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Biodegradation of 2, 4 Dichlorophenol

[1]Taghreed Al-Khalid and [2]Muftah H. El-Naas

[1]Department of Chemical Engineering, UAE University, Al-Ain, UAE
[2]Gas Processing Center, Qatar University, Doha, Qatar

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Corresponding Author:
Muftah H. El-Naas
Gas Processing Center, Qatar University, Doha, Qatar
Email: muftah@qu.edu.qa

Abstract: The present study focuses on the optimization of process parameters of the aerobic biodegradation of 2, 4 Dichlorophenol (2, 4 DCP) by a commercial strain of *P. putida* immobilized in PVA gel matrix, using design of experiments by Response Surface Methodology (RSM). The degradation rates obtained from these experiments were used to evaluate the effects of the main factors and their interactions during the biodegradation of 2, 4 DCP. An effective quadratic regression model predicting the rate in terms of the main independent variables, namely temperature, initial pH and initial concentration of 2, 4 DCP, was developed. The optimum conditions for DCP degradation were obtained as follows: Temperature 32.6°C, pH 5.0 and initial DCP concentration 70.5 mg L^{-1}, resulting in a maximum predicted degradation rate of 41.8 mg L^{-1} h^{-1}. Under these optimized conditions, a degradation rate of 40.1 mg L^{-1} h^{-1} was experimentally obtained, thus validating the model.

Keywords: Biodegradation, Immobilization, Optimization, Response Surface Methodology, Box-Behnken Design

Introduction

Chemical and petroleum industries generate a wide range of highly toxic organic substances, which have led to serious environmental impacts. The effluents of these industries are rich in aromatic organic compounds that are rather difficult for natural degradation and thus continue to exist as an environmental threat. Upon long range transportation, they will bioaccumulate in human and animal tissues. Organic pollutants are a set of chemicals that can put human health at high risk (Chung *et al*., 2003; Nair *et al*., 2008; Liu *et al*., 2009) and many aromatic compounds show carcinogenic, teratogenic or mutagenic properties (Zhao *et al*., 2009). Therefore, these compounds must be pretreated into biodegradable or less toxic compounds.

Chlorophenols are xenobiotic contaminants, which form a significant part of all organic chemicals either produced or used by many industries such as petrochemicals, oil refineries, plastics, insulation materials, pesticides, biocides, pulp and wood preservers (Kusic *et al*., 2011). Some of them (in particular mono-chlorophenols) can be formed during the chlorination of wastewaters and drinking water for disinfection and from the breakdown of pesticides and other chlorinated aromatic compounds (Ye and Shen, 2004; Sahinkaya and Dilek, 2006; Olaniran and Igbinosa, 2011; Majumder and Gupta, 2007; Menale *et al*., 2012). Due to their high toxicity, strong odor emission, persistence in the environment and suspected carcinogenicity, chlorophenols pose critical ecological issues (Kusic *et al*., 2011; Wang *et al*., 2007a). Most of the chlorophenols have been included in the US Environmental Protection Agency EPA list of priority pollutants (Kusic *et al*., 2011; Bhattacharya and Banerjee, 2008; Basak *et al*., 2013). The environmental regulations in many countries specify the maximum allowable concentration of phenols in the industrial effluents to be less than 1 mg L^{-1} (Kusic *et al*., 2011; Bapat *et al*., 2008). However, higher concentrations were frequently found in contaminated environments, with reported levels of chlorophenols ranging from 0.15 to 200 mg L^{-1} and even more (Angelini *et al*., 2011). The UAE legislations are very strict, with a limit of the total phenols in industrial water set to 0.1 mg L^{-1} (Al Zarooni and Elshorbagy, 2006).

Therefore, to save the soils and aqueous ecosystems, pretreatment of industrial wastewater has been mandatory worldwide. Phenolic compounds have been reported to be highly stable due to the difficulty of cleaving the benzene ring (Annadurai *et al*., 2008). However, several microorganisms can tolerate phenols and use them as a source of carbon and energy (Kılıç,

2009; Farrell and Quilty, 2002). As for chlorophenols, they are even more resistant to biodegradation as the enzyme activity needed for ring cleavage could be suppressed by the chloride atom (Wang *et al*., 2007a; Papazi and Kotzabasis, 2007).

Biological treatment of phenols has emerged as an increasingly important method in pollution management (Liu *et al*., 2009; Wang *et al*., 2007b; El-Naas *et al*., 2010a). Compared with physico-chemical methods, the biodegradation method of phenols removal is widely preferred as a more environmental friendly and cost effective approach, due to the possibility of complete mineralization of phenol or its derivative (Liu *et al*., 2009; El-Naas *et al*., 2009; Kargi and Eker, 2005). This results in complete conversion of a compound to its inorganic mineral constituents (Nair *et al*., 2008), with harmless end products and minimum secondary metabolites (Sridevi *et al*., 2011). Novel bioremediation strategies are rising as a favorable and most promising green technology for the treatment of wastewaters (Menale *et al*., 2012; Shourian *et al*., 2009).

Many strains of aerobic bacteria, including *Pseudomonas putida*, have been proven effective in utilizing aromatic compounds as the only source of carbon and energy (El-Naas *et al*., 2010a; 2009; Tziotzios *et al*., 2005). In recent years, *P. putida* has been a focus of research as the most widely used type of bacteria for phenols biodegradation and many studies have been reported on its utilization in free and immobilized forms, using different types of bioreactors (El-Naas *et al*., 2009). Immobilization is an effective technique that provides significant protection to the biomass from the harmful influences of the harsh environment imposed by the high substrate concentrations; reutilization of the biomass is an added value (El-Naas *et al*., 2010a; 2009; 2010b). Compared with free cells, the benefits of immobilization extend to include distinct stability and high degradation efficiency (Sheeja and Murugesan, 2002).

Since the biodegradation of chlorophenols is highly dependent on many conditions, such as pH, temperature, initial substrate concentration and others, it is critical to evaluate the effects of the key influencing parameters. Optimization of the biodegradation conditions is a prerequisite to large-scale applications of biodegradation processes (Basak *et al*., 2013). In recent years, optimization techniques have been employed to tackle important issues of contaminated site management (Bhattacharya *et al*., 2009). It is extremely tedious and time-consuming to go through such work using the conventional "one-factor-at-a-time" technique. Furthermore, this method does not account for the different interactions between two or more factors and therefore the results might be misinterpreted (Basak *et al*., 2013; Sridevi *et al*., 2011; Wu *et al*., 2009). In contrast, statistical design of experiments can optimize all the affecting parameters, saving a lot of time and money by considerably decreasing the number of trials needed to investigate the effects of multiple variables (Basak *et al*., 2013; Rigas *et al*., 2005).

Response Surface Methodology (RSM) is a graphical statistical modeling technique that is the most widely used for bioprocess optimization (Annadurai *et al*., 2002). It requires a prior knowledge of the process to obtain a mathematical model based on the relationship between a set of controllable experimental factors and observed results (Sridevi *et al*., 2011; Bhattacharya and Banerjee, 2008; Annadurai *et al*., 2008), with the great advantage that the interaction among the influencing variables is inherent in the model and can be evaluated with limited number of experiments (Annadurai *et al*., 2002). This optimization method involves three major steps: Performing the statistically designed experiments, estimating the coefficients in a mathematical model and validating the model by response prediction (Annadurai *et al*., 2008; 2002).

Biotreatment process optimization by statistical experimental design has been well documented (Annadurai *et al*., 2008; Sridevi *et al*., 2011; Sheeja and Murugesan, 2002; Wu *et al*., 2009; Rigas *et al*., 2005; Cutright and Meza, 2007; Agarry *et al*., 2008b; Yao *et al*., 2009). However few studies were reported as to chlorophenols removal (Kusic *et al*., 2011; Bhattacharya *et al*., 2009). Kusic *et al*. (2011) applied the Box-Behnken experimental design inherent in the RSM to evaluate the feasibility of UV/ferrioxalate system for the degradation of 4-Chlorophenol (4 CP) as a model wastewater pollutant. Kargi and Eker (2005) used a Box-Wilson design to optimize the removal of 2, 4 DCP from synthetic wastewater in a rotating perforated tube biofilm reactor by activated sludge culture supplemented with *P. putida*. Bhattacharya and Banerjee (2008) and Bhattacharya *et al*. (2009) employed RSM for the study of enzyme mediated biodegradation of 2,4 DCP. Basak *et al*. (2013) employed the Taguchi design of experiments to optimize the degradation of 4 CP by a yeast strain of *Candida tropicalis*.

