Effects of phenol on sulfate reduction by mixed microbial culture: kinetics and bio-kinetics analysis

Mohit Prakash Mohanty, Bharati Brahmacharimayum and Pranab Kumar Ghosh

ABSTRACT

Mixed microbial culture collected from the wastewater treatment plant of Indian Institute of Technology Guwahati (IITG) was further grown in anaerobic condition in presence of sulfate where lactate was added as a carbon source. Sulfate addition was increased stepwise up to 1,000 mg l^{-1} before phenol was added at increasing concentrations from 10 mg l^{-1} to 300 mg l^{-1}. Kinetics of sulfate, phenol and chemical oxygen demand reduction were studied and experimental findings were analyzed using various bio-models to estimate the bio-kinetic coefficients. This is the first detailed report on kinetics and bio-kinetic studies of sulfate reduction in presence of phenol. Experimental results showed that there was no inhibition of sulfate reduction and microbial growth up to 100 mg l^{-1} phenol addition. However, inhibition to different degrees was observed at higher phenol addition. The experimental data of microbial growth and substrate consumption in presence of phenol fitted well to the Edward model ($R^2 = 0.85$, root mean square error = 0.001011) with maximum specific growth rate = 0.052 h^{-1}, substrate inhibition constant = 88.05 mg l^{-1} and half saturation constant = 58.22 mg l^{-1}. The characteristics of the cultured microbes were determined through a series of analysis and microbial tests.

Key words | bio-kinetic coefficients, mixed microbial consortia, reaction rate coefficients, maximum specific growth rate, substrate inhibition constant

Mohit Prakash Mohanty
Pranab Kumar Ghosh (corresponding author)
Department of Civil Engineering,
Indian Institute of Technology Guwahati,
Guwahati-781039
India
E-mail: *pkghosh@iitg.ernet.in*

Bharati Brahmacharimayum
Centre for the Environment,
Indian Institute of Technology Guwahati,
Guwahati-781039
India

INTRODUCTION

Phenol and its compounds, owing to toxicity, have been categorized in the list of priority pollutants by the US Environmental Protection Agency (Huang *et al.* 2014; Yagmur *et al.* 2017). This toxicity is related to two main processes: (i) unspecified toxicity related to hydrophobicity of the individual compound and (ii) formation of free radicals. The concentration of phenol in surface water generally varies between 0.01 and 2.0 μg l^{-1}. The sources of phenol include oil refineries, chemical plants, explosives, resins, coke manufacture, coal conversion plants, pesticide and textile industries (Wang *et al.* 2014; Wirth *et al.* 2015). These compounds have high stability, high toxicity and are carcinogenic in nature even at low concentrations. Hence, phenol-containing wastewater needs careful handling before it is discharged to the receiving water bodies. The bio-reduction using micro-organisms is a viable and well proven method for phenol remediation (Wu *et al.* 2016; Kiliç & Dönmez 2017). However, wastewaters containing phenol in the range of 5–500 mg l^{-1} are generally considered suitable for treatment by biological processes (Monteiro *et al.* 2000). The utilization of phenol as a substrate can be accomplished when the microbial mass can be acclimatized to it for a sufficient time. Although it is toxic, phenol can be utilized as a carbon source by various micro-organisms. For example *Pseudomonas putida* (Ray & Banerjee 2015; Kurzbaum *et al.* 2017; Lin *et al.* 2017), *Candida tropicalis* (Basak *et al.* 2013; Phalgune *et al.* 2013; Mahgoub *et al.* 2014), *Acinetobacter calcoaceticus* (Jiang *et al.* 2013; Lin 2017), and *Alcaligenes eutrophus* (Ahmad *et al.* 2017) have been widely used to study the biodegradation of phenol. However since isolation of single strains is both difficult and limited in field applications, it is urged that mixed microbial consortia could be used for the biodegradation of phenol (Ray & Banerjee 2015). In sulfate bio-reduction, sulfate-reducing bacteria (SRB) utilize sulfate as the electron acceptor in the presence of a suitable electron donor, preferably a carbon source. So

doi: 10.2166/wst.2017.630

far various organic compounds such as lactate, acetate, methanol, ethanol, and molasses have been used as electron donors to study the effect on sulfate reduction (Jing *et al.* 2013; Mattei *et al.* 2014; Xu *et al.* 2014). Phenolics along with sulfate appearing in various industrial sources such as coke oven plants, paper mills, pharmaceuticals, and tanneries pose a huge environmental hazard and hence remediation of such pollutants becomes necessary. However, the investigation of sulfate reduction in presence of phenol is limited. Boopathy (1985) studied sulfate reduction using phenol as a sole carbon source and a sulfate-reducing bacterium obtained from swine manure, which could degrade 51.7 mg l^{-1} (0.55 mM) of phenol. Mort & Dean Ross (1994) found sulfate reduction abilities in bacteria obtained from freshwater river sediments, which consisted of SRB, when phenol was introduced as the carbon source without any prior acclimation. However, the biodegradative phase for phenol reduction by acclimated cultures varied from 25 to 40 days. Lin & Lee (2001) studied sulfate reduction in an anaerobic bio-film process composed of mixed culture of SRB and phenol-utilizing bacteria (PUB). They described that simultaneous removal of phenolic compounds and sulfate follows metabolic pathways in which biodegradation of phenolic compounds to simple organic acids was carried out by PUB, while bioreduction of sulfate to sulfide was carried out by SRB by utilizing simple organic compounds as the electron donor and sulfate as the terminal electron acceptor. To describe substrate biodegradation, it is necessary to evaluate the kinetics and biokinetics. While kinetics deals with the study of substrate degradation through determination of various reaction rate coefficients, bio-kinetics involves determining a relationship between the specific growth rate and the substrate concentration, which affects the biodegradation of any pollutant. Knowledge of such behavior is necessary for improvements in the process control and removal efficiency.

The aim of the present study is to investigate the effect of phenol on sulfate reduction using mixed microbial consortia. Based on the results obtained, kinetic studies pertaining to sulfate, chemical oxygen demand (COD) and phenol reduction were analyzed. In addition, bio-kinetic coefficients were evaluated using different existing models.

