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Treatment of refinery wastewater using *Arthrobacter chlorophenolicus* A6 in an upflow packed bed reactor

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ABSTRACT

In this study, refinery wastewater collected from Guwahati, India, was treated in an upflow packed bed reactor (PBR) with immobilized *Arthrobacter chlorophenolicus* A6. The wastewater was initially characterized using standard methods. The toxicity of the wastewater was determined using aerobic mixed microbial consortia following the resazurin toxicity assay. As the chemical oxygen demand (COD) level in the raw refinery wastewater was very low (190 mg l^{-1}), the raw wastewater was spiked with a mixture of substituted phenol at different concentrations. The batch shake flask experiment revealed that the COD removal efficiency was found to be less when the raw refinery wastewater was not spiked with nutrient. On the other hand, when supplemented with nutrients in the form of mineral salt medium, more than 98% COD removal was achieved. The PBR was operated by varying the influent phenolic concentration in the range of 250–350 mg l^{-1} and at a hydraulic retention time of 12.5 h. Results indicated that more than 99% pollutant removal and 94% effluent toxicity removal were achieved at a combined pollutants loading rate of 1707 mg l^{-1} d^{-1}. However, at higher pollutants loading rate, the reactor performance was found to be unstable, due to the transient accumulation of toxic intermediate products such as chlorocatechol and nitrocatechol.

Keywords: Immobilized biomass; Substituted phenol; Toxicity removal; Pollutant loading rate; Intermediate product

1. Introduction

Oil refinery industries utilize a large quantity of water for desalting, distillation, thermal cracking, and catalytic treatment processes, thus generating wastewater volume in the range of 0.4–1.6 times that of crude oil production in the industry [1]. Discharge of untreated refinery wastewater into the water body results in severe environmental and human health hazard due to the release of toxic pollutants such as phenolics, minerals, and other hydrocarbons [2,3]. Presence of 30–200 mg l^{-1} of phenolic compounds in petroleum refinery wastewater is common [1,4], but the concentration may reach even up to 2,123 mg l^{-1} [5].

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Similarly, the COD level of refinery wastewater increased even up to 7,896 mg l^{-1} [6]. These pollutants are reported to be carcinogenic and highly embryo toxic in nature [7]. Therefore, these phenolic compounds are listed as priority pollutant by the US Environmental Protection Agency (EPA) [8,9]. EPA calls for lowering the phenolic content in potable and mineral waters to 0.5 μg l^{-1}, while the limits for wastewater discharge are 0.5 mg l^{-1} for surface waters and 1 mg l^{-1} for the sewerage system [10]. Thus, it is necessary to remove these phenolics to sufficiently low levels before discharge into the environment. Appropriate strategies of wastewater treatment have to be employed in order to counter balance these growing environmental problems.

Many techniques are available for substituted phenol removal from contaminated wastewater such as flocculation, adsorption, reverse osmosis, and advanced oxidation process [11]. However, high cost, low efficiency, and generation of toxic by-products are the major limiting factors of these methods. On the other hand, biological treatment processes are economical and efficient, that can be used for the treatment of refinery wastewater. Several researchers have investigated on the treatment of refinery wastewater both under aerobic and anaerobic conditions. Jou and Huang [4] achieved a maximum COD removal of 85–90%, using fixed film bioreactor systems at an influent COD concentration of 911.9 mg l^{-1}. On the other hand, Ochieng et al. [12] reported only 34% COD removal using a fluidized bed reactor system for the treatment of refinery wastewater. These results are also well supported by Holubar et al. [13], in which they achieved 35.4% COD reduction from refinery wastewater. Therefore, it is essential to improve the COD removal efficiency to treat real refinery wastewater contaminated with a higher concentration of phenolics.

Among these different bioreactor configurations, packed bed reactor (PBR) offers high volumetric biomass concentration which helps in the degradation of toxic and recalcitrant organics besides providing high toxicity tolerance and stability against shock loadings [14,15]. However, conventional PBR suffers from certain major limitations such as non-uniform distribution of nutrients and oxygen throughout the reactor bed, high pressure drop, gas liquid channeling, and loss of microbial activity over time [14,16]. In order to overcome these limitations, a newly designed packed bed bioreactor was developed [17] and implemented in this study to evaluate its performance in the treatment of refinery wastewater containing phenolics using immobilized *Arthrobacter chlorophenolicus* A6. As the COD level and phenolic concentration in the raw refinery wastewater were found to be low, it was spiked with a mixture of phenolic compounds at different concentrations prior to its feeding into the PBR.

