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Impact of Stationary Algal Biofilm Adhesion on Substrate's Surface for Biomass Harvesting and Treatment of Wastewater: Experimental Investigation

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Algal biofilm system can act as a potential platform for better algal biomass production and easy harvesting. The goal of this study is to investigate a stationary algal biofilm adhesion on various substrate's surfaces with integrated approach for algal biomass harvesting and wastewater treatment. An algal biofilm system in continuous mode with autoclaved and unautoclaved Composite Industrial Wastewater (CIWW) and selected substrates (on the basis of roughness and smoothness) was used to grow the selected algal strain. Maximum algal cell attachment was reported on rough surface when compared to other selected substrates. Nutrient recovery efficiency for TDS, BOD, COD, Nitrate and Phosphate in autoclaved and unautoclaved CIWW was found in the range 31-45%, 60-76%, 98.4-98.6%,17.2-30.7% and 72.9-73.8% respectively. The utilization of discarded materials as an attachment surface for algal biofilm formation was proved to be an effective measure to manage the problem of water choking created by these discarded materials. Such materials with further extensive studies can be suggested for wastewater remediation through algal biofilm cultivation approach under the concept of resource recovery and reutilization.

Keywords: Algal biofilm; substrate; Algal biomass harvesting

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With the rising problem of water pollution, the extensive exploration for remediating agents has picked up a good pace. The demand for present remediation methods involves a multipurpose approach that is capable of serving the purpose of removal of contaminants along with the production of some valuable end products. There have been different types of studies with respect to algae that explored its potential to produce fuel, feed, and other value-added by-products [1-3]. Algae have been cultured in various types of wastewater such as municipal, dairy, and slaughterhouse because wastewater rich in nutrients is considered as a good source for cultivation of the algae [4]. Present popular algal cultivation systems include open suspended ponds, photobioreactors, and surface attached cultivation systems. These biological remediation methods are better in regard to the safety of the surrounding environment, but the issues related to these technologies include high inputs of capital and energy [5]. The harvesting process in these technologies is also a laborious job that is further subjected to more energy-intensive operations.

A potential alternative to these technologies keeping the intactness of the environment in mind involves algal biofilm based cultivation systems. These systems have the efficiency to reduce the cost factor involved in the harvesting process [6]. In algal biofilm based cultivation systems, the algae gets into contact with some substrate and begin to colonize over it. The substrate provides a firm site for its attachment allowing an easy algal growth. Thus, wastewater remediation using algal biofilm cultivation systems involves the growth of algal biofilm onto a substrate present in the wastewater which acts as a source of nutrition for algae [7]. Algae take up the excess nutrients present in the wastewater and produce biomass in the attached form which is much easier to harvest through simple steps such as scrapping. Algal biofilm has additional benefits of overcoming the barriers faced by other algal cultivation methods such as limiting light, and mass transfer of carbon dioxide [8]. The attached algal cells on the substrate when compared to the suspended cells show better light availability making these cultivation systems a good option for attaining high biomass production and wastewater remediation efficiency. Presently, various algal biofilm cultivation systems have been developed to treat a variety of wastewater with high biomass production such as, revolving algal biofilm cultivation systems, vertical algal biofilm cultivation systems, horizontal algal biofilm cultivation systems, totally immersed or partially immersed algal biofilm cultivation systems.

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In this study, a stationary algal biofilm cultivation system was built for biomass production and wastewater treatment (CIWW) with an approach of utilization of discarded materials as a resource for biofilm formation. The reactor has an even distribution of wastewater to maintain equal contact of the substrate with algal cells. Less turbulence of wastewater ensures a stable biofilm formation. The algal strain used in this growth system was *Chlorella vulgaris as* it has anti-asthmatic, anti-cancer, and antioxidant properties. Carotenoids obtained from this strain have successfully shown tremendous results as neuroprotective natural products by slowing down progression of Alzheimer's disease [9]. It is a spherical and unicellular freshwater green alga belonging to the family *chlorellaceaea*. The novelty of this study is assessing the algal biofilm in wastewater with treatment strategies. Real time conditions responsible for problems such as water choking have been mimicked at lab-scale for Composite Industrial Wastewater (CIWW) to validate the research at lab scale.

