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Full Length Research Paper

Isolation, characterization and fingerprinting of some chlorpyrifos- degrading bacterial strains isolated from Egyptian pesticides-polluted soils

Nabil S. Awad[1,2]*, Hussein H. Sabit[1], Salah E. M. Abo-Aba[1,3] and Reda A. Bayoumi[4]

[1]Faculty of Biotechnology, Misr University for Science and Technology, Egypt.
[2]College of Medicine, Biotechnology and Genetic Engineering Unit, Taif University, Taif, Kingdom Saudi Arabia.
[3]Department of Biological Sciences, Faculty of Science, King Abdul-Aziz University, Jeddah, Saudi Arabia.
[4]Department of Biotechnology, Faculty of Science, Taif University, Taif (El-Khorma), Kingdom Saudi Arabia.

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Five bacterial isolates (B-CP5- B-CP6 - B-CP7- B-CP8- B-CP9) were isolated from pesticides-contaminated soil in Egypt. The capability of these isolates to degrade chlorpyrifos was investigated using enrichment mineral salt (MS) medium containing chlorpyrifos. Two different PCR-based techniques, RAPD-PCR and PCR-RFLP for amplified16S rRNA fragment were used to conduct genetic fingerprinting and obtain specific molecular markers for the studied isolates. The isolates exhibited substantial growth in mineral salt medium supplemented with 100-300 mg/L chlorpyrifos as a sole source of carbon and energy. Based on their morphological, cultural and biochemical characters, the isolates have been identified as *Pseudomonas stuzeri, Enterobacter aerogenes, Pseudomonas pseudoalcaligenes, Pseudomonas maltophila* and *Pseudomonas vesicularis* respectively. *Pseudomonas stuzeri* was the most potent degrader strain. Five specific markers for this strain were determined. The highest genetic similarity was observed between CP8 and CP7 (66%), while the lowest genetic similarity was detected between CP8 and CP6 (37%). All isolates had the same pattern after digestion of 16S rRNA amplified fragment with two restriction enzymes (EcoRI and AluI) except *Enterobacter aerogenes,* which generate two monomorphic bands at 420 and 130 bp, respectively. In conclusion, the strain *Pseudomonas stuzeri* could be used to clean up the areas contaminated with Chlorpyrifos. Obtained molecular markers might be used for identifying and tracking the most potent bacterial isolate. The used PCR techniques represent a powerful tool and could be used for rapid typing of this strain.

Key words: Pesticide, chlorpyrifos, biodegradation, 16S rRNA, RAPD-PCR, *Pseudomonas* sp.

INTRODUCTION

Recently the use of microbes for effective detoxify, degrades and removal of toxic chlorinated compounds from contaminated soils has emerged as an efficient and cheap biotechnological approach to clean up polluted environments (Strong and Burgess, 2008). Chlorpyrifos (CP) is one of the most widely used organophosphorous insecticide. Chlorpyrifos-oxon and 3,5,6-trichloro-2-pyridinol (TCP) are the two potent transformation products of chlorpyrifos (Bhagobaty et al., 2007). Unlike other organophosphorous compounds, CP has been reported to be resistant to enhanced degradation since its first use in 1965. Although CP has been reported to be degraded co-metabolically by bacteria in liquid media, several attempts to isolate CP-degrading bacteria from agricultural soil have not been successful (Mallick et al., 1999; Racke et al., 1990). Singh et al. (2004) isolated the first CP-degrading bacterium, it was Enterobacter B-14, which hydrolyzed CP to diethylthiophosphate DETP and TCP, and utilized DETP for growth and energy. Two recently isolated CP-degrading bacteria, *Stenotrophomonas* sp. and *Sphingomonas* sp., could also utilize CP as the sole source of carbon and phosphorous, but they did not degrade TCP (Yang et al.,

*Corresponding author. E-mail: nabilfaris151@yahoo.com or nabilfaris@hotmail.com. Tel: 00966 509891624.

