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Cultivation of Microalgae in Domestic Wastewater for Biofuel Applications – An Upstream Approach

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Abstract

Cultivation of microalgae for biofuels assumes importance when it is grown in domestic/industrial wastewater due to dual benefits; treatment of wastewater and generation of biomass for use in the production of biofuels. In this study, therefore, isolation of microalgae was carried out from the sewage waters in Chennai city. The sewage was enriched and the microalgae, *Chlorococcum humicola*, *Chlorella vulgaris* and *Selenastrum* sp. were isolated and purified. Treatment of sewage was carried out using the isolated microalgae and generation of biomass and lipid contents were monitored. Treatment using *C. humicola* resulted in nearly 60% of BOD and more than 80% of total N. TDS and TS reduction was approximately 65%. Total phosphorus was reduced to nil by all the microalgae. Reduction of all the parameters was in the range of 60-65% by the microalga *C. vulgaris*, except total N which was nearly 85%. The treatment efficiency was less by *Selenastrum* sp. when compared to that by *C. humicola* and *C. vulgaris*. Similarly, *C. humicola* and *C. vulgaris* showed higher biomass measurements and *Selenastrum* sp. showed higher lipid productivity. Thus treatment of industrial and domestic wastewaters coupled with biomass generation is very important in the production of commodity products like biofuels.

1. Introduction

Microorganisms play an important role in secondary/tertiary treatment of domestic wastewater. In particular, microalgae can be grown in facultative or aerobic high-rate ponds for use in treatment of municipal wastewater in small- and middle-scale treatment facilities (Aziz and Ng, 1993; Abeliovich, 1986; Oswald, 1988). They aid in the removal of nutrients, heavy metals and pathogens, and some mixotrophic algae can mineralize organic pollutants. As a consortium with heterotrophic aerobic bacteria, they utilize the CO_2 released from bacterial respiration and in turn furnish oxygen to the bacterial population. It has also been shown that microalgae can indeed carry out degradation of various hazardous contaminants (Munoz and Guieysse, 2006; Safonova et al., 2004; Rao et al., 2011a). Thus microalgae can be very well used for treating industrial wastewaters and the same has also been implemented on a mass-scale (Sivasubramanian et al., 2009a; Rao et al., 2011b). Apart from being used for secondary treatment of sewage, microalgae can potentially be employed for the production of biofuels in an economically effective and environmentally sustainable fashion. Compared with first- and second-generation biofuels, algal fuels have a higher yield. In fact, they can produce 30–100 times more energy than terrestrial crops (Demirbas, 2009). Microalgae biomass has been used for the production of a variety of biofuels including bioethanol, biomethane, biodiesel, bio-syngas, jet fuels and bio-hydrogen. Further research in microalgal cultivation and downstream processing is anticipated to further enhance the cost effectiveness of the above strategy for production of biofuels (Chinnasamy et al., 2012). Microalgae as a biofuel feedstock becomes all too important when they can be effectively

grown in conditions which require minimal freshwater input, and when they can be cultivated in arid/semi-arid/coastal waste lands, which are otherwise non-productive to plant crops, thereby making the process potentially sustainable. There has been keen interest in the recent past to grow microalgae near the sea to utilize hypersaline/saline/brackish water. Hence there is constant urge in developing biofuel technology by growing microalgae under saline conditions (Rodolfi et al., 2009; Takagi et al., 2006). Another potentially sustainable and feasible growth medium for algal feedstock is domestic/industrial wastewater, which also leads to an effective phycoremediation process (Sivasubramanian, 2009b). Thus for successful commercialization of algal biofuel technology, integrated approach such as growth in wastewater, production of biomass/biofuels, and production of value-added products is essential. In this paper, selected species of microalgae were cultivated in sewage for biomass and lipid production. The objectives of this study were to i) collect sewage water samples, ii) isolate, axenize and identify microalgal cultures, iii) cultivate various microalgae in different concentrations of sewage in outdoor conditions, iv) treat the sewage using microalgae and study the physico-chemical parameters and v) measure biomass and lipid productivity of microalgae.

2. Materials and Methods

2.1 Collection of Samples

For the isolation of microalgae, the sewage water samples were collected from Chennai city. The samples were collected with necessary precautions and were taken to the laboratory for further analysis.

