Bioremediation of Atrazine-Contaminated Water and Soils by *Pseudomonas fluorescens*

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Abstract: Biodegradation of atrazine was investigated in water and soil. Atrazine degrading bacterium (designated strain E10) was isolated from maize soil previously treated with atrazine using enrichment technique. Based on morphological, physiological and 16S rDNA, this bacterium was identified as *Pseudomonas fluorescens*. It was capable of using atrazine as a sole source of carbon and nitrogen. *P. fluorescens* biomass and atrazine degradation were found to be optimum at pH 7 and 30°C. Additional carbon sources (i.e., glucose, sucrose and phenol) and nitrogen sources (i.e., ammonium sulfate, sodium nitrate and urea) decreased atrazine degradation. *P. fluorescens* was able to degrade atrazine completely in liquid medium at pH 7 and 30°C after 30 days and its half-life was 9 days compared to 64.2 days in uninoculated medium. There was no toxicity of detected atrazine in the supernatant after 30 days of incubation with *P. fluorescens* on *Azotobacter chroococcum* as microbial bioassay test. Four metabolites viz. hydroxyatrazine, deethylatrazine, deisopropylatrazine and deethyldeisopropylatrazine were identified by gas chromatography coupled with mass spectrometry analysis in the liquid cultures. Results showed also that atrazine was degraded faster by *P. fluorescens* in sandy soil than clay soil compared to their respective uninoculated soils after 30 days and their half-lives were 8.7, 14.4, 119.5 and 150.6 days, respectively. The dissipation of atrazine was coinciding with increasing *P. fluorescens* biomass in both atrazine contaminated soils. This study has shown that *P. fluorescens* could be applied to remediate atrazine contaminated soils and water.

Key words: Atrazine · Biodegradation · Degradation Products

INTRODUCTION

The expanding use of pesticides is a problem especially in the developing nations, where agricultural systems demonstrate an increasing reliance on pesticides, with limited regulation and no measure of environmental contamination, which lead to potentially serious health problems [1]. Once pesticides are applied to the field, they can be carried with runoff to surface water, percolate to groundwater, or be retained in the soil column [2].

Atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino) -s-triazine] is the most commonly used herbicide in the world for controlling broadleaf and grassy weeds and is quite persistent in neutral environment and toxic to various living organisms [3]. Because of its high mobility and persistence in soil and its massive application, atrazine has often been detected in surface and groundwater at concentrations well above the permitted limits [4-6].

Atrazine is considered as a potential environmental contaminant and considered also as one of the worst ground water pollutants [7, 8]. Atrazine can also affect human and other animals directly because it can be up taken by plants and transferred to the food chain [9].

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It can act as an endocrine disrupter, affecting the endocrine system, the central nervous system and the immune system [10]. Atrazine exposed male frogs were both demasculinized and completely feminized as adults [6].

Due to its persistence in the environment, the search for microbial strains capable of degrading it is fundamental to the development of bioremediation processes, as corrective tools to solve the current problems of the conventional techniques of pesticides removal such as chemical treatment, incineration and adsorption. These methods are either very costly producing other toxic substances or are not feasible. Biodegradation is one of the natural processes that help to remove xenobiotic chemicals from the environment by microorganisms [11]. It is one of the most cost effective methods amongst remedial approaches and is also considered as the green route to remove many pollutants from environment [12]. Despite its widespread use, no atrazine-degrading bacterial isolate has yet been reported from Kafr El-Sheikh governorate soils, Egypt. Hence, there was a need to explore microbial population having atrazine-degradation capacity from these soils. Although general atrazine degradation pathways are available, the specific pathways in specific conditions are not yet clearly defined [13].

Therefore, the purpose of this study was to isolate the microbial strain, characterize its degradation potential of atrazine in liquid culture and its use in bioremediation of atrazine-contaminated soils. Atrazine degradation products were also identified by GC-MS analysis.

MATERIALS AND METHODS

Chemicals: Atrazine (98%) and its metabolite standards [i.e., deethylatrazine (98%), deisopropylatrazine (99%), hydroxyatrazine (99%) and deethyldeisopropylatrazine (99%)] were donated by Syngenta (Basil, Switzerland). All other organic solvents and chemicals were of analytical grade and purchased from standard commercial suppliers.

Microbial Degradation of the Tested Pesticide
Media: M9 - Minimal Medium as mineral salt medium (MSL) and Luria Bertani Medium (LB) were used through this study as described by Sambrook *et al.* [14].

