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Isolation of pesticide degrading microorganism from water agriculture drainage

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Abstract

Diazinon is an organophosphorus often used in agriculture as a soil and foliar insecticide. Diazinon could be hazardous as a result of runoff from areas of application into nearby drains or ditches, that typically transport water to streams and lakes. In the present study, bacteria that has a high ability of diazinon degradation was isolated from agricultural drainage ditches (Fayoum, Egypt) by enrichment technique. Based on morphological, biochemical and 16S rDNA gene sequencing, a high isolated bacteria was recognized as *Pseudomonas aeruginosa*. Minimal medium supplemented with diazinon as sole carbon source used for the growth of a pure culture of *P. aeruginosa*. The effect of diazinon concentrations, temperature and pH on the bacterial growth as well as rate of diazinon degradation were studied. It was found that the maximum capability of diazinon degradation (73.46 %) was done at concentration 250 ppm of diazinon at pH value 7.0 and temperature 30 °C within 14 days. Therefore, *P. aeruginosa* could be used efficiently for cleaning up of pesticides in the environment specially contaminated water agricultural usage.

Keywords: *Pseudomonas aeruginosa*, Diazinon, Biodegradation, 16S rDNA.

Introduction

The term “pesticide” includes all chemicals that are utilized to kill or control pests in agriculture, this includes herbicides (weeds), insecticides (insects), fungicides (fungi), nematocides (nematodes), and rodenticides (vertebrate poisons) (Ongley). Due to widespread use of pesticides, environmental matrices such as water, soil and air are exposed to pesticides in large quantities (Rudel *et al.*, 2009). Due to the accumulation of pesticides in water supplies, there is a necessity to develop environmentally safe, convenient and cost- effective techniques for the removal of pesticides (Hussaini *et al.*,2013).

Different methods such as filtration, ozonation, and adsorption using the granular activated carbon are used for the removal of pesticides from aqueous solutions, however these methods have associated with problems as high capital and operating costs, saturation of activated carbon, and production of toxic substances (Rice *et al.*,1989).

Biodegradation is a common method used for the removal (breakdown and detoxification) of organic pesticides as a result of its low cost and little collateral destruction of indigenous animal and plant organisms (Megharaj *et al.*,1994). The microbial breakdown of pesticides is more important than physical and chemical degradation (Garbisu and Alkorta 2003). Microbial degradation of organophosphates has been reported (Singh *et al.*, 2006; Dubey and Fulekar 2012; Pandey and Aparna 2019). Microorganisms can use organophosphorus pesticides as carbon and/or phosphorus sources though their degradation by specific pathways (Ibrahim *et al.*, 2010).

Diazinon (O, O-diethyl O-2-isopropyl-6-methylpyrimidin-4-yl phosphorothioate) is organophosphorus insecticide (Fig. 1). It is commonly used in agriculture as a soil and foliar insecticide. It is an inhibitor of acetylcholinesterase in insects and can affect human nervous system (Chamber).

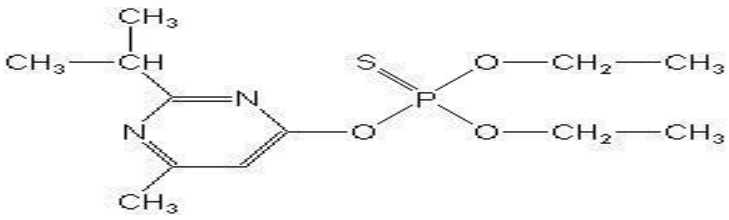


Fig. 1. Structure of the organophosphorus insecticide diazinon

Diazinon could be hazardous as a result of runoff from areas of application into nearby drains or ditches, that typically transport water to streams and lakes (Getzin 1967). Diazinon also induce oxidative stress in animals and humans (Oruç *et al.*, 2007), generate free radicals and cause lipid peroxidation leading to genetic material damage and cell malformations (Čolović *et al.*, 2010). Like other OPs, the key of toxic effect of diazinon is the inhibition of acetyl cholinesterase activity (AChE, EC 3.1.1.7) (Kalantary *et al.*, 2015). Diazinon has short half-life in water, ranging from 70 hr to 12 weeks controlled by pH, temperature, and sunlight as well as the presence of microorganisms while in soil it is effected by the pH conditions in the soil and the soil type (Sethunathan and Yoshida 1973). Researches have demonstrated that diazinon has an immunotoxic, cytotoxic, and genotoxic effects (Abd-Alla 1994). Some techniques for degradation of diazinon in aqueous solution have been advanced as chemical oxidation that was performed using ozonation (Ku *et al.*,1998), aqueous chlorine (Zhang and Pehkonen 1999) and

