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ORIGINAL ARTICLE

Strategic enhancement of *Desertifilum tharense* MSAK01 on dairy wastewater: an integrated approach for remediation and biomass production

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Abstract The present study is an integrated approach to study the potential of *Desertifilum tharense* MSAK01 for treatment of dairy wastewater (DWW) and enrichment of biomass. The present research includes the experiment designed for treatment of DWW. The physical and chemical parameters of wastewater quality, such as nitrate, phosphate, chloride, sulphur, and hardness, were studied. The level of nitrate and phosphate in water bodies was reduced by 94 and 98% in the effluent, respectively. The level of BOD and COD, measure of organic contaminants, were reduced to 70% (BOD5, initial level of 1840 mg O_2 L^{-1}) and 56% (COD, initial level of 2470 mg O_2 L^{-1}). The second module of the experiment was designed for biochemical extractions by harvesting the biomass (algal strain) grown in DWW. The result of this study shows that algal strain *D. tharense* is not only an agent for mitigation of pollutant load, but it can also be used as potential source for lipid, protein and carbohydrate.

Keywords Cyanobacteria · Dairy wastewater (DWW) · *Desertifilum tharense* · Phycoremediation · Principal component analysis

Introduction

Pollution caused by industries has forced more strict environmental regulations throughout the world (Braio and Granhem 2007). Among all industrial activities, the food sector consumes the highest amount of water, and produces the highest effluent per unit of production (Ramjeawon 2000). India ranks first among the maximum major milk producing nation, therefore dairy industry is one of the major food industry in India (Tripathi and Upadhyay 2003). It is estimated that about 110 million tonnes of milk and about 275 million tonnes of wastewater being generated annually from the Indian dairy industries by the year 2010 (Kushwaha et al. 2011) and this will increase every year. Among these, organic compound rich effluents of dairy sector must be removed, before emancipating into the environment. Along with it, dairy effluent is usually composed of sugars, proteins, phosphates, ammonia and/or nitrate, which are easily biodegradable (Demirel et al. 2005). Many technologies are involved with the agro-food industries for the treatment of effluents, physical, chemical or biological, to eliminate organic and inorganic form of nutrients primarily phosphates and nitrogen (Nurdogan and Oswald 1995). Biological treatment involves utilization of microbes for effluent treatment. Microbes had attracted the attention because of their economic importance and biotechnological applications in various fields (Singh and Singh 2013). Microalgae species had been applied either as axenic culture or as mixed consortia as with *Chlorella*, *Scenedesmus* or *Arthrospira* to eliminate organic and inorganic nutrients from different types of effluents, such as pulp and paper industry (Tarlan et al. 2002), brewery effluent (Raposo et al. 2010) or oil drilling industry (Sivasubramanian and Muthukumaran 2012). The concept of phycoremediation (remediation carried out utilizing algae or cyanobacteria) is the removal of heavy metals, such as lead or mercury and incorporation of organic and inorganic nutrients from waste for the production of biomass (Chinnasamy et al. 2010; Wang et al. 2010). Among the various cyanobacteria employed for effluent treatment

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filamentous cyanobacteria is commonly used (Markou and Georgakakis 2011), as it can survive for long time and they are heterotrophic or mixotrophic in nature (Mata et al. 2010). The biomass produced after remediation is rich in organic, and inorganic nutrients can be used as animal feed (in poultry farming, fish, or rotifers), as fertilizer for enrichment of soils with nutrients, also for extraction of valuable pigments, antioxidants, enzymes, antibiotics, or even polysaccharides (Spolaore et al. 2006).

In the present research, the correlation had been studied between dairy wastewater remediation and cyanobacterial *Desertifilum tharense* biomass production. The relation had been studied among physical and chemical characters of *D. tharense* during remediation. It results in significant reduction of phosphate, nitrogen, BOD and COD along with enhanced production of nutrient-rich cyanobacterial biomass.