Isolation of Atrazine Degrading Microorganisms by Enrichment Cultures: Atrazine-degrading microorganisms were isolated by the enrichment technique. Soil samples (200 g) were collected in plastic bags from rhizosphere of maize plants (kafr El-Sheikh Governorate, Egypt) that previously treated with atrazine and were transferred to the laboratory. 100 g of soil samples were treated with 10 μg active ingredient (a.i) atrazine /g and incubated at 30°C for 30 days. Then, 10 grams of soil were added to 90 ml sterilized MSL in 500 ml flask containing 10 ppm from atrazine as a sole source of carbon and nitrogen. Flasks were incubated at 30°C and 150 rpm for 30 days, Thereafter, 10 ml of cultures were transferred into fresh 90 ml MSL containing same concentration from atrazine. This procedure was repeated five times in similar manner. Dilution series were prepared after the final time from enrichment culture in a glass test tube containing 9 ml MSL liquid medium up to 1: 10^{-6} and then 100 μl of the third enrichment culture was transferred onto mineral salt agar plates containing atrazine (10 μg a.i/ml) and spread evenly with sterilized glass beads. The plates were incubated at 30°C for 7 days and monitored for appearance of colonies. The single colonies growing on these diluted plates were selected and were further purified by the standard spatial streaking on LB as complex agar medium for bacterial isolates.

The isolated colonies were then tested for their ability to grow in MSL medium containing (10 μg/ml) of atrazine as a sole source of carbon and nitrogen. The degradation % of atrazine was estimated as described below. One treatment contained atrazine and the other contained the medium and the isolate (no atrazine as control). The cultures were shaken at 150 rpm and 30°C for 30 days. During the experiment, samples were collected periodically at different intervals of time for estimation of viable cell count (cfu/ml) onto mineral salt agar medium (MSA) containing atrazine. All the experiments were done in triplicates.

Identification: The efficient selected atrazine degrading bacterial strain was identified depending upon morphological and physiological characteristics as described by John [15] and 16S rDNA as follows:

P. fluorescens (E10) DNA was extracted from overnight cultures in 10 ml of LB broth (Merck, Germany) by genomic DNA purification Kit (Fermentas, Germany).

PCR Amplification of 16S-rRNA Gene: Primer sequences used to amplify the 16S rRNA gene fragment were: U1 5'-CCA GCA GCC GCG GTA ATA CG-3' and U2 5'-ATC GG(C/T) TAC CTT GTT ACG ACT TC-3'. The PCR master mix contained 10 mol of each primer and 12.5 μl of 2x SuperHot PCR Master Mix (Bioron, Ludwigshafen, Germany) mixed with 50 to 100 ng of DNA template. Sterilized H_2O was added to a final volume of 25 μl.

Thermal cycler (Uno II, Biometra, Germany) program was 94°C for 4 min., 94°C for 1 min., 55°C for 1 min., 72°C for 1.5 min, the number of cycles was 35 cycle and the post PCR reaction time was 72°C for 5 min.

Sequencing of 16S-rRNA Gene: The PCR-products of each isolate were purified from excess primers and nucleotides by the use of AxyPrep PCR Clean-up kit (AXYGEN Biosciences, Union City, California, USA) and directly sequenced using the same primers as described for the amplification process. The products were sequenced using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (ABI Applied Biosystems, Foster City, California, USA) on a 3130XL Genetic Analyzer (Applied Biosystems). The bacterial 16S rDNA sequences obtained were then aligned with known 16S rDNA sequences in Genebank using the basic local alignment search tool (BLAST) at the National Center for Biotechnology Information, United States and percent homology scores were generated to identify bacteria.

Effect of Environmental Factors on Atrazine Degradation by *P. fluorescens* (E10): Experiments on the efficacy of pH and temperature on atrazine biodegradation were conducted in 250 ml flask containing 50 ml MSL containing 10 μg atrazine/ml as a sole source of carbon and nitrogen. MSL medium was inoculated by 1 ml from bacterial cell suspension (10^7cfu/ml). To determine the optimum pH, experiments were carried out at pH 5, 6, 7 and 8. Cultures were incubated on a rotary shaker at 30°C and 150 rpm for 10 days. To determine the effect of temperature, MSL medium with pH of 7 was incubated at 20, 30 and 40°C under 150 rpm for 10 days. The viable cell count (cfu/ml) of *P. fluorescens* was estimated onto MSA medium containing atrazine. All the experiments were done in triplicates.

Effect of Additional Carbon and Nitrogen Sources on Atrazine Degradation by *P. fluorescens* (E10): To determine the effect of additional carbon and nitrogen sources on growth of the tested strain, a 50 ml MSL supplemented with 10 μg/ml of atrazine as a sole source of carbon. Mineral salt medium was inoculated by 1 ml from *P. fluorescens* suspension at 10^7 cfu/ml. To investigate the effect of additional carbon sources such as phenol (100 μg/ml), glucose and sucrose (1gm/l), they were added to atrazine containing medium. The effect of additional nitrogen sources on degradation of atrazine was also studied by adding ammonium sulfate, sodium nitrate and

urea at concentration of 1gm/l to atrazine containing medium. The viable cell count (cfu/ml) of *P. fluorescens* was estimated onto MSA medium containing atrazine. All the experiments were done in triplicates.

