

# Occurrence of Atrazine Biodegrading Bacterium "*Ochrobactrum oryzae*" In Agricultural Wastewater

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## Abstract

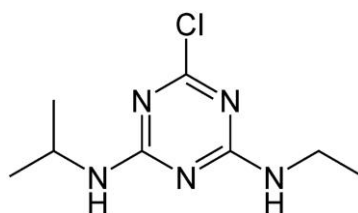
Atrazine is the most extensively used herbicide that restrains photosynthesis of broadleaf and grassy weeds. In consequence of ground and surface water contamination with atrazine, humans and wildlife might be at risk. In the current investigation, an atrazine-degrading bacterium was isolated from agricultural drainage ditches (Fayoum, Egypt) using enrichment technique. According to the morphological, biochemical and 16S rDNA gene sequencing, the bacterial isolate was identified as *Ochrobactrum oryzae*. Using atrazine as sole carbon and nitrogen source, a pure culture of *O. oryzae* was grown in minimum media. The impact of atrazine concentration as well as temperature and pH on the bacterial growth and atrazine degradation rate was studied. The greatest potential for atrazine degradation (83.5%) was attained at concentration 400 ppm of atrazine within 9 days at pH value 9.0 and temperature 30°C. Consequently, *O. oryzae* can be applied conveniently for the cleanup of agricultural wastewater contaminated with high levels of atrazine.

**Keywords:** *Ochrobactrum oryzae* - 16S rDNA - wastewater – atrazine - biodegradation.

## Introduction

The accumulation of toxic pesticides in water and soil represents a prospective environmental concern. The s-triazine herbicides are the most commonly used pesticides in agriculture for several countries and probably it is the most commonly

used herbicide in the world. Atrazine, 2-chloro-4-(ethylamine)-6-(isopropyl amine)-s-triazine, a member of s-triazine group of herbicides is used for the control of annual and perennial grassy or broad leaf weeds in major crops due to its low cost and high effectiveness (Shahitha, 2012). Atrazine strongly reduces photosynthesis via stopping the electron transport in photosystem II resulting in the demolition of chlorophyll (Christopher *et al.*, 2010).



**6-chloro-N 2-ethyl-N 4-isopropyl-1,3,5-triazine-2,4-diamine**

Atrazine is non-volatile and its half-life is about 200 days but it varies from 21 days to 1 year depending on the environmental factors such as pH of the soil, type of soil, moisture content, temperature and the microbial communities (Zhang *et al.*, 2012). Due to the excessive use and the high persistence of atrazine, it is moved to water bodies such as rivers, lakes and drinking water supplies and it had also been found in ground waters (Spalding *et al.*, 1994). The potential contamination of water resources and soil with atrazine may cause pollution to the environment and bring enormous harm to human and other animals as it can be concentrated by plants and transferred to the food chain (Topp *et al.*, 2000).

Some studies had reported atrazine as one of the endocrine disruptors (Moore & Waring, 1998) and as a probable human carcinogen (Luciane *et al.*, 2010). Moreover, atrazine was found to be the reason for low sperm levels in men, the premature birth, miscarriage and various birth defects in humans (Ackerman, 2007; Pathak & Dikshit, 2011). Therefore an efficient remediation strategy for atrazine polluted environments is a vital task for a clean and safe environment. Some

physiochemical methods have been used for the elimination of atrazine from the contaminated environment such as adsorption, reduction-oxidation, dechlorination, photolysis, reverse osmosis (Ahalya *et al.*, 2003). However, these methods are costly and produce many toxic intermediates.

Bioremediation has attracted increasing attention as a harmless, effectual, and economical biotechnological approach for herbicide elimination (Chen *et al.*, 2011; Essa *et al.*, 2016). There have been several reports on atrazine mineralization by wide varieties of bacteria such as *Pseudomonas*, *Acinetobacter*, *Agrobacterium*, *Rhodococcus* and *Arthrobacter* (Vibber *et al.*, 2007; Li *et al.*, 2008; Siripattanakul *et al.*, 2009; Arbeli & Fuentes, 2010; El Sebai *et al.*, 2012). The capability of microorganisms to utilize atrazine as a carbon, energy and/or nitrogen source was clearly demonstrated (Struthers *et al.*, 1998; Rousseaux *et al.*, 2001; Devers *et al.*, 2007). Some bacterial strains metabolized atrazine to diethylatrazine or hydroxyatrazine, while others mineralized it by cleavage the ring. The catabolic pathway, enzymes and corresponding genes involved in atrazine degradation, were characterized in some bacterial strains (Mandelbaum *et al.*, 1995; Sene *et al.*, 2010, Wang *et al.*, 2011).

