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Atrazine mineralization by *Stenotrophomonas maltophilia* and *Agrobacterium tumefaciens* Egyptian soil isolates

Emad K. Radwan[a,*,‡], Talaat N.M. El Sebai[b,‡], Hany H. Abdel Ghafar[a,c], Abd El-Nasser A. Khattab[d]

[a]*Water Pollution Research Department, National Research Centre, 33 El Bohouth St., Dokki, Giza, P.O. Box: 12622, Giza, Egypt, Tel. +202 33370931; Fax: +202 33371211; emails: emadk80@gmail.com (E.K. Radwan), hany_ghafar@hotmail.com (H.H.A. Ghafar)*
[b]*Agricultural Microbiology Department, National Research Centre, 33 El Bohouth St., Dokki, P.O. Box: 12622, Giza, Egypt, email: alaatelsebai@gmail.com*
[c]*Department of Chemistry, Faculty of Science and Arts-Khulais, University of Jeddah, Jeddah, Saudi Arabia*
[d]*Genetics and Cytology Department, National Research Centre, 33 El Bohouth St., Dokki, P.O. Box: 12622, Giza, Egypt, email: khattababdelnasser69@yahoo.com*

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ABSTRACT

Atrazine is a water pollutant that has serious effects to human being and living organisms, thus it should be eliminated for environmental safety. Among the various methods used for its removal, biodegradation gains attraction due to its cost-effectiveness and environmental friendly nature. For this purpose, soil samples having previous history of treatment with atrazine were collected from El-Beheira Governorate, Egypt. The efficiency of the isolates to remove and mineralize atrazine was tested. Two bacterial strains achieved high removal percentage (up to 80% for S3) of atrazine during the first 7 h. These two bacterial strains were identified as *Stenotrophomonas maltophilia* and *Agrobacterium tumefaciens* using molecular identification based on sequencing of 16S rRNA gene. All the 16S nucleotides sequences of bacteria were applied and conserved in GeneBank. *Agrobacterium tumefaciens* mineralizes higher percentage of atrazine at shorter time relative to *Stenotrophomonas maltophilia.* External carbon source plays a key role in atrazine mineralization.

Keywords: Mineralization; Herbicides; Bacterial remediation; Wastewater treatment; Molecular identification; Phylogenetic analysis

1. Introduction

Atrazine is a widely used herbicide in agriculture worldwide. It is used to control the growth of grassy weeds and broadleaf in crops such as sorghum, sugarcane and maize [1,2]. Almost 70,000 to 90,000 tons of atrazine are used annually for this purpose [3]. It is considered as a common pollutant in water and soil. It has gained much attention not only because of its immediate pollution potential but also due to its recalcitrance in the environment. The massive production of atrazine and its high mobility leads to the detection of atrazine in soil, agricultural discharge water, surface and ground water at concentrations much higher than the permissible limits even after a long time past its applications [4,5]. Atrazine can cause damage to future crops, may reduce other organism populations, and may pollute water and food [6,7].

Atrazine was reported as a disruptor for endocrine, regular hormone interrupter, cause birth defects, produce tumors, cause weight loss in humans and has been correlated with the formation of several types of cancer (breast, ovarian, and uterine tumors), as well as of lymphoma and leukemia [5,8].

* Corresponding author.
‡ These authors contributed equally to this work and have rights for both authors.

Consequently, the removal of atrazine is obligatory for saving the environment. Atrazine can be removed from water by several methods such as physical [9,10], chemical [11] and biological methods. Among them, the biological treatment processes have several advantages over chemical and physical treatment methods. Because of its low-cost, eco-friendly, effectiveness and high efficiency features [12].

There are many microorganisms that have been isolated, characterized and reported as atrazine degrader, such as strain of *Pseudomonas* [13], *Acinetobacter* [5], *Rhodococcus* [14], *Arthrobacter* [15], *Bacillus* [8], *Variovorax*, etc. [12]. However, the efficiencies of biodegradation process were not high enough in most cases. Therefore, it is important to isolate highly efficient bacterium strains for atrazine removal [16].

