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Effect of biogenic substrate concentration on the performance of sequencing batch reactor treating 4-CP and 2,4-DCP mixtures

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Abstract

Effect of a biogenic substrate (peptone) concentration on the performance of sequencing batch reactor (SBR) treating 220 mg/l 4-chlorophenol (4-CP) and 110 mg/l 2,4-dichlorophenol (2,4-DCP) mixtures was investigated. In this context, peptone concentration was gradually decreased from 300 mg/l to null in which chlorophenols were fed to the reactor as sole carbon and energy sources. By this way, the effect of peptone concentration on observed yield coefficient (*Y*), biomass concentration, chlorophenols and COD removal performances were investigated. Decreasing peptone concentration accompanied with lower biomass concentration led to increase in peak chlorophenol and COD concentrations within the reactor during each SBR cycle. This, in turn, caused noteworthy declines in the removal rates as chlorophenol degradations followed Haldane substrate inhibition model. Also, increased peak chlorophenol concentrations led to the accumulation of 5-chloro-2-hydroxymuconic semialdehyde (CHMS), which is *-meta* cleavage product of 4-CP. Despite the decreased removal rates, complete chlorophenols and CHMS degradation, in addition to high COD removal efficiencies (>90%), were observed for all studied conditions, even chlorophenols were added as sole carbon and energy sources. Another significant point is that 2,4-DCP at slightly elevated concentrations (>20 mg/l) within the reactor caused a strong competitive inhibition on 4-CP degradation. In SBR, feeding the influent to the reactor within a certain period (i.e. filling period) provided dilution of coming wastewater, which decreased the chlorophenols concentrations to which microorganisms were exposed. Therefore, use of SBR may help to avoid both self and competitive inhibitions in the treatment of 4-CP and 2,4-DCP mixture especially in the presence high biogenic substrate concentrations. In addition, isolation and identification studies have indicated that *Pseudomonas* sp. and *Pseudomonas stutzeri* were dominant species in the acclimated mixed culture.

Keywords: 4-Chlorophenol; 2,4-Dichlorophenol; SBR; Biodegradation kinetics

1. Introduction

Chlorophenols are introduced to the environment through the result of several man-made activities, such as waste incineration, uncontrolled use of wood preservatives, pesticides, fungicides and herbicides as well as bleaching of pulp with chlorine and the chlorination of drinking water [1]. Some typical values of phenolic compounds in chemical industry wastewaters were reported to be 400 mg/l for phenolic resin production, 50 mg/l for refineries, 12 mg/l for naphthalenic acid production and 200 mg/l for shale dry distillation [2].

Some physical and chemical treatment methods including adsorption, air stripping, chemical oxidation, solvent extraction, ultraviolet light, ozone, etc., have been utilized in treatment of wastewaters containing phenolic compounds. However, the high cost and low efficiency of these processes limit their applicability [3]. Therefore, despite the recalcitrant nature of chlorophenols, there are still some efforts toward their biological way of treatment with specialized culture conditions, because of economical reasons and a low possibility of byproduct formation.

In the literature, the major studies on chlorophenol treatment conducted so far have focused on the use of 4-

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chlorophenol (4-CP) as a model non-growth substrate using special strains grown on phenol [4–9] or readily degradable substrate (e.g. glucose) [10,11]. Many pure-culture studies have shown that toxic intermediates accumulate during biodegradation because a single organism may not have the ability to completely mineralize the xenobiotic [12]. However, several studies have showed that mixed bacterial culture has ability to use chlorophenols as sole carbon and energy sources [13–16]. The main advantage achieved by the microbial consortium formed by mixed culture is the interaction between all the species present in the flocks.

A wastewater treatment plant generally receives influent with a mixture of recalcitrant synthetic organic chemicals (SOCs) and biogenic substrates. This means that SOCs and biogenic compounds often coexist in many wastewater reactors. Interactions among these multiple substrates are complex, partially due to the toxicity, competition for enzymes and cofactors [17]. Therefore, the question should be answered is how biogenic substrate concentration affect the removal efficiency of SOCs especially when SOCs are present in mixture. Many researchers claim that a specific competent biomass fraction is responsible for the degradation of specific compound, which is equal to the fraction of COD contributed to the feed by that compound [17–20]. This means that the presence of biogenic substrate does not guarantee the enhanced biodegradation of SOCs [17]. For example, Kulkarni and Chaudhari [21] reported that degradation rate of *p*-nitrophenol decreased with the addition of glucose. In another study, Hu et al. [17] reported that at standard oxygen conditions 4-CP degradation rate decreased with the supplementation of biogenic substrate, whereas, 2,4-DCP degradation rate increased in the presence of biogenic substrate. These results showed that different chemicals may give different response to the presence of biogenic substrate.