Biodegradation of Atrazine by *P. fluorescens* (E10) in Aquatic System: *P. fluorescens* (E10) was cultured onto MSA medium supplemented with atrazine (10 μg /ml) for 7 days and then the growing colonies were washed with 3 ml sterilized MSL medium. The bacterial cell suspension (10^7 cfu/ml) was then used to inoculate 100 ml MSL medium containing (10 μg/ml) of atrazine. The cultures were incubated at 30°C, pH 7 and 150 rpm for 0, 6, 12, 18, 24 and 30 days. The percentage of degradation and the half-life of atrazine were determined as described afterward. Control flasks of equal volume of liquid mineral medium and atrazine without any microbial population were incubated in parallel at all intervals to assess abiotic loss. During the experiment, samples were collected periodically at the aforementioned intervals of time for estimation of viable cell count (cfu/ml) onto MSA medium containing atrazine. All the experiments were done in triplicates.

Toxicity Test: The bioassay of the remaining atrazine toxicity was performed on the aqueous solutions after 30 days of incubation with the tested microbial isolate. *Azotobacter chroococcum*, as the representative soil bacterium and a symbiotic nitrogen fixer was used as the test organism. The toxicity was determined by recording of inhibition zone in growth of *A. chroococcum* comparing to control treatment (untreated). LB agar medium was poured into Petri dishes (9 cm in diameter, 15 ml/dish), after solidification, 100 μl from *A. chroococcum* (10^8 cfu/ ml) were transferred onto LB agar plates and spread evenly with sterilized glass beads, wells (5 mm in diameter) were punched in each plates. After that, 50 μl from the supernatant was put in punched holes (5 mm in diameter) in LB agar medium, where the culture broth was obtained by culturing of the atrazine degrading strain with atrazine in MSL medium after 30 days as mentioned above. The culture broth was filtrated through a sterile membrane filter (0.2 μm). On the other hand, 50 μl from sterilized liquid medium was put in punched holes which were used as control treatments. Experiments were made in three replicates. Plates were incubated at 30°C for 4 days. Diameter of inhibition zone (mm) surrounding each hole was recorded. The toxicity was determined as percentage of inhibition in the growth of tested bacteria comparing to control treatment.

Analytical Procedures

Extraction: Three aliquots of media from each time interval were used, filtered under vacuum through PTFE filter and then subjected to solid phase extraction (SPE). The C_{18} SPE cartridge (OASIS 60 mg Waters cartridges, Milford, MA, USA) was cleaned and conditioned with 10 ml of dichloromethane: methanol (7:3). Then, the sample was pumped through the SPE cartridge at a flow rate of 2-3 ml/min and interstitial water was removed from the cartridge with a vacuum aspirator. The cartridge immediately was eluted with a mixture of dichloromethane and methanol (7/3, v/v). The received solution was concentrated under nitrogen flow for analysis.

Atrazine Determination: A Hewlett-packard, USA serial 6890 gas chromatograph equipped with electron capture detector (ECD, Radioisotope Nuclide ^{63}Ni) and HP PAS-1701column 25 m length x 0.32 mm (i.d.) x 0.52 μm film thickness. Pure nitrogen was used as carrier gas (2ml/min). Detector, injector and column temperature was 250, 240 and 225°C, respectively. Atrazine detection limit was set at concentration where the analyte signal was three times higher than background noise and it was 0.1 μg/l. The recovery of atrazine from liquid medium was 93.3% and was used to correct all obtained values and to examine the reliability of the analytical procedures. The atrazine degradation data were fit to first-order kinetics and the first-order degradation rate constant (k) and the half-life ($T_{1/2}$) were calculated according to this equation $T_{1/2} = 0.693/k$.

Atrazine Metabolites Identification: After extracting the water samples as mentioned earlier, they were derivatized by the addition of N-methyl-N-(*tert*-butyldimethylsilyl)-trifluoroacetamide. The derivatized extracts were analyzed by gas chromatography/mass spectrometry (GC-MS). The identification of atrazine and its degradation products after 12 days from incubation with *P. fluorescens* on MSL medium was performed by using a GC (HP 6890 series) equipped with a mass selective detector MSD (HP 5973). The system was equipped with a TC-5 capillary column (30 m length × 0.25 mm i.d. × 0.25 μm film thickness),

splitless injection and used helium as carrier gas (1ml/min). The GC oven temperature was programmed to initially hold at 90°C for 0.5 min then increased to 160°C at 15°C/min and to 280°C at the rate of 25°C/min then held for 5 min. The injector and GC/MS interface temperature were kept at 280 and 300°C, respectively. Mass spectra were obtained by the electron-impact (EI) mode at 70 eV, using the full scan mode.

