ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

OPEN ACCESS Biotechnology

ISSN 1682-296X
DOI: 10.3923/biotech.2020.1.9

Research Article
Improved Biomass Through Mutualistic Co-culturing of *Chlorella vulgaris* with *Nitrobacter* in Sewage Water

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Abstract

Background and Objective: The mutualistic interaction between microalgae and bacteria when co-culturing aid each other by promoting growth. This work was planned to determine the benefits of co-culturing *Chlorella vulgaris* with *Nitrobacter* sp. in terms of enhanced microalgal biomass and lipid production. **Materials and Methods:** Different growth media viz., Bristol media, synthetic waste water and dilutions of waste water were used for co-culturing for a period of 16 days. Enhancement of microalgal growth in terms of growth rate, biomass, protein, carbohydrates, chlorophyll and total lipid content in microalgae were determined at regular intervals and at the end of cultivation period. **Results:** Specific growth rate was promoted in co-culture after 4th day of cultivation whereas monoculture has resulted in highest growth rate after 13th day of cultivation. Protein and carbohydrate contents of mono- and co-cultured *C. vulgaris* were 20.03 and 9.413 μg mg^{-1}, respectively in 50% sewage water. There was a down trend in dissolved oxygen levels when the microalgae were co-cultured with bacteria. Biomass productivity was 0.0371 g L^{-1}/day in monoculture after 16th day of cultivation and was 0.0285 g L^{-1}/day after 4 days of cultivation as co-culture. Highest lipid content of 20.69% was observed in monoculture and the co-culture has resulted in 17.93%. **Conclusion:** The results indicated that co-culturing of *C. vulgaris* with *Nitrobacter* resulted in enhanced growth promotion as evidenced by increased cellular composition and biomass content. This interaction could be utilized in enhancing microalgal biomass, especially by replacing nitrogen fertilizers in the growth medium.

Key words: Algae, bacteria, co-culture, biomass, effluent, algal growth, nitrogen fertilizers, lipid content

Citation: Sagar Grover, Shaily Rubina Tirkey, Veeramallegowda, Saroj Yadav and G. Sibi, 2020. Improved biomass through mutualistic co-culturing of *Chlorella vulgaris* with *Nitrobacter* in sewage water. Biotechnology, 19: 1-9.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Microalgae and bacteria grow together in aquatic environment as plants and bacteria do in the rhizosphere[1]. In such environments, the interactions been either mutualistic or commensalistic or parasitic and are often considered species specific[2-5]. Both bacteria and algae share a complex ecological relationship and some bacteria can promote the growth and biomass of algae by metabolic aspects[6-8]. During the co-growth of bacteria and microalgae, both the populations promote each other's growth by secreting specific enzymes and growth factors. These mutualisms aid each other and make them adapt to changing environment. Phycosphere is the region where the interaction between bacteria and algae happens. There is evidence that bacteria may derive nutritional benefit from extracellular products released by algae and the negative or positive effect of bacteria on microalgal growth. The nature of association of algae with the bacteria in the phycosphere may well determine the growth characteristics of the algae.

There have been no systematic studies so far that have addressed the nature of growth media in which the co-culturing of bacteria and microalgae was more effective. In view of the beneficial association between bacteria and microalgae, this study attempts to describe the growth promoting ability of nitrogen fixing bacteria while co-culturing with microalgae. It also addresses the mutualistic relationship between *Nitrobacter* and *Chlorella vulgaris* in different growth media by means of enhanced growth rate, biomass, protein, carbohydrates, chlorophyll and total lipid content in microalgae.

MATERIALS AND METHODS

Study area: This study was carried out at Department of Biotechnology, Indian Academy Degree College-Autonomous, Bangalore between January, 2019 and August, 2019.