Fenton treatment of diazinon (Wang and Lemley 2002). Then, irradiation degradation was done by X-ray (Trebše and Arčon 2003) and gamma-ray (Mohamed *et al*.,2009). Recent studies have focused on biodegradation (Cycoń *et al.*, 2009) and ultrasonic technique (Matouq *et al*.,2008). Previous studies have documented that several bacterial species can utilize diazinon as a source of carbon and/or phosphorus such as *Serratia marcescens* (Abo-Amer 2011); *Pseudomonas* sp. (Essa *et al*., 2016), *Agrobacterium* sp. (Yasouri 2006); *Arthrobacter* sp. (Ohshiro *et al*.,1996). Diazinon is one of the common water pollutants in Egypt. In this study (i) A strain of bacteria was isolated from agricultural wastewater that tolerance diazinon, (ii) It was examined the optimum conditions of diazinon biodegradation.

Material and Methods

Chemicals

Diazinon was purchased from Riedel-de Haën, Sigma-Aldrich, Seelze, Germany. All other chemicals purchased are of analytical grade from Fluka AG, Buchs, Switzerland.

Growth Media and Culture conditions

The mineral salt medium (MSM) was used in isolation of bacteria from soil and diazinon degradation studies was consisting of $(NH_4)_2SO_4$ (2.0 g/L); KH_2PO_4 (1.5 g/L); Na_2HPO_4 (1.5 g/L); $MgSO_4 .7H_2O$, (0.2 g/L); $CaCl_2 .2H_2O$ (0.01 g/L); $FeSO_4.7H\ 2O$ (0.001 g/L). The pH of the medium was set at 7.0 ± 0.1 with 2 M NaOH. Diazinon was added to the liquid MSM medium after sterilization. For solid medium, 2% (w/v) agar was added to the same diazinon containing liquid mineral salt medium. Stock solution of diazinon was added with a concentration 500 ppm in acetone and was diluted to the required concentrations for the degradation studies.

Isolation of the most tolerant diazinon degrading bacteria

This experiment was done to select the most tolerant bacteria to high concentration of diazinon. Six hundred milliliter of the agriculture wastewater was collected from El-Batts drain, Fayoum, Egypt. The samples were centrifuged under 10,000 rpm for 10 min and restore with 10 mL Milli-Q water. Forty-five milliliter of liquid MSM medium supplemented with diazinon (100 ppm) was inoculated with 5 mL of bacterial suspension and incubated for 48 hr inside shaking incubator (120 rpm) at 30^{o}C. Aliquots were sub- cultured every 3 days for a total of five passes. The final culture was diluted and plated on diazinon agar plates. Developed colonies were repeatedly streaked on diazinon agar plates for isolation of pure cultures. The bacterial isolate designated AA was chosen for this work because it was the most tolerant strain and can grow under high concentration diazinon up to 300 ppm. A pure culture of AA isolate was then stored in solid MSM containing 200 ppm diazinon for further studies.

Morphological and biochemical tests

The MN isolate was tested for morphology, motility and Gram stain by phase contrast microscopy. Biochemical identification of the MN isolate was done according to (Taha *et al.*, 2018) and using commercially available miniaturized multi test identification systems API (BioMérieux, France). First of all, inoculation of each identification system, a 24hr NA culture of MN isolate was re-inoculated onto Nutrient agar plates to obtain isolated colonies for testing purposes. The API 20NE identification system is created for identifying non-fastidious, non-enteric Gram-negative rods. Test strips were inoculated and incubated according to the instructions provided. Sterile 0.85% saline solution was used as a negative control. APIWEB software was used for identification and was considered acceptable when given a probability of 85% or greater.

