Studies on the Influence of Various Metabolic Uncouplers on the Biodegradation Rate of Toluene in a Biofilm Bio-Filter Reactor

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ABSTRACT: *Biological inhibition of air pollution has vast advantages over physicochemical methods. One of the biggest challenges faced by researchers with traditional bio-filter in controlling Volatile Organic Compounds (VOCs) such as Benzene, Toluene, Ethylbenzene and Xylene (BTEX) is, low degradation rate (elimination capacity) and accumulation of very high biomass. The use of metabolic uncouplers involves uncoupling electron transport from oxidative phosphorylation reactions and thereby ATP production is less efficient, leads to more substrate utilization. So, this research is aimed to study the influence of different metabolic uncouplers on the biodegradation rate of toluene in a biofilm bio-filter reactor. The bio-filter reactor with Pseudomonas putida MTCC 10617 as biofilm in the presence of five different metabolic uncouplers such as Pentachlorophenol (PCP), 2, 4-Dinitrophenol (DNP), 2, 4, 6-Trichlorophenol (TCP), Benzoic Acid (BA) and Malonic Acid (MA) were studied. Results showed that only PCP and TCP increased the Surface Elimination Capacity (SEC) by 87% and 38% respectively. From the SEM analysis, larger and wider air interface cavities were observed in the biofilm subjected to PCP than TCP exposed biofilm. This infers the higher mass transfer in biofilm exposed to PCP.*

KEYWORDS: *Biofilm; Bioreactor; Metabolic uncoupler; Surface elimination capacity.*

INTRODUCTION

Biofiltration is one of the predominant biological Air Pollution Control Technologies (APCTs), which effectively treats the Volatile Organic Compounds (VOCs), mainly high toxic air pollutants such as Benzene, Toluene, Ethylbenzene and Xylene (BTEX) that gets emitted from the industries like petroleum industries, paint industries, automobile industries, etc., In general, Micro-organism present in the bio-filter medium absorbs the biodegradable pollutants and convert it into carbon dioxide, water and salts [1, 2]. The reliability of biofiltration for the treatment of VOCs has been proven as it is more suitable to treat low concentration (even < 1000ppm) and the high volume of VOCs. It is also a cost-effective technology to remove toxic air pollutants because of the low temperature oxidation of the system that eliminates the high costs associated with the combustion and of very less moving parts cut-off its maintenance cost. Although bio-filters, are good at handling pollutants and it also has disadvantages

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that includes poor solubility with water due to higher superficial area available for mass transfer, large size, huge biomass production, low volumetric degradation rate (Elimination capacity) and less efficiency with the traditional bio-filter where soil or compost are used as a bed medium. To overcome such drawbacks, use of biofilm based bio-filter reactor system is preferred, that will greatly benefit in industries in cutting the size of traditional bio-filters and effectively treats the contaminants under controlled environmental conditions. Application of metabolic uncouplers [3] (have proven to treat activated sludge effectively [4, 5]) which indirectly influences the bio-degradation rate of micro-organism and thereby increases the elimination capacity. Metabolic uncouplers are usually weak acids (organic compound) involves in proton transport across cell membrane. It interferes with the Adenosine Tri Phosphate (ATP) production inside microbial cells and thereby increases the metabolic energy of microbes. The mechanism behind metabolic uncouplers is the organism present in the system failed to synthesis ATP by influencing energy production in cells by uncoupling electron transport from oxidative phosphorylation. Hence it is expected to uptake more substrate (biodegradable contaminants) to survive because of its maintenance energy demand. Under steady state condition, it was reported that traditional bio-filter showed 91%, 90%, 84%, and 34% of removal efficiency for xylene, benzene, ethyl benzene and toluene respectively. So, in accordance with all other pollutants of BTEX, concentration of toluene in the polluted region is immense and the same was not potentially treated by traditional bio-filtration method**.** Hence toluene is chosen as a model pollutant for the current study.

