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Research Article

An Integrated Approach of Using Polystyrene Foam as an Attachment System for Growth of Mixed Culture of Cyanobacteria with Concomitant Treatment of Copper Mine Waste Water

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Cyanobacteria have tremendous applications in areas such as production of biofuels and pharmaceutically important pigments and are used as an adsorbent for the removal of toxic metabolites. However, large scale production of Cyanobacteria is not economically feasible due to high cost involved in separation of biomass. In this context, different attachment systems have been developed for the growth of Cyanobacteria on a solid support. In this study, a simple and economical attachment system using polystyrene foam for growth of Cyanobacteria has been presented. Results clearly indicate that high biomass yield can be obtained in attached system when compared to suspended system. In attachment system, the biomass yield showed 21.4%, total protein content showed 29.2%, chlorophyll content showed 11.1%, and carotenoid content showed 13.1% increase as compared to the suspended system. The attachment system can also support the growth of Cyanobacteria in presence of copper mine waste water with concomitant removal of copper ions. These results were corroborated by COD analysis, which indicated significant reduction. Further, copper removal was high in attached system as compared to suspended system. It appears that attachment system offers protection for growing Cyanobacteria and can be effectively employed for growing Cyanobacteria in presence of waste water coming from different sources.

1. Introduction

Copper is considered as a persistent and ubiquitous environmental pollutant, which enters into the environment through anthropogenic and industrial activities [1, 2]. Although copper is an essential trace element in animals, higher levels of copper ingested through contaminated water may lead to disastrous consequences in humans especially in children [3]. Therefore, efficient treatment of copper containing waste water is necessary for reducing its toxicity in living systems [4]. In this regard, many treatment processes have been developed which rely on physical phenomena such as adsorption, electrodialysis, and precipitation [5, 6]. These methods have been shown to be expensive and time consuming. Therefore, biological removal of metal ions using microorganisms has been considered as a cheap and ecofriendly alternative [7, 8]. Studies have shown that exopolysaccharides synthesized by Cyanobacteria act as biological ion-exchange materials [9] and can bind and remove metal ions [8, 10–12].

A major hindrance of utilizing Cyanobacteria is the high cost involved in its harvesting. Cultivation of Cyanobacteria is usually performed in open ponds or enclosed photobioreactors, where the yield is quite low [13]. To harvest Cyanobacteria from this solution, the cell suspension is usually concentrated by sedimentation or by flocculation; water is then removed from condensed slurry by evaporation [14]. Due to the huge size of the algal culture system, harvesting algal biomass from the dilute cell suspension is expensive [13].

Therefore, development of an attachment system, where Cyanobacteria are grown attached to a solid support system, seems to be an attractive alternative [14]. Many attached systems such as Algal Turf Scrubber (ATS) have been developed where Cyanobacteria/algae grow on a solid support [15]. However, this system requires high infrastructure and is practically not feasible in developing countries. There is a need to develop new attachment systems for large scale growth of Cyanobacteria that are economical and show ease in handling and maintenance [16–19]. Johnson and Wen [14] have developed a novel attachment system consisting of polystyrene foam as a solid support for growth of microalgae, which supported growth of *Chlorella* sp. on dairy manure waste water. In the present investigation, we have adopted polystyrene foam as a solid support for the growth of a mixed culture of Cyanobacteria isolated from paddy field. However, major modifications in the culturing of microalgae have been adopted so as to make it feasible for growing in areas where sufficient infrastructure is not available. Further, Cyanobacteria were grown in presence of more toxic copper mine waste water so that growth is achieved with concomitant removal of copper by growing mixed culture of Cyanobacteria [12, 20].

Table 1: Physiochemical properties of copper mine waste water.

