J. Agric. Sci. Mansoura Univ., 33 (10):7477 - 7485, 2008

ATRAZINE DEGRADATION BY *Bacillus megaterium* ISOLATED FROM AGRICULTURAL SOIL

El-Sawah, M.M.A.[1]; Samia M.M. Bauoymy[1]; Eman H. Ashour[1]; Lobna A. Moussa[2] and Samah A. H.Shady[1]

[1] Microbiol. Dept., Fac. Agric., Mansoura Univ., Mansoura, Egypt.

[2] Soil, Water and Environ. Res. Instit., Agric. Res. Center (ARC), Giza.

ABSTRACT

In present study the capability of some microorganisms isolated from Egyptian soil to a triazine herbicide, atriazine, decomposition was assessed. Nine atrazine-degrading bacteria were isolated from soil that received repeated exposures of the commonly used herbicides atrazine. These isolates were belonged to genera *Basilllus, Pseudomonas* and *Micrococcus*. One isolate showed good growth and clearing zone on mineral salts agar media amended with atrazine (at 500 ppm) as a carbon and/or nitrogen source. This most active strain involved in atrazine degradation were selected and identified. The strain was classified as *Bacillus megaterium*. It degraded 45.8% of initial concentration of atrazine concurrent with increasing the population size from 10^5 to 10^8 CFU/ml culture. Atrazine-degrading enzymes by *B. megaterium* appear to be induced. No released chloride was detected from *B. megaterium* culture indicating that the triazine ring may be remained intact and the atrazine-metabolites not hydroxyatrazine. Atrazine metabolites may be deethylatrazine or deisopropylatrazine. These results indicate that *B. megaterium* can play an important role in the bioremediation of atrazine-contaminated sites.

Keywords: atrazine, *Basilllus, Pseudomonas* and *Micrococcus*, degradation, *Bacillus megaterium*, bioremediation.

INTRODUCTION

Atrazine, 2-chloro-4-(ethylamine)-6-(*iso*propylamine)-*s*-triazine, an organic compound consisting of an *s*-triazine-ring, is a widely used herbicide. Atrazine is one of the most environmentally prevalent *s*-triazine-ring herbicides through its global use to stop pre- and post-emergence broadleaf and grassy weeds in major crops, *e.g.*, maize, sorghum and sugarcane. It is also used for non-selective weed control in non-cropland and industrial areas (Sparling *et al.*, 1998). Atrazine acts by binding to the plastoquinone-binding protein in photosystem II, inhibiting electron transport (Ackerman, 2007).

Atrazine poses a potential health threat. Long-term exposure to atrazine-contaminated drinking water can potentially cause cancer. However, it has been classified a class C/possible human carcinogen (Loprieno *et al.* 1980).

Atrazine is though to be persistent especially in aquifers or anaerobic sediments (Widmer and Spalding, 1995). The half-life of atrazine in natural soils ranging from 13 to 261 days (U.S. EPA, 2003). The frequency of the occurrence of atrazine in the environment is related to extensive usage, atrazine's moderate persistence, and its mobility through the soil (Burkart and Kolpin, 1993). However, residues of both the parent compound and its

derivatives have been detected in soils years after application (Schiavon, 1988).

The fate of *s*-triazine compounds in the environment is directly correlated with the ability of microbes to metabolize them. Atrazine biodegradation can occur by two pathways. Atrazine can be dechlorinated followed by removal the other ring substituents *via* amidohydrolases by the enzymes AtzA, AtzB, and AtzC. The end product is cyanuric acid. The best characterized organism that performs this pathway is *Pseudomonas* sp. strain ADP. The second pathway involves dealkylation of the amino groups. subsequent dechlorination yields cyanuric acid. The end result is 2-chloro-4-hydroxy-6-amino-1,3,5-triazine, which currently has no known path to further degradation. This path occurs in *Pseudomonas* species and a number of bacteria (Wackett *et al.*, 2002 and Zeng *et al.*, 2004).