EXPERIMENTAL

Collection of Wastewater Sample

For this study, the CIWW samples were collected from flow channels (*nallah*) of the industrial area, Bari Brahmana, District Samba, Jammu and Kashmir, India (32º38'10" N latitude, 74º56'25" E longitude and 352 m elevation). Due to the coexistence of numerous industries in the area, it became a primary factor for selecting this location for the present experimental investigation. The wastewater samples were collected from the accessible site (confluence point for wastewater from different industries) because access to the main site of origin of the channel was not possible due to army area constraints. The samples were collected using pre-sterilized sampling bottles and kept at 4°C.

Physicochemical Characterization of Wastewater

There were two sets of the same samples that were tested in the present experimental study. One set of the sample was autoclaved (CIWWA) before algal inoculation at 15 psi and 121°C for 20 minutes and other set of the sample was not autoclaved before inoculation (Unautoclaved, CIWWU). The analysis of physico-chemical parameters (color, pH, TDS, and conductivity) was done in the field itself. The greyish color of the sample was observed by naked eyes. The pH, total dissolved solids (TDS), and conductivity were measured by digital Combo pH/Conductivity/ TDS Tester (High Range) - HI98130. The temperature was measured by portable thermo-meter. The rest of the physicochemical parameters, bio-chemical oxygen demand (BOD), chemical oxygen demand, nitrate and phosphate were analyzed by APHA (2012) standard methods. The samples were fixed at the site by adding appropriate chemicals (For COD and Nitrate, 2ml of concentrated H_2SO_4/L, and for phosphate, 40 mg $HgCl_2$/L). The COD of the sample was estimated by titration and the five-day BOD test was used to analyse BOD of the sample by utilizing an incubator for 5 days at 25°C. The nitrate and phosphate were analyzed by UV-Vis Spectrophotometer (Shimadzu, UV-2600, 2020) in 1 cm quartz cuvette. Triplicate samples were used for the physicochemical analysis of the samples.

Algal Strain and Growth Conditions

The algal strain *Chlorella vulgaris* collected from culture laboratory of Department of Environmental Sciences, Babasaheb Bhimrao Ambedkar University, Lucknow (Uttar Pradesh, India) was used in this study. HIMEDIA BG-11 Broth media was used for algal cultivation. The culture media was sterilized in autoclave (Electra A-80, 2023) for 20 min at 15 psi and 121°C prior to cultivation. Subsequently, the media was then cooled down and disinfected by UV light for 20 min. The fresh culture of *Chlorella vulgaris* was then grown in this disinfected and cooled media in 250 ml Erlenmeyer flask at 25 ± 2 °C, to be used as an inoculum. The culture pH was regulated at 7.0±1 by HCl (1N) and NaOH (1N). The culture was cultivated at 8:16 h (light and dark period) by 18 W LED bulb (Philips). Flasks containing BG-11 media and algal inoculum was shaken manually at regular intervals. 30ml of culture with an optical density of 680 nm was then used as an inoculum into the algal biofilm growth system prepared at lab scale.

Lab-scale Algal Biofilm Growth System with Selected Substrates

For this study, two reactors (CIWWA and CIWWU) having 6L capacity with 3L working volume were employed for algal cultivation and wastewater treatment. The reactor was made up of HDPE-grade plastic (15×25 cm). 3L of wastewater was added to this reactor and substrates were placed before algal inoculation. According to the buoyancy properties of the substrates, one substrate was used as a floating substrate for algal cell attachment, the rest of the substrates were heavy enough to be considered under submerged substrates. The reactor was covered with a transparent sheet to ensure a proper supply of light while minimizing the contamination rate. The covering was loosely bounded to the reactor for an easy transfer of gases for biofilm formation. Prior to the experiment, the reactor was disinfected with 5% Hydrogen peroxide. After this, three-times rinsing with distilled water was done to clean any remains of the Hydrogen peroxide. The reactor (Figure 1) was kept at room temperature with 8:16 h light and dark periods. In this study, for biofilm formation, four different substrates were used in each set of wastewater (CIWWA and CIWWU). These substrates were: (i) two plastic ice cream cups (floating type and smooth surface substrate) having initial weights 2.45 and 2.45 gm (ii) glass slides (submerged and smooth surface substrate) having initial weights 5.13 and 5.15 gm (iii) mobile tempered glass (submerged and smooth surface) with initial weights 6.57 and 8.29 gm and (iv) aluminum foil

(submerged and rough surface) having initial weights 11.52 and 9.65gm.