Specific objectives of the study were: (i) characterization of refinery wastewater, (ii) effect of the pollutant loading rate on the reactor performances, (iii) degradation pattern of the mixture of substituted phenol (preferential, sequential, and simultaneous), and (iv) finally, analysis of metabolic intermediate product and the toxicity removal efficiency.

2. Materials and methods

2.1. Refinery wastewater and its characterization

The refinery wastewater used in this study was collected from a petroleum refinery complex located at Guwahati, India. The wastewater was collected from the inlet point to the treatment plant. The wastewater was initially characterized for its phenol content, chemical oxygen demand (COD), pH, dissolved oxygen (DO), heavy metals (nickel, strontium, and lead) along with other parameters. Mono-substituted phenols with different concentrations were spiked with the collected refinery wastewater to make the wastewater contaminated with these pollutants.

2.2. Wastewater treatment experiments

2.2.1. Batch shake flask experiments

The actively growing cells of *A. chlorophenolicus* A6 were obtained as described by Sahoo et al. [18]. Further, the obtained cells were centrifuged (5,000 × g for 20 min at 22°C), washed with 1 × phosphate buffer saline (pH 7.4), and subsequently added to a series of 250 ml shake flasks with 100 ml of raw or suitably diluted refinery wastewater (pH 7.5) to give a final inoculum concentration of 0.1 OD_{600} i.e. equivalent to 23.5 mg l^{-1} of mixed liquor suspended solids (MLSS). Dilution of the raw refinery wastewater was made using optimized mineral salt medium (MSM) as reported by Sahoo et al. [18]. Details of the wastewater considered for its treatment in the batch shake flask study is presented in Table 1.

Since the COD of the raw wastewater was found to be very less, therefore, in the batch shake flask experiments, the refinery wastewater (raw or nutrient supplemented) was spiked with 75 mg l^{-1} each of 4-CP, 4-BP, and 4-NP. The Erlenmeyer flasks were agitated in an orbital incubator shaker set at 30°C and 210 rpm for 20 h [18]. Samples were taken at regular intervals of time during the experiments and were analyzed for biomass and residual COD concentrations. All the experiments in this study were

Table 1
Wastewater dilution considered for its treatment in the batch shake flask study

S. No.	Dilution factor	Wastewater volume taken (ml)	MSM volume used for dilution (ml)	Distilled water volume used for dilution (ml)
1	No dilution	100	0	0
2	1/10th dilution	90	10	0
3	1/5th dilution	80	20	0
4	1/2nd dilution	50	50	0
5	1/2nd dilution	50	0	50

performed in triplicate and the reported results are average with a standard deviation of ±2.5%.

2.2.2. Continuous treatment using the PBR

The packed bed bioreactor used in this study and the results of its start-up as well as abiotic removal of these phenolic compounds have been described in detail in our previous work [17,19]. Based on the results obtained from the previous batch shake flask experiments, the refinery wastewater was mixed with equal volume of MSM and the pH was adjusted to 7.5 before feeding into the PBR. The concentration range of the individual substituted phenols chosen was 250–350 mg l^{-1} and the PBR was operated at 12.5 h of hydraulic retention time (HRT). Whereas, temperature and DO concentration inside the reactor were maintained at 30 (±1)˚C and 4.3–4.5 mg l^{-1}, respectively, throughout the experiments. Samples from the reactor outlet were collected at regular intervals of time specifically at 25, 37.5, and 50 h of the bioreactor run, followed by centrifugation at 6,000 × g for 10 min before analysis of residual phenolics concentration, COD, intermediate product formation, and toxicity removal. Table 2 presents the operating conditions and the schedule followed by the PBR for carrying out this continuous study.

