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Modeling the biodegradation efficiency and growth of *Pseudomonas alcaligenes* utilizing 2,4-dichlorophenol as a carbon source Pre- and Post-exposure to UV radiation

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Abstract

A bacterial strain capable of utilizing 2,4-dichlorophenol as the sole carbon source was isolated by using enrichment and isolation techniques. The strain was identified as *Pseudomonas alcaligenes*. Ultraviolet radiation was used to enhance the biodegradation efficiency of the isolated bacterium. The growth pre- and post-irradiation of the isolated bacterium was modeled using nonlinear equations. Furthermore, the biodegradation efficiency of 2,4-dichlorophenol at temperatures of 25, 30, 35 and 40 ºC and pH values of 6.5, 7 and 8 was modeled using Gompertz type equations. The models indicated that ultraviolet irradiation enhanced the growth capabilities of the *Pseudomonas alcaligenes* and that the highest biodegradation efficiency reached 79% at 35 ºC and pH 7.

الملخص

تم عزل سلالة بكتيرية قادرة على استخدام 2 و4 ثنائي الفينول كمصدر وحيد للكربون والتعرف عليها بأنها Pseudomonas alcaligenes و تم استخدام الأشعة فوق البنفسجية لتحسين كفاءة التحطيم البيولوجي لهذه البكتيريا حيث تم نمذجة نمو هذه البكتيريا قبل وبعد تعرضها للأشعة فوق البنفسجية باستخدام معادلات غير خطية. بالإضافة إلى ذلك تم نمذجة التحطيم البيولوجي لثنائي الفينول عند درجات حرارة 25 و 30 و 35 و 40 درجة مئوية ورقم هيدروجيني 6.5 و 7 و 8 باستخدام معادلات Gompertz وقد أشار النموذج الرياضي إلى أن أعلى كفاءة تحطيم بيولوجي هي عند درجة حرارة 35 درجة مئوية ورقم هيدروجيني 7 حيث بلغت 79.%

Keywords: Nonlinear modeling; Biodegradation; Pseudomonas alcaligenes Gompertz model; Ultraviolet radiation;

1. Introduction

Biodegradation and bioremediation have become the most rapidly developing fields of environmental restoration (Dua *et al*., 2002). It is estimated that 25% of the Earth's biomass is composed of compounds that have a benzene ring as the main structural constituents (Gibson and Harowood, 2002). Chlorophenols are toxic for a wide range of organisms. They are used in pressure treatment in the wood preservation industry, herbicides and fungicides, and are also found in pulp bleaching effluents and industrial wastewater (Bae *et al.*, 2002). Bielicka et al. (2002) have isolated a number of enzymes that catalyze the biodegradation reactions from various microorganisms.

A large number of bacterial and fungal species have the capability to degrade chlorophenolic compounds (Jong and Field, 1997; Reddy and Gold, 2000; Schlosser et al., 2000; Bollag et al., 2003; Steinle *et al*., 1998).

Pseudomonades have been identified to be of importance in bioremediation as a result of their high capacity for biodegradation. *Pseudomonas* species have been used by Premalatha and Rajakumar (1994), Kiyohara et al. (1992) and Farrell and Quilty (2002) for the biodegradation of a variety of chlorophenolic compounds. On the other hand, the efficiency of biodegradation of all bacterial or fungal species is questionable. For example, Koh *et al*. (1997) have reported a 69% dehalogenation of 2,4-dichlorophenol using *Alcaligenes eutrophus*, while Wang *et al*. (2000) reported that the removal of 2,4-dichlorophenol using *Bacillus insolitus* at high concentrations was less than 50% and Tomasi *et al*. (1995) have reported various success rates for the biodegradation of dichlorophenols using *Pseudomonas cepacia*.

Ultraviolet radiation (UV) has been extensively used in the water disinfection and treatment industry. A number of studies have examined the effects, either negative or positive, due to the exposure of different organisms to UV radiation and the potential repair mechanisms of these organisms (Lysetska *et al.*, 2002; Zimmer and Slawson,

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2002; Cooke and Williamson, 2006; Alonso-Saez *et al*., 2006).

The mutagenic capability of UV radiation makes it a useful tool for the genetic modification of microorganisms. Since a study of the capabilities of a bacterial species to grow and utilize an organic compound is a prerequisite for its use in the biodegradation or bioremediation of this compound, the aims of this study are to mathematically model the difference in the growth of *Pseudomonas alcaligenes* utilizing 2,4-dichlorophenol as the sole carbon source pre- and post-exposure to UV radiation and to model the efficiency of biodegradation at different temperatures and pH values.