MATERIALS AND METHODS

Seed sludge enrichment

A mixed microbial culture (total solids = 20.506 g l^{-1}, mixed liquor volatile suspended solids, MLVSS = 16.5 g l^{-1}) was obtained from the wastewater treatment plant of the Indian Institute of Technology (IIT) Guwahati. The culture was grown in a 1-litre Erlenmeyer flask by adding lactate and sulfate. The composition of the media with major nutrients in Milli-Q water was (in g l^{-1}): KH_2PO_4, 0.5; K_2HPO_4, 0.1; $NaHCO_3$, 0.5–0.8; NH_4Cl, 0.1. To this, 1 ml trace nutrient was added to 1 l of media. The composition of trace nutrient media was (g l^{-1}): $NaMo_4.2H_2O$, 0.03; $CaCl_2.2H_2O$, 0.05; $CoCl_2.6H_2O$, 0.02; KCl, 0.05; $MgCl_2.6H_2O$, 0.05; $NiSO_4.6H_2O$, 0.01; $C_6H_7NaO_6$, 0.05 and $C_2H_3NaO_2S$, 0.05. The culture was kept under constant agitation at 180 rpm and temperature of 30 ± 0.2 °C. After due course of time, the culture from the flask was again transferred into a 500 ml aspirator bottle and fed with the same nutrients as described above for a further acclimation phase. This was marked as 'seed sludge' in which the MLVSS was found to be 1,224 mg l^{-1}. During this growth period phenol was not added to the culture.

Analytical methods

Samples drawn from the culture vials were kept inside centrifuge tubes in triplicate for determining various parameters. Dissolved sulfide was determined as per the procedure described by Cord-Ruwisch (1985). Biomass cell concentration was quantified by measuring optical density at 600 nm using a UV/visible spectrophotometer (Model: lambda 45, Perkin Elmer, USA) with distilled water as the reference. The pH of the samples was measured using a digital pH meter (Thermo Scientific, Orion 3 Star, USA). Samples meant for residual sulfate, COD and phenol determination were drawn in separate centrifuge tubes in triplicate. All these samples were centrifuged at 8,000 rpm for 5 minutes to remove the cell debris for further analysis. For sulfate determination, 0.2 ml of zinc acetate and 0.1 ml of NaOH were added to fix the sulfide in the mixture (Somasundaram *et al.* 2009), while samples meant for COD determination were acidified with 2–3 drops of H_2SO_4 and purged with N_2 gas instead of stirring, to release the sulfides present as gaseous sulfide (Sabumon 2008). To reduce the interference of sulfur compounds during phenol estimation, samples were acidified to pH 4.0 by adding H_3PO_4 and stirring briefly. Sulfate was measured by turbidimetric method, COD was measured by closed reflux titrimetric method and phenol was measured using direct photometric method (APHA 2005). To identify the nature of bacteria in the culture, samples were diluted and stained stepwise with crystal violet, iodine solution, 95% ethanol and safranine on a glass plate. The slides were left for air drying for

15 minutes after which they were tested under an upright fluorescence microscope (model: CX 41, Olympus, Japan). The bacterial culture for field emission scanning electron microscopy (FESEM) was fixed with 2.5% glutaraldehyde and then washed with distilled water thrice. The washed sample was then sequentially dehydrated using acetone with concentrations increasing from 30% to 90% in 15% increments, with 10 min exposure time. Finally, the sample was dehydrated with 100% acetone. After dehydration, the dried sample was gold sputtered and then examined under FESEM Σ (Sigma), Carl Zeiss, Germany. For identification of the bacterial colony, the microbial culture was sent to geneOmbio Technologies Pvt. Ltd, Mumbai, India, for terminal restriction fragment length polymorphism (TRFLP) analysis. Using the primers specific to 16S rDNA from the bacterial genome, PCR (polymerase chain reaction) amplification was carried out. The genemapper analysis provided an electropherogram with varied peaks for different bacterial species. The online library tool was used to identify the organisms characterized by the peaks.

Sulfate bio-reduction in batch cultures

Table 1 gives a description of the batch studies carried out in this experimental work. About 10% (v/v) of the mixture from the aspirator bottle containing acclimatized sludge was transferred into eight 1-l aspirator bottles to get around $400 \pm 50\ mg\ l^{-1}$ of biomass concentration (as MLVSS). Phenol of varied concentrations starting from $10\ mg\ l^{-1}$ to $300\ mg\ l^{-1}$ were added into these bottles, while the one with $0\ mg\ l^{-1}$ phenol served as the reference. The contents in aspirator bottles were purged with nitrogen gas to maintain anaerobic conditions. The initial pH was adjusted to 7.0 ± 0.2. The contents from each bottle were then transferred into 60 culture vials (in triplicate) of 15 ml capacity under constant stirring and preventing any prolonged exposure to air. The culture vials thus served as self-sacrificing small batch reactors and were kept in a temperature-controlled horizontal shaker at 180 rpm and $30 \pm 0.2\ ^{\circ}C$. For determination of various parameters as listed in the 'Analytical methods' section, any three tubes were drawn from the shaker at an interval of 24 hours and various parameters were investigated.

Table 1 | Batch studies in presence of phenol

	Initial parameters ($mg\ l^{-1}$)			COD/sulfate		
Batch test	Lactate COD	Sulfate	Phenol	Lactate COD/sulfate	Phenol COD/sulfate	Total COD/ sulfate
BT-1	1,500	1,000	0	1.5	0	1.5
BT-2	1,500	1,000	10	1.5	0.02	1.52
BT-3	1,500	1,000	35	1.5	0.08	1.58
BT-4	1,500	1,000	50	1.5	0.12	1.62
BT-5	1,500	1,000	100	1.5	0.24	1.74
BT-6	1,500	1,000	150	1.5	0.36	1.86
BT-7	1,500	1,000	225	1.5	0.53	2.03
BT-8	1,500	1,000	300	1.5	0.71	2.21

Sulfate bio-reduction and phenol inhibition kinetics

The experimental data for sulfate, COD and phenol reduction were fitted to zero, first and second order reactions. The values of degradation rate obtained from these models gave an idea of the extent of utilization of phenol and lactate for sulfate reduction. For each batch culture with a certain initial phenol concentration, the specific growth rate (μ, h^{-1}) was measured by performing a least squares regression on the semi-logarithmic plot of biomass growth (X, mgl^{-1}) over time (t, h) in the exponential growth phase. Since phenol was a very significant parameter which affected the growth rate and hence sulfates reduction, a plot of μ (h^{-1}) vs phenol concentration ($mg\ l^{-1}$) was developed using various models, Haldane, Webb, Yano, Edward, Aiba and Andrews, in MATLAB 7.0.

