Immobilization of *Bacillus subtilis* on Polyurethane Foam for the Treatment of Pulp and Paper Mill Effluent

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Bacillus subtilis **was immobilized in polyurethane foam (PUF) and subsequently the biofilm was allowed to grow up to twelve days. The developments of microorganisms on the PUF surface were observed in different day's intervals. The optical microscopic observations clearly discriminated the formations of biofilm on the PUF. Afterwards, batch experiments were carried out to study the degradation of pulp and paper mill effluent by using immobilized polyurethane foam (IPUF) under three different circumstances; with and without nutrients to the IPUF and PUF alone *i.e.,* without microorganisms and nutrients. The experimental results revealed that the IPUF with nutrients had effectively reduced the effluent color, biochemical oxygen demand and chemical oxygen demand within the five days of treatment.**

Keywords: *Bacillus subtilis,* polyurethane foam, immobilization, paper mill effluent.

In India, pulp and paper industry ranks 3rd in terms of fresh water usage and it is brought under the list of 17 most polluting sectors as identified by the Central Pollution Control Board. The untreated effluents cause nuisance odor, thermal impacts, slime growth, scum formation and color problems in the surrounding environment and extensive damage to the receiving waters. It leads to increase the toxic substances in the water, causing death to the phytoplankton and fish, as well as extremely affecting the whole terrestrial ecosystem. Apart from the pollution load, there is increasing water scarcity and deterioration in the water quality. Recently, several methods have been made for the treatment of pulp and paper mill effluents. The physical and chemical treatments have been applied to remove the pollutants from these effluents but it requires high cost and removes only high molecular weight compounds, but, low molecular weight compounds are not removed effectively. In view of these problems, recent research has been focused on biological techniques for the effective management of pulp and paper mill effluent[1-6]. Several species of bacteria have been reported for the treatment of pulp and paper mill effluent, among only few of them is able to work effectively. Raj *et al.*[1] find out the highest reduction in color (61 %), lignin (53 %), BOD (82 %) and COD (78 %) by *Bacillus* sp. when compared with *Paenibacillus* sp., and *Aneurinibacillus aneurinilyticus.*

The biological treatment of pulp and paper mill effluent by free microorganisms has been extensively studied. However, the use of free microorganisms for wastewater treatment involves many serious problems such as substrate inhibition, difficult to remove high concentration

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of pollutant and finally separation of microorganism from the treatment systems[2]. Numerous schemes have been developed to avoid these problems, recently immobilization of microorganism on the suitable substrate is the most alternate and attractive strategy[3]. Generally, the immobilized microorganisms are more resistant to high concentrations of pollutants and hazardous toxics compounds[4]. In discrepancy with free microorganisms, immobilization process is able to maintain more microbial population in carriers even in continuous flow mode without losing populations and increase the inoculants storage for continuous treatment[5]. In addition the immobilized microorganisms can withstand high temperature and pH [6]. Different kinds of supporting materials have been reported for the immobilization of microorganisms such as alginate, polymer packing, silica gel, sand, macro-porous cellulose, PVC pipes and nylon mess. Besides, some locally available materials like agricultural wastes, polyurethane foam (PUF) and sawdust have also been reported[2, 7, 8].

In this work, the potential of *Bacillus subtilis* were immobilized in polyurethane foam for the treatment of pulp and paper mill effluent. Initially, optimization of the microbial growth and characterization of immobilized polyurethane foam (IPUF) were carried out using nutrient agar as growth substrates. Afterwards, the more appropriate processes were applied in treatment studies involving pulp and paper mill effluents.

MATERIALS AND METHODS

Collection and preservation of paper mill effluent

The pulp and paper mill effluent samples were obtained from SPB Pvt Ltd. located in Erode, TN, India. The industry uses *Eucalyptus* wood and bagasse pith as raw materials; ozone and hydrogen peroxide was used as bleaching agents. The characteristics of effluent used in this work are presented in Table 1.

Microorganisms and growth conditions

The microorganism used in this study was *B. subtilis* which was originally isolated by Ramachandran [9] from SPB Pvt Ltd., paper mill sludge in the Department of Environmental Sciences, TNAU, Coimbatore. The bacterium was cultivated in nutrient broth medium containing glucose (5g L^{-1}), beef extract (3g L^{-1}), NaCl (5g L^{-1}) and peptone (5g L^{-1}) with pH 7.0 under room temperature (28°C). The microorganism was also maintained on the slants of nutrient medium solidified with 2 % agar (w/v) for stock culture. The propagation of *B. subtilis* was carried out in the nutrient broth medium by using a rotating shaker at 150 rpm under 28°C for 48 h. The cells were harvested when the exponential growth stage by centrifugation at 15,000 rpm for 10 min under 4°C. The pellet was resuspended in 10 ml of fresh nutrient broth medium. A volume of 250 ml of nutrient medium was inoculated with 10 ml of the resuspended cells and used for immobilization study.

