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Isolation and Identification of Herbicide Utilizing Bacteria

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Summary

Soil samples were collected from Baghdad, Babel and Kerkoack were used in order to isolate herbicide utilizing bacteria.

From 20 soil samples, 37 isolates were isolated as 2,4-D utilizer . From 5 soil samples, 5 isolates were isolated as an atrazine utilizer. Another 12 isolates were isolated as a treflan utilizer from a 11soil samples. Among these 54 isolates, 47 isolates succeeded to grow on 2,4-D, atrazine and treflan after confirming their ability to utilize these herbicides.

Among all isolates, only 20 efficient isolates were identified. Results showed that 15 isolates belong to the genus Pseudomonas, 4 isolates belong to the genus Bacillus and 1 isolate belongs to the genus Acinetobacter.

Introduction

Modern agricultural and industrial activities depend on a wide variety of synthetically produced chemicals including insecticides, fungicides, herbicides and other pesticides. The fate of the applied herbicide compounds is of great importance, since disappearance, persistent, or partial transformation of such compounds determines its usefulness or its potential hazardous effects [1].

Microorganisms in the soil, metabolize organic herbicides either aerobically or anaerobically [2,3].Members of the genus Pseudomonas have evolved considerable nutritional versatility and are capable of utilizing a range of complex aromatic compound including oil compounds and xenobiotics [4] .Pseudomonas putida, Pseudomonas fluoreseens and Pseudomonas aeruginosa were found to be the most efficient species of the genus Pseudomonas that have the ability to degrade hydrocarbon compounds [5].

Pseudomonas putida was the most important species of the genus Pseudomonas found to have the ability to convert a variety of environment pollutants, including herbicides, to a small number of structurally simple aromatic compounds that are the starting points for pathways of aromatic ring fusion, such as 2,4-D; 2,4,5-T, MCPA and atrazine [6].

Therefore, in the present study, we investigated the prevalence of bacteria in Iraqi soil which have the ability to utilize herbicide (2,4-D, atrazine, treflan), identified bacterial species and determine the efficient bacterial isolates utilizing these herbicides.

Materials and Methods

Culture media

Amy mineral salt medium [7]

This medium was used with slight modification as mentions.

K_2HPO_4 (1g), $MgSO_4.7H_2O$ (0.2g), NaCl (0.1g), $FeCl_2$ (0.02g) (modified to 0.005g), NH_4SO_4 (1g) (modified to NH_4NO_3) and distilled water 1000ml.pH was adjusted to 7.5 and sterilized by autoclaving at 121 °C for 15min., then (0.1%w/v) of herbicide (sterilized by filtration) was added.

- Enichiyama mineral salt medium [8]

This medium was prepared by dissolving K_2HPO_4 (1.17g), KH_2PO_4 (0.121g), $MgSO_4.7H_2O$ (0.121g), NH_4Cl (2.14g), $FeSO_4.7H_2O$ (0.28g), $MnSO_4.4H_2O$ (0.06g), H_3BO_3 (0.005g), $ZnSO_4.7H_2O$ (0.1g), $CuSO_4.5H_2O$ (0.06l), $Co(NO_3)_2.6H_2O$ (0.06g), $NiSO_4.7H_2O$ (0.00806g) and distilled water 1000ml.pH was adjusted to 7.5 and sterilized by autoclaving at 121°C for 15min, then (0.1%w/v) of herbicide (sterilized by filtration) was added.

-Trypticase soya agar (Biolife)

This medium was prepared as recommended by manufacturing company. pH was adjusted to 7.4 and autoclaved at 121°C for 15min .

Sample collection

Soil samples were collected from different agricultural farms in Baghdad, Babel and Kerkoack governorates. 2,4-D, atrazine and treflan were the main herbicides used their for many years ago. Soil samples were transferred to the laboratory using

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sterile plastic bags to isolate herbicide-utilizing bacteria.

Bacterial isolation

Microbial selection procedure was performed using Amy mineral salt medium amended with 0.1% of herbicide. Twenty milliliter of the culture medium was placed in 100ml Erlenmeyer flask; one percent (w/v) of soil sample were added to the flasks and incubated at 30°C with shaking (180 rpm) for seven days. Samples (0.1ml) from appropriate dilution were spread on plate of trypticase soya agar plates, incubated at 30° C for 48hrs .A single colony was picked with a sterile loop to prepare a pure subculture in a fresh trypticase soya agar plate by streaking.