Materials and methods

Isolation and identification

Desertifilum tharense MSAK01 was isolated from the dairy effluent of local dairies located at Gandhinagar and Ahmedabad city of Gujarat. The isolate was identified morphologically using microscope, and using keys in standard monographs (Iyengar and Desikachary 1981). The sterile synthetic, BG-11 Medium (Stanier et al. 1971) was used for isolation, cultivation and preservation of cyanobacteria. The cyanobacterial inoculation volume (suspended in cyanobacteria growth media) in each system was 10% (v/v), with an initial concentration of approximately 2×10^6 cells/ml. All the cultures were incubated at 25 ± 2 °C under a photoperiod of 16:8 h light dark cycle and illumination (37 µE s^{-1} m^{-2}) with cool white fluorescent lamps (Ram et al. 2013). The cultures were shaken manually once a day (without sparging with air or CO_2) to avoid adherence to the side of the flask.

The purified isolate was further identified by 16S rRNA sequence analysis. For comparative analysis and homologous sequence search, the BLAST program was used. The phylogenetic tree was constructed using the neighbor-joining method (NJ) as implemented in the program MEGA package version 4.1 (Tamura et al. 2007).

The NJ stability of the relationships was assessed by boot-strapping (1000 replicates). The percentage of replicate trees in which the associated taxa clustered together was shown above the branches. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option).

Wastewater sampling

DWW samples were collected from local dairy farms of Gujarat (India), before the final chlorination step and stored in glass bottles. These were studied for physico-chemical analysis. Here, effluent sample is the left over raw whey waste of prepared dairy products, e.g., paneer, butter, sweets, etc. Each sample was used for nutrient analysis, biological oxygen demand for 5 days (BOD5), and chemical oxygen demand (COD). All wastewater was analyzed immediately after collection. If necessary, wastewater was stored at −80 °C.

Characterization of the effluent

Analysis of physico-chemical parameters of DWW sample from different dairy and season were done following APHA Standard Methods (APHA 1985). For the analysis of pH and electrical conductivity (EC), 50 ml of cyanobacterial remediated DWW was used and measured using a pH meter (Systronics, India) and EC (Hanna Instruments, Portugal) meters, respectively. Salinity was also analyzed using salinity meter. The BOD5 and COD were determined using the methods described in Standard Methods for the Examination of Water and Wastewater Gupta (2006). Total nitrogen (TN) (volumetric method using micro-kjeldahl distillation assembly), total phosphorus (TP) (vanadomolybdate colorimetric method), total hardness or calcium and magnesium ($Ca^{2+} + Mg^{2+}$) (versenate method), chlorine (Mohr's titration) and carbonates and bicarbonates Richards (1954) were also estimated as per the methods described in Standard Methods for the Examination of Water and Wastewater. The sodium absorption ratio (SAR) was also calculated.

Optimization of algal growth on DWW

The algal strain was grown at different concentrations (5, 10, 15, and 20%) of autoclaved wastewater (effluent) (Table 1). The experiment was set up in 250 ml of conical flask containing 100 ml of varying wastewater concentration. Homogenous algal suspension (5 ml) was used to inoculate each flask. The dry weight of cyanobacterial biomass was determined gravimetrically and growth was measured as mg l^{-1}.

Treatment of DWW by *Desertifilum tharense*

This step of experiment was carried out in 250 ml conical flask containing 100 ml of wastewater. Most favorable concentration (10%) of DWW (effluent) for algal growth was taken for treatment experiment. Homogenous algal suspension (5 ml) was taken for inoculation. Physico-

Table 1 Biomass production of *D. tharense* in different strength of effluent

Day	Biomass (gm/l)			
	5%	10%	15%	20%
0th	0	0	0	0
3rd	0.09	0.11	0.13	0.11
6th	0.15	0.18	0.23	0.22
9th	0.31	0.53	0.62	0.42
12th	0.53	0.71	0.82	0.58
15th	0.85	1.11	0.94	0.81

chemical parameters of DWW were analyzed at every 3 days interval from zeroth day of inoculation to check the reduction in pollutant load as given in Table 2. All experiments were performed in triplicate. Samples for analysis were taken in triplicate from each system.