2006; Li et al., 2007). DNA-based analyses can contribute significantly to the characterization of bacteria that have been successfully isolated from the polluted environments. RAPD-PCR approach has been shown to be useful in fingerprinting and distinguish between microbial strains within a species (Nowrouzian et al., 2001). An excellent target for bacterial identification and phylogenetic characterization is 16S rRNA gene, the 16S rRNA gene is universally distributed and highly conserved (Woese, 1987). Due to its conserved nature and ease of manipulation, it has been extensively used to establish accurate identification of bacterial isolates. Cleavage of PCR-generated 16S rRNA gene amplicons by a restriction enzyme(s) (RE) results in differentiation by 16S rRNA PCR-RFLP technique. This procedure has been used extensively as a method for bacterial species identification (Wilson et al., 1995; Marshall et al., 1999; Conville et al., 2000; Steinhauserova et al., 2001; Bayoumi et al., 2010). The present study aims to isolate, characterize and fingerprinting of chlorpyrifos degrading bacteria from Egyptian pesticides-polluted soils as well as obtain specific molecular markers of the most potent chlorpyrifos degrading bacterial isolates. In addition examine the effects of various environmental factors which affect the bacterial biodegradation potentiality of chlorpyrifos.

MATERIALS AND METHODS

Pesticide used

Chlorpyrifos (CP) (95%purity) was obtained from Dow-agroscience Co.

Media

Mineral salts (MS) medium enriched with Chlorpyrifos as a sole source of carbon and energy was used as a routine medium for isolation and characterization of different species of chlorpyrifos utilizing bacteria and was prepared as the followings: Solution (A) contained in (g/L): KNO_3, 0.5; KH_2PO_4, 0.68; NaH_2PO_4, 1.79; NH_4NO_3, 1.0; $MgSO_4.7H_2O$, 0.35; $CaCl_2.2H_2O$, 0.4; and agar-agar, 15.0 for solid medium and distilled water up to 1000 ml. Chlorpyrifos was added by 100 to 300 mg/L. Solution (B) trace salt solution (100g / ml): $FeSO_4.7H_2O$, 0.1; $ZnSO_4$, 0.1; $MnSO_4$, 0.1; $CuSO_4$, 0.1, $CaCl_2.2H_2O$, 0.1; H_3PO_4, 0.1, and distilled water up to 100 ml/L. The solid and liquid mineral salts media were supplemented with yeast extract and glycerol (3.0 g/ml) or (3.0 ml/l) respectively for obtaining enrichment media.

Study site

Four Chlorpyrifos polluted soil samples were collected from various agricultural fields belong to Cairo and Giza Egyptian governorates.

Reagent used

The 4-aminoantipyrine was used to measure the production of phenolic compounds in culture solution. It was prepared as following: (1) 5 mM Fe $(NH_4)_2(SO_4)_2$ solution, (2) 100 mM α-ketoglutarate solution (3) 40 mM 4-aminoantipyrine solution. This reagent used for detection of phenolic products of the enzymatic cleavage of 2,4-D and related compounds. 2,4-D α-ketoglutarate deoxygenase, cleaves the phenoxy ether bond of o,o-diethl o-(3,5,6-trichloro-2-pyridyl) phosphothionate (Chlorpyrifos), to form 2,4-trichlorophenol TCP. The red color resulting from a 2 mg/l of TCP solution is capable of being visually distinguished from the control (remains bright yellow).

Isolation of cp-degrading bacteria by enrichment and screening

One gram of each soil sample was added to 99 ml MS medium supplemented with yeast extract and glycerol containing 0.1 ml CP in 250 ml flasks. The suspension was incubated at 30°C with shaking at 100 rpm for 10 days; 10 ml of the culture broth was transferred after 10 days to 90 ml of the fresh media. After the fourth enrichment transfer to fresh medium containing CP as sole source of carbon and energy, the media was supplemented with 1.5% agar and incubated at 30°C for 7 days. Bacterial isolates grown on chlorpyrifos-containing agar were subjected to morphological, cultural and biochemical studies by the aid of Berge's Manual of Determinative Bacteriology (Holt et al., 1994). All bacterial isolates were screened based on the formation of degrading haloes around the bacterial colonies and potential isolates were obtained and tested for their potential degrading ability of CP.