Measurement of bacterial growth

Growth of bacteria was monitored measuring the optical density of liquid culture using spectrophotometer (spectronic 20) at 600 nm.

Ability of bacterial isolates in herbicides utilization

Bacterial isolates were grown in 2,4-D, atrazine and treflan as a sole source of carbon and energy in order to determine their ability in herbicide utilization. A twenty-milliliter of Unichiyama mineral salt medium distributed in 100ml Erlenmeyer flask, the flasks were sterilized by autoclaving, then herbicides (sterilized by filtration) were added. Three replicate were cultured for each bacterial isolate. All the flasks were inoculated with 1% of fresh culture (18hrs), and incubated in shaker incubator (180rpm) at 30°C for 7 days.

Identification of bacteria

Bacterial isolated were then identified depending on its morphological, physiological and biochemical characteristics as recommended by Holt et al.[9].

Results and discussion

Isolation of bacteria

Thirty seven isolates were able to utilize of 2,4-D(termed HD); five isolates were able to utilize atrazine (termed HA) and twelve isolates as utilizers of treflan (termed HT). Results indicated that the numbers of bacteria have the ability to utilize 2,4-D were more than bacteria capable of utilizing atrazine and treflan and that may be attributed to the repeat application of 2,4-D in Iraqi soil more than atrazine and treflan, and that leads to increase the adaptation of soil bacteria to this herbicide [10] or the transfer of the degradative cahapility of this herbicide in the microbial population [11].

Isolates were repeatedly tested for their ability to utilize 2,4-D, atrazine and treflan, in order to ensure their utilization ability. Among 54 isolates, only 47 isolates have the ability to utilize the studied herbicides (table 1). Seven of the total isolates haven't the ability to utilize anyone of the tested herbicides; this may be attributed to that those isolates can benefit from the metabolites of other bacteria which utilize applied herbicide(s) [12].

Table(1).Growth density of the bacterial isolates on 2,4-D, atrazine and treflan after7days of incubation at 37 °C.

Herbicides	Growth after 7 days (O.D.600nm)	Number of isolates	Symbol of isolates
2,4-D	0.05-0.2	10	HD1,HD8,HD10,HD15,HD22, HT1,HT2,HT7,HT8,HA4
	0.21-0.4	21	HD2,HD3,HD13,HD16,HD17, HD18,HD20,HD29,HD33, HD35, HD36,HT3,HT4,HT6, HT10,HT11,HA1,HA2,HA3, HA3,HA5
	0.41-0.8	36	HD6,HD7,HD11,HD13,HD21, HD24,HD25,HD26,HD27, HD28, HD30,HD32,HD34, HD37, HT5, HT9
Atrazine	0.05-0.2	15	HD1,HD2,HD8,HD10,HD15, HD16,HD20,HD22,HD36, HT1,HT7,HT8,HT10,HT11, HA2
	0.21-0.4	19	HD3,HD11,HD17,HD26, HD27,HD29,HD30,HD31, HD34,HD35,HD37,HT2, HT3,HT4,HT6,HT12,HA1,HA3, HA5
	0.41-0.8	12	HD6,HD7,HD13,HD18, HD21,HD24,HD25,HD28,HD32 HD33,,HT5,HT9
Treflan	0.05-0.2	16	HD1,HD2,HD8,HD15, HD16,HD17,HD20,HD22,HD36,HT1,HT3, HT6,HT7,HT10,HA4, HA3
	0.21-0.4	18	HD6,HD11,HD13,HD18, HD29,HD30,HD32,HD33, HT2,HT4,HT8,HT11, HT12,HA1,HA2,HA3, HA5