The16S rDNA identification

The procedure was used for bacterial identification strains. It was found that MN strain that resist high concentration of diazinon and show efficiency to growth in high centration of diazinon. MN bacterial isolate genomic DNA have been extracted using standard bacterial procedures of (Sambrook *et al.*,1989). The primers used in the amplification of the 16S rDNA gene are forward primer (F1; AGA GTT TGA TCC TGG CTC AG) and reverse primer (R1; GGT TAC CTT GTT ACG ACT T). The PCR mixture was prepared as the following; 10 µL (10x) PCR buffer, 3 µL (50 mM) MgCl2, 1 µL (20 pmole/µL) of each primer, 1 µL (10, and the volume is completed to 100 sµL by SDH2O. PCR were carried mM) dNTPs mixture, 0.5 µL (2.5U) Taq DNA polymerase, 2 µL total DNA extract out for 35 cycles under the following conditions: denaturation step at 94°C for 40 sec, annealing step at 55°C for 1 min, extension step at 72°C for 2 min and final extension at 72°C for 10 min. An aliquot of the PCR products (10 µL) was mixed with 2 µL of DNA loading buffer and analyzed by electrophoresis (15 V/cm, 60 min) on 0.7% horizontal agarose gel in TBE buffer having 0.5 µg/mL ethidium bromide, then visualized on an UV transilluminator. Sequencing of the amplified fragments were performed at GATC Biotech, Constance, Germany. DNA Sequences were aligned at NCBI Data Base (www.ncbi.nlm.nlh.gov).

Optimization of the growth conditions and diazinon biodegradation Effect of different concentration of diazinon degrading bacteria were carried out in 250 mL flask containing 50 mL MSM supplemented with different concentrations of diazinon (50 – 300 ppm). The medium was injected by five milliliter of bacterial cell suspension (OD600 nm=0.2) and incubated on a rotary shaker (120 rpm) at 30°C. Cell growth in liquid media was determined spectrophotometrically by determining the cultural optical density at 600 nm at 24 hr meanwhile 21 days. The protein content of the supernatant was assayed using Bradford assay

(Bradford) in order to approve the bacterial growth. Effect of different temperature (30°C, 37°C, 20°C) and different pH value (7.0, 5.0, 9.0) on diazinon degrading bacteria were studied where diazinon was sole carbon source in enrichment media. The optimum temperature and pH for growing of bacterial strain were detected where culture supplement with 250 ppm of diazinon as a sole carbon source. All the experiments were performed in triplicates. Cell growth in liquid media was determined spectrophotometrically as mentioned above. The MSM supplemented with the same concentration of diazinon without bacterial inoculums were performed and incubated under the same conditions in order to measure abiotic degradation of diazinon.

Analysis of the residual diazinon

The ability of *P. aeruginosa* for diazinon biodegradation was determined under different temperatures and pH values after 14 days incubation in order to detect the optimum condition for diazinon degradation. The residual diazinon was extracted according to the method of (Nasiri *et al*., 2016; Hladik *et al*., 2012). About 50 mL of 90% methanol was added to 20 mL liquid culture and was set overnight. Then the mixture was filtrated and extracted twice with 50 mL CH_3Cl. The received solution was concentrated under nitrogen flow to 1 mL to be detected by gas chromatography. A Hewlett-packard, USA serial 6890 gas chromatograph equipped with electron detector (ECD, Radioisotope Nuclide 63Ni) and HP PAS-1701 column 25 m length x 0.32 mm x 0.52 thickness. Pure nitrogen was used as carrier gas (2 mL/min). Detector, injector and column temperature was 250, 240 and 225°C, respectively. Diazinon detection limit was set at concentration where the analyte signal was three times higher than background noise and it was 0.1 µg/L. The diazinon degradation rate was determined according to (Yang *et al*.,2005) by the following mathematical relationship:

$$A = [Ca - Cb / Ca] \times 100$$

where, (A) is the percentage of diazinon degradation, (Ca) is the concentration of diazinon (mg/L) in the medium in absence of diazinon degrading strain, (Cb) is the concentration of diazinon (mg/L) in presence of diazinon degrading strain.

Statistics

The data given here are the mean values of three replicates. Standard errors were calculated for all the values using MS Excel 2010.

