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Isolation and Identification of Atrazine-degrading Bacteria from Corn Field Soil in Fars Province of Iran

[1]M. Dehghani, [1]S. Nasseri, [2]S. Amin, [1]K. Naddafee, [2]M. Taghavi, [1]M. Yunesian and [2]N. Maleky
[1]Department of Environmental Health Engineering,
School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
[2]Department of Water Engineering, Plant Protection and Chemistry, Shiraz University, Shiraz, Iran

Abstract: In this study several agricultural fields with a long history of atrazine application in Fars province of Iran have been explored for their potential of atrazine biodegradation. After several subculturing for a period of 300 days acclimation, leads to an enhancement of atrazine biodegradation rate. A successful enrichment culture with a high capability for atrazine degradation was obtained (88%). A combination of enrichment culture technique, in a basal salt medium containing atrazine and carbon sources under nitrogen limitation and plating on indicator atrazine agar, have permitted the isolation of bacterial consortium with high capability of using atrazine as a nitrogen source. Seven gram-negative and one gram-positive bacterial strain, which were able to use this herbicide as a sole source of nitrogen, were isolated from Darehasalouie Kavar corn field soil. Based on physiological, biochemical and nutritional characteristics, the isolated bacteria were identified as *Pseudomonas alcaligenes*, *Acidovorax* sp., *Pseudomonas putida*, *Ralstonia eutrophus*, *Pseudomonas syiringe*, *Erwinia tracheiphila*, *Entrobacter agglomerans* and *Micrococcus varians*. Therefore, the bacterial consortium in liquid culture containing carbon sources and atrazine as a sole source of nitrogen, degrade added atrazine more than 80%.

Key words: Atrazine, soil, biodegradation, herbicide, Fars

INTRODUCTION

Atrazine (2-choro-4-ethylamineo-6-isopropylamino-1, 3, 5-triazine) has been the most widely used herbicide for nonselective weed control on non-cropped land and for selective weed control in major crops such as corn (Tomline, 1994). Atrazine is a probable human carcinogen (Van Leeuwen *et al*., 1999). However, International Agency for Research on Cancer (IARC), 1991 has concluded that there is inadequate evidence in human and limited evidence in experimental animals for the carcinogenicity of atrazine (Group 2B). Its widespread use has caused environmental concern on the basis of frequent detection of atrazine in water resources at a concentration which exceeds the maximum contaminant level of 3 $\mu g\ L^{-1}$ (Koelliker *et al*., 1986; USEPA, 1991; Goodrich *et al*., 1991; Thurman *et al*., 1992; Solomon *et al*., 1996; Muller *et al*., 1997).

The high incidence of atrazine contaminated water and the increasing concern about this herbicide have led to many studies for decontaminating atrazine polluted soils through bioremediation processes. Biodegradation is recognized as a critical factor affecting the fate of atrazine in the environment, especially in soil ecosystems (Cook, 1987; Adams and Thurman, 1991). This compound was considered as a relative recalcitrant in soils, although microbial degradation has always been recognized as the principle mechanism of atrazine dissipation in soils (Kaufman and Kearney, 1970). Atrazine persistence generally declines with its repeated application on soil, suggesting that soil exposure to atrazine enhances the abundance and activity of atrazine-degrading bacteria (Pussemier *et al*., 1997; Vanderheyden *et al*., 1997; Newcomb and Crowley, 1999; Piutti *et al*., 2002). Complete and rapid mineralization of atrazine, both by bacterial consortium and pure bacterial isolates, is observed (Mandelbaum *et al*., 1995; Radosrich *et al*., 1995; Barriuso and Houot, 1996; Vanderheyden *et al*., 1997; Topp *et al*., 2000). A variety of atrazine degrading bacteria, including members of the genera *Pseudomonas, Acinetobacter*, *Agrobacterium*, *Nocardioides* and *Rhizobium* have been isolated from soils that have come in contact with this

Corresponding Author: M. Dehghani, Department of Environmental Health Engineering, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

chemical (Assaf and Turco, 1994; Yanze-Kontchou and Gschwind, 1994; Mandelbaum *et al*., 1995; Radosrich *et al*., 1995; Bouquard *et al*., 1997; Struthers *et al*., 1998; TOPP *et al*., 2000).

Therefore, the objectives of the study were to (i) enrich of microorganisms capable of atrazine degradation (ii) isolating and detecting them in the agricultural soil samples with a long history of exposure to atrazine.