RESULTS AND DISCUSSION

Time series and kinetics of sulfate bio-reduction at different phenol concentrations

Sulfate degradation profile with time in the batch cultures is shown in Figure 1. It was noticed that the sulfate concentration reduced by more than 80% within 8 days of batch analysis up to $100\ mg\ l^{-1}$ phenol addition. The highest (88%) and lowest (56%) sulfate reduction efficiencies were noticed in batch cultures containing an initial phenol concentration of 100 and $300\ mg\ l^{-1}$. Such efficiency relies on the extent of utilization of phenol and lactate by the mixed microbial consortia as substrates for sulfate reduction. It was further observed that in batch cultures containing 225 and $300\ mg\ l^{-1}$ phenol, sulfate could be used up less efficiently compared to the other concentrations, probably due to these higher doses of phenol imposing an inhibition effect on the sulfate reducers. This observation was also reflected by the fact that even after 192 hours of batch

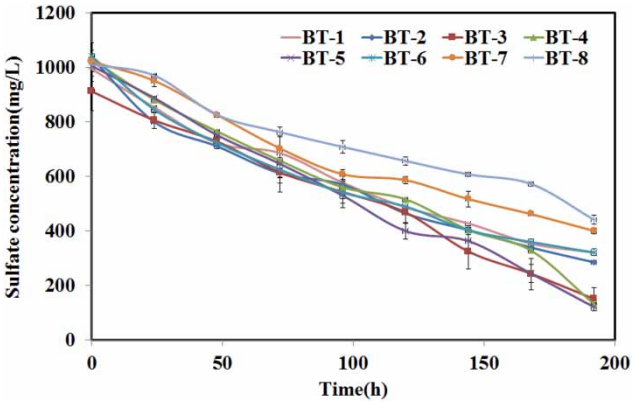

Figure 1 | Time series plot of sulfate reduction at different initial phenol concentrations.

analysis, around $440 \pm 16\ mg\ l^{-1}$ of sulfate concentration still appeared in the batch culture containing $300\ mg\ l^{-1}$ phenol.

To study the kinetics of sulfate reduction, sulfate concentration over time was fitted to various orders of reaction (e.g. zero, first and second) and reaction rate coefficients were determined. It was noticed that the experimental results fitted best to zero order reaction. Table 2 gives the reaction rate coefficients obtained after kinetic analysis. The reaction rate coefficient was found to be the maximum in the case of batch culture containing an initial phenol concentration of $100\ mg\ l^{-1}$ and then declined reaching a minimum of $2.8\ mg\ l^{-1}\ h^{-1}$ in the culture having initial phenol concentration of $300\ mg\ l^{-1}$. The sulfate-reducing potential and reaction rate coefficient thus proved that a concentration of $100\ mg\ l^{-1}$ could give better sulfate-reducing efficiency compared to the other conditions. These results also indicate that beyond a certain phenol concentration of $100\ mg\ l^{-1}$, sulfate reduction efficiencies declined due to inhibition of phenol concentration on the mixed microbial culture for reduction of sulfate. Such observations were also noticed by García-Cruz *et al.* (2010), where acute inhibition to the sulfate-reducing bio-film in terms of IC_{50} (half maximal inhibitory concentration) was observed at $143.8\ mg\ l^{-1}$ phenol.

Table 2 | Kinetics of sulfate reduction with various reaction rate coefficients

Batch test	Equation of plot	Coefficient of determination (R^2)	Reaction rate coefficients ($mg\ l^{-1}\ h^{-1}$)
BT-1	$y = -3.4648x + 935.03$	0.9762	3.46
BT-2	$y = -3.5753x + 924.19$	0.9519	3.57
BT-3	$y = -3.9572x + 911.63$	0.9968	3.95
BT-4	$y = -4.2441x + 992.84$	0.9849	4.24
BT-5	$y = -4.4716x + 981.08$	0.9933	4.47
BT-6	$y = -3.5449x + 934.33$	0.9466	3.54
BT-7	$y = -3.2515x + 986.83$	0.9684	3.25
BT-8	$y = -2.7932x + 996.16$	0.9735	2.79

Phenol reduction and kinetics at initial concentrations

As described earlier in the 'Sulfate bio-reduction in batch cultures' section, phenol was added at increasing concentrations starting from 10 to $300\ mg\ l^{-1}$. The phenol utilization capability of the mixed microbial consortia is well reflected in Figure 2, where phenol could be completely degraded when present at an initial concentrations starting from 0 to $100\ mg\ l^{-1}$ in BT-1 to BT-5. However, in the successive batch tests (phenol concentration: 150, 225 and $300\ mg\ l^{-1}$), phenol concentration of 41.6 ± 0.58, 137 ± 0.86 and $237 \pm 2.12\ mg\ l^{-1}$, respectively, still remained in the system after 8 days of batch study. In the case of batch cultures having the highest phenol concentrations of 225 and $300\ mg\ l^{-1}$, the phenol concentration measured between 168 and 192 hours did not decrease markedly and remained relatively constant. The phenol degradation in all the cultures followed zero order kinetics and the values of reaction rate coefficients increased up to the one containing an initial phenol concentration of $100\ mg\ l^{-1}$ after which it decreased (Table 3). Several researchers also observed that phenol reduction under aerobic conditions followed zero order kinetics in which the reaction rate coefficient decreased with increasing phenol after a certain concentration which proved inhibitory (Lim *et al.* 2013). It was observed that the degradation rates achieved in this study was higher than those reported by Kumar *et al.* (2011). However Wang *et al.* (1988) reported faster phenol biodegradation rates,

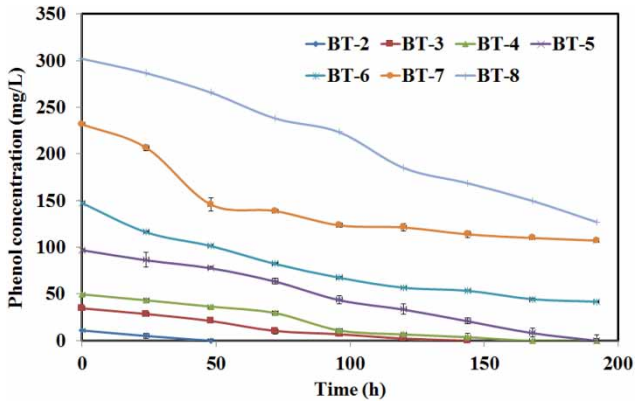

Figure 2 | Time series plot of phenol reduction at different initial phenol concentrations.

