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*Full Length Research Paper*

# Biodegradation of 2,4-dichlorophenol originating from pharmaceutical industries

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**The aims of this work were to isolate a microorganism from the wastewater of pharmaceutical industries, to examine the difference in its growth utilization of 2,4-dichlorophenol as the sole carbon source pre and post-exposure to UV-irradiation and to investigate its efficiency of biodegradation at different temperatures and pH values using a laboratory benchtop bioreactor. Sludge was obtained from the wastewater and standard isolation and identification techniques were used to identify the microorganism. The biodegradability was tested at temperatures of 25, 30, 35 and 40°C and at pH values of 6.5, 7.0 and 8.0. The results indicated that the isolated microorganism was *Pseudomonas alcaligenes*, the maximum concentration of 2,4-dichlorophenol which bacteria can grow on before UV-irradiation was 220 and 380 mg/l after UV-irradiation. The variation in temperature values resulted in different degradation rates and that the degradation of 2,4-dichlorophenol increased at a higher pH value. From these results, it is concluded that *P. alcaligenes* can be used for the degradation of 2,4-dichlorophenol, UV-irradiation can be successfully used for the improvement of *P. alcaligenes* biodegradability and that the best 2,4-dichlorophenol biodegradation was at 35°C and pH 7.**

**Key words:** 2,4-Dichlorophenol, biodegradation, *Pseudomonas alcaligenes*, ultraviolet radiation.

## INTRODUCTION

Highly chlorinated compounds, such as chlorophenols and perchloroethylene (PCE), are recalcitrant pollutants which threaten our environment. 2,4-Dichlorophenol (DCP) is a chlorinated aromatic compound that is a primary reagent in the synthesis of a variety of more highly chlorinated phenols (that is, pentachlorophenol) and pesticides and fungicides (that is, 2,4- dichlorophenoxyacetic acid) (Bae et al., 2002; Annachhatre and Gheewala, 1996; Short et al., 1991). These compounds are present in the wastewater generated from industrial activities such as petrochemical, pharmaceutical, wood preserving, plastic, rubber proofing, pesticide, iron steel and paper and cellulose bleaching industries. Due to their extreme toxicity and persistence in the soil as well as in groundwater supplies, these compounds represent a serious ecological problem and public health risk (ATSDR, 1999; Yee and Wood, 1997). The United States environmental protection agency regulates the phenol content in the wastewater from less than 1 mg/l to the several thousand mg/l (La Rotta et al., 2007; ATSDR, 1999). A number of physical, chemical and biological methods have been used to eliminate chlorophenols from industrial effluents and neither of these methods and their combinations has been used to achieve complete mineralization of chlorophenols (Herrera et al., 2008; La Rotta et al., 2007; Yee et al., 1998). Although, chemical methods are efficient, they may generate undesirable by-products, besides being very expensive. By contrast, biological methods are generally more efficient and relatively cheaper than chemical methods.

The biodegradation of chlorophenols by a variety of microorganisms has been studied by many authors (Reddy and Gold, 2000; Schlosser et al., 2000; Kim et al., 2002; Bollag et al., 2003; Abdel-Haleem et al., 2003; Kargi and Eker, 2005; Herrera et al., 2008). According to these studies and others, the biodegradability of chlorophenols depends on the number and position of halogens in the aromatic ring. Moreover, high chlorophenol concentrations are known to be inhibitory to microbial growth. Hence, prior adaptation to chlorophenols remains the standard strategy used for improving microbial degrading

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ability and for contending with detrimental inhibitory effects (Dua et al., 2002; Wiggins et al., 1987; Annachhatre and Gheewala, 1996). However, variation in the complexity and toxicity of chlorophenols has also promoted the use of fungi, anaerobic bacteria and microbial consortia for their mineralization (Steinle et al., 1998).

Bioremediation is an extremely attractive waste treatment process because microorganisms mineralize a variety of compounds and bioremediation is an *in situ* process that minimizes transportation and disposal expenses. *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 many authors for the biodegradation of a variety of chlorophenolic compounds (Premalatha and Rajakumar, 1994; Kiyohara et al., 1992; Yee et al., 1998; Farrell and Quilty, 2002). On the other hand, the efficiency of biodegradation of all bacterial or fungal species is questionable. Koh et al. (1997) have reported a 69% dehalogenation of 2,4-DCP using *Alcaligenes eutrophus*, while Wang et al. (2000) showed that the removal of 2,4-DCP using *Bacillus insolitus* at high concentration was less than 50% . Matafonova et al. (2006) reported that *B. cereus* significantly biodegraded 2,4-DCP at concentrations between 20 and 560 µM over a 2 days reaction period in submerged culture experiments. Similar reports have been made elsewhere using suspended microorganisms and immobilized cells (Sahinkaya and Dilek, 2002; 2007; Herrera et al., 2008). Tomasi et al. (1995) have reported various success rates for biodegradation of DCP using *P. cepacia*. Degradation of pentachlorophenol (PCP) up to 800 mg/l by *P. aeruginosa* has been reported by Premalatha and Rajakumar (1994).

