Phenotypic and Genotypic Variability among Three *Bacillus megatherium* Isolates. 2- Molecular detection of Orthocide Fungicide Biodegradation

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Abstract: Three B. megatherium isolates (BM1, BM2 and BM3) showed a great ability to degrade the fungicidal orthocide 75 (95% Captan) in vitro and in vivo. They differed in their ability to orthocide degradation in vitro with 14, 10.8 and 9.0% for BM1, BM2 and BM3 isolates respectively in the course 48 hr incubation. In addition they different in their ability to orthocide degradation in soil in the course 30 days earlier the fungicide treatment. Residual fungicide determined by GLC after 10, 20 and 30 days. It is clear that B. megatherium isolate B1 was the most active isolate degrading fungicide. The percentage of orthocide residual were 16, 20, 35 (non-sterilized soil) 43, 46 and 47% (sterilized soil) at 30 days for BM1, BM2and BM3 isolates respectively. On the other hand, the effect of orthocide on B. megatherium growth was little (non-observed). B. megatherium isolates were varied in dehalogenase activity the data showed the level of enzyme activity was found to be considerably higher in BM1 isolate followed by BM2 and BM3. Dehalogenase gene was successfully detected in total DNA genome of three isolates by polymerase chain reaction (PCR), where as showed differences among isolates related to number, density and size of isozymes (bands). In addition base pairs nucleotides (gene) were one band (478 bp) BM1, 3 bands (1059, 560, 478 bp) BM2 and 3 bands (1300, 800, 478 b[) BM3, isolates relative mobility and density. The similarity of dehalogenase activity 20% among three B. megatherium isolates. [Journal of American Science 2010;6(8):116-121]. (ISSN: 1545-1003).

Key words: Fungicide biodegradation, Dehalogenase PCR; B. megatherium, GLC.

1. Introduction

The biodegradational behaviour of pesticides on and in various edible plants was studied by many authors. However, this type of study showed be carried out under the local conditions of every country. They concluded that, the current levels of the pesticides in surface water and plants at a not constitute on acute toxicity hazard to man on a short-term basis, but as a result of its accumulation in man body it causes hazard of large term exposure. On the other hand, some researches were published about the methods of removing pesticides from soil and water by activated carbon (Hegazi et al, 1990), biodegradation by microorganisms (Yakato et al, 1987, Dahrog et al., 2006, Amer, 2008 and Azhar, 2009).

So far haloalkane dehalogenases are the only enzymes known to be capable of direct hydrolytic dehalogenation of chlorinated and brominated hydrocarbons without the requirement for enzymes or oxygen. The enzyme of Xanthomonas autorophicus GIIO's constitutively expressed 2 to 3%, of the soluble cellular protein (Keuning et al, 1985) up to 30% (Janssen et al, 1989). It has a remarkably broad substrate range which includes terminally halogenated alkanes with chain lengths up to 4 carbons for chlorinated and up to at least to carbons for brominated alkans. Other haloalkane dehalogenases of bread substrate range have been found in gram positive haloalkane, utilizing bacteria (Janssen, et al, 1989).

2. Material and Methods

Microbial cultures:

Three B. megatherium isolates (namely BM1, BM2, BM3) from different soils were grown on nutrient broth medium. These isolates were obtained from microbiology Lab. Botany Dept., Fac. Sci., Benha Univ. (Nahid, 2009).

Fungicide:

Orthocide 75 (95% captan): N-Trichloromethyl-mercapto-4-Cyclohexan- 1,2-dicarboximide, supplied by Kumia Chemical Industries Co. Ltd., Japan. Emulsifiable of the active ingredient 40% concentration of this orthocide was used in this investigation.

Detection of orthocide degradation:

In Vitro: Orthocide clearing zone: Assay medium contained (g/L): 6.0 NaCl, 1.0 (NH4)SO4; 0.5 KH2PO4; 0.1 CaCl2, 1.0 yeast extract and d H2O

up to one liter according to Vincent (1970) and supplemented with 0.5% (V/V) of emulsified orthocide (50%). This assay was carried out to determine the most potent degradation on the basis of mean diameters of clean zones (mm) by the testing B. megatherium isolates. After 24 hr petri-dishes were inspected to estimate the efficacy of orthocide degradation by millimeter ruler.