MATERIALS AND METHODS

Sampling sites and preparation: Soil samples were taken from 13 different sites in Fars province and studied for their ability to degrade atrazine under laboratory conditions. The samples were from different agricultural farms with 10 years background of using atrazine for corn cultivation. The farms were located in Kavar (3 sampling sites), Zarghan (3 sampling sites), Bajgah (1 sampling site) and Marvdasht (6 sampling sites). Soil samples were taken in March 2004, after 9 months of atrazine application and were collected with a hand-driven soil auger in 0-30 cm of soil depth at random location at each site and stored at 4°C until they were used. The samples were air dried and passed through 2 mm sieve to be prepared for further microbiological examinations.

Soil analysis: General physicochemical characteristics of soils were determined. Hydrometer was used to determine the soil texture. Other soil characteristics such as soil solution pH (Thomas, 1996), Organic Matter Content (OMC) (Darrel and Nelson, 1996) and Cation Exchange Capacity (CEC) (Summer and Miller, 1996) were also determined.

Preparation of soil suspension for microbial culture: Soil samples (20 g) were suspended three times in 60 mL of 0.1 M phosphate buffer (pH = 7.5) and centrifuged at 6000×g for 15 min at 4°C and the supernatants were discarded to remove the quantity of residual nitrogen sources in soil (Mandelbaum *et al*., 1993).

Enrichment and isolation: Enrichment cultures and basal salt medium were prepared as described in Rousseaux *et al* (2000). Ten gram wet soil was inoculated into 90 mL of basal salt medium and amended with 30 mg L^{-1} of atrazine. The media was supplemented with sodium citrate and delvocid (25 mg L^{-1}) after autoclaving. Delvocid was used to prevent the growth of fungi and pH was also adjusted to 7.5. Cultures were incubated aerobically on a reciprocal shaker (100 rpm) at room temperature in the dark to preclude photolysis reactions. All enrichment cultures were subcultured on the same medium at a 1 week interval. From a one-week-old culture, 10 mL was transferred to 90 mL of freshly prepared atrazine medium. After several subcultures for the period of 30 and 300 days under the conditions of nitrogen limitation, the remained atrazine after 10 days of inoculation, was quantified by square wave voltammetery with hanging mercury electrode. Each culture received a designation that consisted of the abbreviation for the site where the initial inoculums were collected. Controls contained 1 g of sodium azide per liter as growth inhibitor. Samples were kept in laboratory conditions and incubated with shaking at (150 rpm) for 300 days. The solid medium used to indicate atrazine degradation contained the same mineral salts as the liquid medium but at the concentration of 100, 500 and 1000 mg L^{-1} atrazine and 2% (wt/vol) agar. Atrazine was added from a highly concentrated stock solution (500 mg mL^{-1} in methanol). Agar plates were cloudy as a result of particulate atrazine in the medium, enabling visual inspection of clearing zones around atrazine-degrading colonies. Purified colonies were plated on atrazine solid medium (100 mg L^{-1}). Colonies showing a good growth were transferred to solid media containing 500 mg L^{-1} atrazine and then again transferred to 1000 mg L^{-1} atrazine solid medium. A colony showing extensive clearing zone was selected as an atrazine-degrading bacteria. Then the purity of strain was ensured by restreaking isolated colonies on nutrient agar and atrazine medium. Standard biochemical tests such as oxidase reaction, urea hydrolysis, catalase reaction, acid production from differents carbon sources and utilization of differents amino acid were conducted on the basis of standard bacteriological methods (Schaad *et al*., 2001). On the bases of the biochemical differentiative tests and comparing the results with Bergey's manual of systematic and determinative bacteriology, the isolated bacteria were identified.

Chemicals and analytical methods: All chemicals used were of reagent grade and purchased from Merck (Germany). Atrazine standard was supplied by Accua Standard Europe, Switzerland. Electrochemical measurements were carried out using a computer driven M Auto Lab type analyzer equipped with Metrohm VA STAND 663 and GPES 4.9 software. The three-electrode configuration was used comprising an hanging mercury drop electrode (HMDE) as the working electrode, a platinum rod counter electrode as an auxiliary electrode

and an Ag/AgCl electrode as a reference electrode. The electrochemical experiments were carried out in 20 mL at pH = 1.9 of hydrochloric acid solution (0.01N HCl), that was pipetted into a voltammeteric cell containing atrazine sample and mercury droplet. Under optimized conditions for high sensitivity, the square wave voltammeteric experiments were carried out scanning the potential from -0.5 to -1.2 V versus Ag/AgCl using the pulse height of 25 mv and frequency of 10 HZ, with a potential increment of 1.95 mV. Solution inside the voltammeteric cell was degassed with N_2 gas for 10 min before the potential scan, which is performed after 10 sec of equilibrium time. The voltammograms were obtained in HCl (0.01N) at pH = 1.9 (Luciana *et al*., 2004).

