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Isolation and Identification of Atrazine Degrading Microorganisms from Soil of Dera Ismail Khan District of Pakistan

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Abstract: Atrazine; a member of s-triazine group, is a preferred herbicide used globally either individually or as adjunct with other herbicides. Beside its use, problem arises because of its low degradability leading to elevation of Atrazine concentration above the permissible limit resulting in the contamination of soil and water which eventually can greatly affect the living organisms. This contamination level of Atrazine in soil and water can be effectively determined by using high performance liquid chromatography; one of the most effective tool to analyze or quantify the concentration of any compound residues and its byproducts. This study was aimed to isolate the microorganisms capable to degrade Atrazine from soil, obtained from farmed areas of Gomal University D. I. Khan in December, 2011, having history of at least eight years continuous exposure to Atrazine. Isolated fungal and bacterial strains were observed for their potential to degrade Atrazine. Fungal isolates were identified on the basis of colony morphology and spore characteristics while bacteria were identified on the basis of the biochemical parameters. Out of twelve fungal strains, *Aspergilus* fumigates showed the highest biodegradation rate (58.24%) while the lowest biodegradation was shown by *Aspergillus terreus* (17.56%). Among the bacterial isolates three were Gram negative and five were Gram positive. The highest degradation potential (62.77%) was shown by Streptococcus specie while E. coli with lowest degradation rate (26.48%). Bacteria showed more average degradation potential than fungal isolates as the total average of degradation rate observed was 44.62% by bacteria and 40% by fungal isolates.

Keywords: Atrazine, Atrazine Degradation, HPLC, Atrazine Degrading Microorganisms

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Introduction

Atrazine (2-chloro, 4-ethyl amino, 6-isopropyl amino, s-triazine) is one of the selective herbicides belonging to the group of s-triazines (Abigail and Nilanjana, 2012). Atrazine is naturally non-volatile herbicide (Feria-Reyes et al., 2011) which is used worldwide either alone or in combination with other herbicides (Chan and Chu, 2005), to get rid of broad leafy and grassy weeds mainly from corn, sorghum, sugarcane, maize crops, pineapple and also in conifer reforestation plantings (Sene et al., 2010). Atrazine exerts herbicidal effect in susceptible plants upon binding to the quinone-binding protein thus inhibiting the photosynthetic electron transport system (Wang et al., 2011). Its structural activity is enormously affected by several potent environmental factors including pH, humidity, environmental temperature and microbial activity (Feria-Reyes et al., 2011). Atrazine is an active environmental pollutant due to its low biodegradability, having high potential to contaminate both surface as well as ground water (Chan and Chu, 2005). Its continuous use has resulted environmental pollution in soil, ground and aquatic water (Wang et al., 2011). Toxic effects of atrazine not only impacts on human beings but also on other animals directly upon entering in their food chain (Zhang et al., 2009). It has attributed as a strong inducer of mammary gland tumors in Sprague-Dawley (SD) female rats (Stevens et al., 1994), potential disruptor in male frogs during sexual development as well as immune responses in some other animals (Christin et al., 2004; Murphy et al., 2006).

Biodegradation of Atrazine consists of complex physical and biological processes that mainly depend upon the nature and total amount of atrazine available in soil or water. However, some important factors may limit the biodegradation of atrazine in the environment including soil and water and its limited availability to the microorganisms (Abigail and Nilanjana, 2012). While the rate of degradation of Atrazine depends upon certain factors like culture purity, external nitrogen and carbon sources, moisture content, ratio of nitrogen and carbon concentrations, pH as well as the aerobic and anerobic conditions (Ghosh and Philip, 2004). Many sophisticated tools can be used to determine human exposure and the environmental impact of the triazine herbicides like High Performance Liquid Chromatography (HPLC), Gas Chromatography (GC) and Mass Spectroscopy (MS) equipped with highly selective detection systems (Drevenkar et al., 2002).

