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Evaluation of the potential of four microalgal-bacterial symbiotic association on wastewater treatment and lipid accumulation

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Abstract Microalgae-bacteria symbiotic systems have demonstrated greater benefits over the pure culture of microalgae. The existence of certain bacteria will increase the quality of sewage purification and also reduce the associated high capital cost for maintaining the pure culture of microalgae. In this study, the potential of four microalgal-bacterial symbiotic associations to accumulate lipid besides growth rate, biomass concentration, and to eliminate nutrients from secondary treated municipal wastewater was evaluated. The two microalgae strains, namely, *Tetraselmis indica, Chlorella protothecoide* in combination with *Pseudomonus* sp. and *Bacillus pumilus* were observed. *Chlorella protothecoide-Pseudomonas sp.* system achieved the highest lipid productivity of 37.93 ± 2.53 mg $L^{-1}d^{-1}$ with lipid content 25.67 ± 0.95 % after ten days of cultivation. The chlorophyll-a content of *Chlorella protothecoide + Pseudomonas sp.* has 32% higher than the pure *Chlorella protothecoide* culture in wastewater. On the 10th day, *Chlorella protothecoide-Pseudomonas sp.* system removed 83.59 %, 86.76%, and 81.35% of chemical oxygen demand (COD), total dissolved nitrogen (TDN), and total dissolved phosphorus (TDP), respectively. *Chlorella protothecoide + Pseudomonas sp.* could coexist as a consortium has the potential to be utilized in biofuel technology.

Keywords: Microalgae–bacteria consortia, Secondary treated wastewater, Chlorophyll-a

1. Introduction

With the rapid growth of the human population, a large amount of wastewater generated has become one of the major sources of environmental pollution. According to National Green Tribunal, approximately 62% of untreated waste from urban India reaches water bodies such as rivers, resulting in contamination (*NGT Report 2019*, n.d.). This untreated wastewater has immense potential to cultivate microalgae. The municipal wastewater generated is rich in organic concentration and also provides an infinite source of water. Therefore, municipal wastewater can serve the dual purpose for microalgae growth along with coupling wastewater treatment (Molazadeh et al., 2019). Biodiesel production involving conventional microalgae cultivation procedures facing economic constraint. Since traditional microalgae cultivation required the addition of nutrients to freshwater, which used an excessive amount of freshwater (Zhou et al., 2020). In the traditional approach, the pure axenic culture of microalgae needs to maintain in an aseptic condition which increased the cost of cultivation. However, combining biodiesel production with wastewater treatment will significantly minimize production costs while also benefiting wastewater resource recycling (Ji et al., 2018). The concept of biofuel production employing microalgae–bacteria consortia has huge potential to produce sustainable biofuel and recently becoming a research trend (Delgadillo-Mirquez et al., 2016). Bacteria produce essential compounds that promote algal growth and provide resistance against pathogens. Besides, bacterial oxidation of organic compounds results in the formation of other inorganic compounds. The uptake of these inorganic compounds will further enhance microalgae growth in wastewater (Lian et al., 2018). This research paper deals with consortia to reclaim STW, to investigate the removal efficiency of COD, TDP, and TDN. Also, quantify biomass growth and lipid productivity between pure culture of microalgae and the consortia.

2. Materials and methods

2.1. Microalgae, bacteria culture, and the sample collection

T. indica, C. protothecoide, Pseudomonas sp., B. pumilus, were bought *from* National Chemical Laboratory (NCL) Pune, India. Algae inoculums were grown for 7 days in 1000 mL conical flasks in 500 mL BG-11 medium with a 16h/8h light/dark period under 130 mol/(m2 s) light intensity. The bacteria inoculums were grown in an LB nutrient broth for 18 h at 30 ± 2 ° C in a rotary shaker at 120 rpm.

The secondary treated municipal wastewater (STW) was collected from the sewage treatment plant, Yamuna Nagar Harayana, India, and the samples were kept at 4 ℃ to avoid substrate decomposition before treatment. Table 1 shows the physicochemical properties of STW. The pH of the STW was 7.55 ± 0.17.