Properties	Copper mine waste water
Temperature	38°C
PH	6.6
Dissolve oxygen ($mg\ O_2/L$)	14
BOD ($mg\ O_2/L$)	4.0
COD ($mg\ O_2/L$)	604.8
Total dissolve solid (mg/L)	217
TSS (mg/L)	120
Alkalinity ($CaCO_3$ mg/L)	2000
Acidity ($CaCO_3$ mg/L)	500
Hardness ($CaCO_3$ mg/L)	240

2. Materials and Methods

2.1. Strains and Culture Medium. Cyanobacteria employed in the present investigation were obtained from paddy fields situated at Bilaspur, in the state of Chhattisgarh, India. Cyanobacteria were maintained under sterile conditions in nitrogen free BG-11 medium [21] containing (g/L): $K_2HPO_4 \cdot 3H_2O$, 0.04; $MgSO_4 \cdot 7H_2O$, 0.075; $CaCl_2 \cdot 2H_2O$, 0.036; citric acid, 0.006; ferric ammonium citrate, 0.006; EDTA, 0.001; $NaNO_3$, 1.5; Na_2CO_3, 0.02; and trace metal mix A5, 1.0 mL. Trace metal mix A5 solution consisted of (g/L) H_3BO_3, 2.86; $MnCl_2 \cdot 4H_2O$, 1.81; $ZnSO_4 \cdot 7H_2O$, 0.222; $NaMoO_4 \cdot 2H_2O$, 0.39; $CuSO_4 \cdot 5H_2O$, 0.079; and $CoCl_2 \cdot 6H_2O$, 0.05. The culture was incubated at 26–30°C and 16/8 h photoperiod in a culture room under 3000 lux from cool white light.

2.2. Collection of Waste Water Sample. Water samples were collected from the copper mines located at Balaghat Malanjkhand, Madhya Pradesh, India. It is an open pit type mine owned by Hindustan Copper Limited (HCL). Geographical coordinates of Balaghat are 21° 47′ 23.5248″ North, 80° 47′ 28.3344″ East. Effluent from the Malnjkhand (HCL) plant is discharged to the tailing pond. Water samples were collected in autumn (February, 2012) from different spots of outlet1 and outlet2 of the copper mining waste water reservoir. The copper mine wastewater was collected in a reservoir with total area of about 50 m^2 and 1 m depth. Physiochemical properties of copper mine waste water from outlets were determined according to APHA (2005). The average effluent water quality is represented in Table 1.

2.3. Growth of Cyanobacteria in Attachment System. The substrate used for attachment of Cyanobacteria was polystyrene foam EPS 70 (6 cm × 12 cm), fixed on the bottom of a growth chamber. Polystyrene foam EPS 70 was selected because it is of low cost and is widely used in packaging of various industrial and household goods and, after use, it is disposed as waste material. Therefore, it is readily available. The growth chamber was a plastic disposable bowl, which was covered by a polythene sheet. A glass tube was inserted through the sheet to provide aeration (Figure 1). Before inoculation, the whole setup was wiped twice with 70% ethanol and incubated under UV light for 30 min. For suspended system, the same setup was employed without the use of polystyrene foam. The waste water from copper mine was filtered by using Whatman filter paper 1 and used directly without autoclaving. The growth chamber contained wastewater and BG-11 media in different combinations and was inoculated with microalgae culture grown in BG-11 medium at a concentration of 10% v/v. Copper mine waste water was mixed with BG-11 in different combinations, so as to achieve final concentration of 20%, 40%, and 60% copper mine waste water, respectively. Initial concentration of copper in waste water was 78 ppm; therefore, in 20%, 40%, and 60% effluent waste water, the concentration of copper was 13.2 ppm, 29.23 ppm, and 44.18 ppm, respectively. The growth chambers were incubated at 26–30°C and 16/8 h photoperiod in a culture room under 3000 lux from cool white light for 18 days. After 6, 12, and 18 days of incubation, the culture medium was decanted and the biomass was scrapped with the help of a scalpel and was freeze-dried. In suspended system, the medium was centrifuged at 10,000 rpm for 10 min and cell pellet was freeze-dried.

