Studies of the Effects of Co-substrates on Biodegradation Kinetics of 4-Chlorophenol Using Response Surface Methodology (RSM)

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ABSTRACT: *This study aimed to investigate the effect of glucose or sucrose as co-substrate on the kinetics of 4-chlorophenol (4-CP) biodegradation by 4-CP-acclimated activated sludge. The performance of 4-CP biodegradation was enhanced in the presence of co-substrate with greater values of zero-order rate constant observed for sucrose compared to glucose at higher 4-CP concentration. The interaction effects of two factors, namely initial 4-CP and co-substrate concentrations ranging from 100–500 mg l^{-1} and 0–1000 mg l^{-1}, respectively, on the biodegradation kinetics were examined by using response surface methodology (RSM). For glucose, the kinetics was found dependent on the 4-CP initial concentration. The optimised condition for 4-CP biodegradation in the presence of glucose was: 200 mg l^{-1} 4-CP and 750 mg l^{-1} glucose with rate constant of 7.630 mg $l^{-1} h^{-1}$.*

Keywords: Biodegradation, acclimated activated sludge, co-substrate, response surface methodology, kinetics

1. INTRODUCTION

Untreated effluent containing chlorophenols such as 4-chlorophenol (4-CP) from industries such as pesticides and oil refining sectors poses a serious threat to the receiving environment.[1–3] The United States Environmental Protection Agency has listed these chlorophenols as priority pollutants in view of their high toxicity and carcinogenicity.[3] Hence, proper treatment of these wastewaters is of the utmost importance before their discharge into the environment. Various physicochemical

methods such as adsorption, extraction and photodegradation have been developed for the removal of chlorophenols.[4] However, these processes involve high operating cost with secondary waste generated.[5,6] Thus, biodegradation process involving the elimination of the pollutants by the metabolic activities of living microorganisms can serve as an alternative to remove such compounds.[7] This process is eco-friendly and energy-efficient with achievable complete mineralisation of chlorophenols.[8]

Various studies on chlorophenols biodegradation using pure or mixed cultures have been conducted.[9,10] In comparison to pure cultures, mixed cultures which contained several kinds of microorganism offered complete mineralisation of toxic compounds.[10] Activated sludge process is a biological approach that employs mixed culture in treating wastewater. The performance of the activated sludge can be improved by subjecting the activated sludge to acclimation process. Enhancement of biodegradation in terms of rate and efficacy was observed after the activated sludge was pre-adapted to target toxic compound.[9,11] In the study conducted by Sam et al., phenol biodegradation time decreased from 330–1260 min to 35–33 min at the initial phenol concentration of 25–250 mg l^{-1} by using phenol-acclimated activated sludge.[12] In spite of the reported efficacy of acclimated activated sludge in removing phenolic compounds, the microorganism suffered from growth inhibition at high concentration of phenolic compounds.[13]

Co-metabolism is defined as the biotransformation of non-growth substrates by organism that requires an alternative source of carbon and energy for microbial growth.[14] Thus, growth substrate of conventional carbon sources has been proposed to increase the tolerance of the microorganisms towards the substrate inhibition.[6,15] Addition of growth substrate has been shown to improve the biodegradation efficiency of chlorinated chlorophenols and aromatic compounds, respectively.[15,16] The commonly used co-substrates are peptone, glucose and yeast extract.[5,6] In the study by Loh and Tan, the enhancement of phenol biodegradation rate by yeast extract as co-substrate was dependent on the yeast extract concentration.[6] To the best of our knowledge, the studies of interaction effects of co-substrate and 4-CP at various concentrations on the biodegradation performance are still limited.

Response surface methodology (RSM) is a combination of statistical and optimisation method used to assess the relationship between controllable experimental factors and observed results.[16] In this study, RSM was used to examine the interaction effects of two factors, namely initial 4-CP and co-substrate concentrations on 4-CP biodegradation kinetics. It was subsequently employed to optimise the relationships between the factors and the response in the presence of different co-substrates.

The objectives of this study are to: (1) study the effects of different co-substrate, namely glucose and sucrose, on the biodegradation kinetics of 4-CP; (2) investigate the interaction effects of co-substrate concentration and 4-CP initial concentration using RSM; and (3) optimise the 4-CP biodegradation in the presence of co-substrate.

