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Development of Mixed Anaerobic Culture for Degrading High Concentrations of Chlorophenols

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Abstract

This study details the development of mixed anaerobic culture capable of degrading high concentrations of chlorophenols, 4-chlorophenol (MCP), 2,4-dichlorophenol (DCP) and pentachlorophenol (PCP) were used for that purpose. The role of glucose concentration and the relative potential of mixed culture for acclimatization of different chlorophenols under anaerobic conditions were studied. Methane production, pH and their reduction in concentrations of glucose and chlorophenols were measured at regular intervals. It was observed that after 350 days of acclimatization, anaerobic cultures degraded up to 200 ppm MCP, 200 ppm DCP and 250 ppm PCP. It was also found that the biogenic substrate such as glucose increased the rate of chlorophenols acclimatization and degradation.

Key Words: Anaerobic degradation; Chlorophenols; Mixed culture; Glucose;

1. Introduction

Industrialization and urbanization are resulted in release of large concentrations of toxic and harmful chemicals to the water bodies. Consequently, the scientific communities have evolved various treatment options such as adsorption, advanced oxidation processes, constructed wetland and so on for treating these chemicals (Mukesh et al. 2004; Mukesh et al. 2009; Mukesh et al. 2010; Kumar et al. 2013)]. The harmful chemicals such as chlorophenols, aromatic compounds , and flocculants are known environmental hazards, which are usually introduced into the environment from several chemical industries (Mukesh et al. 2009; Xuran et al. 2019a, Xuran et al. 2019b, Xuran et al. 2019c]. They are suspected/confirmed carcinogen and have possible endocrine-disrupting effects (Mukesh et al. 2009; Olaniran and Igbinosa; 2011; Kumar et al. 2013; Tingting et al. 2017).

Chlrophenols are observed in surface as well as ground waters (Sudarsan et al. 2015). The most pertinent issue is that they are not biodegradable and persevere in the environment for significant time (Hema et al. 2009). They are harmful even in small quantity in species. Thus we need holistic and suitable approaches to degrade chlorophenols in wastewaters. The chlorophenols concentration in effluents reaches the optimum or more it makes bioinhibitory by nature in species. And it creates highly toxic and hostile environment for the development and sustenance of all. Thus, it was apparent that biological treatment required a microbial consortium acclimatized of mixed cultures to chlorophenols.

Biological treatment processes involving microorganisms are one among the useful method for wastewater treatment. They are environmentally friendly and generally cost effective. They are also capable of degrading a wide variety of compounds, including PAHs, explosives, chlorinated organics, etc (Titus et al. 20014). The processes depending upon their interaction with oxygen and an anaerobic process occurs in the absence of free or combined oxygen (Feigelson et al. 2004;

Irfanudeen ete al. 2015). The anaerobic degradation resulted in reductive dechlorination of chlorophenols which are succeeded finally as CH_4 and CO_2 (McCarty, 1985; Mukesh et al. 2014). There are reports in literature regarding the application of conservatively cultured anaerobic microbes for dehalogenation (Armenante et al. 1999; Nijenhuis *et al.* 2016; Nijenhuis et al. 2018). Several microbial species are reported to be capable of degrading pesticide 2,4-dichlorophenoxy acetic acid (2,4-D) after proper acclimatization. Since single microorganisms are not so effective in reductive dehalogenation, mixed culture is a viable alternative. In this work, three dissimilar chlorophenols were used - 4-chlorophenol (MCP), 2,4-dichlorophenol (DCP) and pentachlorophenol (PCP). They are highly persisted in the environment. Their history and public health impact are typical of chemicals on USEPA's list of priority pollutants. This work comprised of following objectives: a) develop acclimatized anaerobic cultures for degrading high concentrations of chlorophenols; b) study the effect of glucose concentration on their acclimatization; and c) study the relative potential of mixed culture for degradation of different chlorophenols under anaerobic conditions. In brief, the chlorophenols acclimatization through anaerobic cultures was analyzed with 0 g/L, 5 g/L and 10 g/L for 350 days.

2. Material, Methods and Experimental Procedure

2.1 Materials

All Analar grade chemicals were purchased from Acros Organics, Belgium. Stock solutions of 2000 ppm MCP was prepared by dissolving 2000 mg of MCP in 0.1 N NaOH solutions. Similarly, 1000 mg of DCP and 1000 mg of PCP were dissolved in 0.1 N NaOH to make, 1000 ppm DCP and 1000 ppm PCP stock solution respectively.

