Factors affecting potentials of certain bacterial isolates for atrazine bioremediation

Ayman H. Mansee*, Nabila. M. Bakry, Doaa. M. Abd El-Gwad

(*Pesticide Chemistry and Technology Dept., Faculty of Agriculture, Alexandria University, Egypt*)

Abstract: Different bacterial isolates from two types of soil with different atrazine applications history were propagated. Four isolates from each soil sample (clay loam and calcareous) were classified, counted, and tested for their atrazine degradation efficacy. Isolates showed good capabilities for degrading 0.06 mM atrazine reached to 47.97% after 48 hrs. These percentages were found to be correlated to isolates counts in the soil. Calcareous soil isolates showed high intensity for atrazine degradation than to those of clay loam soil isolates. Optimum conditions such as pH, temperature, atrazine concentration, and addition of cobalt chloride influences isolates atrazine degradation efficiencies have been studied. The optimum temperature degree was recorded as 25ºC; while at 35ºC an enhanced degradation percent was recorded for 0.12 mM of atrazine treatment. Isolates from calcareous soil showed enhancement for atrazine degradation at pH 3 while those of clay loam soil presented the most efficiency at pH 5. Identification of isolate F was conducted using molecular methods indicating the validity of *Pseudomonas* sp.

Keywords: atrazine, bioremediation, soil contamination, *Pseudomonas* sp., temperature –pH, cell abundance

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1 Introduction

Herbicides have great influences on agriculture industry. However; their side effects could possibly threaten different environmental phases and usually resulting soil and water contamination. This gets the attention of scientists to introduce and improve their remediation. One major example is atrazine (2-chloro-4-ethylamino-6-isopropylamino-1, 3, 5-triazine), which is classified as one of the most widely used triazine herbicides to control a variety of upland weeds (Koskinen and Clay, 1997), primarily those growing during corn production. Atrazine and its toxic chlorinated dealkylated metabolites are frequently detected in surface- and ground-waters (Kalkhoff *et al,* 2003). Concerns for atrazine application due to their impact on human health and ecosystems are illustrated (Oh *et al,* 2003). Moreover; recent toxicological studies have raised concern of atrazine being a possible carcinogen, endocrine disrupter and teratogen (Mizota and Ueda, 2006). Consequently, atrazine has been classified by regulatory agencies as one of the major target anthropogenic pollutants requiring immediate attention and effective development decontaminating methods for contaminated matrices (Pandey *et al,* 2009, and Wackett *et al,* 2002).

Egypt and other countries have banned the use of atrazine in 2006 as a result of its effects on mammals as well as other environmental hazards effects. This was mandatory health requirement.

Such situation has stimulated research on efficient bioremediation strategies to decontaminate polluted environments as well as stocks in storage places locally in Egypt and worldwide. Thus, searching for atrazine microorganism capable of metabolizing atrazine was targeted by many authors (Wackett *et al,* 2002; El Sebaï *et al,* 2011; Wang and Xie, 2012; Swissa *et al,* 2014).

Current study aimed to isolate different bacterial isolates from two types of soil that has different atrazine

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***Corresponding author: Ayman H. Mansee,** Pesticide Chemistry and Technology Dept., Faculty of Agriculture, Alexandria University, Egypt. Email: amansee@alex-agr.edu.eg.

application history. Their potentials for atrazine degradation were investigated under different conditions; and finally identify the F isolate that showed promising capability for atrazine decontamination and will be used in further studies.

2 Materials and Methods

2.1 Chemicals

Technical atrazine, standard (≥98%) and formulated (Atrazex 80% W.P) were obtained from Kafer El-Zayat Pesticides and Chemical Company, Kafer El-Zayat, Egypt.

All analytical chemicals were obtained from El-Gomhouria Company for trading chemicals and medical appliances, Alexandria, Egypt. Thiamine-HCl, biotin, folic acid, nicotinamide, and pyridoxine-HCl were obtained from Sigma–Aldrich Chemical Co., USA.

2.2 Soil

Two types of soil samples were collected from the top layer 0-30 cm, air dried, crushed and sieved to pass through a 2 mm sieve. Calcareous soil, (1.54% organic matter, pH 7.92) was collected from corn field in El-Nahda region, Alexandria with different practices with herbicide application including atrazine. The second (Clay loam soil, 1.68% organic matter, pH 7.54) was collected from Kafer El-Zayat region, with a history of long term of atrazine applications.