Although atrazine was prohibited in the European Union in 2003 (Bethsass & Colangelo, 2006), it is still used as a major herbicide in Egypt. Exploration of atrazine-degrading microorganisms is of immense importance to reduce or eliminate the negative consequences of this compound to human health and ecosystem. The objectives of the present study were (i) to isolate and characterize atrazine resistant bacteria from contaminated agricultural wastewater, (ii) to investigate the optimum growth and degradation conditions of these isolates in order to be used for bioremediation of the polluted environments.

## **Materials and methods**

### **Chemicals**

Atrazine (99% pure) was purchased from Riedel-de Haën, Sigma-Aldrich, Seelze, Germany. All other chemicals purchased are of analytical grade from Fluka AG, Buchs, Switzerland.

### **Growth Media and Culture conditions**

The mineral salt medium (MSM) used in this study contained  $K_2HPO_4$  (0.4 g/L),  $KH_2PO_4$  (0.2 g/L), NaCl, (0.1 g/L),  $MgSO_4 \cdot 7H_2O$  (0.5 g/L),  $MnCl_2$  (0.01 g/L),  $Fe_2(SO_4)_3$  (0.01 g/L),  $Na_2MoO_4$  (0.01 g/L) and 1 ml of trace element solution per liter (Singh *et al.*, 2004). Atrazine was used at different concentrations as a sole carbon and nitrogen source. The liquid mineral salt medium was supplemented with 2% (w/v) agar for the preparation of solid medium. A Stock solution of atrazine was prepared with a concentration 500 ppm and was diluted to the required concentrations for the degradation studies.

### **Isolation of atrazine-degrading bacterium**

This experiment was carried out to select atrazine tolerant bacterial isolates. The agriculture wastewater (500 mL) was collected from El-Batts drain, Fayoum, Egypt. The sample was centrifuged at 10,000 rpm for 10 min and reconstructed with 10 mL sterilized  $dH_2O$ . Liquid MSM medium (45 mL) supplemented with atrazine (200 ppm) was inoculated with 5 mL of bacterial suspension and incubated for 48 hrs inside shaking incubator (120 rpm) at 30°C. Aliquots were sub-cultured every 3 days for three times. The final culture was diluted and plated on atrazine agar plates. Developed colonies were repeatedly streaked on atrazine agar plates for isolation of pure cultures. Atrazine-degrading bacterial isolates were identified as colonies surrounded by a clear halo. The bacterial isolate designated ATT was chosen for this

work because it is the most tolerant strain that can grow under elevated levels of atrazine (500 ppm). A pure culture of ATT bacterium was then stored in solid MSM media containing 100 ppm atrazine for further studies.

### **Phenotypic characterization of ATT isolate**

The ATT isolate was tested for morphology, motility and Gram stain by phase contrast microscopy. Biochemical identification of the ATT isolate was performed using commercially identification systems API (BioMérieux, France). Prior to inoculation of each identification system, a 24h bacterial culture re-inoculated onto NA plates to obtain isolated colonies for testing purposes. The API 20NE identification system is used for the identifying non-fastidious, non-enteric Gram-negative rods. Test strips were inoculated and incubated according to the instructions provided.

### **Identification of the bacterial isolate ATT**

The atrazine-degrading isolate was identified using 16S rDNA gene sequencing technique. The genomic DNA was extracted according to Essa (2012). For the amplification of the 16S rDNA gene two primers were used; F1; AGA GTT TGA TCC TGG CTC AG and R1; GGT TAC CTT GTT ACG ACT T. The PCR mixture was prepared as the following; 10 µL (10x) PCR buffer, 3 µL (50 mM) MgCl<sub>2</sub>, 1 µL (20 pmole/µL) of each primer, 1 µL (10 mM) dNTPs mixture, 0.5 µL (2.5U) Taq DNA polymerase, 2 µL total DNA extract, and the volume is completed to 100 µL by sdH<sub>2</sub>O. PCR was carried out for 35 cycles under the conditions described by Essa *et al.* (2016). PCR products (10 µL) were mixed with loading buffer (2 µL) and analyzed by electrophoresis (15 V/cm, 60 min) on 0.7% agarose gel containing ethidium bromide (0.5 µg/mL) using TBE buffer. The amplified DNA fragments were sequenced at GATC Biotech, Constance, Germany. Furthermore, a phylogenetic tree

was established using neighbour-joining technique using TREEVIEW software (1.6.6) derived from 16S rRNA gene sequences of some strains phylogenetically close to the isolated strain.