2.3. Analytical methods

Biomass concentration in the liquid samples was determined by measuring its optical density at a

Table 2
Operational schedule and performance of the newly designed PBR for treating the refinery wastewater spiked with phenolic compounds (HRT = 12.5 h)

		Influent concentration (mgl^{-1})			Effluent concentration (mgl^{-1})			% Removal efficiency		
Operation stage No.	Day of operation	4-CP	4-BP	4-NP	4-CP	4-BP	4-NP*	4-CP**	4-BP***	4-NP
	1	250	250	250	0.800	BDL	BDL	99.68	100	100
1	2	250	250	250	0.900	BDL	BDL	99.64	100	100
	3	250	250	250	0.820	BDL	BDL	99.67	100	100
	4	250	250	250	0.840	BDL	BDL	99.66	100	100
2	5	300	300	300	28.00	5.50	1.15	90.6	98.16	99.5
	6	300	300	300	26	4.5	1.2	91.33	98.5	99.6
	7	300	300	300	25.4	4.2	1.2	91.53	98.6	99.6
	8	300	300	300	25.5	4.25	1.4	91.5	98.6	99.4
	9	300	300	300	25.7	4.3	1.2	91.43	98.56	99.5
3	10	350	350	350	115.5	73.0	7.0	67.64	79.14	98.0
	11	350	350	350	113	71	7.5	67.71	79.7	97.8
	12	350	350	350	111	71.5	7.2	68.28	79.57	97.95
	13	350	350	350	111.5	71.9	7.25	68.14	79.45	97.92
	14	350	350	350	111.8	72	7.15	68.06	79.42	97.95
	15	350	350	350	111.7	71.7	7.25	68.05	79.51	97.92

*4-BP: 4-Bromophenol.
**4-NP: 4-Nitrophenol.
***4-CP: 4-Chlorophenol.

wavelength 600 nm (OD_{600}) using a UV–vis spectrophotometer (Model lambda-45, Perkin Elmer, USA). The absorbance values were expressed as dry cell weight using a calibration curve plotted between optical density (OD_{600}) and MLSS of the culture. One unit of absorbance was equivalent to 235 mg l^{-1} of MLSS. Immobilized biomass concentration on the polyurethane foam (PUF) was measured by the Bradford method of protein assay [20]. About 1 g of PUF was dried at 105˚C for 12 h and soaked in a 1 M NaOH solution, followed by digestion at 90˚C for 10 min. Protein assay was then performed after cooling the dried solution. A calibration curve relating biomass dry weight and protein concentration was used to obtain the immobilized biomass dry weight. COD of the samples was measured by closed reflux methods as per the standard methods [21]. 4-CP, 4-BP, and 4-NP concentrations in the filtered samples were measured using a reverse phase HPLC (Varian Prostar 210) equipped with a UV–vis detector (wavelength 280 nm) and Onsphere C-18 column, (Varian) with *methanol*–water–acetic acid (50:49:1 v/v) as the mobile phase. The retention times of 4-CP, 4-BP, and 4-NP in the column were 5.4, 6.41, and 3.1 min, respectively, at a flow rate of 0.4 ml min^{-1}. The metabolites that were formed during the degradation of the phenolics supplemented refinery wastewater were analyzed using a liquid chromatography-mass spectrometry (LC-MS) system (Q-Tof Premier, USA). A capillary ACQUITY UPLC ® BEH shield RP 181.7 μm C-18 column (10 × 50 mm length) was used for the separation of the metabolites. A mixture of acetonitrile–water–formic acid (1:1:0.1 v/v) was filtered through a 0.22 mm Millipore syringe filter and used as the mobile phase in the experiment. Concentrations of heavy metals, such as strontium, lead, and nickel, in the petroleum refinery wastewater were measured using atomic absorption spectrometer (Spectra AA 240 FS, Varian, USA), at their respective absorbance maxima. Conductivity, turbidity, total dissolved solids, DO, oxidation reduction potential, ammonia, nitrate, and chloride ions were determined using a multi-parameter water quality measurement kit (6,920-M Yellow Springs, Ohio-USA) for characterizing the wastewater. Toxicity removal efficiency in the PBR system was determined by the Resazurin reduction assay method [22].

3. Results and discussion

3.1. Wastewater characterization

Characteristic of the raw refinery wastewater is presented in Table 3. From this table it can be seen that the COD level as well as phenolic concentration in the raw refinery wastewater is low i.e. 190 and 20 mg l^{-1}, respectively. Due to the low COD and phenolic content, the raw refinery wastewater was spiked with a mixture of phenolics at different concentrations for further experiments. The inorganic content of the raw refinery wastewater were found to be significantly high, particularly chloride ion concentration (740 mg l^{-1}) along with heavy metals such as lead and strontium (Table 3). Similarly the pH of the raw refinery wastewater was relatively high i.e. 8.2.

