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ORIGINAL PAPER

Evaluation of various waste substrates for biofilm formation and subsequent use in aerobic packed-bed reactor for secondary treatment of domestic wastewater

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Abstract
Immobilization of bacterial cells on suitable substrates is of utmost importance in the secondary treatment of wastewater using fixed-film reactors. Therefore, screening of efficient and cheaper materials for bacterial surface immobilization was carried out. Eleven waste materials were used as substrates, packed in a column, and bacterial surface immobilization was carried out using cow dung slurry/MLSS mixture. All the chosen substrates were screened for bacterial immobilization/biofilm formation by standard bacterial enumeration technique. The substrate with the highest biofilm-forming ability was used for secondary treatment of raw domestic wastewater. The results showed that high-density polyethylene and aluminium foil sheets have poor immobilizing characteristics with 2.2×10^{8} and 2.4×10^{8} CFU/cm^2 respectively, whereas jute fibres were observed to be the most efficient among the substrates with 5.1×10^{23} CFU/cm^2. The column packed with jute fibres was used for wastewater treatment. Various physico-chemical parameters were analyzed before and after treatment and there was a significant reduction in major parameters after treatment. The bacteria-immobilized jute fibres showed maximum immobilization potential and were highly efficient in wastewater treatment, and therefore these findings offer immense promise in the synthesis of composite polymers for bacterial immobilization and subsequent secondary treatment.

Keywords Bacterial surface immobilization · Biofilms · Bioremediation · Fixed-film reactors · Secondary treatment of domestic wastewater · Sewage

Introduction

Of the major environmental challenges faced by the urban societies across the globe, large-volume generation of domestic wastewater is a serious issue which can prove to be risky if not treated prior to disposal (De-Bashan and Bashan 2010; Rudenko et al. 2018). In the conventional process train of domestic wastewater treatment, secondary treatment is the sequential treatment of the effluent from the primary treatment, primarily for the removal of residual organics and suspended solids (Sonune and Ghate 2004). Most of such methods for secondary treatment of wastewater use biological systems, which can be categorized into suspended systems and immobilized microbial systems (Nicolella et al. 2000). Numerous advantages have been reported in the literature for the immobilized systems such as increased biomass generation, higher metabolic activity and stronger resistance to process and environmental constraints (Wang et al. 2005; Zhou et al. 2008; Cai et al. 2011; Liu et al. 2012; Malovanyy et al. 2015). From the bioremediation perspective, immobilization can be defined as the entrapment or surface attachment of a wide variety of organisms or enzymes (Lopez et al. 1997). Thus, cell immobilization involves transport of cells from the bulk liquid phase to the surface of support, followed by cell adhesion, and colonization on the support surface (Kilonzo and Bergougnou 2012). Immobilization has been reported to occur by any of the following processes: covalent coupling/cross linking, encapsulation, entrapment and adsorption (Mallick 2002; Halecký and Kozliak 2020). Among the above, adsorption is the simplest method, which is also known as reversible immobilization (Martins et al. 2013; Siddiqui et al. 2020).

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The basic principle of adsorption is the physical attraction of microorganisms with the carrier surfaces, which is based on weak forces (Bouabidi et al. 2019). Even though the forces are weak, they are still capable of binding because several of them are involved, including van der Waals forces, ionic and hydrophobic interactions, and hydrogen bonds. A key step in controlling cell immobilization on the support is cell support adhesion, which is governed by both electrostatic and hydrophobic interactions (Górecka and Jastrzębska 2011; Dufrêne 2015). Aggregation of microorganisms on the surface of the solid substrates results in the formation of biofilms. Use of biofilm reactors is very common in wastewater treatment systems, which include trickling filters, high-rate plastic media filters, rotating biological contactors, fluidized bed reactors, airlift reactors, granular filters and membrane-immobilized cell reactors. However, fixed-bed bioreactors include all the treatment systems, which use static media such as plastic profiles, rocks, sponges, granular carriers or membranes for biofilm formation (Lazarova and Manem 2000). In the last few decades, there has been growing interest in the use of immobilized cells in packed bed bioreactors for the treatment of wastewater (Feng et al. 1997; Lupton 2009; Banerjee and Ghoshal 2017).

