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Biodegradation of ciprofloxacin in a continuous anaerobic hybrid reactor conglomerating attached and suspended growth system

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Abstract

The detection of antibiotics in the natural surroundings needs immediate attention to control antibiotic pollution in the precious water resources and avoid generation of antibiotic resistant strains. The present study investigated the biodegradation of ciprofloxacin (CIP) containing wastewater in a continuous lab-scale anaerobic hybrid reactor (AHR), which conglomerates the dual advantages of attached and suspended growth. The reactor was started with the inoculum sludge, historically been exposed to the pharmaceutical residuals and fed with simulated synthetic wastewater for its acclimation to higher COD concentrations (1000 mg/L). CIP was then gradually infused in the bioreactor from 0.5 to 10 mg/L for acclimation of microorganisms till it attained CIP removal >90%. The acclimation study revealed that AHR could sustain CIP concentration as high as 5 mg/L beyond which the process severely got deteriorated. Zone of inhibition study was carried out to assess the tolerance level of bacterial strains to varying CIP concentrations. MS-QToF analysis of effluent samples identified 8 new organic intermediates. The anaerobic hybrid reactor proved to be a significant mode to control the pollution caused by the above group of antibiotics.

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Introduction

Pharmaceutical products are vast group of complex organic molecules which play a crucial role in both human and animal health care. These pharmaceuticals enters into the environment through various pathways and cause severe impacts on to the environment due to its toxicity. Appearance of these pharmaceuticals and their metabolites in the wastewater effluent and surface water is a prime concern as it can lead to the development of resistant microbial stains thus making these antibiotics ineffective when used for medication. The studies reported persistence of around 80-100 pharmaceuticals and their metabolites in both effluent and surface waters in various countries (Ashton *et al.*, 2004; Chen *et al.*, 2008). Fermentation and synthesis operations in pharmaceutical industries usually generate larger volumes of wastewater, which contain higher organic load and variety of pharmaceutical residuals (Oktem *et al.*,2007). Fluoroquinolone are broadly used in anthropological, veterinary, and agricultural applications (Pico and Andreu, 2007). Ciprofloxacin (CIP) is one of the most commonly used antibiotic of fluoroquinolone group, which has been detected in varying concentration in domestic (5.6 µg/l-2.5 mg/l), hospital (3 µg/l-14 mg/l) and industrial (up to 31 mg/l) effluents in India (Larsson *et al.*, 2007; Batt *et al.*, 2007; Diwan *et al.*, 2009). Not only this, the surface water resources have also shown significantly higher concentration of CIP ranging from 2.5-6.5 mg/l (Larsson *et al.*, 2009).

The widespread usage of these pharmaceutical and their ever increasing concern desires a strong drive for developing an efficient treatment process so as to eradicate their concerns (Fent *et al.*, 2006). Conventional treatment processes have their own process constraints and limited applicability. Due to complex nature and structure, these compounds are not readily biodegradable, they are escaped without treatment and raise their appearance in the surface water resources. Enick and Moore (2007) reported that nearly half of the pharmaceutical wastewaters generated worldwide are discharged without specific treatment. This has shifted the attention of various researchers and scientist to evolve more efficient system to deal with these antibiotics. Several studies have been conducted to understand the fate and removal of these antibiotics in wastewater (Arya *et al.*, 2016, Zaviska *et al.*, 2013, Grenni *et al.*, 2013, Falas *et al.*, 2012, Wick *et al.*, 2009). The crucial issues of energy and cost consideration have escalated interest on anaerobic treatment of industrial wastewater in particular to the pharmaceutical effluents. Various reactor configurations have been investigated to study the removal of antibiotics from pharmaceutical effluent, viz., expanded granular sludge bed (EGSB), fluidized bed reactor (FBR), anaerobic continuous stirred tank reactor (CSTR), anaerobic baffled reactor (ABR) , anaerobic filter (AF) and upflow anaerobic sludge blanket (UASB) (Enright *et al.*, 2005; Saravanane *et al.*, 2001; Oz *et al.*, 2003; Zhou *et al.*, 2006; Chen *et al.*, 1994; Ince *et al.*, 2002; Ince *et al.*, 2001). Extensive literature review suggests that the degradation of antibiotics depended on various operational parameters, such as inoculum sludge, reactor configuration, HRT, and substrate-co-substrate concentrations, etc. Chelliapan *et al.* (2006) investigated the performance of upflow anaerobic sludge reactor (UASR) and reported 95% removal of tylosin corresponding to HRT and OLR of 4 day and 1.86 kg COD $m^{-3}d^{-1}$ respectively. In a similar study, Amorim *et al.* (2014) reported 83.3% reduction of ciprofloxacin and norfloxacin in a sequential batch reactor (SBR) at an HRT of 7.9 hrs. Garcia *et al.* (2013) investigated the performance of membrane bioreactor (MBR) and reported CIP degradation of 52.8% at 12 hrs HRT.