Organisms used: *Chlorella vulgaris* was cultivated in Bristol medium ($NaNO_3$-2.94 mM, $CaCl_2.2H_2O$-0.17 mM, $MgSO_4.7H_2O$ -0.3 mM, K_2HPO_4-0.43 mM, KH_2PO_4-1.29 mM, NaCl-0.43 mM, distilled water-1000 mL). *Nitrobacter* NCIM 5062 was cultivated in modified Stanier's medium (Solution-1: $MgSO_4.7H_2O$-0.2 g, K_2HPO_4-1.0 g, $FeSO_4.7H_2O$-50 mg $CaCl_2.2H_2O$-20 mg, $MnCl_2.4H_2O$-2 mg, $Na_2MoO_4.2H_2O$-1 mg, distilled water-1 L, pH 8.5. Sterilization at 121°C for 15 min. Solution-2: $NaNO_2$-6.0 g, distilled water-100.0 mL. Solution 2 is filter sterilized. To 100 mL of solution I, 5 mL of solution 2 is added aseptically) separately.

Co-culture of algae and bacteria: Cells of *C. vulgaris* were harvested by centrifugation at 4,500 g and 25°C for 5 min when they grew to saturation. The algal pellet was washed gently with distilled water. *Nitrobacter* NCIM 5062 was also harvested by centrifugation at 5,000 g and 25°C for 5 min when *Nitrobacter* is grown to saturation (stationary phase) then resuspended in autoclaved water so that OD_{600} = 1.0. Then, 10 mL of the resuspended bacterial sample was added to experimental flasks containing 250 mL of growth media followed by the addition of 0.5 mg algae. The mixed culture in the flask was shaken gently so that bacteria and algae are mixed well.

Experimental setup: Bristol media, synthetic waste water media and sewage water were used to co-culture the bacteria and microalgae. Dilutions of sewage water were prepared in distilled water and the autoclaved sewage water was used for the experiments. Monoculture of *C. vulgaris* alone served as control for the experiments:

- Exp 1: Bristol medium+*Nitrobacter*+*C. vulgaris*
- Exp 2: Synthetic waste water+*Nitrobacter*+*C. vulgaris*
- Exp 3: 25% sewage water+*Nitrobacter*+*C. vulgaris*
- Exp 4: 50% sewage water+*Nitrobacter*+*C. vulgaris*
- Exp 5: 75% sewage water+*Nitrobacter*+*C. vulgaris*

Cultivation conditions: The experimental setups were carried out in Erlenmeyer flasks under controlled laboratory conditions (temperature 25°C, light intensity of 60 µmol photons m^{-2} sec^{-1} and a light/dark cycle of 12/12 h). Water losses through evaporation were maintained by adding required amount of double distilled water to cultures. All experiments were carried out in triplicates for a period of 16 days.

Specific growth rate: Specific growth rate (µ) of the microalgae was calculated according to the following Eq.[9]:

$$\mu = \frac{\ln (N_t / N_0)}{T_t - T_0}$$

where, N_t and N_0 are the total cells at the end of log phase (T_t) and start of log phase (T_0), respectively.

Chlorophyll estimation: Chlorophyll contents of the microalga were estimated according to Becker[10]. Algal cells were centrifuged and extracted with acetone overnight. The extract was centrifuged at 3000×g for 5 min and the chlorophyll content in the supernatant were determined by

measuring the optical densities at 645 and 663 nm in a spectrophotometer and then calculated using the Eq. 4:

$$\text{Chl (mg L}^{-1}) = 8.02\times OD_{663}+20.21\times OD_{645} \quad (1)$$

Protein assay: The extraction of proteins from microalgae was performed using alkali method. Aliquots of algal sample were centrifuged and 0.5 N NaOH was added to the pellet followed by extraction at 80°C for 10 min. The mixture was centrifuged and protein content of the supernatant was estimated using Bovine Serum Albumin (BSA) as standard[11].

Carbohydrate assay: Cellular carbohydrates were estimated using the anthrone method after hot alkaline extraction[12,13]. Briefly, microalgal pellets were resuspended in distilled water and then heated in 40% (w/v) KOH at 90°C for 1 h. After cooling down, ice cold ethanol was added and stored at -20°C overnight followed by centrifugation. The pellet was resuspended in distilled water and then reacted with anthrone reagent. D-glucose was used as standard and the color development was read at 578 nm in a spectrophotometer.