Certain bacteria can adapt to diverse substrate and possess some metabolic pathways. Such bacteria being there in the system has the gene that produces subsequent enzymes required for the TOD (Toluene Degradation) catabolic pathway. Few studies with *Pseudomonos putida* reported metabolic pathway of VOCs especially toluene [6].

So this work is mainly focused on to study the effect of five different metabolic uncouplers such as Benzoic Acid [7], Pentachlorophenol [8], Trichlorophenol [9], Malonic Acid [9] and Dinitrophenol [10] in enhancing the biodegradation rate of toluene using *Pseudomonos putida MTCC10617* as biofilm in biofilm bio-filter reactor. A similar work was carried out earlier in the same research group using a differential biofiltration reactor and soil as a biofilter medium [15]. However, the current work totally replaces the soil with pure cultures of toluene degrading bacteria, *Pseudomonas putida*.

EXPERIMENTAL SECTION

Biofilm bio-filter reactor with water control system (Fig. 1), offline gas chromatography (Mayura analytical model 1100) and carbon dioxide analyzer (Mayura analytical model 7722) for studying the effect of the metabolic uncoupler was designed with reference to the earlier work done [15]. Reactor was fabricated manually and the environmental parameters such as flow rate, inlet concentration of toluene, system temperature, and surface tension [11] were optimized and represented in the Table 1.

Developed reactor was autoclaved at 121^{o}C with pressure 15 psi for 15 min. Then reactor was loaded with the biofilm under sterile conditions.

Screening of Micro-organism

From the previous work [12], only *Pseudomonos putida* was found to be extensively studied and reported to follow Catabolic pathway of degrading toluene [6]. So that *Pseudomonos putida MTCC 10617, Pseudomonos putida MTCC 1194* and *Pseudomonos putida MTCC 7426* were procured from Microbial Type Culture Collection (MTCC), Chandigarh, India. Minimal salt medium (MS medium) [13] with the composition in Table 2 was used to prepare culture plates. Sterility was maintained. All the mentioned organisms were individually inoculated in sterile culture plate and incubated at 37^{o}C in the closed system (3 litres desiccators was used) containing petri-plate with 20 mL vacuum pump oil and 2 µL of toluene (produces 650 ppm of toluene) as carbon source. The plates were monitored every day for its growth and also fresh toluene was added to the vacuum pump oil. *Pseudomonos putida MTCC 10617* was found to have potential to grow in such concentration of toluene. Hence *Pseudomonos putida MTCC 10617* was selected as best toluene degrader for making biofilm.

Loading biofilm in reactor

Pseudomonas putida MTCC 10617 was inoculated in liquid medium and incubated at 37 ^{o}C. The initial concentration of *Pseudomonas putida MTCC 10617* was 1x10^{3} Cells/L. Growth rate was monitored every day and

Table 1: Optimized conditions of environmental parameters.

Parameters	Optimized conditions	Units
Flow rate	20	ml/min
Inlet concentration of toluene	250±5	ppm
Temperature	40	^oC
Surface tension	20	cm_{H2O}
Surface elimination capacity	0.20	g/m^2h
Operational period	28	days

Table 2: Composition of Minimal Salt Medium.

Constituents	Quantity required (g per litre of water)
$NaNO_3$	4
KH_2PO_4	1.5
Na_2HPO_4	0.5
$FeSO_4,7H_2O$	0.0011
$MgSO_4.7H_2O$	0.2
$CaCl_2$	0.01

