

Factors Affecting Biodegradation of the Organophosphorus Insecticide Diazinon by Bacterial Mono-Culture of *Bacillus Sefensis* 7, Isolated From the Rhizosphere of Date Palm Tree

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Abstract: Due to widespread use pesticides for crop protection, they are considered as contaminants in the environmental matrices such soil which are exposed to large quantities. Three isolates from soil samples using enrichment culture technique have been isolated and grown in the minimal growth medium where Diazinon served as a sole carbon source (60 mg/l). Total three bacterial strains were screened for Diazinon degradation. The most active one was isolate BMNF7 (33% degradation). Lower degradation percentages were recorded for the two other isolates, BMRF3 and BMTF 8 (21-30%). The isolate BMNF7 was identified by morphological and biochemical studies as *Bacillus* sp. and identification was confirmed using 16srRNA. It was identified as *Bacillus sefensis* 7 and this isolate was able to degrade up to 63% of Diazinon (60 mg/l) in mineral salt medium (MSM) as a sole carbon source within 10 days of incubation. The bacterial growth and Diazinon degradation were accelerated when MSM supplemented with 0.75 (g/l) yeast extract and 0.5 g/l glucose as an additional carbon and nitrogen sources. The maximum degradation was obtained at pH 7 and 45°C after 10 days of incubation and using 4×10^6 CFU/ml as inoculum size. It was noticed that the best growth temperature was 37°C while the maximum degradation was at 45°C, meaning that high temperature increased degradation process. In conclusion the bacterial strains isolated from the agricultural soil, especially *Bacillus* species showed the ability to degrade Diazinon insecticide and optimization of growth conditions enhanced the percentage of degradation.

Keywords: *Bacillus*, Diazinon, Insecticide, Degradation, 16srRNA.

I. Introduction

Although, *Phoenix dactylifera* L., Date palm, is one of the oldest trees in the world, the effective insect weevil or Oliver (*Rhynchophorus ferrugineus*) destroys the tissues of the trunk, leading to the falling of the tree (Kamil and Najj, 2012). Organophosphate (OP) pesticides specially Diazinon is used worldwide to control this insect through the inhibition of acetyl cholin esterase (Theriot and Grunden, 2011, Singh and Walker, 2006). Diazinon which is widely employed in agriculture (Khani and kafilzadeh, 2014) is highly toxic to man and animals and the residues found in the environment has many health hazards (Chapalamadugu and Chaudhry, 1992), thus its biodegradation by bacteria is very necessary (Ghosh *et al.*, 2010). The non-systemic insecticide Diazinon is synthetic, colorless to dark brown liquid with average half life of 30 days when applied to soil and with chemical structure of O, O- diethyl 0- 2- isopropyl- 4-methyl -6- pyrimidyl phosphorothioate (Mallick *et al.*, 1999, Khani and kafilzadeh, 2014). Diazinon is an organophosphorous compound very effective for the control of ecto-parasites. Biodegradation of insecticides in soil differed with their chemical properties, type of soil and microorganism activities (Kenneth, 1990). Application of pesticides to soil cause changes in the soil microflora, decreased soil fertility, crop production, N₂fixation by soil microorganisms, symbioses in plants, nodulation in legumes and growth of cellulolytic, phosphate solubilizing and nitrifying bacteria (Jarallah *et al.*, 2016). Complete degradation of a parent compound by specific enzymes provides both carbon and energy for the growth and reproduction of microbes (Singh, 2008).

The diversity of bacterial communities associated with the date palm root system was investigated through the analysis of the diversity of the 16S rRNA gene in the rhizosphere and root surrounding soil fractions. It was found that the south region (closest to desert) presents harsher conditions that select a more restricted type of bacteria (Ferjani *et al.*, 2015). These ecological islands represent specific cluster of biological diversity that may contribute to the overall regional bacterial community functionality and furthermore increase the level of resilience to environmental change of the entire system (Anderson *et al.*, 2008). Therefore, the aim of this study was isolation and characterization of indigenous bacteria capable of metabolizing Diazinon as a sole energy and carbon sources and to fulfill this aim, a soil samples were collected from Diazinon - contaminated soil of the rhizosphere of date palm root system. Factors affecting degradation were also studied.

