The International Journal of Biotechnology
2014 Vol.3, No.1, pp.12-23
ISSN(e): 2306-6148
ISSN(p): 2306-9864

DEGRADATION OF THE ORGANOPHOSPHORUS INSECTICIDE DIAZINON BY SOIL BACTERIAL ISOLATE

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ABSTRACT

Microorganisms isolated from soil sample using enrichment culture technique have been grown in the minimal growth media where diazinon served as a sole carbon source. Total three bacterial strains were screened and identified by morphological and biochemical studies as Pseudomonas peli, Burkholderia caryophylli and Brevundimonas diminuta and designated as Pseudomonas peli BG1, Burkholderia caryophylli BG4 and Brevundimonas diminuta PD6, respectively. All these isolates were able to completely degrade 20 mg/l diazinon in mineral salt medium (MSM) as a sole carbon source within 12 days of incubation. The bacterial growth and diazinon degradation were accelerated by these isolates when MSM supplemented with 0.5 % (w/v) glucose as an additional carbon source. The maximum degradation rate by the isolates BG1, BG4 and PD6 were 3.350, 4.265 and 3.140 mg/l/d, respectively. The bacterial growth and diazinon degradation rates were increased by these three isolates when MSM supplemented with 0.5 % (w/v) glucose as an additional carbon source. The maximum degradation rates were 4.556, 5.367 and 5.885 mg/l/d for the isolates BG1, BG4 and PD6, respectively in the presence of glucose. pH of the growth medium decreased more sharply in presence of glucose as a consequence of microbial metabolism of glucose. The results of this study suggested a correlation among diazinon degradation, microbial growth and pH in MSM with or without glucose during diazinon degradation studies.

Keywords: Biodegradation, Diazinon, Glucose, Mineral salt medium, Organophosphorus pesticide, *Pseudomonas peli, Burkholderia caryophylli, Brevundimonas diminuta.*

1. INTRODUCTION

Agricultural practices now rely heavily on the use of pesticides but the contamination of soils and water supplies are now an ever increasing consequence of such farming activities. Bangladesh, as are many other developing countries, is still a largely agricultural based community, with the use of pesticides in such countries likely to suffer from higher than necessary application rates. The wide spread use of these pesticides over the years has resulted in problems caused by their interaction with biological systems in the environment(Kanekar *et al.*, 2004). Excessive and persistent use of pesticides results in deterioration of the environment. The quality of soils, ground water, surface waters as well as the air, is compromised by pesticide contamination(Surekha *et al.*, 2008) .

Diazinon is an organophosphate insecticide is the fifth most commonly used pesticide used by homeowners, with two to four million pounds used annually(Beyond, 2003). Most synthetic OP compounds are highly toxic and are powerful inhibitors of acetylcholinesterase, a vital enzyme involved in neurotransmission, in the form of acetylcholine substitutes (Grimsley *et al*., 1998; Bakry *et al*., 2006). Environmental hazards and health risks caused by obsolete pesticides could therefore potentially affect health and environment. So in situ decontamination of pesticides contaminated water and soils are necessary.

Degradation of pesticides is usually a combination of a number of processes, including chemical hydrolysis and microbial degradation, and is also influenced by some physicochemical properties such as pH, organic carbon and moisture content(Gunther, 1974). However, biodegradation is the primary mechanism of pesticide breakdown and detoxification in many soils. Thus microbes may have a major effect on the persistence of most pesticides in soil(Surekha *et al*., 2008) . Biodegradation is a common method for the removal (breakdown and detoxification) of organic pesticides because of its low cost and low collateral destruction of indigenous animal and plant organisms(Liu *et al*., 2007). Microbial degradation is considered to be a major factor determining the fate of diazinon and other organophosphorus insecticides in the environment. The aims of the present study were to degradation of diazinon in the presence and absence of glucose by the organisms isolated from soil.

2. MATERIALS AND METHODS

2.1. Culture Medium

The Mineral Salt Medium(Cycon *et al*., 2009; Abo-Amer and Aly, 2011)used in both isolation of bacteria from soil and diazinon degradation studies, contained (g/l) $(NH_4)_2SO_4$, 2.0; KH_2PO_4, 1.5; Na_2HPO_4, 1.5; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.01; $FeSO_4 \cdot 7H_2O$, 0.001. The pH of the medium was adjusted to 7.0 ± 0.1with 2 M NaOH. Diazinon was added to the Mineral Salts Medium after sterilization. Diazinon solution was prepared by dissolving diazinon with acetone. Mineral Salts Medium together with Diazinon was used for biodegradation studies.