RESULTS AND DISCUSSION

Atrazine degrading-bacteria: The physicochemical characteristics of soil samples were summarized in Table 1. Log CFU g^{-1} soil for the soil samples was in the range of 8.2-8.7. Initial enrichment results (after 30 days of acclimation) indicated that the rate of atrazine biodegradation was not significant (4.5-15% atrazine reduction) (Table 2). No growth or growth without halo formation around the bacterial growth was observed in the solid media containing atrazine and confirmed the fact that the bacterial growth in the atrazine media was not possible because bacterial concortium had not been able to use the atrazine as nitrogen source. However, repeated subculturing has resulted higher biodegradation rate (Table 3). An enrichment culture with a high capability for atrazine biodegradation was obtained after a period of 300 days of acclimation. Atrazine utilization was indicated by significant growth in liquid culture. Also indicator plate containing atrazine at a concentration of 1000 mg L^{-1} showed clearing zone around bacterial growth. Atrazine concentrations left in the enrichment medium were measured after 10 days of inoculation with the bacterial consortium. Successful enrichment culture at pH = 7.5 exhibited high atrazine removal (88%). However, the atrazine reduction for the rest of the cultures was less than 50%. Control media which was not inoculated and contained 1 g of sodium azide per liter as bacterial growth inhibitor, did not exhibit atrazine removal (<3%). Therefore, atrazine disappearance was determined to be the result of microbial metabolism.

After successive enrichment, the bacterial consortium capable of atrazine degradation was obtained. Purified colonies (a total of 56 isolates) obtained from the mixed culture were tested for the ability to degrade atrazine as a nitrogen source. Eight isolates were selected as the atrazine-degrading bacteria. From these isolated bacteria, seven of them were gram negative and motile rod. Five of these isolated bacteria were strictly aerobic and the rest were facultative anaerobic. One of these isolates was gram positive, strictly aerobic and non-motile. According to the result of differentiative tests and their comparison with Bergey's manual of systematic and determinative bacteriology, the isolates were *Pseudomonas alcaligenes*, *Acidovorax* sp., *Pseudomonas putida*, *Ralstonia eutrophus*, *Pseudomonas syiringe*, *Erwinia tracheiphila*, *Entrobacter agglomerans* and *Micrococcus varians*. Earlier studies have reported *Pseudomonas alcaligenes* (De Souza *et al*., 1998), *Acidovorax* sp. (Santiago-Mora *et al*., 2005), *Pseudomonas putida* (Bheki and Khan, 1986) and *Ralstonia eutrophus* (Radosrich *et al*., 1995) as atrazine degrading bacteria. Hence in this report, *P. syiringe*, *Erwinia tracheiphila*, *Entrobacter agglomerans* and *Micrococcus varians* were identified to be effective in atrazine degradation that earlier studied had not reported them.

Table 1: Physicochemical properties of the agricultural soil samples

Sampling site	pH	OMC (g kg^{-1} soil)	CEC mole (+) kg soil	Particles sand distribution (%) Sand	Silt	Clay	Soil texture
K_1	7.94	8.80	11.12	47.44	31.5	17.06	Loam
K_2	7.49	20.43	17.39	24.70	27.3	40.00	Clay-loam
K_3	7.48	13.60	9.31	51.44	31.5	17.06	Loam
Z_1	7.43	13.62	12.50	28.70	33.3	32.00	Clay-loam
Z_2	7.46	20.43	16.84	12.70	31.3	48.00	Silty clay
Z_3	8.18	14.90	12.64	35.44	41.5	33.06	Loam
B	7.98	14.20	16.66	15.44	47.5	37.06	Silty clay loam
M_1	8.24	19.10	14.88	25.44	49.5	27.06	Loam
M_2	7.85	17.00	13.76	23.44	51.5	25.06	Silty loam
M_3	7.94	17.00	14.96	17.44	53.5	29.06	Silty clay loam
M_4	7.95	19.70	14.44	23.44	51.5	25.06	Silty loam
M_5	7.75	16.34	10.86	22.70	35.3	38.00	Clay loam
M_6	7.95	39.10	20.49	23.44	47.5	29.06	Clay loam

K = Kavar, Z = Zarghan, B = Bajgah, M = Marvdasht

Table 2: Atrazine reduction after inoculation with bacterial consortium at different site sampling locations

Site sampling	Atrazine reduction (%) After 30 days*	after 300 days*
K_1	15	88
K_2	8.5	43.27
K_3	9.5	27.5
Z_1	4.5	26
Z_2	5.5	36.67
Z_3	11.7	37
B	12.9	48
M_1	10	33.33
M_2	11.2	32.5
M_3	13.8	47
M_4	12.5	44.33
M_5	11.3	35
M_6	12.5	47.3