Bioremediation of Atrazine-Contaminated Soils: In order to examine the possibility of practical use of the isolated bacteria, atrazine degradation was tested in two types of soil (i.e., clay and sandy soil: their physicochemical characteristics were shown in Table (1). After sterilization of the soil samples, atrazine was added with 10 mg/kg to each soil. *P. fluorescens* (E10) was cultured onto MSA containing atrazine as mentioned above. One hundred ml from bacterial cell suspension (10^7 cfu/ml) was then used to inoculate 1 kg of each soil sample. Each soil was put into a beaker, covered with aluminum foil and incubated at 30°C for 30 days. Throughout the incubation period, soil moisture was maintained at 40 % of the field water-holding capacity. After the static incubation, 10 g aliquots from each soil were sampled at different time intervals (i.e., 0, 6, 12, 18, 24 and 30 days) and extracted by shaking with 40 ml solution of dichloromethane and methanol (7/3, v/v) for 1.5–2 h using a mechanical shaker and analyzed for atrazine as early mentioned. The mean recoveries of atrazine from clay and sandy soil were 87 and 91%, respectively and then these figures were used to correct all obtained values and to examine the reliability of the analytical procedures. During the experiment, samples were collected periodically at different intervals of time for estimation of viable cell count (cfu/g soil) onto MSA medium containing atrazine. All the experiments were done in triplicates.

Statistical Analysis: Data were calculated as mean ± standard deviation (SD) and analyzed using analysis of variance (ANOVA). Probability of 0.05 or less was considered significant. The statistical package of Costat Program [16] was used for all chemometric calculations.

Table 1: Physicochemical characteristics of clay and sandy soil

Parameter	pH	EC ds/m	OM %	N	P	K	Cd	Ni	Pb	Mn	Cu	Fe
				--- (ppm) ---								
Clay soil	7.3	2.2	1.43	130.2	12.14	0.042	1.72	3.04	3.5	7.98	4.44	13.26
Sandy soil	7.8	1.5	0.14	16	6.23	0.005	0.52	4.1	2	1.9	2.9	3.14

RESULTS AND DISCUSSION

After successive enrichments, several mixed cultures capable of atrazine degradation were eventually obtained in Fig. 1. Purified colonies (a total of 7 isolates) from these mixed cultures were tested for the ability to degrade atrazine as the sole source of carbon and nitrogen. A preliminary classification based on the morphology of the isolates revealed that the pesticide– degrading organisms belong to the group of Gram positive and negative bacteria. Four of 7 bacterial isolates (E10, E11, E12 and E13) were Gram-negative, motile, rods and oxidase positive. Two of 7 bacterial isolates (B14 and B15) were Gram positive, motile, rods and spore former. One of 7 bacterial isolates (St16) was Gram positive and filamentous shaped bacterium. Results in Figure (1) showed that isolates were tested to their growth ability and degradation of atrazine in MSL medium as a sole source of carbon and nitrogen. Among 7 bacterial isolates, one bacterial isolate designated as E10 achieved complete degradation of atrazine comparing with the other isolates. The disappearance of atrazine was coinciding with increasing in the growth rate for all the bacterial isolates. The maximum growth of this isolate was achieved after 30 days of inoculation in atrazine supplemented MSL medium. The obtained results were compared with control treatments (uninoculated). Many pure and mixed triazine-transforming cultures have been isolated by many authors [17-19] who found that enrichment culture technique lead to the isolation of microorganisms, which are able to degrade atrazine. According to Rhine *et al.* [20], the repeated exposure to atrazine can increase biodegradation of atrazine. Silva *et al.* [21] demonstrated that the occurrence of fast atrazine mineralization after an acclimatization period of approximately 28 days. The strain *Delftia acidovorans* D24 mineralized atrazine as a sole source of carbon and nitrogen [22].

Atrazine degradation in media containing atrazine as a sole carbon and nitrogen source showed that the maximum degradation was achieved by bacterial E10 isolate; therefore this isolate was selected for the subsequent studies. This bacterial strain (E10) was identified according to morphological, physiological as well as using analysis of 16S rDNA. This efficient atrazine degrading strain (E10) was Gram-negative, motile, rods and oxidase positive. According to the 16S rDNA analysis, the phylogenetic tree of the atrazine degrader bacteria (E10) and related bacterial species based on the 16S rDNA sequence was provided in Figure (2). It can be clearly seen that the atrazine degrader bacteria was included in the genus *Pseudomonas* and closely related to the species *fluorescens*. It showed the highest sequence similarities with *P. fluorescens* Pf 01 (100 %).

Effect of Some Environmental Factors (pH and Temperature) on Atrazine Degradation by *P. fluorescens*: Optimum pH: The influence of pH on *P. fluorescens* biomass growth and degradation of atrazine was shown in Figure (3). The highest degradation of atrazine was achieved at pH 7 with 68 % of degradation. After ten days of incubation, optimum pH value for *P. fluorescens* biomass growth and atrazine degradation was found to be 7.

Optimum Temperature: The effect of different temperatures on *P. fluorescens* biomass growth and atrazine degradation was shown in Figure (4). Degradation of atrazine occurred at 20, 30 and 40°C while the highest degradation was achieved at 30°C, with 65% of degradation. A temperature of 30°C appeared to be the optimum temperature for growth of the bacterial isolates as well. Our results are in agreement with those reported by Abigail *et al.* [19] who found that the optimum pH and temperature for atrazine degradation by *Cryptococcus laurentii* are 7 and 30°C, respectively.