The objective of the present study is to evaluate the RSM efficiency in optimizing the biodegradation of 2, 4 DCP in wastewater to achieve highest degradation rate. Batch experiments were performed in a Spouted Bed Bioreactor (SBBR) using a commercial consortium, in which *P. putida* is dominant, immobilized in Polyvinyl Alcohol (PVA) gel matrix. The classical single-parameter selection technique was initially used to approach the optimum range of each main factor. Following, the RSM with Box-Behnken design was applied to evaluate the effects of significant factors and their interactions, in the degradation of 2, 4 DCP and to find the optimal conditions for maximized degradation rates as obtained from batch experiments in the SBBR. The goal was to develop an effective quadratic regression model that is capable of predicting the rate in terms of the main independent variables, namely temperature, initial pH and initial concentration.

Materials and Methods

Details of the procedures and techniques utilized in the preparation of the biomass as well as bacterial immobilization and acclimatization have been published in a previous study (El-Naas *et al*., 2009). A brief summary of these procedures is given below.

Reagents

Analytical grade 2, 4 DCP was obtained from Sigma-Aldrich, Germany with purity greater than 99%. Synthetic 2, 4 DCP solutions ware prepared at the required concentrations by adding predetermined amount of the organic substance to nutrient solution, which had a total concentration of 825 mg L^{-1} of the essential mineral nutrients as shown in Table 1. The solid 2, 4 DCP was dissolved by heating at 35°C. The prepared solutions were then stored in dark containers in closed cabinets to avoid light oxidation of 2, 4 DCP. All other used chemicals and PVA powder were of analytical grade and obtained from BDH Chemicals, UK.

Preparation of Microbial Culture

The bacterium used was a specific strain of *P. putida*, obtained in cereal form (AMNITE P300) from Cleveland Biotech Ltd., UK. Detailed description of the bacteria preparation can be found elsewhere (El-Naas *et al*., 2009).

Immobilization

The bacteria cells were immobilized in a homogenous 10 wt% PVA gel mixture, which is reported to result in good quality and high porosity of the polymer matrix (El-Naas *et al*., 2009; 2010b). The immobilization technique aimed at increasing the mechanical strength of the polymer structure by inducing cross-linking with five repeated cycles of freezing-thawing. The frozen molds were divided into three portions, each of about 350 mL and cut to the specified size of 1, 0.5 and 0.25 cm^3 cubes, respectively. Detailed description of the biomass immobilization process can be found elsewhere (El-Naas *et al*., 2009).

Table 1. Composition of nutrient mineral medium

Component	Concentration, mg L^{-1}
$MgSO_4.7H_2O$	300
K_2HPO_4	250
$CaCl_2.2H_2O$	150
$(NH4)_2CO_3$	120
$FeSO_4.7H_2O$	3.5
$ZnSO_4.7H_2O$	1.3
$MnCl_2.H_2O$	0.13
$CuSO_4.5H_2O$	0.018
$CoCl_2.6H_2O$	0.015
Na2MnO.$2H_2O$	0.013
Total	824.98

Acclimatization of Bacteria

Phenol has been reported for its inhibitory effect to bacteria growth at concentrations above 0.05 g L^{-1} (El-Naas *et al*., 2010b; Bajaj *et al*., 2008). Therefore, acclimatization (adaptation) of the microbial cultures to phenols is an important step for efficient biodegradation of these compounds due to their high toxicity to microorganisms (El-Naas *et al*., 2009; 2010b; Bajaj *et al*., 2008; Al-Khalid and El-Naas, 2012; Khan *et al*., 2011). Pre-adaptation of the biomass to the chlorophenols is known to enhance the rate and the extent of biodegradation of those compounds (Bhattacharya and Banerjee, 2008; Kargi and Eker, 2005). The study was carried out with freshly-prepared bacteria adapted directly to 2, 4 DCP. Each portion of the three different sizes of the immobilized bacteria was suspended in a glass bottle to a total volume of about 1100 mL in nutrient solution prepared as shown in Table 1 and containing 25 mg L^{-1} of DCP as a source of organic carbon. The bottles were immersed in a water bath to control the temperature at 30°C. A continuous supply of air into the bottles was required to promote mixing and also secure excess oxygen to foster aerobic condition. The bacteria were then gradually acclimatized to DCP by a stepwise increase in the DCP from 25 to 50, 100, 150, up to 200 mg L^{-1} over a period of 5 days.

Analytical Methods

The concentration of 2, 4 DCP in the biomass-free samples was determined using Shimadzu Gas Chromatograph, Model GC 2014, Japan, equipped with capillary column and Electron Capture Detector (ECD). The injector and detector temperatures were adjusted to 275 and 300°C, respectively. The column temperature program started at 120°C and was increased to 220°C at a rate of 25°C min^{-1}. Measurements were made after filtering the 1 μL sample through 0.45 μm GHP Acrodisc filter, then injecting into the GC. A calibration curve of standard solutions of known concentrations was first established and then used as a basis to determine the residual concentration. The accuracy of the GC was continually checked every 4h of continuous operation by a standard solution. Statistically, averages of duplicate or triplicate experimental results were reported in the study, with the standard deviation ranging from 2 to 5% of the reported average. A portable pH-meter, Sartorius PT 10, Germany, with accuracy of ±0.005 and readability of ±0.01, was used for pH measurements. In the present study, Minitab16 was used for the design of experiments and analysis of the results.

Batch Removal of 2, 4 DCP in SBBR using PVA-Gel Immobilized Bacteria

After acclimatization and before starting the runs in the SBBR, the PVA gel cubes were soaked in the

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substrate solution of required concentration for 10 min, then the soaking solution was decanted. This step is needed to reduce the dilution effect in the reactor due to the water inherent in the PVA gel particles. The reactor was initially filled with standard mineral medium, prepared according to Table 1, which contained the required concentration of the substrate. PVA gel cubes with immobilized bacteria were added in a ratio of 30 vol% of the working volume (about 1 l) in the reactor. The SBBR configuration has the advantage of more efficient mixing over conventional bubble column bioreactors (El-Naas *et al*., 2010a; 2010b). The efficient intense mixing is due to the cyclic motion of particles within the bed, which results from an air jet injected through an orifice in the bottom of the reactor. The spouted bed bioreactor is made of Plexiglas and has a total volume of 1.1 l. The temperature control was achieved by circulating water into a jacket surrounding the reactor, from a water bath set at the desired temperature. A continuous flow of air into the reactor, at a pre-specified flow rate, was introduced to ensure intense mixing and to provide sufficient oxygen to maintain aerobic condition. A schematic diagram of the spouted bed reactor with dimensions is shown in Fig. 1.

When needed, the pH was adjusted by adding few drops of either HCl or NaOH according to the desired value. Samples for analysis of residual 2, 4 DCP concentrations were withdrawn at regular time intervals. To confirm that the removal efficiency was due to the biodegradation effect and not to other abiotic effects such as stripping or adsorption on the carrier, some blank experiments were run under the same operating conditions as explained in the results and discussion section.

Single Factor Batch Experiments to Identify the Optimum Range of Different Factors

This study was based on a classical single-parameter selection technique to approach the optimal range of each main variable (temperature T, initial pH value, initial DCP concentration C_o, size of immobilized particles and inlet air flow rate AFR). These factors were found critical in biodegradation processes by many previous studies (Menale *et al*., 2012; Bhattacharya and Banerjee, 2008; El-Naas *et al*., 2010a; 2009; 2010b; Sheeja and Murugesan, 2002; Bhattacharya *et al*., 2009; Agarry *et al*., 2008b; Yao *et al*., 2009; Agarry *et al*., 2008a). Subsequently, this was followed by optimization of the first three factors by the response surface methodology.