Biomass content and productivity: Biomass (g L^{-1}) of *C. vulgaris* grown under different experimental setups was determined by measuring the optical density of samples at 600 nm (OD_{600}) using UV-Vis spectrophotometer. Biomass concentration was then calculated by multiplying OD_{600} values with 0.6, a predetermined conversion factor obtained by plotting OD_{600} versus dry cell weight (DCW). DCW was determined gravimetrically by centrifuging the algal cells (3,000×g, 10 min) and drying[14]:

$$\text{Biomass concentration} = OD_{680}\times 0.6 \quad (2)$$

The biomass productivity (g L^{-1}/day) was calculated according to Eq. 3:

$$\text{Biomass productivity} = \frac{B_t\text{-}B_0}{T_t\text{-}T_0} \quad (3)$$

where, B_t was the biomass concentration at the end of cultivation period (T_t) and B_0 is the initial biomass concentration at the beginning of the cultivation period (T_0).

Total lipid estimation: Lipid extractions from dried algal cells were carried out by chloroform: methanol extraction method[15]. Dried algal cells added with distilled water were ultrasonicated and mixed with chloroform: methanol (2:1). The mixture was left for 30 min in a water bath (30°C) and filtered through a Whatman No. 1 filter paper. The filtrate was transferred to another screw cap tube containing NaCl solution (0.9%) and the purified chloroform layer was evaporated to a constant weight in a fuming hood under vacuum at 60°C. The total lipid content of dry weight was calculated using the following Eq:

$$\text{Lipid content (\%)} = \frac{m_2 - m_0}{m_1}\times 100 \quad (4)$$

where, m_1 is the weight of the dried algal cells, m_0 is the weight of the empty new screw cap tube and m_2 is the weight of the new screw cap tube with the dried lipids.

Statistical analysis: All the experiments were carried out in triplicate and data are expressed as Mean±SD.

RESULTS

Specific growth rate: The specific growth rate of *C. vulgaris* cultivated as monoculture started increasing significantly after 10 days of cultivation period irrespective of the growth media used (Fig. 1). Highest specific growth rate of 0.061 µ/day was observed in 50% sewage water and the lowest specific growth rate was seen in synthetic waste water throughout the study. In the case of co-culturing, the specific growth rate was in the range of 0.02-0.047 µ/day in 50% sewage water and the increase in growth rate was observed from 2nd day of cultivation. Higher growth rate was also observed in co-culture grown in 50% sewage water and the lowest growth rate was recorded in Bristol and synthetic waste water medium (Fig. 2).

Chlorophyll content: Both chlorophyll-a, -b of the microalgae grown as monoculture and co-culture was measured using UV-Vis spectrophotometer at the end of cultivation period. The content of chlorophyll-a of *C. vulgaris* co-cultured with *Nitrobacter* in synthetic waste water medium was highest with a value of 1.48 µg mg^{-1}. Higher values of chlorophyll-a were seen with co-culture except in 25% sewage water which also recorded the lowest value of 0.25 µg mg^{-1} (Fig. 3). However, highest chlorophyll-b content was observed in monoculture of *C. vulgaris* cultivated in Bristol medium. The other tested growth media resulted in higher values of chlorophyll-b in co-culture which was similar to that of chlorophyll-a content (Fig. 4).