Agar (1.5 %). 1.5 g dissolved in 100 ml of above medium

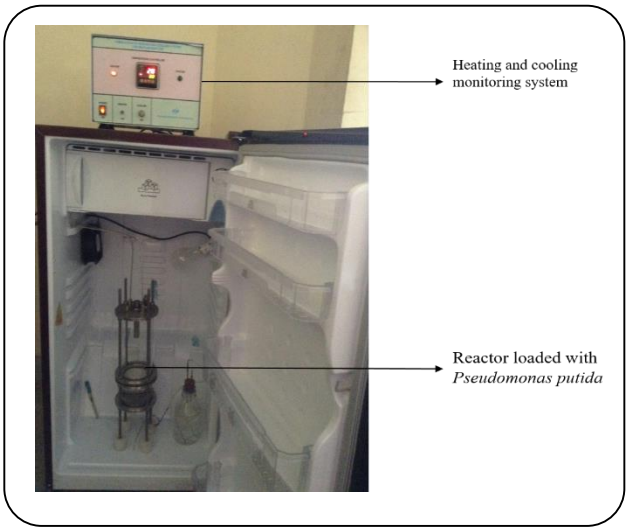

Fig. 1: Biofilm bio-filter reactor with offline gas chromatography and carbon dioxide analyzer.

at late logarithmic phase, culture was used for the biofilm preparation. Nitrocellulose membrane of about 0.2 µm pore size with surface area of 0.0043 m^2 was used as a surface for the biofilm. Under sterile condition, 20 mL of pure culture was poured over the membrane in such a way to form uniform biofilm. Then the membrane with biofilm was placed over the sieve plate of the reactor (i.e., between gas and liquid reservoir).

Generating toluene vapour

Toluene vapour used in this work was generated by simple diffusion system [14], 10 mL diffusion tube with the length 90 mm and 50 mm from the neck were used. Inner diameter of the tube was 3.5 mm. One litre reagent bottle with diffusion tube inside was placed in water bath with temperature control. By adjusting the temperature of the system to 40 ^oC and flow rate, length of the diffusion as constant (As mentioned in the Table 1), 250 ppm concentration of toluene gas was generated and used to study with metabolic uncouplers.

Screening of metabolic uncouplers:

Based on the screening studies conducted with differential reactor by [15], five different metabolic uncouplers BA, PCP, TCP, MA and DNP were selected and used for the current study. All five metabolic uncouplers were buffered with PBS at pH 7 and autoclaved before using in the reactor. Different concentration

Table 3: Metabolic uncouplers used for this study.

Metabolic uncouplers	Concentration tested
Benzoic acid(BA)	6000 µM & 10000 µM
Pentachlorophenol (PCP)	100 µM & 170 µM
2, 4, 6-Trichlorophenol (TCP)	3000 µM & 4000 µM
Malonic Acid (MA)	25 µM & 50 µM
2, 4-dinitrophenol(DNP)	75 µM & 150 µM

of metabolic uncouplers buffered with PBS was tested individually in the biofilm bio-filter reactor on continuous mode as mentioned in Table 3. Experiments were carried out at different concentrations of metabolic uncouplers in order to find out the critical effective concentration at which it enhances the biodegradation rate to a maximum extent in *Pseudomonas putida MTCC 1061*.

Initially biofilm bio-filter reactor was run with PBS without metabolic uncoupler until it reaches steady Surface Elimination Capacity (SEC) in the presence of toluene degrader in the reactor. SEC was calculated using a formula developed in our earlier work (15). Following each metabolic uncoupler was tested individually and further residual metabolic uncoupler was removed by multiple PBS wash. The inlet and outlet toluene concentration of toluene were monitored by using offline Gas chromatography [14]. The CO_2 from the outlet was monitored by offline carbon dioxide analyzer.

RESULTS AND DISCUSSION

Screening of metabolic uncouplers on continuous mode

Effect of Benzoic Acid

Effects of five different metabolic uncouplers were studied in continuous mode. As represented in Fig. 2, initially the modified biofilm bio-filter reactor was run continuously for a period of 22 days with *Pseudomonos putida MTCC 10617* as biofilm along with buffer PBS showed steady state surface elimination capacity of about 0.20 g/m^{2}h. On 22nd day, PBS was replaced with 6000 µM of benzoic acid, SEC of 0.18 g/m^{2}h was observed. Then multiple PBS wash was done to remove the benzoic acid present in a reactor on 25th day. Despite of this, SEC recovery of about 50% (0.10 g/m^{2}h) of initial SEC was found. Subsequently, on 28th day the concentration of benzoic acid was increased to 10000 µM and replaced PBS, the SEC dropped significantly with the addition of higher concentration of benzoic acid and it was found to be 0.16 g/m^{2}h. Further PBS wash was not done as it is evident that benzoic acid is not increasing the SEC. The error bars in Fig. 2 represents the deviations in the triplicate sample injection in Gas Chromatography for testing the toluene in the reactor.