2.4. Analytical Techniques. The protein content of freeze-dried biomass was determined by the modified Lowry method [22]. The chlorophyll a and carotenoid were determined according to Vonshak et al. [23]. For the estimation of copper, samples of 50 mg of the freeze-dried biomass were mixed separately with 1 mL of aqua regia in covered 10 mL beakers. The samples were heated for another 1 hr in water

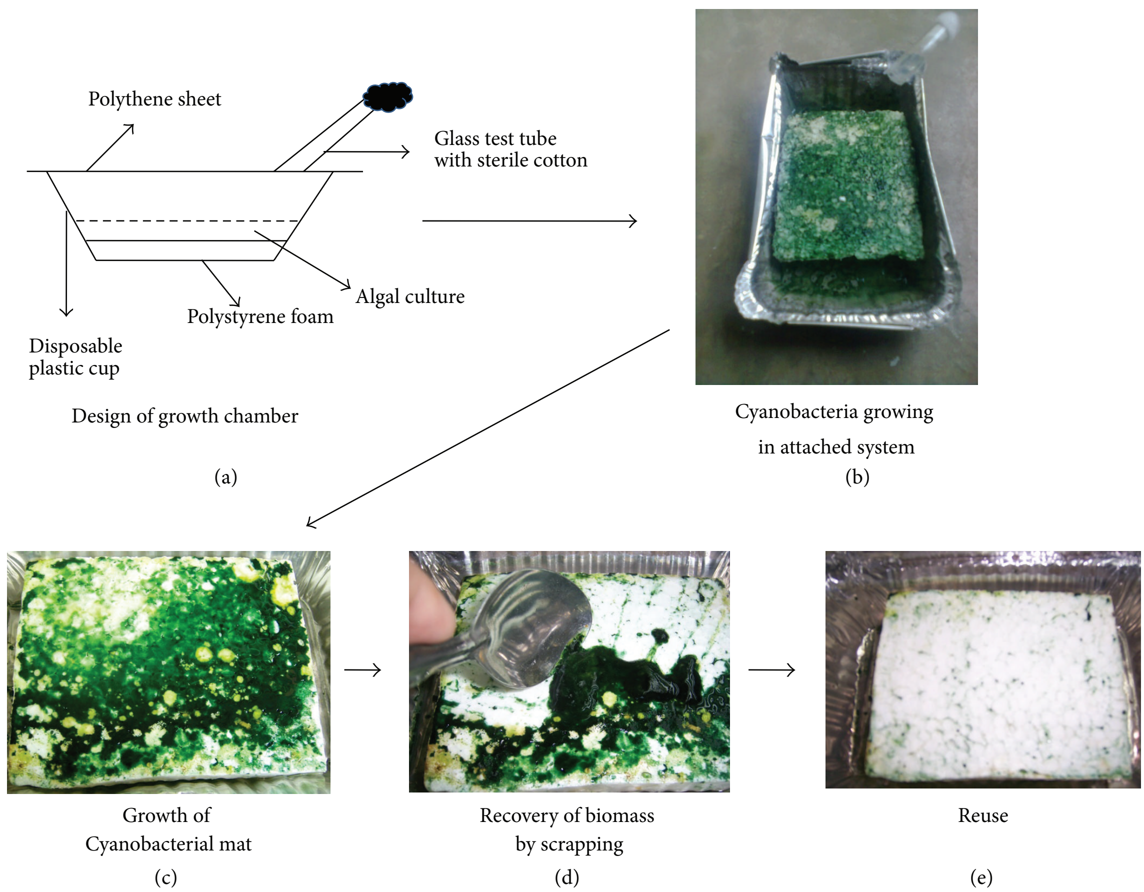


Figure 1: Development of an attachment system for the growth of mixed Cyanobacterial culture incubated at 26–30°C and 16/8 h photoperiod in a culture room under 3000 lux from cool white light. (a) Schematic representation of attachment system, (b) Cyanobacteria growing in the attachment system, (c) development of Cyanobacterial mat on the surface of polystyrene foam, (d) recovery of Cyanobacterial biomass by scrapping, and (e) reuse of attachment system for another cycle of growth.