Effect of Additional Carbon and Nitrogen Sources on Atrazine Degradation by *P. fluorescens*: Cells grown in media with additional carbon sources showed comparatively low atrazine degradation and biomass yield with sucrose and phenol. Glucose showed higher *P. fluorescens* biomass than the other carbon sources and low atrazine degradation comparing with atrazine only. Phenol exhibited lower biomass and atrazine degradation than glucose and atrazine. Sucrose showed the lowest *P. fluorescens* biomass and atrazine degradation comparing with the other carbon sources. The presented results in Fig. 5 illustrated that the highest degradation of atrazine was obtained by *P. fluorescens* at pH 7, 30°C for 10 days in the presence of atrazine only as a sole source of carbon and nitrogen followed by glucose, phenol and sucrose as carbon sources.

Additional nitrogen sources also decreased atrazine degradation as well as biomass yield of *P. fluorescens* comparing with atrazine only as a sole source of carbon and nitrogen (Figure 5). Atrazine was degraded faster by *P. fluorescens* in the presence of atrazine only as a sole source of carbon and nitrogen followed by ammonium sulfate, sodium nitrate and urea. The presence of atrazine in growth media containing other nitrogen sources did not
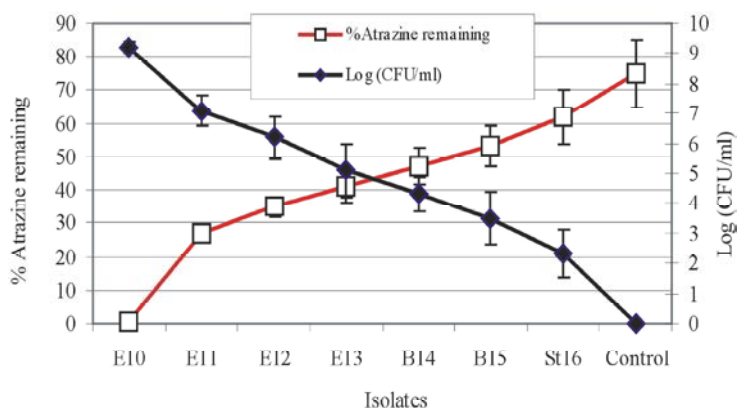
Fig. 1: The growth ability of the bacterial isolates and atrazine degradation in mineral salt medium
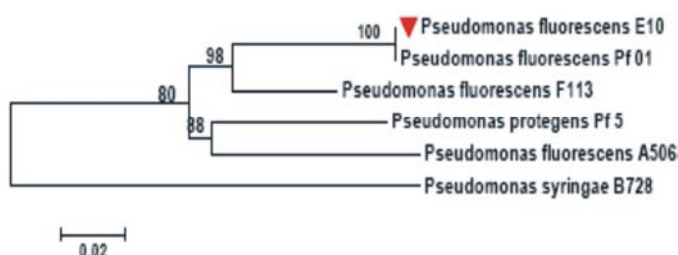

Fig. 2: Phylogenetic dendrogram obtained by distance matrix analysis of 16S rDNA sequences, showing the position of strain *P. fluorescens* (E10) among phylogenetic neighbors. The scale bar indicates 0.02 substitutions per nucleotide position
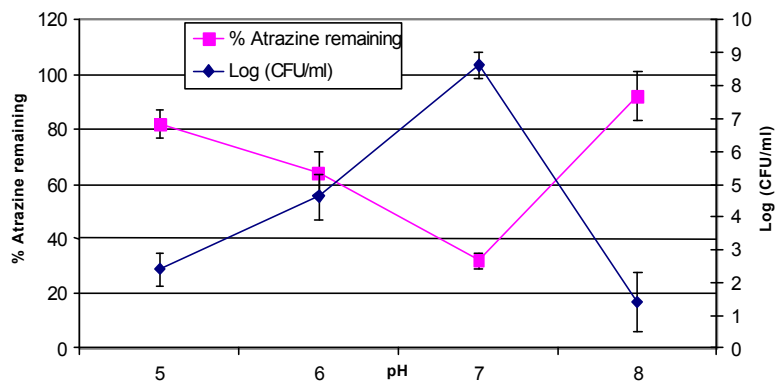

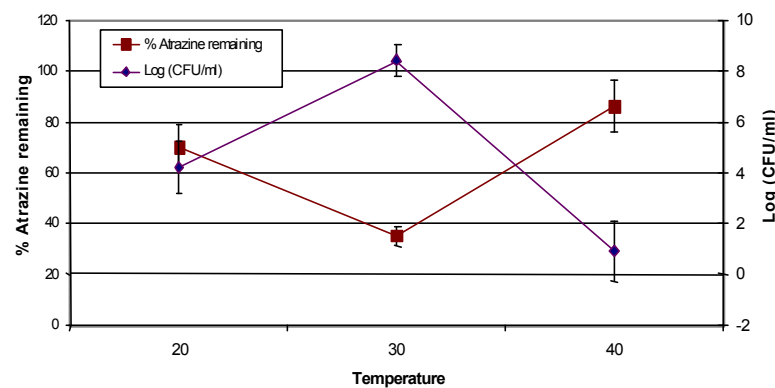Fig. 3: Effect of pH on *P. fluorescens* biomass growth and atrazine degradation in mineral salt medium