Biochemical analysis of cyanobacterial biomass remediating DWW

Exponentially growing (15 days of incubation) cultures in DWW were used for the determination of growth and biochemical variables, such as total protein, total carbohydrate and total lipid. A definite volume of cyanobacterial suspension, after being filtered through a pre-weighed filter paper (Whatman no. 1), dried over night in an oven at 80 °C. The dry weight of cyanobacterial biomass was determined gravimetrically and growth was measured as mg l^{-1}. Total protein was estimated according to Lowry's procedure (Lowry et al. 1951) as modified by Herbert et al. (1971) using bovine serum albumin as standard. Estimation of carbohydrate was done by Anthrone method while lipids were extracted into chloroform and methanol–water layers, respectively, using the chloroform–methanol–water solvent system (Bligh and Dyer 1959). Aliquotes of the chloroform fractions were evaporated and lipids determined by dichromate oxidation method of Pande et al. (1963) using palmitic acid as a standard.

Statistical analysis

The triplicate sets of data for the various parameters evaluated were subjected to ANOVA (analysis of variance) ($p < 0.05$) followed by correlation coefficient (r) analysis using curve expert 1.3 (1997) software and Microsoft office Excel 2007. Principal component analysis was performed using the software Statistical package for Social Sciences (SPSS Version 11.0) and PSPPire software (version 3). The correlation analysis was calculated between parameters and productivity using SPSS software.

Results and discussion

On the basis of morphological characters, the isolate showed resemblance with *Oscillatoriaceae* family which was further confirmed as *D. tharense* MSAK01 by 16S rRNA gene sequencing. Sequence data reported in the present study has been deposited in the gene bank nucleotide sequence database under the accession number KF933830. The phylogenetic tree constructed using sequenced 16S blue-green cyanobacterial strains and the similar sequences belonging to other species taken from the NCBI data base had been revealed in Fig. 1. *D. tharense*

Table 2 Change in the physico-chemical characteristics of DWW inoculated with *D. tharense* during 15 days study

Physico-chemical parameters	0th day	5th day	10th day	15th day
pH	7.5 ± 0.1	8.8 ± 0.1	9.6 ± 0.1	10.02 ± 0.1
Electrical conductivity (EC; dS m^{-1})	1.18 ± 0.26	1.47 ± 0.1	2.01 ± 0.09	1.68 ± 0.02
Total sulphur (TS; mg l^{-1})	143 ± 4.8	116 ± 3	78 ± 1.2	41 ± 0.9
Total hardness (Ca + Mg; mg l^{-1})	83.8 ± 6.9	56.1 ± 1.8	40.2 ± 1.3	28.9 ± 1.05
Chlorides (Cl; mg l^{-1})	53.8 ± 0.94	51.0 ± 1.5	47.4 ± 0.2	48.3 ± 0.6
Sodium (Na; mg l^{-1})	205 ± 9.14	189 ± 4.6	171 ± 2.6	154 ± 2.7
Carbonates bicarbonates (mg l^{-1})	204 ± 2.36	210 ± 1.1	216 ± 1.8	231 ± 1.5
Total nitrogen (TN; mg l^{-1})	160 ± 2.06	125 ± 2.4	89 ± 1.04	54 ± 1.4
Nitrate–nitrogen (NO_3–N; mg l^{-1})	94.3 ± 0.07	78.1 ± 0.8	53 ± 0.26	38 ± 0.08
Nitrite–nitrogen (NO_2–N; mg l^{-1})	48.3 ± 0.18	33.4 ± 0.2	21.1 ± 0.32	14.5 ± 0.06
Total phosphorus (TP; mg l^{-1})	183.4 ± 1.14	158.1 ± 0.1	113.2 ± 0.6	79.7 ± 0.3
Ortho-phosphates (OP; mg l^{-1})	120.6 ± 1.77	95.4 ± 2.2	72.2 ± 1.1	57.2 ± 1.02
Biological oxygen demand (BOD; mg l^{-1})	1840 ± 26	1487 ± 32	1121 ± 21	560 ± 13
Chemical oxygen demand (COD; mg l^{-1})	2470 ± 87	1989 ± 28	1503 ± 16	1103 ± 21