Experiments were conducted with different initial 2, 4 DCP concentrations (25, 50, 100, 150, 200 mg L^{-1}), temperature (22, 25, 30, 35, 40 °C), the initial pH value (5, 6, 7, 8, 9, 10). Three levels were considered for PVA particle size (0.25, 0.5, 1 cm^3) and five levels of air flow rate (1, 2, 3, 4, 5 L min^{-1}) for a total working volume of 1000 mL. In each experiment, the factor under study was changed, with the other factors fixed at their normal values. At the end of this study, the PVA particle size and the inlet air flow rate were fixed, whereas the range of temperature, pH and initial DCP concentration were chosen to be further optimized by RSM.

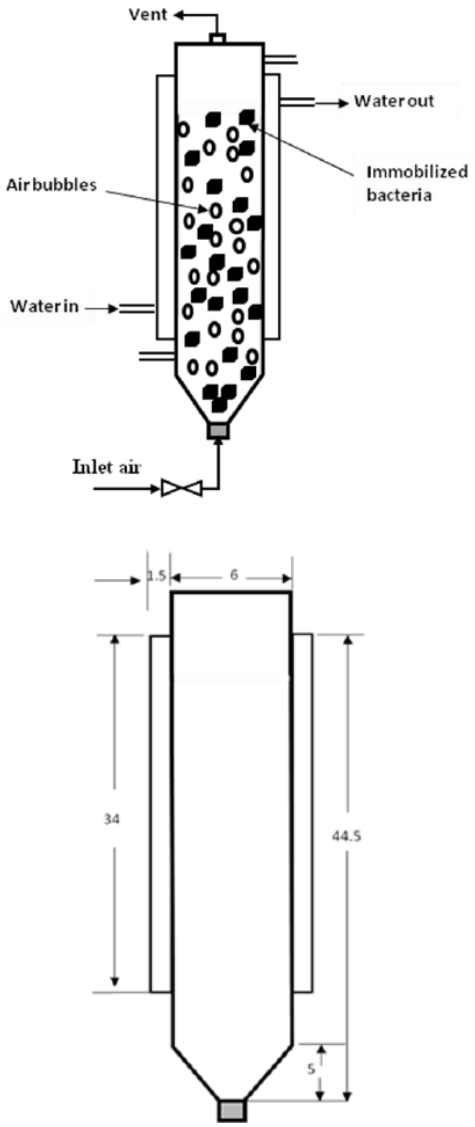


Fig. 1. A schematic diagram of the spouted bed bioreactor (all dimensions in cm)

Table 2. Experimental design range and levels of the design factors

	Coded values		
Factors	-1	0	1
A: Temperature, °C	25	30	35
B: Initial pH value	5	7	9
C: Initial DCP concentration, mg/l	25	75	125

Response Surface Methodology (RSM)

From the above experiments, the optimal ranges of the three main variables were determined to maximize DCP degradation with RSM. Minitab 16 Statistical Software was used to apply a Box-Behnken factorial design with three factors, each at three levels, in addition to three replicates at the center point. A second order response model was obtained that describes DCP degradation based on the main and interactive effects of these parameters. The range and the levels of the process variables under study are given in Table 2: Temperature (25, 30 and 35°C), pH (5, 7 and 9) and initial DCP concentration (25, 75 and 125 mg L^{-1}), which were represented in the model as critical parameters A, B and C, respectively.

Results and Discussion

Single Factor Batch Experiments to Identify the Optimum Range of Different Factors

Confirmation of Biodegradation

Since biodegradation experiments are usually performed at 30°C with continuous aeration, there is a need to investigate and exclude any possible contribution to the overall biodegradation rate by both stripping of 2, 4 DCP evaporation and adsorption. Control experiments were conducted to evaluate the contribution of stripping and adsorption on bacteria-free PVA to the overall biodegradation rate. Some runs involved nutrients added to the DCP solution to check the less likely possible contribution of external bacteria to the biodegradation of DCP. These control tests were performed under identical operating conditions to those employed in the biodegradation experiments. The losses ranged from 2.5 to 7%, which confirms that the 2, 4 DCP removal is mainly attributed to biodegradation by immobilized *P. putida*.

Effect of PVA Particle Size

To investigate the effect of PVA particle size of 1.0, 0.5 and 0.25 cm^3, experiments were conducted for a range of DCP initial concentration (5, 10, 25, 50 and 100 mg L^{-1}). The initial pH was kept at the initial value without any adjustment in the range of 8.2-8.4, while the temperature and AFR were fixed at 30°C and 3 L min^{-1}, respectively. It is expected that mixing inside the reactor would be improved upon reducing the particle size and mass transfer would be enhanced probably by lowering diffusion limitations and increasing the surface area, which makes the biomass more accessible to the substrate (El-Naas *et al*., 2010b; Sheeja and Murugesan, 2002). This was observed to be more pronounced at low initial concentrations (10, 25 and 50 mg L^{-1}) rather than high concentration of 100 mg L^{-1} as shown by Fig. 2. This could be justified by the fact that the highly porous structure of the PVA particles, coupled with high bulk concentration of the substrate, will result in a negligible resistance to mass transfer (El-Naas *et al*., 2009).

Figure 3 presents a comparison of the three particle sizes for an inlet AFR of 1 L min^{-1} (a) and 5 L min^{-1} (b). According to El-Naas *et al*. (2010b), the enhancement of the biodegradation rate was more obvious for high air flow rates as a result of improving the mass transfer inside the reactor by the combined effect of good mixing and reduced particle size. However, this is not significantly shown by the results in Fig. 3. This could be attributed to the fact that these experiments were carried out directly after acclimatization and it is suspected that there was a lot of loss in cells due to the cutting process, which still could not be compensated at the early stage of the process. This is depicted by the degradation by the 0.5 cm^3 particles, which seemed to be the slowest in most of the runs.

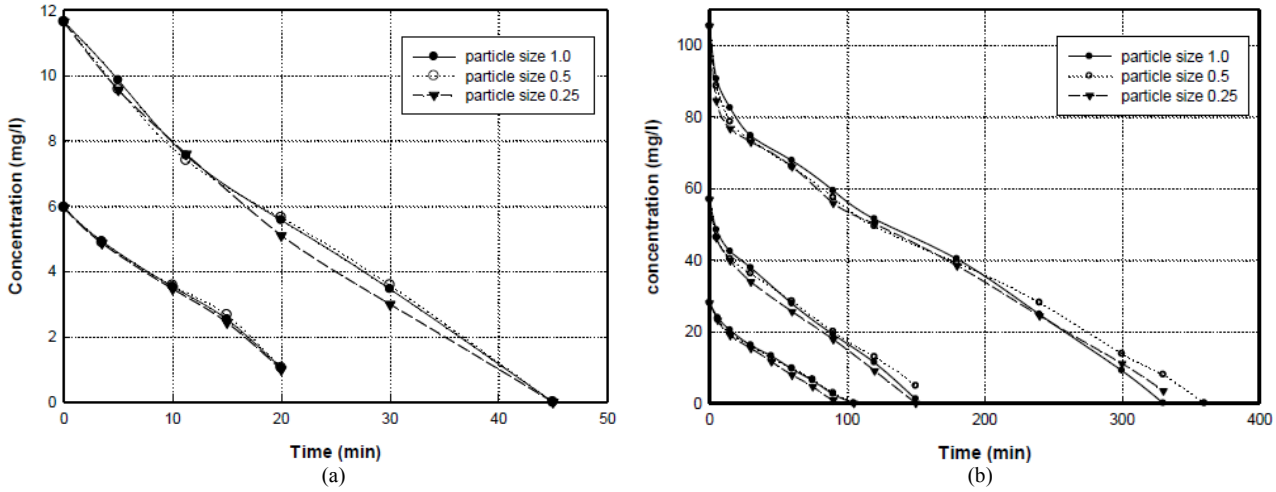


Fig. 2. Effect of PVA particle size at different initial DCP concentration; T = 30°C; pH = 8.2-8.4; AFR = 3 L min^{-1}

