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Biodegradation of *p*-cresol by aerobic granules in sequencing batch reactor

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Abstract

The cultivation of aerobic granules in sequencing batch reactor for the biodegradation of *p*-cresol was studied. The reactor was started with 100 mg/L of *p*-cresol. Aerobic granules first appeared within one month of start up. The granules were large and strong and had a compact structure. The diameter of stable granules was in the range of 1–5 mm. The integrity coefficient and granules density was found to be 96% and 1046 kg/m^3, respectively. The settling velocity of granules was found to be in the range of 2×10^{-2}–6×10^{-2} m/sec. The aerobic granules were able to degrade *p*-cresol upto 800 mg/L at a removal efficiency of 88%. Specific *p*-cresol degradation rate in aerobic granules followed Haldane model for substrate inhibition. High specific *p*-cresol degradation rate up to 0.96 g *p*-cresol/(g VSS·day) were sustained upto *p*-cresol concentration of 400 mg/L. Higher removal efficiency, good settling characteristics of aerobic granules, makes sequencing batch reactor suitable for enhancing the microorganism potential for biodegradation of inhibitory compounds.

Key words: aerobic granules; *p*-cresol; sequencing batch reactor (SBR); haldane kinetics equation

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Introduction

The cresols are xenobiotic contaminants that are often found in waste discharges. *p*-Cresol is an isomeric phenol with a methyl substituent at the para position relative to the hydroxyl group. *p*-Cresol has a wide variety of uses including as a disinfectants, fumigants, explosives, in the manufacturing of synthetic resins, in photographic developers etc. Due to their toxicity, strong odour emission, persistent in the environment and suspected carcinogenity and mutagenity to living organisms, cresols pose a serious ecological problem as environmental pollutants. *p*-Cresol, even at very low concentration, has adverse effects on the central nervous system, cardiovascular system, lungs, kidney and resulting in central nervous system depression (Buckman et al., 1984). US Environmental Protection Agency has classified *p*-cresol as a pollutant of group C (possible human carcinogen) (ATSDR, 1990). The Ministry of Environment and Forest (MOEF), Govt of India has set a maximum concentration level of 1.0 mg/L of *p*-cresol in the industrial effluent for safe discharge in the surface waters (Singh et al., 2008). The World Health Organization (WHO, 1963) recommends the permissible *p*-cresol concentration of 0.001 mg/L in potable waters. Hence the removal of cresols from wastewater is a necessary task to conserve the quality of natural water resources.

Removal of phenolic compounds by solvent extraction, adsorption, chemical oxidation, incineration and other non-biological treatment methods suffer from serious drawbacks such as high cost and formation of hazardous by products (Loh et al., 2000). Biodegradation is the most effective method for complete destruction of organic pollutants and also due to its lower cost. The new technique of cell immobilization in biological wastewater treatment is gaining interest. Aerobic granules are self-immobilized microbial aggregates that are cultivated in sequencing batch reactor (SBR) without adding a carrier material. Aerobic granules could be applied for biodegradation of toxic wastewaters (Jiang et al., 2002). Many factors are involved in the granulation of activated sludge. The main factors are carbon source, hydrodynamic shear force, feast famine regime, feeding strategy, dissolved oxygen, reactor configuration, volume exchange ratio and settling time (de Bruin et al., 2004; Liu and Tay, 2004). Due to the excellent settling capacity of aerobic granules, the land area of settling tank will not be required which in turn reduces the wastewater treatment plant area by 80% (de Bruin et al., 2004). The aim of this study is to evaluate the utility of aerobically grown microbial granules for the biological treatment of *p*-cresol containing wastewater.

1 Materials and methods

1.1 Reactor setup and operation

A laboratory scale SBR with an effective volume of 2 L was used to cultivate aerobic granules. The internal diame-

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ter of the reactor was 5 cm and the working height/diameter was about 20 (Fig. 1). Fine air bubbles for aeration and mixing were supplied by diffusers placed at the reactor bottom. Superficial air velocity was maintained in the range of 2–3 cm/sec. The reactor was operated sequentially in 8 hr cycles which consist of 5 min of influent filing, 447–472 min of aeration, 5–30 min of settling and 3 min of effluent withdrawal. Effluent was discharged at 50 cm from the bottom of the reactor with a volumetric exchange ratio of 50%.