Table 3
Characteristics of the petroleum refinery industry wastewater used in the study

Parameter	Concentration
Turbidity	327 NTU
Total dissolved solids	559 mg l^{-1}
Conductivity	0.825 mS/cm
Dissolved oxygen concentration	0.95 mg l^{-1}
pH	8.20
Oxidation reduction potential	201.8 mv
NH_4^+-N	9.5 mg l^{-1}
NH_3-N	0.753 mg l^{-1}
Cl^-	740 mg l^{-1}
NO_3^-	162 mg l^{-1}
Ni	2 mg l^{-1}
Pb	8 mg l^{-1}
Sr	15 mg l^{-1}
Phenol	20 mg l^{-1}
COD	190 mg l^{-1}

Notes: NTU = Nephelo turbidity unit.

3.2. Batch shake flask wastewater treatment

The pH of the raw refinery wastewater was adjusted to an optimum value of 7.5 for the growth of *A. chlorophenolicus* A6 [18]. Fig. 1 demonstrated the effect of MSM to the refinery wastewater on COD removal efficiency by *A. chlorophenolicus* A6. As shown in the Fig. 1, the COD removal efficiency gradually increased with the addition of MSM to the wastewater. More than 98% of COD removal was observed from the refinery wastewater containing an initial COD of 447 mg l^{-1} within 20 h of culture, when mixed with equal volume of MSM. Hence, for the continuous experiments with the PBR, the refinery wastewater was mixed with equal volume of MSM before feeding into the reactor.

3.3. Continuous wastewater treatment with the PBR

Table 2 shows the operational schedule and performance of the PBR for the treatment of refinery

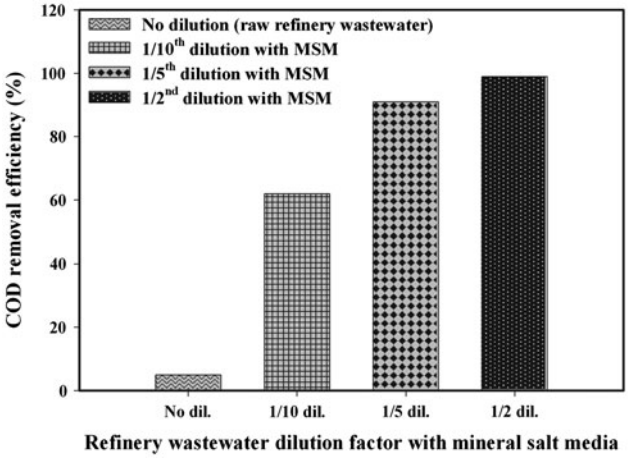

Fig. 1. Effect of MSM on COD removal efficiency (%).

wastewater spiked with mixture of substituted phenols. Before start of the refinery wastewater treatment in the present study, the reactor with immobilized *A. chlorophenolicus* A6 was previously operated nearly about 6 months for performance evaluation of 4-NP and 4-BP removal as a single-substrate system. The reactor was started with 250 mg l^{-1} each of 4-CP, 4-BP, and 4-NP (stage 1), and was continued to run until the achievement of steady-state conditions. Once the effluent concentrations of reactor at any stage, in terms of substituted phenols were constant for 3–4 cycles of run, it was assumed that the reactor had reached a steady-state condition. After achieving the steady-state performance, the influent concentration of these pollutant mixture was increased/changed stepwise from stage 1 to stage 3. HRT of the reactor was kept at 12.5 h. Fig. 2 illustrated the degradation performance of the