Ultraviolet radiation (UV) has been extensively used in the water disinfection and treatment industry (Elkarmi et al., 2008). 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, 2002; Cooke and Williamson, 2006; Alonso-Saez et al., 2006).

The aims of this study were to investigate the efficiency of microbial degradation of 2,4-DCP by isolated a pure culture of *Pseudomonas spp*. from pharmaceutical and healthcare industries wastewater, to examine the difference in the growth of *Pseudomonas* spp. utilizing 2,4-DCP as the sole carbon source pre and post-exposure to ultraviolet (UV) radiation and to investigate the efficiency of biodegradation at different temperatures and pH values.

## MATERIALS AND METHODS

### Isolation and identification of *Pseudomonas* spp

The bacterial species were isolated from wastewater samples taken from 2 pharmaceutical companies that their wastewater have been analyzed to contain phenolic compounds and centrifuged at 3000 rpm for 30 min to obtain sludge. 200 mg of the sludge was added to pre-sterilized flasks containing 20 ml of chlorophenol enrichment culture media supplemented with 100 mg/l of 2,4-dichlorophenol (Zhang and Weigel, 1990; Kiyohara et al., 1992) and incubated in a shaking water bath for 4 days at 30°C. Flasks containing 50 ml of nutrient broth (HiMedia Laboratories Limited, Mumbai, India) were inoculated with 0.5 ml of the first suspension and incubated for 4 days under the same conditions. Nutrient agar plates supplemented with 100 mg/l of 2,4-DCP were inoculated with 0.3 ml from the flasks and incubated for 4 days at 37°C. Biochemical characterization of the bacterial colonies was carried out according to Cowan and Steel's manual for identification of medical bacteria (Barrow, 1993) and Bergey's manual of systematic bacteriology (Krieg and Holt, 1984; Sneath et al., 1986). Confirmation of the identity of the bacterial species was carried out by Jordan university hospital, using a "REMEL" system and API 20NE kit.

### Efficiency of biodegradation of 2,4-DCP isolates prior UV irradiation

Colonies that appeared on the plates were streaked into nutrient agar plates supplemented with cetrimide (1 g/l) and incubated for three days at 37°C. 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-DCP in order to examine the ability of *P. alcaligenes* in using 2,4-DCP as the sole source of carbon. Enumeration of colony forming unit (CFU), at different 2,4-DCP concentrations, was carried out by using a colony counter.

### Modification of the isolated colonies by UV-irradiation

The isolated colonies were streaked into nutrient agar plates in duplicates and irradiated by UV radiation (365 nm, 11 $W/m^2$) for 24, 48, 72 and 96 h. Colonies from UV irradiated plates were used to inoculate nutrient agar plates supplemented with 2,4-DCP concentrations of 240, 260 , 280 , 300, 320 , 340, 360 , 380 and 400 mg/l in order to examine the effect of UV irradiation on the capability of *P. alcaligenes* in utilizing 2,4-DCP for its growth.

### Efficiency of biodegradation of 2,4-DCP by *Pseudomonas alcaligenes* post UV irradiation

The UV irradiated *P. alcaligenes* was cultivated in a benchtop bioreactor (Laboratory benchtop bioreactor with stirrer unit model 300 and EMC unit model 351, Hamburg, Germany). The biodegradebility was tested at different environmental conditions, temperatures of 25, 30, 35, and 40°C and at pH values of 6.5, 7.0 and 8.0. The initial concentration of 2,4-DCP 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-DCP was determined using a UV- spectrophotometer (UV-visible spectrophotometer, 100 Bio:Cary Varian, USA), wavelength was 295 nm.

### Statistical analysis

Experimental mean values were analyzed using the analysis of variance (ANOVA) test, one-way ANOVA test was used to determine the level of significance within single experimental group and LSD (Fisher's least significant difference) test was used to determine the difference between different means and $P < 0.05$ was considered statistically significant.

