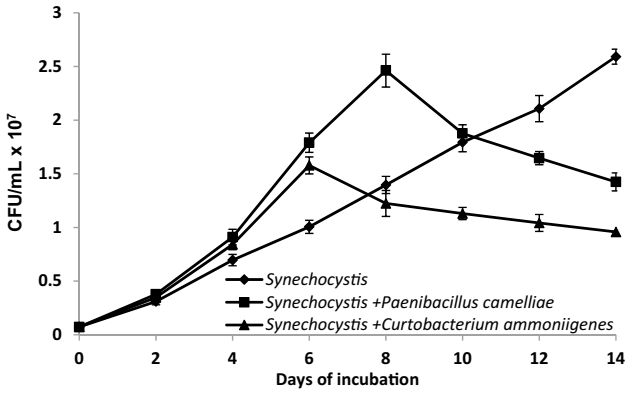

Fig. 4 Cell count of *Synechocystis* sp. in axenic and co-culture with two associated bacterial strains under photoautotrophic condition. Axenic *Synechocystis (filled diamond)*, co-culture with *Paenibacillus camelliae (filled square)* and co-culture with *Curtobacterium ammoniigenes (filled triangle*). All values are mean ± SE, $n = 3$

day of incubation (Fig. 3b). Similarly, co-culture with *C. ammoniigenes* showed maximum carotenoid content of 1.06% dw on 6th day as compared to 0.36% dw under axenic cultures.

Likewise, the phycocyanin content enhanced up to 0.37% dw under co-culture conditions with *P. camelliae* and 0.28% dw with *C. ammoniigenes* under the photoautotrophic condition on 8th and 6th day of incubation, respectively (Fig. 3c).

All the photosynthetic parameters (chlorophyll, carotenoid, and phycocyanin) showed a similar trend of increase in concentration under the photoheterotrophic condition (data not shown).

Estimation of cell count

Cyanobacterial cell count Growth of *Synechocystis* sp. in terms of cell numbers under axenic and co-culture with its two bacterial partners was presented in Fig. 4. *Synechocystis* sp. attained peak cell density in consortium with *P. camelliae* (2.46×10^7 cells ml^{-1}) on 8th day of incubation under the photoautotrophic condition as shown in Fig. 4. On the other hand, co-culture set-up with *C. ammoniigenes* demonstrated a maximum cell density of 1.58×10^7 cells ml^{-1} on 6th day of incubation. The cell number under co-cultured conditions was significantly higher as compared to the axenic culture which have a cell density of 1.39×10^7 cells ml^{-1} and 1.00×10^7 cells ml^{-1} on 8th and 6th day, respectively (Fig. 4).

Bacterial cell count The bacterial cell count was found to increase with the increasing cyanobacterial population under co-culture conditions. Under the photoautotrophic condition, co-culture with *P. camelliae* recorded the highest cell density of 9.9×10^6 cells ml^{-1} on 6th day of incubation

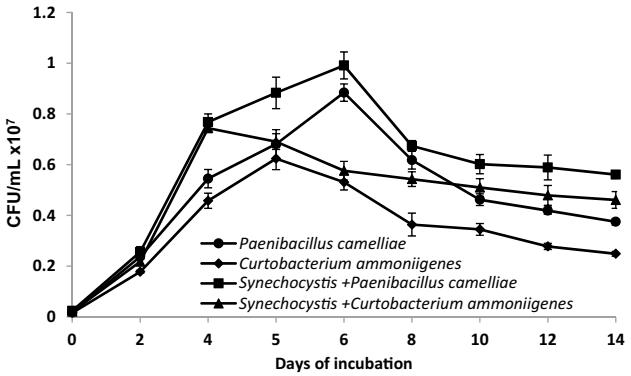

Fig. 5 Bacterial Cell counts in axenic and co-culture under photoautotrophic condition. Axenic *Paenibacillus camelliae (filled circle), axenic Curtobacterium ammoniigenes (filled diamond), Synechocystis* with *Paenibacillus camelliae (filled square)*, *Synechocystis* with *Curtobacterium ammoniigenes (filled triangle)*. All values are mean ± SE, $n = 3$