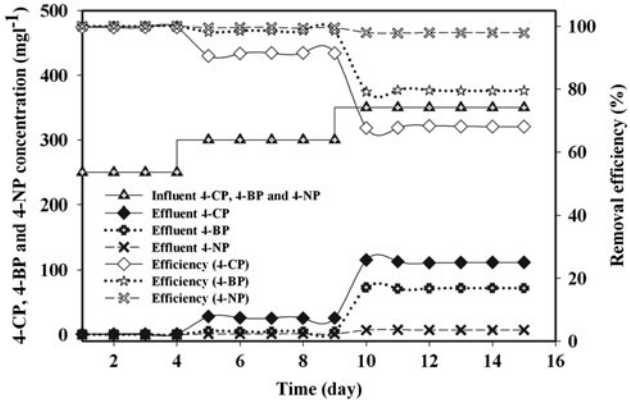

Fig. 2. Degradation performance of PBR in treating petroleum refinery wastewater containing a mixture of 4-CP, 4-BP, and 4-NP at 12.5 h HRT by *A. chlorophenolicus* A6 (*S stage of operation).

bioreactor system in treating refinery wastewater containing mixture of these substituted phenols.

From this figure, it is clear that at stages 1 and 2, (lower concentrations ranges) degradation efficiencies of all the pollutants in the refinery wastewater were considerably high. Complete removal of all the three pollutants (below detection limit: BDL) from the refinery wastewater was achieved at maximum individual pollutant loading rate of 569 mg l^{-1} d^{-1} or combined pollutant loading rate of 1,707 mg l^{-1} d^{-1} (stage 1). In the present study, the phenolics detection limit of the HPLC method was 0.1 μg l^{-1}. Though, complete removal was not achieved, more than 98% removal occurred at stage 2, except for 4-CP, which was removed 90%. Therefore, before proceeding from stage 2 to stage 3, the reactor was brought back to steady-state conditions by operating the reactor at stage 1 for few cycles of run. The reactor performance deteriorated at stage 3, especially with respect to 4-CP and 4-BP removal, which were 68 and 80%, respectively. Further, the efficiency of the PBR on degradation of these three substituted phenols was found to be in the following order: 4-NP > 4-BP > 4-CP (as shown in Fig. 2 and Table 2).

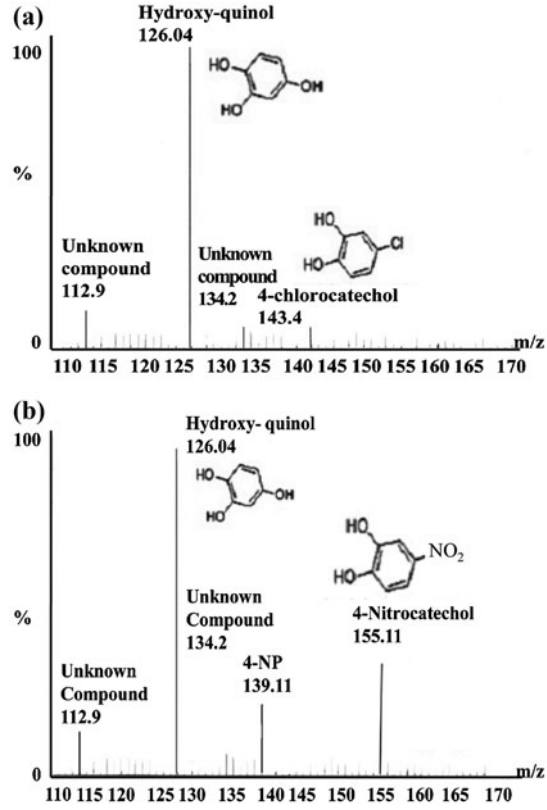

Fig. 3. Metabolites and intermediates analysis of the phenolic biodegradation in the PBR effluent obtained at stage 3 using HPLC mass spectra (a) 4-CP and (b) 4-NP.

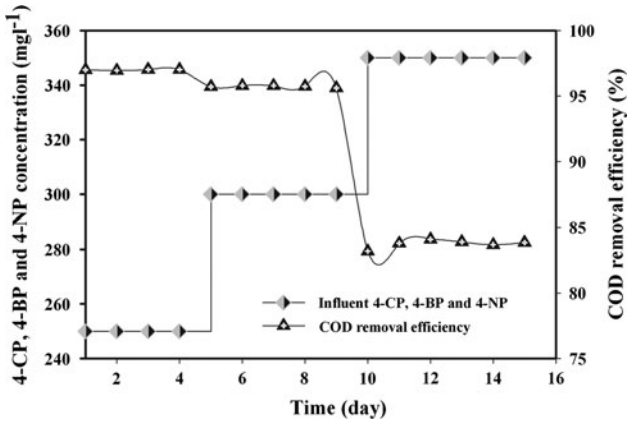

Fig. 4. COD removal performance of PBR in treating petroleum refinery wastewater containing a mixture of 4-CP, 4-BP, and 4-NP at 12.5 h HRT by *A. chlorophenolicus* A6 (*S stage of operation).