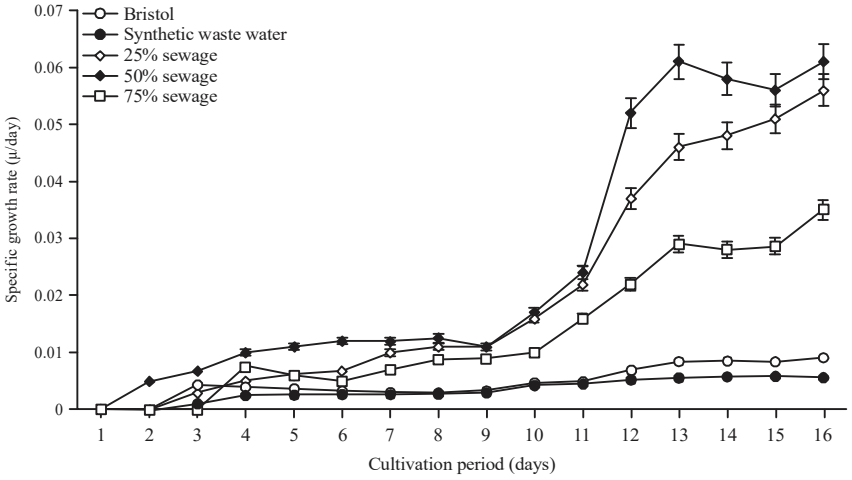

Fig. 1: Specific growth rate of *C. vulgaris* as monoculture in different growth media

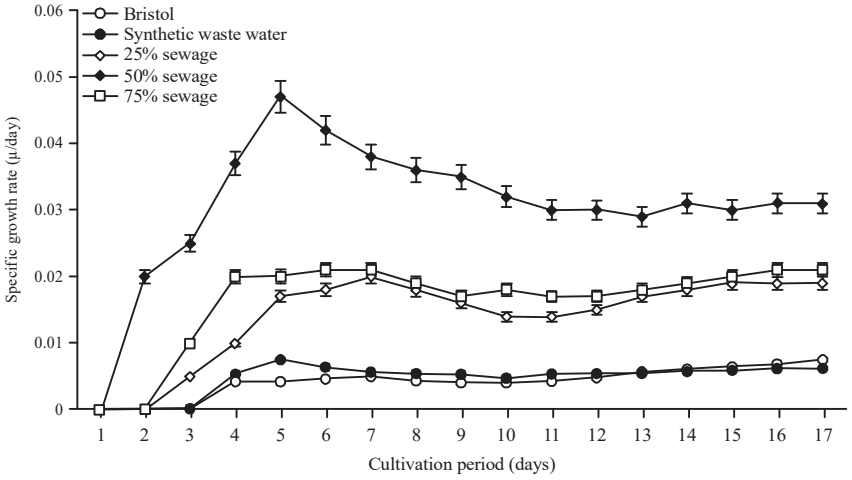

Fig. 2: Specific growth rate of *C. vulgaris* co-cultured with *Nitrobacter* in different growth media

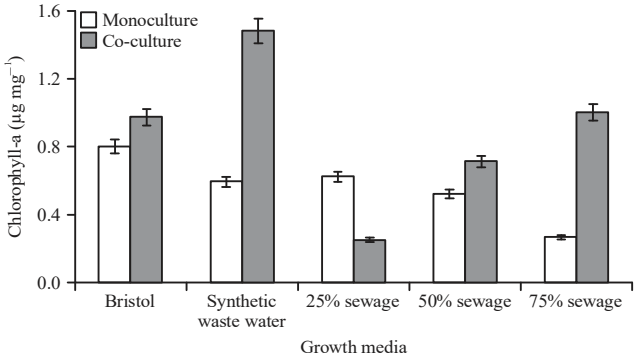

Fig. 3: Chlorophyll-a content of mono and co-culture of *C. vulgaris* in different growth media

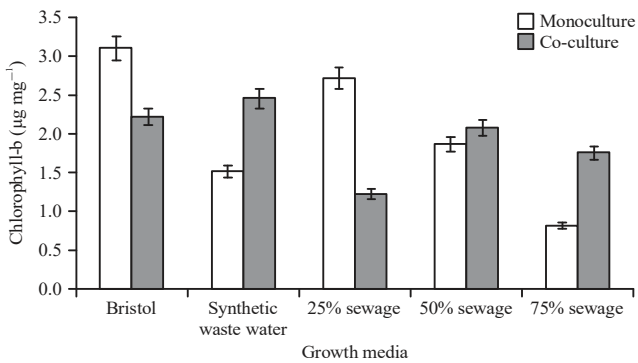

Fig. 4: Chlorophyll-b content of mono and co-culture of *C. vulgaris* in different growth media

Total protein: Total proteins of mono and co-culture of *C. vulgaris* was extracted by alkali method. Monoculture of *C. vulgaris* had protein content in the range of 2.56-6.32 μg mg^{-1} whereas, it was 12.8-20.03 μg mg^{-1} when

co-cultured with *Nitrobacter* (Fig. 5). In both the culture methods, highest protein content was observed in cells grown in 50% sewage water. The protein content of co-cultured microalgae from 50% sewage water was 20.03 µg mg^{-1} which was 3.2 times higher than monoculture (6.32 µg mg^{-1}).