### **Optimization of the growth conditions and atrazine biodegradation by *O. oryzae***

Experiments aiming to study the effect of atrazine concentration on the bacterial growth were carried out in 250 mL flask containing 50 mL MSM enriched with various doses of atrazine (100 – 500 ppm). The medium was inoculated by 5 mL of bacterial cell suspension ( $OD_{600} = 0.6$ ) and incubated on a rotary shaker (120 rpm) at 30°C for 21 days. Cell growth in liquid media was determined spectrophotometrically by measuring the cultural optical density at 600 nm ( $OD_{600}$ ) at 24 hrs intervals over 20 days. In order to confirm the bacterial growth, the protein content of the bacterial cultures was determined according to Bradford (1976). To explore the impact of pH on the bacterial growth, experiments were conducted at different pH values (5, 7 and 9). Cultures supplemented with 400 ppm atrazine as a sole carbon and nitrogen source were incubated as mentioned above. Simultaneously, the influence of temperature on the bacterial growth was investigated on MSM medium enriched with 400 ppm atrazine at pH (9.0). Bacterial cultures were incubated at different temperatures (20°C, 30°C and 37°C). All the experiments were done in triplicates. The bacteria growth in liquid media was assayed spectrophotometrically as mentioned above. In order to measure the abiotic degradation of atrazine, Minimum salt media supplemented with equal concentrations of atrazine without bacterial inoculum were prepared and incubated under the same conditions.

### **Analysis of the residual atrazine**

The capability of *O. oryzae* for atrazine biodegradation was determined at different temperatures and pH values after 12 days incubation in order to identify the optimum condition for atrazine degradation. The residual Atrazine was extracted according to the method of Liu (2003). About 50 mL of 90% methanol was added to 20 mL liquid culture and was left 24hrs. After that, the mixture was extracted two times with 50 mL CH<sub>3</sub>Cl. Then the received solution was concentrated under nitrogen flow to 1mL to be determined by gas chromatography. A Hewlett-Packard, USA serial 6890 gas chromatograph equipped with electron detector (ECD, Radioisotope Nuclide 63Ni) and HP PAS-1701 column 25 m length x 0.32 mm (i.d.) x 0.52 thickness. Pure nitrogen was used as carrier gas (2mL/min). Detector, injector and column temperature was 250, 240 and 225°C, respectively. The atrazine degradation rate was calculated by the following formula:

$$A = [C_a - C_b / C_a ] \times 100$$

where, (A) is the percentage of atrazine degradation, (C<sub>a</sub>) is the concentration of atrazine (mg/L) in the medium in absence of atrazine-degrading strain, (C<sub>b</sub>) is the concentration of atrazine (mg/L) in presence of atrazine-degrading strain.

### **Statistics**

The data presented here are the mean values of three replications. Standard errors were calculated for all the values using MS Excel 2007.

### **Results and discussion**

#### **Isolation of atrazine degrading bacteria**

The atrazine-degrading bacteria was isolated from agricultural drainage ditches in 2013 (Fayoum, Egypt) using enrichment technique. The bacterial isolate ATT was the most tolerant strain against high levels of atrazine (1200 ppm). Most agricultural fields are well drained, whereas hydrologic conditions in ditches can

change widely. Agricultural drainage has been reported to contain high levels of pesticides (Moore *et al.*, 2008; Moore *et al.*, 2011). The microbial populations of the agricultural wastewater usually include some bacterial strains that can tolerate high concentrations of the toxic pesticides (Radwan *et al.*, 2017). Moreover, these bacterial strains might have the capability to degrade and mineralize these compounds (Tyler *et al.*, 2013).