II. Material and methods

Collection of soil sample

Contaminated soil samples were collected from different date palm farms in western region, Saudi Arabia, in sterilized screw-capped test tubes for bacterial isolation. The samples were transferred to the laboratory and 1.0 g of each soil sample was suspended in a 250 ml conical flask containing 50 ml mineral salts (MS) medium enriched with Diazinon (0.1 % v/v) as a sole carbon source. The composition mineral salts medium was (g/l): 1 g (NH₄)₂SO₄, 0.8 g K₂HPO₄, 0.2 g KH₂PO₄, 0.2 g MgSO₄·7H₂O, 0.1 g CaCl₂·2H₂O, and 5 mg FeSO₄·7H₂O. The medium pH was adjusted to pH 7 (Wu *et al.*, 2013). Flasks were placed in a shaking-incubator at 100rpm for 10 days. To ensure the ability of Diazinon degradation by bacteria, subsequent transfer of inoculum (3%) into a fresh MS medium containing increasing concentration of Diazinon was carried out. The used concentration of Diazinon was 0.1-6.0 % where very small colonies were obtained.

Isolation, purification and preservation of the strain

Aliquots (100 µl) from the Diazinon-enriched medium were streaked on both MS agar containing the tested concentration of Diazinon and Nutrient agar plates. All plates were incubated at 45°C for 7 days. Single colonies were picked up and subcultured into fresh MS agar amended with Diazinon until pure colonies were obtained. All pure colonies were preserved on Nutrient agar slants at 4°C or in 10 % sterile glycerol at 70°C.

Measuring Diazinon degradation on solid medium

Each selected bacterial culture was inoculated in the central of agar plate containing MS agar medium with 5% v/v Diazinon which equal to 50 mg/l. All plates were incubated at 45°C for 10 days. All plates were examined and clear zone diameter (cm) was measured three times following by mean value calculation.

Morphology of colony and cells

The morphological characteristics of the colony; pigmentation, diameter, elevation and transparency was determined visually of 24 hrs old colony growing on Nutrient agar medium and incubated at 45°C. Cell shape, arrangement and reaction to the gram staining were assessed.

Biochemical characterization using the API50Ch strip kit

In order to investigate the biochemical characteristics of the strain API50Ch strip kit (Biomerieux, France) was used following the guidelines of the manufacturer. Results were recorded after 48hrs of incubation of API50Ch.

Identification of the strain using Vitek compact II

The bacterial strain DDK6 was identified using the automated system for identification of bacterial strains, Vitek compact II. Gram-Positive Card was used and the data analysis was carried out using the Software version: 05.02 (Biomerieux, Mary L'Etoile, France).

Identification of the isolates using 16S rRNA gene sequencing

16S rRNA gene was amplified using the universal primers 27F 5'-AGAGTTTGATCMTGGCTCAG-3' and 5'-TACGGYTACCTTGTTACGACTT-3' (Weisburg *et al.*, 1991), in 20 µl total PCR reaction. Genomic DNA was extracted from the strain using the InstaGene Matrix (Bio-Rad, USA) following the instructions of the manufacturer. PCR conditions were adjusted as previously described (Khalifa *et al.*, 2015). For sequencing of the 16S rRNA gene, the Big Dye terminator cycle sequencing kit (Applied Biosystems, USA) was used. Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied Biosystems, USA) as described in Chun *et al.*, 2007, Tamura and Nei, 1993, Tamura *et al.*, 2011).

Measuring bacterial growth

Bacterial growth in MS broth medium was determined by measuring the Absorbance at 550 nm. Moreover, after collecting the bacterial growth and centrifugation at 10,000 rpm, cell packed volume (CPV)/l were determined (George, 1990, Agwa *et al.*, 2000)