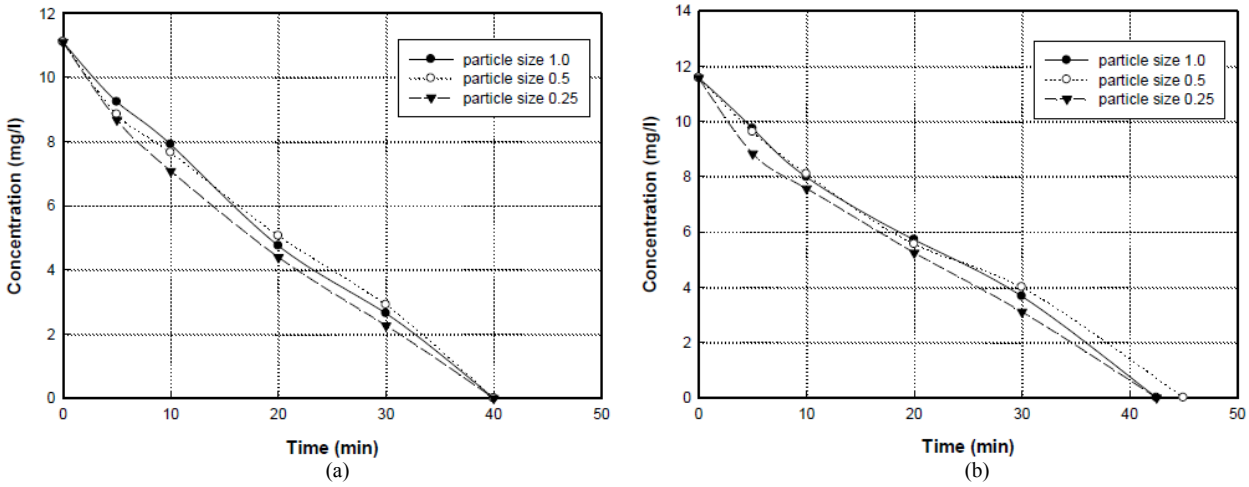


Fig. 3. Effect of PVA particle size at AFR of 1 L min^{-1} (a) and 5 L min^{-1} (b); T = 30°C; pH = 8.2-8.4

Based on the above and in view of the technical difficulty in using the 0.25 cm^3 particles due to the attrition effect, it was decided to continue all subsequent experiments with *P. putida* immobilized in PVA gel particles of 1 cm^3 in size (for simplicity, particle sizes in the figures will be designated as 1.0, 0.5, 0.25).

Effect of Temperature

The enzymatic activity and rate of metabolism are highly dependent on temperature, which makes it an important quantity in bioprocesses (Agarry *et al*., 2008b). Experiments were carried out to assess the effect of temperature in the range from 22°C (room temperature) to 40°C in two phases as shown in Fig. 4a and 4b. Figure 4a for the first phase indicates that the degradation rate increases with temperature up to 30°C and does not change at 35°C. However, a strange phenomenon took place when the replicate run at 35°C was carried out, represented by the suppression of the degradation rate accompanied by the appearance of a peculiar peak in the GC chromatogram, which grew to a maximum before it started to diminish towards the end of the biodegradation time. It took the biomass about one week of daily operation to get rid of this incidental effect. Once the previous biodegradation ability was restored, the second phase of experimentation started to cover the whole temperature range. Since the biodegradation capacity changes due to biomass growth in PVA matrices, it was essential to conduct all the experiments for one factor sequentially within a short period of time. Thus all the experiments at the different temperatures were repeated as shown in Fig. 4b.

It is clear that the performance had even improved as represented by the shorter degradation times at all temperatures. The trend in Fig. 4a is reiterated by the increase in biodegradation rate with temperature to a maximum at 30°C, which does not change much at 35°C (only one run at 35°C). At 40°C the degradation rate decreased and again there was later a rather great suppression in the biodegradation rate, which was even more pronounced than the previous one and it is thought to be caused by the greater production of metabolites beyond 30°C. According to a preliminary GC-MS identification, the likely possibility of the odd peak would be an aromatic material, dimethyl phthalate, or vitamin A aldehyde, which could be a bacterial enzyme released at those conditions. However, it has been pointed out by several studies (Chung *et al*., 2003; Lu *et al*., 1996; Reardon *et al*., 2000; Annadurai *et al*., 2002; Safont *et al*., 2012) that the formation of dead end metabolic intermediates or the toxic effect of the substrate on the cells is often associated with a yellowish to brownish colour change, often resulting from involving the *meta*-pathway for degradation, which is more related to incomplete mineralization (Solyanikova and Golovleva, 2004; Lu *et al*., 1996). Such a colour change has never been detected in any situation in the present study. Rigas *et al*. (2005) reported in their study on biodegradation of lindane that the chromatograms obtained by the GC showed no significant new peaks, which was considered an evidence for a biotransformation pathway proceeding with fast intermediate reaction rates and in favor of complete mineralization of the pollutant. It is believed that this topic of intermediate detection and characterization is worth further investigation.

El-Naas *et al*. (2009) believed that the key step in the biodegradation process, which is benzene ring cleavage, may be adversely affected by sudden exposure to temperatures higher than 35°C.

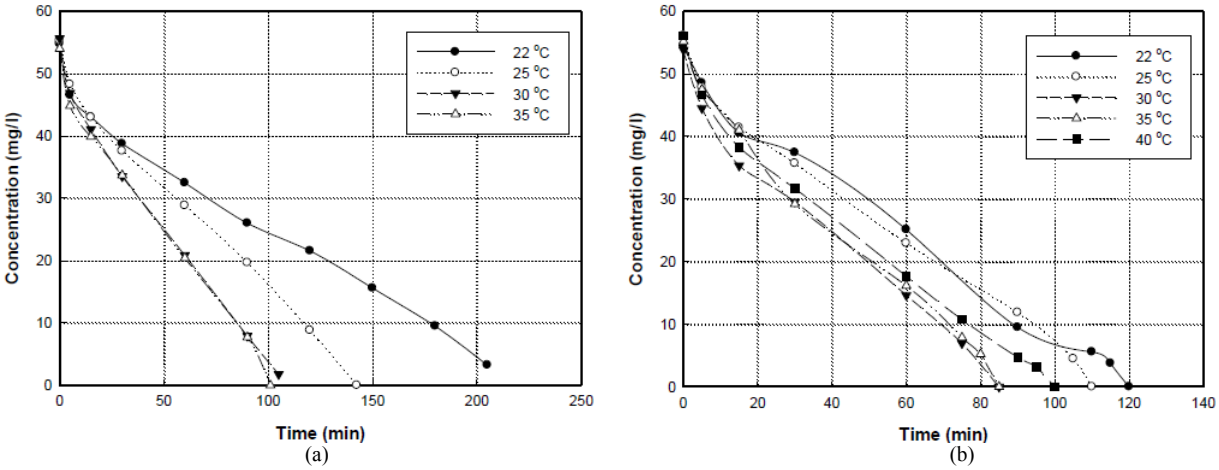


Fig. 4. Effect of temperature: experiments of phase 1 (a) and phase 2 (b); C_o = 55 mg L^{-1}; pH = 8.2-8.4; AFR = 3 L min^{-1}

It is believed that the decrease in the reactivity of the enzyme system within the cell, that is usually responsible for ring cleavage, will result in a decrease in the degradation rate (Agarry *et al*., 2008b; Sheeja and Murugesan, 2002). Additionally, extreme temperatures lower the water solubility of oxygen resulting in insufficient content to favor a higher reaction rate at high temperatures (Menale *et al*., 2012). However, from Fig. 5, it can be seen that the highest degradation rate was at a temperature of 30°C.