3.4. Influence of intermediates formed

In order to probe possible reason for the poor degradation performance at higher pollutant loading rate of the reactor, HPLC-MS analysis of the reactor effluent was performed. In the present study, HPLC-MS analysis of the reactor effluent confirmed the formation of intermediates such as 4-chlorocatechol, hydroxyl-quinol from 4-CP and 4-nitrocatechol along with hydroxyl-quinol from 4-NP as shown in Fig. 3(a) and (b); on the contrary, in case of 4-BP, no such intermediate was noticed in the reactor effluent. Fig. 4 shows the COD removal profile that suggests more than 97% removal at stages 1 and 2.

3.5. Influence of pollutant loading rate

Fig. 5 shows the reactor performance in terms of percentage removal efficiency and pollutant removal rate ($mg\,l^{-1}\,d^{-1}$) at different loading rates of these substituted phenol mixture spiked in the refinery wastewater. Complete removal of all these three pollutants was achieved at the maximum individual loading rate of $569\,mg\,l^{-1}\,d^{-1}$. However, when individual loading rates of the substituted phenols exceeded $569\,mg\,l^{-1}\,d^{-1}$, the removal efficiency was gradually reduced in all the concentration combination tested. On other hand, the degradation rate was increased with the increase in loading rate of the individual pollutant, up to $683\,mg\,l^{-1}\,d^{-1}$. At a loading rate more than $683\,mg\,l^{-1}\,d^{-1}$, the degradation rates of 4-CP and 4-BP were reduced. Whereas, there was a prominent increase in the degradation rate of 4-NP at all the loading rates investigated. This further demonstrated the preferential uptake up 4-NP over the other two pollutants. In the present study, complete removal of the phenolics (BDL) and more than 97% COD removal was observed at an individual pollutant loading rate of $569\,mg\,l^{-1}\,d^{-1}$ (stage 1) when the concentration of individual pollutant was $250\,mg\,l^{-1}$ or the equivalent influent COD concentration of $1{,}268\,mg\,l^{-1}$ in the refinery wastewater mixture.

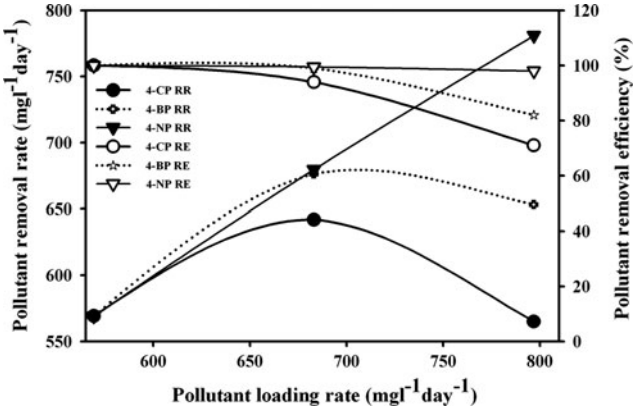

Fig. 5. Substituted phenols removal efficiency and biodegradation rate in the PBR at different loading rates (RR = Removal rate; RE = Removal efficiency).

3.6. Wastewater toxicity removal

The percentage (%) toxicity of influent refinery wastewater containing substituted phenol mixture at stage 1 (at $250\,mg\,l^{-1}$ each of 4-CP, 4-BP and 4-NP) was tested on mixed microbial consortia and found to be 84.23%. Percentage effluent toxicity and % toxicity removal with respect to the above-mentioned influent were estimated to be 4.5 and 94.65%, respectively.

