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Biodegradation of Diazinon by *Serratia marcescens* DI101 and its Use in Bioremediation of Contaminated Environment

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Four diazinon-degrading bacteria were isolated from agricultural soil by using an enrichment technique. The biochemical analysis and molecular method including RFLP indicated that these isolates were identical, and one strain designated DI101 was selected for further study. Phylogenetic analysis based on 16S rDNA sequencing indicated that the strain DI101 clearly belongs to the *Serratia marcescens* group. The ability of the strain to utilize diazinon as a source of carbon and phosphorus was investigated under different culture conditions. The DI101 strain was able to completely degrade 50 mg/l diazinon in MSM within 11 days with a degradation rate of 0.226 day^{-1}. The inoculation of sterilized soil treated with 100 mg/kg of diazinon with 10^6 CFU/g DI101 resulted in a faster degradation rate than was recorded in non-sterilized soil. The diazinon degradation rate by DI101 was efficient at temperatures from 25 to 30°C and at pHs from 7.0 to 8.0. The degradation rate of diazinon was not affected by the absence of a phosphorus supplement, and addition of other carbon sources (glucose or succinate) resulted in the slowing down of the degradation rate. The maximum degradation rate (V_{max}) of diazinon was 0.292 day^{-1} and its saturation constant (K_s) was 11 mg/l, as determined by a Michaelis–Menten curve. The strain was able to degrade diethylthiophosphate-containing organophosphates such as chlorpyrifos, coumaphos, parathion, and isazofos when provided as a source of carbon and phosphorus, but not ethoprophos, cadusafos, and fenamiphos. These results propose useful information for the potential application of the DI101 strain in bioremediation of pesticide-contaminated environments.

Keywords: Biodegradation, diazinon, *Serratia marcescens*, biotic and environmental factors

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Pesticides are synthetic organic compounds and used for pest control. However, when pesticides are distributed in the environment, they become pollutants, with ecological effects that require remediation. Organophosphate pesticides constitute a group of widely used, very heterogeneous compounds that share a phosphoric acid derivative chemical structure. Diazinon (*O*,*O*-diethyl *O*-2-isopropyl-6-methylpyrimidin-4-yl phosphorothioate) is an organophosphate insecticide used throughout the world. Diazinon is a contact insecticide, which means it kills insects by inhibiting the action of enzymes in the nervous system. It is classified as slightly to moderately toxic, depending on the formulation [9]. Diazinon and other pesticides applied directly to the soil or grass can be washed off into nearby storm drains and ditches, which typically transport water to streams and lakes. The persistence of diazinon in soils affects how much of the chemical is available for transport to these surface-water bodies. In the soil, chemicals break down into other substances. The amount of time it takes for half the pesticide to break down is called the half-life of the chemical; the half-life of diazinon in soil is 39 days [1]. Most synthetic organophosphate pesticide compounds are highly toxic and are powerful inhibitors of acetylcholinesterase, a vital enzyme involved in neurotransmission, in the form of acetylcholine substitutes [2, 12]. Pesticides in soil and water can be degraded by biotic and abiotic pathways; however, biodegradation by microorganisms 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 [42]. Isolation of indigenous bacteria capable of metabolizing organophosphate pesticide compounds has received considerable attention, because these bacteria provide an environmentally friendly method of *in situ* detoxification [26, 34]. Biodegradation is a common method for the removal of organic pollutants because of its low cost and low collateral destruction of indigenous animal and plant organisms [24]. Degradation

products of diazinon include diazoxon, a toxic metabolite, and 2-isopropyl-6-methyl-4-hydroxypyimidine (IMHP or oxypyrimidine), a persistent, less toxic product [3, 20]. Microbial degradation is considered to be a major factor determining the fate of diazinon and other organophosphorus insecticides in the environment. Many authors indicated that the bacterial strains belonging to the different taxonomic groups have a great degradation potential of the organophosphorus insecticides and other pesticides [4, 6, 7, 11, 40]. Studies on microbial degradation are useful in the development of bioremediation strategies for the detoxification of these insecticides by microorganisms [29, 30].

The aims of the present work were to isolate and characterize diazinon-degrading bacteria from agricultural soils, characterize their degradation potential and their use in bioremediation of diazinon-contaminated soil, and study the environmental factors that affect diazinon biodegradation in soil. The ability of bacteria to utilize diazinon as a sole carbon source in liquid medium, as well as the effect of additional carbon on diazinon biodegradation, was investigated.

Materials and Methods

Chemicals

Analytical (technical)-grade diazinon (99% purity), *O,O*-diethyl *O*-2-isopropyl-6-methylpyrimidin-4-yl phosphorothioate, chlorpyrifos, coumaphos, parathion, ethoprophos (British Greyhound, Ltd., Birkenhead, UK), cadusafos (FMC, Philadelphia, PA, USA), isazofos, and fenamiphos (Promochem, Teddington, UK) were used in this study. All other chemicals and solvents were of commercially high purity.

Isolation and Identification of Diazinon-Degrading Bacteria

The mineral salts medium (MSM) was used for isolation of diazinon-degrading bacteria from soil collected from diverse fields at Taif Province, Saudi Arabia. The MSM consisted of 2.0 g of $(NH_4)_2SO_4$, 1.5 g of $Na_2HPO_4 \cdot 12H_2O$, 1.5 g of KH_2PO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $CaCl_2 \cdot 2H_2O$, and 0.001 g of $FeSO_4 \cdot 7H_2O$ per liter of distilled water. The pH was adjusted to 7, and the medium was autoclaved at 121°C for 20 min. After cooling, the medium was amended with diazinon as a carbon source at a final concentration of 50 mg/l in the form of an acetone solution and was shaken for a day to evaporate the solvent. Five g of soil sample was added to 50 ml of MSM amended with diazinon in 250-ml conical flasks. The conical flasks were incubated at 30°C on a rotary shaker at 120 rpm for 5 days in the dark to avoid photodegradation of diazinon. After incubation, 1 ml of soil suspension was transferred to conical flasks containing fresh MSM supplemented with 100 mg/l diazinon and incubated under the same conditions for a further 5 days. The above procedure was repeated twice.