2.2. Enrichment of the Soil Samples

The aim of this step was to adapt the soil microflora to insecticide, diazinon. In this step of enrichment procedure the mineral salt medium (MSM) was used. To obtain this effect, collected soil samples (5 g) were mixing with 100 ml of mineral salts medium containing diazinon (Di) at concentration of 50 mg/l. Before mixing the sample soils and diazinon, the MSM was autoclaved and pH maintained at 7 ± 0.1. Diazinon was introduced in a form of acetone solution. After mixing the soil suspension was incubated in the dark at 30 °C. After 10-14 days of incubation the aliquot of contaminated soil suspension was taken for isolation of bacteria.

2.3. Preparation of Samples for Microbial Count

After enrichment of the soil sample, 1 ml of contaminated soil suspension taken and added to 9 ml of sterile saline water (0.85% NaCl) and a serial dilution (10^{-1} - 10^{-5}) technique was followed for culture of the sample.

2.4. Isolation of Diazinon Degrading Bacteria

Different discrete colonies on the plates were selected for isolation. Morphologically dissimilar isolated colonies were picked up from the plates with the help of sterile loop and each individual colony was streaked on diazinon agar plate for pure culture.

2.5. Screening all the Isolated Diazinon Degrading Bacteria

Total 16 isolated colonies were picked from the diazinon agar plates and screened to observe the well and rapid growth as well as resistance to diazinon by replica plate method(Lederberg and Lederberg, 1952) onto diazinon agar plates containing up to 50 mg/l diazinon.

2.6. Identification of the Isolate

The selected bacterial isolate BG4 was identified by cultural, morphological and biochemical tests. Colony characteristics of this isolate were observed after growing on nutrient agar plate after 24 h at 30 °C. Bergey's Manual of Determinative Bacteriology(Buchanan and Gibbons, 1984), Manual for the Identification of Medical Bacteria(Cowan, 1975) and 'ABIS6' an online software (ABIS6, 2012) for bacteria identification were used as a reference to identify the isolates.

2.7. Preparation of Inocula

Bacterial strain was inoculated in nutrient broth (1.3%, w/v) and incubated overnight at 30 °C and at 150 rpm for 24 h. After that the cells were centrifuged at 6000 rpm for 10 min at 4 °C. Pellet cells were washed twice with sterile 50 mM potassium phosphate buffer (pH 7.2). Final pellets were resuspended into 0.85% (w/v) sterile NaCl medium to prepare bacterial suspension (approximately$\approx 10^6$ cells/ml) and that were used as bacterial inoculums in diazinon degradation studies.

2.8. Effects of Glucose on Diazinon Biodegradation

To study the effect of extra carbon source on the bacterial growth and degradation rate of diazinon, 145 ml of MSM containing 50 mg/l diazinon in 250 ml conical flasks amended with 0.5% (w/v) glucose were inoculated with $\approx 10^6$ CFU/ml of the isolates as described previously. Triplicate samples of MSM containing diazinon with glucose (Di+G), non-inoculated with the bacterial suspension were kept as control. Conical flasks were incubated at 30° C on a rotary shaker at 120 rpm in the dark condition. At regular intervals, a portion of the samples were taken aseptically for determination of bacteria growth and the concentrations of diazinon.

2.9. Measurement of Biomass

The biomass concentration was estimated by optical density measurement in 1 cm cuvettes at wavelength of 600 nm using UV-Visible spectrophotometer.

2.10. Assay of Diazinon

Concentrations of diazinon were measured by High Performance Liquid Chromatography (HPLC) (WATERS 486, UK) using the retention times and peaks corresponding to reference standards. For detecting diazinon, HPLC method was used according to (Soleas *et al*., 2000).

2.11. Data Analysis and Software used for Bacteria Identification

Statistical analysis was also performed for determining correlation between the bacterial growth and the diazinon concentration (mg/l) by using the Sigma plot 7. Microsoft Office Excel 2007 also used for data analysis. $p< 0.05$ was considered as the minimal level of statistical significance. An online software 'ABIS6' was used for identification of the isolated bacteria (http://www.tgw1916.net/bacteria_logare.html).