Results

Isolation and identification of diazinon degrading bacteria

Isolation of some bacterial species able to degrade diazinon were applied from agricultural drainage ditches in August, 2018 (Fayoum, Egypt). It was carried out by using enrichment

technique. It was found that, the bacterial isolate MN was the most tolerant strain against high levels of diazinon (300 ppm). A variety of morphological and biochemical assays were carried out to have a comprehensive view of the phenotypic characteristics of the bacterial isolate MN as shown in Table 1. MN isolate was Gram negative motile spore forming rods. This isolate demonstrated positive results with β-galactosidase, arginine dihydrolase, lysine decarboxylase, orenthine decarboxylase, urease, amylase, gelatinase, catalase, cytochrome oxidase, nitrate reduction and acetoin production tests. Meanwhile, negative results were recorded for tryptophane deaminase, lipase, starch, H_2S production, indole production tests. Simultaneously, the MN isolate showed the capability to utilize glucose, sucrose, mannitol, amygdalin, inositol and citrate as carbon source.

Table 1. Morphological and biochemical characters of the diazinon degrading bacterial isolate (MN)

Morphological characters	**Result**	**Sugar fermentation**	**Result**
Gram staining	Negative	Glucose	Positive
Motility	Motile	Sucrose	Positive
Cell shape	Rod	Mannitol	Positive
Endospore formation	Negative	Inositol	Negative
Enzyme profile		Rhamnose	Negative
β-galactosidase	Positive	Melibiose	Negative
Arginine dihydrolase	Positive	Amygdalin	Positive
Lysine decarboxylase	Positive	Arabinose	Negative
Orenthine decarboxylase	Positive	Starch	Negative
Urease	Positive	Citrate utilization	Positive
Tryptophane deaminase	Negative	Sorbitol	Negative
Gelatinase	Positive		
Other tests			
Catalase	Positive	H_2S production	Negative
Amylase	Positive	Acetoin production	Positive
Lipase	Negative	Indole production	Negative
Cytochrome oxidase	Positive		
Nitrate reduction	Positive		
-To nitrite	Positive		
-To N_2	Negative		

Using 16S rDNA gene sequencing technique, The MN isolate was identified as *Pseudomonas aeruginosa* with maximum homology of 99.64% *Pseudomonas aeruginosa DSM 50071* strain. The phylogenetic tree of the diazinon degrading bacterial strain MN and related bacterial species based on the 16S rDNA sequence was given in **Fig. 1.** It is a clear manner that the MN diazinon degrading strain was included in the genus *Pseudomonas* and closely related to the species *aeruginosa*.

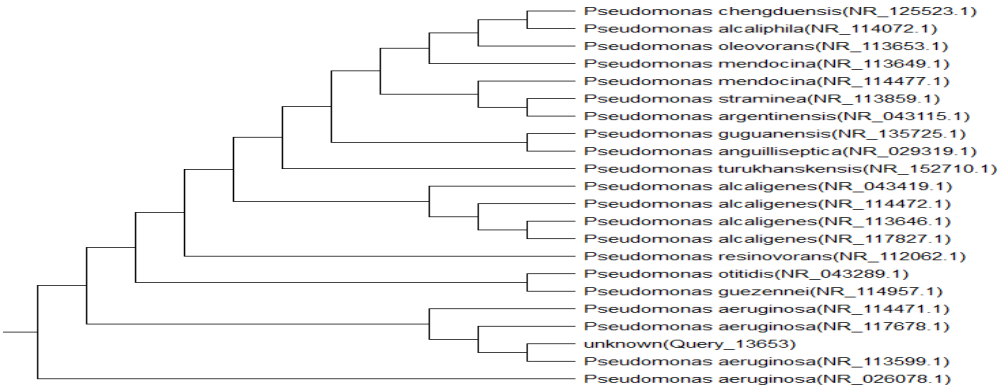


Fig. 1. Phylogenetic dendrogram obtained by distance matrix analysis of 16S rDNA sequences showing the position of the bacterial isolate MN among phylogenetic neighbors

Growth optimization and diazinon degradation of *P. aeruginosa*

The obtained data recorded the ability of *P. aeruginosa* (isolated from agricultural wastewater), to tolerate high levels of diazinon up to 300 ppm. Data in (Fig. 2 and 3) designed the growth of *P. aeruginosa* is in the minimal media supplemented with diazinon as a sole carbon source was increased concentration up to 250 ppm then the growth decreased at 300 ppm. The maximum cell density within 14 days of incubation was (0.8837) which recorded at 250ppm while the maximum protein content (293.397 mg/L) was achieved with 250ppm of diazinon.