Several researchers have investigated the treatment of antibiotics using various reactor configurations. However, none of them have investigated the performance of anaerobic hybrid reactor (AHR) for the biodegradation of CIP. Hybrid reactor used in the study is a conglomeration of the added features of both the anaerobic filter (AF) and upflow anaerobic sludge blanket (UASB) reactor in a single reactor

system. The attached growth media in the upper part of the reactor along with the formation of a granular or flocculent sludge bed in the bottom section enhances significant biomass retention and result into increased process stability, improved gas/solid/liquid separation and higher removal efficiency. Hence, the present study was undertaken to investigate the fate and biodegradation of CIP in AHR.

Materials and methods

Design of experimental set-up

Experimental set-up consisted of 5 litres capacity AHR designed and fabricated as per Lettinga *et al.* (1991). The reactor was made of 10 mm thick transparent acrylic sheet with an internal diameter of 10 cm and effective height of 63.5 cm (Fig. 1). The top most portion of the reactor (10 cm) was filled with the cylindrical corrugated PVC pipes of internal diameter (2.5 cm) and length (2.5 cm) each. This packed section was designed to perform the dual functions of retaining the suspended biomass through its sieving mechanism and providing polishing treatment to the wastewater through the activity of bio-film developed on the surface of these PVC media. The reactor was fed using peristaltic pump through the inlet provided at the bottom of the reactor.

The outlet was provided above the packing media to facilitate collection of effluent samples. The reactor was covered with black sheet to avoid the photo biodegradation and development of algae. The vent pipe provided at the topmost part of the reactor which allows the passage of biogas produced in the process, which was measured with the gas flow meter connected to the vent pipe.

Start-up of the reactor

For achieving early start-up, the pharmaceutical sludge obtained from effluent treatment plant of M/s Bengal Chemicals, Kolkata was chosen as inoculum which had historically been exposed to the pharmaceutical residuals. The sludge was washed with water followed by sieving with a mesh of 150 µm to remove debris and inert impurities. The obtained filtrate (2 L) was transferred to the AHR which contained volatile suspended solids (VSS) concentration of 15 g/l. The reactor was started with synthetic wastewater (Table 1) at influent COD of 1000 mg/l and hydraulic retention time (HRT) of 1 day. The influent pH and alkalinity were 7.5±0.5 and 450±10 mg/l, respectively.

The performance of reactor was monitored on daily basis till it attained pseudo steady state, which is referred to as the state when the effluent COD values lies within ± 5% of average value observed for a minimum operation period of 7 days. In this study, the start-up was achieved in 39 days with COD removal of 89% at an HRT of 1 day.

Acclimatization study

CIP was initially infused in the synthetic wastewater at a concentration of 0.5 mg/l, which was then gradually increased to 10 mg/l keeping the influent COD constant at 1,000 mg/l. The step wise increase in CIP load was done only after achieving > 90% of CIP removal. The influent pH of 7.5±0.5 and HRT of 1 day was kept constant throughout this study.

Identification of intermediates of CIP biodegradation

To assess the fate, biodegradation pathways and intermediates formed in biodegradation of CIP, the effluent samples were analysed using mass spectrometry equipped with ESI source coupled to quadrupole-time-of-flight detector (QTOF). The spray needle voltage-4.5kV and corresponding products, formed during transformation of CIP, were identified using software Mass Lynx version 4.1.

Granulation study

Granulation of the biomass is an important parameter of a bioreactor. In this study, various morphological parameters of the sludge granules, such as volatile suspended solids (VSS) content, sludge settling velocity, sludge volume index (SVI), size of the granules, etc. were determined. Scanning electron microscopic (SEM) was also used as a tool to analyse

the morphology of granules formed in AHR.

Analytical methods and chemicals

The analysis of pH, alkalinity, chemical oxygen demand (COD), suspended solids (SS), volatile suspended solids (VSS) and sludge volume index (SVI) were carried out as per Standard Methods (APHA, 2012). CIP (Fluka BioChemika) was procured from Sigma-Aldrich with a purity of ≥98.0% and was analysed using HPLC (Thermofisher) with UV detector. Reverse phase Acclaim C-18 column (4.6x 250 mm; 5 µm) was used for separation of compounds.

As per the method reported by Gad-Allah *et al.* (2011). Biogas was collected in gas bladders and analyzed in triplicates using GC (Thermofisher Ceres 800 plus), equipped with a Thermal conductivity detector (TCD). The average reading was considered for estimating the percentage of methane in the gas samples. The standard for gas analysis was procured from Chemito India Pvt. Ltd., Mumbai, India. The morphology of the granules was studied using Field Emission Scanning Electron Microscope (FE-SEM). The sludge samples were dehydrated and sputter coated with gold (Quorum Q15ORES) and viewed under a Camera Scanning Electron Microscope (Carl Zeiss Supra-55).

Results and discussion

Performance of the AHR during acclimatization

Acclimation of anaerobic biomass plays a key role in governing process stability and performance of AHR. The study shows that when the CIP (0.5 mg/L) was initially infused in AHR, the percentage removal of CIP was very less (< 10%), however, after 21 days (61st day), there was a sharp increment in CIP removal which escalated to 93.8% on 85th day (Fig. 2). It was also observed that the reactor performance got impaired for a period of 7-10 days with each step increase in the influent CIP concentration, which slowly got recovered.

Table 1. Composition of the synthetic wastewater (Senta *et al.*, 2011).