Diazinon-degrading strains were isolated by plating 10-fold dilutions of the last culture fluids onto mineral salts agar (MSM agar) containing 100 mg/l diazinon as a carbon source and incubated at 30°C for 7 days. According to their morphological characteristics, four individual bacterial strains were selected as diazinon-degrading bacteria and subcultured to obtain pure culture.

These isolates were investigated for their oxidase and catalase activities, Gram stain, shape, and utilization of glucose, lactose, D-galactose, L-(+) arabinose, rhamnose, sucrose, D-sorbitol, and nitrate [14].

Molecular Characterization of Isolated Diazinon-Degrading Bacteria

16S rRNA gene amplification and restriction fragment length polymorphism (RFLP). A colony of each isolate was suspended in 100 µl of sterilized water and boiled for 5 min. One µl of the lysate was used as a template to amplify the 16S rRNA gene by PCR using the universal eubacterial primers P-forward (5'-AGAGTT TGATCCTGGCTCAG-3') and P-reverse (5'-ACGGCTACCTTGTT ACGACT-3'), which correspond to 8–27 bp and 1,495–1,514 bp of the *Escherichia coli* 16S rDNA (Accession No. E05133). PCR mixtures (100 µl) consisted of 10 µl of 10× *pfu* buffer, 5 U *pfu* DNA polymerase (Promega), 200 µM of each nucleotide (Promega), 50 pmol of each primer, and 1 µl of template, adjusted to 100 µl with sterilized ddH_2O. PCR amplification was programmed as follows: 3 min of denaturation at 95°C; followed by 30 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 90 s; with a final extension at 72°C for 10 min [37].

After amplification, 5 µl of each reaction mixture was run on 1% agarose gel to confirm the size and purity of PCR products. The DNA was then purified with a QIAquick PCR purification kit following the protocol provided by the supplier (Qiagen). Five µl of purified PCR product of each isolate was digested in 20-µl reaction mixtures with *Rsa*I (2 U). Digestion was carried out according to the instructions provided by the supplier (Promega), and the digestion products were analyzed on 1% agarose gel.

16S rRNA Gene Sequence Determination of Strain DI101 and Phylogenetic Analysis

The 16S rDNA sequence was determined from the purified PCR product with automated fluorescent Taq cycle sequencing (373A DNA sequencer; Applied Biosystems). The sequencing result was submitted to the GenBank database and a similarity search of the nucleotides done by BLAST. From the search results, the sequences within the genus *Serratia* were included in the subsequent phylogenetic analysis. The genus *Serratia* belongs to the family Enterobacteriaceae, and consequently some sequences from family Enterobacteriaceae were also involved. The sequences were aligned using the MacClade program. Sequences were compared using the packages DNADIST and Bootstrap, and distance trees generated using phylograms from within the PAUP Ver. 4.0b10 suite of packages [43]. Various trees were compared and produced essentially the same results. The 16S rRNA gene sequence of strain DI101 was deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases under the accession number AB571066.

Inoculum Preparation

The inocula for all of the experiments were prepared by growing bacteria in 50 ml of MSM amended with 100 mg/l diazinon at 30°C on a rotary shaker at 150 rpm overnight. Cultures were centrifuged at 12,000 rpm for 5 min at 4°C. Pelleted cells were washed twice with 25 ml of sterile 50 mM phosphate buffer (pH 7.2) and counted

by the dilution plate count technique. For all experiments throughout this study, 10^6 CFU/ml of liquid media or 10^6 CFU/g of soil sample were used, and samples were incubated at 30°C at 150 rpm unless otherwise stated. The cell density (OD) was measured at 600 nm by a spectrophotometer. All experiments were carried out in triplicate.

Degradation of Diazinon by Strain DI101 in MSM

Bacterial growth was carried out in 500-ml conical flasks containing 200 ml of MSM supplemented with 50 mg/1 diazinon. After a day of shaking to evaporate the solvent, the medium was inoculated with strain DI101 at a final concentration of 10^6 CFU/ml. Samples of MSM supplemented with diazinon and non-inoculated with the bacterial strain were kept as controls. Conical flasks were incubated at 30°C on a rotary shaker at 120 rpm in the dark to avoid photo-degradation of diazinon. Samples of liquid medium were taken aseptically at regular intervals for detection of growth (OD_{600}) and diazinon degradation.

Effects of Inoculum Density, pH, and Temperature on Diazinon Degradation

Inoculum densities of the strain DI101 in MSMN amended with 50 mg/1diazinon were prepared as described previously. A serial dilution in MSM was prepared and the inoculum levels of 10^6, 10^4, 10^2, and 50 CFU/ml (final concentrations) were used to inoculate 500-ml conical flasks containing 200 ml of MSM with diazinon. Non-inoculated controls were also included and all cultures were incubated as before and diazinons degradation was measured.