Preparation of carrier material

Commercially available PUF was obtained from local sources and it was cut into small pieces of 1cm x 1cm x 1cm size. The pieces were alternately eluted by HCl (5%) and NaOH (5%) solution to remove the impurity and then they were washed with distilled water and finally dried at 65°C for 3 hr under vacuum. The dried PUF were used as carrier materials for immobilization of microorganisms.

Immobilization of *B. subtilis* into PUF

The harvested *B. subtilis* cells were immobilized in pre prepared PUF by adopting the method of De Ory *et a.l*[10]. Exactly, 250 ml of freshly prepared bacterial cell suspension was added to 1g of pre prepared PUF in a 500 ml conical flask. The flask was kept on a rotary shaker at 160 rpm for two days under room temperature. After that, 125 ml of the medium was removed from the flask and it was refilled with the same volume of fresh growth medium as a substrate for refreshing the liquid medium. Similarly, nine alternating cycles were carried out. At the end of 10^{th} cycle, the carrier was removed and used for wastewater treatment. The developments of bacterial colonies on immobilized PUF were captured by using image analyzer under 40X in a compound microscopy.

Biological reactor setup and configuration

The batch experiment was carried out in a packed bed column reactor made up of a cylindrical glass (38 cm height and 5 cm diameter) with an inlet and outlet facility. The working volume of the reactor was 400 ml. Aeration was maintained throughout the system from the bottom of the reactor and it enabled the recirculation of the liquid into the reactor. The reactor was packed with 60 g of PUF to a height of 28 cm with the support on a stainless steel sieve plate which ensures homogeneous distribution of gas flow over the entire section of the reactor. The apex of the packed column was covered with two round PUF dice to circumvent the evaporation and retention of air into the reactor.

For the treatment, three conditions were employed; 1. Column was filled with immobilized PUF plus pulp and paper mill effluent, 2. Column was filled with immobilized PUF and supplement nutrients such as carbon and nitrogen sources (Glucose @ 5g L^{-1} + Ammonium sulphate @ 1g L^{-1}) plus pulp and paper mill effluent and 3. Column was filled just PUF plus pulp and paper mill effluent. The treatment process was carried out under room temperature for five days. Each day's samples were removed and centrifuged at 8000 rpm for 15 min. The samples were analysed for their physico-chemical properties such as pH, color, BOD and COD as per the standard procedure by APHA[11]. The above experiments were repeated in four times and the data in subsequent sections are based on arithmetic means of three measurements.

RESULTS AND DISCUSSION

Morphological characteristics of IPUF

The microscopic images of the PUF before and after immobilization were shown in Fig. 1. Before immobilization, the samples showed a smooth surface and hollow holes (Fig. 1a) but after immobilization, the surface of the specimens becomes irregular. After 48 hr of incubation, the IPUF showed many spores and lysed cells on the surface (Fig 1a) and some of the suspended cells are transported from liquid to a carrier by physical movement. Furthermore, the physical and chemical attractive force retains more cells and endorses stable multicellular interactions leading to establishment of colonies on the surface of IPUF. In Fig. 1c (after 96 hr of incubation) clearly discriminate the partially developed colonies (one third of the biofilm was covered) on the surface of IPUF. Further the cells were attached to each other and to form microgranules, probably immobilized by mechanically or originating from a complex colonization phase. The final biofilm was irregularly deposited in the aperture of the IPUF, besides a fine layer dispersed on the surface (Fig. 1d). From these different images confirmed that the fully developed colonies were formed on the IPUF and the biofilm so developed were subsequently used for wastewater treatment.

Changes of pH and color during biological treatment of paper mill effluent

The fluctuation of pH was observed during the treatment of effluent (Fig. 2). The initial pH value of 7.82 decreased to 6.1 on the third day of treatment and there after gradually increased up to 7.1 on the fifth day of incubation in the biofilm with nutrient treatment. The same trend was also observed in the remaining two treatments. The reason behind such fluctuations in pH can be directly correlated with bacterial enzyme activity during the alteration of complex organic compound into simple organic acids. Similar observations have been reported when the microbial degradation of alkali lignin and pulp and paper mill effluent by Dominguez *et al.*[12] and Diez *et al.*[13].