Sample preparation and Diazinon detection using HPLC

The liquid chromatographic system (Waters 2690 separation module) with a column (2 cm × 4.0 mm, 5 µm) and a reversed-phase µBondapak 125A C18 column (10 µm, 3.9 × 300 mm) were used (Product Number: WAT027324). µBondapak C18 columns are general purpose, silica-based, reversed-phase C18 columns that are based on 10 µm particle technology. Samples were acidified with 1M acetic acid (pH 5.0). Disposable C18 Sep-Pak cartridges were conditioned with 3 ml of acetonitrile then equilibrated using 3 ml of water prior to use. After vortex for 30 sec, the sample was centrifuged for 5 min at 1000 rpm and the supernatant was loaded into the disposable cartridges for elimination of waste, then washed with 2 ml of water, eluted twice by 2 ml of methanol in a marked small test tube, and reduced to 500 µl using a gentle stream of nitrogen prior to analysis by HPLC. The chromatographic conditions applied were as the following: A 10-µl solution was injected into

the HPLC and the mobile phase was a water–acetonitrile gradient at a flow rate of 1 to 1.5 ml/min. The water was adjusted to pH 3.0 using 1M acetic acid. The gradient started at 1% acetonitrile, increased to 55% acetonitrile at the 6-min mark, and then increased to 80% acetonitrile at 11 min. The system then returned to 1% acetonitrile at 13 min and was kept under this condition for 3 min in order to re-equilibrate. The eluents were monitored by the UV detection of the wavelengths 254 nm. The chromatographic analysis was performed at ambient temperature. Calibration procedures of a Diazinon were prepared in acetonitrile. Their concentrations ranged from 200 to 2000 ng/ml. Linear calibration curve was obtained by plotting the peak areas of the individual compounds as a function of the concentration. The standard curves were used to determine Diazinon, limits of detection (LODs) and limits of quantization were determined at the lowest concentration to be detected and quantified of Diazinon, taking into consideration a 1:3 and 1:10 ratio of the baseline noise and calibration point, respectively. The results were repeated five times for confirmation.

III. Results

The collected soil samples were from 8 date farms, located in western region, Saudi Arabia. About 1.0 g of each soil was used to isolate bacteria in minimal salt broth medium containing Diazinon 0.1% v/v (1.0 ml/l of standard solution). After growth for 10 days, the cells were transferred to new medium containing increasing concentration of Diazinon. After each transfer bacterial growth was checked on NA medium. After increasing concentration of Diazinon, number of bacterial isolates was decreased up to 3 isolates at 50 ml/l equal to 50 mg/l Diazinon. Finally by visual observation, among the well and faster growing bacterial isolates three were selected and designated as (BMR3), (BMN7) and (BMT8). These isolates were picked at 45°C and 50ml/l Diazinon. These isolates were preserved on agar slants at 4°C or in 10% sterile glycerol until used for further details studies.

All bacterial isolates were sub-cultured on both Diazinon mineral salt agar and broth media containing the same concentration of Diazinon and all plate and inoculated flasks were incubated 45°C and the growth, clearance zones of Diazinon degradation and % of degradation were measured for each isolate. The diameter of clear zones were ranged from 4.1-7.0 cm and maximum diameter was for the isolate BMNF7 (Table 1). The % of degradation was ranged from 21-33%. BMNF7 was selected for detail studies and its cultural characteristics were determined. It formed a yellowish white, convex colony, with a diameter of 2 to 3 mm, and entire margin. Cells were rod shape with a terminal endospores and exhibited positive reaction to the Gram staining (Fig. 1). These features were typical to the type strains of the genus *Bacillus*. For confirming the identity biochemical characterization using API50Ch strip kit containing 50 biochemical tests designated to estimate the carbohydrate metabolism ability of a microorganism was carried out. The system is exploited in the current study as a fast, effective a reliable tool to study the metabolic versatility of the strain BMNF7 towards different sources of carbohydrate. It exhibited the ability to utilize a wide range of different carbon including monosaccharides (e.g., D-galactose and L-arabinose) disaccharides (e.g., D-maltose and D-lactose), polysaccharides (e.g., Starch and Glycogen) and sugar alcohols (e.g., D-galactose, D-mannitol). Out of 49 used sugars, it utilizes 35 (~75 %) different carbon source while about 25 % of the carbon compounds were not utilized. The biochemical profile of the BMNF7 using API 50CH kit strip is typical to that of the genus *Bacillus*. Vitek II is a powerful and accurate automated platform with an expanded identification database for rapid microbial identification, and antibiotic susceptibility testing based on biochemical analysis using colorimetric method. The bacterial BMNF7 was identified as *Bacillus* by the Vitek compact II. This indicated that this tool is efficient for bacterial identification at the genus and specific levels. The 16S rRNA gene sequencing is a well-established technique for a robust and accurate bacterial identification and for inferring phylogenetic relationships among species. Comparative analyses of the 16S rRNA gene sequencing revealed that the strain BMNF7 was related to the genus *Bacillus*. Sequence analysis showed that BMNF7 was most closely related to *Bacillus pumilus* (95 % identity), at similarity levels of gene sequences. As can be seen in Fig. 2, the neighbour-joining phylogenetic tree clearly highlighted that the strain BMNF7 grouped with the *Bacillus safenis*. This isolate was able to degrade 60% Diazinon in mineral salt medium (MSM) as a sole carbon source within 10 days of incubation. The bacterial growth and Diazinon degradation were accelerated when MSM supplemented with 0.75 (g/l) of yeast extract (Fig. 3) and 0.5 g/l glucose (Fig. 4) as an additional carbon and nitrogen sources. The maximum degradation was obtained at pH 7 (Fig 5) and 45°C (Fig. 6) after 10 days of incubation (Fig. 7) and using 4×10^6 CFU/ml as inoculum size (Fig. 8). In conclusion the bacterial strains isolated from the agricultural soil, especially *Bacillus* species showed the ability to degrade Diazinon insecticide and optimization of growth conditions enhanced the percentage of degradation.