This work is focused on the isolation of probable atrazine degrading bacterial strains from the soil of River Nile delta in Egypt. The ability of the isolated strains to grow and form clear zone on the liquid atrazine medium was tested and the promising strains were further tested to remove atrazine parent compound. The efficiency of promising strains to mineralize atrazine was investigated as well. This is the first report on the isolation and identification of atrazine degrading bacterial strains *Stenotrophomonas maltophilia* and *Agrobacterium tumefaciens* from Egyptian soil.

2. Materials and methods

2.1. Chemicals

All chemicals used in mineral salts medium (MSM) and atrazine (structure shown in Fig. 1) PESTANAL® analytical standard were obtained from Sigma-Aldrich (Egypt) and used as obtained without further purification.

2.2. Soil sampling sites

The samples were collected from soil cultured with corn in El-Beheira Governorate, Egypt. The soils had been used to grow corn and had been treated with atrazine for weed control according to normal farming practice for at least 2 years.

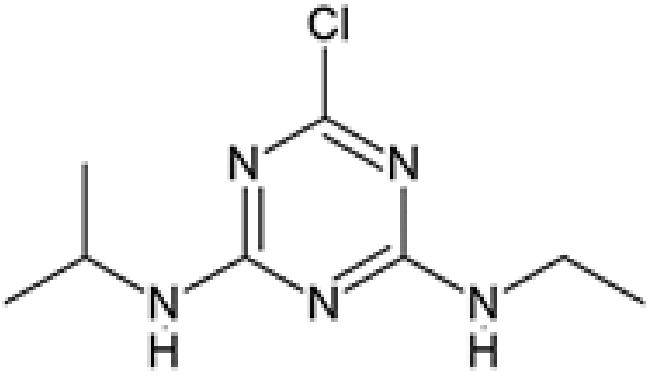

Fig. 1. Structure of atrazine (2-chloro-4-(ethylamino)-6-(isopropylamino)-1,3,5-triazine).

2.3. Analytical methods

2.3.1. Measurement of atrazine

The concentration of atrazine parent compound was followed spectrophotometrically. The atrazine concentration was measured in withdrawn samples from media after filtration through 0.45 um membrane syringe filter and the concentration was measured using Shimadzu (Japan) UV-VIS model UV-240 spectrophotometer at wavelength 223 nm. The samples, blank and control were measured in triplicate.

The mineralization of atrazine was followed by measuring the total organic carbon in filtered samples using TOC instrument (Phoenix 8000–UV/Persulfate-TOC Analyzer). The measurement was performed to indicate the amount of organic carbon present in each step of experiment and to follow up the decay of atrazine by bacteria.

2.3.2. Isolation, enrichment and culture conditions

Enrichment preparations consisted of a MSM containing 50 mg/L atrazine as the sole nitrogen and carbon source, inoculated with soil (15%, w/v) and incubated aerobically with shaking at 30°C [8]. Enrichment medium consisted of MSM and 100 mg/L atrazine as the sole nitrogen source, plus sucrose 3 g/L as carbon source and was autoclaved at 121°C for 30 min. The MSM contained (per liter): 1.6 g of K_2HPO_4, 0.40 g of KH_2PO_4, 0.20 g of $MgSO_4{\cdot}7H_2O$ and 0.10 g of NaCl. Trace element solution contained (per liter): EDTA, 2.5 g; $FeSO_4{\cdot}7H_2O$, 1.0 g; $ZnSO_4{\cdot}7H_2O$, 5.0 g; $MnSO_4{\cdot}H_2O$, 1.0 g; $CuSO_4{\cdot}5H_2O$, 0.40 g; $Na_2B_4O_7{\cdot}10H_2O$, 0.20 g; $Na_2MoO_4{\cdot}2H_2O$, 0.25 g. The pH of the medium was adjusted to 7.0 by 1 M NaOH solution. Atrazine was added from a 100 mg/mL stock solution (in methanol). The solid growth medium contained the same mineral salts and 2% agar. The isolated species, named as S1, S2 …. and S20, were re-enriched in the lab level using the above enrichment technique but the atrazine concentration was elevated up to 1 g/L.