The reduction in color during the course of effluent treatment is shown in Fig. 3. The maximum color reduction was observed when biofilm with nutrient treatment. The percentage of decolorization (17 %) was suddenly arising at first

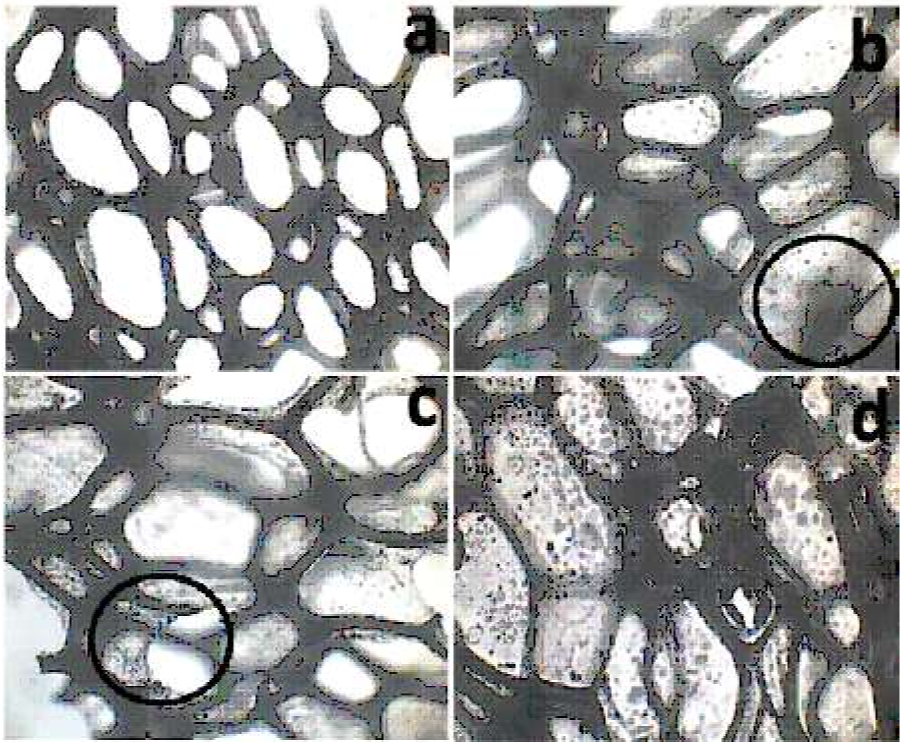
Table 1. Physico-chemical and biological characteristics of the pulp and paper mill effluent

S.No.	Parameters	Concentration
I	Physico-chemical characteristics	
1.	Color	720
2.	Odor	phenolic
3.	pH	7.82
4.	EC (dS m^{-1})	4.91
5.	TSS (mg L^{-1})	755
6.	TDS (mg L^{-1})	3320
7.	BOD (mg L^{-1})	352
8.	COD (mg L^{-1})	998
9.	Phenol (mg L^{-1})	28.4
10.	Lignin (mg L^{-1})	98.2
11.	Calcium (mg L^{-1})	338
12.	Sodium (mg L^{-1})	552
13.	Bicarbonate (mg L^{-1})	396
II	Biological characteristics	
14.	Bacteria (x 10^6 CFU ml^{-1})	19
15.	Fungi (x 10^4 CFU ml^{-1})	9
16.	Actinomycetes (x 10^3 CFU ml^{-1})	2

day of the initial treatment; thereafter steadily color diminished up to 65 per cent at the end of the treatment. The unexpected color reduction was (25 %) observed when PUF alone treatment. The reduction of color at the time of PUF treatment might be attributed to physical absorption by the PUF and chemical oxidation of pollutant due to aeration.

Changes in BOD and COD during biological treatment of paper mill effluent

The concentration of lignin and total phenol are more responsible for BOD and COD of



a) Before immobilization b) 48hr after immobilization
c) 96 hr after immobilization d) At the time of termination

Fig. 1. Morphological characteristics of immobilized polyurethane foam

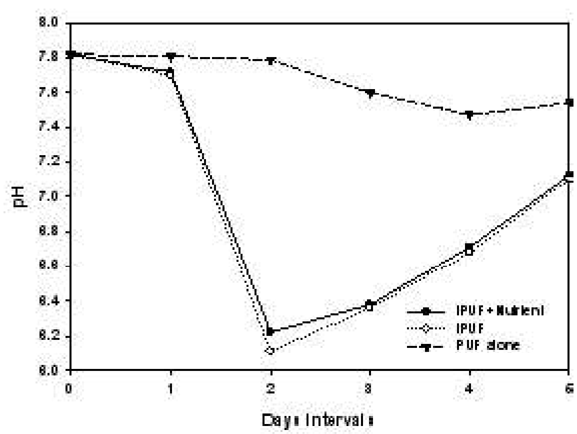


Fig. 2. Changes in pH during biological treatment of pulp and paper mill effluent