According to the growth density at 600nm of the isolates grown on mineral salt medium containing suitable herbicides along seven days of incubation, twenty isolates were identified as the efficient isolates in utilizing the three types of herbicides. However it is difficult to predict which molecular change can be expected by a specific microbe, since each group of microorganisms, even various strains of one genus, can alter a selected molecule differently [13]. Results also indicated that there was, in general, a lag phase of two days before active growth ensued, as it was indicated by an increased in the turbidity of the culture and that may be attributed to the various molecular mechanisms enable microorganisms to recruit genes and modify nucleotide sequences in the structural and regulatory genes to enhance expression [4]. Haugland et al. [14] reported that the specific growth rate at 600nm of 2,4-D degradation by a pure and mixed culture of P. cepacia AC1100 was started after one day of incubation, the efficient growth density would reach more than 0.4. Harris [15] also reported that Pseudomonas sp. strain D have a specific growth density 0.24±0.02 on atrazine as a source of nitrogen and energy after twelve hours of incubation in condition of carbon and nitrogen limitation.

Results also indicated that the most efficient isolates were HD7, HD21, DH24, HD28, HT5, and HT9 (figure 1), and from them, HD24 isolate was rapidly grown after two days of incubation, and that may be attributed to the physiological and genetic properties of these isolates [9].

Results also indicated that the most efficient isolates were HD7, HD21, DH24, HD28, HT5, and HT9 (figure 1), and from them, HD24 isolate was rapidly grown after two days of incubation, and that may be attributed to the physiological and genetic properties of these isolates [9].

Identification of herbicide utilizing bacteria

From the over all isolates only twenty efficient isolates were identified (table 2), these isolates were characterized as. Pseudomonas sp. (15 isolate), Bacillus sp. (4 isolates), and Acinetobacter sp. (1 isolate) The results of morphological, physiology, and biochemical tests for isolates were agreed with Holt et al [9]

The results showed that 75% of identified bacteria belong to genus Pseudomonas; most of them were P. aeruginosa except one Pseudomonas strain, which are known to exhibit a wide range of metabolic activites against most of their extreme nutritional versatility [16].Nasier et al.,[17] recorded that Pseudomonas sp. were the most abundant and the most efficient bacterial species have the ability to utilize crude oil and different hydrocarbon compounds in Iraqi soil.

Bacterial genera such as Pseudomonas, Acinetobacter, Arthrobacter, Corynebacterium, and Alcaligenes have been shown to utilize 2,4-D [18,19]. Harris [15] reported that Pseudomonas sp. also have the ability to utilize atrazine as a sole source of carbon and energy, which can help in removal of atrazine present in the soil or ground water. The ability of microorganisms to utilize the herbicide treflan as a sole source of carbon and energy and help in eliminating their toxicity from the soil was recorded also by Devlin et al. [20].

Microbial degradation of herbicides is usually cheap and environmentally friendly and entails no major technical hurdles compared with physiochemical means, for that the ability to isolate specific type of microorganisms can cleaning a dump site by herbicide is so much important in pollution prevention.