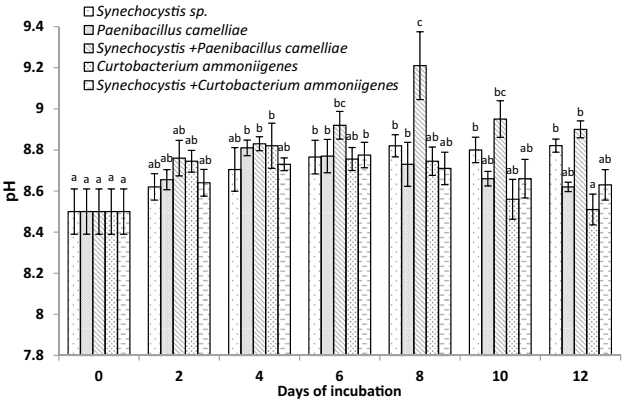

Fig. 6 pH change of culture medium of *Synechocystis* under axenic and co-culture with two associated bacterial strains under photoautotrophic condition. All values are mean ± SE, $n = 3$. Values in the graph superscripted by different letters are significantly ($P < 0.05$) different from each other (Duncan's new multiple range test)

(Fig. 5). Axenic *P. camelliae* also depicted an increase in cell number up to 8.8×10^6 cells ml^{-1} on 6th day. *C. ammoniigenes* both axenically and in association with cyanobacterium recorded its stationary phase on 5th day of incubation with 6.24×10^6 cells ml^{-1} and 6.6×10^6 cells ml^{-1}, respectively (Fig. 5).

pH

The increase in the growth rate of the cyanobacterium was evident from the increasing pH of the co-cultures. The pH of the co-culture with *P. camelliae* increased from 8.5 to 9.21 till 8th day of incubation and then decreased to 8.9 after reaching the stationary phase where no further growth was seen. The presence of *C. ammoniigenes* along with *Synechocystis* sp. also illustrated a rise in pH up to 8.77 as shown in Fig. 6. A slight increase in pH was evident in the axenic cultures of both the bacteria up to 4 days.

Dissolved oxygen (DO)

The amount of dissolved oxygen was found to decrease with the increasing growth of cyanobacterium. A drastic decrease in DO was evident in case of co-culture with *P. camelliae* (8.75–5.45) which reported maximum growth as shown in Fig. 7. Likewise, the co-culture with *C. ammoniigenes* also depicted a considerable decrease in dissolved oxygen levels as compared to that of axenic cyanobacterium (Fig. 7). Thereby, confirming the role of DO in contribution to the increasing growth. However, the axenic bacterial cultures did not show any significant decline in DO levels. A strong negative correlation was evident between DO level and biomass of co-cultures with *P. camelliae* ($r = -0.61$) and moderate negative correlation *with C. ammoniigenes* ($r = -0.43$), respectively.

Confirmation of growth-promoting substance

Production of IAA

The growth-promoting effect under co-cultures was investigated for the presence of IAA which could play an integral role in biomass enhancement. The presence of IAA was evident from the change in color of the supernatant from colorless to pink upon the addition of Salkowski reagent. This was further confirmed spectrophotometrically by measuring