Seeding biomass was obtained from the municipal wastewater treatment plant, Okhla, New Delhi, India. The sludge was acclimatized to phenol in batch culture for a period of one month. The acclimatized sludge was used as inoculum for the reactor. The reactor was fed with cresol as the sole carbon source by using a synthetic wastewater with following nutrient composition: *p*-Cresol, NH_4Cl, $MgSO_4{\cdot}7H_2O$, K_2HPO_4, and KH_2PO_4 at a weight ratio of 1:0.4:0.26:3.3:2.7. The feed was supplemented with 1 mL/L of micronutrients, as described previously (Moy et al., 2002).

1.2 Analytical methods

Measurement of pH, biomass concentration, chemical oxygen demand (COD), Sludge Volume Index (SVI) were conducted in accordance with standard methods (APHA et al., 2002). Dissolved oxygen concentration (DO) was measured using HACH, HQ30d portable meter coupled with LBOD10101 probe. The concentration of *p*-cresol in influent and effluent was measured using DR5000 UV/Vis spectrophotometer (HACH, USA) at 280 nm wavelength. The samples were centrifuged at 10,000 r/min for 10 min (Remi, India) and measured in a HACH square sample cell. A calibration plot (absorbance versus concentration of *p*-cresol) was drawn and used for estimating the concentration of unknown *p*-cresol concentration. The plot was linear (R^2 = 0.98) between 0 and 10 mg/L *p*-cresol solution. The test samples drawn from experiments with higher concentrations of *p*-cresol were adequately diluted and then the absorbance was determined. Samples were taken from the reactor at a predetermined time intervals for analysis.

Granule was analysed using Cenisco binocular petrological microscope with SANYO digital camera. The photographs were analysed using image analysis (IA) system (Averz Software). The morphology of granules in terms aspect ratio was analyzed by using IA technique. Aspect ratio is the ratio between minor axis and major axis of ellipse equivalent to the granule (0 = line, 1 = circle). SEM analysis of aerobic granule was performed by Scanning Electron Microscope (LEO Model 435 VP, UK).

Granule strength is defined as the ability of granules to resist disintegration. It is defined as an integrity coefficient (IC, %) which is the residual volatile suspended solids (VSS) after sample has been agitated for 5 min at 200 r/min on a platform shaker to total VSS of the intact granules prior to agitation (Ghangrekar et al., 1997). The stronger granules have higher ICs. The density of aerobic granules was determined using volumetric displacement method. (Beun et al., 1999).

1.3 Gas chromatography-mass spectrometry

Gas chromatography (GC) and gas chromatography-mass spectrometry (GC/MS) analysis of effluent samples were done in order to identify the number of products formed during aerobic biodegradation of *p*-cresol. For GC/MS analysis, the supernatant was initially acidified to pH 2.0 using 0.5 mol/L H_2SO_4 and subsequently extracted three