Table 1. Physiochemical characteristics of STW before treatment

Characteristics	Value (mg/L)
TDs	458.12 ± 3.12
COD	139.04 ± 3.89
BOD	71.36 ± 2.27
TDN	4.85 ± 0.045
TDP	10.29 ± 1.42

2.2. Experimental set-up for culture

Experiments were carried out with a working volume of 300 ml in four 500-mL Erlenmeyer flasks. Pure culture *Tetraselmis indica, Chlorella protothecoide* were grown in STW as control while both microalgae cultived with each *Pseudomonus* sp. and *Bacillus pumilus*. All the six cultures were grown for 10 days with a 16h/8h light/dark period under 130 mol/ (m^2 s) light intensity at 28^0C.
The aquarium air pump (Sebo SB-548A) was used to ensure a constant air sparging mechanism in the system. (Fig.2.)

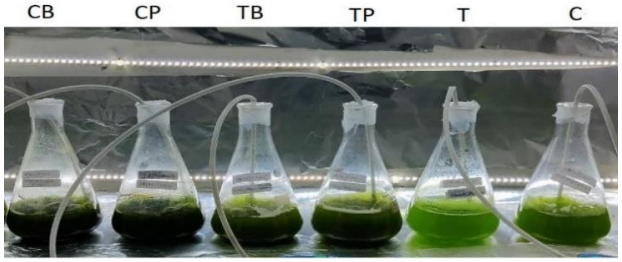

Fig.1. Experimental set-up of culture of (i) CB- *Chlorella protothecoide-Bacillus pumilus* (ii)CP-*Chlorella protothecoide-Pseudomonas sp.*, (iii)TB-*Tetraselmis indica-Bacillus pumilus*, (iv) TP-*Tetraselmis indica-Pseudomonas sp.*, (v) T-*Tetraselmis indica,* and C-*Chlorella protothecoide*.

2.3. Analytical methods

2.3.1. Dry weight

A filter paper with pore size 0.45 m was dried for 6 hours in a dryer at 105 ° C to determine dry cell weight before being weighed with an electronic microbalance (W1). The filter paper was dried to a constant weight after filtering the 10 mL sample (W2). By subtracting the original weight from the final weight, the dry weight of the samples was calculated. The dry cell weight (mg L^{-1}) and biomass productivity (mg $L^{-1}day^{-1}$) were calculated from Eqs. (1) and (2), where T was culture time in days.

$$Dry\ cell\ weight\left(\frac{mg}{L}\right) = (W2 - W1)/V \quad (1)$$

$$Biomass\ productivity\ \left(\frac{\frac{mg}{L}}{d}\right) = Dry\ cell\ weight/T \quad (2)$$

2.3.2. Measurement of water quality

The STW was filtered with 0.45 µm membrane filters. The filtrate was used to determine the water quality parameters. The Hanna multiparameter photometer was used to calculate COD, TDN, and TDP (HI839800). TDN and TDP were determined using the chromotropic acid and vanadomolybdophosphoric acid formulas, respectively. COD was measured using the dichromate equation.

2.3.3. Chlorophyll extraction

10 ml of each sample was centrifuged for 10 minutes at 5000 rpm. Following the removal of the supernatant, the pellet was washed twice with purified water and centrifuged. The pellet was then suspended in 10 mL of 90% methanol. The extraction was carried out using an ultrasonic probe for 2 min (Miko India GOC35, India). The absorbance of the supernatant at wavelengths at 652 and 665 (Simon & Helliwell, 1998).

$$Chl\ a\ \left(\frac{mg}{L}\right) = 16.75 * (OD)665 - 9.16 * (OD)652 \quad 3$$

2.3.4. Lipid extraction and content analysis

1 gm of dried algal biomass is combined with 1 ml of chloroform and 2 ml of methanol and stored at 25°C for 18 hours. The mixture is shaken for 1 min before another 1ml of chloroform is applied and vigorously shaken for 1 minute. 1 ml of purified water is used in the mixture, which is then mixed in a whirlpool for 2 minutes to separate the layers and determine the lipid (Eggers, 2019). The following Eqs. Required to calculate lipid productivity:

$$Total\ lipid\ content(L,\%) = \frac{M2 - M1}{m} * 100\% \quad 4$$

$$Lipid\ yield\ \left(Y,\frac{mg}{L}\right) = DW * L \quad 5$$

$$Lipid\ productivity\left(P,\frac{\frac{mg}{L}}{day}\right) = \frac{Y}{T} \quad 6$$

3. Results and discussions

3.1. Biomass productivity and chl-a concentration

For all microalgae symbiotic systems, the initial dry weight appears to be lower during the initial growth period compared to pure microalgae culture in STW. However, the benefit of biomass accumulation was later observed, implying that the consortium was in the adaptation period at the initial stage of culture to form a symbiotic relationship (Fig. 2). The *Chlorella protothecoide-Pseudomonas sp*. system showed the highest biomass growth as compared with other symbiotic systems. Also, the *Pseudomonas sp*. enhances the growth of *Chlorella protothecoide* to 1574.87 ± 41.37 mg L^{-1}. Although the aseptic *Chlorella protothecoide* culture showed only 1109.38 ± 49.11 mg L^{-1}. The observation is supported by the concentration of Chlorophyll-a content which is highest in *Chlorella protothecoide-Pseudomonas sp*. system (Fig.2).