Effect of Initial pH

The initial pH of the reaction medium is essentially considered a very important parameter in the development of biological processes owing to the critical role it plays in microbial growth and enzyme activity (Basak *et al*., 2013; Yao *et al*., 2009). The effect of the initial pH (from 5.0 to 10.0) on the DCP degradation rate was investigated. As shown in Fig. 6, at each initial pH of the reaction medium, the DCP could be completely utilized within less than 100 min. However, the maximum degradation rate was perceived when the initial pH was 5.0 and the lowest when it was 10.0, while the degradation times were very close in the range 7.0-9.0 of initial pH. Therefore, the optimum pH value occurred at 5.0 in agreement with some other studies which utilized either enzymes or whole cells for DCP biodegradation (Menale *et al*., 2012). When following the pH value along the biodegradation process, it was noticed that the pH followed a similar trend to that of reduction of DCP concentration. This may be attributed to the organic acids formed as a consequence of intermediates formation during the degradation process. Sheeja and Murugesan (2002) reported that the pH will rise following the subsequent degradation of these acids; however, the final pH still remains less than the initial set value and this was evident in all the experiments of the current study.

The decrease in pH may be also due to HCl formation from the biodegradation of chlorophenols (Sahinkaya and Dilek, 2006). El-Naas *et al*. (2009) mentioned that the decrease in pH is caused by the production of CO_2 that results in the formation of carbonic acid.

However, this decrease in pH value was confirmed in the cases where the initial pH was higher than 7.0. The greater the initial pH is, the greater the decrease in its value. On the contrary, when the initial pH was less than or equal to 7.0, the pH increased during the reaction time. The lower the initial pH is, the higher the increase in its value. In both cases, the final pH would be in the range 6.5-8.0, which is the optimum range of the used bacteria to thrive.

Effect of Initial DCP Concentration

In the biodegradation process, initial substrate concentration plays an important role, which is clearly recognized by the inhibitory effect of some hydrocarbon contaminants, including DCP, on the activity of the biomass. Experiments were carried out in the range of 25-200 mg L^{-1} as shown in Fig. 7b, whereas Fig. 7a presents results from the previous group of experiments, which were performed during the study of particle size effect (for particle size 1.0). Complete degradation of DCP was observed at every initial concentration, which means that the inhibition effect was not significantly encountered, thanks to the protection provided to the bacteria within the PVA matrix by immobilization against the high contaminant concentration. It was noticed that DCP concentration decreased almost linearly with time and thus, the degradation rate may be assumed constant, following zero-order kinetics for all initial concentrations of DCP. A similar trend was observed by El-Naas *et al*. (2009) for the biodegradation of phenol.

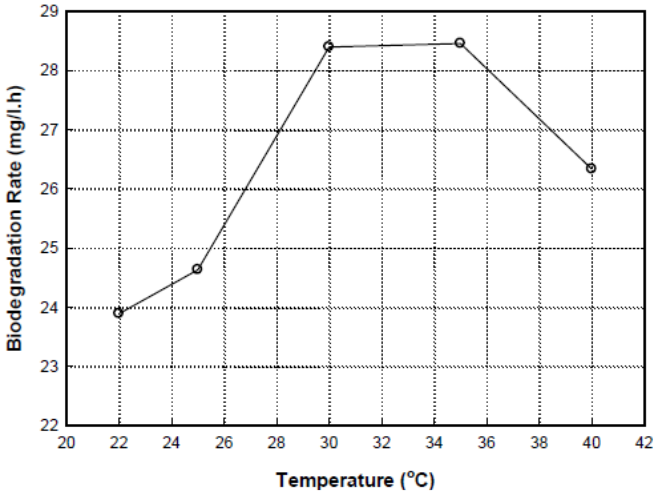


Fig. 5. Effect of temperature on DCP degradation rate

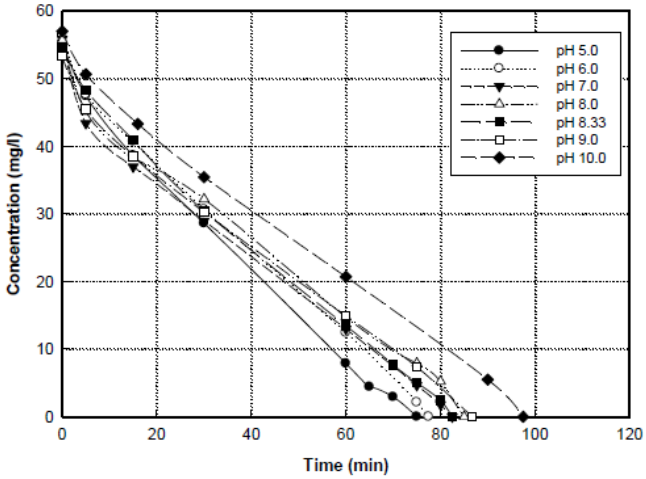


Fig. 6. Effect of initial pH; T = 30°C; C_o = 55 mg L^{-1}; AFR = 3 L min^{-1}

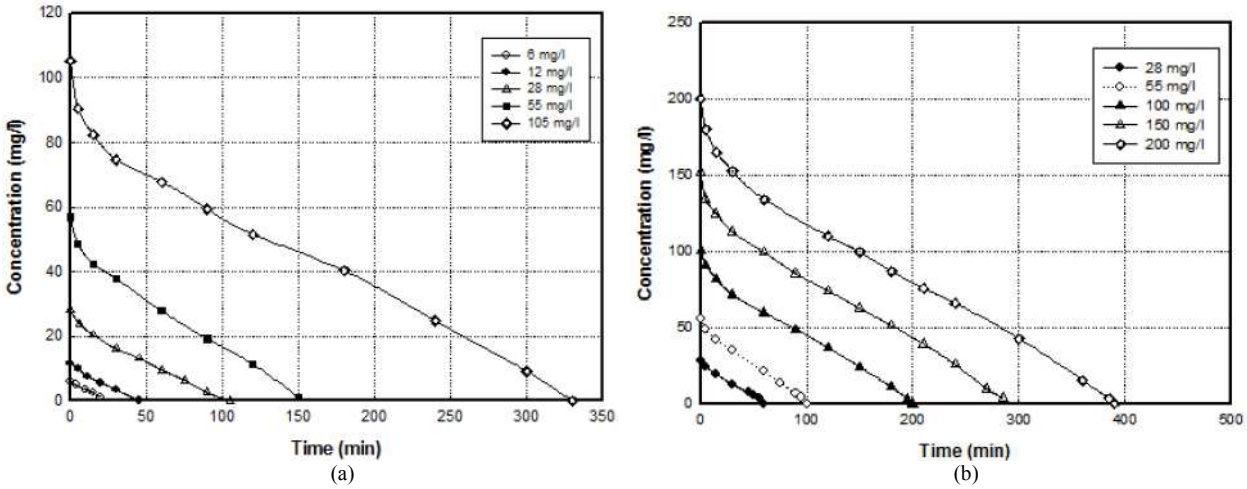


Fig. 7. Effect of initial DCP concentration: First set (a) and second set (b); T = 30 °C; pH = 8.2-8.4; AFR = 3 L min^{-1}

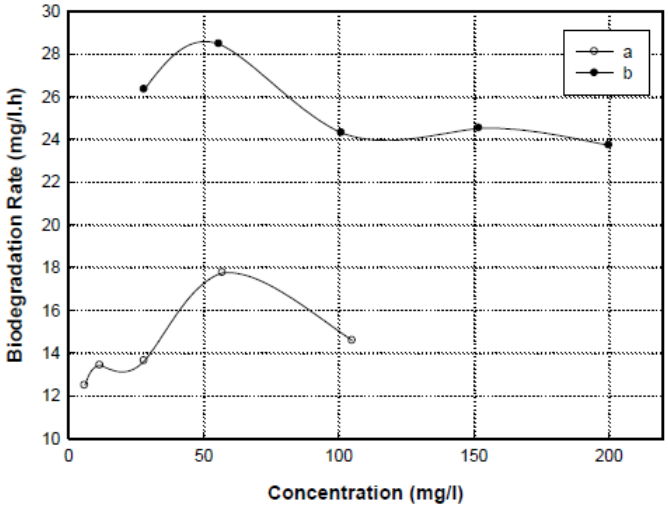


Fig. 8. Biodegradation rates for the data in Fig. 7

Biodegradation rates for the degradation data in Fig. 7a and 7b are presented in Fig. 8, referred to as (a) and (b), respectively. Both curves of the degradation rates reached a maximum at an initial substrate concentration of 55 mg L^{-1} and then decreased slightly and smoothly (b), which can be explained by a minor inhibitory effect toward the cells. On the other hand, the lower biodegradation rate at low DCP concentration could be attributed to mass transfer limitation, where there is less DCP accessible to the biomass. It is worth mentioning that the rates in the experiments of the more recent study (b) are superior to those of the previous study (a), which indicates the increase in the activity of the biomass as a result of cell growth over a time span of almost two months between the two studies.