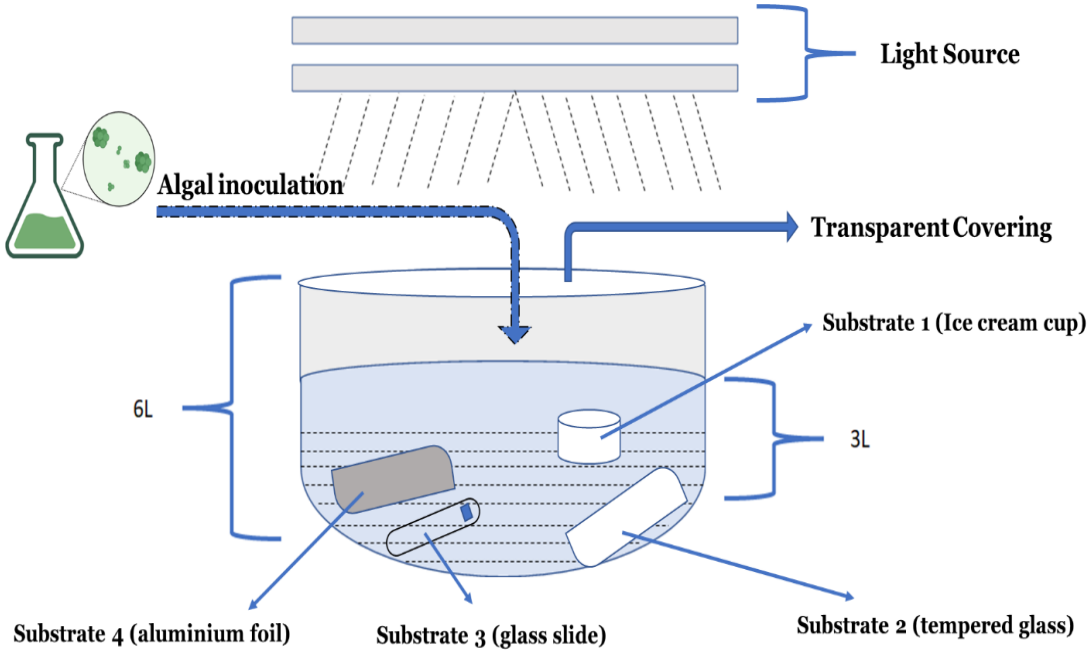

Figure 1. Laboratory-scale biofilm growth system.

Table 1. Physicochemical composition before and after the treatment.

Parameters	Initial Readings	Autoclaved wastewater	Efficiency % (Autoclaved)	Unautoclaved wastewater	Efficiency % (Unautoclaved)	Accepted/ Permissible limits as per CPCB (2019)
Color	Blackish greyish	Light green	-	Green	-	-
pH	9.0	6.9	-	7.0	-	5.5-9.0
Temperature	33	30.7	-	34.0	-	< 5
Conductivity	1.91	1.50	21.4	1.42	25.6	-
TDS	1020	698	31.5	558	45.2	-
BOD	580	250	56.8	160	72.4	30-100
COD	1,160	18	98.4	16	98.6	250
Nitrate	10.54	8.72	17.2	7.30	30.7	10
Inorganic Phosphorus	4.078	1.102	72.9	1.057	73.8	5

Note: Except Colour, Temperature(°C), and Conductivity(S/m), all other parameters are in mg/L

All of these substrates were discarded as waste so these were readily available. These substrates were screened on the basis of their accessibility, durability, and surface properties (rough/smooth). These materials are usually discarded as waste after their one time use such as ice cream cups are thrown after use, mobile tempered glasses are also discarded when there is need to change the tempered glass by the mobile users, the glass slides from laboratories are also discarded after contamination or breakage and aluminium foil has multidimensional utilization from packing eatables to storing materials in laboratories, but after utilization, they need to be discarded as waste. These substrates were tested with algal cultivation in BG-11 Media

before the actual experiment for their algal cell attachment for its durability. The substrates were first cleaned with 5% Hydrogen peroxide and later rinsed thrice with distilled water to remove any unwanted material. These substrates were then stored at 4℃ to keep them contamination-free till the experiment starts. All the substrates were weighed and labelled before running the experiment.