In vivo: Potted experimentally :

The soil taken from the surface layer (20 cm depth) of clay loam soil. Fourty pots (250 ml capacity) were filted with soil (about 200 pot-1). Twenty pots were sterilized and an other twenty pots were left without sterilization. The soil moisture content in all pots was raised up to 60% of WHC at 15 ibs 21 hr during 2 days. All pots were supplemented with 5000 ppm of orthocide and 100 ml broth media and mixed. Twenty ml of each broth medium contained ~ 5x108 cfu of three B. megatherium isolates used for inoculation of the soil and soil without inoculation as control. Soil samples were taken periodically after, 10, 20 and 30 days for the determination of residual orthocide using Gas liquid chromoatography (GLC) according to Vogeler (1968). As well as counted B. megatherium in sterilized and non-sterilized soil using plate count method. The soil samples were kept in freezer until analysis to determine the residual amount of orthocide.

Determination of dihalogenase activity and isozyme using polymerase chain reaction (PCR):

Enzyme activity: Cell cultures were harvested at the end of the experimental growth phase. The protein was extract from cell cultures as described by Bradford (1976). The enzyme activity was assayed according to Janssen et al (1987).

Isolation of genomic DNA:

Bacterial cells were cultured in broth medium and incubated at 28ºC with shaking at 200 rpm for 24 hr. and harvested by centrifugation. The DNA was extracted using CTAB method as described by Owen and Borman (1987).

Amplification of DNA:

The DNA was amplified by polymetase chain reaction (PCR) in 100 µl react mixture containing: 20 µl template DNA (25 mg), 0.2 µl Taq DNA polymerase (unit); 3.0 µl DNTP, (25 mM of each dATP, dCTP, dTTP, dGTP), 3.0 µl MgCl2 (25 mM); 30 µl PCR buffer (10X); 20 µl specific primer (dehalogenase encoding gen) Table (1) and 23.8 µl dH2O. The mixture was assembled on ice overlaid with 2 drop of mineral oil. The amplification was carried out in DNA thermal cycler (MWG-Biko-TECH Primuse) programmed as follows: One cycle at 94ºC for 2 min and then 30 cycles at 94ºC for 30 sec., 55ºC for 30 sec. and 72ºC for 20 sec. On cycle at 72ºC for 5 min then store in 4ºC final mix of PCR.

Table (1): Oligonucleotide sequences specific primers.

Primer sets	5` Sequence 3`
TF	tgggcggatttttggggct
TR	gtacgaaatggccagcgtcc

Gel electrophoresis analysis: Agarose gel (1.5%) was prepared in TAE buffer. Total sample, 10 µl miniprep; 2 µl 6x loading dye and 6 µl dH20) of each isolate was loaded in each well. The electrophoresis was done of 65 V for 1 hr and then stained with ethidium promide solution (10 mg/ml) for about 10-15 min. DNA amplified was visualized on UV transilluminator and photographed.

Determination of orthocide residues:

1-Extraction and clean up:

One gram of each treated soil and untreated soil for each isolates were transferred into 250 ml separating funnel and added 60 ml at 15% methylene chloride/hexane (V/V). The funnel was stoppered and shaken vigorously for 2 min and vented the pressure during shaking. The two layers were separated. The aqueous layer was drawn off into separating funnel. This procedure was repeated using 30 ml of 15% methylene chloride/hexane (V/V). The aqueous layer was discarded and the solvent extracts in both separating funnels were combined and poured on top of anhydrous sodium sulphate column. Before the solution recedes the top of sodium sulphate layer, three 10 ml rinses of 15% methylene chloride/hexane were added. The filtrates were collected for clearn up by using the US.EPA, 1988 procedures.

For clean up on a florisil column chromatography (300 mm long x 25 mm) internal diameter (i.d) with a small glass plug in the bottom was prepared by adding an activated florisil (130ºC/overnight) in small portions, while tabbing until about 10 cm high. About one and a half cm layer at anhydrous sodium sulphate was added to the top carefully without mixing with florisil.