This continued improvement in the performance of the biomass upon repeated use has been highlighted by others (Hsieh *et al*., 2008; El-Naas *et al*., 2013; Varma and Gaikwad, 2009; Ali *et al*., 2013). The biodegradation rates were calculated from the slopes of the best fitted straight line, ignoring the initial segment of fast drop in DCP concentration as it is thought to be much influenced by the dilution effect of water release from the PVA gel particles (water accounts for about 90% of the gel mass).

Effect of Air Flow Rate in SBBR

Air flow rate affects the biodegradation process in a SBBR through its direct relation with two main factors: Mixing and aeration (El-Naas *et al*., 2010b). To determine the effect of AFR, two groups of experiments were carried out at an initial concentration of 55 mg L^{-1} using different air flow rates: One group was conducted without adjustment of the initial pH (original pH of 8.3) as shown in Fig. 9a, whereas the other group was conducted with the initial pH adjusted to 5.0 as illustrated by Fig. 9b. Both figures show a trend of increase in the degradation rate with increase in AFR, being more pronounced at initial pH value of 5.0. This is mainly due to the reduction in external mass transfer resistance being induced by enhanced agitation and the increase in the amount of dissolved oxygen. In their study, El-Naas *et al*. (2010b) proved that at AFR higher than 1 L min^{-1}, the main factor of enhancing continuous biodegradation of phenol in a SBBR at an initial concentration of 30 mg L^{-1} is the availability of sufficient oxygen rather than mixing, highlighting the importance of mixing effect in cases where PVA particles of smaller size are used or when the initial substrate concentration is very low. It was reported that high AFR may produce excessive foaming or air bubbles coalescence (Agarry *et al*., 2008b), which could adversely affect the biodegradation process but this effect was not observed in this study. However, to avoid these adverse effects at high AFR, which may also cause slugging and attrition of the PVA particles and to minimize the role of evaporation/stripping as well, it was decided to continue all the following experiments with AFR of 3.0 L min^{-1}.

Response Surface Methodology (RSM)

The objective of applying the RSM is to determine the optimal operating conditions for the given process. The Box-Behnken design was employed to study the effects and interactions of the main parameters, namely temperature, pH and initial DCP concentration, to be further optimized for DCP biodegradation. The design was applied considering the three parameters at three levels. The experimental conditions and the corresponding results obtained, expressed as global biodegradation rates are summarized in Table 3.

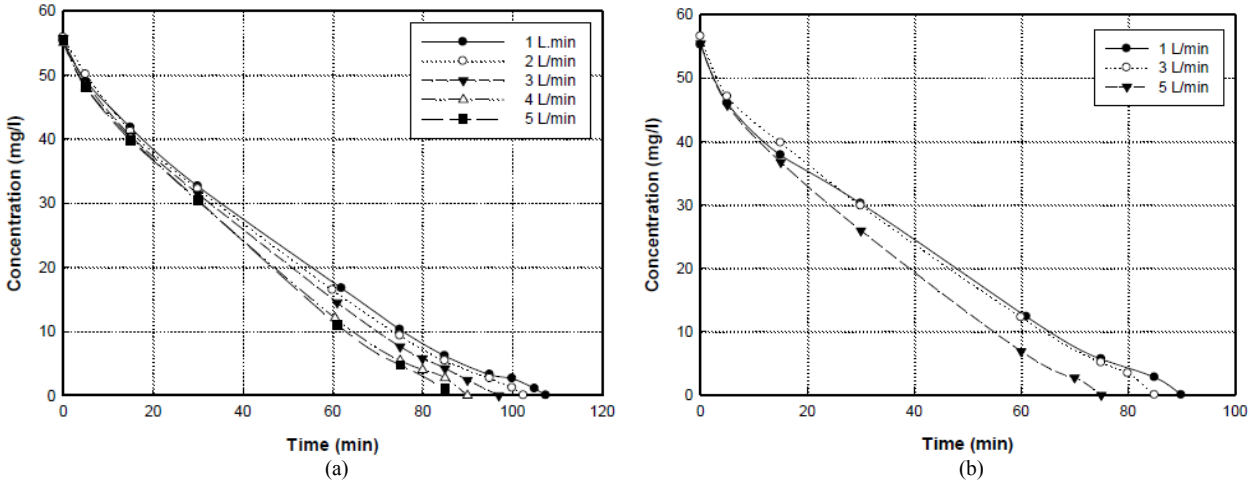


Fig. 9. Effect of inlet air flow rate at initial pH 8.3 (a) and 5.0 (b); T = 30°C; C_o = 55 mg L^{-1}

Table 3. The Box-Behnken experimental design along with the actual and predicted responses

				Global degradation rate, mg L^{-1} h^{-1}	
Run order	A (T)	B (pH)	C (C_o)	Experimental	Predicted
1	0	1	1	34.85	33.30
2	-1	0	1	28.41	29.47
3	1	-1	0	41.71	41.22
4	0	0	0	37.02	37.69
5	-1	1	0	29.70	30.19
6	1	1	0	34.74	34.86
7	1	0	1	33.07	34.50
8	0	1	-1	27.24	28.18
9	0	0	0	37.70	37.69
10	0	0	0	38.35	37.69
11	0	-1	1	37.16	36.22
12	-1	-1	0	36.40	36.28
13	-1	0	-1	29.30	27.87
14	1	0	-1	33.52	32.46
15	0	-1	-1	36.15	37.70

Regression Model

The experimental data were subjected to multiple regression analysis to fit a second order polynomial regression model containing 3 linear, 3 quadratic and 3 interaction terms. Hence, the following second order polynomial equation was obtained to represent the global rate of DCP degradation adequately. The mathematical formulation is given below in terms of coded factors:

$$Y = 37.69 + 2.40A - 3.11B + 0.91C - 2.41A^2 + 0.36B^2 - 0.42C^2 - 0.07AB + 0.11AC + 1,65BC \quad (1)$$

and in terms of uncoded factors:

$$Y = -50.90 + 6.29T - 3.86pH + 0.14C_o - 0.1T^2 + 0.09pH^2 - 0.002{C_o}^2 - 0.01TpH + 0.0004TC_o + 0.017pHC_o \quad (2)$$

Where:

A = The temperature (°C)
B = The initial pH value
C = The initial DCP concentration (mg L^{-1})
Y = The response represented by the global degradation rate of DCP (mg L^{-1} h^{-1}))

A, B, C are the independent factors in the Box-Behnken design. Positive values of A and C in Equation 1 indicated that DCP degradation rate increased as temperature and initial DCP concentration increased, whereas it increased as pH decreased. This was reflected by the negative coefficient of B. The trends for the first-order terms were as expected as they were matching the effects derived from the experimental data.

It is essential to analyze the statistical significance of the regression model of 2, 4 DCP biodegradation to assess its reliability in representing the data. So, the

Analysis Of Variance (ANOVA) of the quadratic equation was performed as presented in Table 4. The coefficients of the regression model as represented by Equation 1 are listed in Table 5.

A *p*-value below 0.05 for any regression coefficient indicates that it is statistically significant. Thus, the model may be assumed highly significant based on a low failure probability (p = 0.014) and a coefficient of determination (R^2) value of 0.9403, as well as a value of the adjusted R^2 of 0.8328. These values confirm the adequacy of the model, with the indication that 94.03% of the total variations in the response could be explained by the model. Moreover, the *p*-value of 0.091 for the lack-of-fit of the model indicates that it is insignificant at the 95% confidence level, in support for the adequacy of the model to represent the experimental results.