Table 3 | Kinetics of phenol reduction with various reaction rate coefficients

Batch test	Equation of plot	Coefficient of determination (R^2)	Reaction rate coefficients (mg l^{-1} h^{-1})
BT-2	$y = -0.2288x + 10.836$	0.9978	0.22
BT-3	$y = -0.2508x + 32.792$	0.9646	0.25
BT-4	$y = -0.3214x + 49.486$	0.9569	0.32
BT-5	$y = -0.5319x + 99.043$	0.9932	0.53
BT-6	$y = -0.5224x + 128.77$	0.9155	0.52
BT-7	$y = -0.4957x + 227.47$	0.9652	0.49
BT-8	$y = -0.326x + 303.71$	0.9926	0.32

i.e. 0.5–7 mg l^{-1} h^{-1}, while using a phenol-enriched methanogenic culture under anaerobic conditions in batch cultures. Such higher degradation rates were possible since the culture derived from a continuous fermentor was previously acclimated with 4,000 mg l^{-1} phenol for 30 days. Rosenkranz *et al.* (2013) obtained a phenol degradation rate as high as 1.47 ± 0.02 mg l^{-1} h^{-1} at a concentration of 200 mg l^{-1} phenol under anaerobic conditions while using an inoculum, obtained from a tobacco wastewater treatment plant in glass bottles, after it had been acclimated with increasing phenol concentrations up to 200 mg l^{-1} for over 100 days. The degradation rates obtained in this study thus show good potential abilities of the mixed microbial consortia for phenol degradation.

COD reduction and kinetics at different phenol concentrations

COD was measured as total COD (COD due to lactate and phenol). The time series profile for COD reduction is shown in Figure 3. The COD utilization reduced as the phenol concentration was increased beyond 100 mg l^{-1}. COD reduction efficiencies reached as high as $75 \pm 2\%$ in batch culture containing phenol concentration of 100 mg l^{-1} after which it decreased to a minimum of 55% in batch culture containing phenol concentration of 300 mg l^{-1}. This observation leads to the fact that, probably due to inhibition caused by higher concentration of phenol, sulfate reducers were unable to utilize the available substrate. The kinetics study for COD reduction showed that it followed zero order kinetics with maximum degradation rate observed in the case of batch culture containing phenol concentration of 100 mg l^{-1}, which declined with increasing phenol concentrations (Table 4). Liu *et al.* (2002) used a sequencing aerobic sludge blanket reactor to determine the utilization

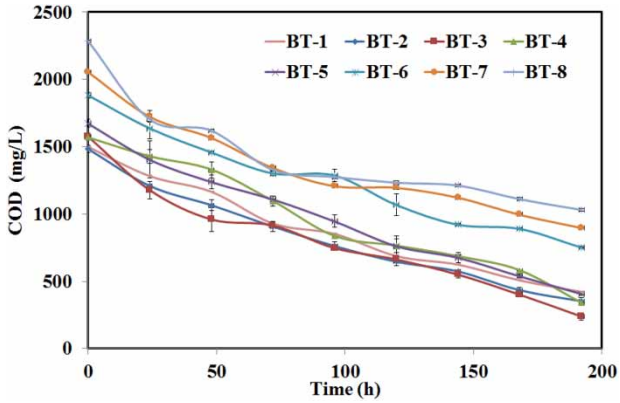

Figure 3 | Time series plot of COD reduction at different initial phenol concentrations.

of acetate in presence of phenol by an acetate-fed aerobic granular sludge. They observed that as the ratio of initial concentration of phenol to initial concentration of biomass was increased from 0 to 0.19 mg phenol/mg MLSS, the zero order rate coefficient of acetate decreased from 1.15 to 0.38 mg l^{-1} min^{-1}. It therefore proves that addition of phenol beyond 100 mg l^{-1} has a significant inhibitory effect on substrate utilization required for sulfate reduction.

Biomass growth at different phenol concentrations

The growth of biomass against time is represented in Figure 4. The absence of a lag phase in the time series plot reveals that the mixed microbial mass could readily utilize phenol for growth. The highest growth rate of biomass was observed in batch culture containing phenol concentration of 100 mg l^{-1}. In this case, the biomass concentration reached as high as $1{,}630 \pm 4.2$ mg l^{-1} from initial value of 417 ± 10 mg l^{-1}, showing almost 75% increase. However, the percentage growth of biomass over

Table 4 | Kinetics of COD reduction with various reaction rate coefficients

Batch test	Equation of plot	Coefficient of determination (R^2)	Reaction rate coefficients (mg l^{-1} h^{-1})
BT-1	$y = -5.5167x + 1{,}416.6$	0.9765	5.51
BT-2	$y = -5.6252x + 1{,}366$	0.9741	5.62
BT-3	$y = -6.0654x + 1{,}502.7$	0.9901	6.06
BT-4	$y = -6.3061x + 1{,}563.7$	0.9793	6.30
BT-5	$y = -6.347x + 1{,}582.8$	0.9877	6.34
BT-6	$y = -5.594x + 1{,}781.7$	0.9732	5.59
BT-7	$y = -5.4489x + 1{,}869.4$	0.9254	5.44
BT-8	$y = -5.3301x + 2{,}062.5$	0.9154	5.33

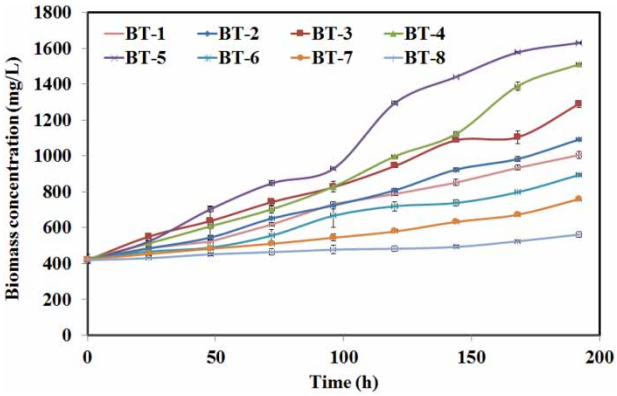

Figure 4 | Time series plot of biomass growth at different initial phenol concentrations.

time declined in the successive batch tests and reached a minimum in the culture containing 300 mg l^{-1}. It could be also inferred from Figure 5 that phenol concentration between 10 and 150 mg l^{-1} did not impose any significant repression on the biomass growth. However, at concentration of 225 mg l^{-1}, a prominent lag phase was observed which co-related with the delay in the utilization of phenol by the bacterial culture. Thus, it could be concluded that phenol concentration above 150 mg l^{-1} exerted a significant toxic effect on the culture growth.