Many studies have reported that there are various microorganisms in water and soil that have the ability to utilize Atrazine as their sole carbon and nitrogen source, and as a result degrade atrazine and their derivatives (Zhang et al., 2009) which include bacteria (members of genera Pseudomonas, *Arthrobacter*, *Acinetobacter*, *Rastonia*, *Agrobacterium* and Norcardioides) and fungi (members of genera *Penicillium*, *Rhizopus*, *Aspergillus*, *Trichoderma*, *Phanerochaete* and *Fusarium*) (Abigail and Nilanjana, 2012). These microorganisms utilize it through various pathways consisting of steps like N-dealkylation, dechlorination, dehalogenation, Ring-cleavage and deamination (Gebendinger and Radosevich, 1999).

Likewise its global use, atrazine is also used extensively in Pakistan for the control of broad leafy and grassy weeds. Due to its low biodegradability and extensive toxic effects upon human beings and other living organisms, there was a need to isolate atrazine degrading microorganisms from soil because no significant literature can be found regarding the atrazine degrading microorganisms investigated from

soil of Dera Ismail Khan, this study was conducted with aims to isolate and identify of the Atrazine degrading microorganisms from the selected fields, to evaluate the potential microorganisms for atrazine biodegradation and to find out the frequency of atrazine degradation by the isolated microorganisms.

Material and Methods

This work was carried out at Microbiology Lab, Department of Microbiology, Kohat University of Science & Technology from December 2011 to August 2012 using the method followed by Zhang et al., (2009) with slender modifications.

Random samples of 1kg soil were compositely collected from the upper soil-surface (10cm in depth), from cultivated lands of Gomal University, Dera Ismail Khan (KPK) Pakistan, with persisted history of atrazine exposure for at least eight consecutive years. Nitrogen contents, pH, electrical conductivity (EC), calcium carbonate ($CaCO_3$), percent nitrates and texture of soil samples were determined using standard protocols and tools.

The soil sample (10gm) was diluted in distilled water (100ml) followed by serial dilution which resulted in concentrations 1/10, 1/100, 1/1000, 1/10000 and 1/100000 in grams per milliliter. These samples were then inoculated on nutrient agar and sabourauds dextrose agar for isolation of bacteria and fungi, respectively. After 24 hours incubation at 37^OC (for bacteria) and 25^OC (for fungi), sub-culturing was performed and pure colonies were obtained. Isolated bacterial species were confirmed with the help of biochemical tests including oxidase test (OT), catalase test (CT), starch hydrolysis (SH), phenylalanine deaminase test (PAD), voges-proskauer (VP), methyl red test (MR), indole test (Ind), nitrate test (Nit), motility test (Mot) and simmons citrate test (SC). Rotary shaker culture (150rev/min) was developed in Mineral Salt Medium supplemented with 1ml of 1/1000 dilution of the samples. After one week, sub-culturing was performed from the broth culture to obtain purified bacterial cultures.

Broth cultures for bacteria and fungi were prepared in 1M solution of high purity grade atrazine (Merck, Germany). After incubation, optical density (OD) was determined using spectrophotometer (BMS). Double autoclaved soil (100gm) was supplemented with 1M atrazine solution (10ml) and pure broth cultures of bacteria (10ml) and fungi (10ml), separately. Each soil sample was treated in three replicates followed by characterization of treated soil for Nitrogen contents, pH, electrical conductivity (EC), calcium carbonate ($CaCO_3$), percent nitrates and texture.