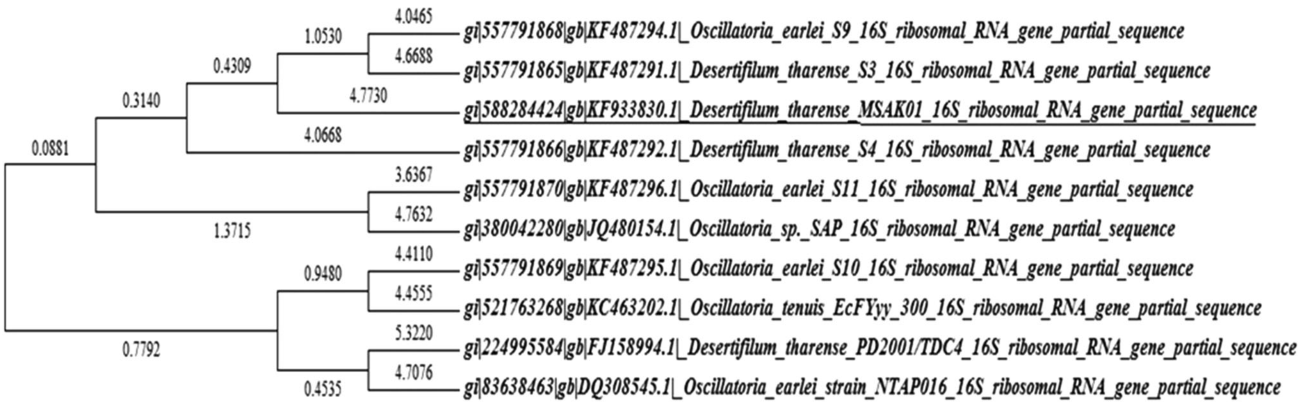

Fig. 1 Phylogram generated using nearly partial 16S-rRNA sequences (approximately 540 bp) from *D. tharense* and available NCBI sequences, constructed by neighbor-joining method. Boot strap values are indicated at the corresponding nodes

has been observed with a rapid maximum growth rate of $1.715 + 0.015$ gm L^{-1} after 7 days of incubation at 25 °C.

Cyanobacterial growth on various concentration of DWW

Cyanobacterial growth as indicated by dry weight of biomass was determined gravimetrically in different concentrations (5, 10, 15, and 20%) of selected sample. *D. tharense* could survive in all of the selected sample concentration. Although when compared between the selected concentrations, it is observed that cyanobacteria grew faster in 10% diluted sample with dairy effluent wastewater samples as shown in Table 1.

DWW characteristics before and after treatment

The effluent of DWW was autoclaved and was analyzed for various physico-chemical parameters as given in Table 2. Generally, effluent has improved water quality parameters after treatment than the effluent before treatment. The decrease in the organic load of the effluent was recorded at the 15th day of incubation. The highest nitrogen removal rate obtained with *Desertifilum* sp. was during 0–5 day and lowest at 10th to 15th day, as shown in Table 2.

Effluent before treatment: the effluent has milky color and acidic pH (6.2), offensive smell. The concentration of nitrate is found to be higher (94.3 mg l) than the permissible limit (5 mg l). Similarly, the nitrite level (48.3 mg l) was also very high. Phosphate concentration is found to be 183.4.0 mg l. Significant amount of sodium (205 mg l) is found in the wastewater. BOD, COD, total alkalinity and hardness are found to be in higher range.