2.3.3. Molecular identification of bacteria

The overnight cultures of bacterial strains (S2 and S3) grown in liquid atrazine medium containing 25 mg of atrazine per liter were used for total DNA extraction according to i-genomic BYF DNA extraction Mini Kit, iNtRON Biotechnology Inc., South Korea. The quantity and purity of the obtained DNA were determined according to the ultraviolet (UV)-absorbance at 260 and 280 nm using spectrophotometer (Shimadzu UV-Vis model UV-240, Japan) according to the study by Maniatis [17]. The PCR amplification of 16S ribosomal gene was done by using Maxima Hot Start PCR Master Mix (Thermo K1051) and the nucleotide sequences of the 16S primers were as follows: forward primer, 5′-AGA GTT TGA TCC TGG CTC AG-3′; reverse primer, 5′-GGT TAC CTT GTT ACG ACT T-3′. The PCR product was cleaned up using GeneJET™ PCR Purification Kit (Thermo K0701). The DNA sequencing of the PCR product was done using ABI 3730xl DNA sequencer (GATC Company, Germany) by forward and reverse primers.

2.3.4. Phylogenetic analysis

The 16S rDNA sequences of the isolates were compared with the sequences available by the BLAST search in the NCBI, GenBank database (http://www.ncbi.nlm.nih.gov). The sequences were aligned together with those of reference taxa retrieved from public databases. The evolutionary distances were generated based on parameter model [18] and phylogenetic trees were constructed by using the neighbor-joining method [19].

2.3.5. Nucleotide sequence accession number

The 16S rDNA sequence of the bacterial strains (S2 and S3) was deposited in the NCBI Gene Bankit nucleotide sequence database under accession numbers MH698831 and MH698832, respectively.

3. Results and discussion

3.1. Bacterial growth

Twenty atrazine-degrading bacterial strains were isolated from two farms cultured with corn and cucumber. All of these organisms were capable for clearing zone on the liquid atrazine medium, but two isolates S2 and S3 gave the highest growth and clearing zones in comparison with the others isolates (Fig. 2). Strain S3 produced obvious clearing zones around the colonies on agar medium amended with atrazine (1 g/L). This phenomenon indicated the ability of the bacterium to metabolize high concentrations of atrazine.

3.2. Biodegradation of atrazine

The disappearance of atrazine parent compound was tracked using spectrophotometer. The percentage removed of atrazine as a function of time is presented in Fig. 3. Both of the bacterial strains S2 and S3 achieved high removal percentage (up to 80% for S3) of atrazine during the first 7 h then the removal percentage remained almost constant. In addition, the presence of external carbon source had insignificant effect on atrazine removal in case of S2 and a significant detrimental effect in case of S3. On the other hand, a trivial removal percentage was observed for the bacterial strain S4 after 20 h both in the presence and absence of external carbon source, only 6.85% and 12.38%, respectively. Based on all the aforementioned results, the two strains designated as S2 and S3 were selected for further study due to its high degradation activity on atrazine.

The mineralization of pollutants is vital to ensure environmental safety because in some cases more toxic and/or recalcitrant intermediates are formed during the degradation [20]. Therefore, mineralization is a key feature to evaluate the efficiency of the treatment process. In this study, the importance of studying the ability of the bacterial strains to mineralize atrazine parent compound stems from the fact that atrazine degradation usually stops at the formation of cyanuric

Fig. 2. Halo zone around bacterial strains growth on solid media containing atrazine (1 g/L) as a sole carbon and nitrogen source.