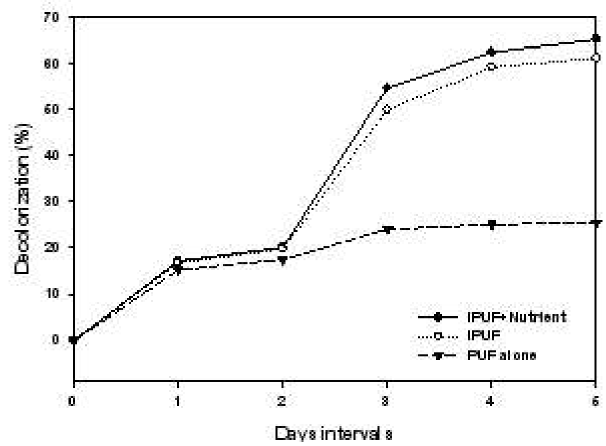


Fig. 3. Decolorization of pulp and paper mill effluent by biological treatment

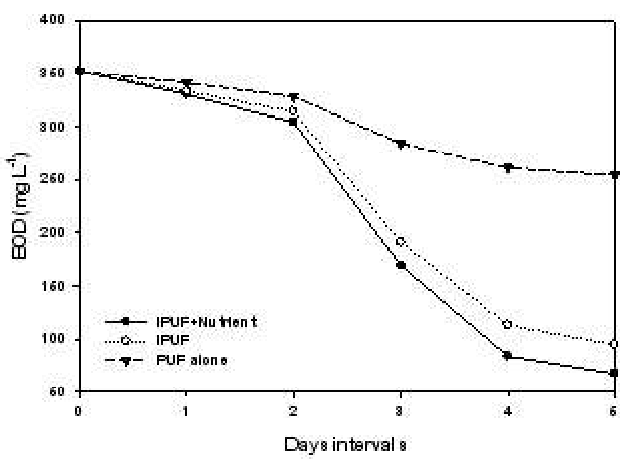


Fig. 4. Reduction of BOD during biological treatment of pulp and paper mill effluent

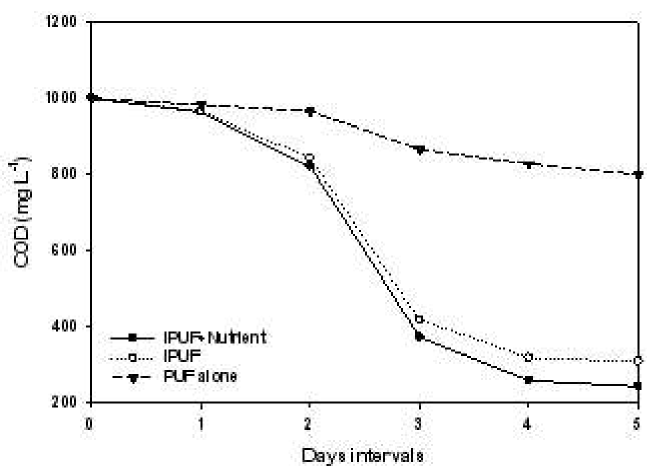


Fig. 5. Reduction of COD during biological treatment of pulp and paper mill effluent

the effluent. The temporal decrease of BOD by different treatment processes is illustrated in Fig. 4. The treatment in the absence of microorganisms showed very little destruction of BOD containing solution from 352 mg L^{-1} to 254 mg L^{-1} corresponding to reduction of 27 per cent, whereas in the presence of microorganisms showed superiorly reduced BOD content. The IPUF with nutrient reduced up to 67 mg L^{-1} followed by IPUF reduced up to 94 mg L^{-1} corresponding to the reduction of 80 and 73 per cent, respectively. The changes in COD during the course of treatment were recorded and the results are given in Fig. 5. The IPUF with nutrient reduced the COD from 998 mg L^{-1} to 243 mg L^{-1} corresponding to reduction of 75 per cent. The IPUF and PUF alone reduced the COD up to 309 mg L^{-1} and 798 mg L^{-1}, respectively. The highest reduction of BOD and COD by the IPUF with nutrient might be due to increased activity of microorganism resulting from increased dissolved oxygen content. The higher microbial activity in the immobilized PUF is more responsible for the degradation of organic matter through enzymes production, which catalyzes the biodegradation reaction. These observations are in close agreement with the findings of Nikolopoulou *et al.*[14] who reported enhanced biodegradation of organic pollutant by the addition of nutrient along with the bacteria.