Total carbohydrates: Total carbohydrates of microalgae grown in different media were between 1.02 and 9.41 µg mg^{-1}. In general, co-culturing of microalgae with nitrogen fixing bacteria had resulted in higher levels of total carbohydrates (Fig. 6). In other words, 2 and 3 fold increase of carbohydrates was observed in co-culture than that of monoculture. Similar to other assays, co-culturing in 50% sewage water has recorded highest value in terms of carbohydrates.

Biomass productivity: The biomass productivity of *C. vulgaris* was calculated for a period of 16 days from biomass concentration. Similar to specific growth rate, biomass productivity of monoculture *of C. vulgaris* increased from 10th day of cultivation (Fig. 7). A higher microalgae biomass production in the co-culture is more likely that this is because *Nitrobacter* decrease the local concentration of photosynthetic oxygen at the surface of microalgae cells thereby improving *C. vulgaris* growth.

Biomass productivity of co-cultured microalgae has started increasing from 2nd day of cultivation in 50 and 75% sewage water with a maximum of 0.028 g L^{-1}/day at the end of 4th day in 50% sewage water. After 7 days of cultivation, there was a decline in biomass however it was higher than other growth media at the end of cultivation period (Fig. 8).

Total lipid content: The total lipid content of microalgae was estimated by gravimetric method and it was noted that the monoculture of *C. vulgaris* has produced higher lipid content than the co-culture at the end of cultivation period under

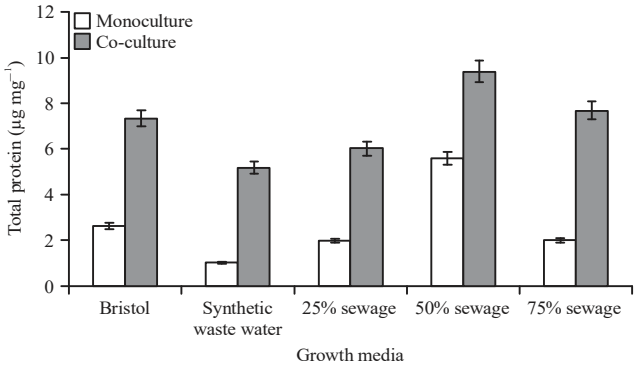

Fig. 5: Total protein content of mono and co-culture of *C. vulgaris* in different growth media

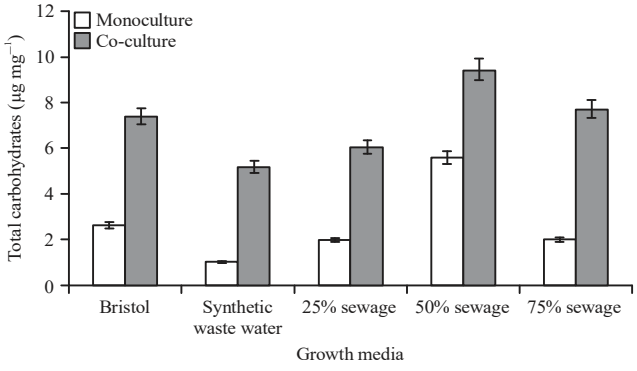

Fig. 6: Total carbohydrate content of mono and co-culture of *C. vulgaris* in different growth media