Parameters controlling cp biodegradation by CP5

Eight parameters were investigated for studying CP biodegradation by CP5 on MS medium supplemented with CP as sole source of carbon and energy viz. incubation period (1, 2, 3, 4, 5, 6 and 7 days), different CP concentrations (1, 2, 4, 6, 8,10, 20,40, 60, 80 and 100 ml/L); inoculum size (0.5, 1, 2, 4, 8, 10 ml/100ml); incubation temperatures (25, 30, 35, 40 and 45°C); different carbon sources (D-Glucose, D-Fructose, D-Mannose, D-Ribose, Maltose, Arabinose, L-Rhamnose, Sucrose, Lactose, and Starch). All different tested carbon sources were applied as equimolecular weight located in 1% (w/v) sucrose except poly-sorbite and starch which were applied by 1%; different pH values (5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5 and 9) using 1 N HCl and 1 N NaOH; different nitrogen sources (sodium nitrate, magnesium nitrate, potassium nitrate, ammonium chloride, ammonium nitrate, EDTA, urea and peptone); under static and shaking conditions. After each parameter, 4-aminoantipyrene reagent was used to measure the production of phenolic compounds.

Molecular genetics fingerprinting

Genomic DNA extraction

Genomic DNA was extracted from five studied isolates using Easy Quick DNA extraction kit (Genomix) following the manufacturer's instructions.

Random amplified polymorphic DNA (RAPD)

RAPD-PCR

PCR reactions were carried out using sixe arbitrary 10-mer primers (Operon Tech., Inc.). PCR reactions were conducted using 2x superhot PCR Master Mix (Bioron; Germany) with 10 Pmol of each

Table 1. List of used primers, their nucleotide sequences, total number of bands, monomorphic and polymorphic bands produced by six primers.

Primer code	Prime sequence	Isolate					Total bands	Amplified bands	Monomorphic bands	polymorphic bands
		CP5	CP6	CP7	CP8	CP9				
A2	5'-TGCCGAGCTG-3'	11	11	11	14	13	60	17	7	10
A3	5'-AGTCAGCCAC-3'	4	11	7	8	7	37	13	6	7
B3	5'-CATCCCCCTG-3'	12	9	13	10	12	56	15	8	7
B5	5'-TGCGCCCTTC-3'	7	10	8	7	8	40	18	4	14
C3	5'-GGGGGTCTTT-3'	8	7	6	8	6	35	10	8	2
C5	5'-TGCGCCCTTC-3'	11	7	9	9	11	47	11	6	5
Total		53	55	54	56	57	275	84	39	45

6 different arbitrary 10-mer primers. The codes and sequences of these primers are listed in Table 1. The 25 µl reaction mixture was (10 Pmol. of each primer, 30-50 ng of DNA template and 12.5 µl of 2x superhot PCR Master Mix). The PCR protocol was initial denaturation, 94°C for 2.5 min and 35 cycles of subsequent denaturation, 94°C for 45 s; annealing temperature, 37°C for 30 s; extension temperature, 72°C for 2 min and final extension, 72°C for 10 min. PCR products were analyzed on 10 x 14 cm 1.5% agarose gel electrophoresis with DNA ladder standard 100 bp (Jena Bioscience, Germany)for 30 min using Tris-borate- EDTA Buffer and visualized by ultraviolet illumination after staining with 0.5 µg/ml ethidium bromide.

16S RRNA PCR-RFLP analysis

The amplification of 16s rRNA gene was performed with universal primer 968F AAC GCGAAGAACCCTAC and 1401R GCGTGTGTACAAGACCC. The reaction mixture was (10 Pmol. of each primer, 50 ng of DNA template and 12.5 µl of 2x superhot PCR Master Mix). The PCR program was as follow; 1 cycle at 95°C, 5 min; 35 additional cycles consisting of 95°C 1 min, 63°C 1 min, and 72°C 1 min and 72°C 10 min as post PCR reaction time. The amplified DNA fragments were digested with two restriction enzymes (EcoRI and. AluI) separately as described by the manufacturer (Jena Bioscience, Germany). The digested DNA fragments were separated on 2.5% agarose gel electrophoresis with DNA ladder standard 100 bp (Jena Bioscience, Germany), stained with 0.5 µg/ml ethidium bromide, visualized on a UV Transilluminator, photographed by Gel Doc. System and analyzed with software data analysis for Bio- Rad Model 620 USA.