IV. Discussion

The soil with previously used pesticide contained different microbes that can use these materials and this may be due to outcompeting, biotic and/or abiotic interacting factors. Therefore, isolation of new bacterial strains adapted to the local conditions of a particular area is crucial for the efficiency of pesticide removal in this area (Wu *et al.* 2013). Many microorganisms can survive in pollutant environment due to the potential to degrade substance that harmful to our environment like chemical fertilizers and pesticides (Mahiudddin, 2014). In this study, soil samples had been taken from date farms in Saudi Arabia and isolation of the microorganisms from soil with a history in using of pesticide was carried out using enrichment culture technique. Many microorganisms were isolated from soil sample using enrichment culture technique and they have been grown in the minimal salt growth medium where Diazinon served as a sole carbon source. The most active isolate was identified according to biochemical characters, API50 CH strip kit. Vitek compact II as a species of the genus *Bacillus*. Three bacterial strains *Pseudomonas peli*, *Burkholderia caryophylli* and *Brevundimonas diminuta* were isolated and screened on medium containing Diazinon as carbon source (Mahiudddin, 2014).

Bala *et al.*, (2012) providing another evidence to the reliable identification using biochemical characterization by applying API50 CH strip kit. Vitek compact II is efficient tool for bacterial identification at the genus and species levels. The efficiency of this system to correctly identify *Staphylococcus* spp. and other bacterial genera has been proven previously (Chatzigeorgiou *et al.*, 2011; Paim *et al.*, 2014). Nonetheless, discordance between Vitek II and 16S rRNA gene sequencing for bacterial identification has been reported. For example, Vitek II identified a bacterial strain from human blood as *K. kristinae* (score of 98%) whereas 16s rRNA sequencing identified it as *Rothia amarae* (Abouseadaet *et al.*, 2016). Many results have been obtained by Hassan *et al.* (2016) which showed that both Vitek2 and 16S rRNA gene sequencing correctly identified certain bacteria (Hassan *et al.*, 2016). Mahiudddin, (2014) used morphological and biochemical studies and 16S rRNA gene sequencing for identification of the Diazinon degrading bacteria obtained from soil. Bacterial isolates, able to grow in medium containing the individual pesticide as the carbon source, were characterized by 16S rRNA gene sequencing and biochemical tests (Naphade, 2012).