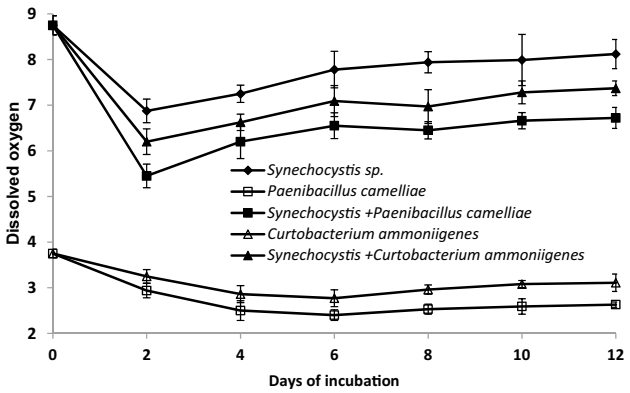

Fig. 7 Dissolved oxygen content of *Synechocystis* under axenic and co-culture with two associated bacterial strains under photoautotrophic condition axenic *Synechocystis (filled diamond)*, with *P. camelliae (filled square)*, with *C. ammoniigenes (filled triangle)*, only *Paenibacillus camelliae (empty square),* only *Curtobacterium ammoniigenes (empty triangle*). All values are mean ± SE, $n = 3$

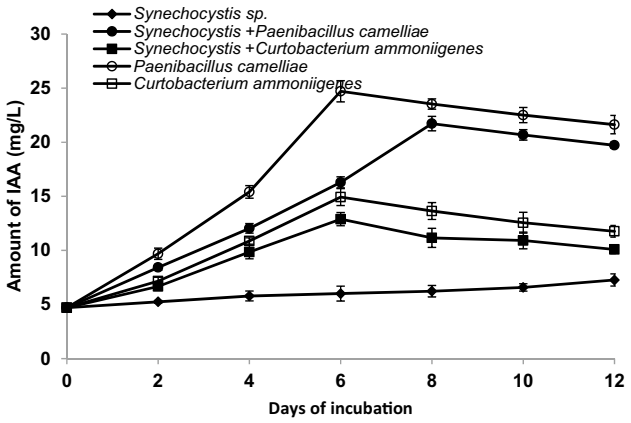

Fig. 8 IAA concentration of cyanobacteria and bacteria in axenic and co-culture conditions without L-tryptophan supplementation. All values are mean ± SE, $n = 3$

the absorbance at 535 nm. Axenic culture of *P. camelliae* showed a maximum IAA concentration of 24.71 mg/L released in the culture medium on 6th day followed by its co-culture with cyanobacteria having 21.62 mg/L of IAA on 8th day of incubation (Fig. 8). The co-culture with *P. camelliae* depicted about a three-fold increase of IAA production whereas, co-culture with *C. ammoniigenes* showed about a two-fold increase in IAA production as compared with that of axenic cyanobacteria. However, both the co-cultures with *P. camelliae* ($r = 0.99$) and *C. ammoniigenes* ($r = 0.96$) demonstrated a stronger correlation between IAA and biomass production, respectively.

The preliminary colorimetric results proved the ability of cyanobacteria and bacteria to synthesize IAA in the absence of tryptophan. The cultures were then treated with tryptophan (500 µg/ml) to validate the presence of IAA through the most widely used method HPLC.