In the present study, the lower COD and phenolic content of the refinery wastewater are well supported by several literature, for instance, COD value of about $200\,mg\,l^{-1}$ along with $20\,mg\,l^{-1}$ phenolic was estimated in refinery wastewater [23,24]. In the batch shake flask study, although individual concentration of the phenolics in the refinery wastewater was not measured, their combined COD was analyzed, and 1 gm of 4-CP, 4-BP, and 4-NP was found equivalent to 1.70, 1.46, and 1.53 gm of COD, respectively. In the batch experiments, the actinomycetes failed to remove COD from raw refinery wastewater (Fig. 1), as well as refinery wastewater diluted with distilled water. This might be due to deficiency of one or more components of MSM in the raw refinery wastewater essential for the growth of *A. chlorophenolicus* A6. The poor performance of the culture can also be attributed to the toxicity exerted by heavy metals present in the refinery wastewater as shown in Table 3. The enhancement of COD removal efficiency might be due to the supplementation of essential nutrient and buffer system for

effective growth of the micro-organism consequently degraded the pollutants. This is clearly indicated in Fig. 1, that when the raw refinery wastewater was not added with MSM, the phenolic degradation performance was found significantly low i.e. below 5%, on the other hand, when added with MSM, the degradation performance was improved significantly i.e. more than 98% when mixed with an equal volume of MSM. Further, addition of MCM causes wastewater dilution, which aided in reducing the toxicity due to the presence of inhibitory substances, such as lead, strontium, and chloride ions in the wastewater, for which *A. chlorophenolicus* A6 does not possess suitable enzymatic system to degrade and/or detoxify. This is well supported by several literatures that the presence of chloride ion and heavy metals inhibited microbial activity [25–27].

Previous studies with the PBR system showed that abiotic removal of the phenolics due to adsorption and volatilization was insignificant when compared with their removal due to biodegradation by *A. chlorophenolicus* A6 [17,19]. SEM characteristics of the immobilized biomass on the PUF also confirmed microbial colonization on the biosupport as well as the presence of dense biofilms embedded within a polymeric matrix. The colonies were also large sized and cocci shaped, typical of *A. chlorophenolics* A6 [17].

During the reactor operation at a higher stage, the removal efficiency was found to be reduced, especially for 4-CP and 4-BP (Fig. 2). The preferential uptake of 4-NP in the refinery wastewater may be reasoned based on the fact that the enzymes of *A. chlorophenolicus* A6 responsible for the phenolics degradation may possess a higher affinity towards 4- NP over the other two compounds. Alternatively, there could be transport-level interactions affecting degradation of the compounds. For example, if *A. chlorophenolicus* A6 takes up only the phenolate ion form of these substituted phenols, the difference in pKa values of the compounds would affect their order of degradation. 4-NP has a pKa value of 7.1, whereas, it is 9.3 and 9.17 for 4-CP and 4-BP, respectively, which essentially means that at pH 7.2–7.5 of the culture media typically used in these experiments, about 55% of the 4-NP was available in its dissociated form, i.e. as a phenolate ion, whereas, in case of 4-CP and 4-BP it was less than 2%. All these inferences could be posited for preferential degradation of 4-NP over the other halogenated substituted phenols. Further, simultaneous degradation of 4-CP and 4-BP in the mixed substrate system could be attributed to their similar bioavailability efficiency owing to their identical pKa values (9.41 and 9.37, respectively). This observation was also in agreement with the results obtained in batch kinetics single-substrate degradation system investigated earlier, where the value of half-saturation constant (K_s) for 4-CP was found to be 30.83 mg l^{-1}, in case of 4-NP, the value of the constant was lower i.e. 20.15 mg l^{-1} [18,28]. In the literature, preferential degradation of one aromatic compound over another in mixed substrate system has been reported for toluene and phenol degradation by *Pseudomonas putida* F1 strain [29]. The authors in that study observed that although the strain followed the same pathway for degrading toluene or phenol, when challenged with a mixture of these compounds, phenol degradation did not start until almost all toluene has been removed from the medium.