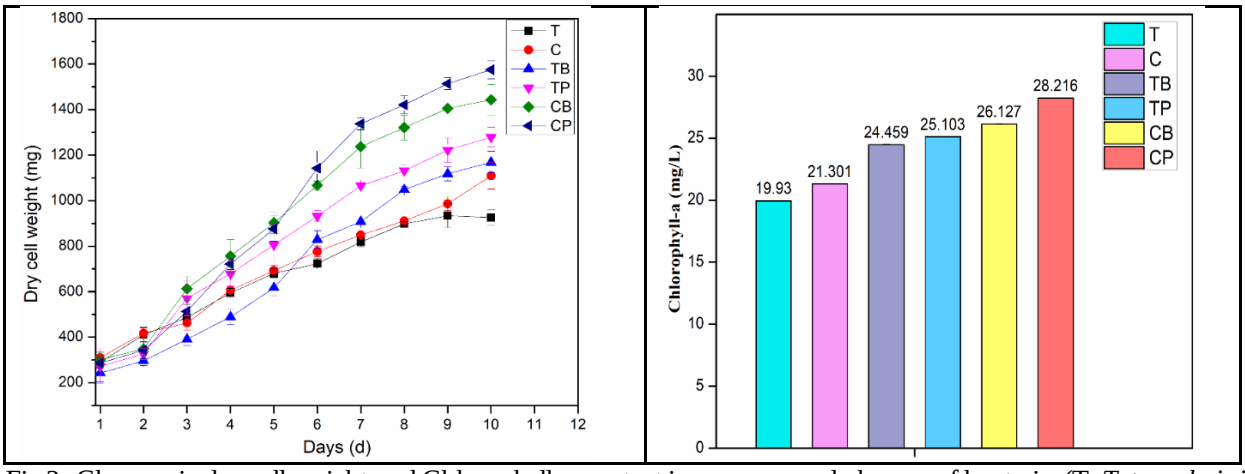

Fig.2. Changes in dry cell weight and Chlorophyll-a content in presence and absence of bacteria. (T- *Tetraselmis indica*, C- *Chlorella protothecoide*, TB- *Tetraselmis indica-Bacillus pumilus*, *TP- Tetraselmis indica- Pseudomonas sp*., CB- *Chlorella protothecoide- Bacillus pumilus*, and CP- *Chlorella protothecoide- Pseudomonas sp*.)

3.2. Removal efficiency of COD, TDN, and TDP

The removal efficiency of COD, TDN, and TDP were 83.59 %, 86.76%, and 81.35% for *Chlorella protothecoide-Pseudomonas sp*. in STW, which is far more than pure culture of *Chlorella protothecoide*. It is clearly observed that the symbiotic association of microalgae-bacteria showed better removal efficiency of COD, TDN, and TDP as compared to the pure culture of microalgae (Fig.3.).

3.3 Lipid content and FTIR analysis of extracted lipid

According to Table 2. *Chlorella protothecoide-Pseudomonas sp*. system has the highest lipid productivity, i.e., 37.93 ± 2.53 mg/L/day. This indicates that the presence of bacteria enhances the lipid content in microalgae (Rajapitamahuni et al., 2019). FTIR analysis was performed to confirm the presence of various functional groups present in extracted lipid. The absorption peak at 1745 cm^{-1} confirms the presence of carbonyl groups in the extracted lipid. FTIR peaks at