Compounds	Concentration(mg/L)
NH_4Cl	43
KH_2PO_4	18
$MnCl_2{\cdot}4H_2O$	2
$FeCl_3{\cdot}6H_2O$	1
Peptone	86
Glucose	940
Yeast extract	1.2
Methanol(ml)	0.397
CIP	0.5-5

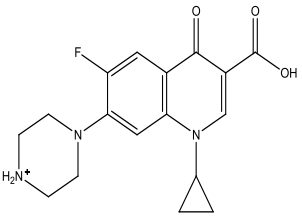
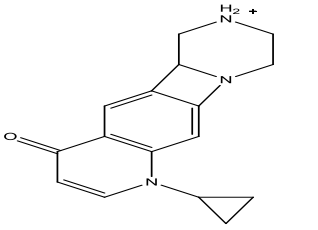
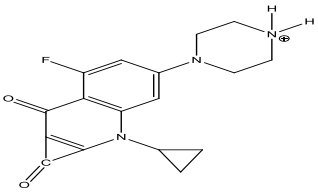
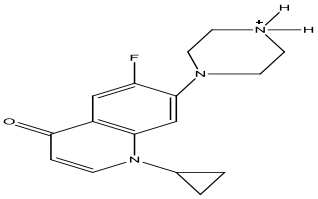
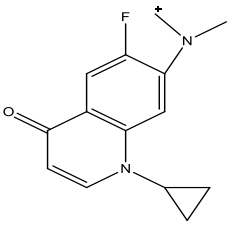
The AHR registered 89.1% removal of CIP at OLR and CIP loading rate (CLR) of 1 kg/m^3.d and 5 mg/L d, respectively. On further increase in CIP concentration to 7.5 mg/l, the AHR registered severe upset as is evident from continuous decline in both CIP and COD removal efficiency. A possible reason could be the elevated toxicity of CIP (beyond 5 mg/L) which might have impaired the bacterial activity resulting into deterioration in the reactor performance.

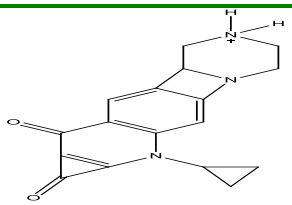
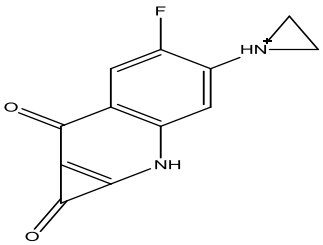
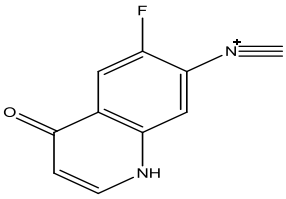
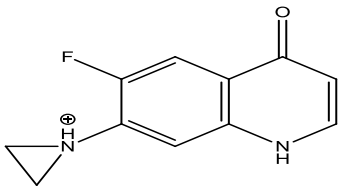
Table 2. Zone of inhibition at varying CIP concentrations.

CIP Conc.(mg/L)	0.5	1.5	5	7.5	10
Zone of Inhibition (mm)	0	10	15	24	32

The study revealed that the reactor took 114 days for its acclimation to CIP concentration of 5 mg/l at which the CIP and COD removal were 89.3% and 93%, respectively. Garcia *et al.* (2013) investigated the removal of quinolones in pilot scale MBR and reported 52.8% biodegradation of CIP at a concentration of 0.5 mg/l. Amorim *et al.* (2014) in a sequencing batch bioreactor reported CIP removal of 83% at corresponding CIP concentration of 32µM. Comparatively, higher percentage of CIP reduction found in our study may be attributed to the reactor configuration which enhances the sludge retention time (SRT) and results into enhanced biodegradation efficiency. Owing to the dual advantages of suspended and attached growth, AHR demonstrated significantly higher removal of TCE and COD (Mitra and Gupta, 2014). Duan *et al.* (2012) also reported that non-woven carrier in hybrid reactor could catch the anammox cells effectively, which stopped biomass loss and improved the NRR in the reactor additionally by 8.1%.

Table 3. Systematic proposed formula, pseudo molecular ions, chemical structures and IUPAC names and its transformation products.

Product	Experimental (m/z)	Proposed formula	Structure proposal	IUPAC name
Ciprofloxacin	332.14	$C_{17}H_{19}FN_3O_3^+$		1-Cyclopropyl-6-fluoro-4-oxo-7-(piperazin-1-yl)1,4dihydroquinoline-3-carboxylic acid
Product 1	268.33 (269.22)	$C_{16}H_{18}N_3O^+$		1-cyclopropyl-4-oxo-4,5b,6,7,8,9-hexahydro-1H-pyrazino[1',2':1,4]azeto[3,2-g]quinolin-7-ium
Product 2	314.33 (311.07)	$C_{17}H_{17}FN_3O_2^+$		4-(2-cyclopropyl-6-fluoro-1,7-dioxo-2,7-dihydro-1H-cyclopropa[b]quinolin-4-yl)piperazin-1-ium
Product 3	288.34 (285.20)	$C_{16}H_{19}FN_3O^+$		4-(1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinolin-7-yl)piperazin-1-ium
Product 4	245.27 (247.23)	$C_{14}H_{14}FN_2O^+$		1-cyclopropyl-6-fluoro-N-methyl-N-methylene-4-oxo-1,4-dihydroquinolin-7-aminium