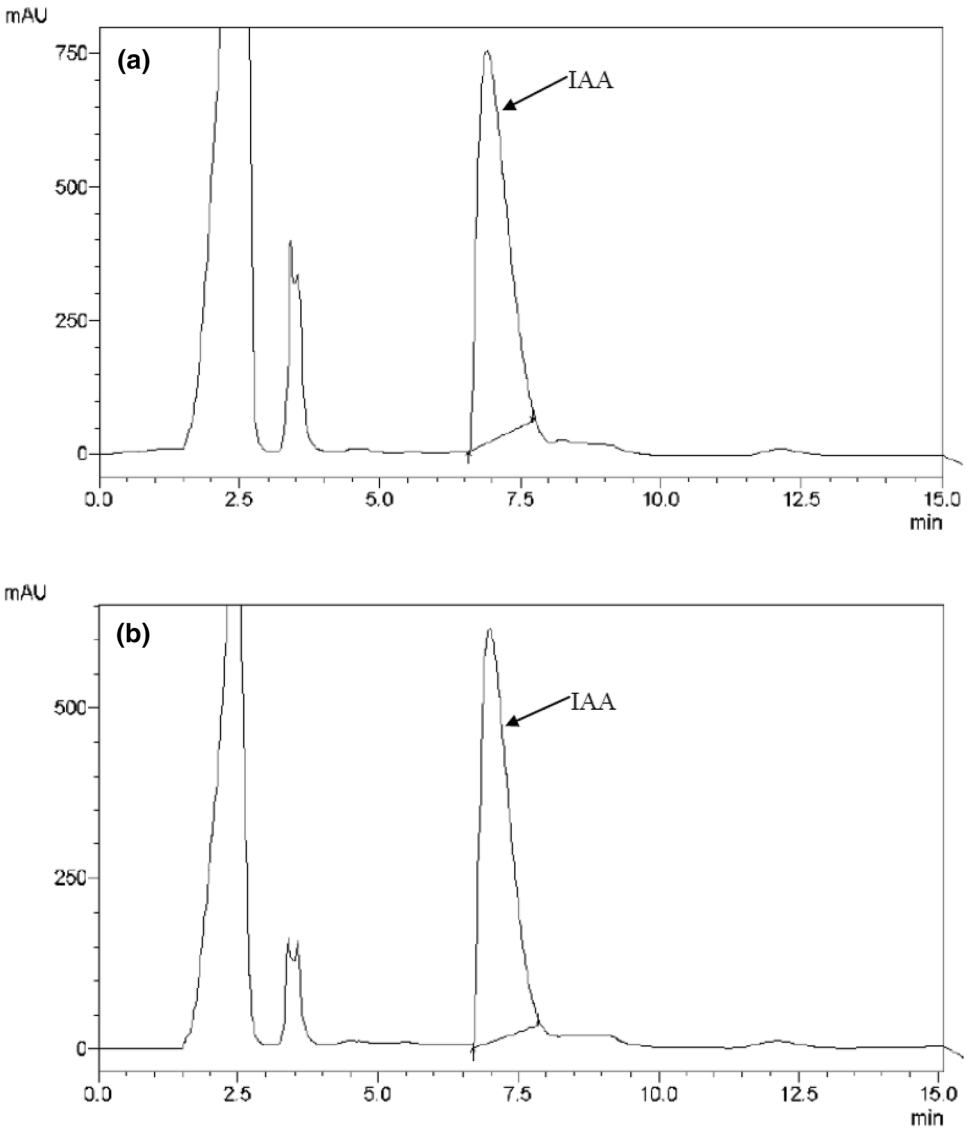

Fig. 9 HPLC Chromatogram for **a** standard IAA and **b** IAA from co-culture of *Synechocystis* sp. and *Paenibacillus camelliae*

The presence of IAA in the co-culture was confirmed by the HPLC chromatogram which produced a peak at 7.29 retention time (Fig. 9). This was similar with the predominant peaks at comparable retention time produced by the axenic *Synechocystis* sp., *Paenibacillus camelliae* and the standard IAA (data not shown). The results of HPLC were in compliance with the outcomes of colorimetric analysis.

The presence of tryptophan increased the IAA concentration of both axenic and xenic culture conditions significantly. Maximum amount of IAA production, i.e., 37.83 mg/L was detected in the presence of *P. camelliae* along with *Synechocystis* sp. under tryptophan supplementation (Table 2). Exogenous addition of IAA was also found to increase the IAA production of axenic and co-culture condition.

Effect of exogenous IAA on biomass

To test the effect of IAA on biomass production of the test organism, exogenous IAA was added to the culture medium and the biomass was estimated till 12 days (in every alternate day) under axenic and co-culture conditions. A 1.5-fold increase in biomass was evident under axenic as well as co-culture condition of cyanobacteria with each bacterial species Fig. 10. Biomass of the test cyanobacterium also illustrated a significant boost in the presence of tryptophan as shown in Fig. 10.