2.12. Calculation of Degradation Rate

The degradation rate by the bacterial isolate under specific conditions was calculated according to (Lin *et al*., 2008). Degradation rate = Δ [Diazinon] / Δ [t]

Where, Δ [Diazinon] was the difference of diazinon concentrations between two sampling times (mg/L) and Δ [t] was time difference in days.

3. RESULTS & DISCUSSION

3.1. Isolation, Screening and Quantification of Diazinon Degrading Bacteria

Based on morphological properties of the bacterial colonies, 16 isolates were selected and repeated sub-culture on diazinon agar (containing 50 mg/l diazinon) was performed untiluniform colonies were found. Finally by visual observation, among the well and faster growing bacterial isolates three were selected and designated as BG1, BG4 and PD6. These isolates were investigated further details studies.

3.2. Identification of Diazinon Degrading Bacteria

By comparing the results of cultural, morphological and various biochemical tests (Table1) with Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1984), Manual for the Identification of Medical Bacteria(Cowan, 1975) and using 'ABIS6' online software(ABIS6, 2012) for bacteria identification. It was found that, the isolates BG1, BG4 and PD6 were *Pseudomonas peli*, *Burkholderia caryophylli* and *Brevundimonas diminuta*, respectively. Identified three different diazinon degrading isolates were then designated as *Pseudomonas peli* BG1, *Burkholderia caryophylli* BG4 and *Brevundimonas diminuta* PD6.

3.3. Biodegradation of Diazinon and Growth by the Isolates in Mineral Salt Medium (MSM)

Growth of bacterial isolates, diazinon degradation and changes in pH when 20 mg/l diazinon was supplied as sole source of carbon are presented in Figure 1. The results showed that *Pseudomonas peli* BG1 degrade diazinon completely within 14 days of incubation. The bacterial growth and diazinon degradation both increased rapidly during 8 days of incubation. Result also showed that, when growth of the bacterial cell increased, diazinon degradation also relatively increased. The results for the isolates *Burkholderia caryophylli* BG4 and *Pseudomonas diminuta* PD6 showed similar patterns of degradation and growth but the isolate *Burkholderia caryophylli* BG4 was able to degrade 20 mg/l of diazinon within 12 days.

It has been reported by (Ramanathan and Lalithakumari, 1999), *Pseudomonas* sp. A3 isolated from soil through enrichment technique able to degrade diazinon, methylparathion, malathion, and monocrotophos. (Ortiz-Hernández and Sánchez-Salinas, 2010)isolated six strains presumptively identified as *Stenotrophomonas malthophilia*, *Proteus vulgaris*, *Vibrio metschinkouii*, *Serratia ficaria*, *Serratia* spp. and *Yersinia enterocolitica*.Results of their experiments showed that only one pure strain *Serratia ficaria* A3 increased during 72 h of culture, and therefore was the most efficient strain for removing TCV (48.48 %). Two bacterial strains *Pseudomonas diminuta* MG and *Flavobacterium* sp. ATCC 27551 were isolated which was able to degrade a considerable number of synthesized OP(Mulbry, 2000).

(Hayatsu *et al*., 2000)previously isolated and characterized a *Burkholderia* sp. strain NF100 capable of utilizing fenitrothion, an organophosphorus insecticide as a solesource of carbon. (Desdpande, 2002) isolated two strains belonged to the genus *Brevundimonas* and identified as *Brevundimonas* sp. It was reported that several bacterial species such as *Pseudomonas* sp. (Ramanathan and Lalithakumari, 1999), *Flavobacterium* sp. (Ghassempour *et al*., 2002), *Agrobacterium* sp. (Yasouri, 2006), can utilize diazinon as a sole source of carbon. Moreover, the same bacterial species may also contribute in biodegradation of other organophosphorus insecticides (Cycon *et al*., 2009; Ortiz-Hernández and Sánchez-Salinas, 2010).