Atrazine degrades in soil by the action of microorganisms. Microbial degradation determines the environmental impact and efficacy of an herbicide. Rates of biodegradation affected by atrazine's low solubility. Atrazine itself is a poor energy source due to the highly oxidized carbons in the ring. It is catabolized as a carbon and nitrogen source in limiting environments. Inorganic nitrogen accelerates atrazine catabolism whereas organic nitrogen decreases it. Low concentrations of glucose can decrease the bioavailability, whereas higher concentrations promote the catabolism of atrazine (Ralebitso *et al.*, 2002). A variety of bacteria and fungi that dealkylate or dechlorinate atrazine but do not mineralize the *s*-triazine ring have been isolated (Donnelly *et al.* 1993, Behki *et al.* 1993; Nagy *et al.* 1995, De Souza *et al.*, 1995 and 1996 and Mougin *et al.* 1997 and Bouquard *et al.* 1997). Several microorganisms including members of the genera *Pseudomonas*, *Acinetobacter*, and *Agrobacterium* capable of atrazine mineralization have been isolated from sediments or soils that have frequently contact with this herbicides (Vanderheyden *et al.* 1997; Struthers *et al.* 1998; Rousseaux *et al.* 2001 and Topp 2001).

The objective of this research was to enrich for microorganisms capable of mineralization of high concentration of atrazine and use it as a carbon and energy source.

MATERIALS AND METHODS

Chemical

Herbicide atrazine (W.P 80% KZ) 2-chloro-4-[ethylamino]-6-[isopropylamino]-1,3,5-triazine (Fig. 1) was a gift from pesticides center lab., Egypt.

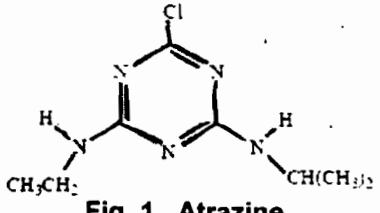

Fig. 1. Atrazine

Enrichment and Isolation

One kg dry weight soil was placed into glass beaker, fortified with commercial atrazine at a double of rate field dissolved in water which adds to save soil moisture at approximately 70% field capacity, thoroughly mixed with the soil and beaker covered with porous aluminum foil to ensure gas exchange. The beaker was, incubated at 30°C for three weeks. Each amendment of atrazine was applied a total of 3 times at 3-weeks intervals, and after the final amendment, the soil remained in the incubator for an additional 2 weeks prior to further analysis. Water was added to replace any water lost during the incubation period.

Ten grams of previously atrazine-treated soil was placed in 250 ml of basal salts medium containing K_2HPO_4, 0.5 g; $(NH_4)_2SO_4$, 0.5 g; $MgSO_4.7H_2O$, 0.5 g; $FeCl_3.H_2O$, 10 mg; $CaCl_2.H_2O$, 10 mg; $MnCl_2$, 0.1 mg; $ZnSO_4$, 0.01 mg; glucose 1 g /l (pH 6.8) prepared as described by (Radosevich *et al.*, 1995). This medium was amended with atrazine as an active ingredient at 50 ppm. The pesticide was solved in acetone as a stock solution and added after autoclaving media.

The enrichment culture was incubated with shaking for 1 week at 30°C, 5 ml was placed in 45 ml of saline solution, and the cultures was isolated by serial dilution and plated onto Petri dishes containing nutrient agar medium. Individual colonies were picked up and purified. Pure isolates were streaked on agar slants and stored at 4°C.

All isolates were tested for growth on atrazine at 100 and 500 ppm as carbon and/or nitrogen source on mineral salts agar medium. The best isolates which show high, rapid growth and clearing zone on agar plates were picked up to complete the experiments.

Isolates identification:

The isolates were identified according to Sneath *et al.* (1986).

The growth ability of selected isolates on atrazine concentrations

A fresh cell suspension of selected isolate (OD 0.5 at 620 nm) was incubated aerobically in 100-ml flasks contain 40 ml of basal salts medium with active ingredient of atrazine as a sole source of both carbon and nitrogen at 0, 50, 100, 300 and 500 ppm and incubated on a rotary shaker (170 rpm) at 30°C. The growth rate was measured by OD at 620 nm (A_{620}) at 0, 4, 8, 12, 16, 20, 24, 48, 72 and 96 h.