Statistical analysis

The presence / absence RAPD and 16S rRNA PCR-RFLP data were analyzed using the SPSS-PC programs of (Nie et al., 1975). Pair-wise comparisons between strains were used to calculate the genetic similarity values (F) derived from Dice similarity coefficient.

RESULTS

Five different bacterial isolates were selected on the basis of production of halo zone around the bacterial colonies and growth in high concentrations of CP (100-300 mg /L). Morphological, cultural and biochemical parameters were determined and illustrated in the Table 2. Degrading bacterial isolates were characterized and identified as *Pseudomonas stuzeri* (B-CP5), *Enterobacter aerogenes* (B-CP6), *Pseudomonas pseudoalcaligenes* (B-CP7), *Pseudomonas maltophila* (B-CP8) and *Pseudomonas vesicularis* (B-CP9), by consulting Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984) and Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). *Pseudomonas stuzeri* (B-CP5) was selected as the most potent CP- utilizing bacterial isolate.

Parameters controlling the Chlorpyrifos biodegradation by *Pseudomonas stuzeri* B-CP5

Data recorded in Table 3 showed that, when the *Pseudomonas stutzeri (*B-CP5) was grown on MS medium supplemented with 0.3 ml/L Chlorpyrifos as sole source of carbon and energy proved to be capable of grown with 7 days as optimum incubation period, preferred Chlorpyrifos concentration 0.1-0.35 ml/L, maximum inoculum size (0.5 ml), optimum temperature (30°C), optimum pH (7), favorite sugar (fructose) and preferred nitrogen source (ammonium nitrate) under shaking rotation of (100 rpm). Below and above optimal incubation period, Chlorpyrifos concentrations, inoculum size, temperature, pH, the phenolic compounds production decreased gradually. Fructose only was induced in biodegradation of Chlorpyrifos whereas all tested carbon sources failed to induce the production of phenolic compounds. Ammonium nitrate was exhibited the most preferd nitrogen source whereas other tested nitrogen sources failed in biodegradation of Chlorpyrifos.

Molecular genetics characterization

RAPD and 16S rRNA PCR-RFLP

RAPD and 16S rRNA PCR-RFLP techniques were used to conduct the genetic fingerprinting, construct the genetic relationship and determine genetic distance between the studied isolates and identify specific molecular markers for most potent isolate. An informative profile was obtained (Figures 1 and 2). The six used primers produced multiple band profiles with a variable number

Table 2. Characteristics of Chlorpyrifos-degrading strains isolated from contaminated Egyptian soils.

Test	CP5	CP6	CP7	CP8	CP9
Gram's reaction	_	_		_	_
Cell shape	Coccobacilli	Short rods	Short rods	Short rods	Short rods
Methyl red	+	+	+	+	+
Motility	+	+	+	+	+
Spores	_	_	-	-	_
Catalase	+	+	+	+	+
KoH (3%)	+	+	+	+	+
Oxidase	+	+	+	+	+
Methyl red	+	+	+	+	+
Levan formation	+	+	+	+	+
Citrate utilization	+	+	+	+	+
Voges-Proskauer test	_	-	_	_	_
Nitrite reduction	_	_	_	_	_
Fermentation of glucose	+	+	+	+	+
Fermentation of mannose	+	+	+	+	+
Fermentation of starch	_	_	_	+	+
Fermentation of arabinose	_	_	_	_	_
Fermentation of fructose	_	_	_	_	_
Fermentation of lactose	_	_	_	_	_
Fermentation of mannitol	_	_	_	_	_
Fermentation of rhamnose	_	_	_	_	_
Fermentation of sucrose	_	+	_	_	_
Production of amylase	+	+	+	_	+
Production of pectinase	+	_	_	_	_
Production of cellullose	+	+	+	+	_
Production of gelatinase	_	_	+	+	+
Production of H_2S	+	_	_	_	_
KCN resistance	_	_	_	+	_

Table 3. Parameters controlling the biodegradation of Chlorpyrifos by *Pseudomonas stuzeri* –B-CP5.