Inoculum: It is a medicine from bacterial cellulose (BC). The inoculums used in this setup are originated from a wastewater treatment plant operating at a relatively short HRT (18 days) and with mixed sludge as a substrate. The biogas plants used for collection of inoculum were mainly fed with thin stillage and agricultural waste, respectively. This was obtained operating in the

laboratory at 37 ^{o}C for the past two years. Liquid and gas samples from the reactors were preserved weekly to determine pH value, methane (gas chromatography), and VFA content (high performance liquid chromatography) (Westerholm et al., 2012).This inoculum was used for seeding the experimental set-up only after ensuring that methane composition of the reactor gas was 60% or more. Chlorophenols degradation and methane production was regularly monitored to evaluate the amount of acclimation. The acclimatization was done in 1 L aspirator bottles fitted with butyl rubber stoppers. The experimental procedure is described elsewhere [3, Movahedyan et al. 2008]. Glucose and chlorophenol were used as the carbon source. 1% glucose media comprises 10 g/l glucose, 0.34 g/l yeast extract, 0.84 g/l NH_4Cl, 0.134 g/l KH_2PO_4, 0.234 g/l K_2HPO_4 and 0.084 g/l $MgCl_2.6H_2O$. All the reactors were maintained at a pH 7.0 and 37 ^{0}C temperature.

2.2. Analytical Procedure

Glucose Measurement: DNS (3,5-dinitrosalicylate) method was used to measure glucose concentration (Marsden et al. 1982).

Measurement of Chlorophenols: HPLC (Waters, USA) equipped with a UV Detector and a C18 Column was used to measure chlorophenols concentrations in the samples. The detection wavelength was 280 nm for MCP and DCP, whereas 210 nm was used for PCP measurement. The mobile phase consists of a mixture of acetonitrile and 1% acetic acid (50 % v/v). Flow rate of 1 ml/min was used for analysis purpose. The injection volume was 20 μl. In order to study the formation of methane, 3,5-dichloro-4-hydroxybenzyl alcohol (0.1 g/liter) was incubated with deuterated 2,6-dichlorophenol (Varhagen et al. 1998).

Methane Gas Measurement: Gas samples compositions were determined using a gas chromatograph (AIMIL-NUCON Series 5700, India). The GC conditions maintained with Injector temperature – 60 ^{o}C; Oven temperature- 40 ^{o}C; Detector temperature – 60 ^{o}C. Hydrogen was used as the carrier gas.

Optical Density: Optical density measurements were carried out using a Spectrophotometer (Jenway 6305, UK).

2.3. Experimental Procedure

In order to start the experiments, three kinds of cultures were prepared with different concentration of glucose. One culture was started with 10 g/L glucose, another with 5 g/L glucose and last one where glucose was not supplemented (Tong et al. 2017).

For differentiating cultures subjected to different conditions, experiments started with 1% glucose were named MCP1, DCP1 and PCP1 for MCP, DCP and PCP respectively. Similarly cultures started with 0.5 % glucose were named MCP2, DCP2 & PCP2 and cultures without glucose supplementation were named MCP3, DCP3 and PCP3. Since, it was anaerobic acclimatization; the prefix *An* was used to indicate anaerobic culture. So culture started with 1% glucose concentration for MCP was named AnMCP1, culture started with 0.5% glucose was named AnMCP2 and cultures without glucose supplementation were named AnMCP3. Detailed information of cultures is mentioned in Table 1. The degradation reaches around 90%, fresh chlorophenol was added from stock solution to increase the chlorophenol content in the medium. An equivalent amount of supernatant would be discarded. Methane gas production, pH, glucose and chlorophenols concentration were monitored at regular intervals to assess the microbial activity of the culture. Depending on uptake rate, chlorophenols concentrations were measured either every day or every three days or every 7 days. Only relevant data points are reported. In all the cultures containing glucose, its concentration was decreased in a controlled manner to acclimatize the culture to chlorophenol.