Therefore, in this study, evaluation of commonly available waste substrates was carried out for efficient biofilm formation with increased cell biomass. All the substrates used for immobilization were already-utilized scrap materials, cheap, robust enough to withstand the flow of wastewater with coarse surfaces, and easily washable and reusable. The substrate with the highest immobilizing ability was used in the secondary treatment of domestic wastewater using the principles of microbial cell immobilization on solid substrates.

Materials and methods

Substrates for immobilization

Not easily-biodegradable and cheaper solid waste materials were used as substrates for biofilm production. Eleven substrates including high-density polyethylene (HDPE), polyethylene terephthalate (PET), coconut shell (CS), waste tyre rubber (WTR), jute fibre mat (JFM), polyvinyl chloride (PVC), broken clay pots (BCP), ceramic tile chips (CTC), wooden chips (WC), aluminium foil sheets (AFS) and polystyrene foam (PF) were collected from a local scrap shop. All the eleven materials were selected because of its easier availability as scrap. Moreover, coarse surface nature and the presence of inert attachment surfaces of the above materials are highly suitable for bacterial immobilization and subsequent biofilm formation (Nerenberg 2016). All the substrates were cut into appropriate sizes (4 × 4 cm^2 approximately) so as to fit the column for packing, and used.

Setup of a packed bed reactor (PBR)

A miniature PBR (Fig. 1) was constructed using acrylic tubes, PVC pipes and a PVC dummy. The column had an outlet at the bottom and a sprayer was fixed on top. The dimensions of the column were measured (height = 0.67 m, diameter = 0.09 m, volume = 0.0043 m^3) and the volume of the reservoir/feeder tank used was 0.0165 m^3. The eleven materials mentioned above (attachment substrates for biofilm) were cut into appropriate sizes (as mentioned earlier) and packed randomly into the column in such a way that all the materials were distributed throughout the column. The outlet of the column was connected to a reservoir/feeder tank containing the effluent/medium/inoculum and a miniature submersible pump with a flow rate of 120 L/h (LPH) was used for recirculation. During recirculation, the medium/effluent was trickled back on to the top of the column, allowing continuous flow and treatment of the sample.

Surface immobilization/biofilm formation

Immobilization was carried out based on the principles of physical adsorption. The PBR column was packed with all the substrate materials, which were placed throughout the

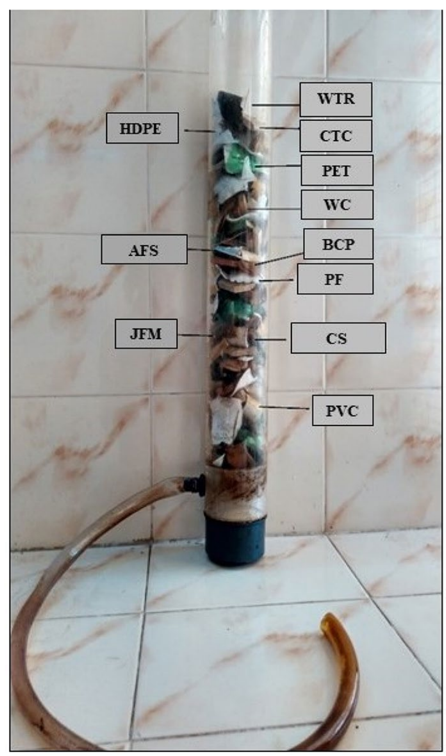

Fig. 1 Packed-bed reactor column with various substrates

entire length of the column. The reservoir/feeder tank was filled with a mixture containing equal volume of 5% fresh cow dung slurry and mixed liquor suspended solids (MLSS). The column was allowed to run with the mix for a week and on the eighth day, 500 mL of rice starch water was added to the inoculum mix as the sole carbon source. For nitrogen and phosphorus sources, urea (250 ppm of N) and diammonium phosphate (DAP) (5 ppm of P), respectively, were added to the mix to enhance the growth and formation of biofilm on the surface of the substrates, following which, the reactor column was allowed to run for 2 days. The carbon, nitrogen and phosphorus concentrations were standardized, the data of which are not shown in this study.