In our previous studies, we have investigated the effect of biogenic substrate availability on 2,4-DCP [22] and 4-CP [23] degradation for short term experiments, however, no study has been conducted so far regarding the effect of biogenic substrate concentration on chlorophenols degradation for long term experiments, especially, when chlorophenols are present in mixture.

Conventional activated sludge systems work generally well for easily degraded components of wastewater, but not for hazardous components which are toxic to bacteria, or slow to degrade [24]. Sequencing batch reactors (SBRs) are an attractive alternative to conventional biological wastewater treatment systems, mainly because of their simplicity and flexibility of operation and cost effectiveness for small-scale treatment facilities [25]. Also, the enforcement of controlled short term unsteady-state conditions may favor the induction of enzymes required for degrading biorefractory compounds [26].

Therefore, there seems to be a need to study degradation of chlorophenols in mixture at varying biogenic substrate concentrations using acclimated mixed cultures in SBR to define substrate interactions in multi-substrate systems.

2. Materials and methods

2.1. Culture medium

Acclimated culture for the startup of SBR was obtained from a fed batch reactor receiving 220 mg/l 4-CP, 110 mg/l of 2,4-DCP and 300 mg/l peptone. Around 200 ml well mixed sludge (1200 mg/l mixed liquor volatile suspended solids (MLVSS)) was used as initial inocula for SBR. As culture inoculum was already acclimated, SBR experiments were directly started with 220 mg/l 4-CP and 110 mg/l 2,4-DCP without the requirement of any further acclimation to such high chlorophenols concentrations.

Primary settling tank effluent of Greater Municipality of Ankara Domestic Wastewater Treatment Plant was used as initial inocula for the fed batch reactor which was used as a biomass source to start SBR operation. The fed batch reactor was fed with growth medium devoid of chlorophenol for 2 weeks in order to produce biomass at a reasonable concentration and adapt biomass to growth medium. Then, 4-CP and 2,4-DCP was added to the feed at low concentrations and the influent concentrations were gradually increased to 220 mg/l 4-CP and 110 mg/l 2,4-DCP, which lasted around 5 months.

The composition of the synthetic wastewater used in the experiments contained 300–0 mg/l peptone; 220 mg/l 4-CP; 110 mg/l 2,4-DCP; 30 mg/l NaCl; 44.6 mg/l $MgSO_4$; 400 mg/l K_2HPO_4; 200 mg/l KH_2PO_4; 46–182 mg/l NH_4Cl; 3.7 mg/l $MgCl_2{\cdot}6H_2O$, $CaCl_2{\cdot}2H_2O$ and $FeCl_2{\cdot}2H_2O$; 0.057 mg/l $MnSO_4$; 0.046 mg/l $ZnSO_4$; 0.049 mg/l $CoSO_4$; 0.076 mg/l $CuSO_4$. Tap water was used in the preparation of feed solution throughout the SBR operation. The total nitrogen (N) content of proteose–peptone (Oxoid), used as a biogenic substrate, was around 12%. As peptone concentration was decreased in the feed solution, the excluded amount of N was supplemented with increased concentration of NH_4CI not to allow culture to be N limited. When peptone was 100 mg/l, nitrification of NH_4CI at an elevated concentration led to the formation of H^+ ion at high enough concentration to decrease the pH value below 6 at the end of the SBR cycle. In order to avoid creation of suboptimal environment for biomass activity, $NaHCO_3$ (300 mg/l) was added to the feed as an extra source of buffer to keep pH between 6.5 and 7 when peptone concentration was 100 mg/l or lower.

2.2. Experimental setup of SBR

A 2.5 l glass vessel with the 2.25 l of working volume was used as SBR. The reactor was operated in a water bath at 26 ± 1 °C and aerated using air pumps to keep dissolved oxygen concentration at least 3 mg/l. Each SBR cycle lasted one day consisting of fill (8 h), reaction (22 h – including fill period) and settle–draw periods (2 h). The sludge retention time (SRT) was kept constant at 10 d removing excess sludge from well mixed reactor content at the end of the each daily cycle. After settling, 2 l of the treated effluent was drawn and

around 250 ml sludge was maintained in the reactor for the next cycle, which gives 1.125 d of hydraulic retention time (HRT). The 2 l of synthetic wastewater was fed to the reactor using a peristaltic pump.