Table 1. Detailed information on anaerobic culture for acclimatization to chlorophenols

Anaerobic Culture	***Culture Started With***
AnMCP1	1% glucose and 20 ppm MCP
AnMCP2	0.5% glucose and 20 ppm MCP
AnMCP3	Without glucose; 20 ppm MCP
AnDCP1	0.5% glucose and 20 ppm DCP
AnDCP2	Without glucose; 20 ppm DCP
AnDCP3	Without glucose; 20 ppm DCP
AnPCP1	1% glucose and 20 ppm PCP
AnPCP2	0.5% glucose and 20 ppm PCP
AnPCP3	Without glucose; 20 ppm PCP

3. Results and Discussion

The removal of chlorophenols from wastewaters started with acclimatization of chlorophenols to grow in mixed culture (McCarty, 1985). The acclimatized culture should be not only capable of growing in the toxic environment, but also of degrading the chlorinated organics present therein.

Acclimatization of Anaerobic Culture to MCP: The amount in MCP was increased in the order: 20, 40, 60, 80, 100, 130, 160 and 200 ppm. For AnMCP1, glucose was decreased in the following order: 10, 8, 6, 4, 3 and 2 g/l. For AnMCP2, glucose was reduced in sequential order from 5 g/l to 2 g/l. Initially glucose was consumed within 24-48 hours. In such cases, the culture

was allowed to grow without glucose in order to increase their capacity to degrade chlorophenols. From day 30 to day 80, 8 g/l glucose was added every 3-4 days. As the culture started to degrade chlorophenols, glucose consumption rate decreased. From day 81 to day 150, 6 g/l glucose was added every 5-6 days in AnMCP1. As glucose consumption rate further decreased, glucose was added every 7 days. From day 151 to the end of acclimatization, glucose addition was maintained every 7 days and from day 270 to the end of acclimatization, initial concentration of glucose used was reduced to 2 g/l.

The chlorophenol degradation for AnMCP1, AnMCP2 and AnMCP3 for the duration of 350 days has been compared in Fig. 1. In AnMCP1 (the culture started with 10 g/L glucose), the first sign of MCP degradation occurred only after 28 days. MCP concentration was appreciably reduced to 16 ppm. By day 48, MCP concentration came down to 4 ppm. The initial acclimatization period is generally high for breaking down complex xenobiotics like chlorophenols. This period is related with multiplication of organisms responsible for catabolism of compound. MCP concentration was made up to 40 ppm on day 48. This time the degradation rate was much faster, resulting in complete removal of MCP in 36 days. On 84^{th} day, the concentration of AnMCP1 was increased to 60 ppm. It got degraded in 26 days.

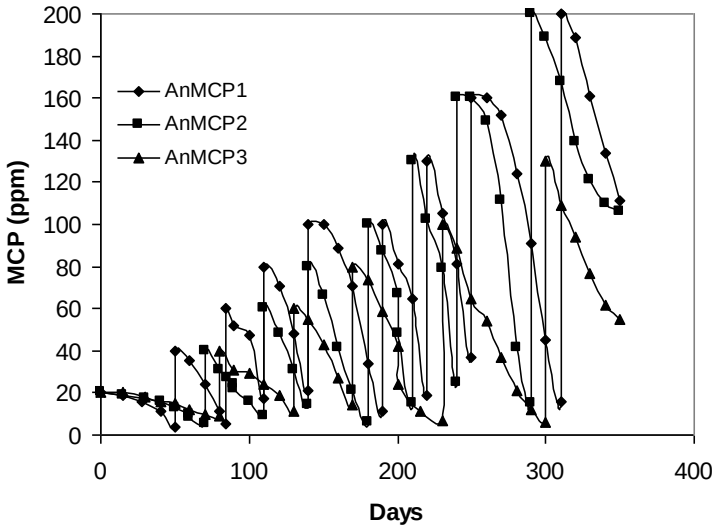

Fig. 1. Effect of glucose concentration on anaerobic acclimatization of MCP

The culture was exposed to 100 ppm MCP on day 140 in the order: 60, 80 and 100 ppm. For 10 days, no degradation was observed and methane was found to be inhibited (Fig. 3). pH was also found to be reduced indicating the inhibition of methanogens (data not shown). In the next 10 days, only 11% removal was observed. Methane composition also came down to 23% from 48%. It was clear that methanogenesis was inhibited due to the presence of MCP. To overcome the inhibition, 25 ml of fresh thick sludge enriched on glucose was added to AnMCP1 on day 160. The inhibition effect gradually subsided and on day 170, 29% reduction in MCP was observed with increase in methane composition. On day 190, MCP degradation was 89%. AnMCP1 was again made up with 100 ppm of MCP. Acclimatization proceeded smoothly, until 160 ppm of MCP was added on the 250th day. Once again for 10 days, no degradation was observed. Similar strategy as done earlier was followed, i.e. adding fresh thick sludge enriched on glucose. Within 3 days of adding fresh sludge, MCP degradation improved. By day 310, 90% removal was observed. On day 311, 200 ppm of MCP was added. AnMCP1 showed the sign of degradation,

but the inhibition effect seems to be dominant as rate of degradation was very slow. In 39 days, only 53% elimination was noted.