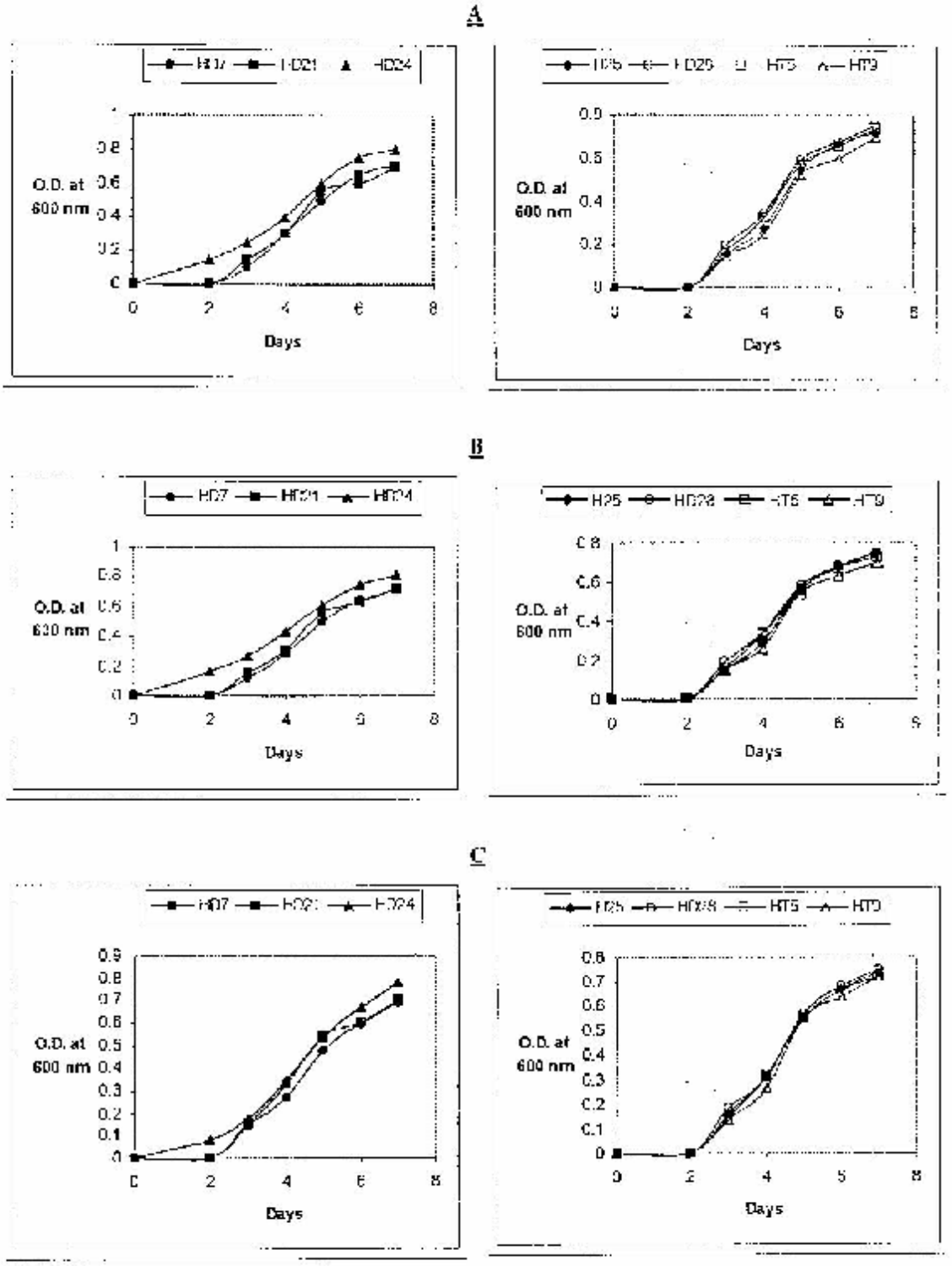

Figure (1). Growth curve of the 7 efficient isolates along 7 days of incubation on (A) 2,4-D;(B) Atrazine and C) Treflan.

Table (2). Morphological, physiological and biochemical characteristics of the locally isolated herbicide utilizing bacteria.

Characters		Strains						
		HD6	HD7	HD11	HD13	HD17	HD18	HD21
Colony color		White	Green	White	Green	White	Green	Green
Cell shape		Rod	Rod	Rod	Rod	Rod	Rod	Rod
Gram's stain		+	-	+	-	+	-	-
Catalase production		+	+	+	+	+	+	+
Oxidase production		-	+	-	+	-	+	+
Growth on king A		-	+	-	+	-	+	+
Growth on king B		-	-	-	-	-	-	+
Growth on Cetrimide		-	+	-	+	+	+	+
Gelatin hydrolysis		+	+	+	+	+	+	+
Growth on 4°C		+	-	+	-	+	-	-
Growth on 42°C		+	+	+	+	+	+	+
Nitrate reduction		+	+	-	+	+	+	+
Urea hydrolysis		-	+	-	+	+	+	+
Starch hydrolysis		+	+	+	+	+	+	+
Citrate utilization		-	+	-	+	-	+	+
Methyl red		-	+	-	+	+	+	+
Voges-proskauer		+	-	-	-	+	-	-
Growth on Kligher's iron agar	Slant	Alkaline	Alkaline	Alkaline	Alkaline	Alkaline	Alkaline	Alkaline
	Butt	Alkaline	Alkaline	Acid	Alkaline	Acid	Alkaline	Alkaline
	H_2S Production	-	-	-	-	-	-	-
	Gas Formation	-	-	-	-	-	-	-