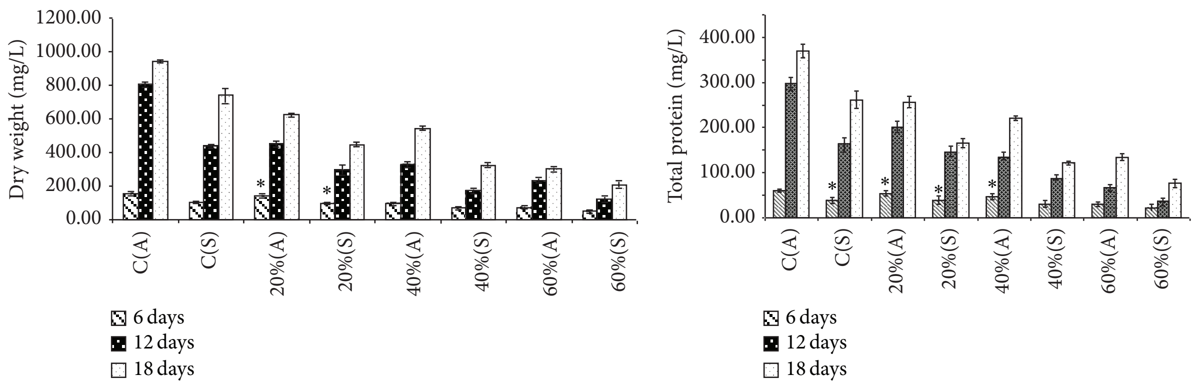
bath and cooled. Then, volumetric flasks were filled with double deionized water to exactly 10 mL mark and left in shaker for overnight. The solution was analyzed with the flame atomic adsorption spectrophotometer 7000 (Shimadzu Corporation).

2.5. Microscopic Studies. Examination of mixed culture was under compound microscope (Nikon Eclipse E400, Nikon Corporation, Tokyo, Japan).

2.6. Statistical Analysis. All the results are expressed as mean ± standard deviation. The results were analyzed using two-way ANOVA followed by post hoc analysis at $P > 0.05$ confidence level.

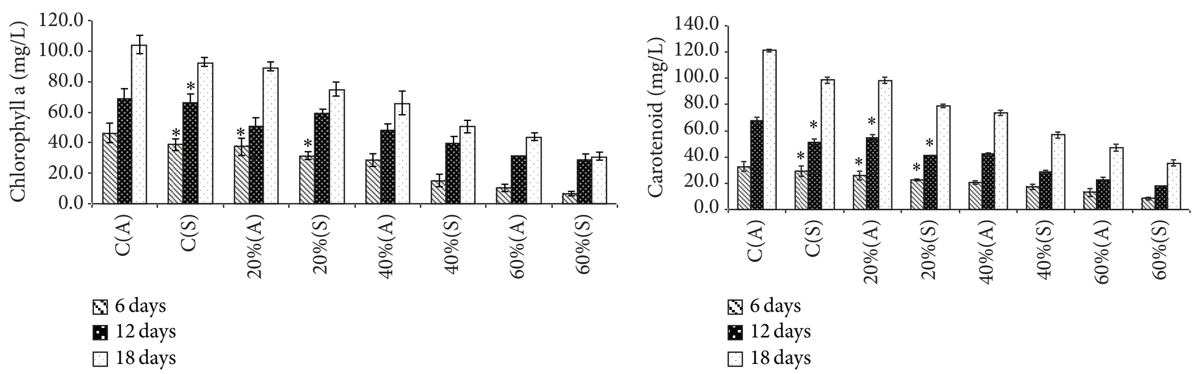
3. Results and Discussion

3.1. Growth of Cyanobacteria. Figures 2(a) and 2(b) summarize the biomass yield and total protein content and Figures 3(a) and 3(b) summarize the chlorophyll a and carotenoid content obtained when algal cells were grown in attached and suspended system in the presence of different combinations of copper mine waste water, respectively. It was observed that in control containing BG-11, the yield in attached and suspended system in all the tested parameters was not significant ($P < 0.05$) after 6 days of incubation; however, after 12 and 18 days, the suspended system and the difference in yield become significant. The yield in suspended system after 18 days of incubation was low as compared to attached system. The biomass yield was 21.4%, total protein content was 29.2%, chlorophyll content was 11.1%, and carotenoid content was 13.1% reduced as compared to the attached system. Similar results have been reported by Johnson and Wen [14]; in their study, *Chlorella* sp. was grown in both attached and suspended systems in presence of dairy manure waste water and high growth yield was obtained in attached system. In presence of different concentrations of copper mine waste water, the biomass yield showed gradual reduction and decreased in concentration dependent manner. It was observed that after 18 days of incubation, in attached system, biomass yield showed 33.8%, 42.2%, and 67.8% reduction, total protein content showed 30.5%, 40.2%, and 63.6% reduction, total chlorophyll content showed 14%, 36.6%, and 57.7% reduction, and carotenoid content showed 18.7%, 39.3%, and 61.2% reduction in the presence of 20%,



(a) (b)

Figure 2: Biomass yield and protein content of mixed Cyanobacterial culture incubated at 26–30°C and 16/8 h photoperiod in a culture room under 3000 lux from cool white light. (a) Biomass yield and (b) total protein content of mixed Cyanobacterial culture in both attachment and suspended systems in presence of BG-11 growth medium and in presence of different concentrations of copper mine waste water. (A) represents attachment system and (S) represents suspended system, respectively. Values are represented as mean ± standard deviation of three independent experiments.