For AnMCP2, instability started only when the culture was made up with 160 ppm. Even after adding fresh sludge, culture continued to show the toxic effect resulting in decreased uptake rate at higher MCP concentration (Fig. 2). Both AnMCP1 and AnMCP2 were therefore not acclimatized beyond 200 ppm. AnMCP3, on the other hand could degrade only up to 130 ppm of MCP.

The obtained results were confirming by calculating chlorophenol uptake rate (q). It is defined as change in chlorophenol uptake per unit time. Fig. 2 gives the uptake rate for AnMCP1, AnMCP2 and AnMCP3. Though AnMCP1 showed higher acclimatization rate in the beginning, the acclimatization rate decreased in the intermediate stage (between 150-210 days) and in the last stage of acclimatization. This was, of course, due to the toxic effect of MCP observed in case of AnMCP1. Higher acclimatization in the beginning could be due to high biomass concentration because of higher glucose concentration (1 %). As discussed earlier, high biomass concentration enables higher secretion of enzymes capable of degrading toxic compounds. Later on, when AnMCP2 as well got affected by toxics, the acclimatization rate decreased (between 300 – 350 days). The value of q came down to 1.6 ppm/day. The highest value of q observed for AnMCP2 was 3.5 ppm/day.

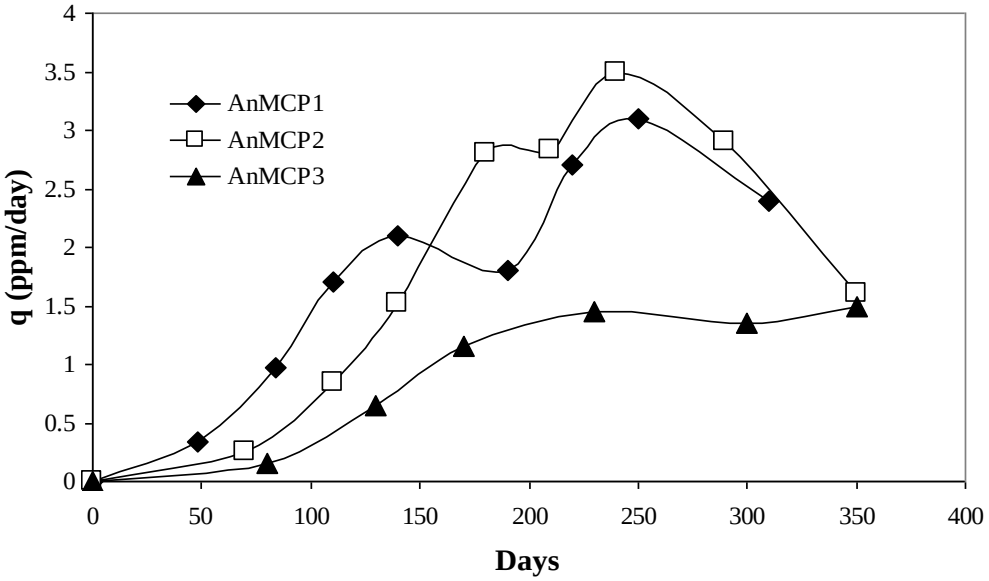

Fig. 2. Variation of chlorophenol uptake rate with respect to time for AnMCP1, AnMCP2 and AnMCP