2.3 Growth media

The used growth media were atrazine medium and nutrient agar medium (NA). The atrazine medium was prepared by dissolving the total of: 1.6 g of K_2HPO_4, 0.4 g of KH_2PO_4, 0.2 g of $MgSO_4.7H_2O$, 0.1 g of NaCl, 0.02 g of $CaCl_2$, 1 g of sucrose, 1 g of sodium citrate, 2.5 mL of atrazine stock solution, 20 ml of a salt stock solution, and 20 ml of a vitamin stock solution in one liter of de-ionized water.

The salt stock solution was prepared by dissolving the following salts in one liter of de-ionized water: 2.5 g of EDTA, 11.1 g of $ZnSO_4$, 5.0 g of $FeSO_4$, 1.54 g of $MnSO_4.H_2O$, 0.4 g of $CuSO_4.5H_2O$, 0.25 g of $Co(NO_3)_2$ $6H_2O$, 0.18 g of $Na_2B_4O_7.10H_2O$, and 5.0 ml of concentrated H_2SO_4 to delay precipitation of salts. Also, vitamins stock solution was prepared in 1 L deionized water: 5 mg of thiamine-HCl, 2 mg of biotin, 2 mg of folic acid, 10 mg of nicotinamide, and 10 mg of pyridoxine-HCl. However, atrazine stock solution was prepared in methanol (20 mg/mL), and the pH was adjusted to 7.3.

2.4 Bacterial Isolation

Two glass jars (40×70×30 cm^3) were filled separately by the two types of soil (Calcareous and Clay loam soil) and treated with half of the application rate (187.5 g/feddan) of formulated atrazine (atrazex 80% W.P, 375 g/feddan). Soil sample (1gm) was suspended in 9 milliliters of sterilized water and vigorously shaken for 10 minutes. Then 1 milliliter of the liquid was transferred to 9 milliliters of sterilized water (five times). From this freshly agitated dilution 1 milliliter was placed onto nutrient agar medium (NA) plates, and incubated for 5 days at 30ºC. Bacterial colonies were isolated and differentiated according to their morphological shape and growth pattern (Bergey's manual of systemic bacteriology; Krieg and Holt, 1984).

2.5 Cells Counting

Cells were counted to investigate their abundance of each isolate in the atrazine treated soil at 0, 7, 14, 21, 28 and 35 days using hemocytometer slide. One colony of each strain was transferred to 5 ml water, 10µl of the suspension were placed in the hemocytometer wells, and then visible cells were counted under microscope using 100X magnification.

2.6 Assaying isolates efficiency for atrazine biodegradation

The method of García-Gonzalez *et al,* (2003) for assaying atrazine degradation was used for evaluating the isolates efficiency. One colony of the isolated bacteria was incubated while shaking overnight at 30°C in 3 milliliters of atrazine medium, with ammonium chloride (4 mM) as the sole nitrogen source. Cells were harvested by centrifugation (15min/5000 rpm) and pellets were washed three times with phosphate-buffered saline solution and resuspended in atrazine treated medium. Cultures were subsequently shaken overnight at 30°C, cells were harvested by centrifugation (15 min/5000 rpm) and pellets were washed three times with U buffer (10 mM sodium phosphate pH 7, 0.1 mM $MgSO_4$) and resuspended to reach an OD_{600} of 0.25. Five milliliters were incubated with 50 µL of 6 mM atrazine to give a final concentration of 0.06 mM for 48 hours. Samples

(1 milliliter each) were centrifuged immediately for 3 min. at 5000 rpm. The atrazine concentration was determined by measuring the absorbance at 221 nm (A221) of the supernatants using UV- visible light spectrophotometer (Nicolet Evolution100 spectrophotometer, Thermo Electron Corporation).

2.7 Factors affecting atrazine biodegradation

2.7.1 Concentration

Three atrazine concentrations (0.03, 0.06, and 0.12 mM) were incubated with isolates for 48 hours, to investigate their biodegradation magnitude. Samples (1 milliliter) were taken and centrifuged immediately for 3 min at 5000 rpm. The remaining atrazine was determined in the supernatants at 221 nm.

2.7.2 Temperature

Effect of temperatures on the efficiency of isolated bacteria for degrading atrazine was tested at 5, 15, 25 and 35ºC for the three tested levels using phosphate buffer at pH 7.