Effect of Pentachlorophenol

Similar to the benzoic acid, study was carried out to observe the effect of pentachlorophenol in the SEC. Biofilm bio-filter reactor was run for 28 days in the absence of any metabolic couplers to attain the stable initial SEC of about 0.20 g/m^{2}h. This is followed by the addition of 100 µM of PCP to replace PBS. After 35 days, increased and steady state SEC of 0.29 g/m^{2}h was observed. Following this, multiple wash by PBS was done and noted 15% (0.17 g/m^{2}h) decreased SEC when compared with the initial SEC. Furthermore, the concentration of PCP was increased from 100 µM to 170 µM, elevated concentration of PCP improved the SEC of 87% of about 0.375 g/m^{2}h (Fig. 3) after 50 days and it was observed stable SEC with further increased concentration. Finally the PBS wash was done once again to find the recovery of SEC and to remove the PCP from the reactor and observed 0.32 g/m^{2}h of final SEC. The error bars in Fig. 3 represents the deviations in the triplicate sample injection in Gas Chromatography for testing the toluene in the reactor.

Effect of 2, 4, 6-Trichlorophenol

Nitrogen source (0.05M sodium nitrate) was given to increase the growth of the organism in the system and to attain the steady state on SEC. This was not done in earlier cases as the growth was very slow in the current study for unknown reasons. Once significant SEC was reached, nitrogen source was replaced with PBS. The reactor was run continuously until it reaches steady state SEC. After

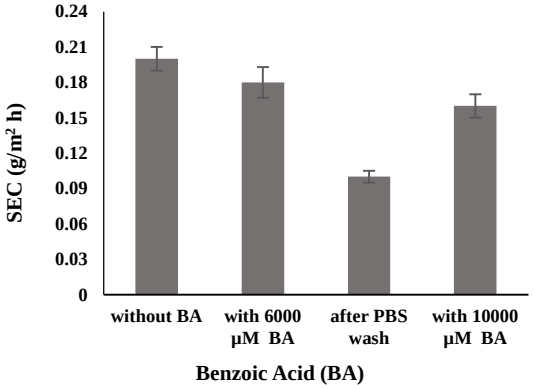

Fig. 2: Effect of benzoic acid on biodegradation rate of toluene in biofilm bio-filter reactor. Error bars are the standard deviation for triple injection of same sample.

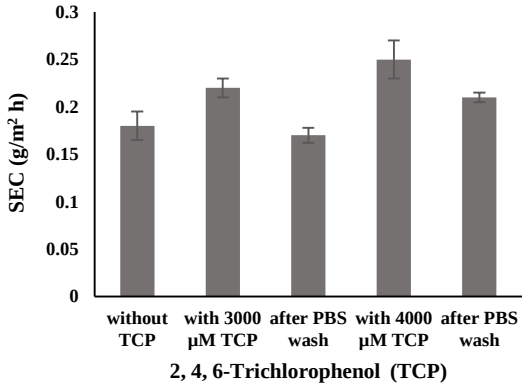

Fig. 4: Effect of 2, 4, 6-Trichlorophenol on biodegradation rate of toluene in biofilm bio-filter reactor. Error bars are the standard deviation for triple injection of same sample.