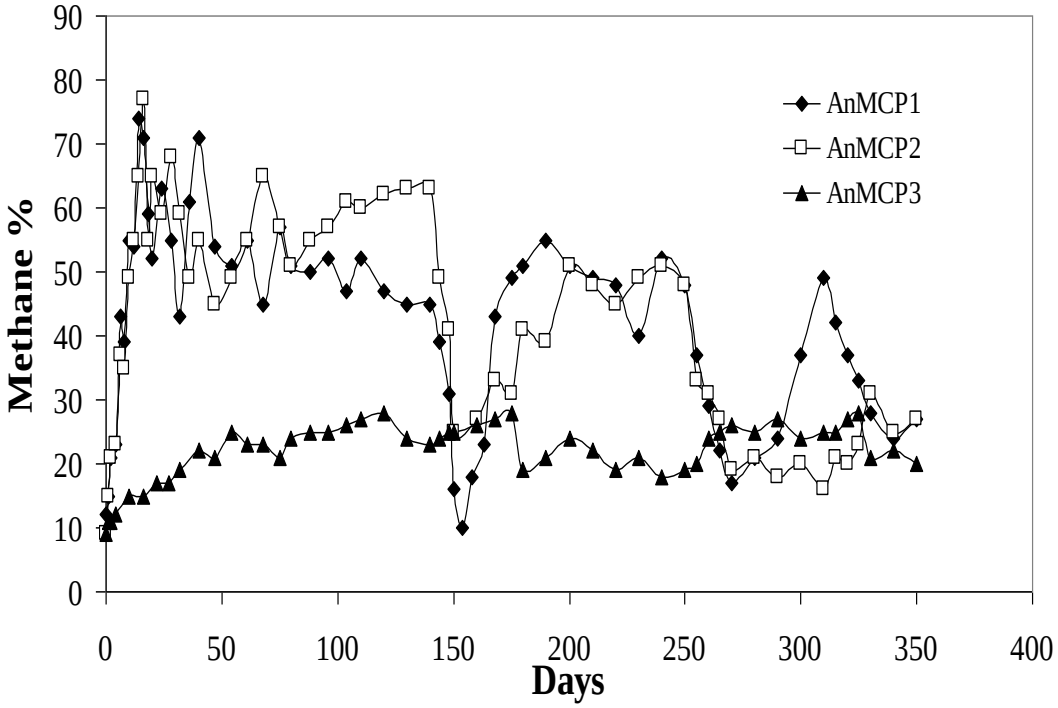

Fig. 3. Methane generation for AnMCP1, AnMCP2 and AnMCP3

The acclimatization of anaerobic culture was also monitored by noting methane production in these cultures (Fig. 3). During acidogenesis, some additional products were formed and these are

converted to acetic acid. The aromatic compounds undergo reductive dehalogenation and form phenol and subsequently benzoate ($C_7H_5NaO_2$). Benzoate reduces to acetate (CH_3COO^-) as per the following reaction, which is consumed by methanogens to produce methane (Evan, 1977; Jothimani et al. 2013).

$$C_7H_5NaO_2 + 7H_2O \rightarrow 3\ CH_3COO^- + 3\ H^+ + 3H_2 + HCO_3^- \quad (1)$$

$$CH_3COO^- + H_2O \quad - CH_4 + HCO_3^- \quad (2)$$

Inspection of the collective data reveals that there is no potential relationship between methane production and chlorophenol transformation. This is mainly due to the presence of high concentration of glucose. The concentration of MCP was less (approx. 1.5 mM at its peak concentration of 200 ppm), than that of glucose (which was in the range, 55.55 mM to 11.11 mM). Evidently the concentration of acetate will be affected more by the presence of glucose than by that of chlorophenols. In the beginning when, glucose was added at 10 g/l, methane concentration reached its peak (approx 74% in day 14). The intermittent decrease in methane concentration was due to the intermittent addition of glucose. As glucose concentration reduced, methane generation also reduced. Nonetheless, the inhibition of mixed culture by chlorophenol resulted in significant inhibition in methane production. Methane production reduced to 16 – 18% between 150 - 160 days. This corresponds to the time, when inhibition was observed in AnMCP1. It also illustrates that methanogenesis is related to the presence of the toxic compounds. Methane production observed in AnMCP3 also indicates that methanogens are active in the presence of MCP, if toxicity effect is not there, that is, if the MCP concentration is not above the threshold limit above which it severely inhibits the methanogens. As the toxicity effect in the culture increased and glucose addition depleted, methane production reduced in both AnMCP1 and AnMCP2.

Acclimatization of Anaerobic Culture to DCP and PCP: The acclimatization pattern of anaerobic culture to DCP and PCP were observed to be of similar nature as that of MCP. The acclimatization was steady in the beginning; the toxic effect was observed only when AnDCP1 was exposed to 130 ppm DCP. Once the effect of toxicity was weakened, acclimatization was more or less smooth till the culture was exposed to 200 ppm DCP. The acclimatization of AnDCP1, AnDCP2 and AnDCP3 is compared in Fig. 4. Uptake rates are shown in Fig. 5.