2.7.3 pH

Influences of pH on the efficiency of isolated bacteria for degrading atrazine were tested at values: 3, 5, 7 and 9 for the three tested levels at 25ºC.

2.7.4 Influence of cobalt chloride

The method of Seffernick *et al,* (2009) was followed with slight modification to assess the effect of cobalt chloride on enhancing the isolates efficiency for atrazine degradation. One colony of isolated bacteria was resuspended in phosphate buffer pH 7, containing 0.1 mM $CoCl_2$ to reach an OD_{600} of 0.25. Five milliliters were incubated with 50 μL of 6 mM atrazine to get a final concentration of 0.06 mM for 48 hours. After incubation, samples (1 milliliter) were centrifuged immediately for 3 min at 5000 rpm, and remaining atrazine was determined at 221 nm as mentioned before.

2.8 Identification of F isolate

2.8.1 Morphological Identification

Culture characterizations including shape of bacterial cells, colony type and gram stain were utilized for identification according to Bergey's manual of systemic bacteriology.

2.8.2 Molecular Identification

The genomic DNA was extracted from the purified isolates following the method of Stock *et al,* (2001). The amplification of the 16S rDNA gene was performed using universal primers F-Start (5'-AGAGTTTGATCMTGGCT CAG-3') and End-R (5'-TACGGYACCTTGT TACGACT-3') to give a PCR product of approximately 1550 bp. The PCR amplification conditions were performed in a thermal cycler (Eppendorf, Germany) by an initial denaturation step at 95ºC for 5 min followed by 34 cycles at 95ºC for 45 sec, annealing at 50 ºC for 1 min and an extension at 72 ºC for 2 min followed by a final extension step at 72ºC for 10 min. The PCR products were purified using PCR purification kit (Qiagen, Germany) and the two strands of each amplicon were sequenced using ABIPRISM model 310 automated DNA sequencer at Sigma Scientific Services Company.

Sequences were then subjected to similarity BLAST searches (www.ncbi.nlm.nih. gov/blast) and the closely related sequences from GenBank were detected. The phylogenetic relationships of the experimental isolates and closely related species were analyzed using the multi-sequence alignment program (MEGA 4.1)

The selected sequences comparison and phylogenetic analyses of the partial sequencing of 16S rDNA were performed using the software MEGA 4.1. Neighbor-joining (Saitou and Nei, 1987) with Kimura 2-paramter (Kimura, 1980) method were used for computing the evolutionary distance, thousand bootstraps replicates were used to build the corresponding phylogenetic trees.

The sequence generated in this study was deposited in the GenBank under the accession U55933.1.

2.8.3 Statistical analysis

All experiments were statistically analyzed using SAS version 9.1.3 statistical software. Statistical differences between treatment means (P>0.05) were determined by LSD test.

3 Results

3.1 Bacteria isolates

Different bacterial isolates were taken place after treating the two types of soil with one half the application rate of atrazine (187.5 gm/feddan). Isolation was

performed using nonselective media technique as mentioned early. Differentiation between isolates was conducted based on morphological and growth feature according to Bergey's manual of systemic bacteriology and illustrated in Table 1. Isolates from calcareous soil were presented as A, B, C and D, while those from clay loam soil were F, G, H and I. Data presented in Table 1 showed that eight morphologically different phenotypes were isolated and purified. Three isolates (B, G, and H) were gram positive, while five isolates (A, C, D, E, and I) were gram negative. According to the cell shape five isolates (A, C, F, G, and I) were rod shape while the other isolates (B, D, and H) were *cocci*. Moreover, all isolates did not form spores; this may indicate the biodiversity of the consortium of isolates of each soil sample.

Table 1 Characteristics of isolated bacterial from calcareous soil and clay loam soil

Isolates	Characteristics		
	Shape of cells	Gram reaction	Spores
A	Rod	Negative	Negative
B	Cocci	Positive	Negative
C	Rod	Negative	Negative
D	Cocci	Negative	Negative
F	Rod	Negative	Negative
G	Rod	Positive	Negative
H	Cocci	Positive	Negative
I	Rod	Negative	Negative

3.2 Effect of Atrazine Applications on Isolated Bacterial Isolates Abundance

Cells of isolated bacteria from the two types of soil were counted to investigate the abundance of each isolate at 0, 7, 14, 21, 28 and 35 days after atrazine treatment. The results are recorded in Tables 2 and 3 for calcareous and clay loam soils, respectively.