2.2 Enrichment of Culture in BG - 11 Medium

Collected sewage water samples were brought to the laboratory and the samples were enriched by inoculating into sterile conical flasks containing BG-11 medium and this was incubated for 15 days by providing required environmental condition such as 12:12 light/dark photoperiods and a temperature of 25°C. This allows further growth and multiplication of organisms.

2.3 Isolation and Purification

Enriched sample culture was isolated by serial dilution and streaking methods. The serially diluted samples were inoculated in BG-11 medium. Isolation was also performed by quadrant streaking method in sterile petri plates containing BG-11 medium. These plates were kept for incubation at a temperature of 25°C and 12:12 light/ dark photoperiods for 15 days.

2.4 Identification

The microalgal sample was subjected to microscopic observation for the identification of microalgae. The microalgae were identified using the manual of Philipose (1967).

2.5 Growth and maintenance in BG-11 Medium

For the maintenance of algal culture, BG-11 broth was prepared and inoculated with the culture. This was kept for incubation at 25°C provided with 12:12 light/dark photoperiods. The culture was maintained both in slants and broth cultures.

2.5 Outdoor Cultivation

Outdoor cultivation of microalgal cultures was performed in tubs, using NMR medium (Mohan, 2012). The tubs used had a total height of 25 cm, diameter of 40.5 cm and a holding capacity of ~33 L. The volume for scale-up culture was 5 L and after proper observable growth, the volume was increased to 20 L. First, various microalgal cultures were cultivated in 50% medium and 50% sewage. The treatment efficiency was studied after 7 days. Harvesting was carried out by autoflocculation methods by increasing the pH (Mohan et al., 2009, 2010). After harvesting, the microalgal cultures were grown in 100% sewage water. Again the treatment period was 7 days; however, the cultivation was continued for a further period of 2 weeks in sewage for studying the biomass production potentials. Daily water loss by evaporation was compensated by the addition of tap water. The study was carried out to fulfil dual purpose; one for screening potential strains, which can grow under varying concentration of sewage and the other for enhancing lipid concentration in higher concentrations of sewage, which in turn can be used for biofuel production. Simultaneously, sunlight intensity was measured and photosynthetic efficiency for effective biomass production was calculated. Similarly, regular monitoring of pH and temperature along with manual mixing of outdoor cultures was carried out. The effluent treatment efficiency was determined by measuring pH, total dissolved solids (TDS), total solids (TS), total suspended solids (TSS), biological oxygen demand (BOD), total nitrogen and total phosphorus before and after treatment. The biomass and total lipid concentration were also monitored at regular intervals.

2.6 Measurement of pH

pH was measured in outdoor culture experiments on a regular basis using a bench-top pH meter.

2.7 Measurement of Temperature

Temperature was regularly monitored in outdoor conditions using a digital thermometer.

2.8 Measurement of Light Intensity

Sunlight intensity was measured during various periods of the day using a lux meter and average intensity of the sunlight radiation was calculated.

2.9 Measurement of Water Levels

In outdoor tub cultures of microalgae, daily water levels were measured using a cleaned, surface-sterilized metal rulers. Separate ruler was used for each of the cultures. The evaporation volume was calculated based on the measurement.

2.10 Physico-chemical parameters

The sewage water was analysed for physico-chemical parameters such as TS, TDS, TSS, BOD, total nitrogen and total phosphorus according to the protocols of APHA (APHA, 2000).

2.11 Growth measurement

Growth was measured by counting cells using a haemocytometer (Neubauer, improved).

2.12 Extraction and estimation of lipid

Ten millilitres of the alga culture was centrifuged at 5000 rpm for 20 min. The pellet was ground in mortar and pestle with 6 mL of Chloroform and Methanol (at a ratio of 2:1). Then 2 ml of 0.9% physiological saline was added and vortexed for a few seconds. This mixture was allowed to remain overnight in the dark condition under room temperature for phase separation. The chloroform layer containing the lipid was aspirated using a pasteur pipette. A volume of 0.5 ml was collected in a glass test tube and the solvent was allowed to evaporate at room temperature and the pellet was collected for the estimation of lipid. Lipid estimation was carried out according to the method of Folch et al (1957).

3. Results and Discussion

3.1 Isolation and Identification

Sewage samples from different locations in Chennai city were collected and labelled. The samples were tested microscopically for the presence of algae. The samples were processed for enrichment of algal growth. After enrichment, the microalgae were isolated and purified. The culture was maintained at the conditions mentioned in the previous section and used for further study. Three microalgae were isolated and identified by microscopic morphology, following the monographs of Philipose (1967). The microalgae were identified as *Chlorococcum humicola*, C*hlorella vulgaris* and S*elenastrum* sp.