Atrazine-degradation by growing cells

Selected isolate was cultured in 100-ml flasks contain 40 ml of basal mineral salts medium with active ingredient of atrazine as sole carbon and nitrogen source at 50 ppm. The culture was grown for 30 days on a rotary shaker (170 rpm) at 30°C. The growth was measured by cell counts and the residue of atrazine was measured by GLC at 0, 3, 7, 10, 15, 21 and 30 days. The control was free from any isolates to notice the impact of photodegradation or hydrolytic effect on atrazine.

To determined if the atrazine-degrading enzymes are produced constitutively or induced, basal mineral medium amended with 50 ppm of atrazine as a carbon and nitrogen source and 100 µg of chloramphenicol ml^{-1} was inoculated with cells and incubated for 10 days. Concentrations of

herbicide were determined by GLC. Population size was determined by plating (Struthers *et al.*, 1998).

Residue analysis

Atrazine were extracted twice by ethyl acetate (1:1, vol/vol). Pooled fractions were dried over sodium sulfate, reduced just to dryness in vacuum, and taken up in a small volume of acetone for GLC analysis (Behki *et al.*, 1993).

Chloride release

Cell culture of selected isolate was inoculated into chloride, carbon, and nitrogen-free liquid medium with atrazine at 100 ppm. Cultures were incubated for 10 days on a rotary shaker (170 rpm) at 30°C. Chloride release was determined by $AgNO_3$ (Smith *et al.*, 2005).

RESULTS AND DISCUSSIONS

Isolation and selection of atrazine-degrading bacteria

Fifteen individual bacterial isolates were obtained after purification. Nine of the isolated bacteria, which show different morphology according to growth on agar plates and to shape of cells under microscope, were chosen for subsequent experiments. These isolates classified according to Gram stain, morphology and some biochemical characteristics to three genera, *Bacillus*, *Pseudomonas*, and *Micrococcus*. Ayansina and Oso (2006) reported that *Bacillus* sp. and *Pseudomonas* sp. were the most frequently isolated bacteria from atrazine treated soils.

These isolates showed a varied ability to grow on mineral salts agar with atrazine (100 and 500 ppm) as a carbon and/or nitrogen sources.

The mineral salts agar medium amended with 100 ppm atrazine was used for the isolation of the individual members, while the medium with higher atrazine concentration (500 ppm) was employed for potential atrazine degradation activity.

The isolates encoding. A1, A5, A8 and A9 showed good growth on atrazine as a carbon and/or nitrogen source at (100 ppm). Only two isolates A8 and A9 were proven to have the activity of degrading and growing on atrazine as a sole carbon and nitrogen at high concentration (500 ppm). Isolate A8 showed clearing zones after 2 weeks while isolate A9 showed clearing zone after 3 week. However, these clearing zones are indicating to atrazine being dissolved and transformed. Since, the isolate A8 was the best one, which showed high ability to grow on atrazine as a carbon and/or nitrogen at high concentration (500 ppm).

Identification of selected isolate

According to morphological and biochemical characteristics isolate A8 was long rode, Gram-positive, spore-forming ellipsoidal and terminal, motile, catalase positive, hydrolysing of casein, gelatin and starch, utilizing of citrate, produce acid from glucose but not gas, V.P test and indole production were negative. On nutrient agar the growth was creamy, moderately dull. This isolate belongs to genus *Bacillus* and fall within *Bacillus megaterium* (Sneth *et al.*, 1986).