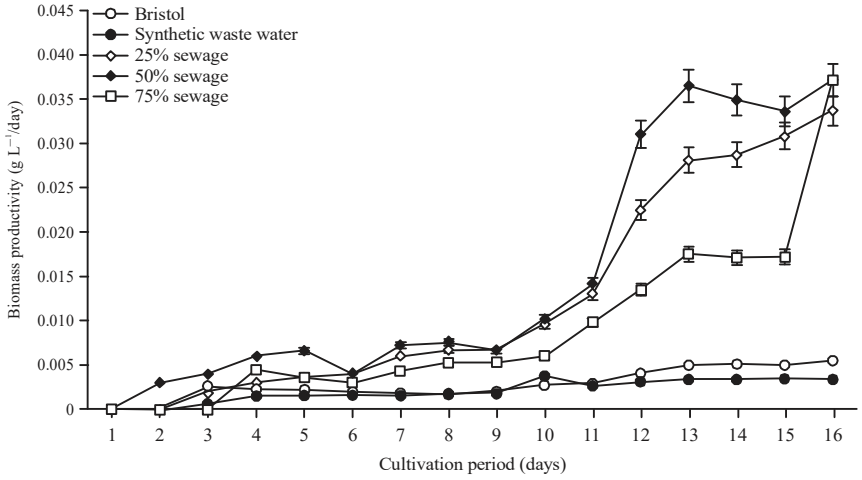

Fig. 7: Biomass productivity of *C. vulgaris* as monoculture in different growth media

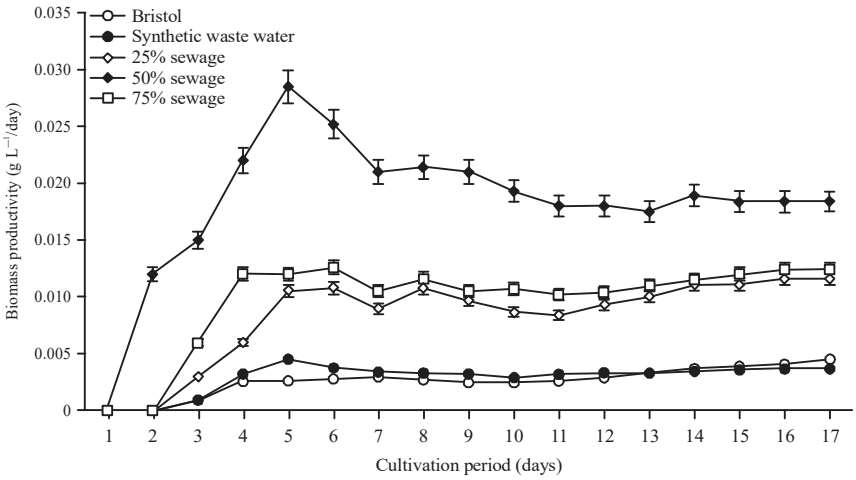

Fig. 8: Biomass productivity of *C. vulgaris* co-cultured with *Nitrobacter* in different growth media

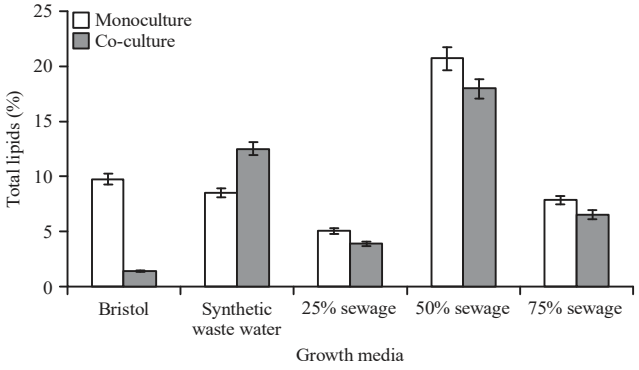

Fig. 9: Total lipid content of mono and co-culture of *C. vulgaris* in different growth media

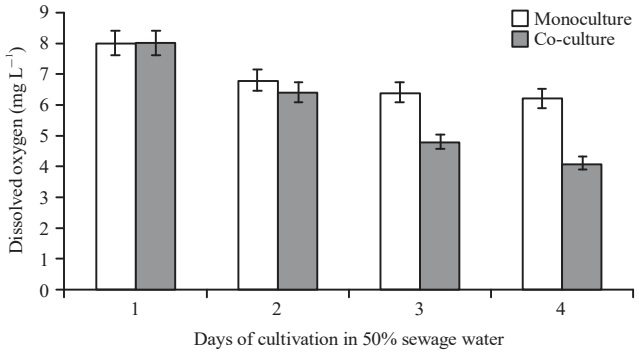

Fig. 10: Dissolved oxygen levels in 50% sewage containing monoculture and co-culture

the experimental conditions (Fig. 9). The highest lipid content of 20.69 and 17.93% was observed in monoculture and co-culture grown in 50% sewage respectively. The lowest lipid content (1.46%) was seen in microalgae co-cultured with bacteria in Bristol media.