To investigate the effect of pH on diazinon degradation by strain DI101 at 30°C, the pH values in MSM were adjusted to 5.0, 6.0, 7.0, 7.5, 8.0, 9.0, and 10.0 with 0.2 M H_3PO_4 and 0.1 M NaOH solutions. To study the effect of temperature on diazinon degradation in MSM at pH 7.0 by strain DI101, five different incubation temperatures (10, 20, 25, 30, and 40°C) were used. The conical flasks containing 200 ml of MSM amended with 50 mg/l diazinon were inoculated with 10^6 CFU/ml of strain DI101 and incubated in a rotary shaker at 160 rpm in the dark. At regular intervals, samples were removed for analysis of diazinon degradation.

Biodegradation of Diazinon in Soil

To investigate biodegradation of diazinon in soil, different microbiologically active soils were used. The soil, from which the strain DI101 was isolated, was sterilized twice by autoclaving at 121°C for an hour. Samples (250 g) of the soil were placed in 500-ml conical flasks and treated aseptically with 100 mg/kg diazinon and mixed. After solvent evaporation, soil samples were inoculated with strain DI101 at 10^6 CFU/g and mixed aseptically. Samples of sterilized soil without inoculation, and non-sterilized soil with and without inoculation, were used as controls. Distilled water was added to adjust the soil moisture to 50% of its water holding capacity. The soil samples were incubated at 30°C in the dark. Water losses exceeding 5% of the initial values were compensated by the addition of sterile distilled water during the incubation period. At regular intervals, samples of 10 g soil treatments were taken aseptically for detection of diazinon degradation.

Effects of Carbon and Phosphorus Sources on Diazinon Biodegradation

To study the effects of other carbon sources on diazinon biodegradation, 200 ml of MSM containing 50 mg/1 diazinon in 500-ml conical flasks amended with 1% glucose or succinate were inoculated with 10^6 CFU/ml strain DI101. Samples of MSM containing diazinon and non-inoculated with strain DI101 were prepared as controls. Conical flasks were incubated at 30°C on a rotary shaker at 120 rpm in the dark. At regular intervals, samples were withdrawn aseptically for detection of diazinon degradation.

In order to investigate the effect of phosphorus on diazinon biodegradation, the MSM at pH 7 was prepared as described previously without two phosphorus sources, KH_2PO_4 and Na_2HPO_4, which were replaced by K_2SO_4 (1.04 g/1) and NaCl (1 g/1), respectively. Conical flasks containing 200 ml of MSM, or MSM minus phosphorus source amended with 50 mg/1 diazinon were inoculated and incubated as before. Non-inoculated controls were also prepared for each medium. Samples were taken as before for detection of diazinon degradation.

Enzymatic Activities of Strain DI101

To investigate the activity of phosphodiesterase and alkaline phosphatase of the isolate DI101, the rates of hydrolysis of *bis*-(*p*-nitrophenyl) phosphate and *p*-nitrophenyl phosphate were determined. Approximately one loop of the overnight culture of DI101 was suspended in 1 ml of 25 mM of either compound and incubated at 37°C for 30 min. One control inoculated with *E. coli* and another control without inoculation were also incubated for both compounds. To stop the reaction, 4 ml of 0.5 M NaOH and 1 ml of 0.5 M $CaCl_2$ were added. Formation of *p*-nitrophenol was detected by optical absorbance of the samples at 420 nm.

Effect of Diazinon Concentration on Diazinon Biodegradation

To examine the effect of diazinon concentration on degradation, the initial concentrations of diazinon in the MSM were set to 20, 50, 100, 120, and 160 mg/l. Conical flasks (500 ml) containing 200 ml of MSM supplemented with different concentration of diazinon were inoculated with strain DI101 and incubated as described above. At designated intervals, samples were withdrawn for the analysis of diazinon degradation.

Degradation of Other Organophosphorus Compounds by Strain DI101

Cross-feeding studies with other organophosphorus insecticides were also carried out. The MSM was supplemented with chlorpyrifos [*O*, *O*-diethyl *O*-(3,5,6-trichlor-2-pyridyl) phosphorothioate], coumaphos (*O*-3-chloro-4-methyl-2-oxo-2H-chromen-7-yl *O*,*O*-diethyl phosphorothioate), or parathion (*O*,*O*-diethyl *O*-4-nitrophenyl phosphorothioate). The pesticide degradation was measured by GC. Cross-feeding with organophosphorus nematicides, ethopropphos (*O*-ethyl *S*,*S*-dipropyl phosphorodithioate), cadusofos (*S*,*S*-di-sec *O*-ethyl-phosphorodithioate), fenamiphos (ethyl 4-methylthio-*m*-tolyl isopropylphosphoramidate), and isazofos (*O*-5-chloro-1-isopropyl-1H-1,2,4-triazol-3-yl *O*,*O*-diethyl phosphorothioate) was also performed. Organophosphorus compounds were added to MSM as a sole source of carbon and energy and incubated at 30°C, pH 7.0, at an intial concentration of 50 mg/l. Samples were collected and analyzed after 11 days of incubation.

Gas Chromatography Analysis

Gas chromatography (GC) was used for detection of concentrations of insecticide. To extract insecticides from MSM, 10 ml of liquid samples were made up to a volume of 50 ml with deionized water and extracted twice with 20 ml of hexane on a rotary shaker for

30 min. To extract insecticide from soil, 10-g soil samples were extracted with 20 ml of hexane on a rotary shaker for an hour. After that, the extracts were dehydrated with anhydrous Na_2SO_4 and evaporated to dryness under a stream of N_2 at 45°C using a rotary evaporator, and subsequently diluted to a final volume of 10 ml with acetone and kept for chromatographic analysis. Analyses were carried out as described previously [7].