3.3 Isolates of calcareous soil

The results obtained from calcareous soil (Table 2) showed that the calculated mean numbers of cells per gram soil for isolates A and B in the treated sample tended to decrease while those for isolate D increased for all tested intervals. The statistical analysis for this data showed significant differences between treated and control samples for isolates A, B, and D while not for C isolate.

Table 2 Effect atrazine application on isolated bacterial abundance in calcareous soil

Time, days	Number of bacterial cells $\times 10^5$/g soil			
	A	B	C	D
0	60	79	1	1
7	29	11	1	5
14	27	18	1	8
21	30	14	1	12
28	31	15	1	16
35	32	13	1	9
Treatment (*Mean)	34.83^{c}	25.0^{d}	1.00^{f}	8.50^{e}
Control (mean)	45.33^{b}	60.00^{a}	1.00^{f}	1.1^{f}
**$L.S.D_{0.05}$ isolates	0.512			
$L.S.D_{0.05}$ interaction	0.872			

Note: *Means labeled by the same letters are not significantly different at 0.05 levels. ** L.S.D.; Least significant difference at 0.05 level of probability.

3.4 Isolates of clay loam soil

The results showed that the numbers of cells per g soil for all isolates decreased at the 7th day (Table 3). At day 14th, the numbers of cells showed an increase for isolates F, G and H while decreased for isolate I. At the 21st day, no significant difference was observed in counts for isolates F and G, while; H and I increased. At the 28th day, isolates F and G started to show an increase while a decrease for H and no change for I was recorded. No change was noticed in the counts for all isolates after the period of 28 days. Statistical analysis showed significant differences between tested isolates as well as between treatment and control.

Table 3 Effects of atrazine application on isolated bacterial abundance in clay loam soil

Time, days	Number of bacterial cells $\times 10^5$/g soil			
	F	G	H	I
0	23	17	2	5
7	16	7	1	3
14	20	10	2	2
21	20	10	3	3
28	22	11	2	3
35	22	11	2	3
Treatment (*Mean)	20.50^{b}	11.00^{d}	2.00^{h}	3.17^{f}
Control (mean)	24.17^{a}	15.17^{c}	2.50^{g}	4.17^{e}
**$L.S.D_{0.05}$ isolates	0.457			
$L.S.D_{0.05}$ interaction	0.845			

Note: * Means labeled by the same letters are not significantly different at 0.05 levels.

Many authors have investigated the impacts of different pesticides applications on microbiological

properties including population densities. (Moreno *et al,* 2007; Littlefield-Wyer *et al,* 2008).

This study showed a reduction in isolates counts after 7 days of atrazine application, then a recovery in isolate counts for the following time intervals was observed. Similarly, Littlefied-Wyer *et al,* (2008) reported that all microbial communities came to a minimum level at day four after atrazine treatment. They also concluded that exposure to atrazine at a concentration above 245 mg/L may irreversibly change the structural and functional status of aquatic microbial communities. However, Moreno *et al,* (2007) observed an increase in microbial biomass due to atrazine application rates of 0.2, 0.6 and 1 mg/kg after 16 day of incubation period. Their atrazine application rates were lower than those applied in the current study. This could explain difference between our and their finding. Vargha *et al,* (2005) cited that bacterial cell count values were reduced by at least two orders of magnitude during filtration of the soil sample through a laboratory scale model system of Danube river sediment.

The present study showed an interesting trend for the two type of tested soil regard in their isolates abundance. This pattern showed four predominant isolates A, B, F, and G which present the highest abundant isolate. Isolates C and H were less in counting and presented secondary isolate type. The last two isolates D and I were obviously small and presented the minor isolates. These differences in abundance of isolates could be due to the specificity of those isolates to atrazine and its metabolites. Further studies for correlating the abundance of each isolate regarding role of target degradation pathway is urgently needed.

3.5 Capability of isolated bacteria for atrazine biodegradation

Each isolate was individually incubated with 0.06 mM of atrazine for 48 hrs to assess its capability for degrading atrazine. The efficiency was presented as percentage degradation in Table 4 for calcareous soil isolates (A, B, C and D) and clay loam soil ones (F, G, H and I). Isolate A was the most active for degrading atrazine (47.97%) followed by C (35.65%); while B and D gave 33.95 and 28.87%, respectively. For clay loam soil, all isolates were less active with atrazine degradation percentages of 22.61, 23.58, 13.98 and 5.15% for F, G, H and I isolates, respectively. It was observed that bacterial isolates from calcareous soil showed higher atrazine degradation efficiency than those of clay loam soil isolates. Also, the highest atrazine degradation percentage was obtained from isolate A (47.97%), while isolate I showed the lowest percentage (5.15%).