### **Identification of atrazine degrading bacteria**

A variety of morphological and biochemical assays were carried out to have a comprehensive view of the phenotypic characteristics of the bacterial isolate ATT as shown in Table (1). ATT Isolate was Gram-negative, motile, and non-spore forming rods. This isolate demonstrated positive results with catalase, cytochrome oxidase, lysine decarboxylase, urease, amylase, arginine dihydrolase, gelatinase, NO<sub>2</sub> production, acetoin production and tryptophane deaminase. Meanwhile, negative results were obtained with indole production, β-galactosidase, H<sub>2</sub>S production, lipase, and ornithine decarboxylase. Simultaneously, the ATT isolate showed the capability to utilize glucose, mannitol, inositol, starch and citrate as a carbon source. The ATT isolate was identified as *Ochrobactrum oryzae* using 16S rDNA gene sequencing technique with maximum homology of 93% to *Ochrobactrum oryzae* strain: SP91A with accession no.AB41136 (Figure1). The phylogenetic tree of the atrazine degrading bacterial strain ATT and related bacterial species based on the 16S rDNA sequence was provided in Figure (2). It can be clearly seen that the atrazine degrading strain (ATT) was included in the genus *Ochrobactrum* and closely related to the species *oryzae*.

The obtained data showed a remarkable capability of a pure culture of *Ochrobactrum oryzae* for atrazine degradation. The capability of *Ochrobactrum* sp.



for the degradation of atrazine was recorded by many authors as a member of mixed atrazine transforming cultures. In 2007, Kolic *et al.* characterized atrazine-degrading bacterial community from contaminated soils of an agrochemical factory. The bacterial community consisted of two *Arthrobacter* strains in addition to *Ochrobactrum* sp. and *Pseudomonas* sp. Furthermore, Udikovic-Kolic *et al.* (2010) identified *Ochrobactrum* strain as a member of the bacterial population consisting of, *Arthrobacter* sp., *Achromobacter* sp. and *Flavobacterium* sp. This bacterial community demonstrated elevated competence for atrazine elimination.

### **Optimization of the growth of *Ochrobactrum oryzae* and atrazine degradation**

The degradation of s-triazine pesticides by bacteria has been found to occur via different pathways ending with cyanuric acid as intermediate compound (De Souza *et al.*, 1996; Sadowsky *et al.*, 1998). The cyanuric acid is further metabolized by bacteria via hydrolytic enzymes that ultimately release the nitrogen as ammonia (Cheng *et al.*, 2005). In order to specify the optimum growth conditions of *Ochrobactrum oryzae*, the effect of atrazine concentration, temperature, and pH value was investigated. Data in Figure (3) showed the effect of atrazine concentration (100 – 500 ppm) as a sole carbon and nitrogen source on the growth of *O. oryzae*. The maximum optical density (0.61) was recorded at 400 ppm of atrazine after 12 days of incubation while the highest protein content (284 mg/L) was observed after 9 days at the same concentration of atrazine. Above or beyond this concentration, an obvious reduction in the optical density and protein content of the bacterial culture was recorded.

Regarding the effect of temperature on the growth of *O. oryzae* (Figure 4) and atrazine degradation (Figure 6 and Table 2), the maximum bacterial optical density, protein content and atrazine degradation were demonstrated at 30°C after 9 days. At

temperatures below (20°C) or above (37°C), clear inhibition in the optical density (0.51 & 0.36) and protein contents (185.6 & 127.3). At the same time, a marked inhibition in the degradation efficiency of atrazine (59.3% & 20.1%) was recorded. These results are in agreement with those reported by Abigail *et al.* (2012) and Belal *et al.* (2013) who found that the optimum temperature for atrazine degradation by *Cryptococcus laurentii* and *Pseudomonas fluorescens* was 30°C.

Simultaneously, the change in the pH value demonstrated a remarkable effect on the growth of *O. oryzae*. The effect of pH value on bacterial growth and atrazine degradation was shown in Figure (5 & 6) and Table (2). The maximum optical density (0.69), protein content (253.4 mg/L) and atrazine degradation (83.5%) was obtained at pH 9.0 after 9 days. In the meantime, the recorded optical density (0.19 & 0.56), protein content (43.9 & 215.7 mg/L) and atrazine degradation (16.2%, 72.3%) were significantly reduced at pH ranges 5.0 and 7.0. These outcomes are in harmony with the studies which clarified that soil pH was the most significantly related parameter for atrazine mineralization. At pH values lower than 6.5, less than 25% of the initial atrazine was mineralized. The optimum pH for atrazine degradation was found to be between 7.0 and 9.0 (Clotaire & Nikolaus, 1994; Abigail *et al.*, 2012 ).