As the waste water supports the growth of cyanobacteria, it is possible to use cyanobacterial culture for waste water treatment. During cyanobacterial growth, low cost nutrients, supplied as wastewater, were used by the cyanobacterial biomass and resulted into reduction in pollutant load of effluent. The conventional wastewater treatment has been unfeasible due to complex process and high energy consumption (Wang et al. 2008). The microalgae have been used as an alternative way to recycle some of the nutrients within these wastewater sources (Pizarro et al. 2002), particularly for the removal of organic carbon, nitrogen and phosphorus which are unsuitable for water bodies. The level of nitrate, nitrite, sulphur, chloride in the effluent was reduced by 60, 70, 71.3, 10.2%, respectively, on 15th day of algal growth compared to untreated control. It has been observed that nitrate removal by alga is better in the effluent. *D. tharense* utilized for DWW treatment remediates waste without destruction of cyanobacterial cell. Adey et al (1996) reported that the alga *C. vulgaris*, reduced ammonium by 72%, phosphorus by 28%, and COD by 61% after 5 days of incubation.

It may be due to the different conditions upon which algal growth depends. Growth of cyanobacteria in the absence of DWW, served as control (with normal growth media prepared using tap water), was negligible. Thus, result suggested that wastewater provides nutrient rich medium for better growth of cyanobacteria. In addition, the results also indicate that effluent with high nutrient load hinders the dissolution of required gases in the media. Kothari and others (Kothari et al. 2012) found the growth of *C. pyrenoidosa* to be better at 75% concentration of the dairy effluent wastewater.

Dairy industry generates high strength wastewaters characterized by high BOD and COD concentrations (Hill and Bolte 2000). DWW that contains the highest value of organic pollutants is cheese-whey, which usually contains 30–50 g L^{-1} BOD and 60–100 g L^{-1} COD (Gelegenis et al. 2007). The BOD value indicating the amount of biologically oxidizable organic matter and COD value indicating toxic conditions due to the presence of biologically resistant organic substances, are decreasing during

15 days remediation study of DWW using *D. tharense*. The significant reduction could be due to high cyanobacterial growth which will consume nutrient from wastewater to synthesize its organic biomass. Though the level of BOD and COD were reduced tremendously through algal cultivation, the effluent cannot be disposed into environment safely. Further treatment is needed to reduced level of BOD and COD to desired level. In the effluent, concentrations of phosphate were almost always higher than nitrogen, probably due to chemicals used in some of the dairy production units Driessen and Vereijiken (2010). The removal rates of phosphorus were higher in the effluent, ranging from 92 to 94% (Gonzalez et al. 1997) and also higher than those observed in the control cultures. Nevertheless, phosphorous removal rates were always lower than those observed for N removal. The values for total N removal ranged between 97 and 98% for the effluent cultures. However, it has to be considered that the organisms involved did not consume the entire N. In fact, despite being present at a higher concentration than nitrate, ammonia is easily removed because of the outgassing to the atmosphere, due to the high pH, which shifts the equilibrium in favor of NH_3 (Wang et al. 2010) which supports the present study. Nitrification process favors in the wastewater treatment for the conversion of nitrite to nitrate. Nevertheless, phosphorus uptake by algae was lower than nitrogen uptake because the nitrogen content of algae was approximately ten times higher than the phosphorus content (Wang et al. 2010). Aslan and Kapdan (2006) had studied that unicellular cyanobacteria and blue-green algae are tolerant to many wastewater conditions and efficient in removing nutrients from wastewater.

Algae appear to offer the most easily exploited biological system for extracting phosphorus from dairy waste. In the present investigation, there was significant removal of phosphorus on 25th day which is in conformity with the data of 54–66% removal of phosphate presented by Raposo et al. (2010). The organisms involved in the biological processes of wastewater treatment, all requires phosphorus for reproduction and synthesis of new cellular material. DWW contains amounts of phosphorus far in excess of the amount needed to stabilize the limited quantity of organic matter present. This fact is demonstrated by the presence of appreciable amounts in effluents from biological wastewater treatment plant.

A high SAR recorded for these dairy waste effluent, ensues limitation of $Ca^{2+} + Mg^{2+}$ due to Na^{+} induced displacement of these cations. Similarly, various cyanobacterial species taken up the salts from the dairy effluent, and thus there was a gradual reduction in hardness level (50%) which is correlated with the uptake of Ca and Mg from DWW.