Data Analysis

Because the diazinon degradation by the strain DI101 during time was a first-order reaction, the degradation rate of diazinon was calculated by plotting ln C (concentration of diazinon) against the time. The slope of the linear regression line indicates the degradation rate. The results were evaluated by analysis of variance and statistical analyses were carried out on triplicates of data obtained from each treatment. The significance ($p<0.05$) of differences was treated statistically by one-way ANOVA and assessed by post hoc comparison of means using lowest significant differences (LSD) using the programme SPSS 11.0.

Results and Discussion

Isolation and Characterization of Diazinon-Degrading Bacteria

Four isolates able to use diazinon as a source of carbon and energy were obtained from soil samples collected from different sites by using an enrichment culture technique. All four isolates were Gram-negative, rod shaped, oxidase-negative, and producing prodigiosin. Moreover, these isolates utilized glucose, lactose, D-galactose, L-(+) arabinose, rhamnose, sucrose, and D-sorbitol, and also reduced nitrate to nitrite. PCR-amplified products (~1,534 bp) corresponding to the 16S rRNA gene were obtained specifically from all strains. The PCR products of the 16S rRNA gene were digested with *Rsa*I, and all four isolates produced identical RFLP profiles. Each PCR product had four DNA fragments of sizes ~884 bp, ~400 bp, ~148 bp, and ~102 bp. These results indicate that all the isolates belonged to the same bacterial strain. Therefore, further analysis was done with only one isolate, designated strain DI101. The 16S rRNA sequence (~1,534 bp) of strain DI101 was determined and a similarity search done by BLAST. It showed similarity to 16S rRNA sequences from members of the order *Enterobacteriales*. Moreover, the results indicated that the greatest similarity was seen for members of the *Serratia* group within the RDP database. A dendrogram illustrating the relationship between strain DI101 and other selected bacteria is presented in Fig. 1. As illustrated, the 16S rDNA sequence of strain DI101 is most closely related to *S. marcescens*, and showed high similarity with other strains in the same genera. To determine the confidence

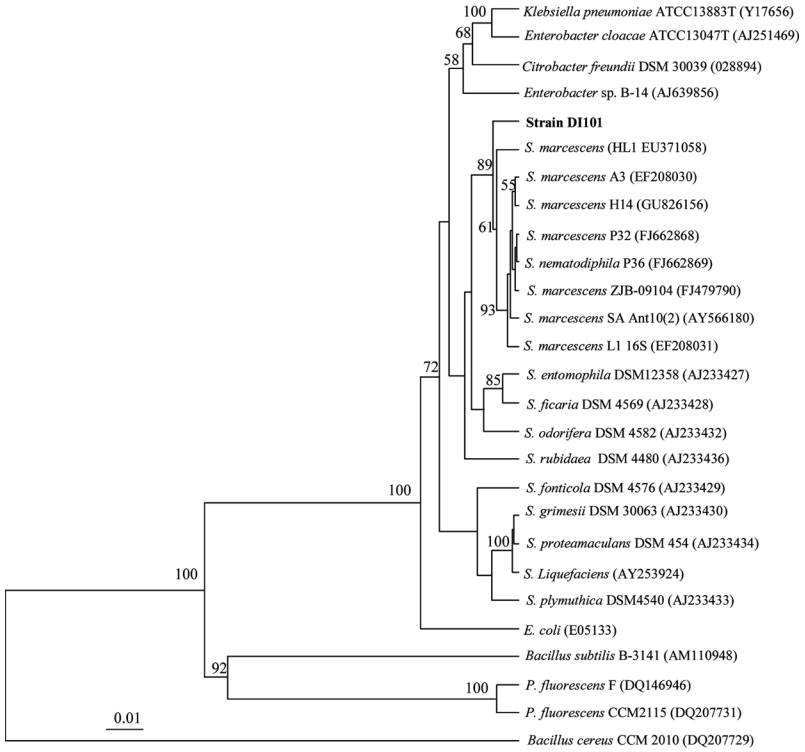

Fig. 1. Phylogenetic tree illustrating the similarity of the diazinon-degrading strain DI101 16S rRNA gene to that of other bacteria in the same family.

The tree was generated by using the neighbor-joining method, and genetics distances were computed by the Jukes-Cantor model. A genetic distance bar is illustrated. The number shown next to each node indicates the percentage bootstrap value of 100 replicates (only values of 50 and above are indicated). The sequence from *Bacillus cereus* was treated as the outgroup.

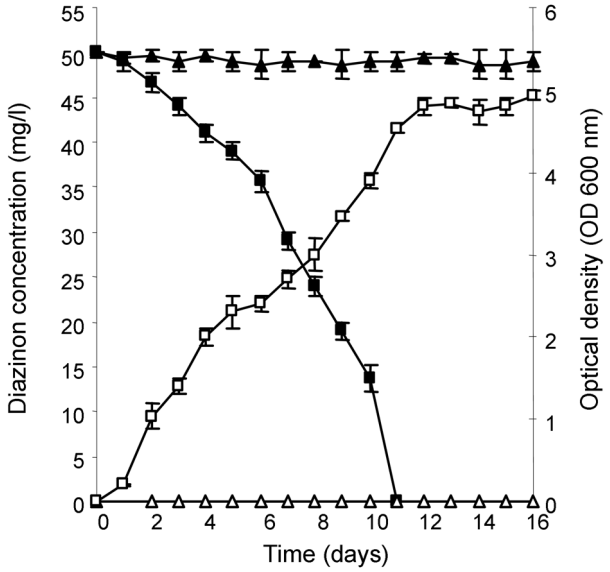

Fig. 2. Growth of bacterial isolate (DI101) and degradation of diazinon.