Table 2 Accumulation of IAA by *Synechocystis* sp. and its associated bacteria in the presence and absence of tryptophan and exogenous IAA

Culture condition	Days of incubation	Without tryptophan[a]	With tryptophan[a]	With exogenous IAA[b]
		IAA (mg/L)	IAA (mg/L)	IAA (mg/L)
Synechocystis sp.	8	6.23[a]	15.17[a]	11.54[a]
Synechocystis sp. + *Paenibacillus camelliae*	8	21.72[c]	37.83[c]	28.6[c]
Synechocystis sp. + *Curtobacterium ammoniigenes*	6	12.89[b]	22.04[b]	16.56[b]

All values are mean ± SE, $n = 3$

Values in the column superscripted by different letters are significantly ($P < 0.05$) different from each other (Duncan's new multiple range test)

[a]Tryptophan concentration – 500 µg/ml

[b]IAA concentration – 100 µg/ml

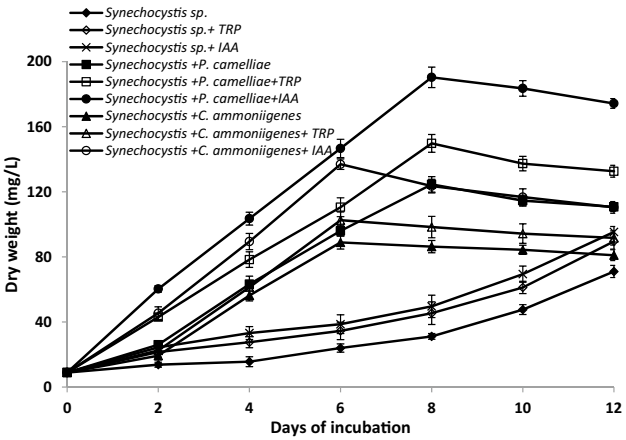

Fig. 10 Growth of *Synechocystis* sp. in axenic and co-culture with associated bacteria strains in the presence of L-tryptophan(TRP) and IAA axenic *Synechocystis (filled diamond)*, with *P. camelliae (filled square)*, with *C. ammoniigenes (filled triangle)*, axenic *Synechocystis* with tryptophan *(empty diamond)*, with *P. camelliae* and tryptophan *(empty square)*, with *C. ammoniigenes* and tryptophan *(empty triangle)*, axenic *Synechocystis* with IAA (cross), with *P. camelliae* and IAA *(filled circle)*, with *C. ammoniigenes* and IAA *(empty circle)*. All values are mean ± SE, $n = 3$

Production of siderophore

Both the bacterial isolates viz. *P. camelliae* and *C. ammoniigenes* produced siderophore as evident from the positive reaction with the $FeCl_3$ test. The color change of the supernatant from yellow to reddish orange on the addition of $FeCl_3$ confirmed the presence of siderophore. The axenic

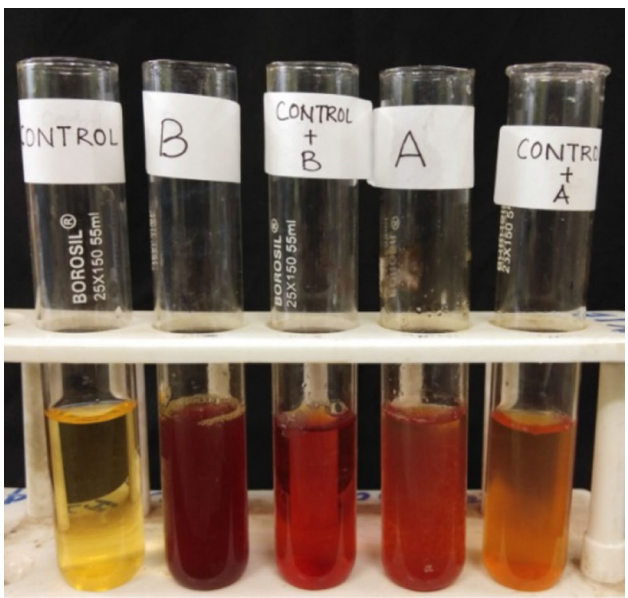

Fig. 11 Siderophore production in bacterial isolates (control: *Synechocystis* sp. A: *Paenibacillus camelliae* B: *Curtobacterium ammoniigenes*)

cyanobacterial culture did not depict any color change on $FeCl_3$ addition as shown in Fig. 11. Out of the two axenic bacterial cultures, *C. ammoniigenes* was found to produce a higher amount of siderophore (dark reddish color) as compared to *P. camelliae* (Fig. 11). The co-cultures also gave positive results to the test, thus establishing the presence of siderophore and its role in growth enhancement.