Incubation periods (days)	Phenolic compounds g/ml	CP Concentration (ml/L)	Phenolic compounds g/ml	Inoculum size (ml/l)	Phenolic compounds g/ml	Temperature (ºC)	Phenolic compounds g/ml	pH	Phenolic compounds g/ml
1	0.024	0.1	0.256	0.1	0.199	20	0.123	5	0.0
2	0.045	0.15	0.233	0.2	0.298	25	0.199	5.5	0.0
3	0.084	0.2	0.21	0.4	0.244	30	0.229	6	0.199
4	0.122	0.25	0.188	0.5	0.20	35	0.21	6.5	0.25
5	0.157	0.3	0.132	1	0.142	40	0.12	7	0.299
6	0.190	0.35	0.122	2	0.121	45	0.11	7.5	0.312
7	0.234	0.4	0.02	2.5	0.122	50	0.11	8	0.12
8	0.20	0.45	0.01	5	0.1			8.5	0.12
9	0.14	0.5	0.01					9	0.11

and molecular weights of amplified DNA fragments (Table 1). Different polymorphic and monomorphic markers were obtained across RAPD profiles (Table 4 and 6). As regards genetic relationships, data presented in (Table 5) showed that, the highest genetic similarity was between CP8 and CP7& (66%), while the genetic similarity between CP8 and CP6 was the lowest (37%). All isolates gave the same pattern after digestion with two

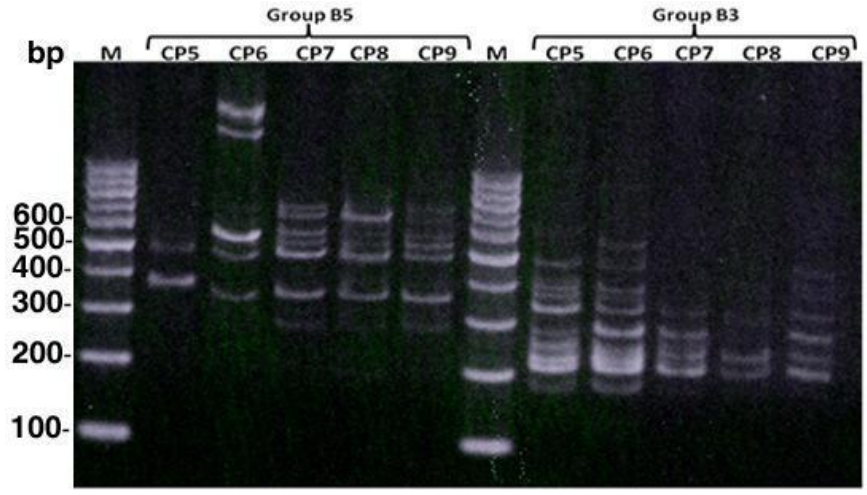

Figure 1. RAPD profile of soil bacterial isolates (CP5, CP6, CP7, CP8 and CP9) revealed from RAPD B5 primer (Group B5) and B3 primer (Group B3).

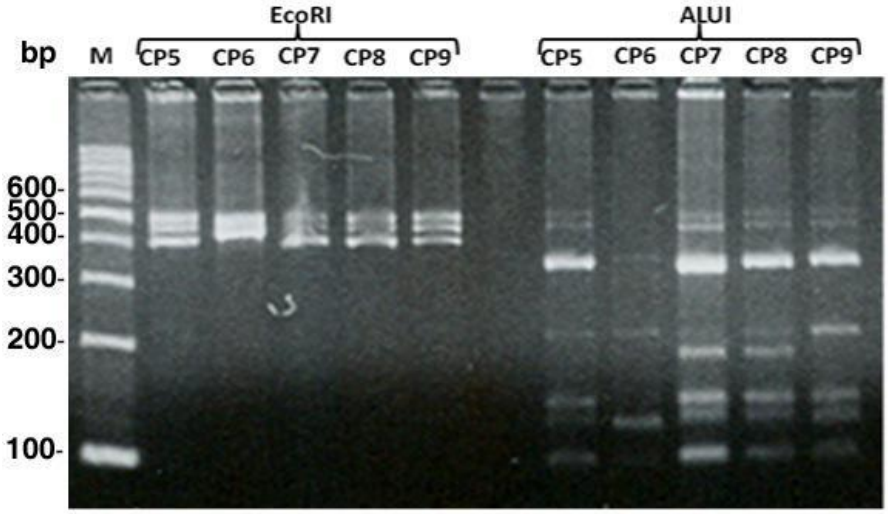

Figure 2. Restriction analysis of a 16SrRNA gene fragment amplified by PCR after digestion with *EcoR1* and *ALU1.*

restriction enzymes (*EcoRI* and *AluI*) separately except CP6 isolate (Figure 2).Two monomorphic bands were obtained (200 and 500 bp) among five bacterial isolates after digestion. Two specific bands for CP6 were identified (420 and 130 bp) with *EcoRI* and ALUI respectively (Figure 2).