Evaluation of biofilm formation

On day 10, the column was stopped, drained off and total heterotrophic bacterial load on the surface of each of the substrate was determined by plating technique. The substrate materials were taken out from the column with care and lined up on a tray. For sampling, the conventional method of swabbing was used, which involved the use of a sterile cotton swab with an applicator stick for releasing microorganisms from surfaces (Pérez-Rodríguez et al. 2008). The swabs were then immersed into the extracting solution (0.1% sterile peptone water), vortexed, serially diluted in sterile saline and spread plated on to the plates containing standard medium (nutrient agar) for estimating total heterotrophic bacteria. The plates were incubated overnight at 37 °C and the CFU/cm^2 of each substrate was calculated.

Secondary treatment of domestic wastewater

Use of PBR column with single substrate

The substrate which showed the maximum immobilizing property in terms of heterotrophic bacterial biomass was packed inside the entire column (Fig. 2). As mentioned earlier, the single-substrate column was also allowed to run with MLSS/cow dung mix for biofilm formation; once the biofilms are established on the substrate by surface immobilization after running for 10 days (standardized based on multi-substrate column study), the column containing active substrate was used for treatment of raw sewage.

Collection of samples

MLSS (used for biofilm formation) sample was procured from the Chennai Metro Water Supply and Sewerage Board (CMWSSB), Sewage Treatment Plant (STP), Koyambedu, Chennai. Raw untreated sewage sample was collected from the Madras Christian College (MCC) farm STP; the source of the effluent was from the entire campus of MCC, including academic and residential blocks.

Fig. 2 Packed-bed reactor column with single substrate (JFM)

Treatment

The effluent was allowed to stand for 1 h to allow the sediments/larger flocs to settle down and was run through the column packed with immobilized single substrate for 4 h for secondary treatment. The treatment process was aerobic; sparged column was not used in this study as the column used was miniature in size, the substrates were less densely packed so as to allow natural convection of air, and lastly the wastewater was sprayed to aid the aeration. The treatment time was based on standard retention times employed for most of the biological treatment processes (Pepper et al. 2011). The pre-treated and post-treated effluent samples were analyzed for various physicochemical parameters such as pH, total dissolved solids (TDS), total suspended solids (TSS), alkalinity, hardness, chloride, ammonia, nitrite, nitrate, total kjeldahl nitrogen (TKN), total phosphorus and biochemical oxygen demand (BOD). The analyses protocols followed were according to the Standard Methods for Water and Wastewater Examination

(American Public Health Association (APHA) 2005). The results were tested for significant reduction compared to those of untreated effluent.

Statistical analysis

All analyses were performed as experimental triplicates. Mean values of all replicate sets of data are presented with standard deviation values. The one-tailed paired Student's *t* test was used to determine statistical significance between the parameters of untreated and treated effluent samples at $P<0.05$.

Results

Surface immobilization/biofilm formation

Biofilm formation on all the substrates was observed after running through the mixture of MLSS and cow dung. The immobilized biofilms onto the solid surfaces are shown in Fig. 3. The total number of heterotrophic bacterial CFU per unit area was calculated for each substrate with countable colonies in different dilutions, the values of which are as follows: HDPE: 2.2×10^{8} CFU/cm^2; PET: 3.8×10^{17} CFU/cm^2; CS: 4.6×10^{23} CFU/cm^2; WTR: 4.6×10^{17} CFU/cm$^{2.}$; JFM: 5.1×10^{23} CFU/cm^2; PVC: 3.7×10^{23} CFU/cm^2; BCP: 3.7×10^{23} CFU/cm^2; CTC: 3.2×10^{23} CFU/cm^2; WC: 4.7×10^{23} CFU/cm^2; AFS: 2.4×10^{8} CFU/cm^2; PF: 3.6×10^{23} CFU/cm^2. The above results were obtained from the multi-substrate column, and the values along with standard deviation are given in Table 1. The substrates such as jute fibre mat, coconut shell, polyvinyl chloride, broken clay pots, ceramic tile chips, wooden chips and polystyrene foam have very good surface immobilization potentials and thus showing high heterotrophic bacterial biomass. High-density polyethylene and aluminium foil sheets showed poor immobilizing characteristics with low heterotrophic bacterial biomass densities. Polyethylene terephthalate and waste tyre rubber showed moderate biofilm forming capabilities. However, among all the substrate materials, jute fibre mat was observed to be the best for immobilization of biofilms. In terms of difference compared to the second best substrate—wooden chips—, jute fibre mat showed 8.5% increase in immobilized heterotrophic bacterial biomass. The percent increase in biomass levels on substrates with high-immobilizing potentials compared to CTC (the substrate showing the lowest CFU/cm^2 among the high-immobilizing substrates) is shown in Fig. 4. Likewise, JFM showed 2.3×10^{15} times increase compared to high-density polyethylene, which showed the lowest immobilization potential.