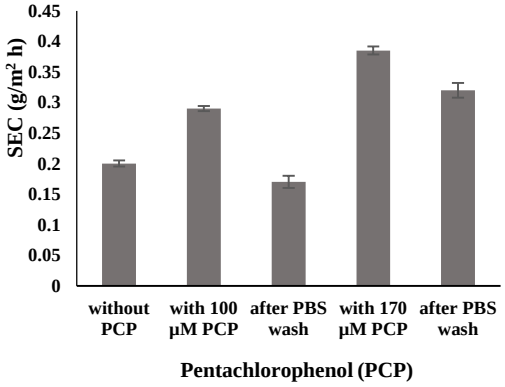

Fig. 3: Effect of pentachlorophenol on biodegradation rate of toluene in biofilm bio-filter reactor. Error bars are the standard deviation for triple injection of same sample.

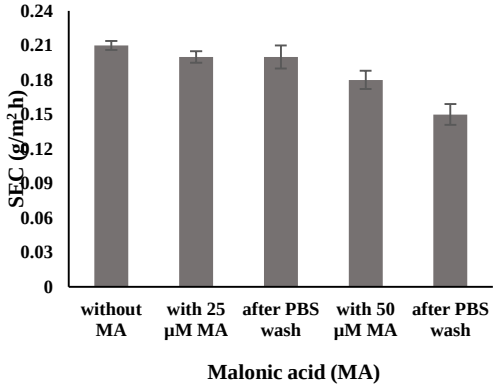

Fig. 5: Effect of malonic acid on biodegradation rate of toluene in biofilm bio-filter reactor. Error bars are the standard deviation for triple injection of same sample

steady state SEC of 0.18 g/m^{2}h was observed on day 20,PBS in a reactor system was replaced with the 3000 µM concentration of TCP showed SEC of 0.22 g/m^{2}h. After that, the system was washed with PBS and SEC of 0.17 g/m^{2}h was calculated which is more or less equal to initial SEC. Further the higher concentration of about 4000 µM was added to the system and was observed for 11 days, that showed SEC increased by 38% when compared with initial EC. Similar to PCP, after removing TCP from the system by PBS wash, SEC dropped only by 5% (Fig. 4) when compared with the SEC observed at the maximum concentration of 4000 µM TCP. The error bars in Fig. 4 represents the deviations in the triplicate sample injection in Gas Chromatography for testing the toluene in the reactor.

Effect of malonic acid:

As like above process, the bio-filtration system with biofilm and PBS was run continuously till it reached the stable SEC of about 0.21g/m^{2}h. With reference to the earlier work, concentration 25 µM and 50 µM was chosen. Initially 25 µM was added to the system and SEC value slowly dropped to 0.2 g/m^{2}h from initial SEC. Then the metabolic uncoupler was replaced by PBS wash and shown same SEC without any changes. Furthermore, second concentration of about 50 µM malonic acid was added, along with the biofilm further decrease in SEC of about 0.18 g/m^{2}h was observed. After next PBS wash, 0.15 g/m^{2}h SEC (Fig. 5) was attained. The system was run for 12 days at 25 µM malonic acid concentration and for 10 days at 50 µM malonic acid concentration. The error bars in Fig. 5

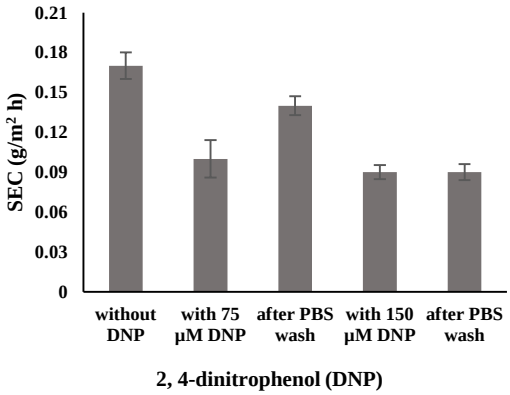

Fig. 6: Effect of 2, 4-dinitrophenol (DNP) on biodegradation rate of toluene in biofilm bio-biofilter reactor. Error bars are the standard deviation for triple injection of same sample.

represents the deviations in the triplicate sample injection in Gas Chromatography for testing the toluene in the reactor.