Fig. 4: Effect of temperature on *P. fluorescens* biomass and atrazine degradation in mineral salt medium
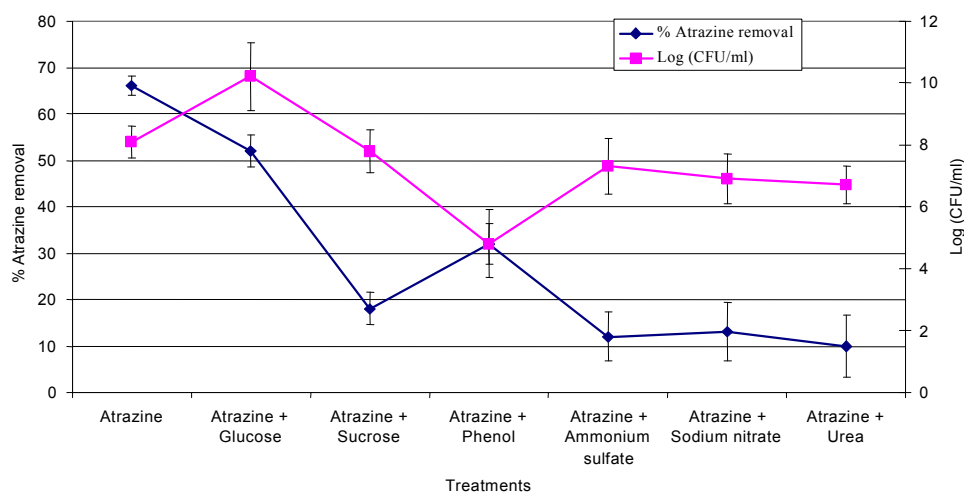
Fig. 5: Effect of additional carbon sources such as (glucose, sucrose and phenol) and nitrogen sources such as (ammonium sulfate, sodium nitrate and Urea) on *P. fluorescens* biomass and atrazine degradation

stimulate degradation. Similar results were observed in case of *Agrobacterium radiobacter* J14a, where the additional carbon sources like sucrose did not significantly increase the atrazine degradation rate [23]. García-González *et al.* [24] demonstrated that atrazine degradation is inhibited in resting cell suspensions previously cultured in the presence of nitrogen sources that support fast growth of *Pseudomonas* sp. ADP, such as ammonium, urea, proline or nitrate. Similar results were reported in atrazine biodegradation by indigenous soil populations where the presence of preferential nitrogen sources was reported to be detrimental to atrazine degradation [25]. Nitrogen amendments were found to decrease the atrazine degradation rates in bacterial strains such as *Pseudomonas* and *Klebsiella* [26]. Atrazine mineralization was suppressed by nitrogen supplements in fungi, suggesting that additional nitrogen could alter the microbial process and carbon uptake and thus decreased atrazine degradation rate [27]. In contrast, atrazine degradation by *Agrobacterium radiobacter* J14a was not influenced by the simultaneous presence of ammonium, nitrate, urea or glycine in the growth medium [28].

Degradation of Atrazine in Liquid Medium by *P. fluorescens*: In the present study, atrazine was significantly declined from the initial concentration (10 ppm) with increasing the incubation period in the medium amended with *P. fluorescens*, while medium without any amendment (i.e., uninoculated control) showed less dissipation of atrazine (Figure 6). The rate of atrazine dissipation was found to be very slow and only

7% dissipation was achieved after 6 days of incubation which increased to 71, 87 and 99% after 12, 18 and 24 days of incubation with the tested bacteria, respectively. *P. fluorescens* was able to degrade atrazine completely in liquid culture after 30 days of incubation revealing that pure culture application is quite promising detoxification technique through bioaugmentation. Atrazine half-life values were found to be 9 and 64.2 days in inoculated and uninoculated medium, respectively. Many investigators reported that atrazine was degraded by individual strains such as *Pseudomonas* sp. [29, 30], *Aerobacterium* sp., *Microbacterium* sp., *Bacillus* sp., *Micrococcus* sp., *Deinococcus* sp. and *Delftia acidovorans* [22], as well as by species consortia including *Agrobacterium tumefaciens*, *Caulobacter crescentus Pseudomonas putida*, *Sphingomonas yaniokuyae*, *Nocardia* sp., *Rhizobium* sp., *Flavobacterium oryzihabitans* and *Variovorax paradoxus* [31].