Fig. 3 Biofilm formation on various substrates

Treatment of domestic wastewater

Biofilm-immobilized jute fibre mat was used for the treatment of raw sewage sample as the substrate showed the highest immobilization characteristics. Table 2 shows the mean values of all the pre- and post-treated physicochemical parameters of the effluent, which was treated in a single-substrate column. All the major physicochemical parameters showed reduction following treatment. The results from the student's *t* test show a *P* value of <0.05 for all the significant parameters. The significant difference indicates that results are statistically significant and shows only a 2% chance of error in the sample being tested if the null hypothesis was actually true. The percentage

Table 1 Total heterotrophic bacterial count in biofilms on various substrates

S. no.	Substrate	Heterotrophic bacterial count (CFU × dilution factor/cm^2)		
		×10^8	×10^{17}	×10^{23}
1	High-density polyethylene (HDPE)	2.2 ± 0.16[a]	TFTC	TFTC
2	Polyethylene terephthalate (PET)	TNTC	3.8 ± 0.32[a]	TFTC
3	Coconut shell (CS)	TNTC	TNTC	4.6 ± 0.26[a]
4	Waste tyre rubber (WTR)	TNTC	4.6 ± 0.14[a]	TFTC
5	Jute fibre mat (JFM)	TNTC	TNTC	5.1 ± 0.5[a]
6	Polyvinyl chloride (PVC)	TNTC	TNTC	3.7 ± 0.13[a]
7	Broken clay pots (BCP)	TNTC	TNTC	3.7 ± 0.35[a]
8	Ceramic tile chips (CTC)	TNTC	TNTC	3.2 ± 0.28[a]
9	Wooden chips (WC)	TNTC	TNTC	4.7 ± 0.15[a]
10	Aluminium foil sheets (AFS)	2.4 ± 0.09[a]	TFTC	TFTC
11	Polystyrene foam (PF)	TNTC	TNTC	3.6 ± 0.08[a]

TNTC too numerous to count, *TFTC* too few to count

[a]Values are mean of n samples (n = 3 ± SD)