Bacterial growth in MSM supplemented with diazinon inoculated with bacterial isolate (□) and non-inoculated medium (△). Also shown, degradation of diazinon in the medium inoculated with bacterial isolate (■) and non-inoculated medium (▲). Each value is the mean of three replicates and error bars represent the SD of the mean, which was within 5% of the mean.

values of branches, 100 bootstrap replications were carried out for each generated tree. Various phylogenetic trees obtained showed that strain DI101 branched deeply with the *Serratia* cluster consisting of *S. marcescens*. From the above results, the isolated diazinon-degrading bacterium was identified as *S. marcescens* DI101. It is well known that *Pseudomonas* species, known as a very metabolically active bacteria, able to use many synthetic organic compounds, were isolated from different soils contaminated with diazinon and other organophosphorus pesticides [7, 11, 22, 29, 41, 44], whereas *Serratia* sp. seems to be a new bacterium that may contribute in complete degradation of diazinon. There is little information concerning the ability of bacteria belonging to *Serratia* spp. to utilize diazinon. Nevertheless, some recent studies reported the ability of *Serratia* to complete the degradation of other organophosphorus insecticides [7, 22, 33].

Bacterial Growth and Biodegradation of Diazinon

The degradation kinetics of diazinon in MSM and growth of strain DI101 monitored by optical density at 600 nm (OD_{600}) are shown in Fig. 2. The growth of strain DI101 in MSM supplemented with diazinon as a carbon source was the most effective within 11 days of incubation. The growth curve of the DI101 reached a maximum OD_{600} after 12 days. On the other hand, non-inoculated control exhibited no change in growth throughout the experiment time. The growth of strain DI101 in MSM amended with diazinon reflected the degradation kinetics of diazinon. The results showed that the DI101 degraded diazinon completely at day 11. Moreover, the degradation process of diazinon by strain DI101 was characterized by a degradation rate of 0.227 day^{-1} while in the absence of the strain was 0.0015 day^{-1}. The degradation rate of diazinon in MSM inoculated with the strain was significantly different as compared with the same medium without the strain ($F=8842.2$, $p<0.001$). Previous results have been reported that several bacterial species such as *Pseudomonas* sp. [32], *Agrobacterium* sp. [11, 44], *Arthrobacter* sp. [28], and *Flavobacterium* sp. [11] can utilize diazinon as a source of carbon. Moreover, the

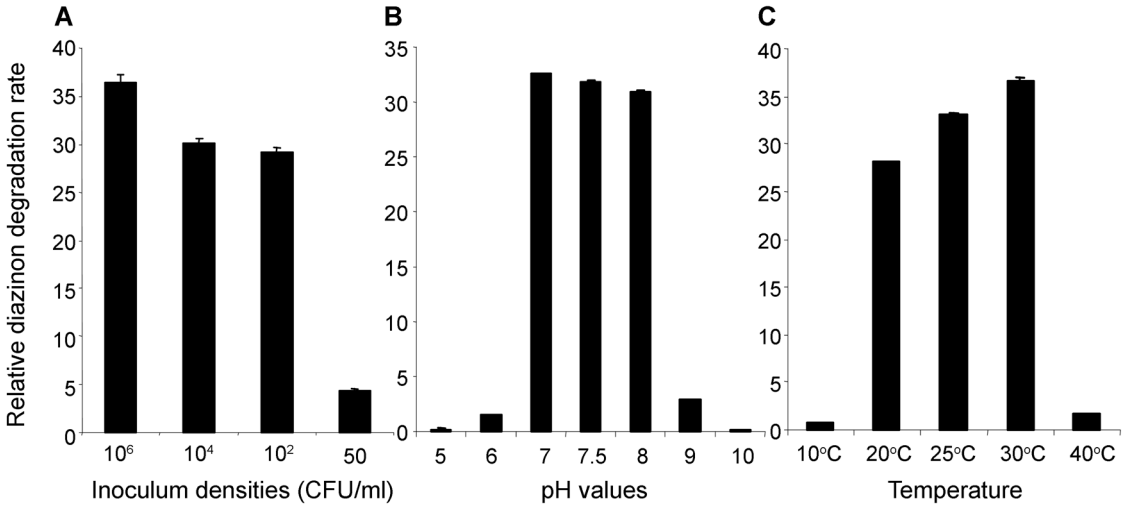

Fig. 3. Relative diazinon degradation rates in MSM inoculated with strain DI101 at various inoculum densities (**A**), pHs (**B**), and temperatures (**C**).

Data represent means of three independent measurements and error bars represent the SD of the mean, which was within 5% of the mean.

same bacterial species may contribute in biotransformation of other organophosphorus insecticides [7, 13, 19, 22, 29].

Effects of Inoculum Density, pH, and Temperature on Diazinon Degradation

The effect of the inoculum density of the strain on the degradation of diazinon is illustrated in Fig. 3A. The data obtained from the present study on degradation kinetics indicated that there were significant differences ($F= 1985.4$, $p<0.001$) in the relative degradation rates of diazinon between inoculum densities. At the high inoculum density (10^6 CFU/ml), diazinon was degraded completely on day 11, with a relative degradation rate of 36.379 day^{-1}. At moderate inoculum densities of 10^4 and 10^2 CFU/ml, degradation of diazinon was complete on days 13 and 16, respectively, having relative degradation rates of 30.107 and 29.150 day^{-1}, respectively. However, the relative degradation rate (4.364 day^{-1}) was slower at lower density (50 CFU/ml); that is only 37% of diazinon degradation on day 16 was detected. No apparent diazinon removal was detected in the control cultures.