Toxicity Evaluation: Toxicity of the remaining atrazine in the aqueous solution after 30 days of incubation with the tested microbial strain was evaluated using *A. chroococcum* as a microbial bioassay test. The results showed that the supernatant of atrazine after 30 days of incubation with *P. fluorescens* had no toxicity which could be detected against *A. chroococcum* as a test organism. The obtained results were compared with control treatment (atrazine only) which revealed 100% of inhibition against *A. chroococcum* under the same conditions. This implies that the aqueous solution spiked with atrazine was completely detoxified after 30 days of treatment with *P. fluorescens*.
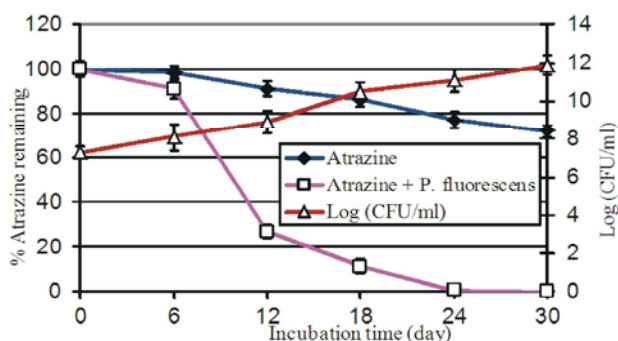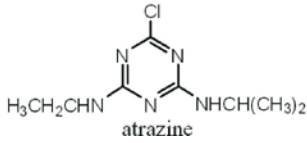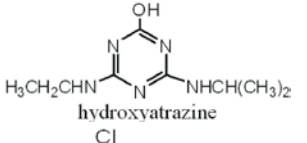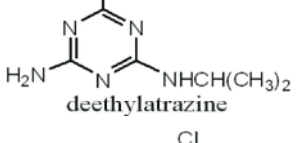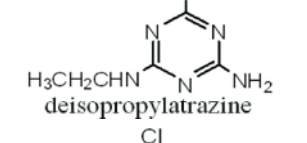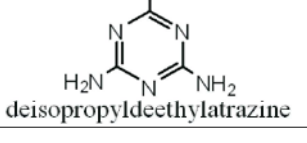
Fig. 6: Atrazine degradation by *P. fluorescens* as compared to uninoculated mineral salt medium at different time intervals

Table 2: Atrazine and its identified degradation products

No.	Molecular weight (m/z)	Empirical Formula	Retention time (min)	Compound
1	215.69	$C_8H_{14}ClN_5$	11.95	atrazine
2	197.24	$C_8H_{15}N_5O$	13.19	hydroxyatrazine
3	187.63	$C_6H_{10}ClN_5$	11.31	deethylatrazine
4	173.60	$C_5H_8ClN_5$	14.21	deisopropylatrazine
5	145.55	$C_3H_4ClN_5$	14.79	deisopropyldeethylatrazine

Identification of Atrazine Degradation Products: Four degradation products (i.e., hydroxyatrazine, deethylatrazine, deisopropylatrazine and deisopropyldeethylatrazine) were identified by the molecular ion and mass fragmentation pattern and through comparison with NIST library data and also by comparing retention times and spectra with commercially available authentic standards (Table 2). The similarities of these compounds to the NIST library data were more than 80%. In addition to these compounds, other degradation products still possibly would exist but were not detected because of their low concentration, extraction efficiency and limited sensitivity in GC–MS. In the present study, *P. fluorescens* aerobically transformed atrazine via dealkylation, dechlorination, deamination and ring cleavage. Once atrazine is dechlorinated, further enzymatic hydrolytic transformations result in atrazine-ring cleavage, mineralization and subsequent release of CO_2 and NH_3 [32]. Biodegradation of atrazine has been reported to proceed via different pathways, resulting in the formation of metabolites like hydroxyatrazine, deethylatrazine, or deisopropylatrazine. All pathways finally lead to the central metabolite, cyanuric acid, which is further mineralized to CO_2 [12]. Of the pathways, one has been elucidated fully in *Pseudomonas* sp. strain ADP [33] and is known to be hydrolytic rather than oxidative and consists of four main steps: dehalogenation; *N*-dealkylation; deamination; and

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ring cleavage. The catabolic enzymes were identified by many authors [34-36]. The location of the genes *atzABC* which encode these enzymes on a self-transmissible plasmid was identified [37].

Bioremediation of Atrazine Contaminated Soil by *P. fluorescens*: Since the previous results shown above indicated that biodegradation removed successfully atrazine in aquatic system and biodegradation ability of *P. fluorescens* revealed its potential for remediation of atrazine contaminated soils (clay and sandy). Atrazine was degraded faster by *P. fluorescens* in inoculated sandy soil (94%) than clay soil (78%) compared to their respective uninoculated soils after 30 days of incubation