Effect of different temperature on the growth of *P. aeruginosa* was recorded at (Fig. 4 and 5) and biodegradation rate of diazinon (Fig. 8 and Table 2). The maximum optical density (0.9103333) and protein content (298.83 mg/L) and diazinon degradation 72.971% was demonstrated at 30^{o}C after 14 days of incubation. At higher temperature, a decreasing in the optical density, protein contents and percentage of diazinon degradation was recorded. At the same time, moderate bacterial growth and diazinon biodegradation rate was recorded at 20^{o}C.

Effect of the change in the pH value was recorded, a remarkable effect on the growth of *P. aeruginosa* (Fig. 6 and 7). The maximum optical density (0.927) and protein content (326.08 mg/L) and diazinon degradation (73.46%) was recorded at pH 7.0 within 14 days. While the recorded growth parameters and diazinon degradation were significantly decrease at the pH levels 5.0 and 9.0 (Fig. 8B and Table 2).

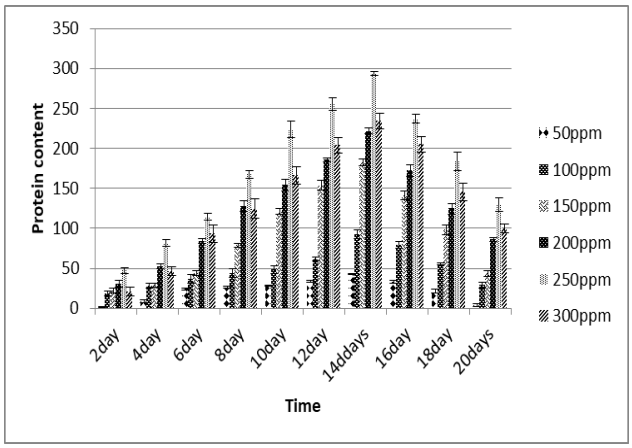


Fig. 2. Effect of diazinon concentration on the growth of *Pseudomonas aeruginosa*, in terms of protein content(mg/l). Diazinon was utilized as sole carbon source. Data represent the means of three replicates and error bars represent

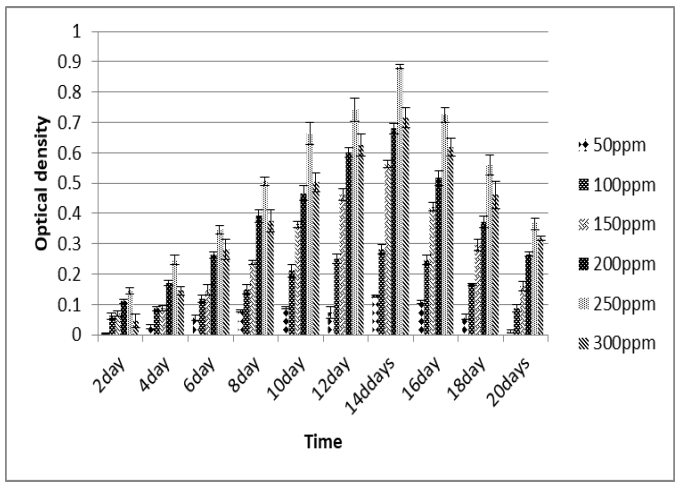


Fig. 3. Effect of diazinon concentration on the growth of *Pseudomonas aeruginosa*, in terms of optical density(600nm). Diazinon was utilized as sole carbon source. Data represent the means of three replicates and error bars represent the standard errors of the mean