Discussion

In the natural environment, cyanobacteria–bacteria consortium is prevalent in almost all conditions. However, in this study, efforts were made to isolate the associated bacteria from the twenty-month-old photo-bioreactor culture of *Synechocystis* sp. PCC 6803 and study its effect on the growth and metabolism of the test cyanobacterium. Two associated bacteria were isolated from the old culture of cyanobacteria and both were found to be Gram-positive, rod-shaped as represented in Table 1. Based on the phylogenetic analysis of 16S rRNA gene sequences, the isolates were clad under the genera *Paenibacillus camelliae* of phylum Firmicutes and *Curtobacterium ammoniigenes* of Actinobacter, respectively (Fig. 1). The isolation of these strains at higher dilution levels suggested their numerical abundance in the old culture. The bacterial and cyanobacterial cells were co-cultured under different ratios (1:1, 1:2, 1:3, 1:4, 2:1 and 3:1); however, the best results were obtained for 1:3 condition which was then chosen for further experimentation. The axenic cyanobacteria biomass was lower than the ones inoculated with both cyanobacteria and bacteria. There were nearly no differences between bacteria cyanobacteria biomass with 1:3 and 1:4, whereas 1:1 and 1:2 ratios did not depict any such significant rise on the biomass. This result suggests that inoculation with both cyanobacteria and bacteria increases the production of biomass in comparison with axenic cyanobacteria, but a higher ratio of bacteria added could decrease the growth of the biomass. No correlation was observed between initial ratio and growth enhancement. Different species showed different initial ratio of algae–bacteria for maximum biomass yield *viz.* 1:1 (Pham et al. 2019), 4:1 (Roudsari et al. 2014), 5:1 (Su et al. 2012). In this work, bacterial strains, both axenically and in association with the test cyanobacterium, were cultured under autotrophic and heterotrophic conditions to discover the relationship between them. For heterotrophic condition, cultures were supplemented with 0.4% sodium acetate as it is a precursor of acetyl-CoA and stimulates accumulation of biotechnological products (Panda et al. 2006). The association of test cyanobacterium and both the isolated bacteria studied here was found to be mutualistic in nature. The presence of bacterial isolates stimulated the cyanobacterial growth and no inhibitory effect was observed as reported by Fisher et al. (1998). Variations in culture conditions and changes in environmental parameters were reported to alter the relationship between the bacteria and its partner (Rier and Stevenson, 2002; Gurung et al. 1999). Conversely, in this study, no change in the relationship between the two partners was evident under different cultural conditions.

Growth of cyanobacteria was measured in terms of dry weight. The presence of isolated bacterial partners along with *Synechocystis* sp. stimulated the biomass accumulation in co-culture experiments. Out of the two bacterial isolates, *P. camelliae* depicted a four-fold higher cyanobacterial biomass [$F_{(2,6)} = 108.92$ at $P < 0.05$] on 8th day of incubation under photoautotrophic condition (Fig. 2). The presence of *C. ammoniigenes* also demonstrated a significant increase [$F_{(2,6)} = 123.03$ at $P < 0.05$] as compared to axenic cyanobacteria on 6th day of incubation. The decrease in time to reach the stationary period and increase the biomass in the presence of bacterial isolates were the major outcomes of this experimental setup. The addition of heterotrophic bacteria might have supported the cyanobacterial growth utilizing the photosynthetically produced carbon compounds, whose accumulation is known to inhibit photosynthesis (Abed 2010).