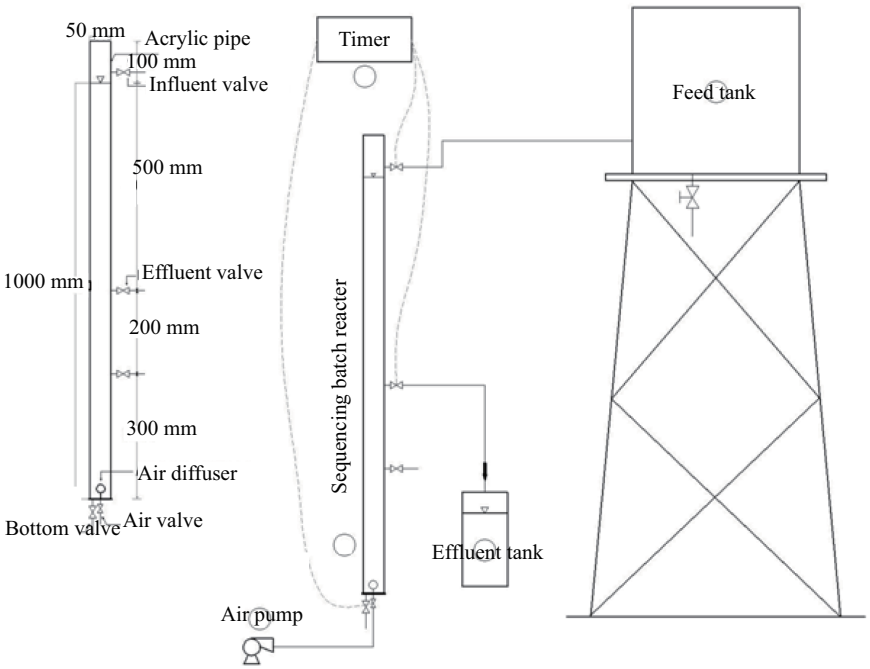

Fig. 1 Experimental setup.

times using dicholoromethane (99.5%) in 1:1 (V/V) ratio in a separating funnel by intermittent shaking. The 1 μL of control and degraded sample were injected in GC-MS equipped with a split/splitless injector and a Perkin Elmer Clarus 680 gas chromatograph interfaced with a Turbomass spectrometric (Perkin Elmer 600T, USA) mass spectrometric mass selective detector system were used.

The MS was operated in the EI mode (70 eV). Helium was employed as carrier gas and its flow rate was adjusted to 1 mL/min. The analytical column connected to the system was a PE-5MS capillary column (length 30 m, diameter i.d. 250 μm). The GC column temperature was programmed at 250°C. A solvent delay of 3.5 min was selected. The injector temperature was set at 200°C, and all injections were carried out on the splitless mode. The GC-MS interface was maintained at 250°C. The oven programme was 50°C and hold for 5 min, and 10°C/min to 250°C and hold for 10 min. The MS was operated in the total ion current (TIC) mode, scanning from m/z 30 to 500. The metabolic intermediates were derived from degradation of *p*-cresol identified by comparing their retention time (RT, min) and mass spectra with that of the National Institute of Standard and Technology (NIST) library available.

1.4 Extracellular polymeric substances

The extracellular polymeric substances (EPS) of the sludge flocs and granules were extracted following a heat extraction procedure as described previously (Li and Yang, 2007). The granules were harvested by centrifugation at 4000 r/min for 5 min and washed twice with a 0.1% NaCl solution prior to extraction. For the granular sludge, the sample was homogenised by grinding to break down the structure of the granules. The sludge suspension was then heated to 60°C in a water bath for 30 min. After centrifugation at 4000 r/min for 15 min, the supernatant was collected, which was regarded as the EPS extract. In addition, the carbohydrate content of the extract was determined by the phenol-sulphuric method and expressed as the glucose equivalent, and its protein substance content were measured according to the modified Lowry method (Li and Yang, 2007).

1.5 Biodegradation kinetics

The ability of granules to degrade *p*-cresol was evaluated in 1 L column reactor which contained the nutrient medium mentioned above and targeted *p*-cresol predetermined concentrations ranging from 100 to 800 mg/L. A kinetic analysis of biodegradation data was performed on the basis of Haldane's equation for describing biodegradation of inhibitory substrate (Yi et al., 2006).

$$V = \frac{V_{max} S}{K_s + S + \frac{S^2}{K_i}} \quad (1)$$

where, V (g *p*-cresol/(g VSS·day)) and V_{max} (g *p*-cresol/(g VSS·day)) are the specific and the maximum specific biodegradation rates, respectively, and S (mg/L), K_s and K_i are the substrate concentration, half saturation constant and inhibition constant, respectively.

2 Results and discussion

2.1 Reactor performance

The biomass acclimatization process to inhibitory compounds is critical step to induced microbial selection and physiological transformation of metabolic pathways required for biodegradation (Tomei et al., 2003). In order to have an effective biomass acclimatization, gradual biomass exposure to the *p*-cresol has been maintained, the concentration was step wise increased when the stable *p*-cresol removal efficiency was achieved.