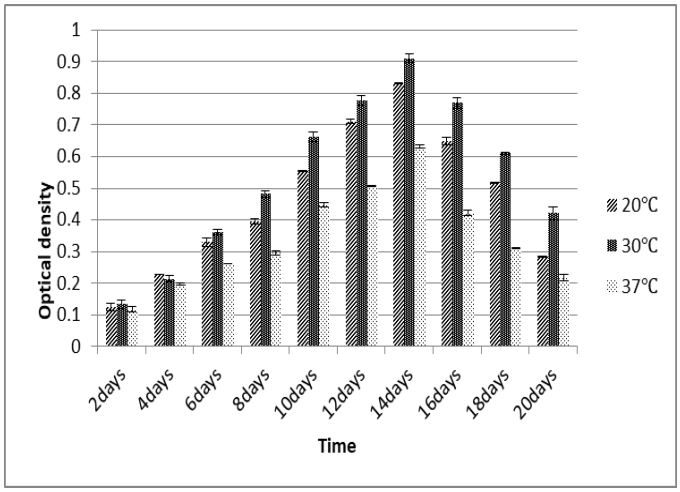


Fig. 4. Effect of different temperatures on the growth of *Pseudomonas aeruginosa*, in terms of optical density(600nm). Diazinon was utilized as sole carbon source. Data represent the means of three replicates and error bars represent the standard errors of the mean

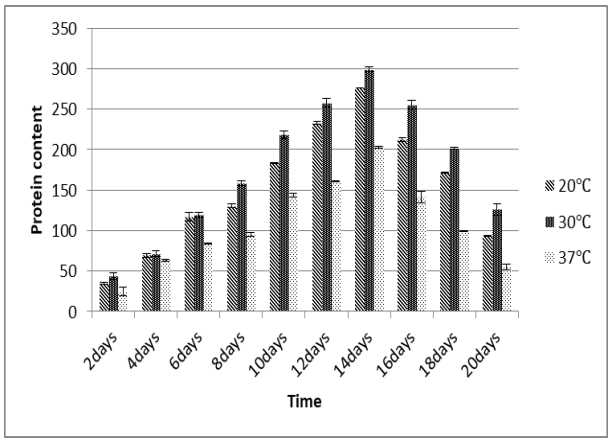


Fig. 5. Effect of different temperatures on the growth of *Pseudomonas aeruginosa*, in terms of protein content(mg/l). Diazinon was utilized as sole carbon source. Data represent the means of three replicates and error bars represent the standard errors of the means

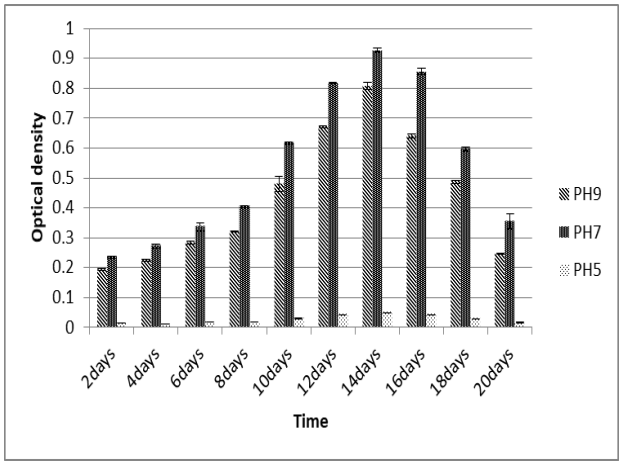


Fig. 6. Effect of different pH value on the growth of *Pseudomonas aeruginosa*, in terms of optical density (600nm). Diazinon was utilized as sole carbon source. Data represent the means of three replicates and error bars represent the standard errors of the means

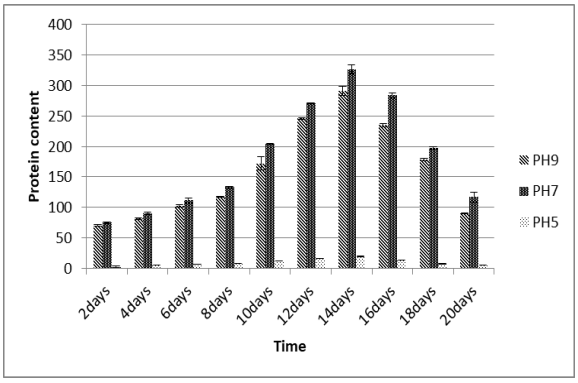
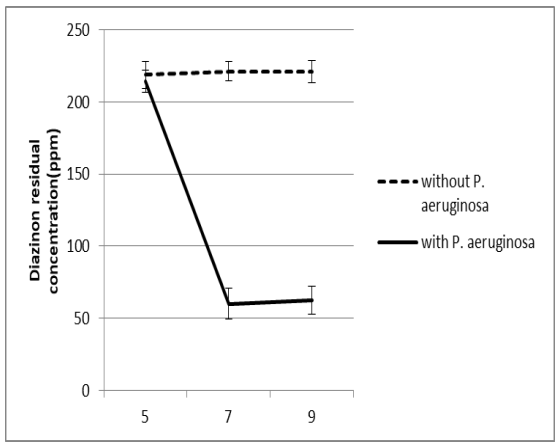
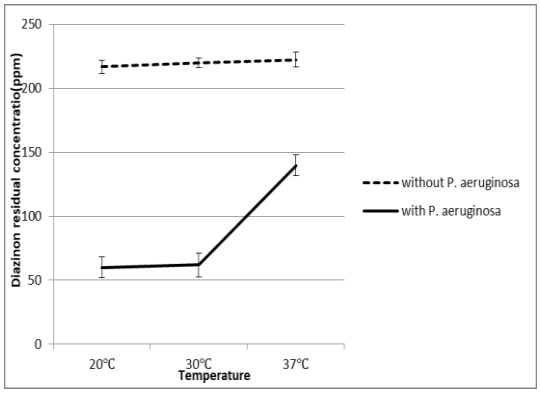