The changes in relative degradation rates of diazinon by strain DI101 in the presence of different pHs were significant ($F=39165.8$, $p<0.001$). Diazinon was completely degraded by strain DI101 on day 11 with relative degradation rates of 32.623, 31.822, and 30.860 day^{-1} when pH values were 7, 7.5, and 8, respectively (Fig. 3B). However, the relative degradation rates of diazinon were very slow (1.470 and 2.853 day^{-1}) at pH 6 and pH 9, respectively. Degradation of diazinon was negligible at pH 5 and pH 10 as well as in the control. Diazinon biodegradation was approximately inhibited in relatively acidic (pH 5) and alkaline (pH 10) conditions. These data indicated that strain DI101 was adaptable to neutral or slightly alkali media.

The effect of temperature on diazinon degradation by strain DI101 is shown in Fig. 3C: at the different temperatures examined, there were significant differences ($F=23470.89$, $p<0.001$) between the relative degradation rates of diazinon. The most rapid degradation of diazinon by strain DI101 was recorded at 20°C, 25°C, and 30°C, with relative degradation rates of 28.082, 32.998, and 36.572 day^{-1}, respectively. On the other hand, the slowest degradation was determined at the two extreme temperatures (10 and 40°C) with relative degradation rates of 0.724 and 1.624 day^{-1}, respectively. Complete diazinon removal was observed on day 11 when incubated at 25°C and 30°C, but on day 13 at 20°C. Degradation of diazinon was negligible in the control. Therefore, the optimal incubation temperature for DI101 cells to degrade diazinon was between 25°C and 30°C. These results were expected, since most members of the family Enterobacteriaceae grow well at 25–37°C. Similar results reported that the ranges of pH value and temperature for satisfactory growth of *Serratia marcescens* strain HL1 cells and hexachlorobutadiene degradation were from 7.0 to 8.0, and 25 to 30°C, respectively [23]. Similar results reported that the addition of *Burkholderia* sp. FDS-1 at 2×10^6 CFU/g soil was suitable for fenitrothion degradation at a slightly alkaline pH (7.5) and over a temperature range of 20–40°C [15]. Moreover, *Pseudomonas putida* epI with inoculum density of 10^4 CFU/g soil was able to rapidly degrade ethoprophos at 20 and 35°C within 16 days at pH 5.4 [18]. A genetically modified bacterium (carbofuran-degrading *Sphingomonas* sp. CDS-1) could degrade methyl parathion and carbofuran efficiently in a relatively broad range of pH values from 6.0 to 9.0, temperatures from 20 to 30°C, and with all inoculum densities (10^5–10^7 CFU/ml). The optimal temperature and pH value for this bacterium to simultaneously degrade methyl parathion and carbofuran was at 30°C and pH 7.0 [17]. The previous results indicated that the degradation of diazinon was affected by the bacterial inoculum ($p<0.001$), pH ($p<0.001$), and temperature ($p<0.001$).

Biodegradation of Diazinon in Soil

Diazinon degradation in different microbiologically active soils is presented in Fig. 4. The degradation rates of diazinon in inoculated sterilized and nonsterilized soils were significantly different ($F=1456.7$, $p<0.001$) as compared with non-inoculated sterilized and nonsterilized soils. Daizinon was completely degraded by DI101 within 14 and 16 days in sterilized and nonsterilized soil samples, with degradation rates of 0.275 and 0.238 day^{-1}, respectively.

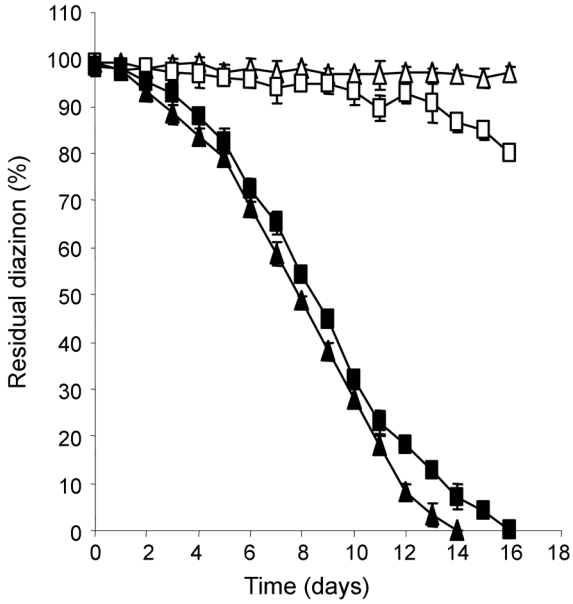

Fig. 4. Degradation of diazinon in sterilized soil inoculated with strain DI101 (▲) and without bacteria inoculum (△) and in non-sterilized soil inoculated with the DI101 (■) and without bacteria inoculum (□).
Each value is the mean of three replicates and error bars represent the SD of the mean, which was within 5% of the mean.