Product 5	294.33 (293.08)	$C_{17}H_{16}N_3O_2^+$		2-cyclopropyl-1,10-dioxo-1,2,5,6,7,8,8a,10-octahydrocyclopropa[b]pyrazino[1',2':1,4]azeto[3,2-g]quinolin-7-ium
Product 6	231.20 (233.23)	$C_{12}H_8FN_2O_2^+$		1-(5-fluoro-1,7-dioxo-2,7-dihydro-1H-cyclopropa[b]quinolin-4-yl)aziridin-1-ium
Product 7	189.17 (187.04)	$C_{10}H_6FN_2O^+$		6-fluoro-N-methylidyne-4-oxo-1,4-dihydroquinolin-7-aminium
Product 8	205.21 (207.17)	$C_{11}H_{10}FN_2O^+$		1-(6-fluoro-4-oxo-1,4-dihydroquinolin-7-yl)aziridin-1-ium

Study on zone of inhibition of CIP

This study was performed to assess the extent of inhibition caused to the bacterial strains on account of infused CIP at various concentration levels. The study elucidated that the zone of inhibition increased with increase in the influent CIP concentration and was found more prominent (Diameter=24 and 32 mm) at CIP concentration of 7.5 mg/L and 10 mg/l, respectively (Table 2). This might have led to upset in the bacterial activity as is evident from continuous decline in both COD and CIP removal efficiency of AHR beyond a CIP concentration of 5 mg/l during acclimation. Parry *et al.* (2010) also showed the susceptibility of *S. Typhi* strain towards CIP at a concentration of 5µg/ml and detected disk inhibition zone diameter ≤ 30 mm with a sensitivity of 94% and specificity of 94.2%. Singh *et al.* (2014) investigated photo and UV degradation of CIP and deciphered photo degradation of CIP based on the decreasing diameter of zone of inhibition with increase in the exposure to light from 1 to 7 hours. In our study, the anaerobic bacteria showed pronounced inhibition beyond a CIP concentration of 5 mg/L (Fig. 3).

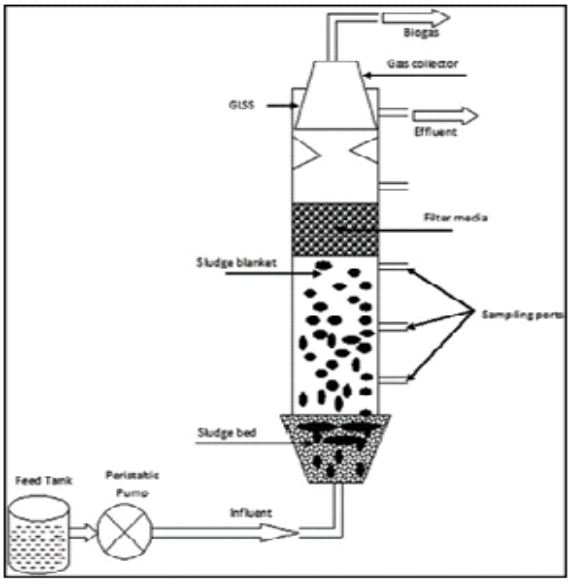


Fig. 1. Schematic diagram of the AHR.

Identification of degradation products

The major degradation intermediates formed during biodegradation of ciprofloxacin by anaerobic bacteria were analyzed by QToF in scan and production scan modes. The proposed chemical structures and the QToF scan data of the detected degradation products are given in Tables 3.

Calza *et al.* (2008) investigated and gave a schematic fragmentation pathway of ciprofloxacin in four possible pathways. Eight new organic intermediates were detected and identified on the basis of accurate mass determination (Fig. 4). Product 1 in our study showed m/z 269.22 which is approximately similar to the intermediate as reported by Maia *et al.* (2014) and Calza *et al.* (2008), which has resulted through the breakdown of CO_2 and HF. Product 2 in our study gave m/z value of 311.07 which shows close structural similarity with m/z 314.12 as reported by Calza *et al.* (2008).