Co-culture kept the dissolved oxygen concentration reducing during the 4 days cultivation period in 50% sewage water. The level of dissolved oxygen was reduced from 8-4.1 mg L^{-1} when both *C. vulgaris* and *Nitrobacter* was cultivated together. Monoculture of *C. vulgaris* did not reduce the dissolved oxygen much when compared to co-culture (Fig. 10).

DISCUSSION

This study has been conducted to identify the positive effects of *Nitrobacter* on *C. vulgaris* growth in co-culture as an alternate to nitrogen fertilizer. The findings indicated that co-culturing of *C. vulgaris* with *Nitrobacter* resulted in enhanced growth promotion as evidenced by increased cellular composition and biomass content. Bacterial association with phytoplankton involves wide range of interactions including metabolite/nutrient uptake, provision and remineralization[16,17], cell differentiation[18] and is also species specific[19]. One such example is that *Marinobacter* is promoting algal assimilation of iron which in turn releases organic molecules that are used by the bacteria for growth[20]. Growth promotion of microalgae in the presence of *Azotobacter vinelandii* was reported earlier by Ortiz-Marquez *et al.*[21]. When *Pseudomonas* was added to the monoxenic culture of *A. glacialis*, the growth rate was about 0.64/day which was about 2.3 times higher than that of control[7]. Bacterial community of *Roseobacter* group, *Hyphomonas* and Flexibacteriaceae had promoted the growth of *Thalassiosira rotula* but also led to a rapid decline after the growth phase[22]. In this study, growth promotion of *C. vulgaris* by *Nitrobacter* was seen at earliest days of cultivation. Though the highest growth rate obtained in

co-culture was lower (0.047 μ/day) than monoculture (0.061 μ/day), the time taken to achieve the highest growth rate was comparatively shorter (5th day) than monoculture (13th day). The results were similar to the findings of Xu *et al.*[23], where the OD_{600} value of *A. chroococcum* co-cultured with algae increased significantly and reached the maximum value of 1.16 on day 7. In another study, *Rhizobium* sp. was co-cultured with green algae and has enhanced the growth by 72% more than the control cultures[24]. The specific growth rate of *C. vulgaris* was increased from 0.47-0.51/day when it was co-cultured with *Rhizobium* sp. It was also concluded that algal symbionts particularly bacteria not only promote algal growth but also offer advantages in downstream processing[25].

Chlorophyll content of the microalgae co-cultured with *Nitrobacter* was determined. The results revealed that synthetic waste water medium has led to highest chlorophyll-a and b content of 1.48 and 2.46 mg L^{-1}, respectively. Sewage water diluted to 50% has increased the chlorophyll-b to 2.08 mg L^{-1} whereas it was 1.76 mg L^{-1} in 75% sewage water. In a study by Xu *et al.*[23] the chlorophyll content of *C. reinhardtii* co-cultured with *A. chroococcum* increased to 39.13 mg mL^{-1}. Increase in chlorophyll-a concentrations during the first 5-7 days of *C. vulgaris* cultivation along with *Sphingomonas* sp. in photobioreactors was reported by Lakaniemi *et al.*[26].

Nitrobacter oxidizes nitrite into nitrate and in earlier studies, nitrate has produced higher biomass and lipid productivity in *C. vulgaris*[27]. It was also noted that growth promotion induced by *B. pumilus* on *C. vulgaris* is related to nitrogen fixation[28]. The increase in biomass and lipid content in this study is evidenced by the availability of nitrate in the growth medium when *Nitrobacter* was co-cultured with microalgae. When bacteria and algae co-grow, the growth of bacterium might exert external stress on algae such as oxidative stress which is known to induce lipid droplet synthesis[29].