The sludge acclimated at 200 mg/L phenol was used as inoculum for the SBR. The feed *p*-cresol was increased gradually from 100 mg/L to 200, 400, 600, 800 mg/L (Fig. 2a) after achieving stable state conditions under each feed concentration. The corresponding COD concentrations against various *p*-cresol concentration feed were 251, 503, 1006, 1509 and 2012 mg/L respectively (Fig. 2b). The

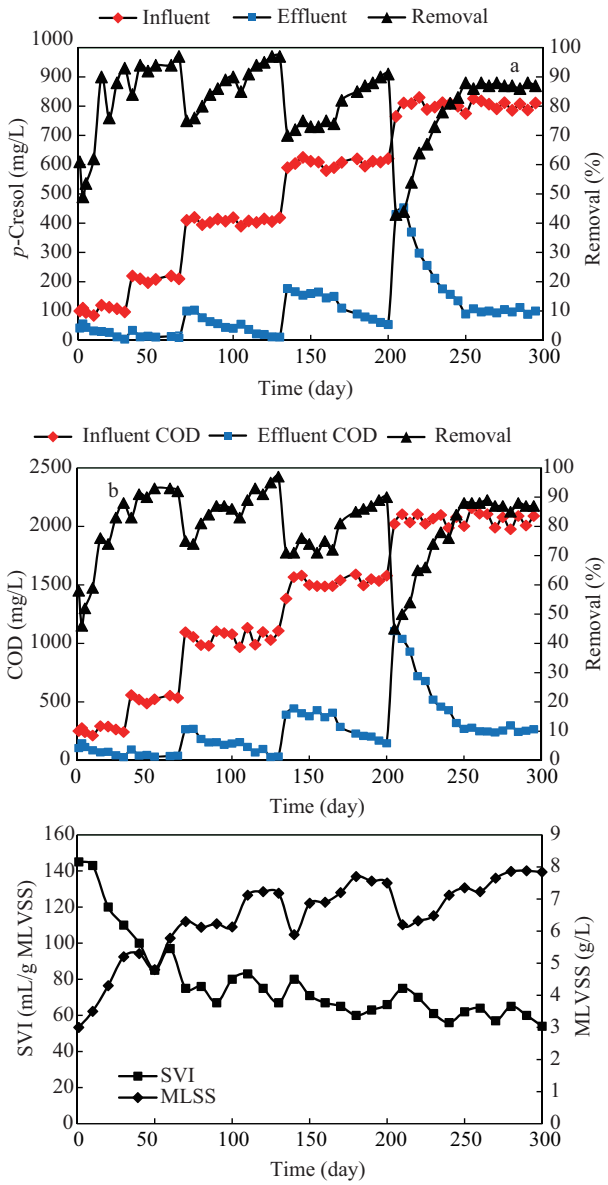

Fig. 2 Performance of SBR during 300 days operation. (a) Influent, effluent cresol and removal; (b) influent and effluent COD, and removal; (c) biomass concentration and sludge volume index (SVI).

main operational parameters and results of the sequencing batch reactor are presented in Table 1.

The influent *p*-cresol and COD concentration in the initial phase was 100 and 251 mg/L respectively which resulted in an organic loading rate (OLR) of 0.15 kg *p*-cresol/(m^3·day) and 0.37 kg COD/(m^3·day). After 30 days operation 90% COD removal efficiency and 96% *p*-cresol removal were achieved. The starting sludge in the reactors had a biomass concentration of 3120 mg/L MLSS and an SVI value of 145 mL/g MLVSS (Fig. 2c). In the starting of reactor operation settling time was kept for 30 min, to retain the biomass in the reactor. The effluent *p*-cresol concentration was 4 mg/L on day 30. After 20 days of operation at *p*-cresol concentration of 100 mg/L the settling time was reduced to 5 min, due to this selection pressure the wash out of poor settling flocs took place. After 45 days of operation at a *p*-cresol concentration and OLR of 200 mg/L and 0.3 kg *p*-cresol/(m^3·day) respectively the aerobic granules were clearly visible in the reactor. The granules surface was covered with filamentous growth. *p*-Cresol removal efficiency was 95% on day 125 at concentration of 400 mg/L (Fig. 2a). MLSS of the reactor was observed to be around 7 g/L. At this stage the biomass was in acclimatized phase, which is essential for the microbial selection and physiological transformation of the metabolic pathways for biodegradation of xenobiotic compounds. At day 205 the cresol concentration was increased to 800 mg/L OLR of 1.2 kg *p*-cresol/m^3 and 3 kg COD/(m^3·day). The *p*-cresol removal efficiency was dropped to 43% (Fig. 2a). A two-month period was required to regain an efficiency of 88%. The reactor was operated for three months at this concentration to achieve a steady state.