Effect of 2, 4-Dinitrophenol:

Finally, effect of 2, 4-dinitrophenol in the biodegradation rate of toluene was studied by following same procedure followed earlier. After steady SEC of about of 0.17 g/m^{2}h reached, the PBS buffer was replaced with the 75 µM metabolic uncoupler 2, 4-dinitrophenol. On day 25, it showed the surface elimination capacity of 0.10 g/m^{2}h which was around 41% decrease with reference to the initial SEC obtained in the presence of PBS. Then the whole system was washed with PBS until metabolic uncoupler was removed. Then SEC was calculated, it was found to have slight increase in SEC of about 0.14 g/m^{2}h. Subsequent addition of 150 µM DNP showed very less SEC (Fig. 6) of about 0.09 g/m^{2}h. Stable SEC was maintained by the system even after PBS wash. The error bars in Fig. 6 represents the deviations in the triplicate sample injection in Gas Chromatography for testing the toluene in the reactor.

Post experimental analysis of PCP and TCP

In comparison with overall study, PCP and TCP have shown enhanced surface elimination capacity. Therefore after experimentation, PCP and TCP containing solution in the water reservoir were analysed to find the residual concentration of metabolic uncoupler. From the results it was observed that reduction in concentration of about 0.53% PCP and 0.76% TCP from the initial concentration used as mentioned in Table 4. The negligible reduction further confirmed that the organism present in the biofilm consumed toluene as a sole carbon source and not metabolic uncouplers. However, the reason behind the negligible reduction is unknown.

SEM analysis of biofilm:

Finally the biofilm treated with PCP and TCP were subjected to SEM analysis to analyse the surface of the biofilm and also to observe the thickness of the biofilm. Larger and wider air interface cavities were observed in the biofilm treated with PCP than biofilm treated TCP as shown in Fig. 7 (A). This may be due to the reason that the cells were in more stress under the presence of PCP than TCP. The slight variation in biofilm thickness before and after treatment with metabolic uncouplers PCP and TCP was observed from SEM analysis as shown in Fig. 7. This confirmed that the system did not build up any significant biomass (non-growth system) over the nitrocellulose membrane.

In the developed biofilm bio-filter reactor, overall effect of five different metabolic uncouplers with *Pseudomonos putida MTCC 10617* as biofilm in enhancing the surface elimination capacity of toluene was studied. As like activated sludge treatment, it was assumed that the use of metabolic uncouplers is expected to reduce the biomass growth with increase in specific substrate utilization rate due to its maintenance energy demand. To compare the effect of different metabolic uncouplers, all five metabolic uncouplers were individually tested with constant operational parameters. Overall study explicitly conveyed that the initial elimination capacity was in the range of 0.17 to 0.2 g/m^{2}h.

The study with benzoic acid showed a decrease in SEC with both the concentration 6000 µM and 10000 µM. It suggested hypothetically that the higher concentration of benzoic acid might have killed the cells present in the system. Hence it is clear that the effect of benzoic acid in biofiltration and activated sludge treatment are not same. Therefore, benzoic acid was not considered as the potential metabolic uncoupler in enhancing surface elimination capacity for non-growth system like biofiltration. Unlike benzoic acid, concentration of 100 µM & 170 µM PCP and concentration of 3000 µM and 4000 µM TCP have shown increased surface elimination capacity. In case of PCP, due to its lower solubility, higher concentration was not tested

Table 4: Post experimental studies on PCP and TCP.