Table- 1. Colony characteristics, Morphological and Biochemical characteristics of 3 different diazinon degrading isolates grown on nutrient agar at 30 °C for 24 hours

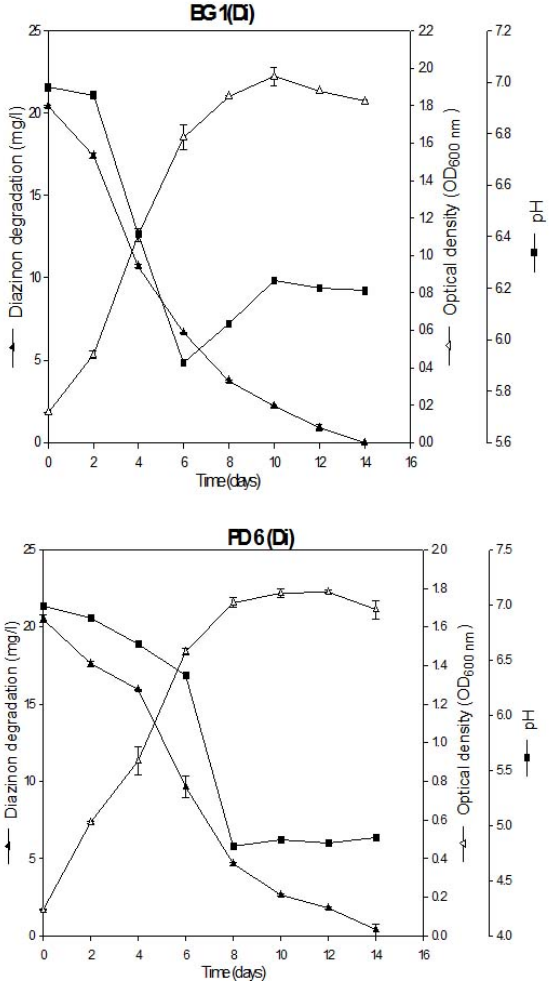
Isolates		BG1	BG4	PD6
Carbohydrate Utilization	Adonitol	-	+	-
	Arabinose	-	+	-
	Fructose	-	+	-
	Galactose	-	+	-
	Glucose	-	+	-
	Innocitol	-	+	-
	Lactose	-	+	-

	Maltose	-	+	-
	Mannitol	-	+	-
	Mannose	-	+	-
	Rhamnose	-	+	-
	Salicin	-	+	-
	Sorbitol	-	+	-
	Sucrose	-	+	-
	Xylose	-	+	+
Biochemical Characteristics	Oxidase	+	+	+
	Catalase	+	+	+
	MR	-	-	-
	VP	-	+	-
	Gas form glucose	-	+	-
	H_2S production	-	-	-
	Indole	-	-	-
	Motility	-	+	-
	OF	-	+	-
	Nitrate reduction	+	+	+
	Gelatin hydrolysis	-	-	-
	Citrate utilization	-	+	-
	Starch Hydrolysis	-	-	-
	Lysine hydrolysis	+	+	+
	Urease	-	-	-
	Arginine dihydrolase	-	+	-
	Esculin hydrolysis	+	+	+
Microscopic Examinations	Gram Reaction	G -	G -	G -
	Microscopic view	Short Rod	Short Rod	Rod
	Size	1.75 mm	2.25 mm	1.75 mm
	Colony Morphology	Circular, Light yellowish, Flat, Mucoid, Regular	Circular, Creamy white, Elevated, Mucoid, Regular	Circular, Off white, Flat, Non-mucoid, Regular

MR: Methyl Red; VP: Voges-Proskauer; OF: Oxidative Fermentative; + for positive reaction, - for negative reaction.

Abo-Amer and Aly (2011)isolated a diazinon degrading bacterium was identified as *Serratia marcescens* D1101. They showed that the isolate *Serratia marcescens* D1101 degraded diazinon completely in MSM supplemented with diazinon as sole carbon source at initial concentrations of 50 mg/l. *Pseudomonas* species, known as a very metabolically active bacteria, enable to utilize many synthetic organic compounds such as many agrochemicals, were isolated from different soils contaminated with diazinon and other organophosphorus pesticides(Ghassempour *et al.*, 2002; Lakshmi *et al.*, 2008; Ortiz-Hernández and Sánchez-Salinas, 2010), whereas *Burkholderia* seems to be a new bacterium that can contribute a complete degradation of diazinon.