Sulfate, phenol and COD reduction at different phenol concentrations

To discuss the nature of sulfate, phenol and COD reduction in batch cultures at varied initial phenol concentrations, a surface plot was fitted between these variables, as shown in Figure 5(a) and 5(b) under two different cases. The nature of degradation was explained by fitting a polynomial as mentioned below.

For case (a): Phenol reduction reaction rate coefficient at varied initial phenol concentrations, with corresponding COD reduction reaction rate coefficient:

$$f(x, y) = p_{00} + p_{10}x + p_{01}y + p_{20}x^2 + p_{11}xy + p_{02}y^2 \quad (1)$$

$f(x, y)$, is the phenol reduction reaction rate coefficient (mg l^{-1} h^{-1}), with x and y representing different initial phenol concentrations starting from 0 to 300 mg l^{-1} and COD reduction reaction rate coefficient (mg l^{-1} h^{-1}), respectively; p_{ij}, where i, j = 0, 1, 2 is the individual variable coefficient.

$$p_{00} = 0.1639,\ p_{10} = 0.004928,\ p_{01} = 0.107,$$
$$p_{20} = -1.37 \times 10^{-5},\ p_{11} = -3.137 \times 10^{-5} \text{ and } p_{02} = 0.02304$$

For case (b): Phenol reduction reaction rate coefficient at varied initial phenol concentrations, with corresponding sulfate reduction reaction rate coefficient:

$$f(x, y) = p_{00} + p_{10}x + p_{01}y + p_{20}x^2 + p_{11}xy \quad (2)$$

where $f(x, y)$, is the phenol reduction reaction rate coefficient (mg l^{-1} h^{-1}), with x and y representing different initial phenol concentrations starting from 0 to 300 mg l^{-1} and sulfate reduction reaction rate coefficient (mg l^{-1} h^{-1}), respectively; p_{ij}, where i = 0, 1, 2 and j = 0, 1 is the individual

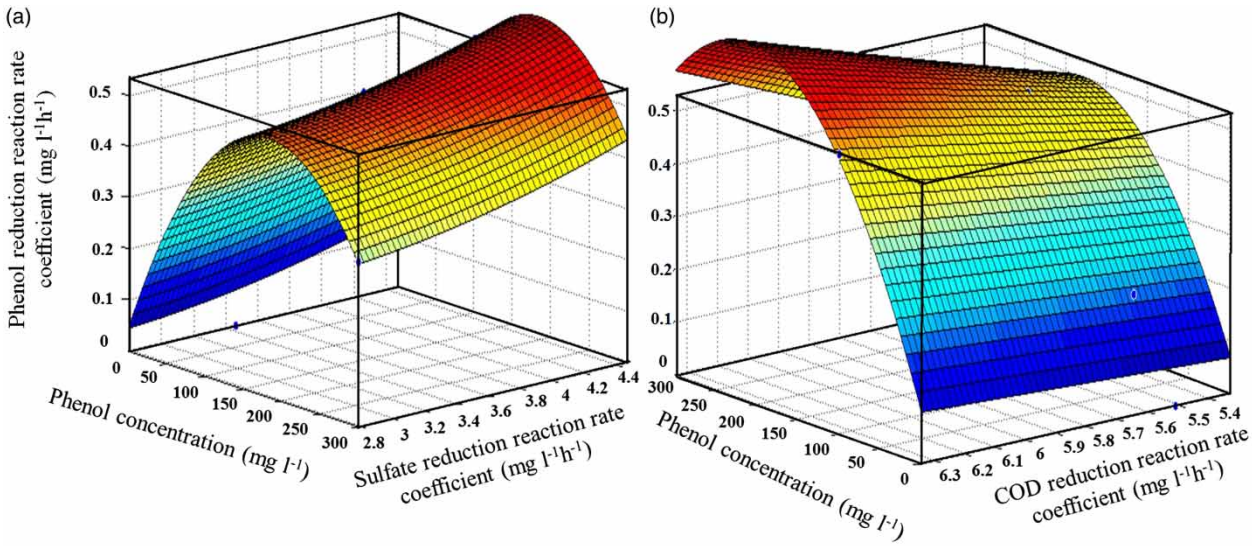

Figure 5 | Surface plots for (a) phenol and sulfate reduction reaction rate coefficients, and (b) phenol and COD reduction reaction rate coefficients with varied concentrations of phenol.

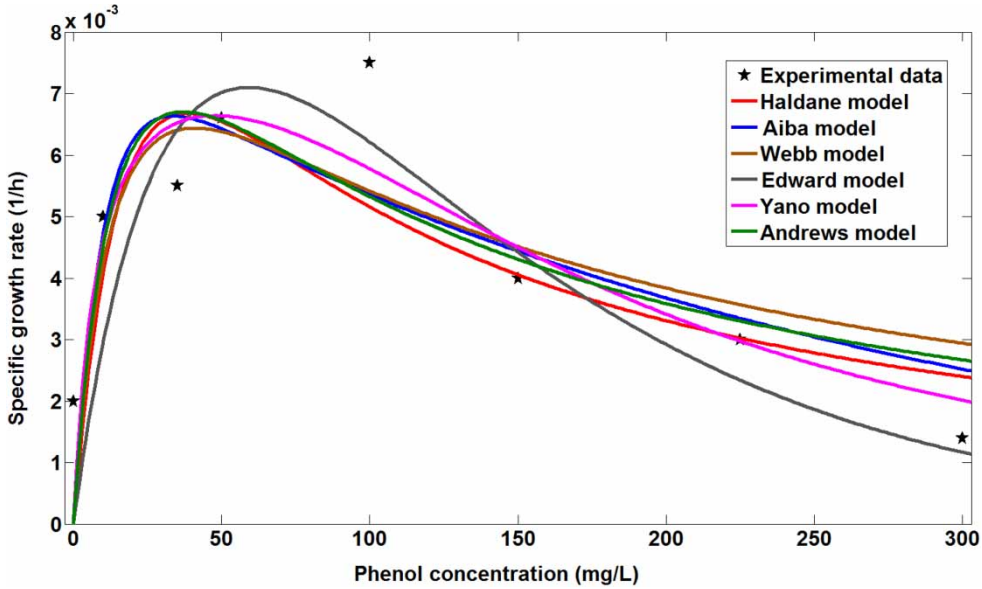

Figure 6 | Bio-kinetic analysis using various bio-kinetic models at different initial phenol concentrations.