Atrazine, from the treated soil was extracted using analytical grade chloroform ($CHCl_3$) through Whatman filter paper (No. 42). The Extracts were then concentrated to near dryness by recycling chloroform from the samples in a rotary evaporator at 40^oC under reduced pressure till the final volume of the extract was 1ml. The extracted atrazine was dissolved in 30ml of HPLC grade methanol and analyzed by a normal phase high performance liquid chromatography (SCHIMADZU SPD-20A) with the chromatographic conditions as 5-µm octadecylsilane (C18) column, width (4.6mm) and length (25cm), isocratic elution of acetonitrile and monobasic potassium phosphate (0.01M) in a mixture of 70:30 respectively at pH 2.0, with the flow rate of mobile phase as 1.0ml/min, at 230nm of detection wave length in isocratic mode as described by Rustum et al. (1990). The amount of atrazine degradation rate was calculated by using the following formula as described by Zhang et al. (2009):

$$X\% = \frac{C_{ck} - C_x}{C_{ck}} \times 100\%$$

Where, "X" is atrazine degradation rate; "Cx" is the concentration of atrazine (mgL^{-1}) in the medium with atrazine degrading strain; "Cck" is the concentration of atrazine (mgL^{-1}) in the medium that does not contain atrazine degrading strain. All the results were analyzed using Statistix9.

Results

Characterization of initial soil samples revealed as the soil sample possessed neutral pH (7.36), electrical conductivity (EC) $0.25Sm^{-1}$, 14% calcium carbonate, 0.04% Nitrates with clay texture. After inoculation of the soil sample, 12 fungal and 8 bacterial species were identified on the basis of morphological characteristics by comparing with standards as bergey's manual, colony morphology and spore characteristics. Fungal species included *Aspergilus* fumigates, *Trichophyton schoenleini, Aspergilus niger, Trichophyton verrucosum, Trichophyton tonsurans, Aspergilus terreus, Madurella grisea, Rhizopus spp., Helminthosporium, Alternaria* spp., *Sepedonium* spp. and *Trichoderma harzianum*, while bacterial species contained 3 Gram negative (Pseudomonas spp., Salmonella spp., Escherichia coli) and 5 gram positive (*Staphylococcus aureus*, Two *Bacillus* spp., *Staphylococcus* Spp., *Streptococcus* spp.). These species were confirmed by detailed biochemical tests and the results are shown in Table 1.

Table 1. Results of biochemical tests performed for identification of bacterial species

SN	Gram Stain	OX	CT	SH	PAD	VP	MR	Ind	Nit	Mot	SC	Identified Bacteria
1	G -ve Rods	-	+	-	-	-	+	+	+	M	-	*E. coli*
2	G +ve Cocci	-	+	-	+	-	+	+	+	NM	+	*S. aureus*
3	G +ve Rods	-	+	-	-	-	-	-	-	M	+	*Bacillus* spp.
4	G +ve Rods	+	+	+	-	-	+	-	+	NM	-	*Bacillus* spp.
5	G -ve Rods	+	+	+	-	+	-	-	+	M	+	*Pseudomonas* spp.
6	G +ve Cocci	-	-	+	-	-	+	-	+	NM	-	*Streptococcus* spp.
7	G +ve Cocci	-	+	+	-	-	+	-	+	NM	+	*Staphylococcus* spp.
8	G -ve Rods	-	_	+	-	-	+	+	+	M	+	*Salmonella* spp.

Results from degradation experiment indicated that atrazine was significantly degraded by the fungal isolates. The highest atrazine degradation rate (58.24%) was

observed by *Aspergilus* fumigates followed by *Aspergillus niger* (55.35%), *Trichophyton verrucosum* (45.41%), *Trichoderma harzianum* (42.6%), *Madurella grisea* (40.06%), *Trichophyton tonsuraus* (37.40%), *Helminthosporium* (34.78%), *Rhizophus* spp. (33.45%), *Trichophyton schoenleini* (29.26%), *Alterneria* spp. (27.74%) and *Aspergillus* terreus (17.56%). Significance of results was determined by calculating error bars with percentage as shown Fig. 1.

Among the eight bacteria, maximum atrazine degradation rate (62.77%) was shown by Streptococcus spp. followed by Staphylococcus aureus (59.57%), Bacillus spp. (54.94 %), *Pseudomonas* spp. (54.58%), *Stapylococcus* spp. (54.42%), *Salmonella enteridis* (54.26%), *Bacillus* spp. (51.79%) and E. coli (26.48%). Results are graphically presented in Fig. 2.