Fig- 1- Diazinon degradation, bacterial growth and change in pH by *Pseudomonas peli* BG1, *Burkholderia caryophylli* BG4 and *Pseudomonas diminuta* PD6 in mineral salt medium supplemented with diazinon as a sole carbon source (MSM + Di) during biodegradation studies.



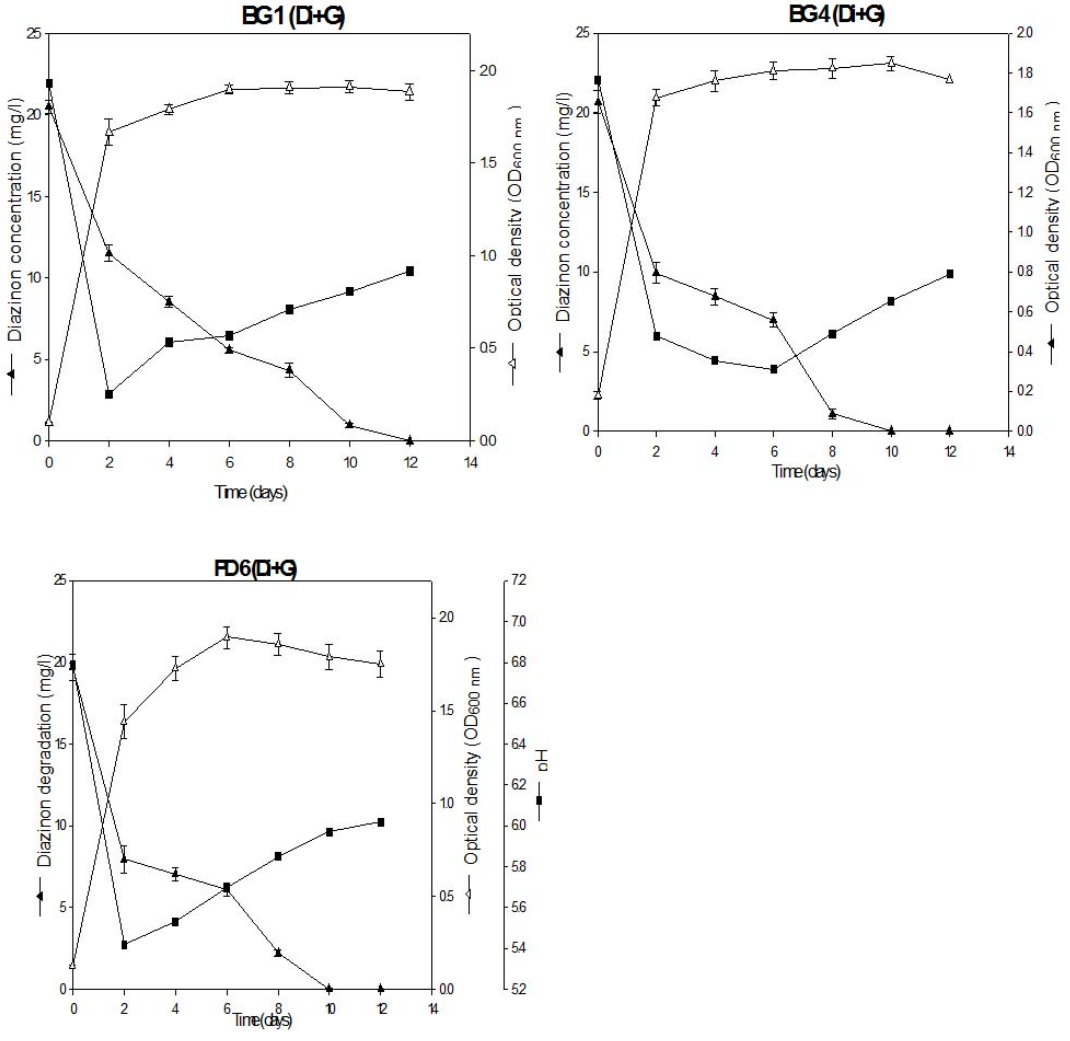
3.4. Effects of Glucose as an Additional Carbon Source on Diazinon Degradation and Bacterial Growth in Mineral Salt Medium (MSM)