In this study, *Bacillus* cells use Diazinon as carbon source in mineral salt medium. Similarly, the bacterial isolates *Pseudomonas peli*, *Burkholderia caryophylli* and *Brevundimonas diminuta* were able to completely degrade 20 mg/l Diazinon in mineral salt medium (MSM) as a sole carbon source within 12 days of incubation (Mahiudddin, 2014). More degradation was obtained by the selected *Bacillus* isolate 60% of 50 mg/l. Optimization of growth conditions enhanced growth and Diazinon degradation by *Bacillus*. Growth was determined using both absorbance at 550 nm and CPV/l. Cell packed volume is ideal for the monitoring of the growth and cell mass (George 1990, Agwa *et al.*, 2000). Addition of yeast extracts (0.75 g/l and glucose enhancing Diazinon degradation. Similarly, the bacterial growth and Diazinon degradation by the bacterial isolates *Pseudomonas peli*, *Burkholderia caryophylli* and *Brevundimonas diminuta* were accelerated and the isolates were able to completely degrade 20 mg/l Diazinon in mineral salt medium as a sole carbon source within 12 days of incubation (Mahiudddin, 2014). When MSM supplemented with 0.5 % of glucose and/or yeast extract as an additional carbon and nitrogen sources, degradation rate was increased and there is a correlation among Diazinon degradation, microbial growth and pH of the medium (Mahiudddin, 2014).

Several bacteria have been isolated to use Diazinon compound as a source of carbon, nitrogen or phosphorus (Phugare and Swapnil Suresh, 2011, Siddavattam *et al.*, 2003, Phugare and Swapnil Suresh, 2011). The genera *Neisseria*, *Veillonella*, *Micrococcus* and *Staphylococcus* were recorded for bioremediation technique in degrading pesticide (Adawiyah, 2008). In the presence of carbosulfan pesticide, *Pseudomonas* was isolated from *Solanum melongena* (brinjal) cultivated soil having a history of pesticide usage and this isolate was valuable for bioremediation of pesticide contaminated soils (Sharif, 2013) and there is positive correlation between pesticide degradation and tolerance to metals and antibiotics (Naphade, 2012).

Microorganisms play a major role for saving our environments by degrading xenobiotic compounds and chemical wastes and the rate of biodegradation by microbes depends not only on the presence of degradative enzymes, but also on a wide range of environmental parameters. Temperature, pH, water potential, nutrients and the amount of pesticide or metabolite in soil may also act as limiting factor for pesticide degrading microorganisms (Singh, 2008). Microbial degradation of pesticide and other hydrocarbon is attributed to cytochrome P450, haemthiolate monooxygenases, which catalyze the oxidative addition of atomic oxygen to the C-H or C-C bond of the organic compound (Van Beilen and Funhoff, 2007). Many bacterial species possess Cytochrome P450 proteins and testing these species is crucial to confirm the ability to degrade pesticide. Other enzymes such as soluble and particulate methane monooxygenases are involved in hydrocarbon degradation (Das and Chandran, 2011).

A few consortia with the capability to metabolize Diazinon have also been reported (Singh *et al.*, 2006, Singh *et al.*, 2003). In general, Diazinon or Organophosphate (OP) compounds do not adversely affect bacteria, because bacteria do not possess acetylcholin esterase and some microorganisms can even use OPs as an energy source (Singh, *et al.*, 2006) due to the presence of Phosphotriesterases. There is a correlation between treatment

duration and % degradation of chlorpyrifos by bacterial mono- and mixed-cultures (Kumar, 2011). A bacterial consortium of *Proteus vulgaris*, *Vibrio* sp., *Serratia* sp. and *Acinetobacter* sp. which degraded Dichlorvos was isolated from agricultural soil in medium and these isolated strains can be used for waste biodegradation or bioremediation of organophosphate pesticide- contaminated soil or water (Agarry, 2013). *Pseudomonas aeruginosa* and *Flavimonas oryzihabitans* grow on mineral medium amended with ground coffee beans and 50 mg/l endosulfan (Barraga n-Huerta, 2007). Degradation rate was slower in lower pH in comparison with natural and alkaline soils and the bacterial isolates were able to rapidly degrade fenamiphos and chlorpyrifos at 15-35°C but their degradation ability was sharply reduced at 5 and 50°C (Singh, 2006). In India; extensive applications of persistent organochlorine pesticides like endosulfan on cotton have led to the contamination of soil and water environments at several sites and microbial degradation using several strains of bacteria and fungi offers an effective approach to remove such toxicants from the environment (Reddy, 2012).