The aforementioned substrate materials were used as the site for adhesion for algal cells for wastewater treatment and formation of algal biofilm. Two reactors each containing 3L wastewater were inoculated with 30 ml of fresh *Chlorella vulgaris* suspension. Before inoculation, one set of wastewater (3L) was autoclaved to remove other organisms while the other set was not autoclaved. The analysis of algal growth was conducted between 1st to 20th days of inoculation. The detailed characteristics of wastewater are given in Table 1. The COD and BOD was 1160 and 580 mg/L which exceeded the permissible discharge limits [10]. The phosphate level (4.078 mg/L) before treatment was also found at the borderline of the discharge limit prescribed by the CPCB (2019) [10]. These findings highlighted the need for effective wastewater treatment to ensure compliance with environmental regulations.

Analysis of Algal Biofilm Growth

The algal biofilm growth was analysed on the basis of biomass production which was detected by using UV-Vis Spectrophotometer (Shimadzu, UV-2600, 2020). The absorbance was noted at a wavelength of 680 nm as it resonates to the cell number change in unicellular microorganisms [11]. Precisely, after the completion of one cycle, the materials having the algal biofilm were meticulously taken out. The biomass adhered to the substrates was scrapped off gently to ensure minimal biomass loss during harvesting process. The scrapped biomass was weighed to obtain wet biomass and then dried weight was determined by drying at 105℃ for 24 h. Dewatering is very important step because before obtaining valuable products, good quality of algal biomass is required, and dewatering process helps in obtaining quality biomass [12]. The amount of biofilm formation depends upon the surface properties of the substrate, if the properties compliment the algal biofilm growth, then majority of the algal cells colonize over the substrate and secrete a layer over cells known as extracellular polymeric substances (EPS) that prevents the washing away of algal cells. These algal cells attach over one another and thus there is less water content left in the biofilm which is needed to support the normal cellular functioning and nutrient movement.

This formed algal biofilm can be harvested manually by scrapping which reduces the costly dewatering step, operated in suspended cultivation [13]. Algal biomass adhered to the substrates in biofilm form already has very less moisture compared to the suspended biomass but there is always some remaining moisture content in the biofilm itself. Biomass concentration (g) in the form of moisture content after dewatering for biomass harvesting was quantified by the following modified formula [14]:

$$B(g) = \frac{Dw - Dd}{Dw}$$

Where, *Dw* is the harvested wet biomass and *Dd* is the weight of the oven dried harvested biomass.

Analysis of Nutrient Resource Recovery (NRR)

The nutrient resource recovery (NRR) showing the percentage of pollution reduction of wastewater via selected algal biomass with the help of biofilm is as follows [15]:

$$NRR\ \% = \left(\frac{C_0 - C_t}{C_0}\right) \times 100$$

Where, C_0 is the initial concentration of nutrient and C_t is the concentration of nutrient after a specific time duration (t) respectively and *NRR* is the pollution reduction efficiency.

RESULTS AND DISCUSSION

As per the objectives designed for present experimental study, observations in the form of results are discussed here in this section with impacts of substrate's surface on biofilm growth and wastewater treatment.