Fig. 7. Effect of different PH value on the growth of *Pseudomonas aeruginosa*, in terms of protein content (mg/L). Diazinon was utilized as sole carbon source. Data represent the means of three replicates and error bars represent the standard errors of the means



(A)



(B)

Fig. 8. Optimization of diazinon degradation by *Pseudomonas aeruginosa* where (A) is the effect of pH value and (B) is the effect of temperature on the biodegradation process after 14 days. Initial concentration of diazinon was 220 ppm. Data represent the means of three replicates and error bars represent the standard errors of the means

Table 2. The percentage of diazinon removal by *Pseudomonas aeruginosa* under different temperature and pH values after 14 days of incubation where the initial concentration was 220 ppm

Treatment		Retention time (min)	Residual concentration of diazinon(ppm)	
			With *P.aeruginosa*	Without *P. aeruginosa*
pH value	5.0	2.051	213.77 ± 7.709	218.4 ± 9.145
	7.0	2.044	63.68 ± 7.0434	221 ± 6.6583
	9.0	2.039	62.72 ±11.231	220.697 ± 7.398
Temperature	20°C	2.048	88 ± 13	356 ± 12
	30°C	2.046	82 ± 10	364 ± 9
	37°C	2.061	201 ± 11	358 ± 8

Discussion

The microorganisms able to degrade organic pollutants may be found in and isolated from many environments including contaminated sites or marine sediments (Ferreira *et al.*,2016). The vulnerability of OP compounds to microbial adaptation has been reported for several compounds including the insecticides. Considering the role of pesticides in the development of modern agriculture and their effects on ecosystems, it seems that microorganisms are the best option for metabolization and elimination of them to improve soil conditions, and the quality of life in the ecosystem (Hassanshahian 2016). Pesticide residues in soils and their following movements in the water–soil system are key aspects in their environmental behaviour (Benimeli *et al*.,2003). The pesticides reach the water drift outside of the intended area when it is sprayed, percolate, or leach through the soil, or carrying to the water as runoff, or It may be spilled accidentally (Drahansky *et al*.,2016). The routes by which diazinon pass into the soil or water can include direct application, spray drift, run-off from treated animals, spills, accidental releases, rinsing of containers and disposal (Cycoń *et al*.,2012) The microbial communities of the agricultural wastewater usually certain bacterial strains that could tolerate high concentrations of the toxic pesticides and might have the capability to mineralize these compounds (Tyler *et al*.,2013). In this study it was able to isolate MN strain that tolerate high concentration of diazinon. A variety of morphological and biochemical assays were carried out to have a comprehensive view of the phenotypic characteristics of the bacterial isolate MN in addition to 16S rDNA gene sequencing. Results show that the genus *Pseudomonas* is closely related to the species *aeruginosa.*

Optimization of the growth of Pseudomonas aeruginosa and diazinon degradation.

The bacterial isolate *P. aeruginosa* tolerate high levels of diazinon concentration up to 300 ppm. It was reported that *Pseudomonas aeruginosa* degrade different type of pesticides (Senthilkumar *et al*.,2011; Barragán-Huerta *et al*.,2007; Fernandes *et al*.,2014). Many studies indicated the capability of various bacterial strains isolated from soil or water polluted with diazinon for partially or completely degradation of this compound such as *Pseudomonas sp*. (Ramanathan and Lalithakumari 1999), *Arthrobacter sp*. (Ohshiro *et al*.,1996), and *Flavobacterium sp*. (Ghassempour *et al*.,2002).