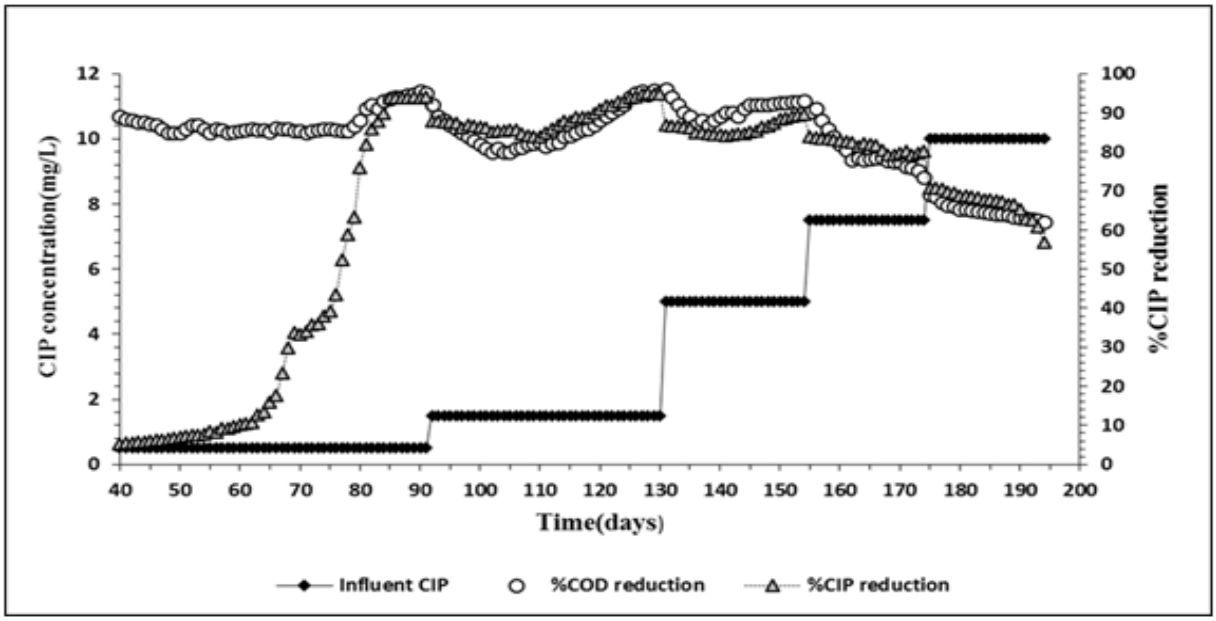


Fig. 2. CIP and COD reduction profile of AHR during acclimation.

They reported that the degradation process was routed through the breakdown of HF bond resulting products whose m/z was nearly 294.12 (similar to product 5 in our study with m/z of 293.08) and also m/z 231.05 (product 6 as shown in our study at m/z 233.23) which routed through the loss of NH attached cyclopropane. Product 3 as detected in m/z spectra of our study is 285.20 which shows a similar structural moiety of m/z 288.15 in Preito *et al.* (2011) and Vasconcelos *et al.*(2009) and in schematic fragmentation shown in Calza *et al.* (2008).The fourth product in our study showed m/z 247.23,that is similar to the m/z 245.10 product as found by Calza *et al.*(2008) and Maia *et al.*(2014) where breakdown of NH attached cyclopropane and CO_2 is predicted. In the present work, product 7 showed m/z 187.04 which shares a common structural similarity with m/z 189.04 as reported by Calza *et al.* (2008). The schematic fragmentation shown by Calza *et al.* (2008) also shows structural similarity with our Product 8 (m/z 207.17). Product 5 in our study has previously been detected as the degradation product of ciprofloxacin both in biological systems by the brown-rot fungus *G. striatum* and also by ozonation (Witte *et al.*, 2008; Wetzstein *et al.*, 1999).

Characterization of the sludge

VSS profile of the sludge

Biomass concentration in the AHR plays a key role in governing process efficiency and the bioreactor performance. Fig. 5(a) depicts the VSS profile of AHR during various phases of acclimation.