The biodegradation of diazinon in nonsterilized soil was characterized by a longer lag phase than in sterilized soil. This lag phase noticed in this study and other studies involves the time required for propagation of a small population of pesticide-degrading microorganisms to reach the essential level for efficient degradation of pesticide [19]. In addition, diazinon was applied to nonsterilized soil at high concentration (100 mg/kg soil), which might cause the initial inhibition of original microflora activity. In contrast, this effect was not observed in sterilized soil samples inoculated with the DI101. The results also revealed that a significant amount of diazinon (about 98–80% of the initial dose) was present in noninoculated sterilized and non sterilized soil samples, respectively, within 16 h. Degradation of diazinon in noninoculated sterilized and non sterilized soils was characterized by degradation rates of 0.0017 and 0.010 day^{-1}, respectively. In the present study, 10^6 CFU g of DI101 was used to inoculate soils, and this inoculum density appeared to be able to degrade diazinon completely. As revealed in other reports, inoculum density is a significant factor determining the effective biodegradation of applied pesticides. Lower inoculation densities of strain *Sphingomonas chlorophenolica* RA2 (10^6 and 10^4 CFU/g soil) resulted in strongly reduced mineralization rates of pentachlorophenol, with 10^4 CFU/g soil not significantly differing from the control [25]. The introduction of 10^4 CFU/g soil of *Chelatobacter heintzii* Cit1 resulted in a 3-fold increase of atrazine mineralization capacity [36]. The addition of *Enterobacter asburiae* B-14 (10^6 CFU/ g) to soil with a low indigenous population of chlorpyrifos-degrading bacteria supplemented with 35 mg/kg of chlorpyrifos resulted in a higher degradation rate than was observed in non-inoculated soils [40]. In addition, Ramadan *et al*. [31] reported that when lower inoculum densities (<10^4 CFU/g soil) were applied, only a small part of introduced bacteria could survive the initial competition and contribute in pesticide degradation. *Enterobacter* sp. did not degrade chlorpyrifos when introduced into soil below an inoculum density of 10^3 CFU/g soil [39]. A higher initial inoculum density can compensate for the initial population decline, and the survivors can multiply and degrade pollutants [5, 8]. Moreover, these results supported the view that particular species of soil microorganisms fluctuate in their general activity responding to degradation of pesticides. It is very difficult to precisely determine the part of particular microorganisms in pesticide destruction, since there are many factors that have an effect on this process. Moreover, microorganism activity may be specific to chemical structure, chemical binding, or a group of selected substances [10]. In this study, the bacterial systems of the DI101 successfully degraded diazinon in sterilized and nonsterilized soils, suggesting that these bacterial systems can compete with and live with the indigenous microorganisms.

Effects of Carbon and Phosphorus Sources on Biodegradation

The relative degradation rate patterns of diazinon in MSM or MSM supplemented with succinate or glucose are presented in Fig. 5. Diazinon was completely degraded by strain DI101 on days 11 and 15, with relative degradation rates of 40.724 and 29.391 day^{-1} in MSM and MSM supplemented with carbon source, respectively. The degradation pattern of the strain was greatly affected by the presence of other carbon sources. There was almost no degradation of diazinon through the first 6 days in the presence of succinate or glucose. However, after 7 days, diazinon was degraded rapidly in these two modified media. The relative degradation rates of diazinon in MSM without addition of carbon source was significantly different ($F=2879.9$, $p<0.001$) as compared with MSM supplemented with carbon sources.

Degradation rates of diazinon in MSM supplemented with phosphorus and MSM without phosphorus source were not significant ($F=35$, $p<0.005$). Diazinon in both media was completely degraded by the strain DI101 on day 11, with degradation rates of 0.241 day^{-1} for MSM with phosphorus and 0.221 day^{-1} for MSM without phosphorus. Therefore, the degradation of diazinon was quite similar in both media and was not affected by the absence or presence of phosphorus source. Moreover, enzymatic assays of the strain DI101 gave positive results for both phosphodiesterase and alkaline phosphatase activities. The utilization of organophosphorus insecticides as a source of phosphorus by DI101 is a significant observation. There are few reports in which an organophosphorus compound was used as a source of carbon and phosphorus by a single species such as *Enterobacter* strain B-14, which could use organophosphorus chlorpyrifos as a source of carbon and phosphorus [30]. However, *Flavobacterium* species could

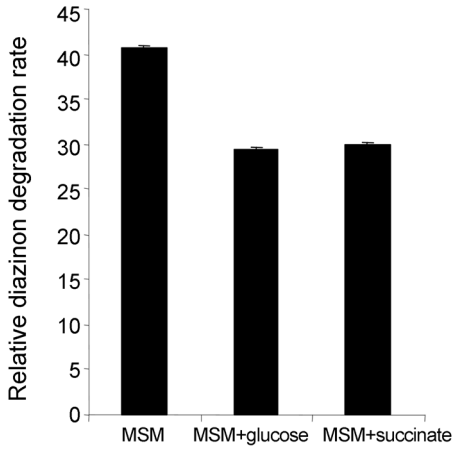

Fig. 5. Relative degradation rate of diazinon by strain DI101 in MSM and MSM supplemented with glucose or succinate.
Data represent means of three independent measurements, and error bars represent the SD of the mean, which was within 5% of the mean.

use parathion as a source of phosphorus but not carbon and diazinon as a carbon source. Nevertheless, a *Flavobacterium* strain was not able to utilize other organophosphorus pesticides either as a source of carbon or phosphorus [38]. Moreover, a variety of bacteria that could utilize phosphorothionate or phosphorodithionate compounds as a source of phosphorus were unable to use these compounds as a carbon source [35]. The conditions under which the environmental bacteria are isolated are crucial in selecting for strains not only with the desired degradative enzyme systems, but also with specific regulation mechanisms for the degradation pathways [21]. However, the utilization of diazinon as a source of phosphorus attributed to the presence of phosphodiesterase and phosphomonoesterase activities in the DI101 strain. Phosphotriesterase degrades the triester bond of organophosphorus compounds, and phosphodiesterase and monoesterase are essential to make the phosphorus atom available for uptake as a source of phosphorus (inorganic form) and to release ethanol for utilization as a carbon source. Moreover, previous reports suggested that organophosphorus-degrading bacteria mainly degrade these compounds co-metabolically [6, 16]. Some species of isolated bacteria can utilize organophosphates as a source of carbon or phosphorus from the hydrolysis products [27, 35]. The strain isolated in this study had phosphotriesterase activity and hydrolyzed 50 mg/l concentrations of diazinon within 11 days when inoculated with 10^6 CFU/ml. However, the addition of other carbon sources such succinate or glucose stopped the degradation of diazinon. When these carbon sources were exhausted, it then degraded diazinon as a source of carbon, suggesting the environmental adaptation of this strain. In the natural environment, the competition for carbon sources is massive and the utilization of diazinon as an energy source by this strain provides it with a significant competitive advantage over other indigenous microorganisms.