2.   EXPERIMENTAL

2.1   Culturing of 4-CP-acclimated Activated Sludge

The 4-CP acclimated activated sludge was cultured in a 1-l sequencing batch reactor (SBR). The SBR was operated with 24-h cycle consisting sequential stages of fill (2 h), react (12 h), settle (1.5 h), draw (1 h) and idle (7.5 h). The reactor was fed with the base mix containing (in mg l^{-1}): peptone (Merck) (32), sucrose (109), KH_2PO_4 (Bendosen) (32), K_2HPO_4 (Systerm) (180), $(NH_4)_2SO_4$ (Systerm) (212), $NaHCO_3$ (R&M) (500), $MgSO_4$ (R&M) (49), $FeCl_3 \cdot H_2O$ (18.8) (Systerm) and $CaCl_2$ (Bendosen) (40). During fill stage, 0.6 l of base mix solution was introduced into the reactor. Equal volume of treated effluent was withdrawn during the draw stage. During the acclimation period, the sucrose and peptone was gradually replaced by increasing concentration of 4-CP as the sole carbon until final 4-CP acclimation concentration of 250 mg l^{-1}. The acclimated activated sludge was used for subsequent batch biodegradation studies.

2.2   Design of Experiment using RSM

In this study, glucose or sucrose was used as the co-substrate during the 4-CP biodegradation. For each co-substrate, the effects of independent variables, namely initial 4-CP concentration (100–500 mg l^{-1}) and co-substrate concentration (0–1000 mg l^{-1}), on the response of 4-CP biodegradation kinetics were studied. The experiment design was conducted using central composite design (CCD). The experimental set of 13 runs with five replications at the design centre is shown in Table 1. The interactions between the variables with the response were analysed using Design Expert 10.0.

Table 1: The experimental runs based on CCD design.

Run	Initial 4-CP concentration (mg l⁻¹)	Co-substrate concentration (mg l⁻¹)
1	200	250
2	400	250
3	100	500
4	300	500
5	500	500
6	400	750
7	300	500
8	200	750
9	300	500
10	300	0
11	300	1000
12	300	500
13	300	500

2.3   Batch Biodegradation Studies

Based on the CCD design, 500-ml reaction vessels containing nutrients, 4CP and co-substrate at different concentrations were prepared. Subsequently, 200 mg l⁻¹ acclimated activated sludge was added into each reaction vessel followed by immediate aeration. At suitable time intervals, sample solution was withdrawn and filtered through Whatman No. 1 filter paper. The filtrate was analysed for the residual 4-CP, following the colorimetric 4-aminoantipyrine method at the λ_{max} of 504 nm using Shimadzu UV Spectrophotometer, until 4-CP concentration was less than 1.0 mg l⁻¹.[10] The procedure was repeated in the absence of co-substrate at various 4-CP initial concentrations.

The kinetic data of 4-CP biodegradation was into zero-order equation:

$$[C]_t = k_0 t + [C]_0 \qquad\qquad\qquad (1)$$

where $[C]_0$ (mg l⁻¹) is the initial 4-CP concentration, $[C]_t$ (mg l⁻¹) is the 4-CP concentration at time t and k_0 (mg l⁻¹ h⁻¹) is the zero-order rate constant.

3.   RESULTS AND DISCUSSION

3.1   Biodegradation of 4-CP

Table 2 shows the kinetic data of 4-CP biodegradation in the absence and presence of co-substrate fitted into zero-order kinetics. In the absence of co-substrate, the k_0 values were found to decrease from 4.3809 l⁻¹ h⁻¹ to 2.6904 mg l⁻¹ h⁻¹ when the initial 4-CP was increased from 100 to 500 mg l⁻¹. At the same time, a reduction of

biomass growth was observed (data not shown). This plausibly explain the toxicity effect of the 4-CP in deteriorating the cell growth and consequently degradation ability.[17]

Table 2: Kinetic parameters for zero-order 4-CP biodegradation in the absence and presence of co-substrate.

Initial 4-CP concentration (mg l^{-1})	Co-substrate concentration (mg l^{-1})	Glucose		Sucrose	
		k_0 (mg $l^{-1} h^{-1}$)	R^2	k_0 (mg $l^{-1} h^{-1}$)	R^2
100	0	4.3809	0.9950	4.3809	0.9950
100	500	7.8773	0.9944	7.5670	0.9954
200	0	4.2526	0.9780	4.2526	0.9780
200	250	8.0135	0.9756	6.6119	0.9650
200	750	8.8138	0.9837	7.5699	0.9835
300	0	4.1379	0.9985	4.1379	0.9985
300	500	4.7202	0.9479	6.1910	0.9940
300	500	5.8065	0.8765	6.0939	0.9720
300	500	4.8518	0.9009	6.1165	0.9493
300	500	4.2459	0.9555	6.3451	0.9986
300	500	4.5919	0.9673	5.7712	0.9548
300	1000	6.6685	0.9922	4.6980	0.9459
400	0	4.0031	0.9972	4.0031	0.9972
400	250	3.0807	0.8534	6.7759	0.9947
400	750	2.5724	0.8895	7.3204	0.9723
500	0	2.6904	0.9673	2.6904	0.9673
500	500	1.0009	0.8852	5.1992	0.9533