Characters	Strains						
	HD24	HD25	HD26	HD28	HD30	HD31	HD32
Colony color	Green	Green	Green	Green	Green	Green	Green
Cell shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Gram's stain	-	-	-	-	-	-	-
Catalase production	+	+	+	+	+	+	+
Oxidase production	+	+	+	+	+	+	+

Growth on king A		+	+	+		+	+	–
Growth on king B		-	+	-	-	-	-	-
Growth on Cetrimide		–	–	+	+	+	–	+
Gelatin hydrolysis		+	+	+	+	+	+	+
Growth on 4°C		-	-	-	-	-		-
Growth on 42°C		+	+		+	+	+	–
Nitrate reduction		+	+	–	+	+	+	
Urea hydrolysis		+	+	–	+	+	+	
Starch hydrolysis		+	+	+	+	+	+	+
Citrate utilization		+	+	+	+	+		+
Methyl red		+	+	+	+	+	–	+
Voges-proskauer		-	-	-	-	-	-	-
Growth on Kligler's iron agar	Slant	Alkaline	Alkaline	Alkaline	Alkaline	Alkaline	Alkaline	Alkaline
	Butt	Alkaline	Alkaline	Alkaline	Alkaline	Alkaline	Alkaline	Alkaline
	H_2S Production	-	-	-	-	-	-	-
	Gas Formation	-	-	-	-			-
Characters		Strains						
		HD33	HD34	HD35	HD37	HT5	HT9	
Colony color		Green	Green	White	Green	Green	White	
Cell shape		Rod	Rod	Rod	Rod	Rod	Coco-bacilli	
Gram's stain		-	-	-	-	-	-	
Catalase production		–	+	+	-	+	+	
Oxidase production		–	+	+	+	+	-	
Growth on king A		+	+	-	-	+	-	
Growth on king B		-	-		-	-	-	
Growth on Cetrimide		+	+	+	+	+	-	
Gelatin hydrolysis		+	+	+	-	+	-	
Growth on 4°C		-	-	-	-	-		
Growth on 42°C		+	+	+	–	+	+	
Nitrate reduction		+	+	+	+	+	+	
Urea hydrolysis		+	+	+	+	+	+	
Starch hydrolysis		+	–	+	+	+	+	

Citrate utilization		+	−	+	−	+	+	
Methyl red		+	+	-	+	+	-	
Voges proskauer		-	-	−	+	-	-	
Growth on Kligler's iron agar	Slant	Alkaline	Alkaline	Alkaline	Alkaline	Alkaline	Alkaline	
	Butt	Alkaline	Alkaline	Alkaline	Acid	Alkaline	Alkaline	
	H_2S Production	-	-	-	-	-	-	
	Gas Formation	-	-	-	-	-	-	

Bacillus = HD6, HD11, HD17, HD35.

Pseudomonas aeruginosa = HD7, HD13, HD18, HD21, HD24, HD25, HD26, HD28, HD30, HD31, HD32, HD33, HD34, HT5.

Pseudomonas sp. = HD37.

Acinetobacter sp. = HT9.

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الخلاصة

جمعت عينات تربة من حقول مختلفة استخدمت فيها مبيدات الاعشاب وبشكل متكرر ولسنوات طويلة في كل من محافظة بغداد وبابل وكركوك.

تم عزل 37 عزلة بكتيرية من 20 عينة جمعت من الحقول المعرضةلمبيد 2,4-D و5 عزلات من 5 عينات جمعت من الحقول المعرضة لمبيد الاترازين و12 عزلة من 11 عينة جمعت من الحقول المعرضة لمبيد التريفلان.

أظهرت 47 عزلة بكتيرية من مجموع البكتريا المعزولة (54) قدرة على استهلاك مبيدات الاعشاب الثلاثة بعد اعادة التأكد من قابليتها على استهلاك المبيدات الثلاثة ، وشخصت 20 عزلة بكتيرية منها والتي تميزت بقابليتها الجيدة على النمو على المبيدات الثلاثة، وتبين ان 15 عزلة منها تعود لجنس Pseudomonas. وأربع عزلات تعود لجنس Bacillus وعزلة واحدة تعود لجنس Acinetobacter.