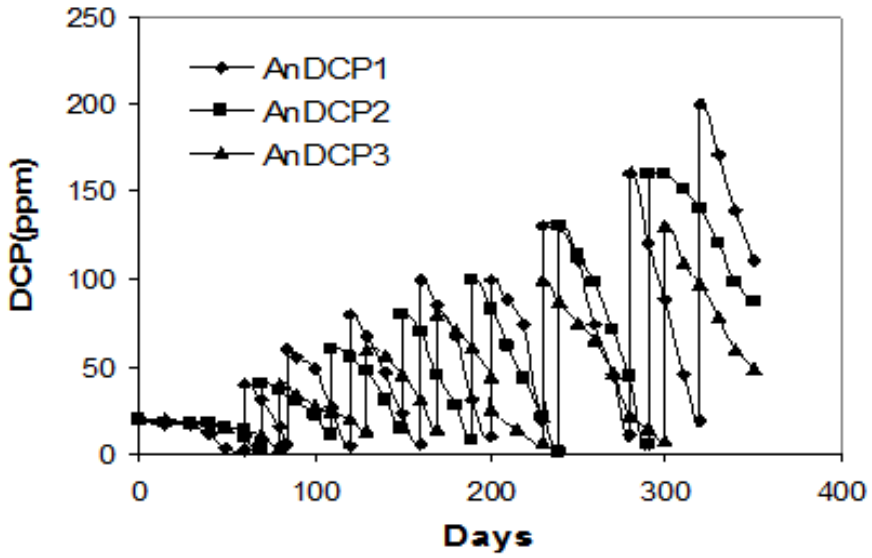

Fig. 4. Effect of glucose concentration on anaerobic acclimatization of DCP

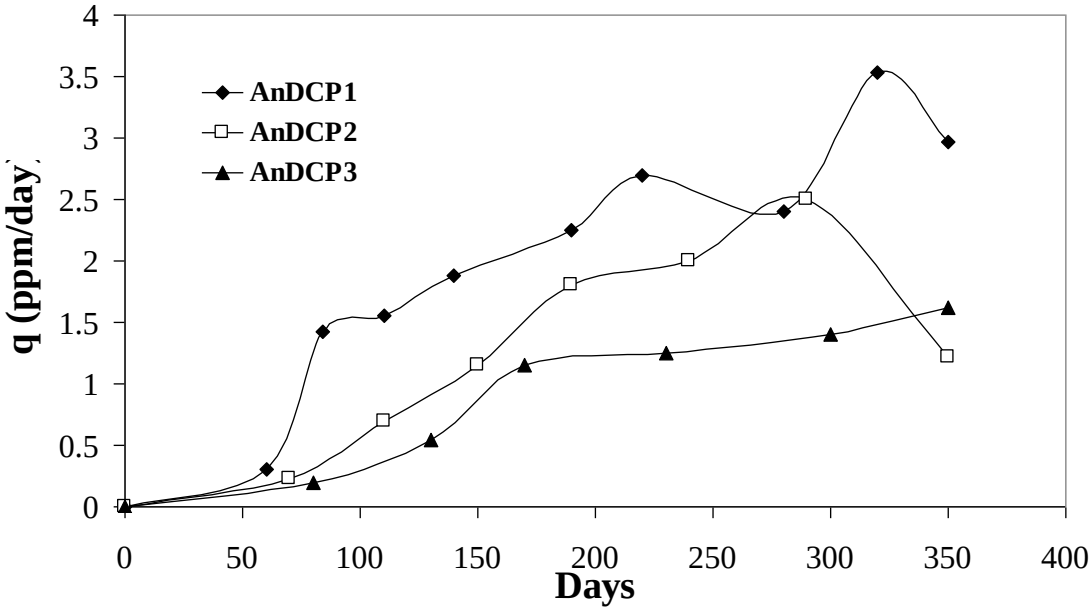

Fig. 5. Variation of chlorophenol uptake rate with respect to time for AnDCP1, AnDCP2 and AnDCP3.

But it can be seen from the Fig. 5 that fluctuations observed in DCP acclimatization was less compared to that observed for MCP acclimatization. The highest value observed was around 3.5 ppm/day.