(a) (b)

Figure 3: Chlorophyll a and carotenoid content of mixed Cyanobacterial culture incubated at 26–30°C and 16/8 h photoperiod in a culture room under 3000 lux from cool white light. (a) Chlorophyll a and (b) Carotenoid content of mixed Cyanobacterial culture in both attachment and suspended systems in presence of BG-11 growth medium and in presence of different concentrations of copper mine waste water. (A) represents attachment system and (S) represents suspended system, respectively. Values are represented as mean ± standard deviation of three independent experiments.

40%, and 60% copper mine waste water, respectively. A similar trend was observed in suspended system also. In suspended system, after 18 days of incubation, biomass yield showed 39.4%, 56.2%, and 71.7% reduction, total protein content showed 36.6%, 53.6%, and 70.7% reduction, chlorophyll a content showed 18.7%, 45%, and 66.5% reduction, and carotenoid content showed 20.1%, 42.3%, and 64.1% reduction which was observed in presence of 20%, 40%, and 60% copper mine waste water, respectively. It is noteworthy to mention that after 6 days of incubation, the reduction in 20% and 40% copper mine waste water was not so significant. However, after 12 days of incubation, the results were more pronounced. It is clear from the results that the reduction in all the parameters was more pronounced in suspended system than in attached system. This indicates that copper present in copper mine waste water is toxic to Cyanobacteria [2] and hence a dose-dependent reduction in growth occurs in the presence of copper ions [24]. However, our results also indicate that attached system offers protection for Cyanobacteria against copper toxicity. This is due to the fact the Cyanobacteria are attached to a solid support and hence encounter a less concentration of copper and therefore growth is favored. It clearly indicates that attached system is suitable for growth of Cyanobacteria in presence of toxic waste materials and aids in their removal. This is a significant advancement in the growth system developed by Johnson and Wen [14]. In their study, *Chlorella* sp. was grown in presence of less toxic dairy manure waste water with subsequent biomass production. In the present study, Cyanobacteria were grown in presence of more toxic copper mine waste water showing its removal.

3.2. Microscopic Examination of Cyanobacteria. The state of Chhattisgarh is well known for intense paddy cultivation.

Table 2: Copper uptake from different concentrations of copper mine waste water by Cyanobacterial biomass after different time intervals in both attachment and suspended growth systems, incubated at 26–30°C and 16/8 h photoperiod in a culture room under 3000 lux from cool white light.

Culture system	Different effluent concentration	Copper uptake (%)* Incubation (days) 6	12	18
Attachment system	20%	26.8 ± 3.31	44.7 ± 3.18	85.6 ± 1.61
	40%	34.2 ± 2.21*	56.9 ± 1.15*	88.9 ± 1.25
	60%	30.4 ± 1.60	42.5 ± 0.91	65.8 ± 1.64*
Suspended system	20%	20.5 ± 1.32	38.2 ± 1.12	70.3 ± 1.54*
	40%	24.8 ± 2.31	45.3 ± 1.12*	74.9 ± 0.85
	60%	19.2 ± 1.41*	28.9 ± 1.99*	53.8 ± 1.27*

Values are represented as mean ± standard deviation of three independent experiments.
The values indicated by (*) are not significantly different from control at $P > 0.05$, determined by two-way ANOVA followed by post hoc test.
In negative control, copper uptake by polystyrene foam was not detected in all combinations of copper mine waste water and BG-11.