The isolated microalgae, after purification by streak plate technique, were maintained in the laboratory in BG-11 medium and subcultured at an interval of 15 days. The cultures were axenized by the addition of triple antibiotic solution. Repeated scaling up was performed until the culture volumes were increased to 2 L. Sewage water was collected and the treatment was carried out using the above microalgae at 50% and 100% concentrations.

3.2 Outdoor Cultivation

The outdoor cultivation cum sewage treatment was carried out in plastic tubs. The sunlight intensity, pH and temperature were monitored, which are shown in Tables 1 and 2. In 50% sewage treatment, remaining volume was made up with medium containing commercial chemicals as indicated earlier. In both the treatment, daily evaporation loss was made up with tap water. Biological oxygen demand (BOD), total nitrogen, total phosphorus, TS, TSS and TDS were measured pre- and post-treatments. Before carrying out treatment using 100% sewage, 50% treatment was carried out to test the feasibility of growing microalgae in wastewaters. As some of the microalgae can grow under mixotrophic conditions, the microalgae can adapt to growing in the sewage/medium mix as well as in the sewage itself. In this study, the feasibility experiments were not carried out in the laboratory as there is a lot of difference in bioremediation efficiencies between outdoor and indoor treatments due to the difference in sunlight intensities (Rao et al., 2011b). Moreover, treatment was carried out by suspended systems; even though the harvesting is easier in attached growth systems, treatment efficiency and microalgal growth rate are more in suspended systems.

Table 1: Outdoor cultivation of microalgae in 50% sewage

Days	Culture temperature			pH	Average Sunlight intensity (Lux)
	Chlorococcum humicola	*Chlorella vulgaris*	*Selenastrum* sp.		
0	32°C	32°C	32°C	7	40,000-50,000
1	32°C	33°C	32°C	7	
2	32°C	33°C	33°C	7	
3	33°C	33°C	33°C	7.5	
4	33°C	33°C	33°C	7.5	
5	32°C	32°C	33°C	8	
6	34°C	34°C	34°C	8	
7	33°C	33°C	34°C	8.5	

Table 2: Outdoor cultivation of microalgae in 100% sewage

Days	Culture temperature			pH	Average Sunlight intensity (Lux)
	Chlorococcum humicola	*Chlorella vulgaris*	*Selenastrum* sp.		
0	35°C	35°C	35°C	8	40,000 – 50,000
1	37°C	38°C	38°C	8	
2	37°C	37°C	37°C	8.5	
3	36°C	35°C	35°C	8.5	
4	36°C	36°C	37°C	8.5	
5	37°C	37°C	37°C	8.5	
6	37°C	37°C	36°C	8.5	
7	36°C	37°C	37°C	8.5	
8	35°C	36°C	36°C	8.5	
9	37°C	37°C	38°C	8.5	
10	36°C	37°C	36°C	8.5	
11	37°C	37°C	38°C	8.5	
12	36°C	36°C	36°C	8.5	
13	36°C	37°C	37°C	8.5	
14	36°C	35°C	35°C	9	
15	36°C	35°C	35°C	9	
16	37°C	38°C	38°C	9	
17	36°C	35°C	35°C	9	
18	36°C	36°C	36°C	9	

3.3 Sewage treatment

In 50% sewage treatment, the treatment efficiency was measured after an incubation period of 7 days. The values are given in Tables 3-5. *Chlorococcum humicola* reduced total N to about 76% and total phosphorus to almost nil. BOD reduction was nearly 40% which is very good in terms of phycoremediation efficiency. TDS and TS reduction was approximately 60%. When treated with *C. vulgaris*, there was a drastic reduction in total N and total P and the BOD reduction was also 43%. *Chlorella* spp. are commonly found in sewage with faster growth rates and a good organic nutrient uptake capacity. When 50% sewage was treated using *Selenastrum* sp., all the parameters were reduced to nearly 60%, and total N showing 84% reduction. Phosphorus uptake was again 100%. Almost all microalgae require N and P for their growth and hence excellent removal of these nutrients takes place in

phycoremediation systems. Phosphorus is found nearly in nutrient-limiting conditions and therefore 100% removal of phosphorus is feasible. When the microalgae are grown in 100% sewage, there was a slight decrease in the growth rates, but the treatment efficiency is not reduced. This is because all the above microalgae were isolated from sewage and they have the inherent capacity to grow in heavy organic load.