Soil was analyzed prior to the initiation of the degradation experiment and the important parameters were recorded as Electrical Conductivity (EC) 2.7Sm^{-1}, Calcium Carbonate 12.50% and 0.0172% Nitrates. The soil texture was noted as silty clay loam.

Total of 24 bacterial samples and 36 fungal samples along with their respective controls were analyzed for quantification of atrazine by high performance liquid chromatography. The retention time for Atrazine was 05 minutes by the mobile phase consisting of 70:30 mixtures of acetonitrile and monobasic potassium phosphate at pH 2.0 at detection wavelength of 230nm using Octadecylsilane (C18) column. Following are the chromatograms obtained for standard, control, bacterial sample and fungal sample (Fig. 3).

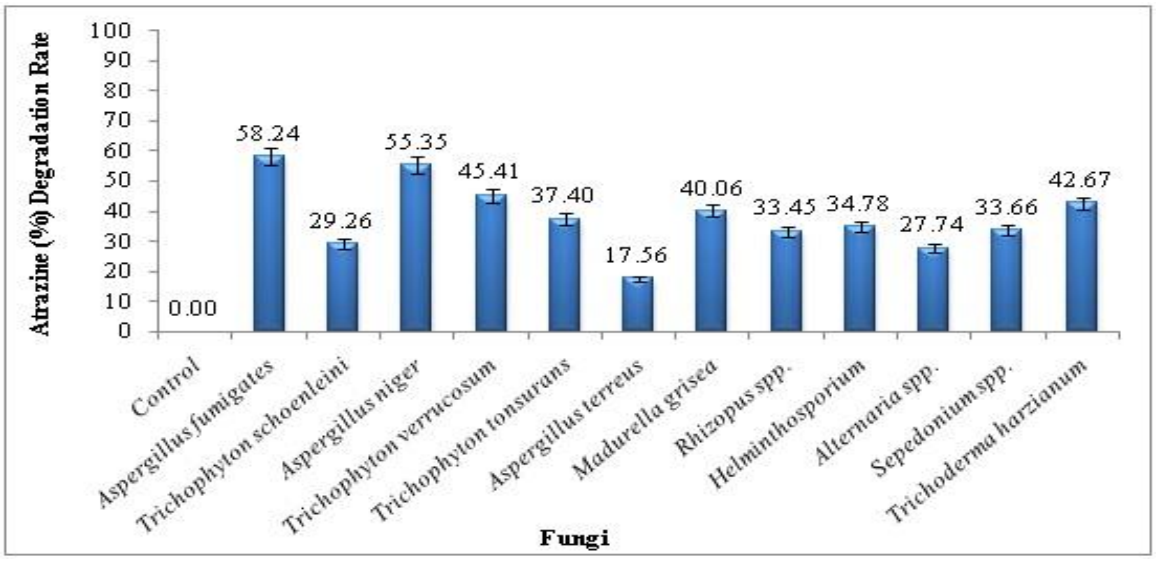

Fig. 1. Amount of atrazine degraded (%) by fungal isolates from soil sample.