Impact of Substrate on Algal Biofilm Adhesion on Substrate's Surfaces

Biomass growth was observed visually on almost all the substrates. A large number of substrates were reviewed as per their suitability for algal biofilm growth in the collected wastewater. Four substrates were chosen in this study as per their availability, reusability and longevity. It was found that among four types of selected substrates i.e., ice cream cups, glass slides, mobile tempered glass and aluminium foil, best growth was achieved over the surface of aluminium foil in both the reactors. In reactor 1 (autoclaved wastewater), aluminium foil showed highest (53.5%) growth while the glass slide showed the lowest growth. This can be due to the roughness of the substrate surface as microbial colonisation rises with the surface roughness (roughness increases surface area and decreases shear forces). On the glass slide, there was the least growth. In reactor 2 (unautoclaved wastewater), aluminium foil showed the highest growth (65%). It has been shown that, when assessing the extent and rate of attachment, microbes adhere to nonpolar, hydrophobic surfaces like Teflon and other plastics more quickly than they do to glass and other substances with hydrophilic nature [16]. Algal-bacterial cells attachment to the substrates is a dynamic inter-play between surface chemistry, hydrophobicity, and

biological processes. This approach provides an eco-friendly way to manage Low density polyethylene (LDPE)-grade plastic materials like ice cream cups, mobile tempered glass, etc, which can cause choking of water bodies. In this study, set up of both autoclaved and unautoclaved CIWW has been used to analyze algal biofilm growth for the purpose of comparative assessment in this study. It was observed that the wastewater which was used as media (unautoclaved) before algal culture inoculation showed maximum algal growth when compared to the wastewater subjected to autoclaving prior to the inoculation. This could be due to the presence of other organism such as bacteria in the wastewater that helped in enhancing the algal growth. Algal-bacterial biofilm has demonstrated elevated free energies of cohesion and adhesion energy [17]. These factors play a crucial role in the establishing and stabilizing the biofilm growth at a rapid pace.

Formation of algal biofilm in unautoclaved CIWW is species-dependent and a complicated process but attachment to the surface of the substrate via adhesion, which is due to secretion/formation of extra-cellular polymeric substances (EPS), can help the algal cells to colonize on the substrate surface via hydrogen bonding. According to Allen et al., 2015 [18] algal biofilms are made up of 90% EPS and 10% of algal cells. Biofilm growth depends very much on constituents of wastewater, algal species, type of substrate surface, nutrient available in wastewater, temperature, pH and light intensity. The substrate plays a very important role in growth of algal biofilm, as clearly indicated in the present study that substrate having rough surface was visible with more algal biofilm attachment as compared to the substrates with smooth surfaces, where there was negligible visualization of the algal biofilm adherence. This could result from the textured grooves present on the substrate materials, preventing algal cells from being washed away by the flow of the nutrient medium/wastewater. Therefore, this approach on the basis of comparative biomass production could provide appropriate guidelines for the selection of suitable substrate as site for algal cells for their adhesion and for better generation of algal biomass.

Algal Biomass Harvesting

From a waste minimization and cost-effective cultivation perspective, discarded waste materials were selected as substrate so that the cost of substrate became negligible in this study. Ice cream cups, tempered glass and aluminium foil were discarded as waste material, so these were selected to investigate whether these discarded materials can be used to address their single use nature. As the growing plastic pollution is capable of altering the water chemistry of the water bodies to a greater extent, microplastics originating from these can hinder the normal function of aquatic ecosystem by entering the food chain and food web, thus causing an overall harm to ecosystem [19, 20]. The maximum growth was observed on aluminium foil which was taken into consideration for further analysis. From the perspective of nutrient removal, algal biofilm in both setups (autoclaved and unautoclaved) was able to remove the excess nutrient load. On the visual observation, the accumulation of algal cells as biofilm biomass on the substrates was patchy during the initial days marking the lag phase of the algal cycle in which pioneer algal cells were showing adherence for colonization process. This was followed by formation of homogenous biofilm over the substrates in increasing manner showing the cells have entered in the exponential or log phase [21]. Biochemical parameters were analyzed during the growth phase in terms of optical density at 680 nm with COD, BOD (organic load), nitrate and phosphate (nutrient load) and pollution load removal [22]. This reactor has an even distribution of wastewater to ease the formation of even algal biofilm. This growth system was efficient enough to make the harvesting process very easy. The suspended algal cells were harvested by centrifugation while the attached cells were harvested by manual scrapping.