Table 4 Degradation percentages (0.06 Mm) of atrazine by isolated bacteria

Calcareous soil Isolates	Degradation, %	Clay loam soil Isolate	Degradation, %
A	47.97[a]	F	22.61[e]
B	33.95[b]	G	23.58[e]
C	35.65[c]	H	13.98[f]
D	28.87[d]	I	5.15[g]
**L.S.D$_{0.05}$ interaction			0.915

Note: * Means followed by the same letters are not significantly different at 0.05 levels.

The obtained results were parallel to those reported by Singh *et al,* (2004) who isolated an *Acinetobacter* species from a soil heavily contaminated with atrazine that capable for degrading up to 250 ppm. Also, Vaishampayan *et al,* (2007), isolated different species of bacteria from different agriculture soil that had history of atrazine applications. They reported that their isolates had the capabilities for metabolizing atrazine. Getenga *et al,* (2009) observed a very high capability of a tropical soil from a Kenyan sugarcane-cultivated field to mineralize ^{14}C-ring-labeled atrazine in laboratory experiments. This soil mineralized about 90% of the applied atrazine within 98 days. Moreover; Wang *et al,* (2010) introduced an *Arthrobacter* sp. strain HB-5 from industrial waste water that exhibited faster atrazine degradation rates in atrazine-containing mineral media than the well-characterized atrazine degrading bacteria isolate *Pseudomonas* sp. ADP. Strain ADP catalyzes the sequential hydrolytic removal of s-triazine ring substituent from the herbicide atrazine to yield cyanuric acid. Zhang *et al,* (2012) studied the metabolic ability and individual characteristics of an atrazine-degrading consortium DNC5 isolated from corn-planted soil. They illustrated the individual metabolic characteristics and the mutualism of the cultivable members in the consortium DNC5. Wang and Xie (2012) isolated high-efficiency

atrazine degrader whose degradation ability was not inhibited by organic matters and fertilizers in the soils. This finding is important for bioremediation of contaminated soils. They isolated a bacterial strain (DAT1) which was capable of utilizing atrazine as a sole nitrogen source for growth, from an agricultural soil in Hebei Province.

Finally, Wang *et al,* (2013) used a previously isolated atrazine-degrading bacterium, *Arthrobacter* sp. strain DAT1 for remediation of heavily atrazine-contaminated soil as bioaugmentation approach. Their results indicated that this bioremediation process could achieve a high removal rate in about 2-3 days.

3.6 Detecting Optimum Conditions for Isolates Efficacy

3.6.1 Effect of Atrazine Concentration and Applied Temperature on Its Biodegradation

Different concentrations of atrazine 0.03, 0.06 and 0.12 mM were incubated with the bacterial isolates of calcareous soil at four different temperature degrees (5, 15, 25 and 35ºC) to assess their biodegradation efficiencies for different concentration levels (Table 5). Raising the temperature from 5 to 35ºC increased atrazine degradation only for the highest tested concentration (0.12 mM). However, no effect was noticed for the lower concentrations (0.03 and 0.06 mM). The results also showed that isolate A was the most active isolate for degrading atrazine (63.23%) at 35ºC followed by B (37.45%), C (28.86%) and D (22.77%). Also, the data presented in Table 6, showed no significant differences in degradation rates at 35 ºC for 0.12 mM atrazine.

The overall trend showed an increase in biodegradation with increasing targeted atrazine concentration. Also, the maximum efficiency was achieved at 25ºC for both soil types' isolates, while slight enhancement was achieved at 35ºC especially for the 0.12 mM.

The optimum temperature from the current investigation was found to be 25ºC. However; Singh *et al,* (2004) and Vaishampayan *et al,* (2007) suggested 37ºC was the optimum temperature degree for atrazine biodegradation while 55ºC was cited by Bouquard *et al,* (1997). This difference in optimum temperature could be due to the variation in isolates types, targeted atrazine concentration, as well as other conditions prevailed.