Mature solvents of 6% eiethyl ether-petrolum ether was added then 10 ml of 15% diethyl ether/petroleum ether and 50% diethyl/petroleum ether were used for elution.

The filtered elution mixture was evaporated using rotary evaporator 40ºC for gas chromatographic determination.

2. Determination:
Separating of the residues was done on a borosilicate glass column 2 meters long, 3 mm internal diameter containing 3% oV-17 (phenyl methyl silicone) on chromosorb W, H.b. 100/120 mesh. The operating conditions were column oven temperature (150ºC) carrier gas (Nitrogen) (1.2 kg/cm), Burner gas (Hydrogen) (1.0 kg/cm), and air (1.0 kg/cm). Phillips PU4410, computerized (Gas Chromatography with FPD was used.

Soil free from Linuron was used to estimate rate of recovery by using the previous producers. The mean of the obtained recovery was 88%. All the obtained data for the residues of Linuron on treated soil were corrected by using such rate of recovery.

A series of concentration 10, 20, 30, 40, 50, 60, 70 mg of A falon analytical standard to 10 and toluene were prepared for obtaining the standard curve (Fig. 1). A suitable aliquot (5 µl) was injected from each concentration.

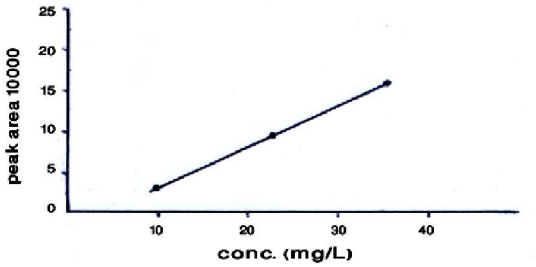

Fig. (1). Standard calibration curve orthocide.

3. Results
Bacillus megatherium isolates under study showed a great ability to degrade fungicide orthocide. They differed in their ability to orthocide degradation in vitro. Data concerning the amount of orthocide degradation with 0.70; 0.54 and 0.44 gm with 14, 10.8, 0.9% for B1, B2 and B3 isolates respectively (Table, 2).

Table (2).The potent ability of B. megatherium isolates to orthocide degradation *in vitro*.

B. megatherium isolates	Diameter clearing zone (cm)	Amount of orthocide degradation	Percent of degradation (%)
BM1	3.5	0.70	14
BM2	2.7	0.54	10.8
BM3	2.2	0.44	9.0

Orthocide amount in plate 5%.

The biodegradation of orthocide in vitro in sterilized and non-sterilized soil inoculated with B. megatherium isolates (BM1, BM2, BM3) showed that the amount of orthocide was decreased gradually through 30 days in sterilized and non sterilized soils (Table 3). The residual concentration of orthocide were 35, 40 and 47 ppm in sterilized soil for inoculated, B1, B2 and B3 respectively (Table 3). While the orthocide concentration was completely disappeared in non-sterilized inoculated soils were 16, 20 and 35 ppm after 30 days post inoculation (Table 3).

On the other hand, the total count of B. megatherium isolates were decreased in the first days then increased gradually under sterilized and non-sterilized soils (Table 3). The number of B. megatherium isolates were 5x107, 4x107 and 3x107 cfu at 30 day post inoculation with BM1, BM2 and BM3 respectively.

Table (3): Biodegradation of orthocide fungicide in soil inoculated with B. megatherium isolates.

Isolates	Non-sterilized soil			Sterilized soil		
	Orthocide (ppm)	% of orthocide residue	Total count	Orthocide (ppm)	% of orthocide residue	Total count
BM1 10th	4250	89	1 x 106	4525	91	2 x 106
20th	2750	55	2 x 106	3375	68	3 x 106
30th	775	16	5 x 107	1750	35	5 x 108
BM1 10th	4325	88	2 x 106	4500	90	2 x 106
20th	2975	60	3 x 107	3605	82	3 x 107
30th	1050	20	4 x 107	2000	40	3 x 108
BM1 10th	4350	87	1 x 106	4625	93	2 x 106
20th	3.50	61	2 x 107	3627	60	4 x 107
30th	1520	35	3 x 107	2375	47	4 x 108

* After one hour from addition.
** % of orthocide residual related to control (without inoculated).30 days post inoculation.