This study showed that the growth of *P. aeruginosa* was increased as increasing diazinon concentration up to 250 ppm, then growth was decreased at used concentrations. These results are in agreement with those obtained with (Essa *et al*.,2016) who studied the effect of diazinon concentration on the growth of a diazinon degrading bacteria *P. aeruginosa*. The ability of *P. aeruginosa* to use diazinon as a sole carbon source. There is remarkable effect in different

temperatures and different pH values. Results also in harmony with that reported by (Abo-Amer and Aly 2011). He showed a high ability *Serratia marcescens DI101* for using Diazinon as a carbon and phosphorus source. At low diazinon concentrations, complete degradation has been achieved within 9 days while at higher concentrations only 40% was degraded within 16 days and record effect of the different temperatures. The optimal incubation temperature for diazinon degradation was between 25℃ and 30℃. Meanwhile, the slowest degradation was determined at the two extreme temperatures (10℃ and 40℃).

Similarly, *Alcaligenes faecalis* DSP3 was found to be able to degrade almost 100% of the initial concentration (100 mg/L) of chlorpyrifos, diazinon and parathion within 10 days (Cycoń *et al*., 2013). A recent study confirmed an efficient diazinon degradation capability of some bacterial isolates *Pseudomonas peli, Burkholderia caryophylli* and *Brevundimonas diminuta* when it was used as a sole carbon source within 12 days of incubation (Mahiudddin *et al*.,2014). In addition, (Cycoń *et al*., 2009) reported a high rate of diazinon degradation (80-92%) by the bacterial strains *S. liquefaciens, S. marcescens, Pseudomonas sp*. within 14 days when it was added to MSM at low concentration. In most published, diazinon was hydrolysed in non-sterilized soils to DETP and 2-isopropyl-4-methyl-6-hydroxypyrimidine (IMHP). The subsequent breakdown of the pyrimidine ring of IMHP to CO2 is microbial in nature (Singh *et al*., 2006).

Temperature, pH, water potential, nutrients and the amount of pesticide or metabolite in soil might act as limiting factors for pesticide degrading microorganisms, that requires further exploration in relation to total microbial population and their biochemical activities (Singh and Dileep 2008). The present study showed that the maximum bacterial growth and diazinon degradation were achieved at 30℃ after 14 days of incubation and decrease in bacterial growth at 25℃ and 37℃. These results are in harmony with (Abo-Amer and Aly 2011). Where Abo-Amer and Aly (2011) reported that the optimal incubation temperature for diazinon degradation by *Serratia marcescens* was recorded between 25℃ and 30℃ while, alow degradation rate was recorded at extreme temperatures. Essa *et al*., 2016 showed that optimum incubation temperature for diazinon degradation by *P. aeruginosa.* was recorded at 30℃ and significant decrease at 37℃ and 25℃.

In addition, the highest bacterial growth rate and diazinon degradation were recorded at pH 7.0 within 14 day. These are in agreement with that mention by Abo-Amer and Aly (2011) who showed remarkable changes in degradation rates of diazinon by *Serratia marcescens* at different pH values. Diazinon was completely degraded on within 11 days at pH values between 7.0 and 8.0, while the degradation rate was sharply decrease at pH 5.0 and pH 10.0.

In the same way, Essa *et al*., (2016) reported that there are remarkable changes in degradation rates of diazinon by *P. aeruginosa* at different pH values. Diazinon degradation rate was optimum at pH 7 equal to (86.3%) and show significant decrease at pH 9.0 and 5.0.

Conclusion

Isolation bacterial strains from agricultural drainage ditches by enrichment technique, and has the ability to utilize diazinon as a source of carbon. The isolated strain was identified by 16S rDNA techniques as *Pseudomonas aeruginosa.* A significant diazinon degradation was showed at pH value 7.0 and temperature 30°C within 14 days. Therefore *P. aeruginosa* can be used effectively in bioremediation technology to clean-up of agricultural wastewater contaminated with high levels of diazinon.

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