Table 3: Treatment of 50% sewage using *Chlorococcum humicola*

Parameters	Untreated sewage (mg/L)	Treated sewage (mg/L)	% Reduction
BOD	70 ± 2.85	43 ± 1.08	39
TDS	2052 ± 95.87	876 ± 45.97	57
TSS	212 ± 18.65	170 ± 10.5	20
TS (TDS + TSS)	2264 ± 132.02	1046 ± 77.98	54
Total Nitrogen	187 ± 11.12	44 ± 3.01	76
Total Phosphorous	11 ± 0.86	Nil	100

All values are presented as mean ± SD of triplicate analyses. Untreated and treated values are statistically significant at $P < 0.05$ according to one-tailed paired Student's t-test.

Table 4: Treatment of 50% sewage using *Chlorella vulgaris*

Parameters	Untreated sewage (mg/L)	Treated sewage (mg/L)	% Reduction
BOD	70 ± 2.85	40 ± 2.55	43
TDS	2052 ± 95.87	744 ± 38.88	64
TSS	212 ± 18.65	133 ± 14.9	37
TS (TDS + TSS)	2264 ± 132.02	877 ± 98.2	61
Total Nitrogen	187 ± 11.12	32 ± 2.86	83
Total Phosphorous	11 ± 0.86	Nil	100

All values are presented as mean ± SD of triplicate analyses. Untreated and treated values are statistically significant at $P < 0.05$ according to one-tailed paired Student's t-test.

Table 5: Treatment of 50% sewage using *Selenastrum* sp.

Parameters	Untreated sewage(mg/L)	Treated sewage (mg/L)	% Reduction
BOD	70 ± 2.85	56 ± 3.35	20
TDS	2052 ± 95.87	812 ± 45.57	60
TSS	212 ± 18.65	153 ± 13.89	28
TS (TDS + TSS)	2264 ± 132.02	965 ± 65.55	57
Total Nitrogen	187 ± 11.12	67 ± 5.5	64
Total Phosphorous	11 ± 0.86	Nil	100

All values are presented as mean ± SD of triplicate analyses. Untreated and treated values are statistically significant at $P < 0.05$ according to one-tailed paired Student's t-test.

Pre- and post-treated parameters were estimated on Day 0 and Day 7. The values are given in Tables 6-8. Treatment using *C. humicola* resulted in nearly 60% of BOD and more than 80% of total N. TDS and TS reduction was approximately 65%. Total phosphorus was reduced to nil by all the microalgae. Reduction of all the parameters was

in the range of 60-65% by the microalga *C. vulgaris*, except total N, which was nearly 85%. The reduction of all the parameters, except total N and total p, was less by *Selenastrum* sp. when compared to *C. humicola* and *C. vulgaris*.

Table 6: Treatment of 100% sewage using *Chlorococcum humicola*

Parameters	Untreated sewage (mg/L)	Treated sewage (mg/L)	% Reduction
BOD	145 ± 12.34	60 ± 4.82	59
TDS	3543 ± 234.5	1200 ± 56.73	66
TSS	454 ± 23.33	232 ± 18.25	49
TS (TDS + TSS)	3997 ± 45.87	1432 ± 102.25	64
Total Nitrogen	342 ± 13.7	67 ± 5.55	80
Total Phosphorous	19 ± 0.98	Nil	100

All values are presented as mean ± SD of triplicate analyses. Untreated and treated values are statistically significant at $P < 0.05$ according to one-tailed paired Student's t-test.

Table 7: Treatment of 100% sewage using *Chlorella vulgaris*

Parameters	Untreated sewage (mg/L)	Treated sewage (mg/L)	% Reduction
BOD	145 ± 12.34	48 ± 2	67
TDS	3543 ± 234.5	1156 ± 97.08	67
TSS	454 ± 23.33	180 ± 12.65	60
TS (TDS + TSS)	3997 ± 45.87	1336 ± 145.5	67
Total Nitrogen	342 ± 13.7	54 ± 5.52	84
Total Phosphorous	19 ± 0.98	Nil	100

All values are presented as mean ± SD of triplicate analyses. Untreated and treated values are statistically significant at $P < 0.05$ according to one-tailed paired Student's t-test.

Table 8: Treatment of 100% sewage using *Selenastrum* sp.