The growth rate on atrazine concentrations

The growth rate of *B. megaterium* showed that *B. megaterium* could to grow on atrazine as a source of carbon and nitrogen at 50 ppm with a specific growth rate 0.0623 hr^{-1} and doubling time was 11.13 hr (Fig. 2). The concentration 100 ppm had similar effect; the specific growth rate was 0.0596 hr^{-1} with doubling time 11.63 hr. By increasing atrazine concentration to 300 and 500 ppm the specific growth rate was greatly reduced to reach 0.0176, 0.0102 hr^{-1} respectively. Doubling time was 39.38 hr at 300 ppm atrazine and 67.96 hr at 500 ppm atrazine. Noteworthy *B. megaterium* could tolerate high concentration of atrazine and the stationery phase was continued after 96 hr. Atrazine decomposition by soil microorganisms was recently reported by Marecik *et al.* (2008)

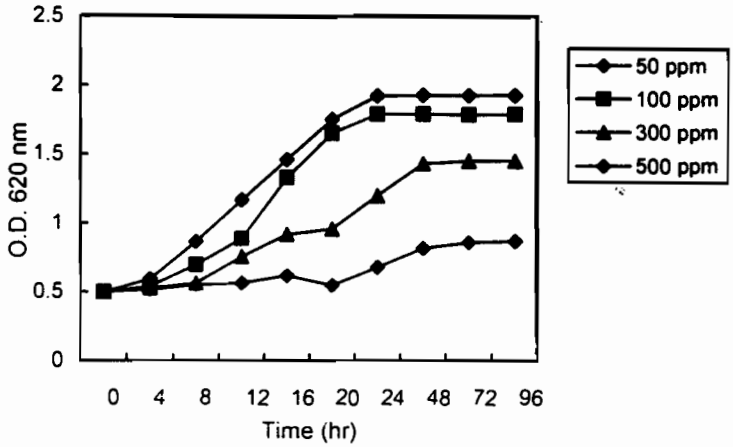

Fig. 2. The growth rate of *B. megaterium* on concentrations of atrazine.

Degradation of atrazine

B. megaterium could to grow successfully in liquid media with atrazine as a carbon and nitrogen source at 50 ppm. Under these conditions viable counts increased from 10^5 to 10^8 CFU ml^{-1} culture. 45.8% of atrazine was transformed within 30 days concurrent increase in the population size (Fig. 3). Viable cell count was measured, with initial inocula density of 30.2 × 10^5 CFU ml^{-1} culture, where it increased until 10 days to reach 45.1 × 10^8 CFU ml^{-1} culture then reduced gradually to 33 × 10^2 CFU ml^{-1} culture at 30 days of incubation.

The loss of atrazine in uninoculated treatment was negligible, only 5.0% of atrazine was disappeared within 30 days. Such loss may be due to hydrolytic effects. This result is in agreement with Behki *et al.* (1993), Mandelbaum *et al.* (1993) and Radosevich *et al.* (1995).

Atrazine-degrading enzymes by *B. megaterium* appear to be induced. The persistence rate of atrazine in medium with 100 µg chloramphenicol ml^{-1} after 10 days was 98.9% and cells population was decreased from 10^6 to 10^4 CFU ml^{-1} culture. However, the persistent rate of

atrazine in medium without chloramphenicol after 10 days was 68.8% with increase the population size from 10^6 to 10^8 CFU ml^{-1} culture.

The persistence rate of atrazine and growth activity for *B. megaterium* results in contrast with what mentioned by Marecik *et al.* (2008) that the mesophilic bacteria *B. megaterium* has lower counts of cells was observed in nutrient broth with atrazine at 5.00 mg l^{-1}. The long sequential enrichment technique with high concentration of pesticides used to make an adaptation to the microorganisms. In addition, free- carbon and nitrogen media used obligate the adaptation bacteria to use the pesticide. However, according to Bergey's Manual Systymatic Bacteriology *B. megaterium* has many strains. Hence, as in our knowledge that was the first report about atrazine-degradation by *B. megaterium* isolated from Egyptian soil.