2.3. Experiments

A stock solution of 4-CP and 2,4-DCP mixture (Merck Chemical Co., Germany) dissolved in 0.02 M NaOH was used to adjust desired concentrations of chlorophenols in reactor feed. Feed solution was prepared freshly every day. The filling time was kept constant at 8 h throughout the experiments. In order to understand the effect of biogenic substrate concentration on the performance of the reactor, peptone concentration was gradually decreased from its initial value of 300 mg/l, while the chlorophenol concentrations were kept constant at 220 and 110 mg/l for 4-CP and 2,4-DCP, respectively. After any change in reactor operation, at least two SRT was allowed to reach steady-state conditions, which were also checked measuring MLVSS and COD concentrations at the end of at least three successive cycles. After ensuring the steady-state conditions, samples were drawn from the reactors at predetermined time intervals and analyzed immediately for COD, chlorophenols and 5-chloro-2-hydroxymuconic semialdehyde (CHMS). MLVSS concentrations at the end of the cycles were also determined.

Following the centrifugation of samples, chlorophenols on biomass was extracted using 0.1 M NaOH to evaluate the degree of removal via adsorption, which was not detected throughout the SBR operation.

2.4. Isolation of pure cultures

In the isolation of bacteria the procedure given by Wang et al. [27] was used. Mixed culture samples, obtained from SBR, were diluted to have maximum 30 colonies on solidified agar medium. The growth medium used in SBR was solidified and used during the isolation of pure strains. Solidified agar medium supplemented with mixture of 50 mg/l 4-CP and 25 mg/l 2,4-DCP. Streaked agar plates were incubated at 30 °C. After development of colonies, single colonies were selected and streaked to new agar plates. This procedure was repeated at least five times in order to ensure the purity of culture. API 20 NE identification kits were used to define the isolated cultures.

2.5. Analytical techniques

CHMS concentration, the -*meta* cleavage product of 4-chlorocatechol, was followed by measuring OD at 380 nm (ABS_{380}) [28] at which CHMS gives maximum absorbance. MLVSS and chloride (titration method) measurements were carried out according to Standard Methods [29]. COD measurements were carried out using Hach COD vials according to the EPA approved reactor digestion method [30]. In this method, after 2 h digestion, COD values of samples were directly read using Hach Spectrophotometer (Model No. 45600-02, Cole Parmer Instrument Co., USA).

A high performance liquid chromatography (HPLC) method was used to determine the 4-CP and 2,4-DCP concentrations. The HPLC (Shimadzu, LC-10AT) used was equipped with a Nucleosil C18 column (4.6 mm × 250 mm), LC-10Atvp solvent delivery module, an SC/L0Avp system controller and a SPD-10Avp UV–vis detector set at 280 nm. Retention time of 4-CP and 2,4-DCP was 7.5 and 12 min, respectively. Solvent used in the analyses was methanol (60%), pure water (38%) and acetic acid (2%) at the flow rate of 0.75 ml/min [31]. The sample injection volume was 20 μl.

All the experiments and measurements were done in duplicate and arithmetic averages were taken throughout the data analysis and calculations. Coefficient of variations for COD and MLVSS measurements was less than 10%, whereas it was less than 5% for chlorophenol measurements.

3. Results and discussion

In order to investigate the effect of readily degradable substrate concentration on SBR performance, influent peptone concentration was gradually decreased from 300 mg/l to null in which chlorophenols were fed as sole organic carbon sources. Initial 4-CP and 2,4-DCP was kept constant at around 220 and 110 mg/l, respectively, and reactor was fed at 8 h of filling time. At the start of each cycle, the volume of settled sludge was 0.250 l and at the end of the filling period the reached total volume of the reactor was 2.25 l. Therefore, for each cycle, SBR received 2 l influent to be treated (HRT = 1.125 d), which was fed to the reactor within 8 h of filling period.