The coefficients listed in Table 5 are three linear, three quadratic, three interaction terms and one block term. Also listed in Table 5 are the corresponding Student's *t*-test and *p*-values, which serve as a measure to determine the significance of each coefficient; the greater the *t*-value and smaller the *p*-value, the more significant is the corresponding coefficient. Accordingly, it is found out that the first order main effects of A (temperature) and B (pH) and the second order effects of A and C (initial DCP concentration) are highly significant as implied by their respective *p*-values <0.05. The quadratic effect of C was more pronounced than its linear effect. All the interaction effects had *p*-values >0.05, making them insignificant. This has the implication that some parameters under study for the biodegradation of 2, 4 DCP are not highly affected by each other.

The normal probability and parity plots further verified the model adequacy, as depicted in Fig. 10 and 11, respectively. The residuals analysis reveals that there were no outliers as all the standardized residuals fell within the range of -2 to +2, being also randomly distributed around zero. This is an evidence of a high degree of correlation between the observed values and predicted values. This was also demonstrated by the parity plot, with the points clustering very closely around the diagonal line confirming the little discrepancy between the observed and predicted values (as obtained from Equation 1) for rate of DCP degradation.

Table 4. Analysis of Variance (ANOVA) for the regression model

Source	Degree of freedom	Sum of squares	Adjusted sum of squares	*p*-value
Model	9	225.244	225.244	0.014
Linear	3	130.288	130.288	0.006
A	1	46.224	46.224	0.010
B	1	77.439	77.439	0.003
C	1	6.625	6.625	0.189
Square	3	84.000	84.000	0.016
A^2	1	17.089	21.512	0.041
B^2	1	1.740	0.482	0.699
C^2	1	65.171	65.171	0.005
Interaction	3	10.957	10.957	0.378
A*B	1	0.018	0.018	0.939
A*C	1	0.048	0.048	0.902
B*C	1	10.890	10.890	0.109
Residual rror	5	14.305	14.305	
Lack-of-fit	3	13.342	13.421	0.091
Pure error	2	0.885	0.885	
Total	14	239.549		
R^2 = 94.03%		Adjusted R^2 = 83.28%		

Table 5. Coefficients for the quadratic regression model

Term	Value	Standard error	*t*-value	*p*-value
Constant	37.69	0.9766	38.594	0.000
Temp: A	2.4038	0.5980	4.019	0.010
pH: B	-3.1113	0.5980	-5.203	0.003
DCP Conc: C	0.9100	0.5980	1.522	0.189
Temp*Temp: A^2	-2.4137	0.8803	-2.742	0.041
pH*pH: B^2	0.3612	0.8803	0.410	0.699
DCP conc*DCP conc: C^2	-4.2013	0.8803	-4.773	0.005
Temp*pH: AB	-0.0675	0.8457	-0.080	0.939
Temp*DCP conc: AC	0.1100	0.8457	0.130	0.902
pH*DCP conc: BC	1.6500	0.8457	1.951	0.109

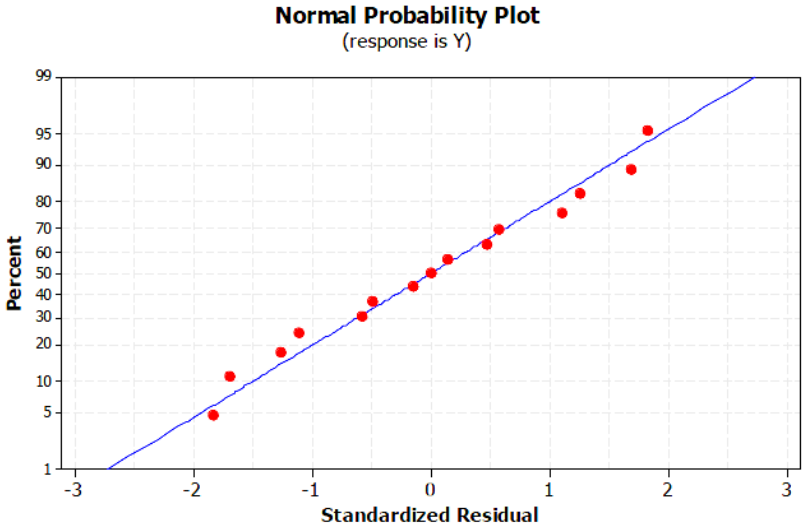


Fig. 10. Normal probability plot of standardized residuals

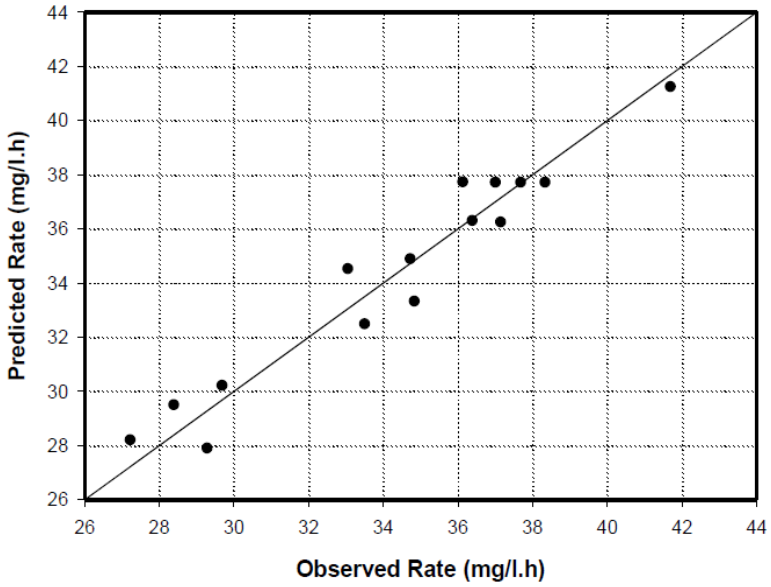


Fig. 11. Parity plot: Predicted versus observed rate

Model Optimization for Maximum Degradation Rate

Model optimization targets the identification of the optimum conditions for maximum degradation rate. The contour plots and their respective three-dimensional surface plots provide a visual explanation of the interaction between two variables and help in locating optimum experimental conditions (Wu *et al*., 2009). Figure 12 and 13 represent the contour and surface plots, respectively, for the optimization of DCP degradation. The mutual interactions between the variables are depicted by the shapes of the contour plots, tending to an elliptical shape for a significant interaction between variables (Wu *et al*., 2009; Agarry *et al*., 2008b). Also, the more the curvature in the surface plots, the more significant the interaction. This is depicted by the elliptical shape of the contour plots and their respective curved surface plots of the mutual interactions AB, AC and BC. Figure 12 and 13 suggest the location of the optimum range of the process variables. Using response optimizer by Minitab, the stationary point of maximum aerobic biodegradation rate inside the experimentation region had the following critical values: Temperature 32.58°C (0.5152), pH 5.0 (-1) and initial DCP concentration was 70.46 mg L^{-1} (-0.0909). Under these conditions, the predicted maximum aerobic global biodegradation rate was 41.82 mg L^{-1} h^{-1}.

Validation of the Model

Model validation is an important step to consider a model practically reliable and fit, so as to ensure that the predicted responses reasonably match the results obtained in the field (Bhattacharya *et al*., 2009). The suitability of the model, as represented by Equation 1 and 2, was tested by performing batch biodegradation experiments at the predicted optimum conditions. Also, other selected conditions were tested to compare the experimental degradation rates with those predicted by the model. The validation experiments were replicated. Table 6 summarizes the results on experimental and predicted responses at the 95% confidence interval. It shows the closeness of the model-predicted responses to the experimental results, confirming the model reliability for predicting the aerobic biodegradation of DCP by *P. putida*. Recalling the results on temperature effect, the observation was that on carrying out the replicate run at 35°C there was a suppression of the degradation rate accompanied by the appearance of a peculiar peak in the chromatogram: It is noteworthy that the bacterial activity was found to be at risk even at a temperature of 33°C. Replicated rates at this temperature could be achieved only when the suppression effect had been reduced.