2. Materials and Methods

2.1. Isolation and Identification of *Pseudomonas spp.*

The bacterial species were isolated from wastewater samples taken from two pharmaceutical companies that their wastewater have been analyzed to contain phenolic compounds and centrifuged at 3000 rpm for 30 minutes to obtain sludge. Two hundred milligram of the sludge was added to pre-sterilized flasks containing 20 ml of chlorophenol enrichment culture media supplemented with 100mg/L of 2,4-dichlorophenol (Zhang and Weigel, 1990; Kiyohara *et al*., 1992) and allowed to grow in a shaking water bath for four days at 30 ºC. Flasks containing 50 mL of nutrient broth (HiMedia Laboratories Limited, Mumbai, India) were inoculated with 0.5 mL of the first set suspension and grown for four days under the same conditions. Nutrient agar plates supplemented with 100 mg/L of 2,4-dichlorophenol were inoculated with 0.3 ml from the flasks and incubated for four days at 37 ºC. Biochemical characterization of the bacterial colonies was carried out according to Cowan and Steels manual for identification of medical bacteria (Barrow, 1993). Confirmation of the identity of the bacterial species was carried by Jordan University Hospital, using a "REMEL system and API 20 NE kit".

2.2. Examination of the Efficiency of Biodegradation of 2,4-dichlorophenol isolates prior to UV irradiation

Colonies that appeared on the plates were streaked onto nutrient agar supplemented with cetrimide (1g/L) and inoculated for three days at 37 ºC. Pink colonies were then transferred to nutrient agar plates supplemented with 120, 140, 160, 180, 200, 220, 240, 260, 280 and 300 mg/L of 2,4-dichlorophenol in order to examine the ability of *Pseudomonas alcaligenes* in using 2,4-dichlorophenol as the only carbon source. Enumeration of the bacterial colonies, at different 2,4-dichlorophenol concentrations, was carried out by using a colony counter to count the bacterial colonies.

2.3. Pseudomonas alcaligenes Exposure to Ultraviolet radiation

The isolated colonies were streaked onto nutrient agar plates in duplicated and irradiated by UV radiation (365 nm, 11 W/m2) for 24, 48, 72 and 96 hours. Colonies from UV irradiated plates were transferred to nutrient agar plates supplemented with 2,4-dichlorophenol concentrations of 240, 260, 280, 300, 320, 340, 360, 380 and 400 mg/L in order to examine the effect of UV radiation on the capability of *Pseudomonas alcaligenes* in utilizing 2,4-dichlorophenol for its growth.

2.4. Biodegradation of 2,4-dichlorophenol

The UV irradiated *Pseudomonas alcaligenes* was cultivated in a benchtop bioreactor (Laboratory benchtop fermenter unit model 300, and EMC unit model 351, Hamburg, Germany) at temperatures of 25, 30, 35 and 40 ºC and pH values of 6.5, 7 and 8. The initial concentration of 2,4-dichlorophenol was 340 mg/L and aeration was kept constant at 0.6 L/min and continuous agitation of 40 rpm. Samples were taken daily and the concentration of the 2,4-dichlorophenol was determined using a UV-spectrophotometer (UV-visible spectrophotometer, 100 Bio:Cary Varian, USA) .

2.5. Modeling the Growth of *Pseudomonas alcaligenes*

A nonlinear regression model is used to determine the relationship between the concentration of 2,4-dichlorophenol and the number of colonies of *Pseudomonas alcaligenes* that can grow on this concentration. This relationship is modeled as:

$$DCP = A / e^{(k*CFU)}$$

Where,

DCP = the concentration of 2,4-dichlorophenol (mg/L)

A = A theoretical value of the maximum the concentration of 2,4-dichlorophenol that Pseudomonas alcaligenes can use as the carbon source

k = regression model parameter

CFU = number of colonies (log 10 of CFU/mL)

Levenberg-Marquardt, a nonlinear least squares method, is used to calculate the A and K parameters. This method is a modification of the Gauss-Newton algorithm which in using the least-squares loss function, the second order partial derivative do not have to be computed in order to find the least-square estimates; instead, the algorithm in each iteration solves a set of linear equations to compute the gradient.

2.6. Modeling the biodegradation of 2,4-dichlorophenol

Two nonlinear models are used to model the biodegradation of 2,4-dichlorophenol. The first, models the nonlinear relationship between each temperature used in the biodegradation (25,30,35,40 ºC) and time of biodegradation. Therefore, four temperature models are formulated. The second, models the nonlinear relationship between each pH (6.5, 7, 8) and time of biodegradation, thus three models are formulated. These nonlinear relationships are modeled as Gompertz equation. The general formulae for these models are:

$$BDCPT = A*(e^{-e(-k*T)}) \quad \text{and,} \quad BDCPH = A*(e^{-e(-k*T)})$$

where,

BDCPH = the percentage of 2,4-dichlorophenol biodegraded at a specific pH value

BDCPT = the percentage of 2,4-dichlorophenol biodegraded at a specific temperature

T = time of biodegradation

A = a theoretical value of the maximum concentration of 2,4-dichlorophenol that can be biodegraded

k = model parameter

Quasi-Newton method is used to calculate the model parameters (A and k). This method uses an algorithm that approximates the second-order derivatives of the loss function to guide the search for the best parameter estimates, given the respective loss function.