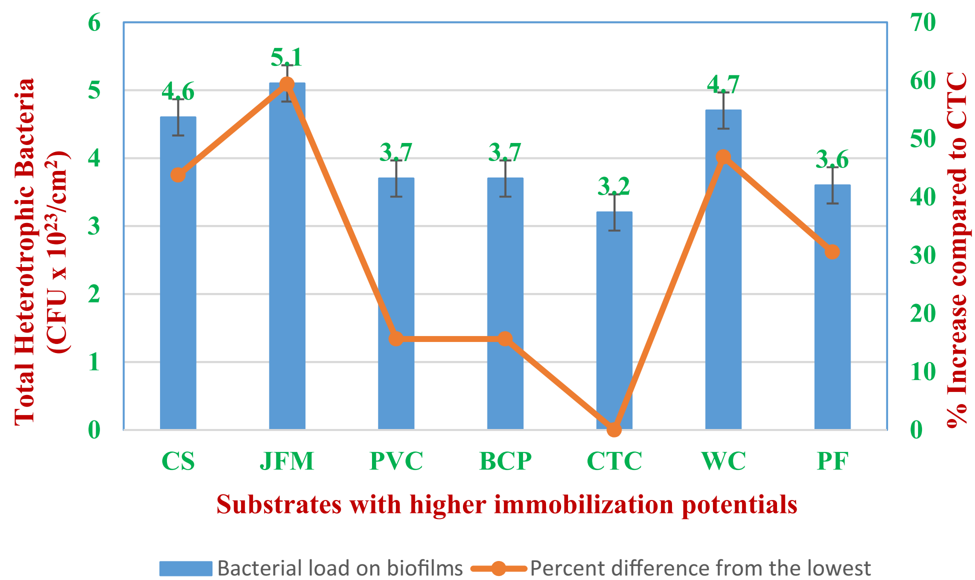

Fig. 4 Total heterotrophic bacterial count on substrates with higher immobilization potentials. *CS* coconut shell, *JFM* jute fibre mat, *PVC* polyvinyl chloride, *BCP* broken clay pots, *CTC* ceramic tile chips, *WC* wooden chips, *PF* polystyrene foam

reduction between the parameters of the treated and the raw untreated sample is shown in Table 3.

Discussion

In wastewater treatment, technologies that depend on immobilized system have several advantages over those with the suspended system. They are highly cost effective as the immobilized microbial systems can be used several times without any significant loss of activity (Devi and Sridhar 2000; Nzila et al. 2016). Moreover, there is no need to replenish biocatalysts as immobilized biofilms can be used in continuous and semi-continuous production processes (Mrudula and Shyam 2012; Tikhomirova et al. 2018). Apart from the above, the other benefits are operational stability and flexibility, ease of handling, smaller space needs, minimal retention times, resistance to environmental changes, higher biomass concentration, enhanced uptake rate, increased ability to degrade recalcitrant compounds and lesser sludge production (Kourkoutas et al. 2004).

In the present study, waste substrates were used that are cheap and readily available in the urban society. However, the substrates were chosen from different groups of materials including the synthetic and natural ones. Moreover, the materials used in the study had coarse surface nature with inert attachment surfaces, which are highly suitable for biofilm formation and subsequent wastewater treatment (Nerenberg 2016). Jute fibre mat was found to be the most efficient substrate for immobilization of bacteria and

Table 2 Physicochemical analysis of pre- and post-treated sewage using a single-substrate JFE column

S. no.	Parameters	Untreated sewage	Treated sewage
1	pH*	7.1 ± 0.17	7.83 ± 0.28
2	TDS (mg/L)*	3380 ± 26.92	1316 ± 20.32
3	TSS (mg/L)*	280 ± 12.13	52 ± 3.86
4	Alkalinity (mg/L)*	356 ± 7.07	380 ± 10.2
5	Hardness (mg/L)*	290 ± 14.14	176.66 ± 11.54
6	Chloride (mg/L)*	315 ± 7.77	273.33 ± 5.77
7	Ammonia (mg/L)*	5.3 ± 0.31	2.3 ± 0.17
8	Nitrite (mg/L)	2 ± 0.06	1.66 ± 0.12
9	Nitrate (mg/L)*	104 ± 8.65	5.5 ± 0.46
10	TKN (mg/L)*	72 ± 5.23	11 ± 0.82
11	Total phosphorus (mg/L)*	5.8 ± 0.49	1.2 ± 0.09
12	BOD_5 (mg/L)*	280 ± 16.56	46.38 ± 2.35

All values are presented as mean ± SD of triplicate analyses. Treated and untreated values are statistically significant except nitrite. The non-significance is due to the low initial concentration of nitrite

TDS total dissolved solids, *TSS* total suspended solids, *TKN* total kjeldahl nitrogen, *BOD* biochemical oxygen demand

*Significant at $P < 0.05$ according to one-tailed paired Student's *t* test

Table 3 Percentage reduction/increase in the physico-chemical parameters in treated sewage