Thus, although the RSM predicted an optimum operating temperature of 32.5°C, the sustainability of the bacterial activity is doubtful at this temperature. Bioprocesses are rather sensitive in terms of achieving reproducibility, which imposes some limitations on the applicability of the model.

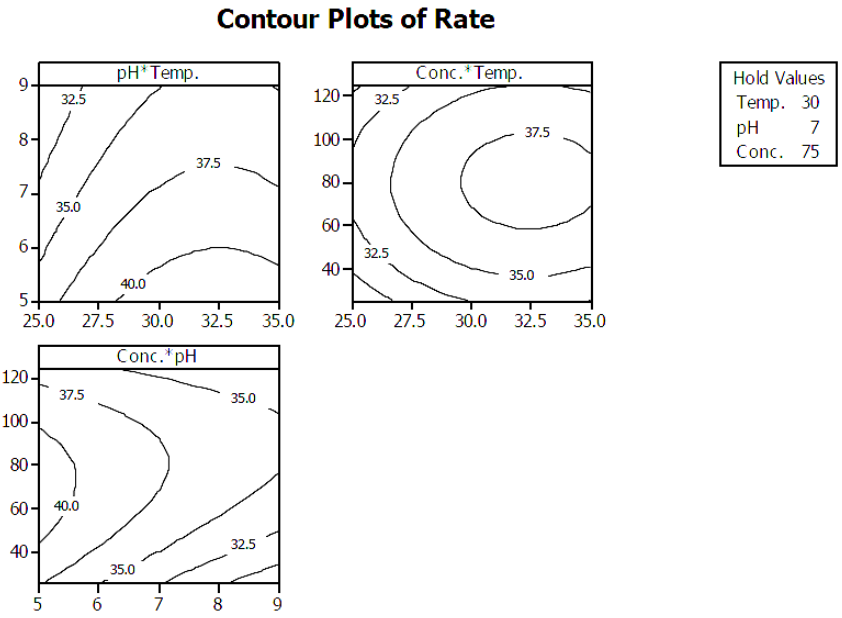
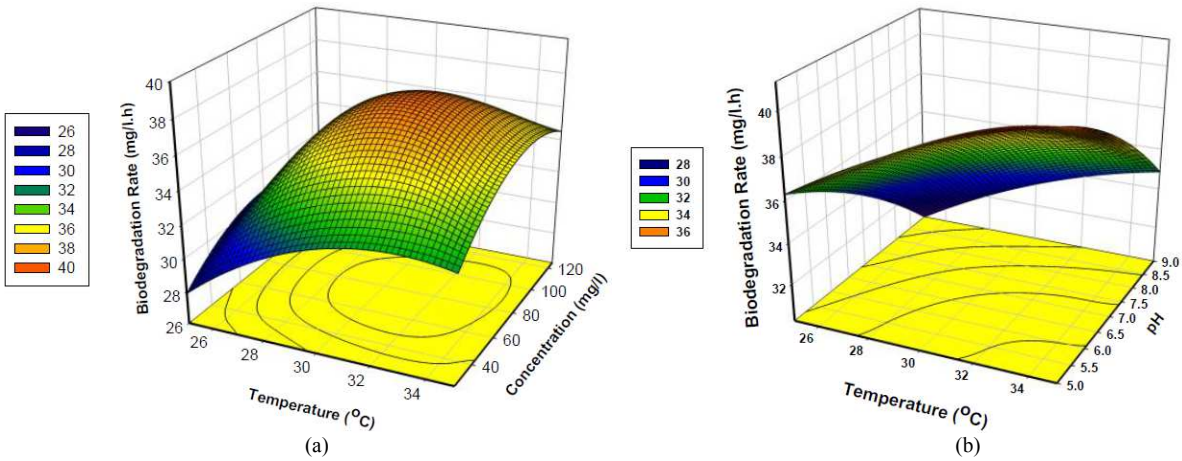
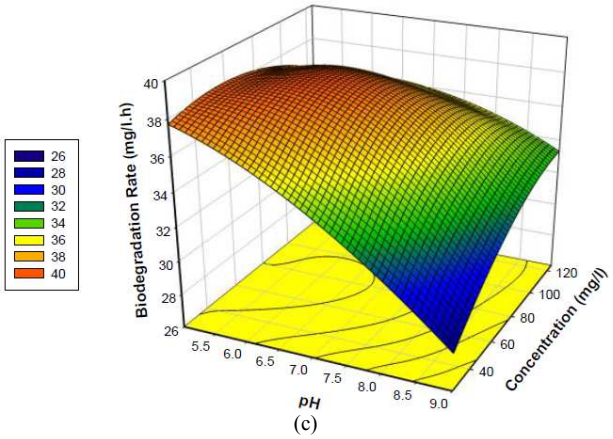


Fig. 12. Contour plots of response versus the different interactions of independent variables



(a) (b)



(c)

Fig. 13. Response surface plots of response versus the different interactions of independent variables (pH, initial concentration and temperature were held constant at their respective center levels in (a), (b) and (c), respectively)

Table 6. Validation of the RSM regression model

T, °C	Initial pH	C_o, mg/l	Observed rate, mg/l.h	Predicted rate, mg/l.h	95% Confidence interval
30	9.0	53.67	35.38	33.08	30.27; 35.90
30	8.4	28	27.67	30.06	27.12; 32.95
32.5	5.0	71.17	40.05	41.82*	38.96; 44.69
27	6.0	81.18	33.91	36.94	34.63; 39.26
27	6.0	41	31.96	34.93	32.57; 37.55
30	8.3	55.17	34.37	34.39	32.04; 36.70
30	5.0	54.37	42.87	40.75	37.94; 43.56
30	5.0	75	39.4	41.16	38.43; 43.90

*Degradation rate at the optimum conditions

Conclusion

The present study showed the effective degradation of 2, 4 DCP, up to 200 mg L^{-1}, by a special consortium of *P. putida* immobilized in PVA gel structure, using a spouted bed bioreactor. Planned design of experiments by response surface methodology with a Box-Behnken design, coupled with the classical single-parameter selection technique, was successfully applied to identify the optimal conditions for 2, 4 DCP removal based on the degradation rates obtained from batch experiments. The key process parameters were identified and their effects were evaluated, along with the interactions amongst the different variables. A true functional relationship, based on a quadratic model, between the dependent variable, which is the response expressed as a global degradation rate of 2, 4 DCP and the main independent variables, namely temperature, initial pH and initial concentration of 2, 4 DCP has been developed.

The model showed a good fit with R^2 and adjusted R^2 94.03 and 83.28%, respectively, which indicates a reasonably adequate model for practical application. The optimum conditions for 2, 4 DCP biodegradation by *P. putida* were as follows: PVA particle size of 1 cm^3 and inlet air flow rate of 3 L min^{-1}, as determined from the single-factor experiments; temperature 32.58 °C, pH 5.0 and initial DCP concentration 70.46 mg L^{-1}, as determined by the regression model; resulting in a maximum predicted degradation rate of 41.82 mg L^{-1} h^{-1}. These optimized conditions, as well as other conditions, were further experimentally validated and a degradation rate of 40.05 mg L^{-1} h^{-1} was obtained at the optimized conditions. Hence, the model prediction is confirmed to be close to the experimental results. By this model, the response for the key factors can be predicted at any time. The optimum operating conditions obtained give a basis for further study with continuous-mode operation for the degradation of DCP in industrial wastewater.

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Author's Contributions

Taghreed Al-Khalid: She made considerable contributions including carrying out the experimental work, analyzing and modeling the experimental data. She also prepared the initial draft of the manuscript.

Muftah El-Naas: He made considerable contributions including designing the research plan and experimental procedure. In addition, he supervised the experimental work and revised the draft of the manuscript.

Ethics

This paper is original and includes unpublished materials. The corresponding author authorizes that the other author has read and approved the manuscript and there is no ethical issues involved.

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