Table 5 Effect of temperature (ºC) on degradation of different atrazine concentrations by calcareous soil bacterial isolates

Isolates	Conc., mM	Degradation of atrazine, %			
		5ºC	15ºC	25ºC	35ºC
A	0.03	44.22^{d}	46.85^{c}	46.30^{c}	38.46^{e}
	0.06	46.67^{c}	47.68^{bc}	48.98^{b}	47.66^{bc}
	0.12	36.69^{f}	46.85^{c}	49.24^{b}	63.23^{a}
B	0.03	35.03^{g}	34.85^{g}	34.82^{gh}	12.61^{q}
	0.06	26.80^{k}	30.25^{i}	32.98^{h}	31.22^{i}
	0.12	18.24^{p}	25.77^{kl}	28.33^{j}	37.45^{ef}
C	0.03	22.09^{n}	33.12^{h}	34.28^{g}	12.49^{q}
	0.06	23.29^{nm}	26.93^{k}	28.81^{j}	27.62^{j}
	0.12	21.00^{no}	25.94^{kl}	28.81^{j}	28.86^{j}
D	0.03	12.96^{q}	25.24^{l}	26.81^{k}	8.86^{r}
	0.06	21.39^{n}	20.47^{o}	23.63^{m}	$24.95l^{m}$
	0.12	19.62^{p}	17.46^{p}	27.98^{j}	22.77^{n}
$L.S.D_{0.05}$ interaction					1.34

Table 6 Effect of temperature (ºC) on degradation of different atrazine concentration (mM) by clay loam soil bacterial isolates

Isolates	Conc., mM	Degradation of atrazine, %			
		5ºC	15ºC	25ºC	35 ºC
F	0.03	22.31^{b}	20.82^{b}	22.72^{b}	1.71^{j}
	0.06	14.81^{e}	16.33^{de}	19.46^{c}	17.93^{c}
	0.12	18.14^{c}	16.31^{de}	19.70^{c}	22.59^{b}
G	0.03	15.54^{de}	25.47^{a}	25.42^{a}	0.10^{k}
	0.06	12.51^{f}	10.87^{f}	14.45^{e}	13.90^{ef}
	0.12	16.86^{d}	15.88^{de}	20.07^{c}	22.65^{b}
H	0.03	3.98^{i}	13.64^{ef}	13.79^{ef}	13.58^{ef}
	0.06	11.26^{f}	8.91^{g}	10.28^{g}	8.10^{g}
	0.12	17.73^{d}	13.97^{ef}	15.70^{de}	22.82^{b}
I	0.03	6.92^{h}	4.65^{i}	4.00^{i}	22.87^{b}
	0.06	13.98^{ef}	14.88^{e}	5.36^{h}	4.21^{i}
	0.12	16.35^{de}	12.56^{f}	15.23^{e}	22.54^{b}
*$L.S.D_{0.05}$ interaction					1.58

3.6.2 Effect of pH and concentration on atrazine biodegradation

The results presented in Table 7 for calcareous soil isolates showed that the atrazine degradation reached maximum at pH 3, while decreased at pH 9. Moreover, atrazine degradation percentage decreased at the level 0.12mM for most of the tested pH values (3, 5, 7 and 9).

Isolates of clay loam soil showed that the highest degradation percentage was recorded at pH 5, while the lowest degradation percentage was recorded at pH 3 (Table 8).

This difference of pH influence on the efficiency of isolates for atrazine degradation between the two tested soils could be due to soil type and types of microorganisms' as well. Similar trends were found by several authors (Mulleller *et al* 2010; Houot *et al* 2000; Wang and Xie 2012)

Table 7 Effects of pH on the Efficiency of Bacteria Strains Isolated from Calcareas Soil for Atrazine Biodegradation

Isolates	Conc., mM	Degradation of atrazine, %			
		pH 3	pH 5	pH 7	pH 9
A	0.03	57.07	44.99	45.84	36.32
	0.06	65.11	52.47	48.91	33.74
	0.12	44.59	51.00	50.93	51.02
B	0.03	62.16	35.62	35.38	27.26
	0.06	56.47	34.71	33.14	14.74
	0.12	21.17	29.87	31.02	15.85
C	0.03	56.40	33.67	34.23	18.83
	0.06	48.85	32.33	29.20	9.18
	0.12	24.41	31.45	31.89	29.39
D	0.03	67.44	29.24	28.07	12.34
	0.06	47.17	25.60	23.79	N.D
	0.12	11.43	28.13	30.44	14.75
**$L.S.D_{0.05}$ interaction				1.306	

Note: ** L.S.D.; Least significant difference at 0.05 level of probability.