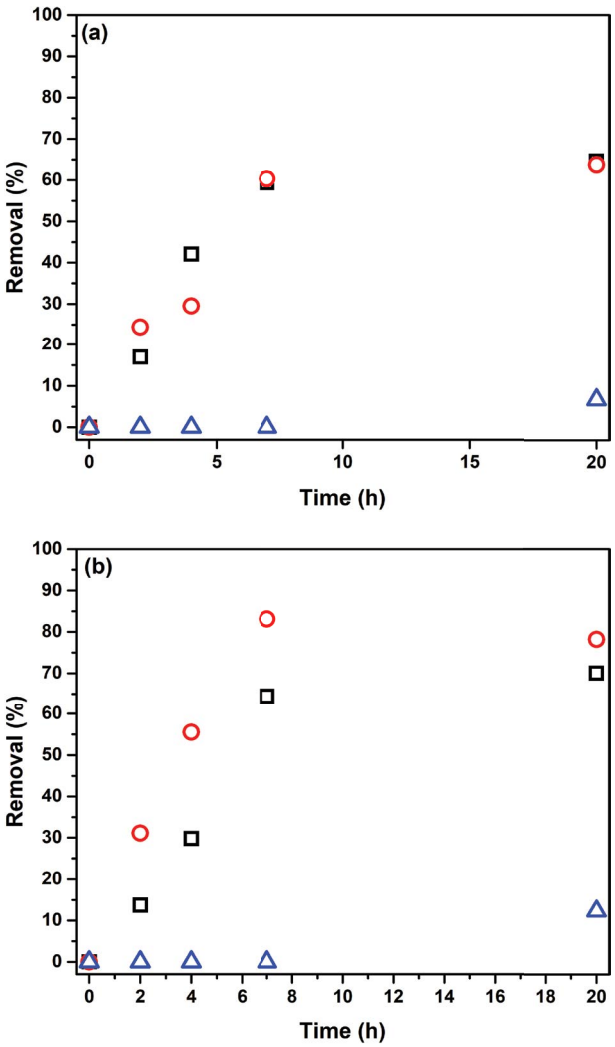

Fig. 3. Decay of atrazine by S2 (square), S3 (circle) and S4 (triangle) isolates in presence (a) and absence (b) of external carbon source (sodium citrate).

acid [11]. The mineralization of atrazine was monitored using a TOC analyzer and the results are shown in Fig. 4.

Because the MSM contains a source of organic carbon, the time profile of the mineralization of the MSM was evaluated and compared with that of the MSM containing atrazine. Fig. 4a shows that the presence of external carbon source enhanced significantly the mineralization of atrazine relative to the MSM. It seems that the mineralization process is fast in the first 2 d (38% and 53% for S2 and S3, respectively). This sharp increase might be due to the degradation of (carbonaceous part) aliphatic part of atrazine which is easily degraded [21]. In case of S3, the fast stage is followed by a steady stage (mineralization remained constant at ~55% until the 7th day) then increased to 78% in the 10th day. This steady stage might be explained by the recalcitrance of the heterocyclic ring for degradation [21]. While for S2 the fast stage is followed by a slower progressive stage that reach 69% mineralization in 10 d. Also, it is obvious that S3 strain achieves higher mineralization of atrazine at shorter time relative to S2. Notably, for both S2 and S3 the level off state was not reached after 10 d indicating that longer time might result in complete mineralization. Thus, it can be concluded that S2 and S3 strains are capable of mineralizing atrazine. On the other hand, Fig. 4b shows that in absence of the external carbon source no significant difference in atrazine mineralization was observed for the MSM and the MSM containing atrazine. This observation implies that the bacterial strains could not propagate quickly when atrazine was the sole carbon and energy source. It also signifies the importance of the presence of external carbon source to mineralize atrazine. Similar trends have been reported before [22,23].

The results of parent compound disappearance (Figs. 3a and b) and mineralization (Figs. 4a and b) seem contrasting. However, it can be explained as follows. It is likely that in absence of external carbon source the degradation process stops after transforming the atrazine parent compound to another intermediate, might be cyanuric acid. The degradation process stops probably due to lack of carbon source necessary for bacterial strains growth. While, in presence of external carbon source the bacterial strains grow and the degradation process proceeds to mineralization. This explanation is consistent with the literature [24,25].