Biochemical analysis of cyanobacterial biomass remediating DWW

The biochemical analysis of cyanobacterial biomass cultivated on DWW results in increased value of biochemical components, such as biomass (280%), chlorophyll (224%), total lipids (380%), carbohydrates (560%) and proteins (467%) (Fig. 2). It has been reported that microalgal biomass and protein increases with increase in nitrogen concentration of wastewater (Piorreck et al. 1984). These results have also demonstrated that protein and residual fraction in chlorella biomass grown on effluent was higher than that grown on effluent and growth medium. This indicates incorporation of the macro nutrients, e.g., TN, TS, carbon, etc., from the DWW in the cyanobacterial cell which is to be converted to protein, carbohydrate and lipid exclusively.

Statistical analysis of the data of wastewater samples

During 25 days study performed, the significant change was noted in the nine physico-chemical parameters measures. Four major clusters had been formed in the PCA plot which suggests that there are four major physico-chemical variables that could significantly affect the remediation of DWW. The PCA analysis revealed two principal components (PC 1 and PC 2) with percentage variances of 66.55 and 22.75%, respectively. Bi-plot analysis of stability measures revealed that stability measures can be distributed into four clusters (Fig. 3): (1) biomass, pH and SAR, (2) TN, TP and Na, (3) $Ca^{2+\cdot} + Mg^{2+}$ alone and (4) $CO_3^{2-} + HCO^{3-}$ and E.C. Among the different parameters examined, total nitrogen and total phosphorus constitutes the largest fraction of wastewater and are exchangeable component in the water. Thus, it possesses the most obvious influence on cyanobacterial growth which is indicated by the long distance from the point of origin in the PCA plot.

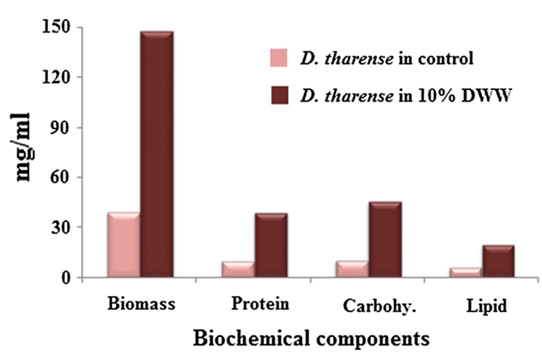

Fig. 2 Comparative analysis of various biochemical content (Mg ml^{-1}) of the cyanobacterial isolates associated with treatment of dairy wastewater when cultivated in medium and medium amended with dairy wastewater

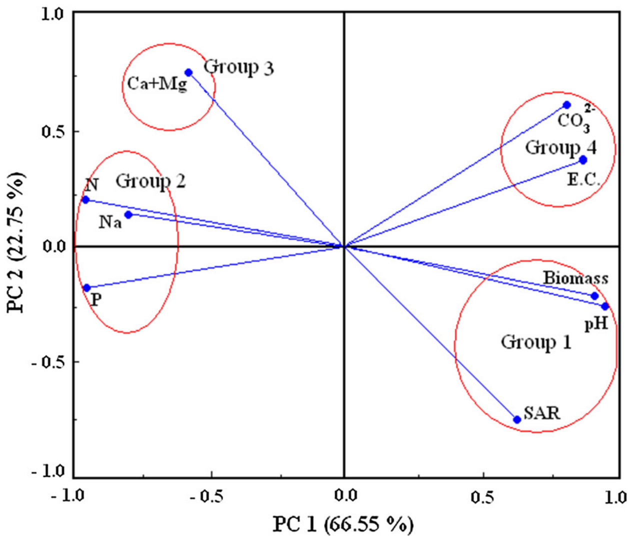

Fig. 3 Statistical analysis of the data of wastewater samples: the principal component analysis of the physico-chemical properties of DWW