Metabolic uncouplers	Biofilter bed	Quantity tested in biofiltration reactor (mg)	Quantity reported from the liquid sample given for analysis (mg)	% Change
PCP	Biofilm	38	37.8	0.53%
TCP	Biofilm	790	784.4	0.76%

and vice versa for TCP. Subsequent PBS wash dropped the surface elimination capacity less than that of initial SEC due to revised lag phase following the effect of metabolic uncoupler. Results observed with PCP and TCP clearly proved that the uncoupling mechanism is exclusively by the toluene degrader. As like benzoic acid, study with lower concentration of malonic acid shown decreased SEC. Hence, it was concluded that the response for uncoupling mechanism was similar to the benzoic acid. Therefore, malonic acid was also considered as not suitable uncoupler. Further study with DNP has shown reduced surface elimination capacity than that of initial SEC. This confirmed that the growth of the microbial cells was inhibited by the action of DNP, though it has 'N' group.

Overall study concluded that the reactor with PCP and TCP shown increased surface elimination capacity. Rest of the metabolic uncoupler such as BA, MA and DNP did not increase the surface elimination capacity but it inhibited the growth of the toluene degrading cells present in the system at respective tested concentration. In biofilm bio-filter reactor, at the PCP concentration of 170 µM, 87% increase in SEC of about 0.375 g/m^2h was observed. Similarly at concentration of 3000 µM of TCP, the observed SEC was found to be 0.22 g/m^2h. It is of about 38% increase in SEC from initial steady state SEC, whereas the work with differential bioreactor [15] showed 37% and 18% elimination capacity with 140 µM PCP and 4051 µM TCP respectively. Post experimental analysis of PCP and TCP showed reduction in 0.53% and 0.76% respectively in comparison with the initial concentration tested in reactor. Hence it further confirmed that the metabolic coupler was not consumed by the metabolic uncoupler thought it has 'C' group. Therefore the toluene was utilized as the sole carbon source by the organism present in the biofilm.

Pseudomonos putida biofilms with PCP and TCP were analysed in SEM. Results revealed that the biofilm subjected to PCP and TCP had larger and wider air interface voids which proved the cells were subjected to stress. Thickness of the biofilm exposed to PCP before and after study were also observed from SEM analysis and found to be, 2.542 µm and 2.641 µm as represented in Fig. 7 (C) and (D) respectively, which is not a significant increase. However, the voids and the thickness of the biofilm might have also increased the mass transfer in biofilm exposed to PCP than biofilm exposed to TCP and thereby increasing the surface elimination capacity. Hence in comparison with differential reactor (15), 2.35 and 2.11 folds increase in SEC with PCP and TCP respectively were observed with biofilm bio-filter reactor. Therefore, PCP and TCP were found to be potential metabolic uncouplers in enhancing the volumetric biodegradation of the toluene in the biofilm bio-filter reactor. In the reactor system, there was no concentration gradient because of the cells in the bio-film was uniformly distributed so that the distance between the cells was also insignificant. Similarly toluene used in the system did not get accumulated both in biofilm and the water phase as system is at steady state. This reactor can also be used to calculate the energy uncoupling coefficient for non-growth systems to quantitatively represent the uncoupling mechanism.

Nomenclatures

APCTs	Air Pollution Control Technologies
VOCs	Volatile Organic Compounds
BTEX	Benzene, Toluene, Ethyl benzene and Xylene
MTCC	Microbial Type Culture Collection
ATP	Adenosine Tri Phosphate
PCP	Pentachlorophenol
DNP	2, 4-Dinitrophenol
TCP	2, 4, 6-Trichlorophenol
SEM	Scanning Electron Microscope
BA	Benzoic Acid
MA	Malonic Acid
PBS	Phosphate Buffer Saline