Photosynthetic pigments like chlorophyll, carotenoid, and phycocyanin were also assessed to study the growth pattern of the test cyanobacterium. The maximum chlorophyll content of 3.32% dw was seen in the case of co-culture with *P. camelliae* on 8th day followed by 2.48% dw in *C. ammoniigenes* on 6th day of incubation as shown in Fig. 3a. Different accessory pigments like carotenoids and phycocyanin also illustrated a similar trend of increase in co-culture conditions than axenic culture (Fig. 3b and c). This significant increase of pigments in co-culture conditions could be explained in light of supplementation of various growth-promoting factors released by bacterial partner (Watanabe et al. 2005).

Cell density was another factor investigated to confirm the growth enhancement effect by bacteria. The addition of bacterial isolates to the cyanobacterial culture media showed a marked increase in the cell density of cyanobacteria when compared to its control (Fig. 4). Population of *Synechocystis* sp. under photoautotrophic conditions increased up to 2.46×10^7 cells ml^{-1} and 1.58×10^7 cells ml^{-1} in association with *P. camelliae* and *C. ammoniigenes* on 8th and 6th day, respectively (Fig. 4). The increase in cell density of cyanobacteria in co-cultures might be due to the consumption of photosynthetic oxygen by bacteria (Mouget et al. 1995).

The bacterial cell count was found to increase with the increasing cyanobacterial population under co-culture conditions. The bacterial population in the co-culture attained stationary phase of growth on 6th and 5th days of incubation for *P. camelliae* and *C. ammoniigenes*, respectively (Fig. 5). The stimulatory effect in the growth of bacteria might be due to the exopolysaccharide in the culture media which

is naturally secreted by the cyanobacteria (Sadchikov and Marakov 2000).

The elevation in pH was observed under both axenic and co-culture conditions (Fig. 6). The maximum pH change was found in case of co-culture with *P. camelliae* which varied from 8.5 to 9.2. Panda et al. (2006) reported a maximum biomass of *Synechocystis* sp. PCC 6803 under pH 8.5 followed by pH 9.5, whereas more alkaline and acidic pH did not support the cyanobacterial growth. Co-culture with *P. camelliae* also depicted highest increase in growth as compared to the other co-culture set-ups. Our results are quite in tune with the reports of Guo and Tong (2014) where a similar increase in pH was observed for *Chlorella vulgaris* co-cultured with *Pseudomonas*. The rise in pH might be due to cyanobacterial photosynthesis (Albertano et al. 2000) which in turn had a negative effect on bacterial growth (Guo and Tong 2014).

Cyanobacteria are promising candidates for carrying out oxygenic photosynthesis, thus releasing oxygen to the surrounding for consumption by aerobic bacteria. Bacteria in return produce carbon dioxide which is a vital requirement for cyanobacterial growth (Borde et al. 2003; Tison and Lingg 1979). Utilization of cyanobacterial exudates by the bacterial isolate results in increased oxygen consumption, thus reducing photosynthetic oxygen concentration which may have a detrimental effect on various metabolic processes of cyanobacteria. Thus, under co-culture conditions, bacteria decrease the DO concentration preventing its partner from the detrimental effect of high oxygen concentration (Mouget et al. 1995). In this study, DO was also found to decrease with the increasing growth of cyanobacterial (Fig. 7) which might be due to the consumption of DO in the medium by the bacterial partner. This is in well compliance with the report of (Guo and Tong 2014). Further, reduction in DO was more distinct for co-culture with *P. camelliae* having a stronger correlation coefficient than that of *C. ammoniigenes*, where the former showed higher biomass than the latter.