Fig. 1 gives time course variations of the 4-CP, 2,4-DCP, ABS_{380} and COD concentrations for each peptone concentration studied. The initial feed COD concentration was around 800 mg/l when reactor received 300 mg/l peptone, 220 mg/l 4-CP and 110 mg/l 2,4-DCP. A 100 mg/l exclusion of peptone caused around 100 mg/l decrease in the COD of reactor influent and the COD concentration was around 500 mg/l when reactor was fed with chlorophenols as a sole carbon and energy sources. Complete degradation of chlorophenols was observed for studied conditions, although the trend of chlorophenol concentrations showed variation depending on the biogenic substrate concentration. Although reactor was fed with a high concentration of 4-CP and 2,4-DCP mixture, 4-CP and 2,4-DCP concentrations were observed to be below 2 mg/l during the filling period of the SBR for influent peptone concentration of 300 mg/l, which were increased to around 12.5 and 5 mg/l in respective order with decreasing influent peptone concentration to 200 mg/l (Fig. 1a). When the peptone concentration was decreased to 200 mg/l, chlorophenols started to exhibit a noticeable peak value which corresponds to the very beginning of the cycle. Then, it can be said that decreasing peptone concentration adversely

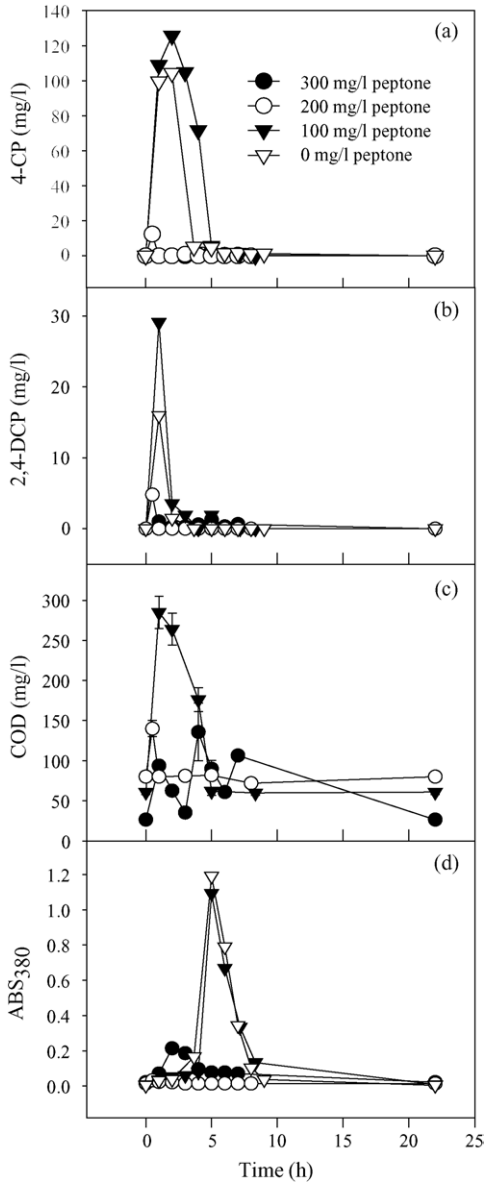

Fig. 1. Time course variation of 4-CP (a), 2,4-DCP (b), COD (c) and ABS_{380} (d) in SBR at different initial peptone concentrations (influent 4-CP and 2,4-DCP were 220 and 110 mg/l, respectively).

affected chlorophenol removal rate, which led to a slight increase in observed maximum chlorophenol concentrations within the reactor. However, the maximum COD concentration within the cycle was not affected from the decreasing peptone concentration from 300 to 200 mg/l as it was observed to be 135 and 140 mg/l in respective order (Fig. 1c). When peptone concentration decreased further to 100 mg/l, the 4-CP peak concentration increased to 126 mg/l and a slight decrease in the peak 4-CP concentration to 104 mg/l was observed when chlorophenols were fed to the reactor as sole carbon and energy source (Fig. 1a). The decrease in 4-CP peak value when peptone concentration decreased from 100 to 0 mg/l is not known, but the possible reason is thought as change in community structure with the complete elimination of peptone from the influent.