Parameters	Untreated sewage (mg/L)	Treated sewage (mg/L)	% Reduction
BOD	145 ± 12.34	53 ± 1.85	63
TDS	3543 ± 234.5	1835 ± 68.74	48
TSS	454 ± 23.33	210 ± 22.2	54
TS (TDS + TSS)	3997 ± 45.87	2045 ± 130.5	49
Total Nitrogen	342 ± 13.7	62 ± 4.86	82
Total Phosphorous	19 ± 0.98	Nil	100

All values are presented as mean ± SD of triplicate analyses. Untreated and treated values are statistically significant at $P < 0.05$ according to one-tailed paired Student's t-test.

3.4 Biomass/Lipid productivity

Aside from performing bioremediation of sewage using algae, growth in terms of cell numbers and lipid content was measured. The values are given in Tables 9-12. In 50% treatment, the growth and lipids were measured only up to

Day 7. However, the growth was continued up to 18 days in 100% sewage to measure the growth and lipid productivity. Therefore, after completion of the treatment, the cultivation was extended to a period of 18 days. The growth of all the microalgae increased with the increase in incubation period. *Chlorococcum humicola* had the highest growth measurements compared to the other two species. However, the lipid content was more in *Selenastrum* sp., which had lower growth measurements in both 50% and 100% sewage. The lipid levels increase with time because of the stress created due to the depletion of nutrients.

TABLE 9: Biomass in terms of cell number (X 10^4/ml) (50% sewage water)

Days	*Chlorococcum humicola*	*Chlorella vulgaris*	*Selenastrum* sp.
0	352	360	312
1	224	240	384
2	344	288	392
3	192	296	384
4	256	480	336
5	472	656	352
6	408	520	408
7	304	936	672

TABLE 10: Biomass in terms of cell number (X 10^4/ml) (X 10^4/ml) (100% sewage water)

Days	*Chlorococcum humicola*	*Chlorella vulgaris*	*Selenastrum* sp.
0	80	80	64
1	104	96	88
2	184	280	144
3	304	160	128
4	408	600	120
5	640	416	132
6	664	680	160
7	728	672	216
8	755	936	376
9	744	760	384
10	1048	864	464
11	1072	848	472
12	1144	1024	528
13	1272	1168	600
14	1216	1144	640
15	1376	1208	728
16	1424	1320	816
17	1576	1344	904
18	1680	1648	1056

Table 11: Total lipid content in mg%

Days	Lipid concentration in 50% sewage water		
	Chlorococcum humicola	***Chlorella vulgaris***	***Selenastrum sp.***
3	0.2801	0.5602	0.2801
7	1.1204	1.6806	0.8403

Table 12: Total lipid content in mg%

Days	Lipid concentration in 100% sewage water		
	Chlorococcum humicola	***Chlorella vulgaris***	***Selenastrum* sp.**
3	0.2801	0.5602	0.5602
6	2.2408	1.4005	2.2408
9	2.5490	1.3725	1.4005
12	4.4817	2.5210	1.9607
15	5.6022	3.3613	2.8011
18	5.6022	4.4817	3.3613

According to Koh & Ghazoul (2008), biofuels are renewable fuels derived from biological feedstocks it becomes significant if it is cultivated in wastewater. Mata et al (2009) demonstrated that many microalgae species can be induced to accumulate substantial quantities of lipids thus contributing to a high oil yield. The average lipid content varies between 1 and 70%, but under certain conditions, some species can accumulate up to 90% of dry weight. Thus treatment of industrial and domestic wastewaters coupled with biomass generation is very important in the production of commodity products like biofuels. Moreover, cycling of carbon is a phenomenon that the society is in dire need in the present-day scenario, which can be effectively carried out using microalgae technology. Intense research is needed on the concomitant bioremediation/biofuel production processes.

4. Conclusion

Growth of the three algal species isolated from sewage water was achieved successfully and could be further used for sewage treatment and cultivation, which is highly beneficial due to scarcity of fresh water. It is found that nitrogen and phosphorous play an important role in stimulating the growth of microalgae and nutrient limitation, particularly, nitrogen and phosphorous, can slow down the growth of microalgae. Increase in growth rate enhances the biomass yield, whereas decreased growth rate leads to lipid accumulation, which may be due to nitrogen starvation and low phosphorous. Overall, the phycoremediation efficiency is good with excellent growth rates, which is very important for biomass generation for the production of biofuels.

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