DISCUSSION

Obtained molecular genetic data documented that the studied isolates belongs to two different genera as showed in Figure 3. The dendrogram showed phylogenetic tree which divided into two main groups. The first main group consisted of two subgroups. The first subgroup contains CP7, CP8 and CP9 isolates. The closest genetic distance was found between CP7and CP8 isolates, which were first clustered together and then with CP9 isolate. The second subgroup of the first main group includes CP5 isolate. On the other hand, the second group includes CP6 isolate. These two genera have focused on its wide range of diverse degrading capabilities and potential application in bioremediation. This is might be due to the great genetic diversity among these two genera which confirmed by different pattern of amplification and restriction enzyme digestion based on RAPD and PCR-RFLP. Chlorpyrifos has been reported to be degraded co-metabolically by bacteria, which needs extra carbon sources (Richins et al., 1997; Mallick et al., 1999). (Singh et al., 2004) reported isolation of

Table 4. Specific markers for each chlorpyrifos degrading bacterial isolate across RAPD-PCR analysis.

Primer	Bacterial strain					Total
	CP5	CP6	CP7	CP8	CP9	
A2		5(350,520,780,850,870bp)		1(580 bp)	1(320bp)	7
A3		4(230,280,630,680bp)		1(430bp)	1(850bp)	6
B3	2(680,550 bp)	4(500,530,630,700 bp)		2(580,620 bp)		8
B5	1(600 bp)	3(150, 620, 670 bp)				4
C3	2(280,680 bp)	4(480,520,620,740 bp)			2(270, 800bp)	8
C5		6(250,280,320,430,550,740 bp)				6
Total	5	26	0	4	4	39

Table 5. Similarity coefficient values among the five studied bacterial isolates.

Isolates	CP 5	CP 6	CP7	CP8	CP 9
CP 5	1.00				
CP 6	0.40	1.00			
CP 7	0.44	0.44	1.00		
CP 8	0.53	0.37	0.66	1.00	
CP 9	0.54	0.40	0.55	0.62	1.00

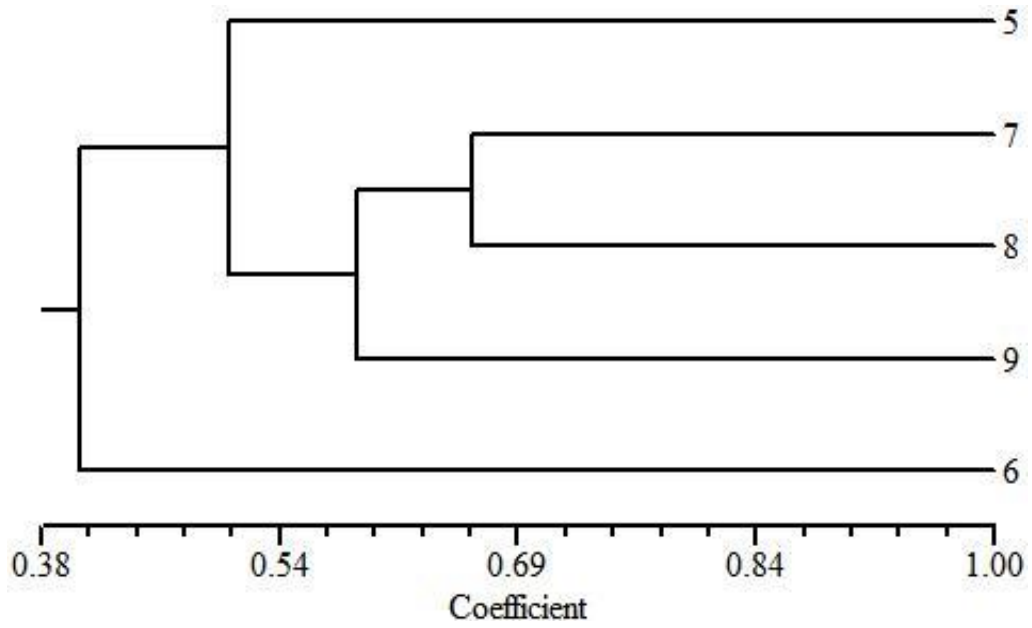

Figure 3. Dendrogram demonstrating the relationships among the five studied bacterial isolates.