## **Conclusion**

An atrazine tolerant bacterial strain was isolated from agricultural drainage ditches by enrichment technique. This strain that was identified by 16S rDNA techniques as *Ochrobactrum oryzae*, showed a high capability to utilize atrazine as a sole carbon and nitrogen source. A remarkable rate of atrazine degradation was achieved at pH value 9.0 and temperature 30°C within 9 days. As a consequence, *O. oryzae* could be used efficiently for the environmental cleanup of agricultural

wastewater contaminated with high levels of atrazine and limiting the amount of this herbicide carried into downstream ecosystems.

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## Figure captions

**Figure 1.** Partial DNA sequences of the 16S rDNA gene of the bacterial strain ATT isolated from agricultural wastewater and the corresponding gene of *Ochrobactrum oryzae* SP91A (accession number: AB841136).

**Figure 2.** Phylogenetic dendrogram obtained by distance matrix analysis of 16S rDNA sequences showing the position of the bacterial isolate ATT among phylogenetic neighbors. The black arrow indicates the position of ATT strain.

**Figure 3.** Effect of atrazine concentration on the growth of *Ochrobactrum oryzae*, (A) represents the optical density (OD<sub>600</sub>) while (B) represents the protein content (mg/L) of the bacterial growth. Atrazine was used as sole carbon and nitrogen source. Data are the means of three replicates and error bars represent the standard errors of the means.

**Figure 4.** Effect of temperature on the growth of *Ochrobactrum oryzae*, (A) represents the optical density (OD<sub>600</sub>) while (B) represents the protein content (mg/L) of the bacterial growth. Atrazine (400 ppm) was used as sole carbon and nitrogen source. Data are the means of three replicates and error bars represent the standard errors of the means.

**Figure 5.** Effect of pH value on the growth of *Ochrobactrum oryzae*, (A) represents the optical density (OD<sub>600</sub>) while (B) represents the protein content (mg/L) of the bacterial growth. Atrazine (400 ppm) was used as sole carbon and nitrogen source. Data are the means of three replicates and error bars represent the standard errors of the means.

**Figure 6.** Optimization of atrazine degradation by *Ochrobactrum oryzae*, (A) represents the effect of temperature while (B) represents the effect of pH value on the biodegradation process after 9 days. Initial concentration of atrazine was 386.3 ppm. Data are the means of three replicates and error bars represent the standard errors of the means.

**Table 1.** Biochemical characterization of the atrazine degrading bacterial isolate ATT.

<b>Reaction</b>	<b>Result</b>	<b>Reaction</b>	<b>Result</b>
<b>Morphological characters</b>		<b>Fermentation of sugars</b>	
Gram staining	-ve	Glucose	+ve
Motility	+ve	Sucrose	-ve
Cell shape	Rod	Mannitol	+ve
Endospore formation	-ve	Inositol	+ve
<b>Biochemical characters</b>		Sorbitol	-ve
<b>Enzyme profile</b>		Rhamnose	-ve
$\beta$ -galactosidase	-ve	Melibiose	-ve
Arginine dihydrolase	+ve	Amygdalin	-ve
Lysine decarboxylase	+ve	Arabinose	-ve
Orethine decarboxylase	-ve	Starch	+ve
Urease	+ve	Citrate utilization	+ve
Tryptophane deaminase	+ve	<b>Other tests</b>	
Gelatinase	+ve	H <sub>2</sub> S production	-ve
Catalase	+ve	Acetoin production	+ve
Amylase	+ve	Indole production	-ve
Lipase	-ve		
Cytochrome oxidase	+ve		
Nitrate reduction			
-to nitrite	-ve		
-to N <sub>2</sub> gas	+ve		



**Table 2.** The percentage of atrazine removal by *Ochrobactrum oryzae* under different temperatures and pH values after 9 days of incubation. The initial concentration was 386.3 ppm.

<b>Treatment</b>		<b>Retention time (min)</b>	<b>Residual (ppm)</b>	<b>Removal (%)</b>
<b>pH value</b>	<b>5.0</b>	4.612	323.6	16.2
	<b>7.0</b>	4.605	123.2	72.3
	<b>9.0</b>	4.644	63.8	83.5
<b>Temperature</b>	<b>20°C</b>	4.616	157.7	59.3
	<b>30°C</b>	4.609	125.9	67.4
	<b>37°C</b>	4.638	307.3	20.1