From Table 2, it is observed that the addition of sucrose improved the 4-CP biodegradation rate for all the 4-CP initial concentrations investigated. It was reported that the presence of co-substrate would increase the biomass concentration and led to higher degradation rate.[15,18] However, when glucose was added as co-substrate, the enhancement of 4-CP biodegradation was only observable at the lower 4-CP concentrations of 100–300 mg l^{-1}. The k_0 value deteriorated at higher 4-CP concentration of 400 mg l^{-1} and 500 mg l^{-1}. At 400 mg l^{-1} 4-CP, the k_0 value decreased from 4.0013 mg l^{-1} to 3.0807 mg l^{-1} h^{-1} with the addition of 250 mg l^{-1} glucose. The value was further reduced to 2.5724 mg l^{-1} h^{-1} in the presence of 750 mg l^{-1} glucose. Similar observation was reported by Loh and Tan, in which better performance of phenol degradation was observed in the presence of 0.2 g l to 2 g l yeast extract as co-substrate, however the biodegradation rate declined at higher concentration of yeast extract.[6] The results from this study indicated that the effects of co-substrate on 4-CP biodegradation not only dependent on the type of co-substrate but also the concentrations of 4-CP and co-substrate.

3.2   Optimisation of 4-CP Biodegradation Using RSM

For each co-substrate. the interaction effects of two variables, namely 4-CP initial concentration (x_1) and co-substrate concentration (x_2) on the response of k_0 value (y) were analysed using CCD. The best models suggested were quadratic (Equation 2) and linear (Equation 3) for cases involving sucrose and glucose, respectively:

$$y = 5.85406 - 7.50920 + 8.49290\text{E}^{-3}x_2 - 4.13500\text{E}^{-6}x_1x_2 \\ + 9.26519\text{E}^{-6}x_1^2 - 6.37817\text{E}^{-6}x_2^2 \tag{2}$$

$$y = 10.38794 - 0.020626x_1 + 1.78440\text{E}^{-7}x_2 \tag{3}$$

Analysis of variance (ANOVA) was performed to analyse the statistical significance of the regression model and its reliability. The results are presented in Tables 3 and 4. Regression coefficient with p-value below 0.05 is statistically significant.[16] For sucrose, the model (Equation 2) was insignificant as evident from the failure probability of 0.2531 that is > 0.05. Also, low values of adequate precision (5.105), R^2 (0.5479) and adjusted R^2 (0.2250) were exhibited. The predicted versus experimental values graph for 4-CP biodegradation in the presence of sucrose is shown in Figure 1(a). In conclusion, this model is insignificant to represent the experimental results and thus further investigation on the optimisation of 4-CP biodegradation with addition of sucrose was not conducted.

Table 3: ANOVA results of the quadratic model for 4-CP biodegradation kinetics in the presence of sucrose.

Source	Sum of Squares	Degree of freedom	Mean Square	F Value	p-value Prob >F	
Model	7.25	5	1.45	1.70	0.2531	Not significant
x_1	1.94	1	1.94	2.27	0.1759	
x_2	0.57	1	0.57	0.67	0.4398	
x_1x_2	0.043	1	0.043	0.050	0.8294	
x_1^2	0.20	1	0.20	0.23	0.6460	
x_2^2	3.64	1	3.64	4.26	0.0779	
Residual	5.98	7	0.85			
Lack of fit	5.81	4	1.94	43.81	0.0016	Significant
Pure error	0.18	3	0.044			
Cor total	13.23	12				

Notes: $R^2 = 0.5479$; $R^2_{adj} = 0.2250$; adequate precision = 5.105

Table 4: ANOVA results of the linear model for 4-CP biodegradation kinetics in the presence of glucose.