2.2 Formation of aerobic *p*-cresol degrading granules

Activated sludge from a municipal wastewater treatment plant was initially acclimatized over a period of 45 days solely on the phenol as a substrate at a concentration of 200 mg/L. This acclimatized sludge was used as inoculum for cultivation of aerobic *p*-cresol degrading granules. The initial biomass concentration and SVI in the reactor was 2.9 g/L and 145 mL/g MLVSS respectively (Fig. 2c). After the application selection pressure on day 20, by reducing the settling time from 30 to 5 min, all the biomass having lesser velocity was washed out from the reactor. Due to this selection pressure there is an enhanced formation of aerobic granules only heavier biomass was retained in the reactor. In next 10 days formation of aerobic granules took place having size less than 1 mm. After day 40, there is a sudden increase in the biomass concentration in the form of aerobic granules (Fig. 3b). The biomass concentration generally showed an upward trend and it remained in between 7–8 g/L MLSS and SVI reduced to 80–60 mL/g MLSS (Fig. 2b) after day 100. The biomass concentration dropped when higher *p*-cresol concentration was introduced in the reactor (Fig. 2c). Stable granules were obtained in the SBR after 100 days of operation (Fig. 3c, d). On day 200 the diameter of stable granules was in the range of 1–5 mm. In earlier studies Chiu et al. (2006) found that as the size of granule increased mass transfer limitation takes place, the biomass inside large granules may not be able to receive sufficient substrate and oxygen to contribute to waste organic degradation. Xiao et al. (2008) proposed that it can be assumed that the biomass within the penetration depth (δ = 200 μm) from the surface is active biomass, whereas the sludge below δ inside the granule is largely inactive due to substrate and oxygen limitation.

The integrity coefficient (IC) and granules density was found to be 96% and 1046 kg/m^3 respectively. The settling velocity of granules was found to be in the range of 2×10^{-2}–6×10^{-2} m/sec. Proteins and carbohydrates were found to be 7.81 and 2.78 mg/g VSS respectively in fully developed granules on day 200. In this study the protein/carbohydrate ratio was approximately 2.8 for *p*-cresol fed granules. Earlier researches showed the dominance of proteins over the carbohydrates (polysaccharides) for aerobic granules (Zhang et al., 2007; McSwain et al., 2005; Wang et al., 2005). The presence of protein in extra cellular polymeric substance suggested its important role in aerobic granule formation. Since protein has a high content of negatively charged amino acids, protein is more involved than sugars in electrostatic bonds with multivalent cations, a key factor in stabilizing aggregate structure (Laspidou and Rittman, 2002).

Table 1 Operational parameters and results of the sequencing batch reactor (average value)

Parameter	Operational parameter				
	1st period day 0–30	2nd period day 31–65	3rd period day 66–130	4th period day 131–200	5th period day 201–295
p-Cresol (mg/L)	100	200	400	600	800
p-Cresol loading rate (kg *m*-cresol /(m^3·day))	0.15	0.3	0.6	0.9	1.2
COD loading rate (kg COD/(m^3·day))	0.37	0.754	1.509	2.263	3.018
p-Cresol removal (%)	93	97	97	91	87
COD removal (%)	88	92	97	90	87
SVI (mL/g MLSS)	110	97	67	67	54
MLVSS (g/L)	3–5.2	5.2–5.78	6.3–7.18	7.18–7.5	7.55–7.88
Size of granules (mm)	–	0.5–1.5	1–2.5	1.5–5	1.5–5
Integrity coefficient (%)	–	–	95	98	98