3. Results

The model for the biodegradation of 2,4-dichloropheno indicates that there are differences between the UV irradiated *Pseudomonas alcaligenes* and the non-irradiated species. A comparison of the calculated A values by the model indicates that the UV irradiated *Pseudomonas alcaligenes* can utilize, as a carbon source, a much higher concentration of 2,4-dichlorophenol than the non-irradiated *Pseudomonas alcaligenes* as shown in table (1).

Table 1: Parameters of the Pseudomonas spp. growth models

Model Parameters	*Pseudomonas spp.*	UV irradiated *Pseudomonas spp.*
A	412.67	1228.85
K	0.0967	0.169
r	0.8783	0.9504

r = correlation coefficient of the model

This is further supported by the value of the k parameter, which is higher in the UV irradiated *Pseudomonas alcaligenes* than the non-irradiated. The model can determine the concentration of 2,4-dichlorophenol that can be utilized as a carbon source by inputting the number of bacteria in Log $_{10}$ of CFU/mL and the model parameters A and k (refer to table 1). The relationship between the number of *Pseudomonas alcaligenes* and the concentration of 2,4-dichlorophenol is indicated in figure (1) for the non-irradiated and UV irradiated species, respectively.

The biodegradation models show that the temperature that resulted in the highest biodegradation percentage is 35 ºC and the pH value that resulted in the highest biodegradation percentage is pH 7. This is indicated by the A values shown in table (2). The models can accurately determine the biodegradation percentage for each temperature and each pH value. For example, the model for the temperature of 35 ºC is:

*DCPT = 79 * e(-e-0.0166*35)*

The equation for pH 7 is:

*DCPH = 68 * e (-e-0.0192*7)*

The analysis of variance (ANOVA) test showed that there are statistically significant differences between the mean values of the temperatures (25, 30, 35 and 40 ºC) tested in the biodegradation (p=0.0108) and the pH values (6.5, 7 and 8) tested in the biodegradation (p=0.0059). The mean values for both the temperature and pH biodegradation results are shown in figures (2) and (3).

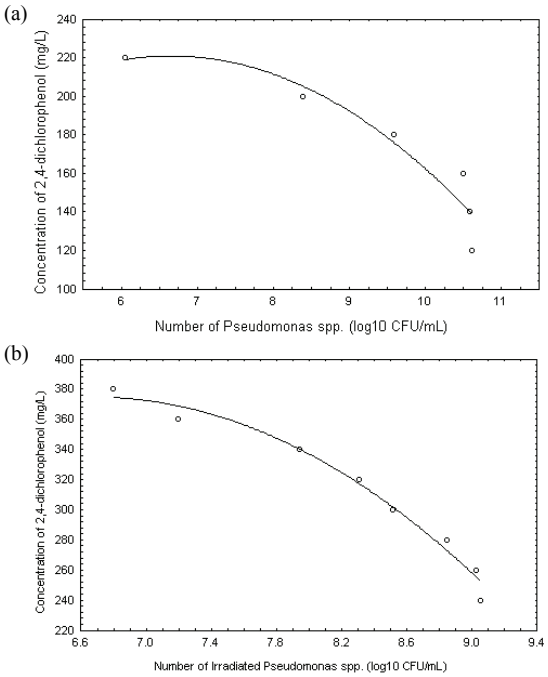

Figure 1: Comparison of the maximum concentration of 2,4-dichlorophenol that can be used as a carbon source by the non-irradiated (a) and irradiated (b) *Pseudomonas spp.*

Table 2: Biodegradation model parameters for the temperature and pH values

		Parameters of the Model		
		A	k	r
Temperature ºC	25	68	0.0192	0.8793
	30	76	0.0183	0.8818
	35	79	0.0166	0.8642
	40	64	0.0107	0.7473
PH values	6.5	59	0.0153	0.8581
	7	68	0.0192	0.8793
	8	48	0.0116	0.8116

r = correlation coefficient of the model

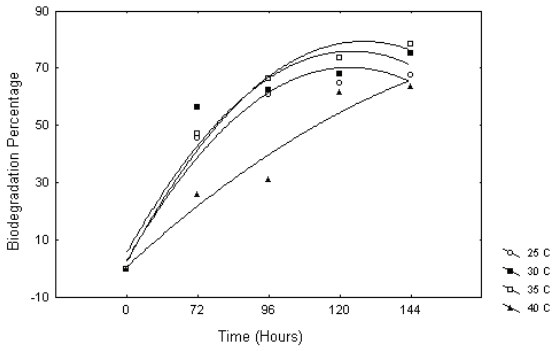

Figure 2: Biodegradation percentages of 2,4-dichlorophenol by *Pseudomonas spp.* at 25, 30, 35 and 40 ºC