Source	Sum of squares	Degree of freedom	Mean square	F Value	p-value Prob >F	
Model	54.17	2	27.08	33.14	<0.0001	Significant
x_1	51.78	1	51.78	63.35	<0.0001	
x_2	2.39	1	2.39	2.92	0.1182	
Residual	8.17	10	0.82			
Lack of fit	6.81	6	1.14	3.33	0.1321	Not significant
Pure error	1.36	4	0.39			
Cor total	62.34	12				

Notes: $R^2 = 0.8623$; $R^2_{adj} = 0.8348$; adequate precision = 18.594

In contrast, the regression model for glucose (Equation 3) was significant based on a low failure probability ($p < 0.0001$) and adequate precision of 18.594. The value of the predicted coefficient (a measure of goodness of fit to the model) indicates that about 86.23% of the total variations could be explained by the model. These values showed the adequacy of the model in explaining the response. The coefficient of variation (CV) indicates the degree of precision, in which higher reliability of the experiment is indicated by lower value of CV.[16] The effect of initial 4-CP concentration (x_1) is significant as the p-value was < 0.0001. Whereas the effect of glucose concentration (x_2) is not significant as indicated by the p-value > 0.05. The R^2 and adjusted R^2 values are 0.8623 and 0.8348, respectively. The predicted versus experimental values graph for 4-CP biodegradation in the presence of glucose is shown in Figure 1(b).

The perturbation plot (Figure 2) shows the comparative effects of 4-CP initial concentration (x_1) and glucose concentration (x_2) on 4-CP biodegradation rate. The sharp line pattern exhibited by x_1 suggests the significant effect of 4-CP initial concentration, in which increasing initial 4-CP concentration decreased the 4-CP biodegradation rate. In contrast, the almost plateau plot of x_2 indicates the less significant effect by glucose concentration. Only little increment in the rate constant was observed with increasing glucose concentration. The influence of 4-CP concentration on biodegradation kinetics is again exhibited in the 3-D and 2-D plots showing the interaction effects of factors x_1 and x_2 on k_0 value (Figure 3). The optimum conditions for the 4-CP biodegradation in the presence of glucose was: 200 mg l^{-1} 4-CP, 750 mg l^{-1} glucose with k_0 value of 7.630 mg l^{-1} h^{-1}.

(a)

**Predicted vs. actual**

(b)

Predicted vs. actual
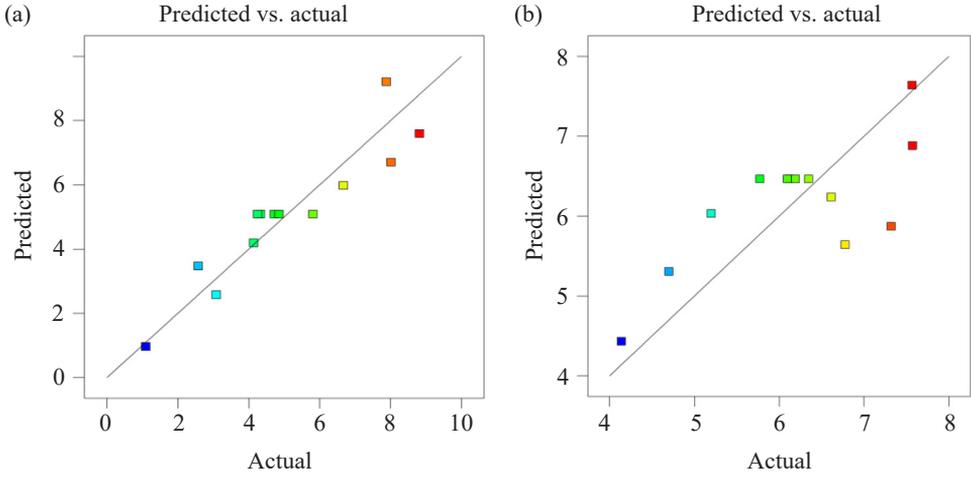
Figure 1:   Predicted versus experimental results of biodegradation rate in CCD design experiments for 4-CP biodegradation in the presence of (a) glucose, and (b) sucrose.
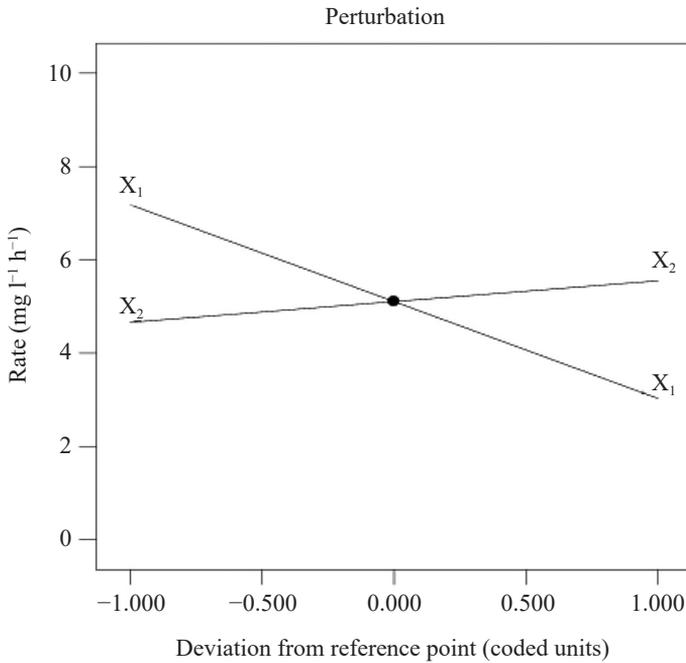
Perturbation