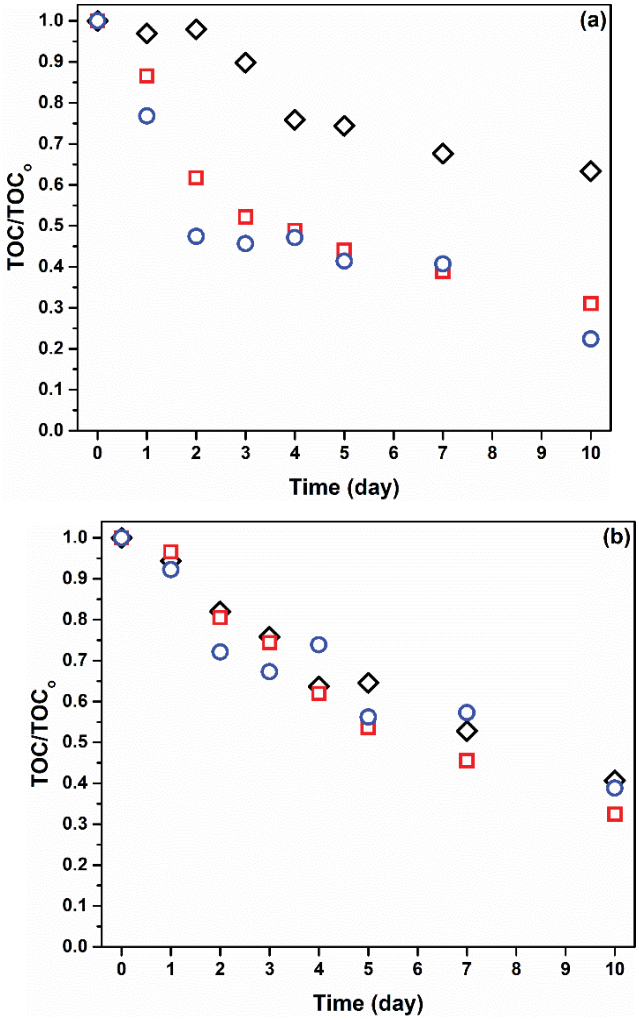

Fig. 4. Mineralization of MSM (diamond) and atrazine by S2 (square) and S3 (circle) isolates in presence (a) and absence (b) of external carbon source (sodium citrate).

3.3. Identification of bacterial isolates

The 16S primer F and R primers were used to amplify the region of the 16S ribosomal RNA gene from the genomic DNA of the bacterial isolates (S2 and S3). After the amplification by

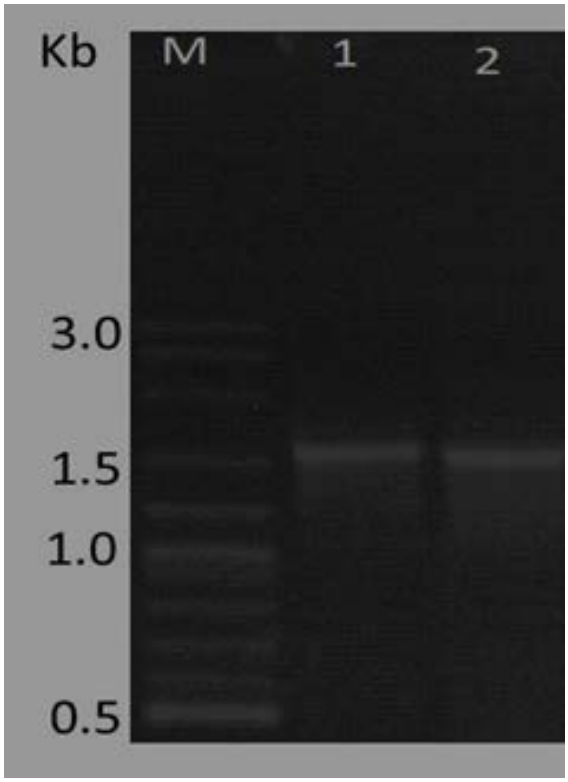

Fig. 5. Photograph of 16S-DNA amplified band for bacterial strains S2 (lane1) and S3 (lane 2) using 16S primers against ladder DNA marker which has three distinct bands: 500; 1,500; 5,000 bp (lane M).

PCR, a product of approximately 1,500 bp was obtained as shown in Fig. 5.