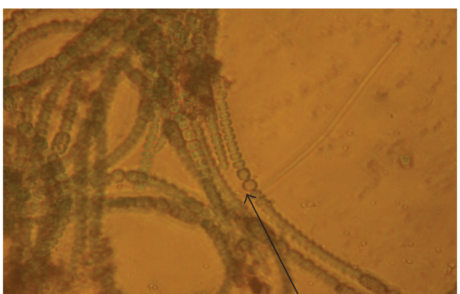


Figure 4: Microscopic examination of mixed Cyanobacterial culture in attachment system showing dominance of *Anabaena* sp. at 40X magnification.

Table 3: Percent of reduction in COD from copper mine waste water by Cyanobacterial biomass.

Culture system	Different effluent concentration	% COD reduction
Attachment system	20%	56 ± 1.8
	40%	53.2 ± 2.2
	60%	35.2 ± 1.3*
Suspended system	20%	54.2 ± 1.2
	40%	47.8 ± 1.6
	60%	30.2 ± 1.9*

Values are represented as mean ± standard deviation of three independent experiments.
The values indicated by (*) are not significantly different from control at $P > 0.05$, determined by two-way ANOVA followed by post hoc test.

Therefore, a mixed culture of Cyanobacteria was obtained from a paddy field situated at Bilaspur in the state of Chhattisgarh. Microscopic examination revealed that mixed culture contained *Nostoc* sp., *Anabaena* sp., and *Chlorella* sp. However, in the presence of different combinations of copper mine waste water, it was observed that *Anabaena* sp. was the dominant Cyanobacteria (Figure 4).

3.3. Removal of Copper Ions. The amount of copper removed by growing culture was also determined by estimating the uptake of copper in Cyanobacterial biomass after 6, 12, and 18 days of incubation. The results are represented in Table 2. It is clear from the results that growing Cyanobacteria effectively removed copper from culture medium. In both attached and suspended systems, an increase in copper removal was observed with an increase in growth of Cyanobacteria. Copper removal did not show significant increase ($P < 0.05$) with the increase of the copper concentration in the culture medium, that is, 20% and 40% concentration. However, at 60% concentration, a significant decrease ($P < 0.05$) in copper removal was observed. A similar trend was also observed in suspended system, but the amount of copper removed was significantly lower ($P < 0.05$) than attached system. This observation can be attributed to a decrease in growth of Cyanobacteria in both systems in presence of high concentration of copper. When amount of copper removed in attachment and suspended systems was compared, it was observed that amount of copper removed was more pronounced in attached system, when compared to suspended system. This was clearly due to high growth yield in attached system. Many reports have confirmed that exopolysaccharides synthesized by various Cyanobacteria are effective in removal of metal ions [4, 10, 25]. In attached system, the growing Cyanobacteria form a dense mat-like structure in which they retain the exopolysaccharides. However, in suspended system, these exopolysaccharides are lost in the culture medium and therefore they are not effective in absorbing the metal ions. In negative control, no adsorption of copper on polystyrene foam was observed. In order to estimate the efficacy of this process, percent of COD removal was estimated after 18 days of incubation. The results are depicted in Table 3. It is clear from the results that percent of COD removal in attachment system at 20% and 40% concentration ranged from 56 to 53.2% and in suspended system ranged from 54.2 to 47.7%; at 60% concentration, the values were significantly

($P < 0.05$) lower in both attachment and suspended systems indicating toxicity of copper mine waste water to growing Cyanobacteria.

4. Conclusions

In this study, we have developed a simple and inexpensive attachment system for growth and harvesting of Cyanobacteria which can be employed for large scale culturing of Cyanobacteria in areas where sufficient infrastructure is not available. Cyanobacteria were grown in presence of copper mine waste water and showed significant removal of copper ions. Cyanobacterial biomass containing copper can be used for recovery of copper thus generating a value added product. This result clearly indicates that attached system offers protection for growing Cyanobacteria against toxic copper ions leading to a high biomass production with removal of copper from waste water. This result is an advancement of previous studies, where Cyanobacteria were grown on attached system in presence of less toxic wastes such as dairy manure waste water. We feel that this system can be effectively involved in treatment of toxic waste water with subsequent biomass production. Biomass can be employed for recovery of metal or for the extraction of value added products such as Cyanobacterial pigments and biofuel, thus generating employment opportunities.

Conflict of Interests

All the authors declare that they have no potential conflict of interests regarding the submission and publication of this paper.

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