V. Conclusion

In conclusion, the bacterial strains isolated from the agricultural soil showed the ability to degrade the insecticide Diazinon and the cultural; biochemical and molecular characteristics were used for the strain BMNF7 identification which exhibited a metabolic versatility and ability to degrade pesticide, indicating ecological and environmental merits for its application in bioremediation of pesticide pollutants. Overall, this study provides the complete insight of the Diazinon degradation with the help of bacterial strains, which could be helpful to devise a proper strategy for the degradation of pesticides under field conditions. Nonetheless, further studies are required to investigate the efficiency of the strain BMNF7 for biodegradation of different organic pollutants and enzymes involved in this process. Bearing in mind the efficiency of local bacterial strains in pesticide degradation was found to be substantially higher than that of the introduced strains which may be attributed to outcompeting, biotic and/or abiotic interacting factors.

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Table 1. Growth and degradation of Diazinon in solid (diameter of clear zone cm) and liquid minimal salt medium and the three selected bacterial isolates

Tested isolate	Growth in broth medium		Degradation	
	A _{440nm}	Cell Packed Volume (CPV)	In solid medium	In liquid medium
			Diameter of the clear zone (cm)	% degradation
BMR F3	0.68 ± 0.2	22	5.1±2.1	30
BMN F7	0.54 ± 0.10	25	7.0 ±1.1	33
BMT F 8	0.49 ± 0.19	24	4.1±1.5	21



Fig. 1. The selected isolate on minimal agar medium containing Diazinon as carbon source (A), on Nutrient agar (B), and under light microscope after Gram staining (C)

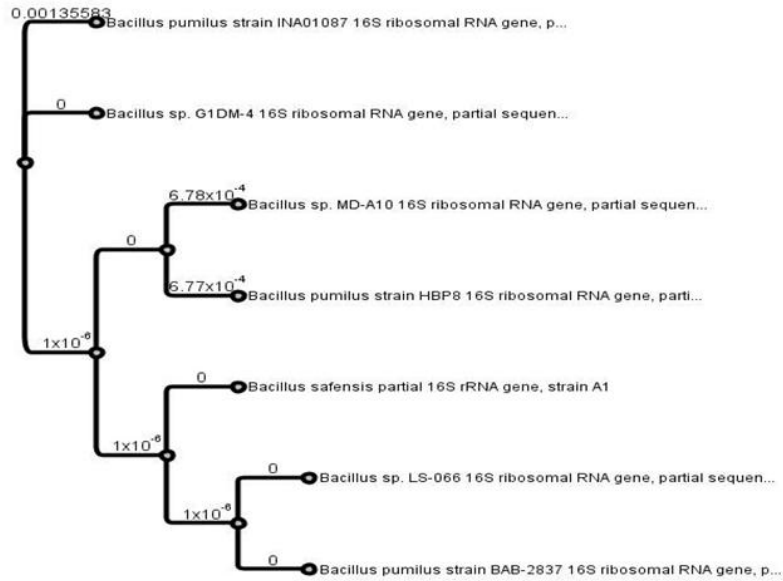


Fig. 2. Phylogenetic tree based on 16S rRNA gene comparison of the bacterial strain and other bacteria

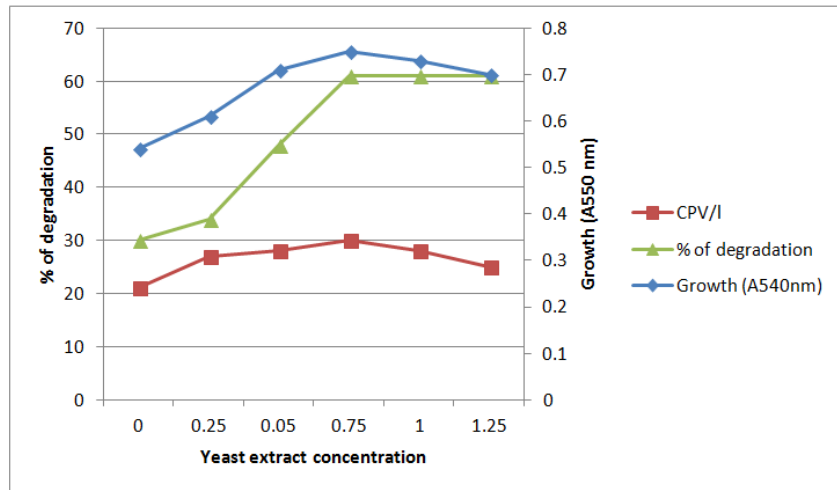


Fig.3. Effect of different concentrations of yeast extracts on growth and % of Diazinon degradation by the isolate BMNF7