The BLAST analysis of the amplified 16S ribosomal RNA gene sequence of the bacterial isolates S2 99% similarity to the partial 16S ribosomal RNA gene *Stenotrophomonas maltophilia* strain (accession number: MH000623.1). Based on this result, the bacterial strain (S2) was identified as *Stenotrophomonas maltophilia* as shown in Fig. 6. On the other hand, the BLAST analysis of the amplified 16S ribosomal RNA gene sequence of the bacterial isolates S3 revealed 99% similarity to the partial 16S ribosomal RNA gene *Agrobacterium tumefaciens* train (accession number: MH236272.1). This result confirmed that the bacterial strain (S3) was *Agrobacterium tumefaciens* as shown in Fig. 7.

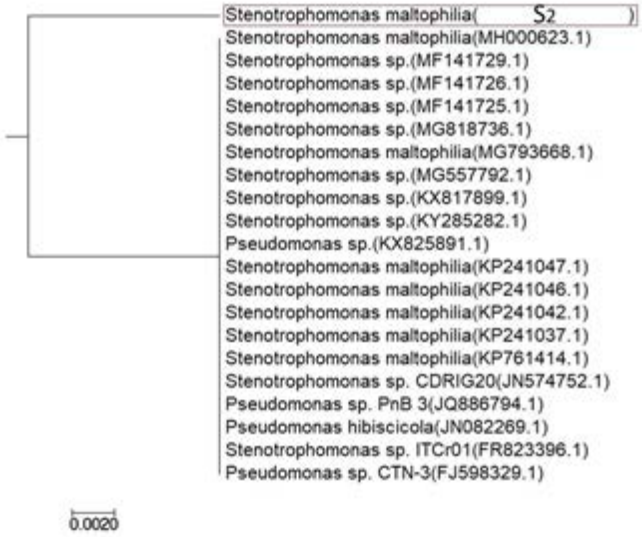

Fig. 6. Phylogenetic tree of the *Stenotrophomonas maltophilia* (S2) strain under study compared with the most related bacterial database.

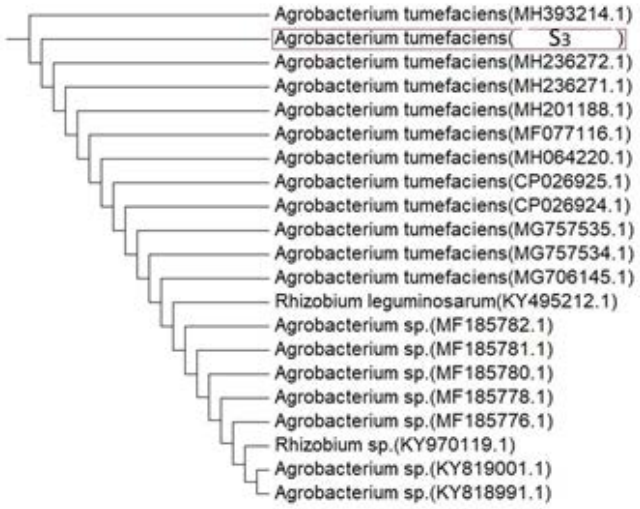

Fig. 7. Phylogenetic tree of the *Agrobacterium tumefaciens* (S3) strain under study compared with the most related bacterial database.

4. Conclusions

In the present study, bacterial strains were isolated from the Nile delta soils in Egypt. Biochemical and 16S rRNA sequencing data suggested that the isolated strains (S2 and S3) belonged to *Stenotrophomonas maltophilia* and *Agrobacterium tumefaciens*, respectively. The experiments in mineral salt medium supplemented with atrazine with and without external carbon source revealed that the isolated bacterial strains were capable to degrade the atrazine. The results that illustrate the degradation of atrazine, indicated that the bacteria degrade more than 80% of atrazine parent compound in the first 7 h of treatment and mineralize 78% during 10 d. In general, the outcomes of this study suggested that the isolated strains can be utilized for the bioremediation of atrazine polluted water. Further study of the mechanism and the degradation pathway of atrazine by *Stenotrophomonas maltophilia* and *Agrobacterium tumefaciens* will be conducted in near future.

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