Table 8 Effect of pH value on the Efficiency of Isolated Bacteria Strains of clay loam soil for Atrazine Biodegradation

Strains	Conc., mM	Degradation of atrazine, %		
		pH 3	pH 5	pH 7
F	0.03	50.94	27.02	23.14
	0.06	37.33	21.43	18.34
	0.12	10.15	27.70	23.21
G	0.03	47.82	26.40	25.30
	0.06	16.19	18.20	14.69
	0.12	16.91	28.34	22.91
H	0.03	41.88	16.79	13.83
	0.06	21.29	14.88	10.81
	0.12	11.43	26.39	19.88
I	0.03	39.23	7.50	4.84
	0.06	66.79	8.02	4.92
$L.S.D_{0.05}$ interaction			1.531	

Note: * L.S.D.; Least significant difference at 0.05 level of probability.

3.6.3 Effect of cobalt chloride on atrazine biodegradation

Cobalt chloride was used in this experiment to determine its effect on enhancing isolates efficiencies for atrazine degradation as a coenzyme factor (Table 9) Isolates A, B, C, D, F and G are expressed atrazine degradation percentages as 56.20, 48.22, 47.46, 37.51, 18.36 and 28.45%, respectively when cobalt chloride is added. The corresponding degradation percentages are 56.49, 47.50, 52.46, 49.04, 31.36 and 33.29% for the same isolates in cobalt chloride media, respectively. The results indicated that cobalt chloride didn't alter the efficiencies of the tested isolates for atrazine biodegradation. However, Shapir *et al,* (2002) mentioned that Co (II) and Ni (II) restored activity of metal-depleted AtzC to nearly the same level as that of the native enzyme.

Table 9 Effect of Cobalt Chloride on Atrazine Biodegradation

	Degradation, %	
Strains	Standard media	Media enriched with cobalt chloride
A	56.49[a]	56.20[a]
B	47.50[d]	48.22[c]
C	52.46[b]	47.46[d]
D	49.04[c]	37.51[e]
F	31.36[g]	18.36[i]
G	33.29[f]	28.45[h]
$L.S.D_{0.05}$ interaction		0.981

Also, they reported that mixture of either Co(II) or Ni(II) metal with 1 mM Mg(II) increased activity to more than 100% of that of the native enzyme. Moreover, Shapir *et al,* (2006) investigated the assumption of certain studies that a metal was not required for activity of TrzN, the broad-specificity triazine hydrolase from Arthrobacter and Nocardioides spp. In the current results showed cobalt didn't affect isolates efficiency for atrazine degradation. They concluded that TrzN is not a metalloenzyme. While exogenously added zinc or cobalt restored activity to metal-depleted TrzN, cobalt supported lower activity than did zinc.

3.7 Molecular characterization of the bacterial isolate

The molecular identification of F isolate was performed by amplification of 16S rDNA gene and sequencing of the purified PCR product. The obtained sequences were compared to the 16S rDNA database in the GenBank. The results showed that the F 16S rDNA gene was 100% similar to *Pseudomonas* ADP with accession no. of U55933.1.

The phylogenetic relationships of the F isolate and closely related species were analyzed using the multisequence alignment program (MEGA 4.1) and the results were presented in phylogenetic tree (Figure 1).

Based on the obtained data, isolate F was designated as *Pseudomonas* ADP.

Several researchers have reported the enrichment and isolation of bacteria able to degrade atrazine from compromised sites in different geographical regions (Yanze-Kontchou and Gschwind 1994; Topp *et al*, 2000) and found to be *Pseudomonas* sp. with molecular identification.