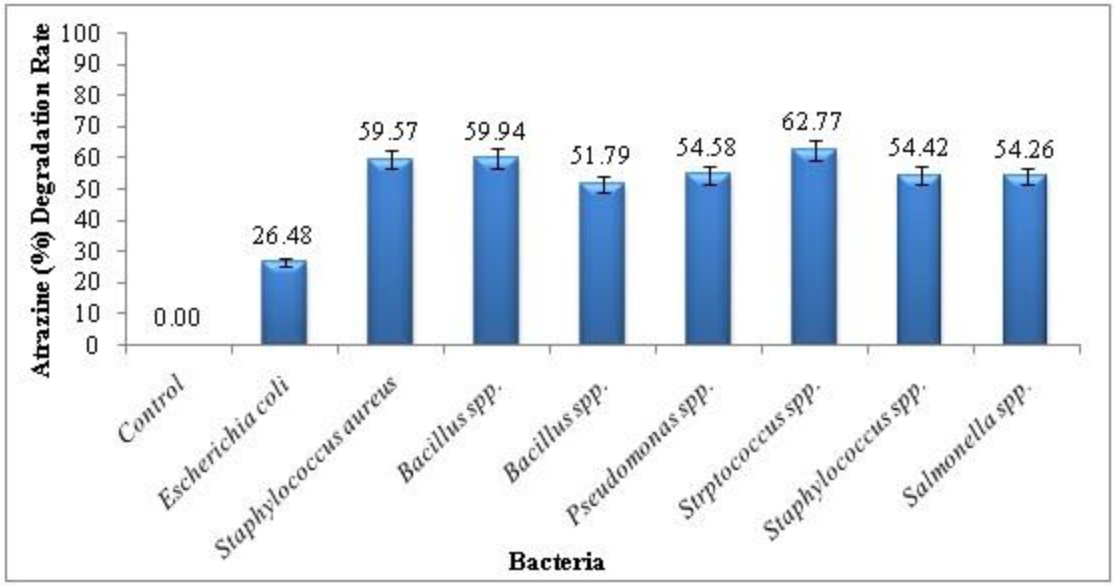

Fig. 2. Amount of atrazine degraded (%) by bacterial isolates from soil sample

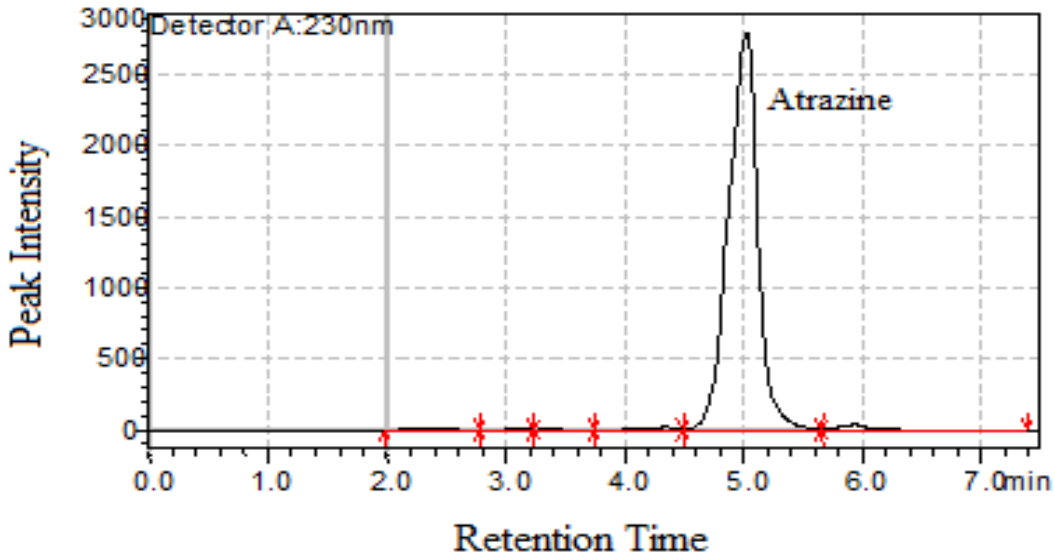

Fig. 3. Fungal Sample Chromatogram

Discussion

Biodegradation is one of the complex and irreversible process causing structural alterations in the subject compounds by the enzymatic activities yielding some by-products which are the eventual outcomes of the metabolic processes ensured in the systemic process of the biodegradation (Ma et al., 2009). Because of the structural complexity, Atrazine undergoes slow degradation process resulting in the contamination of the water reservoirs at different levels which may be either surface water or ground water (Chan and Chu, 2005). Because of the long half life and its high mobility derivatives and parent compound residues are detected in ground water, surface water as well as in

soil even after years of its application (Schiavon, 1988).