(Figure 7). The abiotic loss in uninoculated caly and sandy soil was 13 and 16%, respectively. Atrazine half-lives were 8.7 and 14.4 days in treated sandy and clay soils by *P. fluorescens* while they were 119.5 and 150.6 days in uninoculated sandy and clay soils, respectively. The application of atrazine increased the number of cultivable atrazine-degrading cells in both soils during the 30 days of incubation (Figure 8). This increase of population in clay soil was much higher than in sandy soil, despite of the disappearance of atrazine in sandy soil was much more than in clay soil and this may be due to increasing the organic matter, other nutrients and the highest content of nitrogen in clay soil than sandy soil.
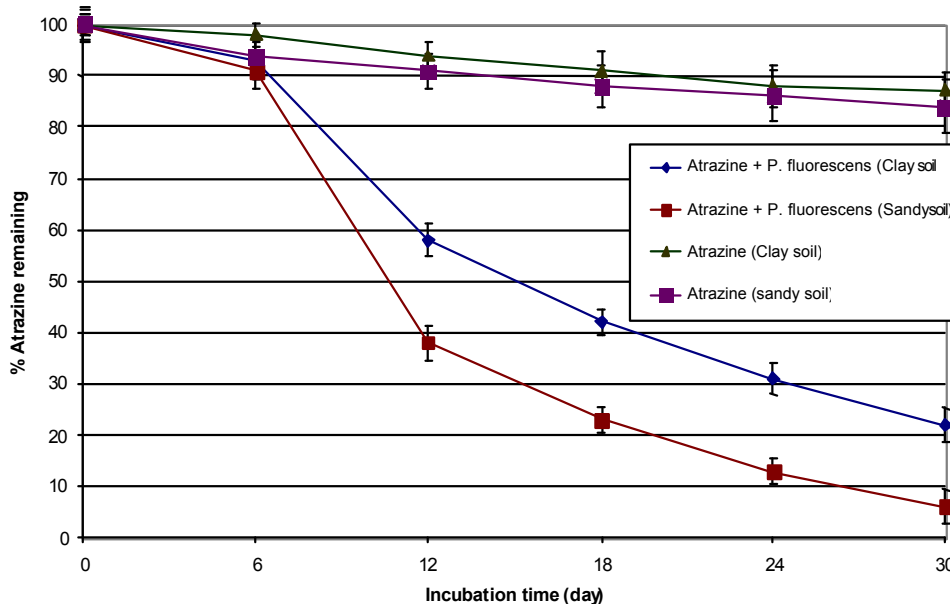
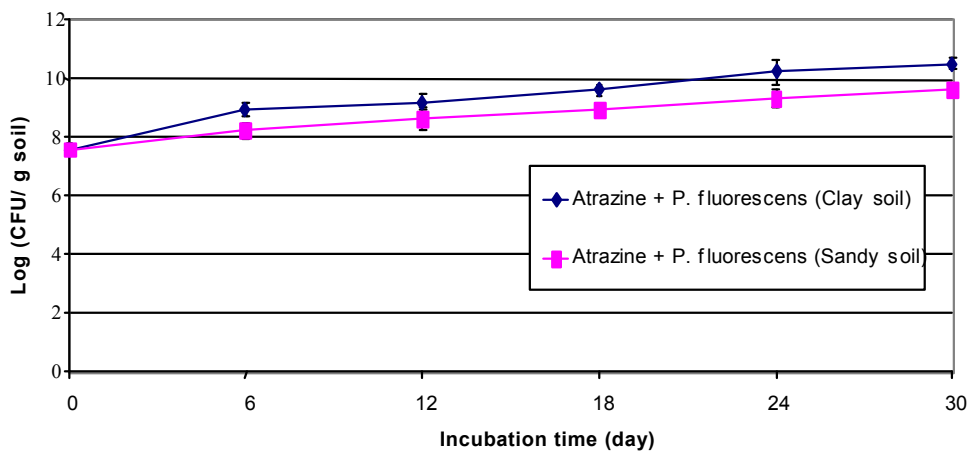Fig. 7: Atrazine degradation in two soils by *P. fluorescens* (E10)

Fig. 8: Bioremediation of atrazine-contaminated soil by *P. fluorescens* (E10) and its effect on population of microorganisms in soil

Atrazine losses in the sterilized control may have been due to abiotic CO_2 formation, abiotic hydrolysis to hydroxyatrazine and inefficiency of detection [38]. These results showed that this strain is advantageous for the degradation of atrazine in the natural environments. Biological degradation of atrazine depends upon various factors like the operating environment, external carbon and nitrogen sources, carbon/ nitrogen ratio (C/N), water content and the microorganism involved in degradation [13]. The half-life values of atrazine ranged from 5 to 18 d in the surface layers of the Northern Greece adapted soils and the higher biotransformation rates of atrazine were simultaneously observed with the abundance of Gram-negative bacteria [39]. *Agrobacterium radiobacter* J14a accelerated atrazine degradation rate in soils without and with previous herbicide application [23]. Conversely, *Pseudomonas* sp. strain ADP was unable to increase atrazine or simazine degradation in *s*-triazine polluted soil [40]. Previous findings pointed out that atrazine degradation in agricultural soils may be limited by the effect of nitrogen added as fertilizer on catabolic or transport gene expression [24, 41].

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