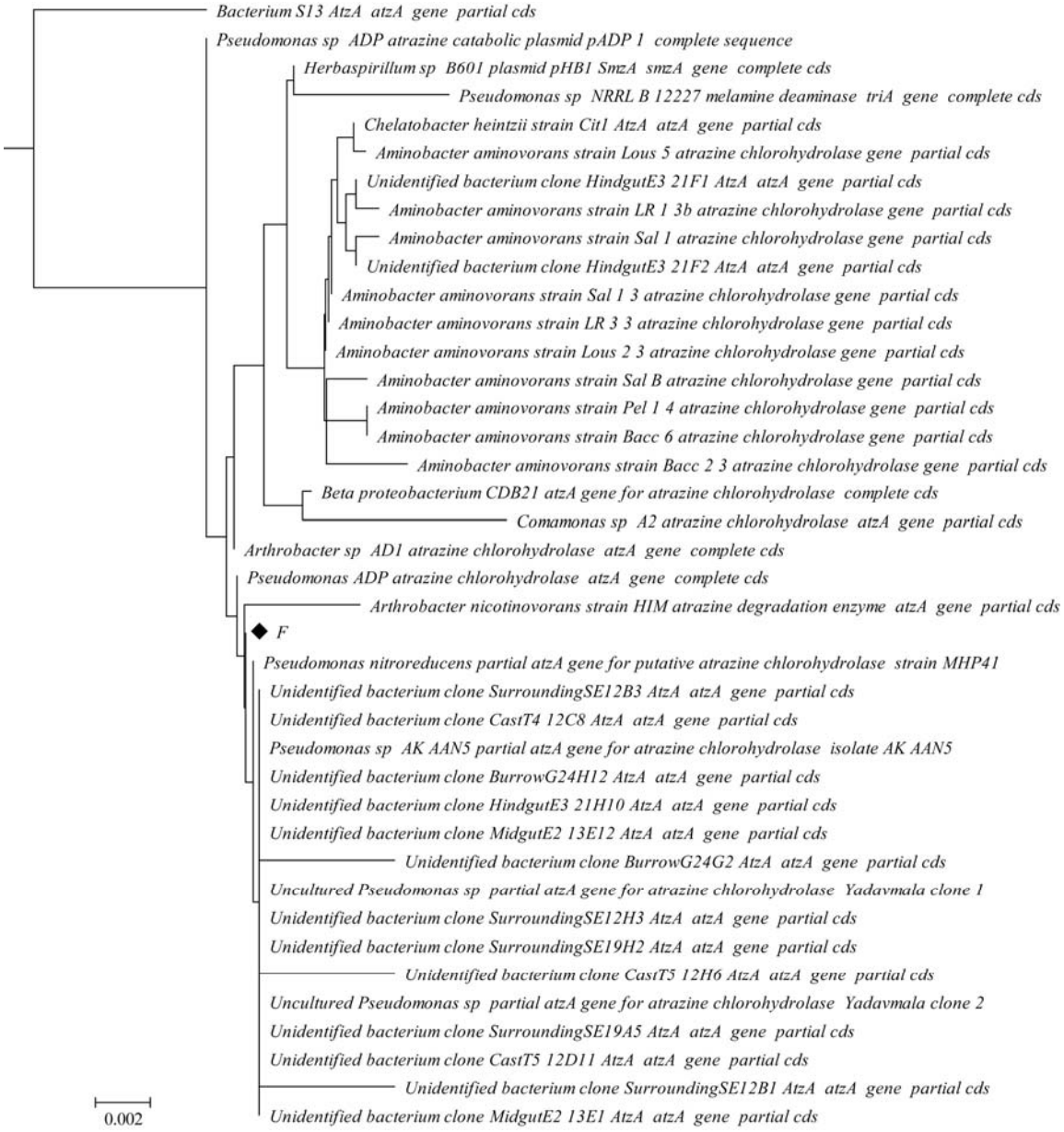

Figure 1 Neighbor-joining tree represents phylogenetic relationship isolate F based on 16S rDNA partial sequences isolated in this study. Evolutionary distances were calculated using the Kimura 2 model using MEGA4.1 software

4 Conclusion

It could be concluded that bacterial isolates of the calcareous soil recorded higher atrazine degradation efficiencies than those of clay loam soil. The highest atrazine degradation percentage was obtained from isolate A (47.97%), while isolate I showed the lowest percentage (5.15%). Biodegradation efficiency increased with increasing targeted atrazine concentration. Also, the maximum efficiency was achieved at 25ºC for both soil types isolates. However, the efficiency of isolates showed slight enhancement at 35ºC for the higher level of targeted atrazine (0.12 mM).

Counting isolates showed that some of them being major members while the others are minors according to their abundance in the consortium. Such majority and minority were correlated and presented in the efficiency of atrazine bioremediation. The optimum pH for

calcareous and clay loam soil isolates recorded as 3 and 5, respectively. Adding cobalt chloride didn't affect the isolates efficiencies for atrazine bioremediation. Identification of the F isolate using the sequencing of 16S rRNA gene technique showed that isolate F 16S rDNA gene was 100% similar to *Pseudomonas* ADP with accession no. of U55933.1.

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