The methodology adopted in the current study might be correlated with Shaner et al., (2007) who used easy setup of the degradation experiment, rapid extraction of atrazine and simplest method for the quantification of atrazine via HPLC. They used 50mg of soil in a 250ml caped glass jar, treated with the 7.5ml of water having atrazine at a concentration of 5µg ai/ml and after treatment, quantified with the help of HPLC. Findings of Vibber et al. (2007) shown that the novel Gram positive microorganisms are capable to degrade atrazine which were characterized and isolated from soil having the history of repeated exposure to acetochlor and atrazine for herbicidal purposes. Our results included both gram positive as well as gram negative bacteria, isolated from the atrazine treated soil. While working on cornfield soil in Iran, Deghani et al. (2007) reported the isolation of both Gram negative and Gram positive bacterial isolates.

In the present study, different bacteria were used for atrazine degradation with varying degrading ability and it was found that the highest degradation rate (62.77%) was shown by Streptococcus spp. which may be due to favorable environmental conditions as well as high production of enzymes responsible for atrazine biodegradation. Our findings are supported by Topp (2001) who observed biodegradation ability of Pseudomonas sp. Zhang et al., (2009) used *Microbacterium* spp. and *Arthrobacter* spp. with degradation rate of 77.7% and 65.5%, respectively. Degradation experiment from water samples have also been reported by Udikovic et al., (2003) who conducted a study with the objective to enrich and weigh up the biodegradation activity of atrazine degrading bacteria from waste water and herbicide manufacturing plant soil, focusing on measuring the efficacy of bacterial strains on effluents treatment from atrazine production. Besides documented the atrazine degrading activity of bacterial isolates, several fungi were isolated and subjected to atrazine degradation. Our results showed that atrazine was significantly degraded by all the fungi, but the highest atrazine degradation rate (58.24%) was observed by Aspergillus fumigates followed by *Aspergillus niger* (55.35%). Ojo (2007) reported the atrazine degrading activity of the bacteria as well as fungi and found that in mineralization the hydrocarbon component of atrazine is utilized by the microbial consortium. The reported atrazine degraders were Micrococcus spp., Bacillus spp., Pseudomonas spp., *Aspergillus fumagitus*, *Penicillium* and *Fusarium* spp. The highest atrazine degrading activity by *Streptococcus* spp. and *Aspergillus* fumigates may be due to increased expression of genes associated with atrazine degradation.

For quantification of atrazine normal phase HPLC was used in isocratic mode having mobile phase flow as 1ml/minute consisting of acetonitrile and monobasic potassium phosphate in a specific composition and retention time recorded was 5 minutes. Rustum et al. (1990) used the reversed phase HPLC using mobile phases of different compositions in gradient elution mode with the different retention times which goes even up to 27 minutes also indicating that our optimized method was simple and easy method for atrazine quantification. The retention time may vary between 3 to 7.5 minutes depending upon the pH of the mobile phase which may be altered by environmental conditions. pH of the mobile phase also affects the shape and the structure of the chromatographic tail of the atrazine as it interferes greatly with the elution mode of the mobile phase.

Conclusion

It can be concluded from our findings that the reported microorganisms both bacteria and fungi play an active role in biodegradation of atrazine and as a whole s-triazine group of herbicides. Specifically, bacterial strains are more potent in biodegradation of atrazine than fungal isolates.

It is evident from the percent degradation rate of fungal and bacterial isolates that these microorganisms can be efficiently used for the biodegradation of the atrazine and as a whole s-triazine group of herbicides in order to protect the humans as well as other living organisms from the toxic effects of atrazine. In these perspectives, the possible further study will need to focus on the isolates obtained should be identified on molecular basis, screening of enzymes involved in the biodegradation of atrazine and genes involved in atrazine degradation should be characterized.

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