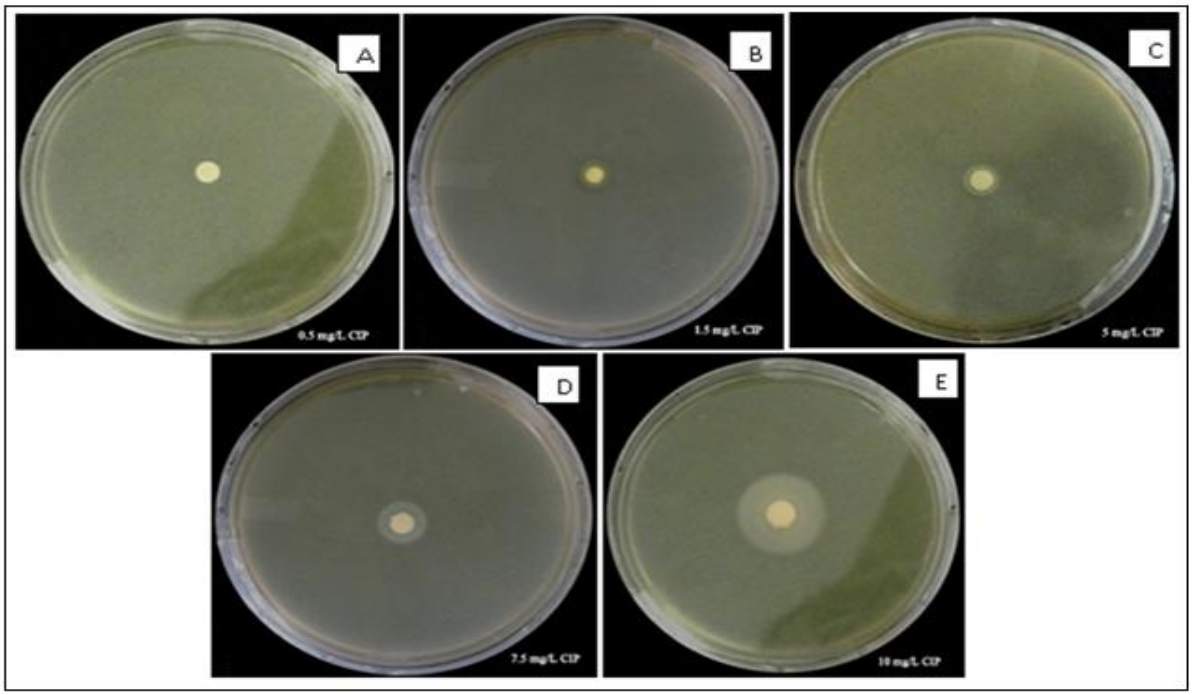


Fig. 3. Zone of Inhibition at different concentration of ciprofloxacin **(A)** 0.5 mg/L **(B)** 1.5 mg/L **(C)** 5 mg/L **(D)** 7.5 mg/L **(E)** 10 mg/L

Initially the biomass concentration of AHR was found as 15 g/l, which increased gradually during the course of acclimation, and was found to be 24 g/l at the end of the operational period. This indicated gradual increase in microbial concentration inside the reactor. Gupta *et al.* (2007) investigated the performance of AHR and UASB reactor treating distillery spent wash and reported that VSS concentration in AHR was comparatively higher (14,000 to 32,000 mg/l) than UASB reactor(14,000–30,000 mg/l).

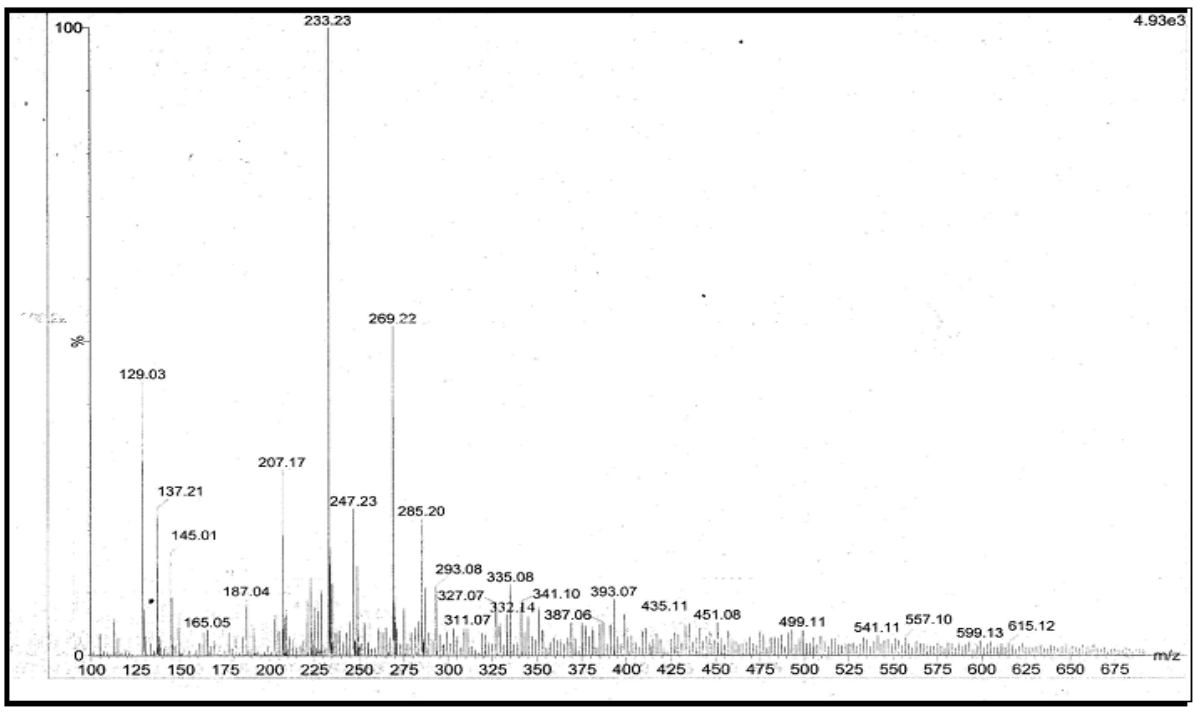


Fig. 4. MS Q-TOF scan of Ciprofloxacin.

The relatively lower increment in the VSS profile in our study might be attributed to the inhibitory effect of CIP on the growth microbial consortia in AHR.

Average settling velocity and SVI of the sludge

The average settling velocity of the sludge increased from 27.55 during start-up phase to 62.24 m/h during first phase of acclimatization (Fig. 5 b).