Oxygen mediated microalgae inhibition is reported earlier by Molina *et al.*[30] and Park *et al.*[31]. It is possible to reduce the aerobic toxicity in the growth medium by capturing the dissolved oxygen. Bilanovic *et al.*[32] reported that the dissolved oxygen concentration in axenic cultures of *C. vulgaris* was higher than the mixed cultures cultivated with nitrifiers. One of the reasons for higher biomass productivity of co-cultured microalgae in this study may be due to the elimination of oxygen by *Nitrobacter*.

Bacteria can modify microalgal growth by affecting either growth rate or biomass accumulation. Maximal growth rate of microalgae is likely to be enhanced by bacterial population[33,34]. Maximal biomass of *Dunaliella* sp. was enhanced by 22 and 26% when associated with *Alteromonas* sp. and *Muricauda* sp., respectively[19]. Growth experiments performed by Tanabe *et al.*[35] indicated that biomass of *Botryococcus braunii* was increased 1.8-fold in the presence of bacterial endosymbiont. In this study, biomass productivity of 0.028 g L^{-1}/day was observed in bacterial co-culture which was 21.4% higher than algal monoculture. Co-culturing *Phaeodactylum tricornutum* with *Escherichia coli* promotes biofilm formation by benthic diatoms[36]. Cho *et al.*[37] has cultivated *C. vulgaris* along with bacterial consortium (*Rhizobium* sp., *Hyphomonas* sp., *Terrimonas* sp., *Flavobacterium* sp. and *Mesorhizobium*) and has reported that the specific growth rate, cell density and lipid content of *C. vulgaris* was 0.29/day, 2.49 g L^{-1} and 28%, respectively. However, cultivation of *C. vulgaris* in the absence of bacterial consortium has resulted in 0.22/day, 1.3 g L^{-1} and 22.4% of specific growth rate, cell density and lipid content. The bacterium *Mesorhizobium loti* supplies vitamin B_{12} to *Lobomonas rostrataas* its micronutrient for growth. In return, the bacterium receives photosynthate from the alga[38].

Another important observation in this study is the amount lipid accumulation in mono and co-culture. The lipid content was lower in co-culture grown in most of the media used except in the cells grown in 50% sewage water and synthetic waste water. It was reported earlier that biomass and lipid productivity are not depending each other[39]. Similar results were obtained in this study also that co-culture has produced higher biomass but lesser lipid content. However selection of appropriate nitrogen sources will promote the growth of both biomass and lipid content simultaneously[27]. It was widely reported that lipid accumulation occurs when there is a nitrogen deprivation in the growth medium and nitrogen starved cells has produced more lipid content. The presence of nitrogen in the growth medium throughout the cultivation period due to the nitrogen fixing ability of *Nitrobacter* has resulted in lower lipid content in *C. vulgaris* cells under the experimental conditions. This mutualistic interaction between *C. vulgaris* and *Nitrobacter* as revealed in this study could be utilized in enhancing microalgal biomass, especially by replacing nitrogen fertilizers in the growth medium. At the same time, strategies to increase the lipid productivity while co-culturing need to be explored to utilize this interaction in terms of both improved biomass and lipid production.

CONCLUSION

Co-culturing of *Chlorella vulgaris* with *Nitrobacter* has promoted the growth of microalgae. The reason for higher biomass in this study could be due to the growth promoting substances produced by the bacteria. At the same time, lower lipid content might be due to fixing of nitrogen by the *Nitrobacter* in the medium.

SIGNIFICANCE STATEMENT

Based on the findings of this study, it was clear that co-culturing of *Nitrobacter* and *C. vulgaris* resulted in enhanced growth promotion as evidenced by increased cellular composition and biomass content. Further, the benefit of microalgae derived from nitrogen fixing bacteria indicates the positive effects of co-culturing. However, the type of interaction between these two populations and the major reason for higher biomass need to be explored.

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