Similar to 4-CP, increase in 2,4-DCP peak concentrations were observed with the decreasing peptone concentration from 300 to 100 mg/l (Fig. 1b), whereas, a slight decrease in the peak 2,4-DCP concentration from around 30 to 16 mg/l was observed with the complete elimination of peptone from the feed solution (Fig. 1b). It is important to note that 4-CP is much more accumulated in the medium compared to 2,4-DCP. The reason of this observation is that 2,4-DCP caused a strong competitive inhibition on 4-CP degradation and 4-CP degradation started only after complete 2,4-DCP removal, which was also verified by batch experimental data (data not shown). This observation can also be verified from the evaluation of time course variation of chlorophenols concentrations given in Fig. 1a and b. The accumulation of 2,4-DCP at lower biogenic substrate concentrations decreased the removal rate of 4-CP further due to competitive inhibitory effect of 2,4-DCP on 4-CP degradation. Therefore, the accumulation of 4-CP in the growth medium is not solely due to the decreasing 4-CP degradation ability of the culture with the elimination peptone from growth medium. Despite the increased peak values of chlorophenols with decreasing readily degradable substrate concentration, it was observed that the mixture of 4-CP and 2,4-DCP were effectively degraded even in the absence of a biogenic substrate and biomass can grow on the mixture of 4-CP and 2,4-DCP.

In 4-CP degradation, the first step is the attack of phenol hydroxylase to 4-CP, which result in production of 4-chlorocatechol. The conversion of 4-chlorocatechol via -*meta* cleavage pathway yields CHMS, and complete removal of the intermediate may further be observed. CHMS has been widely reported to be a dead-end metabolite when pure culture is used in the degradation process [4–6,28]. However, we have observed complete degradation of 4-CP and CHMS with acclimated mixed culture. In the case of 2,4-DCP degradation, the first step is the production of 3,5-dichlorocatechol with the use of 2,4-dichlorophenol hydroxylase. Then 3,5-dichlorocatechol is converted to 2,4-dichloro-*cis*,*cis*-muconate, which is catalyzed by 3,5-dichlorocatechol-1,2-dioxygenase [32]. It was reported that phenol hydroxylase has a broad substrate specificity and it can also catalyze the turnover of 2,4-DCP [33]. It was known that 2,4-dichlorophenol hydroxylase has also ability to convert 4-CP to 4-chlorocatechol [34]. Therefore, the use of the same enzymes for the initiation of 4-CP and 2,4-DCP degradation led to observation of competitive inhibition.

The peak COD concentration increased from around 140 to 285 mg/l with the decreasing peptone concentration from 200 to 100 mg/l. Although different peak values were observed depending on the initial peptone concentration, high COD removal efficiencies ($\geq$90%) were observed at the end of cycle for all studied conditions. When chlorophenols were fed to the reactor as sole carbon and energy sources, only initial and final concentrations of COD were measured as the COD concentration in the reactor at any time may be calcu-

lated using the time course data for 4-CP and 2,4-DCP. The influent COD concentration decreased from 503 to 53 mg/l, corresponding to around 90% COD removal efficiency, which shows that acclimated mixed culture have ability to use mixture of 4-CP and 2,4-DCP as sole carbon and energy sources. The observed COD concentration at the end of the cycle was thought to be due to biomass lyses products, although it seems to be slightly higher (53 mg/l).

During the degradation, a yellow color accumulated in the medium, which was completely disappeared at the end of the cycle (Fig. 1d). The peak value of ABS_{380} increased with decreasing peptone concentration similar to other measured parameters. It was believed that this color was due to CHMS, the -*meta* cleavage product of 4-chlorocatechol as also claimed by Farrell and Quilty [28] and Bali and Şengül [35]. An interesting observation was that CHMS concentration increased steadily with time course decreasing 4-CP concentration and the peak value was reached when 4-CP was just completely removed from the medium (Fig. 1a and d). Observing high COD removal efficiencies (≥90%) and complete removal of CHMS confirm the removal of chlorophenols with acclimated mixed cultures for all studied conditions, which was also verified by HPLC data and released chloride ion concentration, which indicated 102 ± 5% chlorophenols removal.