Figure 2:   Perturbation plot for 4-CP biodegradation kinetics showing the effects of initial 4-CP concentration and glucose concentration.
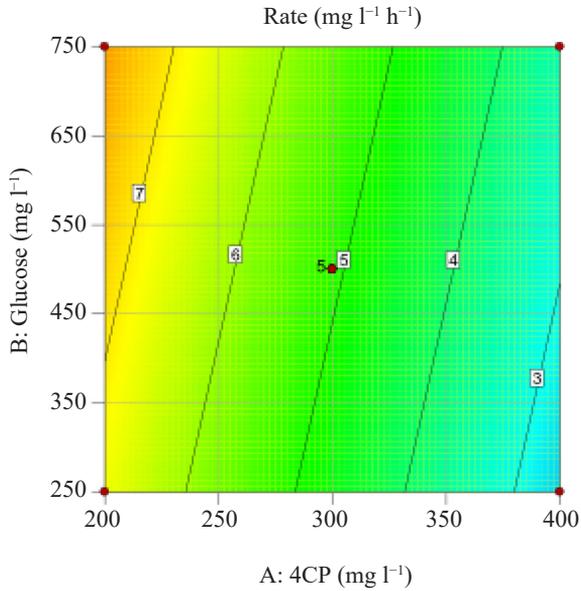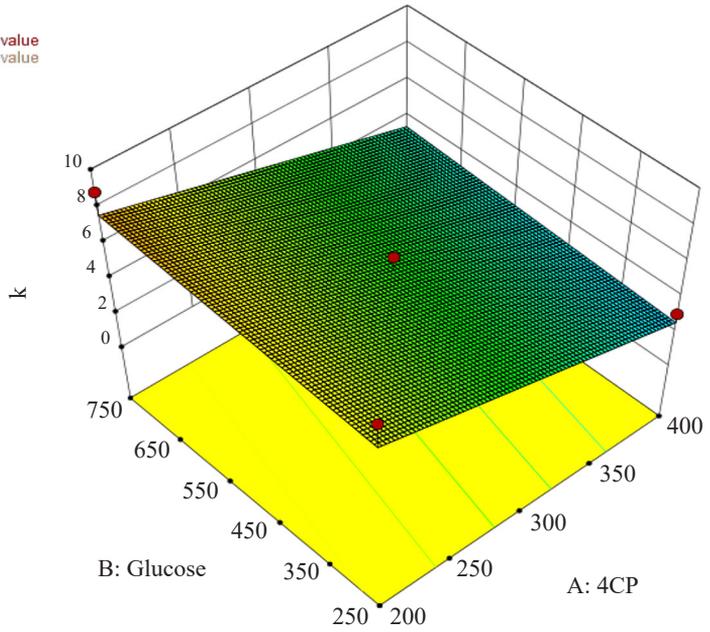
Figure 3: Three-dimensional plot and its contour plot showing the effects of initial 4-CP concentration and glucose concentration on 4-CP biodegradation rate.

4.   CONCLUSION

This study investigated the effect of glucose or sucrose as co-substrate on 4-CP biodegradation kinetics. The 4-CP biodegradation performance was found dependent on the type and concentration of co-substrate used. Sucrose was more effective in enhancing 4-CP biodegradation, especially at higher 4-CP concentration. From RSM analysis, the best model in relating the variables of 4-CP concentration and co-substrate concentration with 4-CP biodegradation kinetics was linear for case of glucose and quadratic for sucrose. For glucose, the 4-CP biodegradation kinetics was only dependent on the initial 4-CP concentration and the k_0 value decreased with increasing 4-CP initial concentration. The optimised 4-CP biodegradation was successfully determined for glucose as co-substrate.

5.   ACKNOWLEDGEMENTS

6.   REFERENCES

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