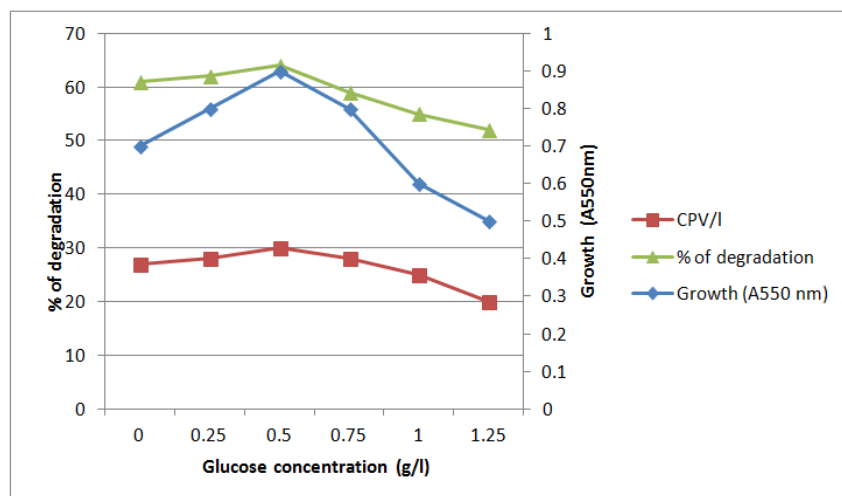


Fig.4. Effect of different glucose concentrations on growth and % of Diazinon degradation by the isolate BMNF7

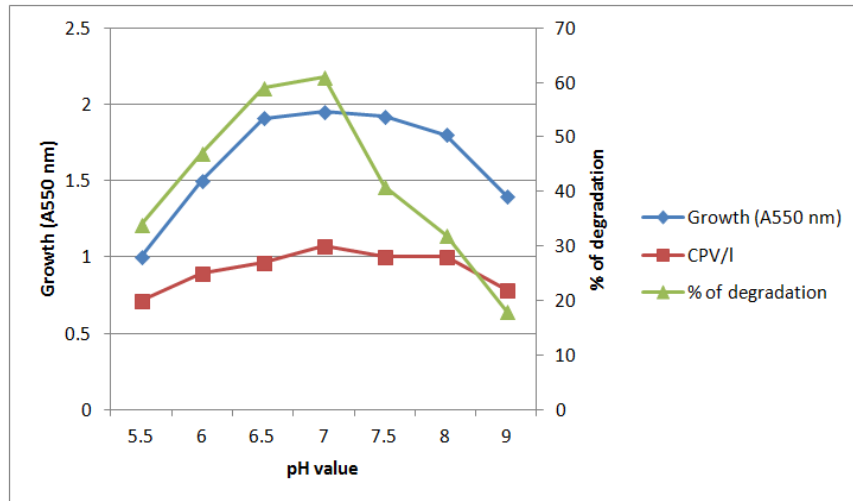


Fig.5. Effect of different pH values on growth and % of Diazinon degradation by the isolate BMNF7