The reason for the increases in peak concentrations of measured parameters with decreasing peptone concentration is thought to be due to decreasing biomass concentration from 1120 to 953 mg/l (Fig. 2). Effect of peptone concentration on the observed yield coefficient (*Y*) and MLVSS values are depicted in Fig. 2. Another striking conclusion, which can be drawn from Fig. 2, is that *Y* did not correlate with peptone concentration and the average value was observed to be 0.155 ± 0.028 mg MLVSS/mg COD. The observed *Y* value is significantly lower than one expected for a readily degradable substrate (0.4–0.6 MLVSS/mg COD). In our laboratory, *Y* value on peptone only was observed to be 0.42 ± 0.008 mg MLVSS/mg COD in a fed batch reactor operated at 10 d of SRT (data not shown). Therefore, the reason of decrease in *Y* can be attributed to the fact that chlorophenols are not good substrate for biomass and they have a strong inhibitory effect on the biomass growth. Similarly, Rutgers et al. [36]

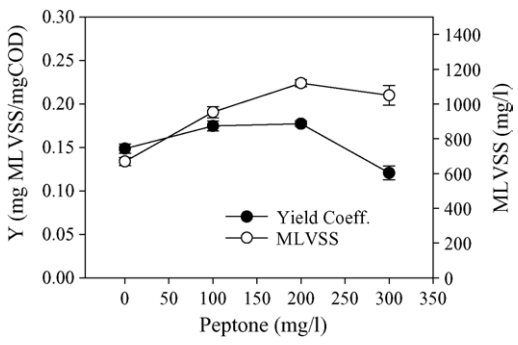

Fig. 2. Effect of peptone concentration on *Y* values and biomass concentrations.

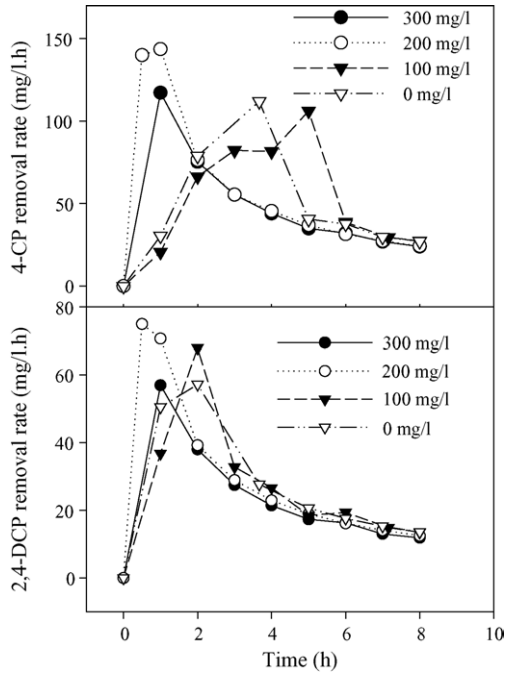

Fig. 3. Effect of peptone concentration on removal rates of 4-CP and 2,4-DCP.

reported that the growth yield coefficients on chlorinated phenols are lower than those of heterotrophic growth on non-chlorinated compounds. They studied growth yield coefficients on various chlorophenols in chemostat culture and reported the growth yield coefficients within the range of 0.255–0.11 molC/molC.

Effect of peptone concentration on time course degradation rates of 4-CP and 2,4-DCP are given in Fig. 3. In the calculation of degradation rates the following equation was used and the change in volume of the reactor was taken into account:

$$\frac{dc}{dt}V = QC_0 - rV \quad (1)$$

where C and C_0 are chlorophenol concentrations (mg/l) in SBR and influent, respectively. V (l) is total reactor volume and r is chlorophenol removal rate (mg/l h). Total volume during the filling period of the reactor is:

$$V = V_0 + Qt \quad (2)$$

where V_0 is settled sludge volume at the start of the cycle. Hence, degradation rate can be observed as:

$$r = \frac{QC_0}{V_0 + Qt} - \frac{dc}{dt} \quad (3)$$

Degradation rates of 4-CP and 2,4-DCP were very high at the start of the experiments for 300 and 200 mg/l of influent peptone concentrations. The observed initial degradation rates for 4-CP decreased to very low values for peptone concentrations of 100 and 0 mg/l, which caused accumulation of chlorophenols within the reactor. The maximum observed

4-CP degradation rate increased slightly with decreasing peptone concentration from 300 to 200 mg/l. The reason of this increase in degradation rate is due to the slight increase in peak 4-CP concentration from 2 to 12.5 mg/l. Degradation of chlorophenols obeys Haldane kinetics, which means chlorophenol degradation rate increases up to a certain concentration (critical substrate concentration) at which degradation rate is maximum, whereas, after this point degradation rate decreases with increasing 4-CP concentration. In our laboratory, 4-CP critical substrate concentration for the biomass used in this study was observed to be close to 53 mg/l (data not shown). Therefore, up to this point increase in 4-CP concentration results in increase in degradation rate.