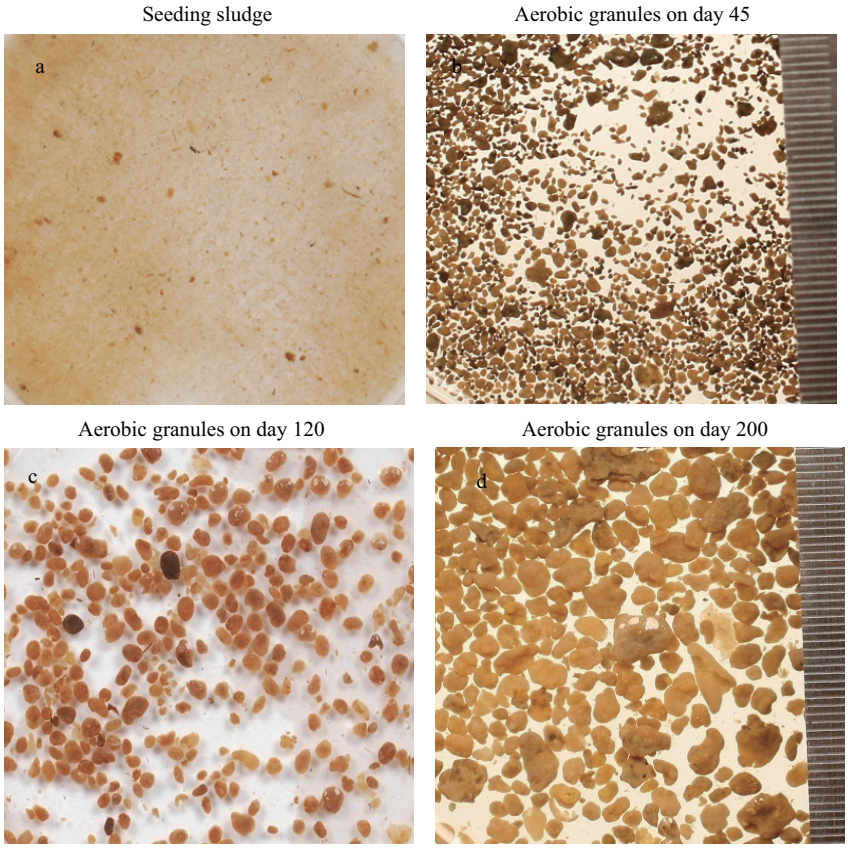

Fig. 3 Images of sludge in the granulation process (1 division = 1 mm).

2.3 UV/GC MS spectral analysis

The changes of structure and quantity of substrate during the biodegradation process are reflected in Fig. 4. As shown in Fig. 4a, the shapes of absorption peak and absorbance values at the wavelength range from 250 to 350 nm decreased quickly during the first 2 hr, shows the fast changing molecular structure of substrate and high biodegradability of *p*-cresol in the acclimatized aerobic granular sludge. From the UV spectra (Fig. 4a), it is evident that there is a new peak at 2 hour (Lambda max 260) which is of catechol. GC-MS anlaysis of effluent also shows the presence of catechol; phenol, 2-4 bis(1-1, dimethyletyl); 3-carboxy-*cis*, *cis*-muconate; 3-oxoadipate; N-tetracosanol-1; E-15-heptadecinal; trimethylne oxide; octadecane, 6-methyl; acetic acid, 1-methylpropylester. Chromatographic analysis was done in order to determine the number of products formed during biodegradation and the results are shown in Fig. 4b and Table 2. GC-MS/UV spectra analysis chromatograph of effluent shows the presence of catechol, *cis*, *cis*-muconate, 3-oxadipate. This shows that *p*-cresol follows the ortho cleavage degradation pathway. Lee et al. (2010) reported that bacterial genera, such as *Bacillus*, *Acinetobacter*, *Corynebacterium*, and *Nocardioides*, were predominant in granules under high cresol stress. These strains can likely endure the toxicity