To determine the effects of additional carbon source on diazinon degradation and bacterial growth, 0.5 % (w/v) glucose was added to MSM supplemented with Diazinon. Diazinon degradation, corresponding bacterial growth and change in pH by *Pseudomonas peli* BG1, *Burkholderia caryophylli* BG4 and *Pseudomonas diminuta* PD6 in mineral salt medium (MSM) supplemented with 20 mg/l diazinon (D) and glucose (G, 0.5% w/v) are shown in Figure 2. Degradation of diazinon and bacterial growth in MSM supplemented with glucose (Di+G) was different as compared to supplemented with only diazinon (Di) as sole carbon source. The growth of bacteria was significantly stimulated and approximately three to four times in first two days of the incubation period as compared to growth of isolates in MSM without glucose. Maximum

growth of the bacterium was found in 6 days with the proportionate degradation of substrates. Diazinon degradation and bacterial growth were different as compared to degradation and growth in MSM without additional carbon source. During the first 2 days of incubation period about 45 %, 52 %, and 60 % of the initial dose of diazinon were degraded by BG1, BG4, and PD6 respectively, while in the same period degradation of diazinon in MSM without glucose was significantly lower and reached at 15 %, 8 %, and 15 % for BG1, BG4 and PD6, respectively (Figure 1) when diazinon was used as sole source of carbon. Similar enhanced diazinon degradation in the presence of glucose in MSM was reported (Drufovka *et al*., 2008; Cycon *et al*., 2009). Significantly faster diazinon biodegradation when easily degradable low molecular weight substrates (i.e. glucose or ethanol) were present in the growth medium was also reported (Drufovka *et al*., 2008). (Gunner *et al*., 1966) showed that soil microorganisms did not degrade diazinon significantly unless an additional carbon source like glucose, ethanol, and hexane was added to the medium. (Sethunathan and Yoshida, 1973)also showed that, microbial degradation of diazinon is enhanced in the presence of an organic carbon source like glucose and alcohols.

The measurement of pH values in MSM during experimental period showed that this parameter was closely related with the bacterial growth and corresponding degradation rate of diazinon. The pH of the medium was dropped from 6.98 to around 6.15 ± 0.03 when diazinon was degraded completely as sole carbon source by the isolates BG1 and BG4 in MSM. When isolates BG1 and BG4 inoculated in the medium with glucose (Di+G), the pH was dropped sharply from 6.8 to 5.41 and from 6.8 to 5.63 respectively during 2 days of incubation period. The results also showed that, the pH decreasing rate was the maximum when bacterial cell growth and diazinon degradation rate were faster (Figure 1 and 2). The changes in relative pH and diazinon degradation were significantly different for the isolate PD6 when inoculated in MSM supplemented with diazinon. The pH was slightly decreased from 6.99 to 6.36 after 6 days of incubation but it was sharply dropped from 6.36 to 4.91 during 6 to 8 days of the incubation period (Figure 2). When isolate PD6 was inoculated in MSM medium supplemented with glucose (Di+G), the pH was decreased as like the isolates BG1 and BG4 during the same period of incubation. The influence of pH and diazinon degradation was reported by previous investigators. It has been reported that in medium with pH below 4.5, diazinon degradation was predominantly driven by chemical process. But it also reported that the diazinon degradation is carried out mainly by biologically when the medium pH was above 5 (Drufovka *et al*., 2008).

Fig- 2. Diazinon degradation, corresponding bacterial growth and change in pH by *Pseudomonas peli* BG1, *Burkholderia caryophylli* BG4 and *Pseudomonas diminuta* PD6 in mineral salt medium (MSM) supplemented with 20 mg/l diazinon (Di) and glucose (G, 0.5% w/v) during biodegradation studies