variable coefficient.

$$p_{00} = -0.08941,\ p_{10} = 0.0006846,\ p_{01} = 0.02949,$$
$$p_{20} = -1.37 \times 10^{-5},\ p_{11} = 0.0007625$$

It was observed that the phenol reduction reaction rate coefficient increased with the corresponding sulfate and COD reduction reaction rate coefficients, with increasing phenol concentrations in the batch culture up to a concentration of 100 mg l^{-1}. However, a further increase in phenol concentration decreased the reaction rate coefficients, thus proving inhibitory.

Bio-kinetic modeling and estimation of bio-kinetic coefficients

In order to establish the effect of phenol on the biomass growth abilities, specific growth rates of the culture at different initial phenol concentrations were calculated as per the following relationship:

$$\mu = \frac{1}{X}\frac{dX}{dt}$$

where X is biomass concentration (mg l^{-1}) at time t (h) and μ is the specific growth rate (h^{-1}).

The observation made on biomass growth due to phenol inhibition was modeled using a suitable substrate inhibition model described in literature. During the course of batch studies, it was observed that the specific growth rate increased with addition of up to 100 mg l^{-1} phenol and then decreased in the successive batch tests. These models were analyzed in MATLAB 7.0 to give an idea of the extent and effect of phenol concentration on the mixed microbial culture. The experimental specific growth rate and predicted values using various models are presented in Figure 6. Table 5 gives the detailed values of various bio-kinetic coefficients obtained using the models along with the root mean square error (RMSE) between the predicted and experimental values. The Haldane model ($R^2 = 0.81$, RMSE = 0.001321) was able to describe the bio-kinetic study; however, the Edward model proved to be a better fit ($R^2 = 0.85$, RMSE = 0.001011). The values of the inhibitory constants given in Table 5 showed a good tolerance of the mixed microbial culture to grow in presence of phenol, and hence proceed to sulfate reduction in the process.

Bacterial images and Gram staining

The mixed bacterial mass was observed in FESEM Σ (Sigma, Carl Zeiss) Germany in the Central Instruments Facility, IIT Guwahati. A cluster of bacterial cells, mostly rod shaped, was observed at different magnifications, whose size ranged from 1.312×0.43 μm to 1.53×0.54 μm (Figure 7(a)–7(c)). Gram staining of the culture was done and is shown in Figure 7(d). The bacteria retained the stain and appeared purple when viewed under an oil immersion microscope and hence proved to be Gram-negative. For identification

Table 5 | Bio-kinetic parameters for various models

Model	Model description	μ_{max} (h^{-1})	K_s ($mg\,l^{-1}$)	K_i ($mg\,l^{-1}$)	K ($mg\,l^{-1}$)	R^2	RMSE
Haldane	$\mu_g = \frac{\mu_m S}{K_s + S + \frac{S^2}{K_i}}$	0.04	58.22	36.25	–	0.81	0.001321
Edward	$\mu_g = \mu_m \left[\exp\left(\frac{-S}{K_i}\right) - \exp\left(\frac{-S}{K_s}\right)\right]$	0.052	58.22	88.05	–	0.85	0.001011
Webb	$\mu_g = \frac{\mu_m S\left(1 + \frac{S}{K}\right)}{K_S + S + \frac{S^2}{K_i}}$	0.026	53.67	48.5	150.4	0.63	0.002544
Aiba	$\mu_g = \frac{\mu_m S\left\{\exp\left(\frac{-S}{K_i}\right)\right\}}{K_s + S}$	0.03	56.45	52.15	–	0.786	0.002325
Yano	$\mu_g = \frac{\mu_m S}{K_S + S + \left\{\frac{S^2}{K_i\left(1 + \frac{S}{K}\right)}\right\}}$	0.045	48.65	100.6	103.2	0.82	0.00135
Andrews	$\mu_g = \frac{\mu_m}{\left\{1 + \frac{K_s}{S} + \frac{S}{K_i}\right\}}$	0.040	52.872	112.34	–	0.808	0.00187

μ_g: specific growth rate (hr^{-1}), μm: maximum specific growth rate (hr^{-1}), S: substrate concentration ($mg\,l^{-1}$), K_s: half velocity constant ($mg\,l^{-1}$), K_i: substrate inhibition constant ($mg\,l^{-1}$), K: Webb constant and Yano constant ($mg\,l^{-1}$) respectively.

of organisms present in the mixed bacterial mass, the culture was subjected for TRFLP analysis. The TRFLP analysis is a culture-independent, rapid, sensitive and reproducible method of assessing diversity of complex communities without the need for any genomic sequence information. The presence of SRB such as *Desulfarculus*, *Pseudomonas*,