Effect of Diazinon Concentration

As presented in Fig. 6, the strain DI101 was able to degrade 20 mg/l diazinon completely on days 9, with a degradation rate of 0.291767 day^{-1}, and of concentrations 50 and 100 mg/l on days 11, with degradation rates of 0.254 and 0.254 day^{-1}, respectively. However, when the concentrations of diazinon reached 120 and 160 mg/l, the strain was able to degrade only 70% and 40%, respectively, of diazinon on day 16, with degradation rates of 0.079 and 0.035 day^{-1}, respectively. These results indicated that the concentration of diazinon had a considerable effect on degradation rate ($F=732.6$, $P<0.001$). This is the first report for isolation of soil bacteria with the capability to degrade diazinon completely in such time. Previous results reported that the isolates *S. liquefaciens*, *S. marcescens*, *Pseudomonas* sp., and their consortium could degrade 80–92% of diazinon within 14 days, when diazinon was added to MSM at 50 mg/l final concentration [7].

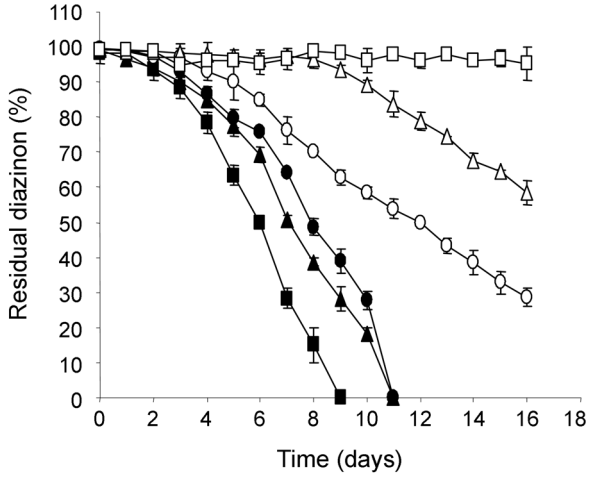

Fig. 6. Diazinon degradation in MSM inoculated with strain DI101 at various initial concentrations of diazinon: 20 mg/l (■), 50 mg/l (▲), 100 mg/l (●), 120 mg/l (○), and 160 mg/l (△). Degradation in non-inoculated sample (□) is also represented. Data represent means of three independent measurements, and error bars represent the SD of the mean, which was within 5% of the mean.

The relationship between the degradation rate of diazinon and concentration of substrate (diazinon) was analyzed by the Michaelis–Menton kinetic equation: $v=V_{max}(S)/K_s+(S)$ where S=diazinon concentration (mg/l), K_{max}=maximum degradation rate of diazinon (day^{-1}), and K_s=diazinon concentration (mg/l) at half the maximum degradation rate. At low diazinon concentrations (less than 20 mg/l), the degradation followed first-order reaction, in which the degradation rate increases with the increase of diazinon concentration. However, at high diazinon concentrations (20–100 mg/l), the degradation rates followed zero-order reaction and approached a maximum value that was independent of diazinon concentration. At higher diazinon concentrations (more than 100 mg/l), the degradation rate became inhibited. The kinetic values were K_{max}=0.291767 day^{-1} and K_s= 11 mg/l.

Biodegradation of Other Organophosphorus Compounds

Strain DI101 had the ability to degrade the diethylthiophosphate-containing organophosphates chlorpyrifos, coumaphos, parathion, and isazofos, when provided as a source of carbon and phosphorus, but not ethoprophos, cadusafos, and fenamiphos, which have different side chains (Table 1). All compounds were hydrolyzed at a phosphoester bond. Because of its wide specificity against a range of organophosphorus compounds, the strain DI101 has a great potential to give an adaptable enzyme system that may be utilized in the remediation of highly toxic organophosphate nerve agents.

Table 1. Degradation of other organophosphorus compounds by strain DI101.

Organophosphorus compounds	Degradation (%)[a]
Chlorpyrifos	91
Coumaphos	89
Parathion	85
Isazofos	87
Ethoprophos	ND[b]
Cadusafos	ND
Fenamiphos	ND

[a]Degradation compared with controls without the strain DI101.
[b]ND: Not degraded. Data are means of triplicate experiments.

An enrichment technique was used to isolate a bacterial strain belonging to *Serratia* genus that may contribute in efficient degradation of the organophosphorus insecticide diazinon. Results of the degradation kinetics for diazinon in sterilized and nonsterilized soils by the strain indicated that this strain has bacterial systems that can compete with and live with the indigenous microorganisms. The strain could utilize diazinon as a source of carbon and phosphorus at pH from 7.0 to 8.0 and at temperature from 25 to 30°C. The strain degraded the diethylthiophosphate-containing organophosphates chlorpyrifos, coumaphos, parathion, and isazofos when provided as the sole source of carbon and phosphorus, but not ethoprophos, cadusafos, and fenamiphos.

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