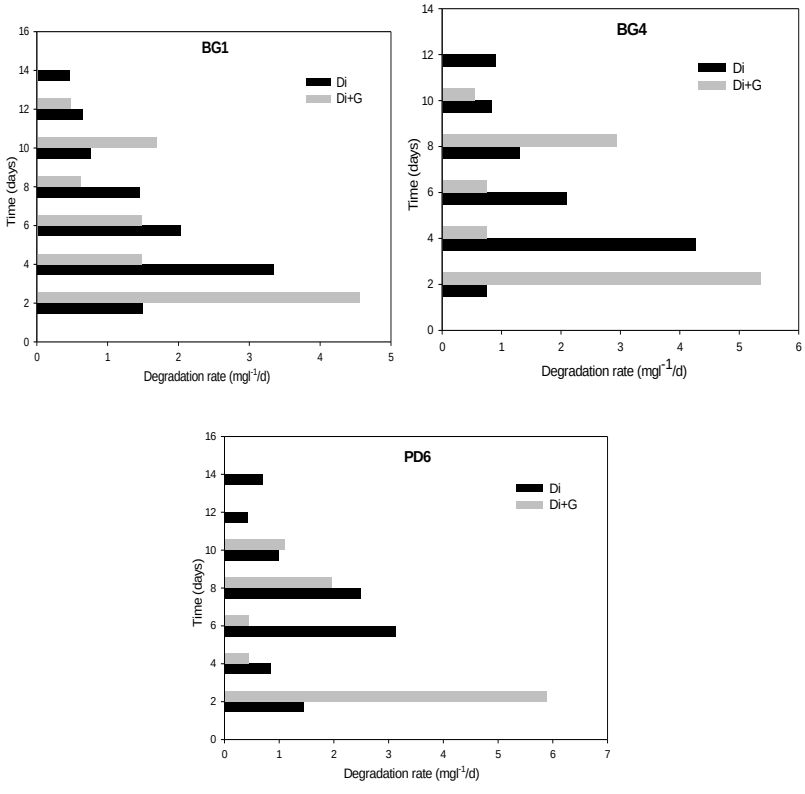


3.5. Diazinon Degradation Rate

The relative degradation rate patterns of diazinon in MSM supplemented with diazinon and glucose (Di+G) are presented in Figure 3. It was observed that the diazinon degradation rate for these three isolates were different at the same incubation period when inoculated in MSM supplemented with or without glucose. Maximum diazinon degradation rates by isolates BG1 and BG4 were 3.35 and 4.265 mgl^{-1}/day in 4 days of incubation when inoculated in MSM supplemented with only diazinon. In this study it was found that the diazinon degradation rates by the isolates *Pseudomonas peli* BG1, *Burkholderia caryophylli* BG4 and *Brevundimonas diminuta* PD6 was greatly increased in the presence of glucose. The maximum degradation rates were

obtained by the isolates BG1, BG4 and PD6 with 4.556, 5.367 and 5.885 mg/l/d, respectively in the presence of glucose after 2 days of incubation. On the other hand in absence of glucose the maximum degradation rates by BG1, BG4 and PD6 were 1.500, 0.760 and 1.460 mg/l/d, respectively in the same period of incubation.

Fig- 3. Diazinon degradation rate in mineral salt medium supplemented with diazinon (MSM+Di) as sole source of carbon and glucose as an additional source of carbon (MSM+Di+G)inoculated with *Pseudomonas peli* BG1, *Burkholderia caryophylli* BG4 and *Brevundimonas diminuta* PD6



(Cycon *et al.*, 2009) showed that, degradation rate of diazinon in MSM supplemented with glucose was accelerated by the isolates *Serratia liquefaciens*, *Serratia marcescens*and *Pseudomonas*sp. when glucose was used as additional carbon source. Their study also reported that, growth rate of bacteria was significantly stimulated and approximately two times faster at the beginning of incubation period (0–2 d), as compared to growth of isolates in MSM without additional carbon source. Ethanol, hexane and glucose provide available source of organic carbon for soil microorganisms, and can be metabolized to acidic products, which in turn increase diazinon hydrolysis (Drufovka *et al.*, 2008). Other studies showed that, diazinon degradation rate were inhibited in presence of glucose. (Abo-Amer and Aly, 2011) studied that diazinon was completely

degraded by the *Serratia marcescens* DI101 on days 11 and 15, with relative degradation rates of 40.724 and 29.391 mg^{-1}/day^{-1} in MSM and MSM supplemented with glucose, respectively.

4. CONCLUSION

Three bacterial isolates belonging to *Pseudomonas*, *Burkholderia* and *Brevundimonas* genera were isolated and identified, which had efficient degradation capability of the organophosphorus insecticide diazinon. Addition of glucose enhanced the growth of the isolated and degradation of dizinon. Results of the biodegradation for diazinon indicated that apart from chemical processes microbial degradation is considered to be one of the main mechanisms of diazinon dissipation in water. Moreover, obtained results have implications for the development of a bioremediation strategy of diazinon-polluted water.

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