Enterobacter sp. B14, which can use CP for the supply of carbon and phosphorous sources, which it stopped degrading CP in the presence of other carbon sources. However, strain *Pseudomonas stuzeri* (B-CP5) shows a more rapid degradation in the presence of an additional fructose as carbon source. This indicates that CP could also be degraded co-metabolically by *Pseudomonas stutzeri* (B-CP5), which might signify the environmental adaptation of this bacterium. *Pseudomonas stutzeri* (B-CP5) was distinguished and characterized by three random primers (B3, C3 and B5) among six used primers. Primer B3 generate two polymorphic bands (550 and 680 bp), C3 obtain two polymorphic bands (280 and 680 bp) while B5 release one polymorphic band (600 bp).These polymorphic bands considered as specific markers for *Pseudomonas stutzeri* with the three mentioned primers Table 4. On the other hand CP6 belongs to other genera so it was generate 26 polymorphic bands

Table 6. Specific markers for bacterial isolate across RAPD and16SrRNA PCR-RFLP analysis.

Primer	Isolate	Specific marker (M.W. bp)
C5	CP6:*Enterobacter aerogenes*	250, 280, 320, 430, 550 and 740
B3	CP5: *Pseudomonas stuzeri*	550 and 680
	CP6:*Enterobacter aerogenes*	500, 530 and 680
	CP8:*Pseudomonas maltophila*	520, 580 and 630
C3	CP5: *Pseudomonas stuzeri*	280 and 680
	CP6:*Enterobacter aerogenes*	480, 520, 620 and 740
	CP9:*Pseudomonas vesicularis*	270 and 800
B5	CP5: *Pseudomonas stuzeri*	600
	CP6:*Enterobacter aerogenes*	150, 620 and 750
A2	CP6:*Enterobacter aerogenes*	350, 520, 780, 850 and 870
	CP8:*Pseudomonas maltophila*	580
	CP9:*Pseudomonas vesicularis*	320
A3	CP6:*Enterobacter aerogenes*	230, 280, 630 and 680
	CP8:*Pseudomonas maltophila*	430
	CP9:*Pseudomonas vesicularis*	850
EcoRI	CP6:*Enterobacter aerogenes*	420
AluI	CP6:*Enterobacter aerogenes*	130

among six used random primers and two polymorphic bands (420 and 130 bp) with EcoRI and AluI, respectively. These polymorphic bands considered as specific markers for *Enterobacter aerogenes*.

Chlorpyrifos has been reported to be degraded in liquid media by *Flavobacterium* sp. (Sethunathan et al., 1973), *Pseudomonas diminuta* (Serdar et al., 1982) and *Arthrobacter* sp. (Mallick et al., 1999), which were initially isolated to degrade other organophosphate compounds. However, these microorganisms do not utilize CP as a source of carbon but as a co-metabolite (Singh et al., 2003; Yang et al., 2006). Recently a *Stenotrophomonas sp*. has been isolated, which is capable of degrading both CP and TCP (Yang et al., 2006; Lakshmi et al., 2008)

In conclusion, among the five studied isolates *Pseudomonas stuzeri* (B-CP5) was the most potent isolate to degrade the Chlorpyrifos. The results of this study demonstrate the usefulness of the RAPD-PCR analysis for detecting DNA polymorphism in bacterial isolates and establishing the relationships among different isolates. The majority of random primers used gave distinctly reproducible patterns in the entire isolates studied. However, primers are varied in the extent of information. Primers B5 and A2 revealed highly polymorphic patterns. Whereas, less polymorphic products were generated by C3 primer. Some DNA fragments were apparently similar in size among the studied isolates, whereas others were unique to a particular isolate. Therefore, different polymorphic and monomorphic bands were given and some of monomorphic considered as specific markers for identifying and tracking these bacterial isolates.

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