The removal of pollutant load during IPUF without nutrient also followed the same trend of IPUF with nutrients treatment, but all the pollutant removal efficiency was moderately low. The batch treatment without microorganisms and nutrient also reduced the pollutant load, but when compared to biological treatment the least reduction of the pollutant was recorded and its performance is deprived when compared to the biological treatments.

CONCLUSIONS

Polyurethane foam has been proved as a good carrier material with high capacity for the immobilization of *B. subtilis* under optimum conditions. The microscopic images clearly discriminated the formation of biofilm on the immobilized PUF. The enhanced and potential degradation of pollutant from the paper mill effluent was achieved by addition of Glucose @ 5 g L^{-1} and Ammonium sulphate @ 1 g L^{-1}. Results on the treatment of pulp and paper mill effluent revealed that the biofilm with nutrients was able to remove 65.2, 80.8 and 75.6 per cent of color, BOD and COD, respectively within five days of treatment. The operational stability and longevity of the immobilized PUF was significantly higher due to addition of carbon and nitrogen sources.

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REFERENCES

1. Raj, A., Reddy, M.K., Chandra, R. Decolourisation and treatment of pulp and paper mill effluent by lignin-degrading *Bacillus* sp. *J. Chem. Technol. Biotechnol.,* 2007; **82**: 399-406.
2. Manohar, S., Kim, C.K., Karegoudar, T.B. Enhanced degradation of naphthalene by immobilization of *Pseudomonas sp.* strain NGK1 in polyurethane foam. *Appl. Microbiol. Biotechnol.,* 2001; **55**: 311–316.
3. Zhou, L.C., Li, Y.F., Hou, Y.F., Ma, P.C. Study on immobilized microorganism based on macroporous carrier and application for wastewater treatment. *Ion Exch. Adsorpt.,* 2007; **23**: 531–539.
4. Wang, J.L., Quan, X.C., Han, L.P., Qian, Y., Werner, H. Microbial degradation of quinoline by immobilized cells of *Burkholderia pickettii*. *Water Res.,* 2002; **36**: 2288–2296.
5. Hsu, C.L., Ouyang, C.F., Weng, H. T. Purification of rotating biological contactor (RBC) treated domestic wastewater for reuse in irrigation by biofilm channel. *Resour. Conser. Recyc.,* 2000; **30**: 165-175.
6. Wang, L., Luo, Q.F., Zhao, J.H., Zhang, X.H., Huang, L.J. Preparation of seeding type immobilized microorganisms and their degradation characteristics on Di-n-butyl phthalate. *Biomed Environ Sci.* 2006; **19**(2): 147-52.
7. Kahraman, S., Yesilada, O. Industrial and agricultural wastes as substrates for laccase production by white-rot fungi. *Folia Microbiol.* 2001; **46**(2), 133-136.
8. Huang, F.M., Xia, S.C., Xiao, X.T., Luo, B.H., Xia, S.B., Gong, W.Q. Immobilization of phosphorus-dissolving bacteria on sawdusts for removing phosphorus in iron ores. *Adv. Mater.*

Res. 2012; **343**-344: 7-10.

9. Ramachandran, N. Biocomposting of paper mill solid wastes, their ecofriendly agrocycling and biodecolourisation and biomethanation options for paper mill effluent. *Ph.D. Thesis,* Tamil Nadu Agric. Univ., Coimbatore, 2007.
10. De Ory, I., Cabrera, G., Ramirez, M., Blandino, A (ed): Methods in Biotechnology: Immobilization of Enzymes and Cells, 2nd edn. Humana Press, 2006; pp. 357-365.
11. APHA: American Water Works Association and water Environment Federation, standard Methods for the Examination of water and Wastewater, 20th edn. American public health association, Washington, DC, p. 83.
12. Dominguez, A., Rivela, I., Couto, S.R. Sanroman, M.A. Design of a new rotating drum bioreactor for ligninolytic enzyme production by *Phanerochaete chrysosporium* grown on inert support. *Process Biochem.,* 2001; **37**: 549–554.
13. Diez, M.C., Castillo, G., Aguilar, L., Vidal, G., Mora, M.L. Operational factor and nutrient effect on activated sludge treatment of *Pinus radiata* kraft mill wastewater. *Bioresour. Technol.,* 2002; **83**: 131–138.
14. Nikolopoulou, M., Pasadakis, N., Kalogerakis, N. Enhanced bioremediation of crude oil utilizing lipophilic fertilizers. *Desalination,* 2007; **211**: 286–295.