Growth-enhancing effect may be due to the complex network of interchangeable materials which are important for the continued nutrition of both the organisms (Kumar et al. 2014). One such growth-promoting factor is IAA, the phyto-hormone responsible for inducing root elongation and growth in plants. IAA has been detected in a large number of microorganisms including cyanobacteria. IAA synthesis can be stimulated by the addition of exogenous tryptophan in the media. However, reports of Mazhar et al. (2012) proved the presence of IAA in some cyanobacteria (including *Synechocystis*) without tryptophan supplementation. In this study, IAA production was found to be less in cultures without tryptophan. The increase in IAA concentration was apparent on addition of exogenous tryptophan to the culture media (Table 2). Thus, this increase in IAA concentration confirmed that *Synechocystis* sp. undergoes a trp-dependent pathway for IAA synthesis (Mazhar et al. 2012). The pathway involves the conversion of tryptophan into indole-3-acetonitrile which subsequently leads to the formation of IAA (Tsalvkelova et al. 2005). The addition of exogenous IAA to the culture media also elevated the biomass accumulation, thus proving its role in promoting biomass production (Fig. 10).

Both the bacterial partner responded positively to the Salkowski test but the amount of IAA was found to be considerably more in case of *P. camelliae* (24.71 mg/L) as shown in Fig. 8. Similar findings were reported by Aswathy et al. (2013) for two different strains of *Paenibacillus* that synthesized IAA. The co-culture with *P. camelliae* also showed the presence of IAA proving it to be the main driving force in growth promotion. This study corroborates with the report of Lee et al. (2019) where *Achromobacter* was found to supplement IAA to its algal partner *Haematococcus pluvialis*. The colorimetric study was further confirmed by HPLC analysis which demonstrated the presence of IAA in co-culture conditions (Fig. 9).

Siderophores are low molecular weight compounds secreted by microorganisms to combat iron stress (Yeole et al. 2001). It is mainly synthesized by bacteria and acts as growth-promoting agent for algae and plants (Benderliev and Ivanova 1994). Siderophore production in the axenic bacterial cultures and co-culture was confirmed by the $FeCl_3$ test through change in color of the supernatant to reddish orange as shown in Fig. 11. This is in well agreement with the reports of Dave et al. (2006). Thus, it established the role of bacteria in influencing cyanobacterial growth and metabolism.

A comparative account of various growth-promoting factors and their effect on *Synechocystis* sp. under the co-culture with *P. camelliae* and *C. ammoniigenes* is represented in Table 3. Co-culture with *P. camelliae* depicted higher biomass yield due to the more positive effect of three growth-enhancing factors IAA, DO and pH. On the other hand, co-culture with *C. ammoniigenes* also tested positive

Table 3 Comparative account on the production of growth-promoting factors in the co-culture of *Synechocystis* sp. with *Paenibacillus camelliae* and *Curtobacterium ammoniigenes*

Co-culture condition	Growth-promoting factors			
	pH	Dissolved oxygen (DO)	IAA	Siderophore
Synechocystis sp. + *Paenibacillus camelliae*	+ +	+ +	+ +	+
Synechocystis sp. + *Curtobacterium ammoniigenes*	+	+	+	+ +

+ +: High + : Moderate

for all the growth-promoting factors; however, siderophore production was more pronounced than the other factors (Table 3). Therefore, the presence of combination of such growth-enhancing factors proved to be the main driving force for boosting up cyanobacterial growth under co-culture conditions.

Conclusion

In conclusion, this work isolated two mutualistic bacteria from the long-term culture of *Synechocystis* sp. Selective infestation of the bacterial isolates in the cyanobacterial phycosphere led to a remarkable decrease in the stationary phase to attain more biomass yield at less span of time. The cyanobacterial–bacterial consortia offer a cost-effective and promising system for biomass enhancement in less time. Among all the growth-promoting factors studied here, IAA was found to be more effective for enhancement of cyanobacterial biomass. Further investigations should extrapolate the molecular basis of co-culture conditions to study the nature of interaction between the partners and extend their application to other fields.

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Author contributions ST and BP carried out the conceptual design of the study. Data acquisition, analysis and interpretation were performed by ST, KD and BP. All the three authors contributed to manuscript writing and reviewing.

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Data availability All the data analyzed during the study are included in this article.

Declarations

Conflict of interest The authors declare that they have no competing interest.

Consent for publication All co-authors consent to the present version of the manuscript for submission for publication.

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