Among all chlorophenols, PCP is the most vulnerable to undergo reductive dehalogenation. Because of the presence of five chlorine atoms in the benzene ring, it is easily susceptible to removal of chlorine. The uptake rate for AnPCP1, AnPCP2 and AnPCP3 are compared in Fig. 6. The acclimatization of PCP to mixed anaerobic culture is illustrated in Fig. 7. It was expected that PCP would show higher acclimatization than MCP and DCP. However, PCP could acclimatize up to 250 ppm, little higher than obtained for MCP and DCP. Acclimatization was quite steady till 250 days. It was only when culture was exposed to 200 ppm, inhibition occurred. Uptake rate drastically reduced from 4 ppm/day to 2 ppm/day. Because of the inhibition, it took about 70 days to degrade 90% of PCP. On observing Figs 6 and 7, it can be easily deduced that

acclimatization rate was same in both AnPCP1 and AnPCP2. However, the presence of glucose was definitely beneficial since AnPCP3 showed lower acclimatization levels.

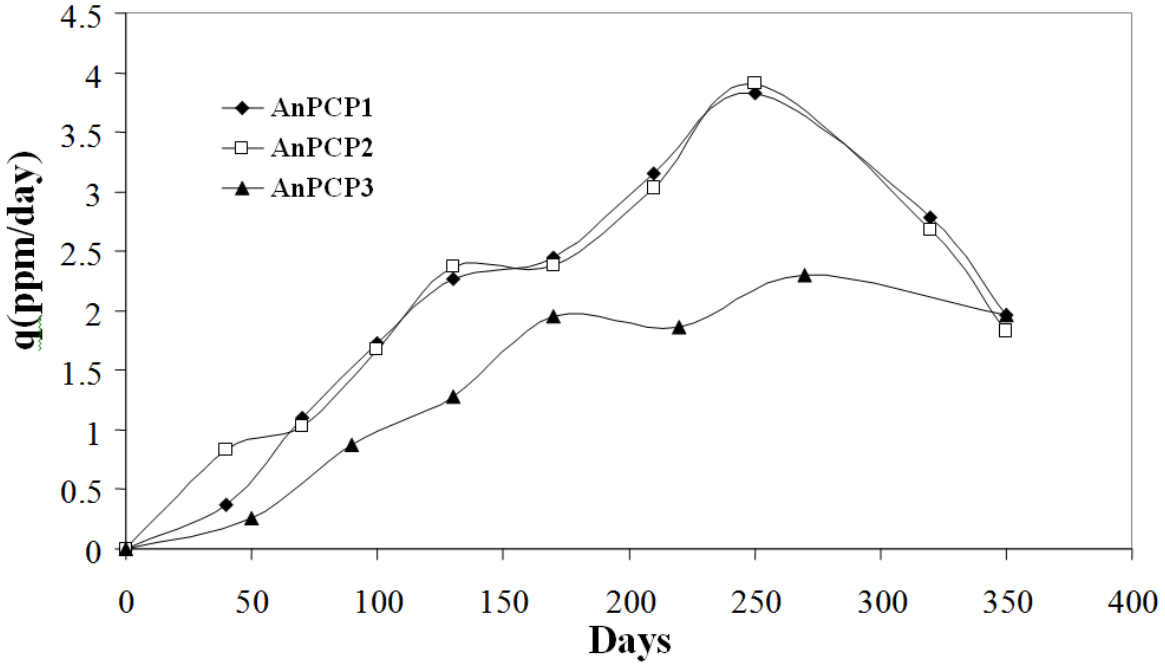

Fig. 6. Variation of chlorophenol uptake rate with respect to time for AnPCP1, AnPCP2 and AnPCP3