S. no.	Parameters	% reduction/increase in treated sewage
1	pH	↑ 10.28
2	Total dissolved solids	↓ 61.07
3	Total suspended solids	↓ 81.43
4	Alkalinity	↑ 6.74
5	Hardness	↓ 39.08
6	Chloride	↓ 13.23
7	Ammonia	↓ 56.6
8	Nitrite	↓ 17
9	Nitrate	↓ 94.71
10	Total kjeldahl nitrogen	↓ 84.72
11	Total phosphorus	↓ 79.31
12	Biochemical oxygen demand	↓ 83.44

formation of biofilms. It is due to the reason that fibrous matrices provide adequate supporting surfaces for cell adsorption (Talabardon et al. 2000; Chu et al. 2009). The natural fibres such as jute possess high specific surface area, void volume, mechanical permeability, less toxicity, low cost and high availability (Huang and Yang 1998; Saleem et al. 2020). Therefore trapping of cells occurs more naturally than other materials (Yang and Lo 1998). In addition, the JFM was also rigid with its integrity intact even after several weeks of running the reactor. Among fixed-film reactors, very commonly used technologies like rotating biological contactor (RBC) are very effective for moderate-scale treatment plants. These reactors use materials such as polyethylene and expanded polystyrene for constructing the immobilized surfaces (Antonie 2018). Based on the results obtained in this study, it can be suggested that the sturdiness of the plastics and the efficiency of natural fibrous materials can be combined to synthesize fibre-reinforced polymers. Although many fibre-reinforced polymers including jute fibre-reinforced polymers are in use, not many studies have been conducted in waste treatment systems. Further studies are warranted on pilot-scale studies involving novel composite polymers.

Mixed liquor suspended solids (MLSS) was used for bacterial immobilization as it consists of mostly of microorganisms in the active state, which thus ensures that there is sufficient quantity of active biomass available for biofilm formation (Pepper et al. 2011). Cow dung was mixed with MLSS as it contains partially digested high fibre thus making the MLSS thick and increases the efficiency of immobilization on the surface of the substrates. Moreover, biofilm formation happened at an increased rate due to supplementation of carbon, nitrogen and phosphorus sources along with cow dung, which also lots of microbial populations and certain nutritive value (Akpomie Olubunmi and Ejechi Bernard 2016). For enumeration of total bacteria on the immobilized surfaces, many methods have been recommended, but in this study, swab method was used. It is an easy to perform, inexpensive and highly reliable technique. Enhanced microbial recovery is achieved by dipping the swab in sterile diluent prior to swabbing under sterile conditions (Pérez-Rodríguez et al. 2008).

With regard to sewage treatment, aerobic processes always have an advantage of rapid treatment rates and higher treatment efficiency (Pronk et al. 2015). Thus the flow rate set was adequate enough to have sufficient sloughing of the biomass to ensure efficient aerobic treatment. There was a significant reduction in most of the parameters and the reduction pattern, particularly for BOD, nitrate, phosphorus and TKN, is in line with treatment efficiency of domestic wastewater using immobilized systems (Pepper et al. 2011). Increase in pH might be due to the changes in carbon dioxide, carbonate–bicarbonate equilibrium caused by closed and compact physicochemical conditions (Dickson 2010). Enormous reduction in nitrate levels was observed in the treated effluent which may be due to nitrate assimilation, nitrate respiration, and nitrate dissimilation (Pepper et al. 2011). Total dissolved solids (TDS) is a measure of the combined content of all the contaminants and in this study, TDS seems to have reduced because of the reduction of other parameters. Results also show a significant increase in alkalinity, which may be attributed to the presence of sulfate-reducing organisms, which in the presence of organic matter reduce sulfate thereby increasing alkalinity of wastewater (Ayangbenro et al. 2018).

Conclusion

From this study, it can be concluded that search for a better attachment substrate for surface immobilization of microorganisms is inevitable. Development of novel synthetic and composite substrates is essential to improve the bioremediation rate, both in the construction of fixed-film reactors and also in the development of integrated reactors, which use both suspended and immobilized systems. Further research is warranted to use natural fibrous substances in the making of composite carrier-immobilized systems.

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Author contributions SZ performed research, analyzed data, and wrote, reviewed and edited the paper. TSK performed research, and reviewed and edited the paper; NM performed research, and reviewed and edited the paper; and PHR designed the study, performed research, analyzed data and validated the findings, supervised research, and wrote, reviewed and edited the paper.

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Compliance with ethical standards

Conflict of interest There are no conflict of interest among the authors.

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