The comparison of loading and degradation rates is also depicted in Fig. 4. As it can be seen from the figure, the ratio of removal/loading rates were almost one during the filling of the reactor when the initial peptone concentration was 300 and 200 mg/l. Whereas, for 100 and 0 mg/l of peptone, initial 4-CP removal/loading rates were at very low levels, which led to the accumulation within the reactor. There seems to be two possible reasons of observing higher 4-CP removal rates at high concentrations of peptone. First, higher biomass concentrations associated with high peptone concentrations (Fig. 2) may cause to decrease inhibitory effect of chlorophenols on biomass. Also, increasing 2,4-DCP degradation rate at high biomass concentrations may prevent the accumulation of 4-CP due to competitive inhibitory effect of 2,4-DCP on 4-CP degradation. Second, the utilization of peptone may lead to increase in the production of NADH, which may increase the initial degradation rate of chlorophenols. In the case of 2,4-DCP, although the observed initial removal rates were lower for 100 and 0 mg/l of peptone, the decrease in removal rates were not noteworthy compared to 4-CP case (Fig. 4).

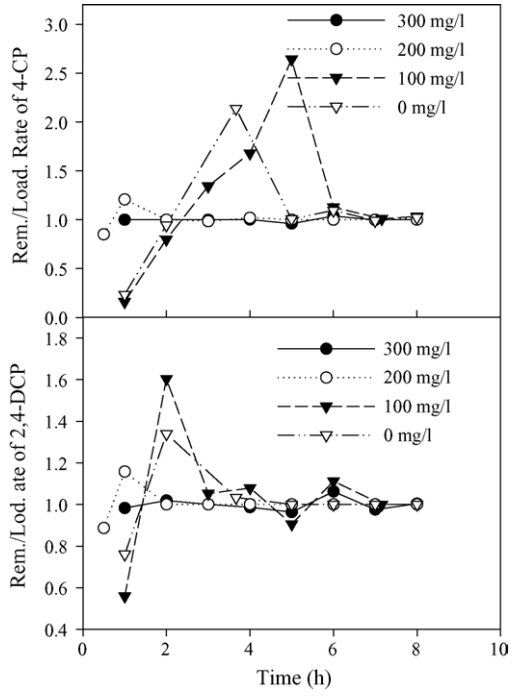

Fig. 4. Effect of peptone concentration of removal/loading rates of 4-CP and 2,4-DCP.

Table 1
Characteristics of isolated and identified species

Isolated and identified specie	Gram reaction	Colony characteristic	Relative amount
Pseudomonas stutzeri	G−	Yellow to transparent	+++
Pseudomonas sp.	G−	Brown	++

Isolated strains from acclimated mixed culture and their colony characteristics are given in Table 1. Two colonies, which are brown and yellow to transparent colonies, were isolated from the agar plates. Similar observation was also reported in the study of Wang et al. [27]. In their study, three different colonies (yellow, brown and transparent) were identified from the mixed culture grown in the presence of chlorophenols.

4. Conclusions

Treatment of toxic organic compounds at high concentrations is a difficult task, especially when they are present in mixtures due to exerted toxic effect on substrate degradation and/or biomass growth. Hence, the use of SBR in the treatment of toxic organic mixture gain importance as feeding the influent to the reactor within a certain period (i.e. filling period) provided dilution of coming wastewater, which decreased the toxic organic concentrations to which microorganisms were exposed. The aim of this study was to investigate effect of biogenic substrate concentration on the performance of SBR treating 220 mg/l 4-CP and 110 mg/l 2,4-DCP mixture. It was observed that decreasing peptone concentration associated with decreasing biomass concentration led to the observation of lower degradation rates, which caused accumulation of chlorophenols within the reactor. Accumulation of chlorophenols further decreased the removal rate due to self inhibitory effect of chlorophenols on their own degradation and strong competitive inhibition of 2,4-DCP on 4-CP degradation. Although peak chlorophenol concentrations within the reactor showed an increasing trend with decreasing peptone concentrations, complete removal of chlorophenols and associated intermediates along with high COD removals were observed even chlorophenols were fed to the reactor as sole carbon sources. Therefore, use of SBR may help to avoid both self and competitive inhibitions in the treatment of 4-CP and 2,4-DCP mixture especially in the presence high biogenic substrate concentrations.

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