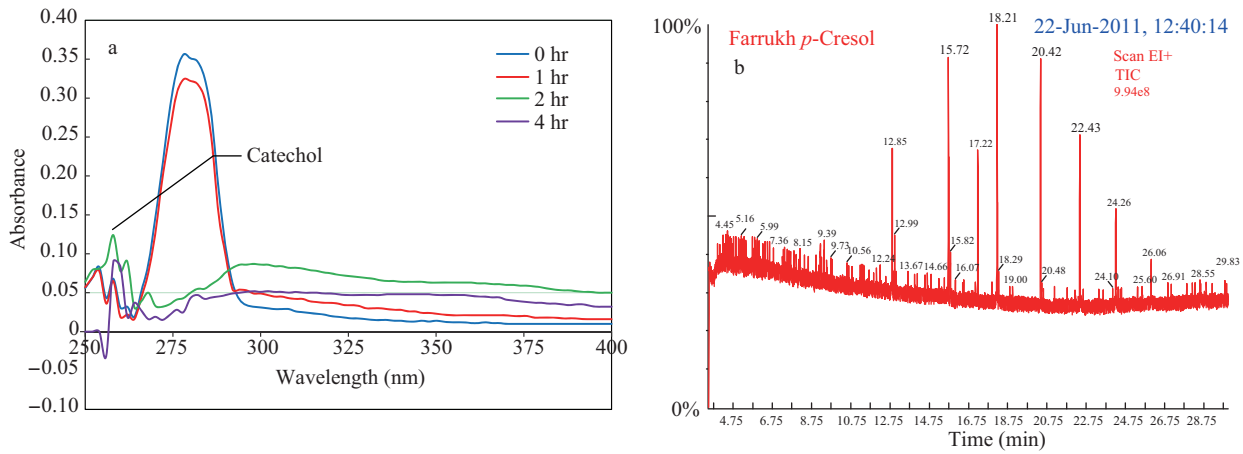

Fig. 4 (a) UV-Vis spectra for the biodegradation of *p*-cresol by aerobic granular sludge at initial *p*-cresol concentration of 300 mg/L; (b) chromatographic analysis of intermediates during biodegradation of *p*-cresol. The MS-identified compounds with respect to their retention time are listed in Table 2.

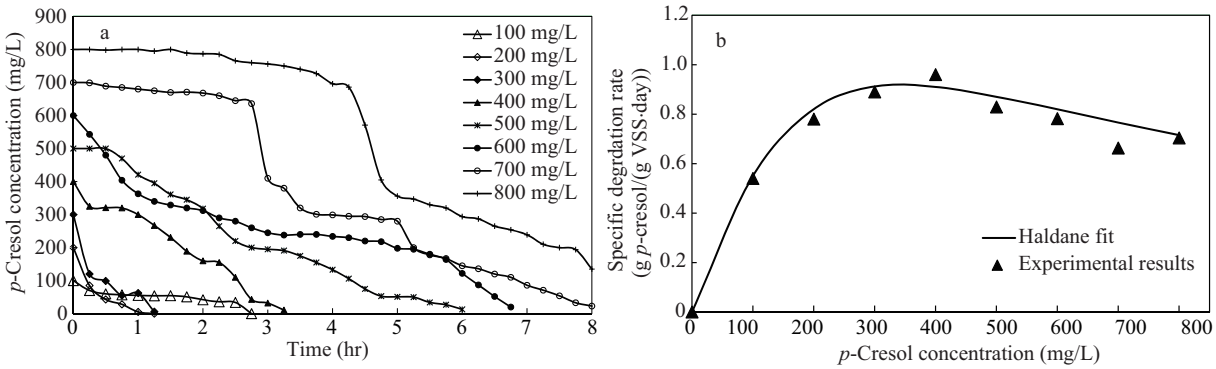

Fig. 5 (a) Time courses of *p*-cresol concentration at different *p*-cresol contents; (b) specific *p*-cresol degradation rates of the aerobic granules at different *p*-cresol concentrations.

derived under high cresol concentrations and/or metabolize cresol. This stable community may be attributed to the potential for cresol degradation, yielding a high degradation rate. The *Acinetobacter* sp. genus can degrade phenolic compounds (Hao et al., 2002) and transform catechol into *cis*, *cis*-muconate, 3-oxoadipate, via the ortho-cleavage pathway to TCA cycle (Nardo et al., 2009).