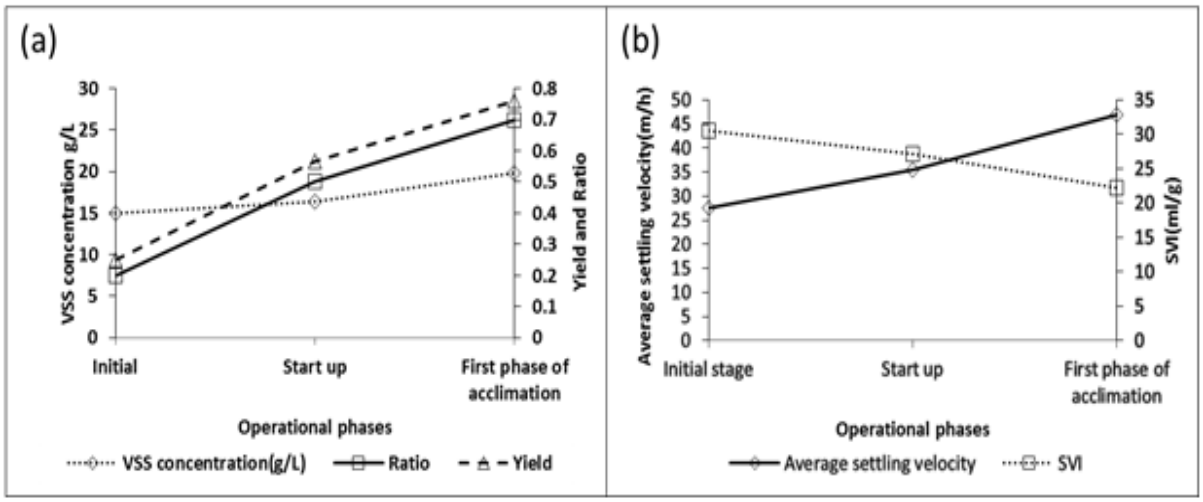


Fig. 5(a). VSS, Yield and Ratio profile of the AHR during different phases of the study **(b)** Average settling velocity and SVI profile of AHR.

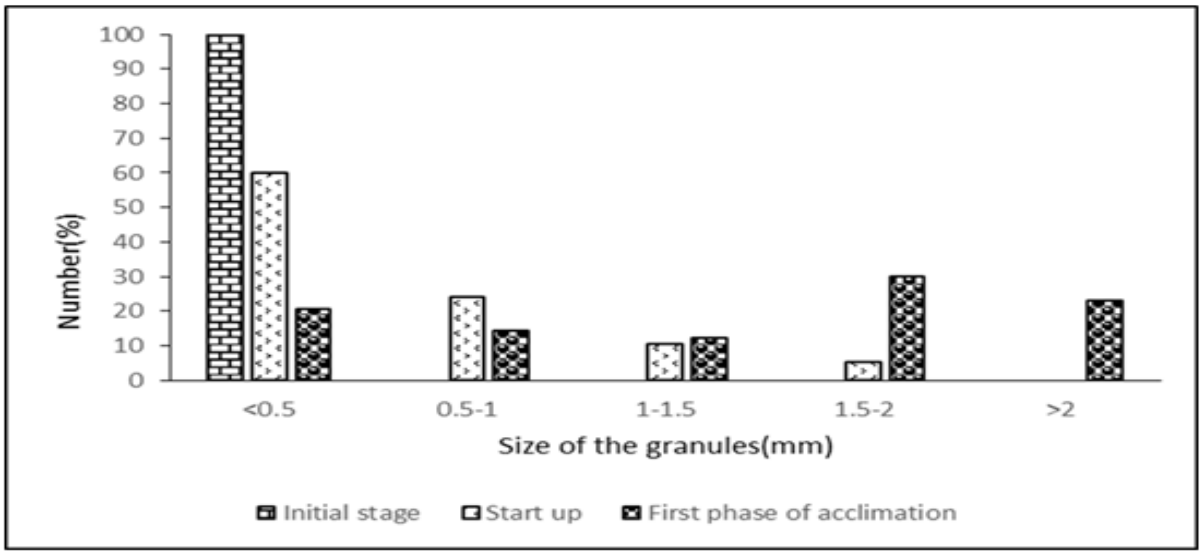


Fig. 6. Granules size distribution of the sludge at different phases of the study.

This might be attributed to the conversion of flocculent sludge to compact granular sludge. The gradual decrement in SVI of the sludge during the start-up (27 ml/g SS), first phase of acclimatization (21.58 ml/g SS) also indicated the gradual transformation of flocculent sludge into compact granular sludge (Fig.6). The values of SVI found in this study are comparable to the values of SVI (10–20 ml/g SS), reported for the good quality of granules (Maat and Habbets, 1987; Gupta and Gupta, 2005).

SEM analysis of the sludge granules

After operating the reactor for 194 days the granular sludge was collected from the reactor bottom for measuring the characteristics using FE-SEM Visual examination of granular biomass revealed that

granules were of black colour and had spherical shape (Fig. 7a). The overall surface of the granules was rough and uneven. SEM study of the granules showed heterogeneous bacterial population of both cocci and rod shaped bacteria with a predominance of cocci shape intermingling on the surface of different granules (Fig.7b, c).