Dehalogenase enzyme analysis:

Dehalogenase activity was varied among three B. megatherium isolates. Theresults in Table (4) reveal the enzyme activity of three isolates. It was found higher in isolate BM1 240 followed by BM2 180 and BM2, 120 sec. (Fig. 2).

Table (4). Rate of dehalogenase activity among B. megatherium isolates.

Isolates	Dehalogenase activity		
	BM1 isolate	BM2 isolate	BM3 isolate
0 time	1.25	1.05	0.70
60 sec.	1.75	1.50	1.10
120 sec.	2.20	1.95	1.50
180 sec.	2.65	2.10	1.50
240 sec.	3.25	2.10	1.50
300 sec.	2.25	2.10	1.50
360 sec.	2.25	2.10	1.50

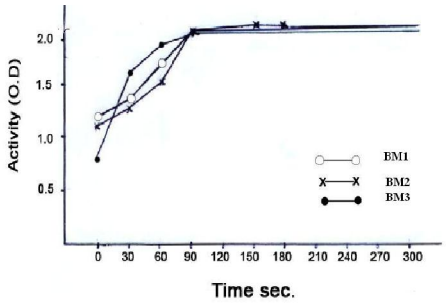

Fig. (2). Showing the rate of dehalogenase activity among three B. megatherium isolates.

Dehalogenase isozymes :

The quantity of DNA purified extracted from 3 B. megatherium isolates were conformed by UV spectrophotometer. The DNA concentration were about 30 µlg/0.5 g cells. The purity of DNA was measured on 260/280 absorbance 1:65, 1:75 and 1:50 for 3 isolates respectively, these results indicating high yield and DNA purity:

The dehalogenase gene of 3 isolates were amplified of DNA, of 3 isolates using specific primers, taq polymerase and PCR reaction mixture using one set PCR method.

PCR amplification of dehalogenase gene appeared variation among 3 B. megatherium isolates, i.e. number, density and size of PCR products. The results in Table 5 and Fig. 3 showed total 7 bands of dehalogenase isozymes in all isolates as well as one bands of dhl clone (505 bp) of dehalogenase. B. megatherium isolate B1 has one isozyme band with 478 bp and both isolates BM2, BM3 has three isozyme bands with (1059; 560; 478) and (1300, 800 and 478 bp). On the other hand, these isozyme bands were differed in density (percent of fraction). The variability among 3 isolates, one monomorphic (common) amplified band (590 bp with 14% percentage and two polymorphic (specific) amplified bands (1540 and 725 bp) with 29% percentage. The different among three B. megatherium isolate related to genetic variability.

Table (5).Genetic variability (DNA markers) of dehalogenase gene among, three B. megatherium isolates using PCR.

Rf	Dhl %	Clone MW	B. megatherium isolates						Polymor-phism
			BM1		BM2		BM3		
			%	MW	%	MW	%	MW	
0.28	-	-	-	-	-	-	54.7	1300	Unique
0.32	-	-	-	-	15.5	1059	-	-	Unique
0.34	-	-	-	-	-	-	18.3	800	Unique
0.39	100	505	-	-	78.7	560	-	-	Unique
0.42	-	-	100	478	5.8	478	27.0	478	Mono-morphic

Rf : Relative mobility. % : Percent of amplified band (DNA fraction)
MW: Size of expect band (bp). Unique : Genetic marker
Monomorphic or common amplified band.

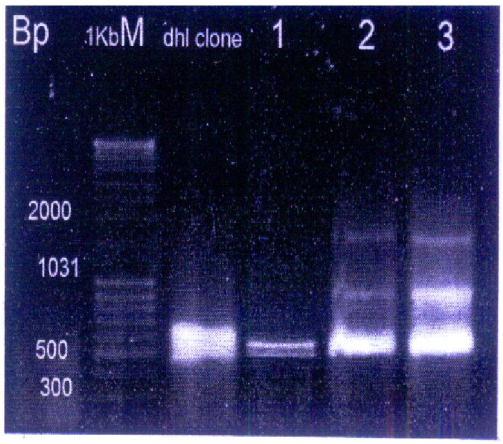

Fig. 3. Agarose gel electrophoresis stained with ethidium bromide showing the PCR products of amplified dehalogenase gene of three isolates using specific primers, Lane M: DNA molecular weight marker, Lanes gene clone, BM1, BM2 and BM3 B. megatherium isolates.