**Table 1.** Microorganism growth at different 2,4-dichlorophenol concentrations.

| 2,4-DCP concentration ( mg/l ) | Microorganism growth ( log $_{10}$ of CFU/ml ) |
|---|---|
| 120 | 10.14 |
| 130 | 10.06 |
| 140 | 10.03 |
| 150 | 9.85 |
| 160 | 10.51 |
| 180 | 9.60 |
| 200 | 8.40 |
| 220 | 6.07 |

**Table 2.** Bacterial growth at different 2,4-dichlorophenol concentrations after UV-irradiation for 96 h.

| 2,4-DCP concentration (mg/l) | Bacteria growth (log $_{10}$ of CFU/ml) |
|---|---|
| 180 | 8.61 |
| 200 | 9.08 |
| 240 | 9.06 |
| 260 | 9.03 |
| 280 | 8.85 |
| 300 | 8.52 |
| 320 | 8.31 |
| 340 | 7.95 |
| 360 | 7.20 |
| 380 | 6.80 |

## RESULT

### Bacterial strain

The wastewater samples studied contained $10^2$ - $10^3$ *P. alcaligenes* cells/g dry weight of sludge. This strain was gram-negative rod, catalase positive, oxidase positive, do not produce acid from sugars, motile and aerobic. Further confirmation by using API 20NE system (the 7 digits numerical profile) this bacteria possessed the number of (1 000 055), which is related to *P. alcaligenes*. The identification was confirmed by the Jordan university hospital. The growth of the isolated strain decreased as the 2,4-DCP concentrations increased. Weak growth was observed when the isolated microorganism was grown at 180 mg/l 2,4-DCP and at higher concentrations of 200 and 220 mg/l 2,4-DCP (Table 1).

Before UV-irradiation the maximum concentration of 2,4-DCP which bacteria could grow on was 220 mg/l. A good level of bacterial growth was detectable after UV-irradiation (Table 2). The effect of UV-irradiation was the same for the colonies irradiated for 48 and 96 h. The 24 and 72 h irradiation showed no considerable increase in the tolerance to 2,4-DCP concentrations. UV-irradiation for 96 h resulted in the best response to the modification of the colonies and growth was detected at 380 mg/L of 2,4-DCP concentration.

**Table 3.** Bacterial growth in bioreactor at different temperatures and constant pH (log $_{10}$ of CFU/ml).

| pH | Temperature | Time (h) | | |
|---|---|---|---|---|
| | | 72 | 120 | 168 |
| 7.0 | 25ºC | 6.84 | 6.85 | 6.48 |
| | 30ºC | 7.14 | 7.10 | 6.84 |
| | 35ºC | 7.11 | 7.18 | 6.93 |
| | 37 ºC | 7.06 | 7.11 | 6.87 |
| | 40ºC | 6.56 | 6.66 | 6.41 |

**Table 4.** Bacterial growth in bioreactor at different pH and constant temperature (log $_{10}$ of CFU/ml).

| Temperature | pH | Time (h) | | |
|---|---|---|---|---|
| | | 72 | 120 | 168 |
| 25ºC | 6.5 | 5.61 | 6.16 | 5.42 |
| | 7.0 | 6.82 | 6.81 | 6.46 |
| | 7.5 | 5.72 | 5.81 | 5.47 |
| | 8.0 | 5.47 | 5.73 | 5.11 |

The UV-irradiated bacteria were cultivated in a bio-reactor to study the optimal growth conditions for the growth of the isolated bacteria. The growth results of these bacteria grown in the presence of 380 mg/l of 2,4-DCP concentration to test the effect of temperature variation analyzed using one-way ANOVA showed that at different temperatures and constant pH(7) the bacterial growth varied depending on temperature value ($p = 0.0108$) (Table 3). Furthermore, there are statistically significant differences between bacterial growth at varying pH and constant temperature (25°C) ($p = 0.0059$) (Table 4).

The results of the degradation of 2,4-DCP at different temperatures and constant pH (Table 5) analyzed using one-way ANOVA and LSD (Fisher's least significant difference) showed a correlation between the temperature value and concentrations of 2,4-DCP ($p < 0.05$). This means that the variation in temperature values resulted in different degradation rates of 2,4-DCP (Figure 1). Moreover, the results of degradation of 2,4-DCP at different pH values and constant temperature (Table 5) showed a correlation between the variation of pH values and 2,4-DCP concentrations ($p < 0.05$), indicating that the degradation of 2,4-DCP increased at higher pH values (Figure 2).