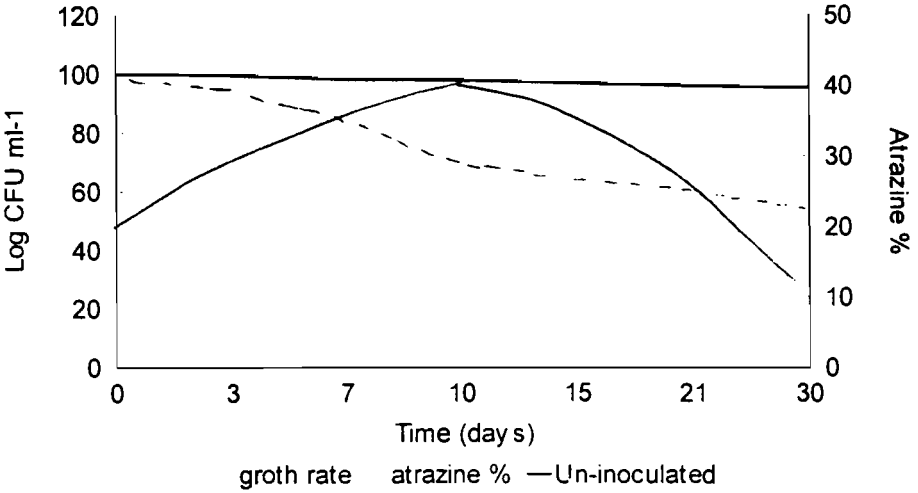

Fig. 3. Persistence of atrazine and growth rate of *B. megaterium*.

Agrobacterium radiobacter J14a grown in nitrogen-free medium with citrate and sucrose as carbon sources mineralized 94% of 50 µg of [^{14}C-U-*ring*] atrazine ml^{-1} in 72 h with increasing in the population size from 7.9×10^5 to 5.0×10^7 cells ml^{-1}. While in the medium with 50 µg of [^{14}C-U-*ring*] atrazine ml^{-1} as a sole carbon and nitrogen source, only 11% of the atrazine was mineralized. Populations declined from 7.6×10^5 to 3.1×10^5 cells ml^{-1} after 120 h (Strurhers *et al.* 1998).

Chloride release

No released chloride was detected from *B. megaterium* culture indicating that the chlorohydrolase enzymes may be not found in this bacterium. Also, this result indicating that the triazine ring may be remained intact. [1]Also, this result a signal to that the atrazine-metabolites not hydroxyatrazine, it may be deethylatrazine or deisopropylatrazine.

Microbial utilization of the lateral-chain carbon and of both the lateral and ring nitrogen has been observed in few microorganisms. *Rhodococcus* strain TE1 metabolized atrazine under aerobic conditions to produce deethyl- and deisopropylatrazine, which were not degraded further and which

accumulated in the incubation medium (Behki *et al.* 1993). Bacterial growth on atrazine typically involves mineralization of the alkyl-side chains that can be used as a nitrogen, carbon and energy sources, whereas the triazine ring is fully oxidized and cannot be used as an energy source (Radosevich *et al.* 1995; Struthers *et al.* 1998 and Bichat *et al.* 1999).

REFERENCES

Ackerman, F. (2007). ase.tufts.edu/gdae/Pubs/rp/EconAtrazine.pdf The Economics of Atrazine. Int. J. Occup. Environ. Health.

Ayansina, A. D. V. and Oso, B. A. (2006). Effect of two commonly used herbicides on soil microflora at two different concentrations. African J. Biotechnol. 5: 129-132.

Behki, R.M., E. Topp, W. Dick, and P. Germon (1993). Metabolism of the herbicide atrazine by *Rhodococcus* strains. Appl. Environ. Microbiol. 59: 1955-1959.

Bichat, F.; Sims, G. K. and Mulvaney, R. L. (1999). Microbial Utilization of Heterocyclic Nitrogen from Atrazine. Soil Sci. Soc. Am. J. 63: 100-110.

Bouquard, C., J. Ouazzani, J.-C. Prome, Y. Michel-Briand, and P. Plesiat (1997). Dechlorination of atrazine by a *Rhizobium* sp. isolate. Appl. Environ. Microbiol. 63: 862-866.

Burkart, M.R., and D.W. Kolpin. (1993). Hydrologic and land-use factors associated with herbicides and nitrate in near-surface aquifers. J. Environ. Qual. 22: 646-656.