4. Discussion

In the recent years, great quantities of fungicides are consumed annually for the control of plant pathogenic fungi during our chemical warfare against a multitude of noxious organisms in the soil. It is necessary to avoid the injury of these fungicides together with their various carriers diluents and solvents on the various beneficial soil microorganisms and their biological activities contributing to soil fertility.

B. megatherium isolates showed great ability to degrade orthocide fungicide in vitro and in vivo which assimilation as a source of carbon. The analysis of orthocide residual by GLC at 10, 20 and 30 days post-inoculation showed the decrease gradually of orthocide at 20 days in sterilized and non-sterilized soils.

The results indicate that, the biodegradation of orthocide residues in inoculated sterilzized soil was high rate (16, 20, 25) followed by non-sterilized soil (43, 46, 47%) with B1, B2, B3 isolates respectively. This difference due to microbial flora in soil may be antagonistic with B. megatherium as well as it had the role in biodegradation of orthocide. In addition, this may be attributed to the concentration of soil (from drift occurrence of this fungicide.

Biodegradation of orthocide was detected in vitro with bacterial isolates but in different values (0.70, 0.54 and 0.44 ppm) of B1, B2 and B3 isolates respectively.

This obtained previous results are in agreement with those obtained by Hashish et al. (1990); Dahrog, et al. (2006), Amer, (2008) and Azhar (2009) studied the ability Pseudomonas, Bacillus, Rhizobium and Streptomyces to degrade the Linuron, Diuron, Ktozin and dichlofunanid. The actual degrade the fungicides and herbicides by microorganisms is caused by the release of enzymes that breakdown of them. The role of fungicide, herbicide and pesticide on bacterial population in treated soil was intensively studied the reported by Berger (1998), bacteria capable of significant biotransformation and reduced phenylurea concentrations in liquid culture.

Shin and Cheney (1989) and Amer, (2008), conduced a trial to determine the effect of Linuron, Simazine, alcohol and nonselective parquet on Bradyrhizobium japonicum and Rh. Leguminosarum bacteria. The alcohol and Linuron were decreased significantly by 27.4 and 57.8% respectively, while little effect was observed in simazine and marked reduction of survivals observed in 200 ppm of parquet.

The degradation of orthocide by B. megatherium isolates proceeds through the concerted enzyme actions that are specific for halogenated compound and enzymes that are involved in the metabolism of natural compounds. The former are the dehalogenase that catalyze hydrolysis of orthocide. The enzymes show a broad substrate range and one only produced by isolates that utilize halogenated substrates and thus can be considered enzymes that are acquired by this specific strain of B. metherum during genetic adaptation to degradation of chlorinated hydrocarbons. Thispaper describe present a further are lysis of the data alkane dehalogenase encoding gene dhlA. The absence of dehalogenase activities in natural of B. megatherijm allowed the identification of clones containing the dehalogenase gene. Isolation of genes involved in methanol-dehalogenase and chloroacetaldhyde dehydrogenase activity was possible by screening for complementation of mutants lacking the dehalogenase activities. In this way; harboring genes were identified and the genes were localized on different DNA segments.

The efficient expression of the halkinase dehalogenase gene in other gram-positive bacteria in not surprising in view of the fact that two regions with the consensus E. coli promoter sequence were present. Copy number probably also plays a role, since expression levels were higher in Xanthobacter autotropicus G10 (p 120) than in the wild type

isolates G110. The E. coli consensus promoter sequence is known to stimulate transcription in B. megatherium, (Dpyle et al, 1984 and Jeenes, et al. 1986) and our data suggest that it might also do so in Pseudomonas sp. In order to determine which of these sequences is the actual cause of the higher expression and whether the promoter can be used for expression of others genes in Pseudomonas spp., it well be necessary to identify the transcription short site of the gene and to study expression of different genes linked to the promoter regions.

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