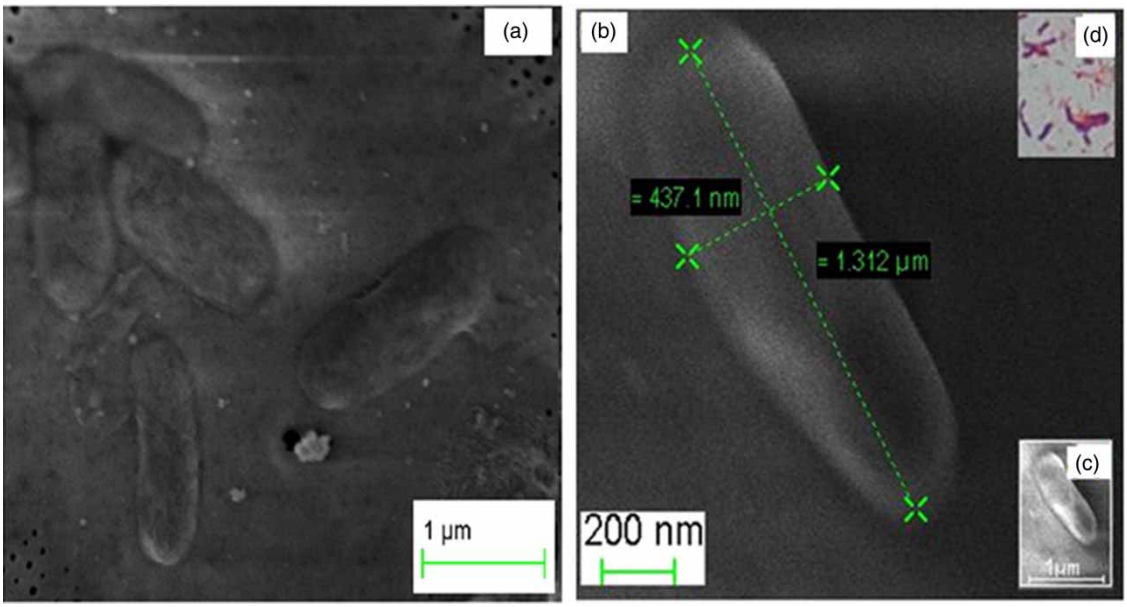

Figure 7 | Images of mixed microbial culture using FESEM at a magnification of (a) 39 KX, (b) 69.45 KX, (c) 14.01KX. (d) Gram staining image.

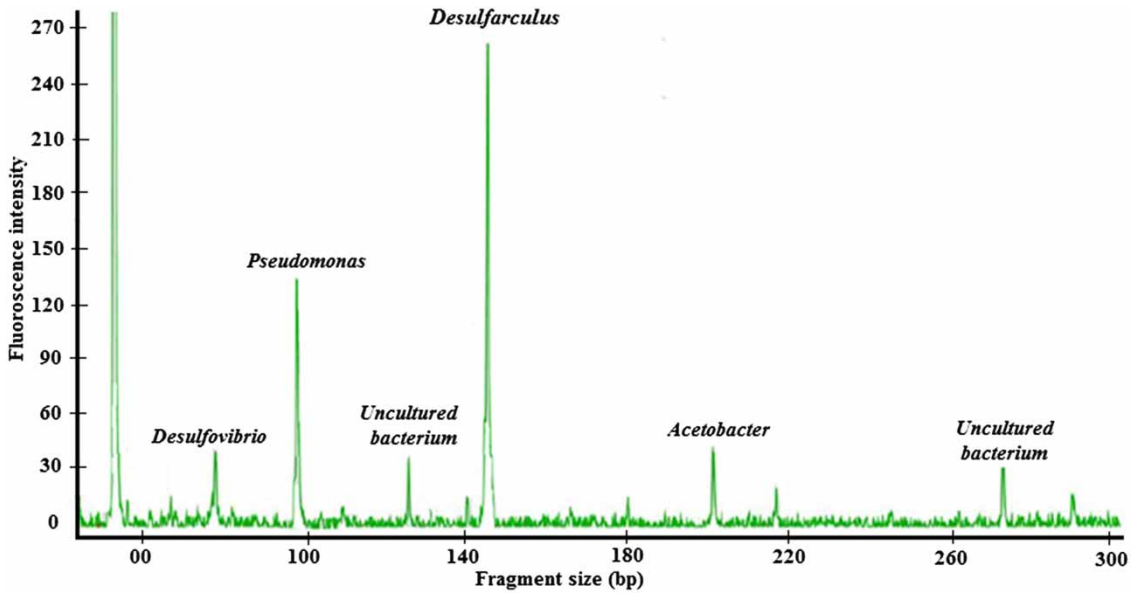

Figure 8 | TRFLP analysis of bacterial sample.

Desulfovibrio and *Acetobacter* species was noticed in the bacterial mass as shown in Figure 8.

CONCLUSION

Sulfate reduction behavior was investigated using mixed microbial consortia in presence of phenol. As observed from kinetic studies, higher concentrations of phenol affected the process of sulfate and COD reduction. The highest sulfate, phenol and COD reduction efficiencies were observed at 100 mg l^{-1} phenol concentration. The substrate inhibition due to phenol was explained using various models, among which the Edward model described the phenomenon with maximum specific growth rate of 0.052 h^{-1} and substrate inhibition constant of 88.05 mg l^{-1} The bio-kinetic constants evaluated showed good tolerance of SRB to varied phenol doses. The outcome of this study suggests that further research on sulfate reduction with prior acclimation using phenol can be carried out. However, the applicability of the microbial culture in a suitable large-scale reactor system for treating phenol- and sulfate-containing wastewater should be studied, which would help in its better application.

ACKNOWLEDGEMENTS

This research was supported by Council of Scientific and Industrial Research (CSIR), India (Project No. 22(0609)/12/EMR-II). The authors are thankful to the Head, Central Instruments Facility (CIF) of IIT Guwahati for allowing the analysis of samples.

REFERENCES

Ahmad, S. A., Shamaan, N. A., Syed, M. A., Khalid, A., Arina, N., Rahman, A., Khalil, K. A., Dahalan, F. A. & Shukor, M. Y. 2017 Meta-cleavage pathway of phenol degradation by *Acinetobacter*. *Rendiconti Lincei* **28** (1), 1–9.

APHA 2005 *Standard Methods for the Examination of Water & Wastewater: Centennial Edition*. American Public Health Association/American Water Works Association/Water Environment Federation, Washington, DC.

Basak, B., Bhunia, B., Mukherjee, S. & Dey, A. 2013 Desalination and water treatment optimization of physicochemical parameters for phenol biodegradation by *Candida tropicalis* PHB5 using Taguchi methodology. *Desalination and Water Treatment* **51** (34–36), 6846–6862.

Boopathy, R. 1985 Isolation and characterization of a phenol degrading, sulfate-reducing bacterium from swine manure. *Bioresource Technology* **54** (1), 29–33.

Cord-Ruwisch, R. 1985 A quick method for the determination of dissolved and precipitated sulfides in cultures of sulfate-reducing bacteria. *Journal of Microbiological Methods* **4** (1), 33–36.

García-Cruz, U., Celis, L. B., Poggi, H. & Meraz, M. 2010 Inhibitory concentrations of 2 , 4D and its possible intermediates in sulfate reducing biofilms. *Journal of Hazardous Materials* **179** (1), 591–595.

Huang, Z., Wang, P., Li, H., Lin, K., Lu, Z., Guo, X. & Liu, Y. 2014 Community analysis and metabolic pathway of halophilic

bacteria for phenol degradation in saline environment. *International Journal of Biodeterioration and Biodegradation* **94**, 115–120.