When the optical density of the cells present in the wastewater began to decrease, it was clear that both systems have entered a declining phase (Figure 2). The algal biomass present in suspended form as well as on the substrates as algal biofilm was harvested in the declining phase of algal growth on the 20th day of the experimental analysis. Suspended biomass was harvested by taking the aliquots in 50 ml falcon tubes and centrifuged (Thermo Scientific SL 8R, Centrifuge, 2014) at 5800×g and 4 ℃ for 10 min. Concentrated wet algal biomass from both set up was weighed. After this, the biomass was dried in hot air oven (UQ-2021101942, 2023) at 40**°C** for 24 hrs. The wet algal biomass from reactor 1 (autoclaved wastewater) was found to be 8.362 g while after drying the biomass, 1.34 g of dry biomass was reported. Similarly, in the reactor 2, after harvesting, the wet biomass was reported to be 11.77 g while after drying in hot air oven, 2.69 g of dried biomass was reported. Furthermore, harvesting of the single species biofilm are of great concern in present research era for bioproducts formation as a future application.

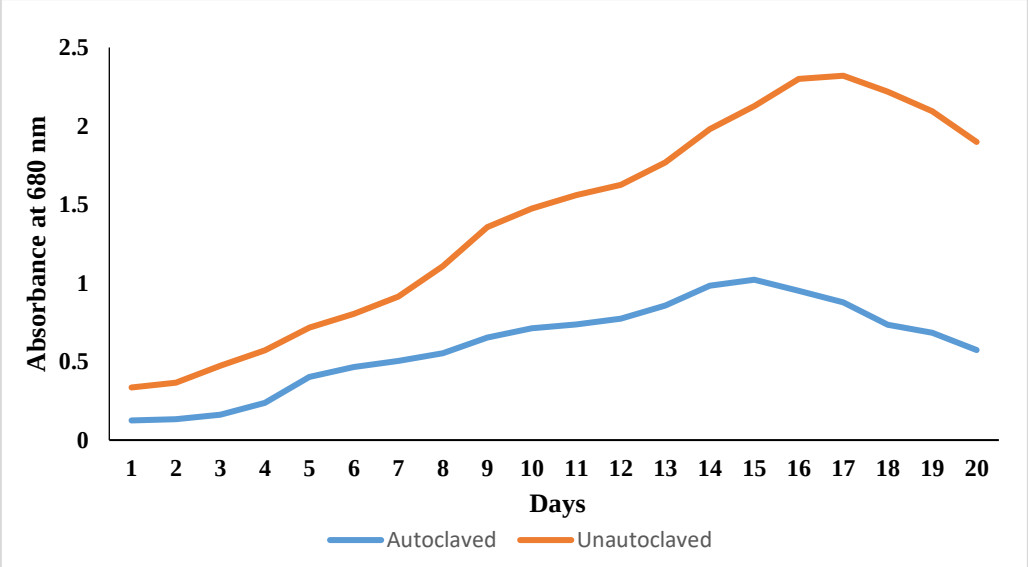

Figure 2. Algal biofilm growth.

CONCLUSION

Algal biofilm growth systems have a high potential for easy biomass harvesting option with wastewater treatment. The discarded materials were screened to act as appropriate substrate on the basis of roughness and smoothness of their surfaces. This study clearly indicated that rough surfaces are suitable for an easier attachment of algal cells with the substrate, thus providing a potential solution for cultivation and harvesting of the algal strains. In the designed experimental setup, algal biofilm was formed in both autoclaved and non-autoclaved wastewater. Rough surface (aluminium foil), showed maximum attachment of algal cells with CIWWU. The findings in this study indicated that unautoclaved wastewater was more effective at treating the wastewater since there was more algal growth in it than in autoclaved wastewater. The possible reason of the same may be presence of algal-bacterial consortium that enhanced the biofilm growth. Unautoclaved wastewater and autoclaved wastewater both showed a good removal capacity for physico-chemical parameters, responsible for organic load of contamination. Besides phycoremediation, cost-effective harvesting process of algal biomass through scrapping was achieved which can provide a sustainable and easy alternative to conventionally available high cost and energy-intensive harvesting methods with multiple solutions of environmental issues like plastic waste, choking of flowing water bodies, biomass growth based on nutrient's available in wastewater and harvesting of biomass for bioproducts formation. However, this research is in infant stage but this approach in near future can prove to be a novel sustainable, clean and green technology that can further reduce the excessive cost factor included in other types of treatment.

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