The performance of the PBR in treating the refinery wastewater is found superior to the bioreactor systems such as fixed film bioreactor, expanded anaerobic sludge bed reactor reported in the literature. Maximum COD removal percentages reported using these systems were 85 and 90%, respectively, for correspondingly influent COD concentrations of 912 and 1,500 mg l^{-1} [4,5]. Ochieng et al. [12] used a fluidized bed reactor system for treating refinery wastewater and observed only 34% organic degradation. Similar results were also reported by Holubar et al. [13] with 35.4% COD reduction from the refinery wastewater. However, the poor performance of the reactor at stage 3 (Fig. 2 and Table 2) could be attributed to the increased toxicity caused due to presence of high level of phenolic mixture. For example, phenolics exert their toxicity by uncoupling oxidative phosphorylation [30], and the formation of dimers between two different phenolic compounds can result in an uncoupling activity that is higher than the sum of these single compounds Escher et al. [31]. In addition, HPLC-MS analysis confirmed the transient accumulation of toxic intermediate products such as 4-chlorocatechol, hydroxyl-quinol from 4-CP, and 4-nitrocatechol along with hydroxyl-quinol from 4-NP (Fig. 3(a) and (b)) when the reactor was operated at higher pollutants loading rate. Thus, the inferior performance at stage 3 of the reactor run (350 mg l^{-1} each of the three substituted phenols) could be attributed to the combined toxic effect of 4-chlorocatechol, chloride, and the presence of high-level phenolic mixture (Fig. 3). This is well supported by literature that the accumulation of chlorinated catechol and even the presence of 100 mg l^{-1} of chloride not only increased the toxicity on growth of the micro-organism but also enhanced the resistance of the aromatic ring for biodegradation by *Sphingomonas* as well as *Arthrobacter* species [26,27,32]. Furthermore, it is reported that, the formation of different intermediates and their combined toxic effects might have inhibited secretion of enzymes vital for their

degradation and/or competition for uptake into the cells [25]. In addition, plausible reason for inferior degradation performance of the PBR towards 4-CP could be due to toxicity exerted by the higher concentration of chloride ion (740 mg l^{-1}) present in the raw refinery wastewater (Table 3). Similar observation is also reported in literature for 2,4-DNT removal by mixed culture that when the pollutant loading rate was increased from 19 to 60 mg l^{-1} d^{-1}, the bioreactor performance dropped steadily from 85 to 65% [33]. Rezouga et al. [34] reported 99% removal of 4-NP in an aerobic stirred tank bioreactor from an influent 4-NP concentration of 528.73 mg l^{-1}. However, when the influent 4-NP concentration increases to more than 528.73 mg l^{-1}, the reactor performance was found to be reduced significantly. In the present study, better 4-NP degradation performance achieved may be due the fact that the major intermediate product formed during 4-NP degradation i.e. 4-nitrocatechol exerted less toxic effect on the growth of *A. chlorophenolicus* A6 in comparison with 4-chlorocatechol [35,36].

The improved efficiency of the PBR system is mainly attributed to the enhancement in hydrodynamic conditions and gas liquid superficial flow velocity in the newly designed PBR [17] consequently facilitated better diffusion of the carbon source (substituted phenols) and oxygen deep inside the biosupport material (PUF), which guided higher biomass growth and pollutants degradation rate. The biomass concentration (416.5 mg of MLSS per gram of PUF) estimated in this study was also much higher than the literature-reported value of 136 mg MLSS g^{-1} of PUF obtained in a fixed bed reactor treating polychlorinated biphenyl [37]. Further, the toxicity associated with the refinery wastewater spiked with the phenolic compounds was also well removed using the PBR system. In the present study, the toxicity removal profile is found to be better than that obtained by other researchers treating nitrophenol and chlorophenol even at lower influent concentrations of 10 and 390 mgl^{-1}, respectively, where they achieved only 72 and 70% toxicity removal of 4-CP and 4-NP, respectively [38,39].

4. Conclusion

This study demonstrated effective biodegradation of phenolics in refinery wastewater by *A. chlorophenolicus* A6 using the PBR system. The best performance i.e. 99% pollutant removal and without accumulation of any toxic intermediate was achieved at a combined optimum pollutants loading rate of 1,707 mg l^{-1} d^{-1}. Further, at this optimum loading rate removal of 94% effluent toxicity proved the potential of the PBR system not only for continuous degradation of refinery wastewater spiked with substituted phenolics mixture but also for the removal of toxicity associated with the refinery wastewater. Therefore, the present bioreactor system with immobilized *A. chlorophenolicus* A6 cells could be implemented for efficient treatment of real refinery wastewater that is discharged with higher level of COD and phenolic content in the range of 500–2,123 mg l^{-1} as reported in many literatures [1,4,5,40].

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