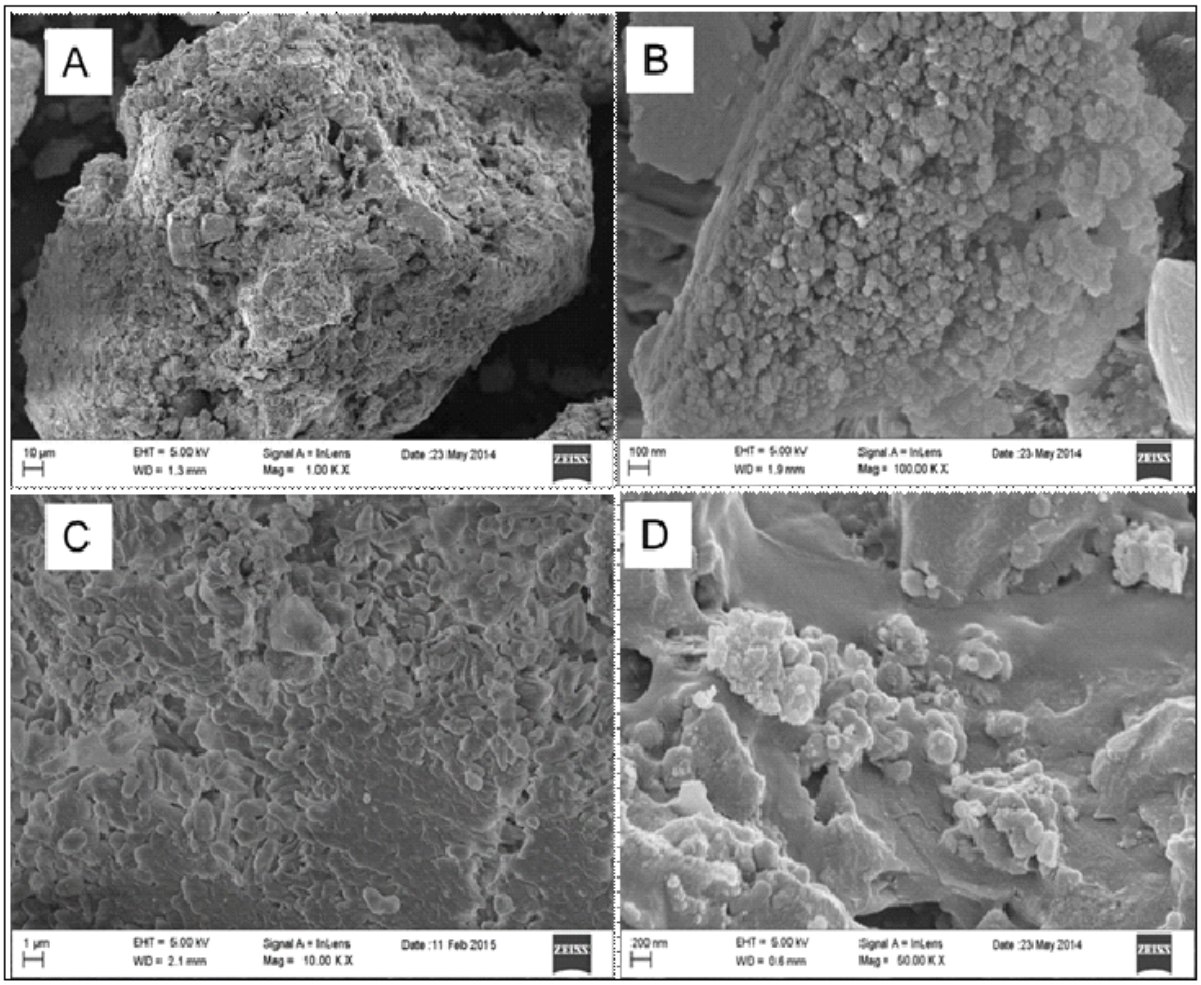


Fig. 7. SEM photographs of the sludge granules. (a) Shape of granule (b) Cocci-shaped bacterial species (c) Heterogenous mixed of both cocci and rod shape bacteria (d) Closer view of the cocci-shaped bacterial species.

From the literature above we could assume for our study that there might be the cocci-shaped (typical morphology of Methanosarcina types of bacterial species as mentioned in Prakash and Gupta, 2000; Gupta and Gupta, 2005 over the surface of the granules.

Presence of cracks & cavities were seen on the surface of the granules which serve as channel for transport of substrate and nutrient (Fig. 7d). Gupta and Gupta (2005) and Zinatizadeh *et al.* (2007) have also reported that presence of both cocci and rod shape bacteria over the surface of the granules. According to Du J. *et al.* (2015) intact granules have more tolerance to antimicrobials than disrupted granules. Xie *et al.* (2009) deciphered that high resistance of granules is attributable to the layered structure of granules.

Conclusion

AHR demonstrated remarkable tolerance and could sustain influent CIP as high as 5 mg/l delivering significantly higher CIP (96.2%) and COD (94.5%) removal efficiencies at an HRT of 1 day. Biodegradation of CIP resulted 8 new organic

intermediates which opens the door for further investigations. Zone of inhibition increased with increase in the CIP concentration which severely inhibited the process at higher CIP concentrations. SEM analysis of the sludge delineated presence of heterogeneous bacterial population of both cocci and rod shaped bacteria with a predominance of cocci shape.

Sludge granules of size >2 mm in diameter, with high VSS content (24 g/l), having a high settling velocity (62.25 m/h) and low SVI (15.88 ml/g SS), were developed in the AHR. The study elucidated that AHR may prove to be a promising treatment option for CIP containing pharmaceutical wastewater.

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