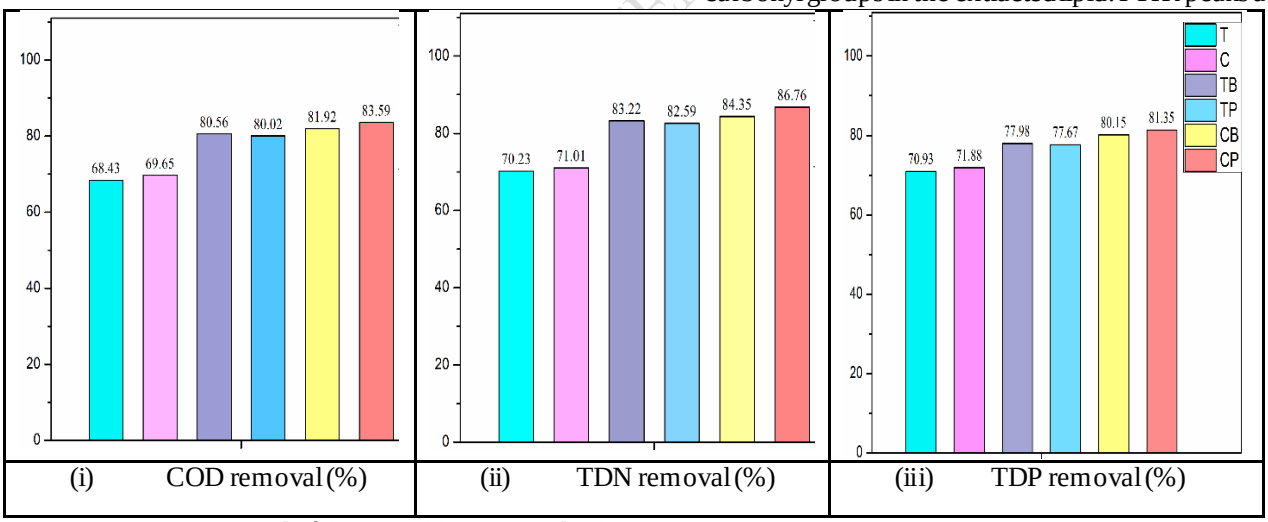

Fig.3. Percentage removal of (i) COD, (ii) TDN, and (iii) TDP in STW.

Group	Lipid content (%)	Dry weight (mg/L)	Lipid yield (mg/L)	Lipid productivity (mg/L/day)
T	16.33 ± 0.15	925.71 ± 32.85	151.17 ± 6.75	14.45 ± 0.68
C	20.43 ± 1.11	1109.38 ± 59.04	226.65 ± 24.38	20.29 ± 2.44
TB	21.16 ± 0.75	1168.19 ± 47.47	247.19 ± 18.81	22.87 ± 1.88
TP	21.69 ± 0.46	1278.92 ± 42.74	277.40 ± 15.15	26.24 ± 1.52
CB	23.27 ± 1.15	1443.46 ± 67.34	335.89 ± 32.27	30.44 ± 3.23
CP	25.67 ± 0.95	1574.87 ± 40.34	404.27 ± 25.32	37.93 ± 2.53

Table 2. Lipid productivity in a different group of microalgae and consortium systems.

2844.5 and 2958.9 cm^{-1} were observed due to the presence of $-CH_2$ and CH_3 groups, respectively. The presence of hydroxyl groups was assured by a broad peak around 3416.6 cm^{-1} observed in the FTIR spectrum (Fig.4). The peak at 1248.1 cm^{-1} was observed because of the C-O stretching vibration of ester groups. The above FTIR peaks confirm the extracted molecule was lipid.

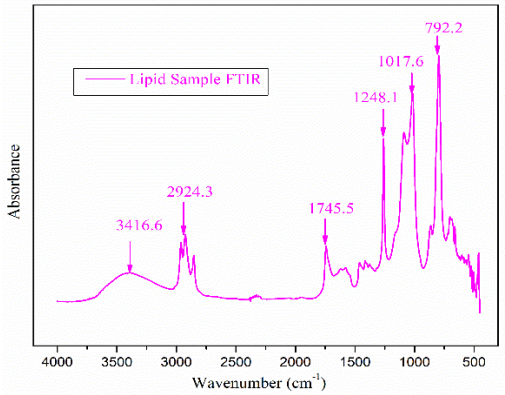

Fig.4. FTIR spectrum of extracted lipid.

4. Conclusion

The *Chlorella protothecoide-Pseudomonas sp*. in STW achieved maximum removal efficiency of COD, TDN, and TDP of 83.59 %, 86.76%, and 81.35% respectively as compared to the other three symbiotic systems. The biomass growth of the consortium was attributable to algae growth rather than other microorganisms as supported by Chlorophyll-a concentration analysis. Symbiotic association of microalgae and bacteria enhances the lipid content as compared to the pure culture of microalgae.

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