*Atrazine concentration was measured in 10 days of inoculation at pH = 7.5

Table 3: Physiological, biochemical and nutritional characteristics of atrazine-degrading bacteria

Characteristics	Isolates 1	2	3	4	5	6	7	8
Gram/KOH	-	-	-	-	-	-	-	+
O/F	O	O	O	O	O	FA	FA	O
Oxidase reaction	+	+	+	+	-	-	-	-
Yellow pigment on YDC	-	-	-	-	ND	-	-	+
Levan formation from sucrose	-	-	-	-	+	+	+	-
Citrate utilization	+	+	+	+	ND	+	+	+
Starch hydrolysis	-	-	-	-	-	+	+	-
Casein hydrolysis	-	-	-	-	ND	-	+	+
Esulin hydrolysis	-	+	+	-	ND	ND	+	-
Motility	+	+	+	+	+	+	+	-
Methyl red	-	-	-	-	ND	-	-	+
Voges-Proskaur	-	-	-	-	ND	-	+	-
Lecithinase	-	+	-	-	+	+	-	+
Urea hydrolysis	+	+	+	+	+	-	-	+
Lipase (tween 80 hydrolysis)	+	+	-	+	-	ND	-	-
Gelatin liquification	-	+	-	-	+	-	-	+
Phosphates activity	-	+	-	+	ND	+	+	-
Nitrate reduction	+	-	-	+	-	-	+	-
Indole production	-	-	-	+	-	-	-	-
H_2S from cysteine	+	+	+	+	ND	+	+	+
3-Ketolactose production	-	-	-	-	ND	-	-	-
Fluorescent pigment on King-B	-	-	+	-	+	-	-	-
Phenyl alanine deaminase	-	-	-	-	ND	-	-	-
Gas from Glucose	-	-	-	-	ND	-	-	-
Acid from glucose	-	+	-	-	ND	+	+	+
Pectolytic activity	-	-	-	-	-	-	+	-
Arginine dihydrolase	+	-	+	-	-	-	-	-
Lysine decarboxylase	-	-	-	-	ND	+	-	-
Catalase reaction								
Growth at temperature (°C)								
4	-	+	-	+	+	+	+	+
37	-	-	+	-	+	-	-	ND
41	+	+	-	+	-	+	+	+
NaCl tolerance (%)	+	+	-	-	-	-	+	+
3	+	+	+	+	+	+	+	+
5	+	+	+	-	ND	-	+	+
7	+	+	-	-	ND	-	+	+
Acid production from								
Maltose	-	+	-	-	-	-	+	+
Raffinose	+	-	+	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-
Trehalose	-	-	+	+	-	-	+	-
L (+) arabinose	-	+	+	-	-	-	+	-
L (+) rhamonose	-	-	+	-	-	-	+	-
D (+) mannitol	-	-	+	-	-	-	+	-

Table 3: Continued

Characteristics	Isolates 1	2	3	4	5	6	7	8
D (-) fructose	-	+	+	+	-	-	-	-
D (+) mannose	-	+	+	+	-	-	+	-
Levulose	+	+	+	+	+	-	-	-
Cellobiose	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-
D (-) ribose	-	+	+	-	+	-	-	-
Dulcitol	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	
Xylose	-	+	+	-	+	-	+	-
Adonitol	-	-	-	-	-	-	-	-
D (+) glucose	-	+	+	+	+	+	+	+
Arginine	+	-	+	-	-	-	-	-
Mellibiose	-	-	+	-	-	-	-	-
Ethanol	+	+	+	-	+	-	-	-
Starch	-	-	-	-	-	-	-	-
Galactose	-	-	-	-	+	-	-	-
Glycerol	-	-	+	+	+	-	-	-
Sucrose	-	-	-	-	-	-	+	-
Utilization of								-
Serin	-	-	-	-	-	-	-	
Salicin	-	-	+	-	-	-	-	-
Inulin	-	-	-	-	-	-	-	-
Sodium citrate	ND	-	+	-	-	ND	ND	-
Sodium tartrate	-	-	-	-	-	-	-	-
Sodium lactate	-	-	+	-	-	+	-	-
Sodium malonate	-	-	+	-	-	-	-	-

In conclusion, the bacterial consortium in liquid culture containing carbon sources and atrazine as a sole source of nitrogen, degrade added atrazine more than 80%. The bacterial consortium consisted of 56 isolates which only eight of them were selected as the atrazine-degrading bacteria.

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