De Souza M.L., L.P. Wackett, K.L. Boundy-Mills, R.T. Mandelbaum and M.J. Sadowsky (1995). From Atrazine to Hydroxyatrazine, Appl Environ Microbiol 61(9): 3373-8.

De Souza, M.L., M.J. Sadowsky and L.P. Wackett (1996). Atrazine Chlorohydrolase from *Pseudomonas* sp. Strain ADP: Gene Sequence, Enzyme Purification and Protein Characterization. J. Bacteriol., 178(16): 4894-4900.

Donneley, P.K., J.A. Entry, and D.L. Crawford (1993). Degradation of atrazine and 2,4-dichlorophenoxyacetic acid by mycorrhizal fungi at three nitrogen concentrations in vitro. Appl. Environ. Microbiol. 59: 2642-2647.

Erickson, L.F., and K.H. Lee (1989). Degradation of atrazine and related *s*-triazine. Crit. Rev. Environ. Cont. 19: 1-15.

Goetsch, W.D., G.C. Kirbach, and W.F. Black (1993). Pesticides in well water and ground water at agricultural facilities in Illinois: An initial investigation. In Agrichemical facility site contamination study. Illinois Dept. Agriculture.

Loprieno N, R. Barale, L. Mariani, S. Presciuttini, A. M. Rossi, I. Sbrana, L. Zaccaro, A. Abbondandolo and S. Bonatti (1980). Results of mutagenicity test on the herbicide atrazine. Mutat. Res. 74: 250-258.

Mandelbaum, R. T.; Wackett, L. P. and Allan, D. L. (1993). Mineralization of the s-triazine ring of atrazine by stable bacterial mixed cultures. Appl. Environ.Microbiol. 59: 1695-1701.

Marecik, R., P. Kro´liczak, K. Czaczyk, W. Białas, Anna Olejnik and P. Cyplik (2008). Atrazine degradation by aerobic microorganisms isolated from the rhizosphere of sweet flag (*Acorus calamus* L.) Biodegradation. 19:293–301.

Mougin, C., C. Laugero, M. Asther, and V. Chaplain (1997). Biotransformation of *s*-triazine herbicides and related degradation products in liquid cultures by the white rot fungus *Phanerochaete chrysosporium*. Pestic. Sci. 49: 169-177.

Nagy, I., G. Schoofs, F. Compernolle, P. Proost, J. Vanderleyden, and R. De Mot (1995). Degradation of the thiocarbamate herbicide EPTC (s-ethyl dipropylcarbamothioate) and biosafening by *Rhodococcus* sp. strain NI86/21 involve an inducible cytochrome P-450 system and aldehyde dehydrogenase. J. Bacteriol. 177: 676-687.

Radosevich, M.; Traina, S. J.; Hao, Y. and Tuovinen, O. H. (1995). Degradation and mineralization of atrazine by a soil bacterial isolate. Appl. Environ. Microbiol. 61: 297-302.

Ralebitso, T.K., E. Senior, H.W. van Verseveld (2002). Microbial aspects of atrazine degradation in natural environments. Biodegrad. 13: 11–19.

Rousseaux, S., A. Hartmann, and G. Soulas (2001). Isolation and characterization of new Gram-negative and Gram-positive atrazine degrading bacteria from different French soils. FEMS Microbiol. Ecol. 36: 211-222.

Schiavon, M. (1988). Studies of the leaching of atrazine, of its chlorinated derivatives, and of hydroxyatrazine from soil using ^{14}C ring-labeled compounds under outdoor conditions. Ecotoxicol. Environ. Saf. 15: 46-54.

Smith, D.; Alvey, S. and Crowley, D. E. (2005). Cooperative catabolic within an atrazine-degrading enrichment culture isolated from soil. FEMS Microbiol. Ecol. 53(2): 265-275.

Sneath, P.H.A. (1986). Endo-sporeforming Gram-positive rods and cocci. In: Bergy's Manual of Systematic Bacteriology. Sneath, P. H. A, N. S. Mair, M. E. Sharpe and J. G. Holt. Volume 2 Williams and Wilkins. Baltimore, USA.