## DISCUSSION

Isolation of indigenous microorganisms from wastewater of pharmaceutical industries and the examination of their

**Table 5.** The concentration of 2,4-DCP (mg/l) from bioreactor samples at different pH and temperature values.

| pH | Temperature (°C) | Time (h) | | | | |
|---|---|---|---|---|---|---|
| | | 0 | 72 | 96 | 120 | 144 |
| 6.5 | 25 | 380 | 241 | 207 | 172 | 159 |
| 7.0 | 25 | | 207 | 147 | 135 | 122 |
| 7.0 | 30 | | 164 | 142 | 122 | 92 |
| 7.0 | 35 | | 202 | 124 | 98 | 81 |
| 7.0 | 40 | | 281 | 262 | 146 | 136 |
| 8.0 | 25 | | 295 | 253 | 232 | 198 |

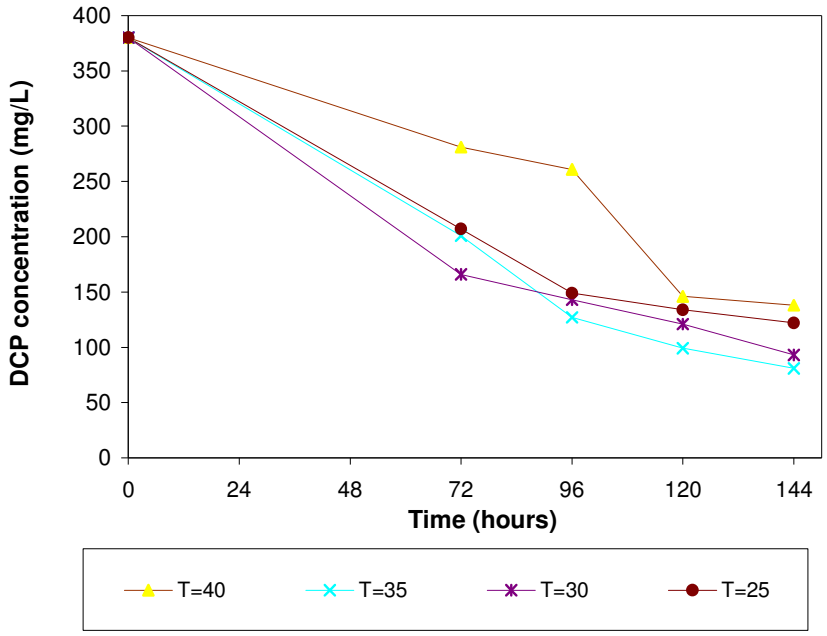


**Figure 1.** Comparison between the degradation of 2,4-dichlorophenol with time in bioreactor samples, at different temperatures at pH 7.

ability to biodegrade of 2,4-DCP could be of great advantage. Several factors, such as temperature, pH, oxygen concentration and the composition of the microbial community, influence the degradation of organic toxic compounds such as chlorophenols (Tiirola et al., 2002). The results presented in this paper showed that prior to UV-irradiation the maximum concentration of 2,4-DCP which *P. alcaligenes* could grow on was 220 mg/l. Comparing with other studies the minimal inhibitory concentration (MIC) of 2,4-DCP to *P. pickettii* was 15.6 mg/l, while MIC was 125 mg/l for *P. putida* and for *P. aeruginosa* the MIC was 62.5 mg/l (Kiyohara et al., 1992). Leontievsky et al., (2002) showed that flavorbacterium spp. were capable of removing 300 mg/l pentachlorophenol (PCP) while *Streptomyces rochei* could remove 400 mg/l PCP. Moreover, PCP was degradation by *P. aeruginosa* in shake-cultures and the bacteria were able to completely degrade pentachlorophenol PCP up to 800 mg/l in 6 days with glucose as a co-substrate (Premalatha and Rajakumar, 1994).

Pseudomonades have been identified to be of importance in bioremediation as a result of their tremensdous capacity for biodegradation (Environmental health and safety publications, 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-DCP ( Perkins et al., 1990; Bhat et al., 1994; Xun, 1996; McFall et al., 1997; Tarao and Seto, 2000). UV-irradiation in this study was used to produce alterations in order to increase the ability of *P. alcaligenes* to degrade DCP. Without UV-irradiation the maximum concentration of 2, 4-DCP which bacteria could grow was 220 mg/l 2,4-DCP. After irradiating the bacteria by UV-irradiation at wavelength 365