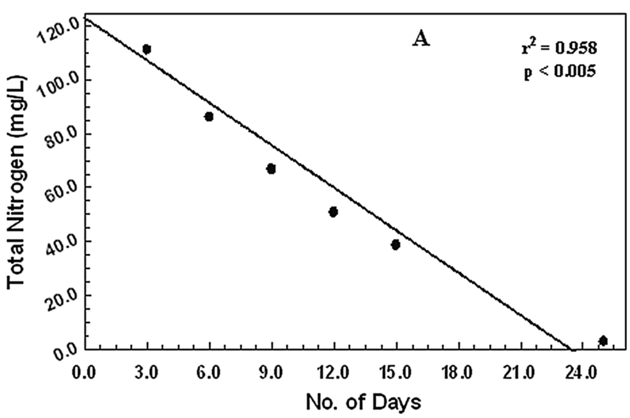

Fig. 4 Regression analysis between number of days (time period) and total nitrogen. Analyses depict the effects of growth period on the physico-chemical characteristics of the wastewater

The regression analysis between TN and number of days revealed coefficient of determination, $r^2 = 0.958$ (Fig. 4) at $p < 0.05$. The regression analysis between pH and number of days ($p < 0.05$) also showed a positive significant correlation due to the removal of various salts or metallic ions. Blue-green algae use CO_2 in their photosynthesis activity and this removal is responsible for such high pH conditions. Blue-green algae, however, can reduce the free CO_2 concentration below its equilibrium concentration with air, and consequently can cause an even greater increase in pH. It continues to extract CO_2 for water until an inhibitory pH is reached, which is usually in the range of pH 10–11 (Rao et al. 2011). Electrical conductivity decreases in initial remediation due to the removal of conducting metallic ions like, Na but then it increases due to increase in exchangeable ions in the effluent. The

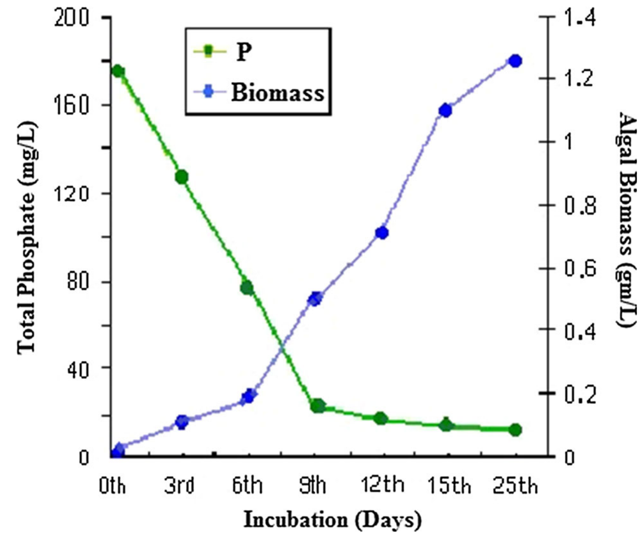

Fig. 5 Cyanobacterial biomass and total phosphate concentrations over days in the wastewater treatment of experiment 25 days period

regression analysis revealed a strong positive correlation with $R_2 = 0.971$ at 95% significant level supported by Muthukumaran et al. (2012) which reveals 41.42% reduction in E.C. supported by 50% Na removal.

The remediation efficiency of phosphorus in effluent by cyanobacteria is observed up to 93.14% at the end of 25th day of inoculation. Total phosphorus level in effluent decreases as the cyanobacterial biomass increases, as incubation period increases. Figure 5 represents the graph indicating the effect of incubation period on phosphorus removal with biomass productivity.

Conclusion

In the present study, *D. tharense* demonstrate appreciable nutrient removal capacity. Combining the nutrient removal from wastewater with the increased biomass provide an economically useful system to enhance biomass production. The remediation performance of *D. tharense* was high in accumulating TP (94%), TN (98%), and reducing BOD (70%), and COD (56%) in the form of biochemical components, such as biomass (280%), chlorophyll (224%), total lipids (380%), carbohydrates (560%) and proteins (467%). Thus, wastewater treatment utilizing cyanobacteria presents an excellent alternative for biological treatment of DWW.

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