2.4 *p*-Cresol biodegradation kinetics

The ability of the aerobic granules to biodegrade *p*-cresol was evaluated by monitoring *p*-cresol removal at different concentration in batch column reactor on day 260. Time course of *p*-cresol concentration is shown in Fig. 5a. *p*-Cresol concentration in the reactor decreased with time steeply at a lower initial *p*-cresol concentration, as compared to those at higher concentration of *p*-cresol.

Figure 5b shows the initial *p*-cresol degradation rate with initial concentration. The specific degradation rate increased with the increasing initial *p*-cresol concentration upto 400 mg/L, peaked at 0.96 g *p*-cresol/(g VSS·day), but decreased when the *p*-cresol concentration was increased from 400 to 800 mg/L, this indicates the inhibitory effect of *p*-cresol concentration exceeding 400 mg/L. The kinetic parameters estimated with the least square error method were V_{max} = 2.97 g *p*-cresol/(g VSS·day), K_i = 210 mg/L and K_s = 490 mg/L. These high values K_s and K_i obtained from the Haldane kinetic equation for *p*-cresol degradation showed that aerobic granules have developed a *p*-cresol uptake system to resist the inhibitory effect of *p*-cresol. Due to this aerobic granule resistance to *p*-cresol toxicity, high values of specific *p*-cresol degradation rate was maintained upto concentration of 400 mg/L. Table 3 shows some of the Haldane kinetics parameters for biodegradation of phenolic compounds using aerobic granular sludge.

Table 2 Metabolites peaks identified in the biodegradation *p*-cresol

Retention time (min)	Identification from mass spectra	Molecular formula	Molecular weight	Peak area*
9.196	1-Decene	$C_{10}H_{20}$	140	Small
9.391	Octadecane, 6-methyl	$C_{19}H_{40}$	268	Small
10.636	1,4-Dioxane	$C_4H_8O_2$	88	Small
12.852	3-Tetradecene	$C_{14}H_{28}$		Medium
12.987	Deodecane	$C_{12}H_{26}$	170	Large
15.723	1-Tridecene	$C_{13}H_{26}$	182	Large
15.823	Decane, 6-ethyl-2-methyl	$C_{13}H_{28}$	184	Medium
16.073	Trimethylne oxide	C_3H_6O	58	Small
17.224	Phenol, 2,4-bis(1,1-dimethyl)	$C_{14}H_{22}O$	206	Medium
18.209	1-Hexadecene	$C_{16}H_{32}$	224	Large
18.289	Hexadecane	$C_{16}H_{34}$	226	Small
19.05	5-Eicosane	$C_{20}H_{40}$	280	Small
20.480	Octadecane	$C_{18}H_{38}$		Small
22.426	1-Octadecanol	$C_{18}H_{38}O$	270	Medium
26.062	1-Heneicosanol	$C_{21}H_{44}O$	312	Small

* Relative area of each peak.

Table 3 Haldane kinetics parameters for biodegradation of phenolic compounds using aerobic granular sludge

Substrate	Concentration range (mg/L)	Haldane model			Reference
		V_{max} (g/(g VSS·day))	K_s (mg/L)	K_i (mg/L)	
p-Cresol	100–800	2.97	490	210	This study
2,4-DCP	50–300	11.472	587	19.1	Wang et al., 2007
PNP (4-nitrophenol)	10–300	0.876	17.9	89.7	Yi et al., 2006
Phenol	0–1900	1.7	40.3	980.7	Tay et al., 2004
Phenol	0–2000	5.6	481	212	Jiang et al., 2002

3 Conclusions

The SBR used in the present study was able to degrade *p*-cresol upto concentration of 800 mg/L with an efficiency of 88%. At steady state the aerobic granules were having the size, settling velocity and SVI of 4.5–5.5 mm, 0.06 m/sec and 60 mL/g VSS respectively. The kinetic analysis was in accordance to the Haldane model. High specific *p*-cresol degradation rate upto 0.96 g *p*-cresol/(g VSS·day) were sustained upto *p*-cresol concentration of 400 mg/L. The SBR was proved to be the most suitable technology when system simplicity, low land area requirement and short start-up time were considered as critical parameters for decision making. This work contributes to a better understanding of the ability of aerobic granules to handle high-strength industrial wastewaters containing chemicals that are normally inhibitory to microbial growth.

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