Jiang, L., Ruan, Q., Li, R. & Li, T. 2013 Biodegradation of phenol by using free and immobilized cells of *Acinetobacter* sp. BS8Y. *Journal of Basic Microbiology* **53** (3), 224–230.

Jing, Z., Hu, Y., Niu, Q., Liu, Y., Li, Y. & Wang, X. C. 2013 UASB performance and electron competition between methane-producing archaea and sulfate-reducing bacteria in treating sulfate-rich wastewater containing ethanol and acetate. *Bioresource Technology* **137**, 349–357.

Kiliç, N. K. & Dönmez, G. 2017 Dual bioremediation of phenol and Cr(VI) by mixed microbial cultures in the presence of molasses. *Water Science and Technology* **73** (10), 2324–2331.

Kumar, M., Liao, W., Tsai, J. & Lin, J. 2011 Versatility of fluorene metabolite (phenol) in fluorene biodegradation by a sulfate reducing culture. *International Journal of Biodeterioration and Biodegradation* **65** (3), 522–526.

Kurzbaum, E., Raizner, Y., Cohen, O., Suckeveriene, R. Y., Kulikov, A., Hakimi, B., Iasur, L., Armon, R. & Farber, Y. 2017 Encapsulated *Pseudomonas putida* for phenol biodegradation : use of a structural membrane for construction of a well-organized con fined particle. *Water Research* **121**, 37–45.

Lim, J., Seng, C., Lim, P., Ng, S., Tan, K. & Kew, S. 2013 Response of low-strength phenol-acclimated activated sludge to shock loading of high phenol concentrations. *Water SA* **39** (5), 695–700.

Lin, J. 2017 Stress responses of *Acinetobacter* strain Y during phenol degradation. *Archives of Microbiology* **199** (2), 365–375.

Lin, Y. H. & Lee, K. K. 2001 Verification of anaerobic biofilm model for phenol degradation with sulfate reduction. *Journal of Environmental Engineering* **127** (2), 119–125.

Lin, Y., Chen, G. & Cai, Y. 2017 Biodegradation of phenol and sodium salicylate by *Pseudomonas putida* BCRC 14365 in batch and packed-bed bioreactors: model development and verification. *Journal of Environmental Engineering* **143** (5), 04017003.

Liu, Y., Woon, K., Yang, S. & Tay, J. 2002 Influence of phenol on cultures of acetate-fed aerobic granular sludge. *Letters in Applied Microbiology* **35** (2), 162–165.

Mahgoub, S., Abdelbasit, H. & Abdelfattah, H. 2014 Removal of phenol and zinc by *Candida* isolated from wastewater for integrated biological treatment. *Desalination and Water Treatment* **53** (12), 37–41.

Mattei, M. R., Acunto, B. D., Esposito, G. & Frunzo, L. 2014 Mathematical modeling of competition and coexistence of sulfate-reducing bacteria, acetogens, and methanogens in multispecies biofilms. *Desalination and Water Treatment* **55** (3), 1–9.

Monteiro, Á. A. M. G., Boaventura, R. A. R. & Rodrigues, E. 2000 Phenol biodegradation by *Pseudomonas putida* DSM 548 in a batch reactor. *Biochemical Engineering* **6** (1), 45–49.

Mort, S. L. & Dean Ross, D. 1994 Biodegradation of phenolic compounds by sulfate-reducing bacteria from contaminated sediment. *Microbial Ecology* **28** (1), 67–77.

Phalgune, U. D., Rajamohanan, P. R., Gaikwad, B. G., Varma, R. J. & George, S. 2013 Biodegradation of phenol by the yeast *Candida tropicalis*: an investigation by NMR spectroscopy. *Applied Biochemistry and Biotechnology* **169** (7), 2029–2037.

Ray, S. & Banerjee, A. 2015 Molecular level biodegradation of phenol and its derivatives through dmp operon of *Pseudomonas putida*: a bio-molecular modeling and docking analysis. *Journal of Environmental Sciences* **36**, 144–151.

Rosenkranz, F., Cabrol, L., Carballa, M., Cruz, L., Chamy, R. & Lema, J. M. 2013 Relationship between phenol degradation efficiency and microbial community structure in an anaerobic SBR. *Water Research* **47** (17), 6739–6749.

Sabumon, P. C. 2008 Development of enhanced sulphidogenesis process for the treatment of wastewater having low COD / SO_4^{2-} ratio. *Journal of Hazardous Materials* **159** (2), 616–625.

Somasundaram, V., Philip, L. & Bhallamudi, S. M. 2009 Experimental and mathematical modeling studies on Cr(VI) reduction by CRB, SRB and IRB, individually and in combination. *Journal of Hazardous Materials* **172** (2), 606–617.

Wang, Y., Suidan, M. T., Pfeffer, J. T. & Najm, I. 1988 Effects of some alkyl phenols on methanogenic degradation of phenol. *Applied and Environmental Microbiology* **54** (5), 1277–1279.

Wang, Y., Wang, X., Li, H., Lin, K., Wang, P., Yang, J., Liu, Y., Sun, Z., Fan, L. & Wu, Z. 2014 Treatment of high salinity phenol-laden wastewater using a sequencing batch reactor containing halophilic bacterial community. *International Journal of Biodeterioration and Biodegradation* **93**, 138–144.

Wirth, B., Krebs, M. & Andert, J. 2015 Anaerobic degradation of increased phenol concentrations in batch assays. *Environmental Science and Pollution Research* **22** (23), 19048–19059.

Wu, C., Zhou, Y. & Song, J. 2016 The activated sludge metabolic characteristics changing sole carbon source from readily biodegradable acetate to toxic phenol. *Water Science and Technology* **73** (10), 2324–2331.

Xu, X., Chen, C., Wang, A. & Guo, H. 2014 Kinetics of nitrate and sulfate removal using a mixed microbial culture with or without limited-oxygen fed. *Applied Microbiology and Biotechnology* **98** (13), 6115–6124.

Yagmur, E., Turkoglu, S., Banford, A. & Aktas, Z. 2017 The relative performance of microwave regenerated activated carbons on the removal of phenolic pollutants. *Journal of Cleaner Production* **149**, 1109–1117.

First received 23 July 2017; accepted in revised form 10 December 2017. Available online 18 December 2017