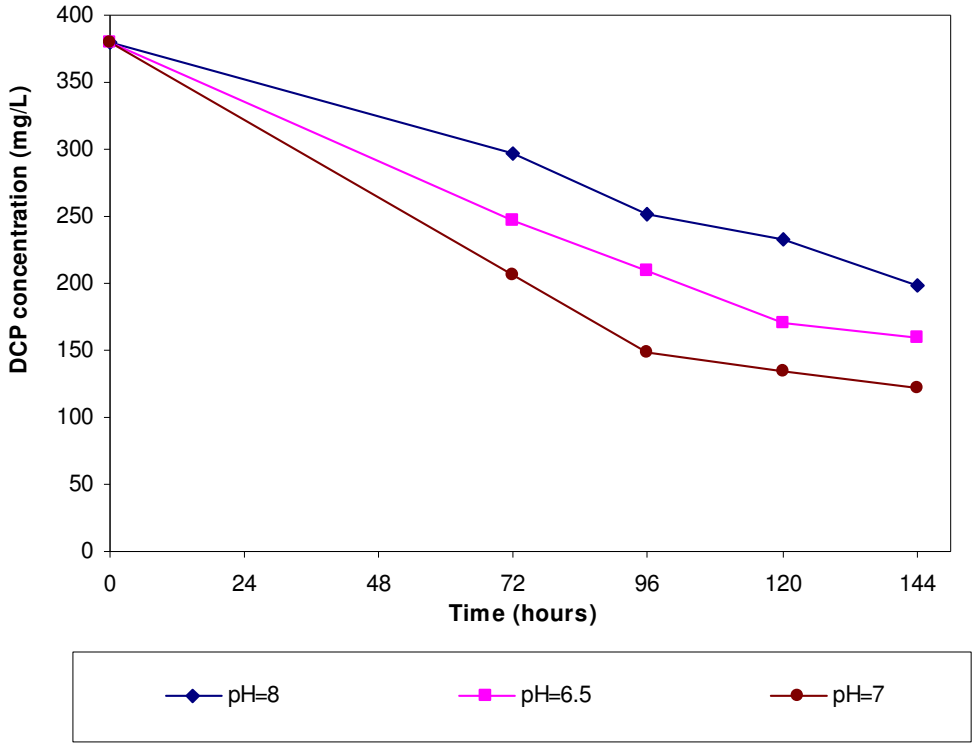


**Figure 2.** Comparison between the degradation of 2,4-dichlorophenol with time in bioreactor samples, at different pH and temperature of 25°C.

nm for 96 h which was the best response to the modification of colonies the concentration increase to 380 mg/l of DCP. This result can be interpreted as the direct effect of the UV irradiation on the genetic material of this microorganism that resulted in a positive mutation in the gene or genes that control the metabolism of chlorophenol degradation. The aim of this study was not to find out the genetic modification that occurred to the *P. alkaligenes* after the UV irradiation, although it is clear that a large improvement in the biodegradation efficiency occurred due to this UV irradiation. It is well-known that the effect of UV irradiation usually results in the formation of pyrimidine dimers. Fortunately, mutations caused by UV irradiation in our isolated microorganism were favorable in the sense that it might have blocked the normal metabolic pathways of these bacteria that utilize carbon sources other than the chlorophenols. There are a number of studies that explain the genetic bases of biodegradation. For example, *Flavobacterium* degrades PCP through a catabolic pathway encoded by multiple genes one of which is *pcp*A, that encodes information for a 30 kDa polypeptide *pcp*A, found in periplasm of the bacterium (Chanama and Crawford, 1997). Further studies in this area are needed to elucidate the genetic alterations and the new biodegradation pathway that occurred due to UV irradiation.

Several factors, such as the genetic potential and certain environmental factors such as temperature, pH, oxygen concentration and the composition of the microbial community, influence the degradation of organic compounds (Tiirola et al., 2002). Cultivation of microorganism was also reported by other researchers. For example, *Ralstonia* was enriched in a fixed-bed reactor fed with 2, 6-dichlorophenol as the sole source of carbon and energy at pH 7.5 and room temperature (Louie et al., 2002; Steinle et al., 1998). It has been reported that the amount of 2,4-DCP 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 showed that the best biodegradation occurred at pH 7 and not 8 as evident in Figure 2 which showed that the lowest concentration of 2,4-DCP was at pH 7. 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 highest at pH 7. Temperature affects biological reaction rates, species mortality rates and biological growth rates. Hence, it is expected that biodegradation efficiency will increase with the increase in temperature, which is indicated by our results. The results of this study indicated that the degradation of 2,4-DCP was high at temperature 35°C and pH 7 during the first 5 days, while the degradation rate was slower in the next 3 days which is confirmed with other studies that used *P. putida* and *P. aeruginosa*

which showed that all compounds are degraded with different speed and the final amount of non-degraded compounds is most often lower for *P. putida* (Bielicka et al., 2002).

In conclusion, the biodegradation efficiency of 2, 4-DCP by *P. alcaligenes* was shown to be enhanced up to 380 mg/l after UV irradiation for 144 h and that the highest biodegradation efficiency occurred at temperature of 35°C 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-DCP biodegradation.

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