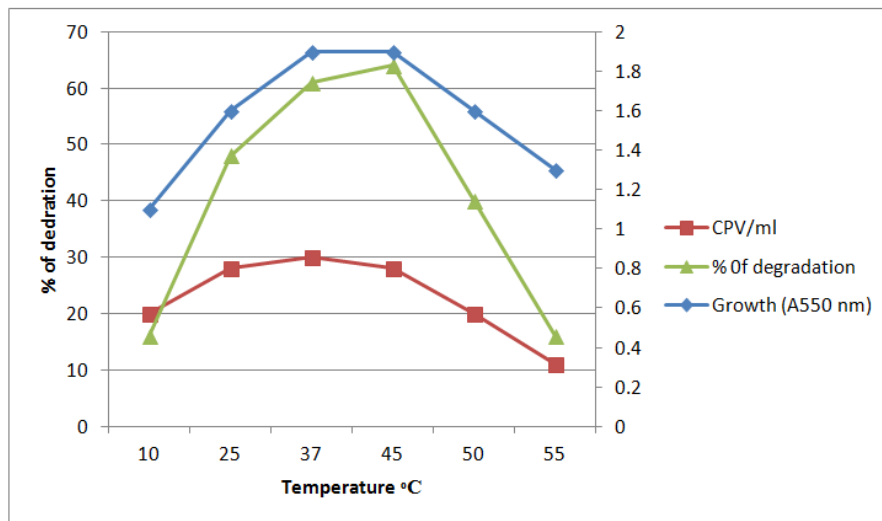


Fig.6. Effect of different incubation temperature on growth and % of Diazinon degradation by the isolate BMNF7

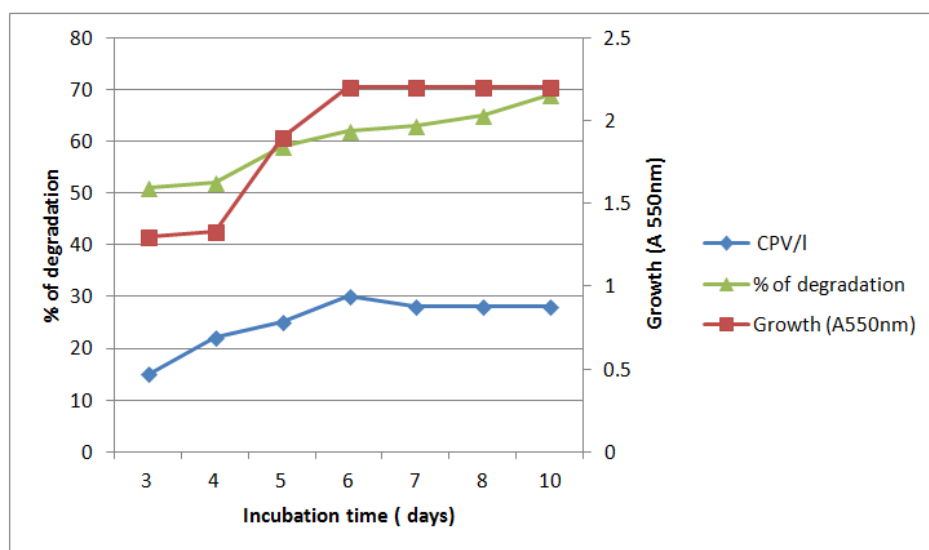


Fig.7. Effect of different incubation time on growth and % of Diazinon degradation by the isolate BMNF7

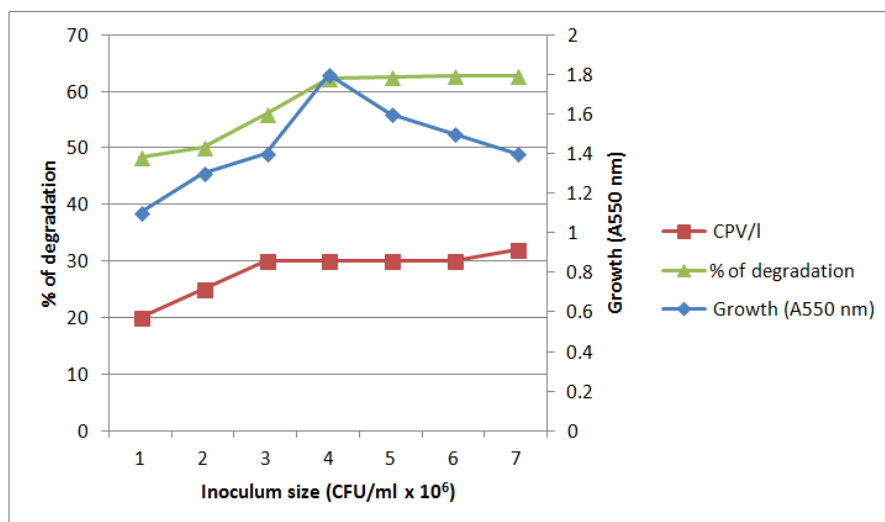


Fig.8. Effect of different inoculum size on growth and % of Diazinon degradation by the isolate BMNF7