Sparling, C, R., Dragten, J. Aislabie, and R. Fraser (1998). Atrazine mineralization in New Zealand topsoils and subsoils: Influence of edaphic factors and numbers of atrazine-degrading microbes. Aus. L. Soil Res. 36: 557-570.

Struthers, J.K., K. Jayachandran, and T.B. Moorman (1998). Biodegradation of atrazine by *Agrobacterium radiobacter* J14a and use of this strain in bioaugmentation of contaminated soil. Appl. Environ. Microbiol. 64: 3368-3375.

Topp, E. (2001). A comparison of three atrazine-degrading bacteria for soil bioremediation. Biol. Fert. Soils. 33: 529-534.

Topp, E.; Mulbry, W. M.; Zhu, H.; Nour, S. M and Cuppels, D. (2000). Characterization of s-triazine herbicide metabolism by a *Nocardioides* sp. isolated from agricultural soils. Appl. Environ. Microbiol. 66: 3134-3141.

U.S. EPA (2003). Interim Reregistration Eligibility Decision for Atrazine, U.S. EPA, January, 2003.

Vanderheyden, V., P. Debongnie and L. Pussemier (1997). Accelerated degradation and mineralization of atrazine in surface and subsurface soil materials. Pestic. Sci. 49: 237-242.

Wackett, L.P., M.J. Sadowsky, B. Martinez and N. Shapir (2002). Biodegradation of atrazine and related s-triazine compounds: from enzymes to field studies. Appl. Microbiol. Biotechnol. 58(1):39–45.

Widmer, S.K., and R.F. Spalding (1995). A natural gradient transport study of selected herbicides. J. Environ. Qual. 24: 445-453.

Zeng, Y., C.L. Sweeney, S. Stephens and P. Kotharu (2004). Atrazine Pathway Map. Wackett L.P. Biodegradation Database.

تحليل الأترازين بواسطة بكتيريا باسيلس ميجاتريم المعزولة من التربة الزراعية

محمود محمد عوض الله السواح [١] وسامية مرسى بيومى [١] وإيمان حسين عاشور [١] ولبنى أحمد موسى [٢] وسماح عبدالله حافظ شادى [١]

[١] قسم الميكروبيولوجيا – كلية الزراعة – جامعة المنصورة – المنصورة – مصر .

[٢] قسم بحوث الميكروبيولوجيا – معهد الأراضى والمياه والبيئة – مركز البحوث الزراعية – الجيزة – مصر .

استهدف البحث دراسة مقدرة بعض الميكروبات المعزولة من التربة المصرية على تحليل مبيد الحشائش الأترازين ، حيث تم عزل تسعة عزلات بكتيرية محللة للأترازين من التربة باستخدام تكنيك المزارع المقواه ، وقد أتبعت هذه العزلات لأجناس الباسيلس والسيدوموناس والميكروكوكس ، وقد أظهرت العزلة ٨ نمو جيد ومنطقة رائقة على بيئة الاجار المحتوية على أملاح معدنية والمدعمة بمبيد الأترازين بتركيز ٥٠٠ جزء فى المليون كمصدر للكربون والنيتروجين ، ومن ثم فقد تم انتقائها وتعريفها على أنها باسيلس ميجاتريم ، وقد وجد أن بمقدورها تحليل ٤٥,٨% من التركيز الأولى للأترازين (٥٠ جزء فى المليون) مع زيادة فى أعدادها من $١٠^{٥}$ إلى $١٠^{٨}$ وحدة مكونة للمستعمرة / ملل مزرعة ، ولم تتمكن الدراسة الحالية من الكشف عن إطلاق الكلوريد فى مزارع هذه البكتيريا بما يدل على بقاء حلقة الترايازين سليمة ، وأن نواتجها الأيضية لتحليل الأترازين ليست هيدروكسى أترازين ، وأنها قد تكون داى إيثيل أترازين أو دى أيزوبروبايل أترازين ، وتدل هذه النتائج على أن بكتيريا باسيلس ميجاتريم قد تلعب دورا هاما فى العلاج الحيوى للمواقع الملوثة بالأترازين .