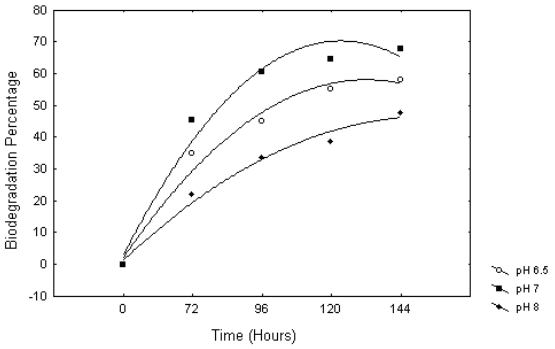

Figure 3: Biodegradation percentages of 2,4-dichlorophenol by *Pseudomonas spp.* at pH values of 6.5, 7 and 8

4. Discussion

There are a number of studies that explain the genetic bases of biodegradation. For example, *Flavobacterium* ATCC biodegrades pentachlorophenol by a catabolic pathway encoded by many genes one of which is pcpA that encodes information for a 30-kDa polypeptide pcpA, found in the periplasm of the bacterium (Chanama and Crawford, 1997). Although several studies have been carried out on the biodegradation capabilities of a number of *Pseudomonas spp.* and still others have determined the biodegradation pathway of 2,4-dichlorophenol (Perkins *et al*., 1990; Bhat *et al*., 1994; Xun, 1996; McFall *et al*., 1997; Tarao and Seto, 2000), the scarcity of studies that used UV radiation to enhance the biodegradation efficiency of this bacterium makes it difficult to compare their results with our results. Even though the aim of this study was not to find out the genetic modification that occurred to the *Pseudomonas alcaligenes* after the UV irradiation, it is clear that a large improvement in the biodegradation efficiency occurred due to this UV irradiation. The model even predicts about three times increase in the theoretical maximum concentration of 2,4-dichlorophenol that can be biodegraded (table 1) by the UV irradiated *Pseudomonas alcaligenes* compared to the non-irradiated species. Further studies in this area are needed to elucidate the genetic alterations and the new biodegradation pathway that occurred due to UV irradiation.

Temperature and pH values obviously affect the biodegradation efficiency. It has been reported that the amount of 2,4-dichlorophenol absorption to microbial cells increased with decreased pH values (Gillian *et al*., 1999). Therefore, at pH 4 there was a great deal more absorption than at pH 8. This could explain our results, which show that the best biodegradation occurred at pH 7 and not 8 (table 2). Furthermore, an increase in hydrogen ion concentration at lower pH than 7 influences and limits the growth rate of microorganisms (Armenante *et al*., 1993). Thus, it is reasonable to expect biodegradation to be the highest at pH 7. Temperature affects biological reaction rates and biological growth rates. Hence, it is expected that biodegradation efficiency to increase with the increase in temperature, which is indicated by our results (table 2). The models of the influence of the temperature indicated that 35 ºC had the highest maximum biodegradation percentage. On the other hand, a higher temperature than the optimal temperature for the microorganisms could have a fast drop in the biological reaction rates and thus, decrease the biodegradation efficiency.

The use of mathematical model to determine the growth of microorganisms has been carried out successfully. For example, Ng and Schaffner (1997) formulated mathematical models to test the effects of pH, temperature and sodium chloride on the growth of *Bacillus stearothermophilus*. They used quadratic polynomial models for determining the germination, outgrowth and the growth rate of the bacterium. They concluded that the models provided an estimate of bacterial growth in response to combinations of the variables studied within the specified ranges. Our results also indicate that the use of the growth and biodegradation models successfully predicted *Pseudomonas alcaligenes* growth and the variation in growth after UV exposure and the biodegradation efficiency at different temperature and pH values. It is worth mentioning that the highest biodegradation percentage obtained in our study was 79% at temperature 35 ºC and pH 7 is higher than the reported percentage of 50% by Tomasi *et al*. (1995) and the 69% reported by Koh *et al*. (1997).

In conclusion, modeling the biodegradation efficiency of 2,4-dichlorophenol by *Pseudomonas alcaligenes* has shown that the biodegradation efficiency was enhanced by UV irradiation for 144 hours and that the highest biodegradation efficiency occurred at temperature of 35 ºC and pH 7. Further studies are needed to explain the genetic alterations that occurred due to UV irradiation and the alterations in the catabolic pathways responsible for the 2,4-dichlorophenol biodegradation.

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