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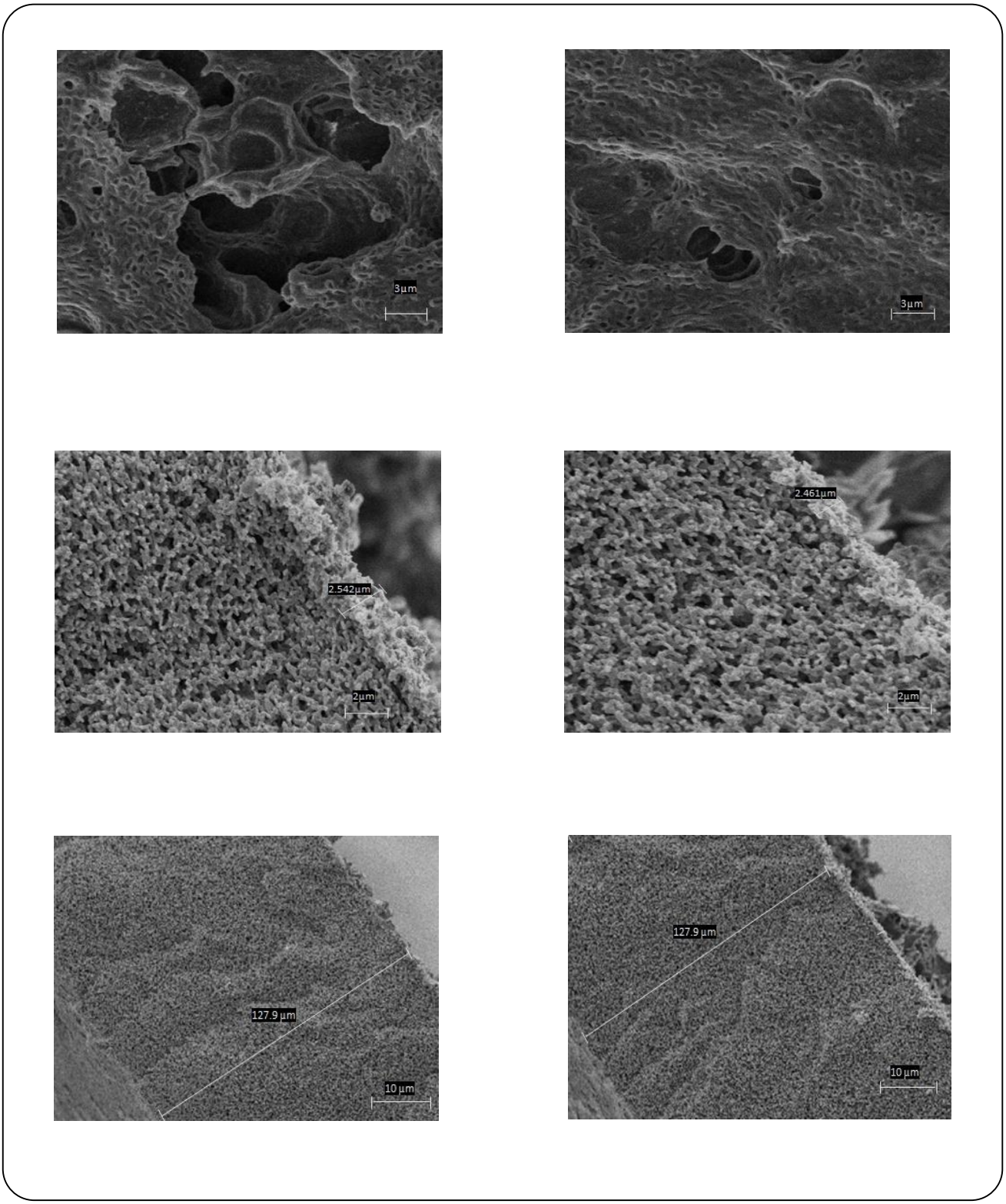

Fig. 7: Results of SEM analysis (A) Microscopic view of biofilm treated with PCP having larger air interface cavities. (B) Microscopic view of biofilm treated with TCP with minimal air interface cavities. (C) Microscopic view of biofilm having thickness 2.542 µm before exposed to metabolic uncoupler. (D) Microscopic view of biofilm having thickness 2.461 µm after exposed to metabolic uncoupler PCP. (E) (F) Microscopic view of nitrocellulose membrane having thickness of 127.9 µm.

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