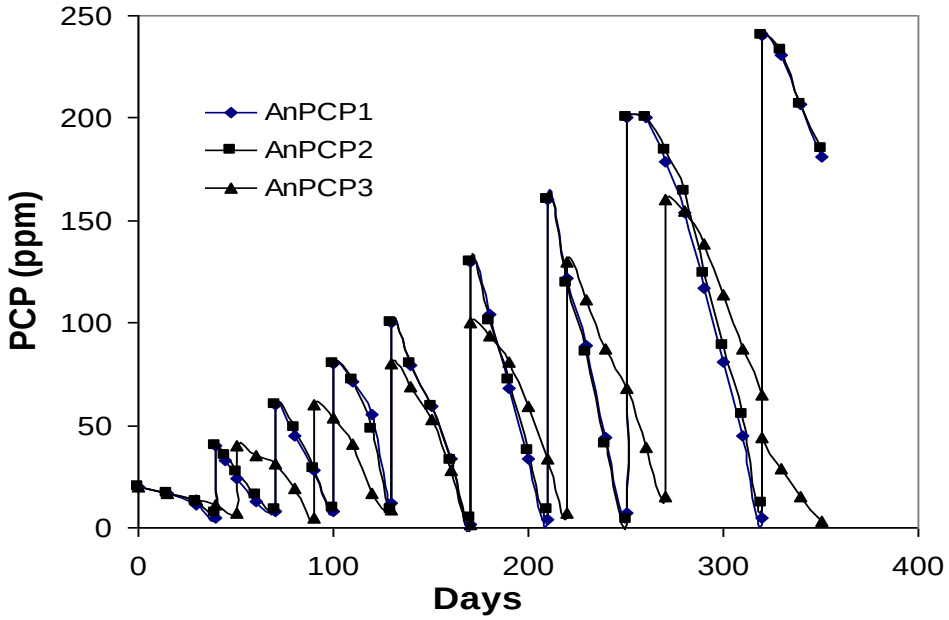

Fig. 7. Effect of glucose concentration on anaerobic acclimatization of PCP.

Effect of Glucose Concentration on Acclimatization of Chlorophenols in Anaerobic Environment: Many reports indicate that the presence of biogenic substrates increases the degradation rate of organics (Hu et al. 2005; Lopez et al. 2013). Menke and Rehm (1992) found that the addition of phenol increased the 3-CP degradation by *Alcacligenes sp* (Greer et al. 1990; Menke and Rehm, 1992). Similarly, naphthalene was reported to be instrumental in biodegradation of phenanthrene and flourene (Peng and Jia, 2013) but not glucose (Wang and Loh, 1999). The glucose represses transcription of the genes for phenol degradation in *Pseudomonas putida* H (Muller et al. 1996). Also, toxic substrates like PAHs affected phenanthrene degradation (Guha et al. 1999; Sahinkaya and Dilek, 2006). This could be due to the increased toxicity caused by some additional compounds or some intermediates generated, which could inhibit certain enzymes necessary for the biodegradation.

In case of mixed substrates, it is reported that substrate consumption generally follows diauxic or sequential growth pattern. However, there are increasing reports indicating that simultaneous consumption of two or more different carbon sources is a commonly observed phenomena, irrespective of growth conditions (Stringfellow and Aitken, 1995; Muller et al. 1996; Unell et al. 2008). 4- nitrophenol was found to be preferred substrate in mixtures of 4-nitrophenol and 4-chlorophenol. On other sides these two are degraded simultaneously. On comparing the glucose consumption and MCP degradation pattern, it was seen that in the initial phase of acclimatization, glucose was consumed at a faster rate.

For anaerobic degradation, biogenic substrates acts as electron donor as well as inhibitor in dechlorinated and chlorinated organics (Muller et al. 1996). Reductive dechlorination of chlorophenols yields the following reactions:

$$2,4,6\text{ - TCP} + 2H \xrightarrow{\text{Biomass}} 2,4\text{-DCP} + H^{+} + Cl^{-} \qquad (3)$$

$$2,4 - DCP + 2H \xrightarrow{Biomass} 4\text{-}MCP + H^+ + Cl^- \quad (4)$$

$$4 - MCP + 2H \xrightarrow{Biomass} Phenol + H^+ + Cl^- \quad (5)$$

where 2H is an electron donor in the medium (David et al, 1992; Feigelson et al. 2000; Jan and Igor, 2015). The presence of this hydrogen increases the rate of dechlorination. Under anaerobic conditions, existence of glucose in the medium facilitates favorable intermediates and also decreased lag phase. The favorable intermediates act as the source of electron donor resulted in increased biomass leading to increased stability. We observed greater acclimatization for culture supplemented with 1% glucose concentration as noted in acclimatization of all the three chlorophenols, MCP, DCP and PCP (Figs.1, 4, 7). However, literatures indicate complex interaction between multiple substrates leading to opposing effects (Juteau et al. 1995).

4. Conclusions

Detailed experiments on the acclimatization of anaerobic mixed cultures to chlorophenols were conducted. It was observed that after 350 days of acclimatization, anaerobic cultures were able to degrade up to 200 ppm MCP, 200 ppm DCP and 250 ppm PCP. Highest uptake rate was found to be 4 ppm/day for PCP acclimatization. It was also found that the presence of biogenic substrate increased the rate of chlorophenols acclimatization. Cultures started with 10 g/L glucose concentration showed higher acclimatization rates as compared to those started with 5 g/L and cultures without glucose.

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