

Acetanilide herbicide degradation using indigenous soil microorganisms

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Abstract

A lab scale study was conducted to investigate the degradation of S-Metolachlor (82.1% W/W) using indigenous soil culture. S-Metolachlor is a pre-emergent acetanilide herbicide for the control of annual grasses and broadleaf weeds. Health advisory level (HAL) of S-Metolachlor by US EPA is 0.525 mg/L in drinking water. The bacterial culture capable of degrading S-Metolachlor was isolated from soil with the help of an enrichment technique. Isolated bacterial cultures were identified as *Pseudomonas* sp. by using morphological and biochemical characteristics. Molecular identification of *Pseudomonas* sp. with the help of Polymerase Chain Reaction (PCR) was also conducted. Pure culture of *Pseudomonas aeruginosa* (ATCC 27853) was used to determine its degradation capacity. By using incubator an orbital shaker bacterial degradation capacity at 10, 20 and 50 µg/ml were determined. Gas chromatography and total organic carbon (TOC) were used to determine the concentration of S-Metolachlor at varied time intervals. TOC of S-Metolachlor was found to be 169.6 ppm. Isolated *Pseudomonas* sp. and standard culture of *Pseudomonas aeruginosa* (ATCC 27853) showed higher degradation capacity of S-Metolachlor.

Keywords: *S-Metolachlor, biodegradation, Pseudomonas sp., acetanilide herbicide, polymerase chain reaction, gas chromatography, enrichment technique.*



1 Introduction

Agriculture is the mainstay of Pakistan's economy as it contributes 21 percent to GDP and employs about 44 percent of the workforce. Weeds have always proved to be one of the main limiting factors in crop production (Avery [1]). Globally it damages 13.2% of agriculture production or about \$75.6 billion per year (Oerke *et al.* [2]). If herbicide use is abandoned damages to the agricultural sector would increase about 500% Bridges [3, 4]. In 2004 world-wide herbicides accounted for 45.4% of the pesticide market (Dinham [5]).

But unfortunately after application of pesticides, it disseminate in the environment and this in-turn results into health issues of un-protected agricultural and industrial workers. Pesticide pollution, pests' resistance and accumulation of residues of pesticides in the body of animals and human beings are the common problems faced by today's world.

Chloroacetanilide herbicide, Metolachlor (2-Chloro-*N*-(2-ethyl-6-methyl-phenyl)-*N*-(1-methoxypropan-2-yl) is a pre-emergent sprayed to control broadleaf and annual grassy weeds in different kinds of crops. Metolachlor has been replaced in 2003 by the S-Metolachlor, composed of 80% of the isomer S of the Metolachlor. The S-Metolachlor is the most effective and active form of Metolachlor for weed controls (Shaner *et al.* [6]). This is widely used to control weeds in maize, cotton, and soybean crops between others (Cao *et al.* [7]). This herbicide poses high toxicity and can be percolated, signifying a potent source of groundwater pollution (Liu *et al.* [8] and Scribner *et al.* [9]). In close vicinity to agricultural soils where these herbicides have been applied generally, acetanilide residues and their metabolites are common in aquifers (Stamper and Tuovinen [10]). Though the degradation of Metolachlor in soils is because of microbial activity predominantly (Buggard *et al.* [11]), little is known about the microorganisms that carry out this process and the mechanisms by which this occurs. Metolachlor is considered to be more persistent and recalcitrant to degradation than the other chloroacetanilide herbicides in soils and water (Stamper and Tuovinen [10]). The basic aim of this study is to isolate S-Metolachlor degrading bacteria from the agriculture soil. Results of this study will be very helpful for the bioremediation strategy of this novel compound.

2 Materials and methods

2.1 Chemicals

Herbicide S-Metolachlor (trade name: Dual Gold) was obtained from Syngenta Crop Production Franchise in Islamabad. FLUKA S-Metolachlor PESTANAL[®] (33859), analytical standard was used in current study. The relative molecular mass of herbicide is 283.8 g mol⁻¹. It's solubility in water at 20°C is 500 µg mL⁻¹. All other chemicals used in the study were analytical grade reagents.

Table 1: Physiochemical properties of S-Metolachlor.

Molecular formula	$C_{15}H_{22}ClNO_2$
Molecular weight	283.46 g/mol
Colour	Clear straw-colored liquid
Melting point	61.1°C
Solubility	530 mg/L (20°C)
Half-Life	15–132 days
Boiling Point	334°C

2.2 Sampling

Sampling was done from cotton field from the Cotton Research Centre Multan. A soil sample was taken from the top 5 cm of soil. Soil was kept at 4°C in polythene bags before bacterial isolation. The soil was regularly sprayed with Dual Gold (S-Metolachlor 82.1% W/W). The pH of soil sample was measured. With the help of an X-ray fluorescence analyser qualitative and quantitative elemental analysis of soil was done.

2.3 Isolation

Isolation of specie S-Metolachlor degrading specie was done through an enrichment technique. In this technique Minimal Media (0.5 g $(NH_4)_2HPO_4$; 0.2 g $MgSO_4 \cdot 7H_2O$; 0.1 g K_2HPO_4 ; 0.001g $FeSO_4 \cdot 7H_2O$; 0.01 g $Ca(NO_3)_2$ in 1 L distilled water pH=6.2) (Hashmi and Kim [12]) having 6 µl/ml of S-Metolachlor was utilized. A bacterium showing highest level of growth in Minimal Media at 30°C was selected for the degradation studies. Potential bacterium was labelled as SF. An isolated strain was cultured on nutrient Agar slants having S-Metolachlor. The strain was kept at 4°C and sub-cultured after every 3 months.

2.4 Identification and characterization of S-Metolachlor degrading bacteria

Isolated strain SF was then identified through morphological characters, gram staining, biochemical characterization and analysis of 16S rDNA. Biochemical characterization was accomplished by using API E20 kit.

DNA from the isolate was extracted with the help of DNA extraction kit (Norgen Biotek Corporation). The 16S rDNA gene was amplified by Polymerase Chain Reaction using universal bacterial primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (ACCTTGTTACGACTT) (Lane *et al.* [13]). PCR reactions were carried out using TE Thermocycler (Extragen). For the amplification, an initial denaturation step of 10 min at 95°C was followed by 40 cycles of amplification consisting of 1 min at 95°C, 1 min at 57°C and 10 min at 72°C. Then PCR product and 1 kb DNA ladder was run on gel electrophoresis.

2.5 Degradation studies

Minimal media having different concentration of S-Metolachlor (10 μ l/ml, 20 μ l/ml, and 50 μ l/ml) was used to determine degradation efficiency of isolated bacterium. Three Erlenmeyer flask of 250 ml for each concentration was kept in an incubator shaker at 30°C and 150 rpm for 5 days. Control of the study was minimal media having bacterial culture but no carbon source. The sample was taken at 0, 24, 48, 72, and 96 hr. the degradation potential was determined with the help of total organic carbon (TOC) and gas chromatography.

2.6 Analytical studies

For total organic carbon 15 ml sample was filtered in a labelled test tube. Test tubes were kept at 4°C until analysis take place. For gas chromatography sample was prepared by adding few drops of 3% H₂SO₄ to kill bacteria present in sample. The sample was then kept at -20°C in dark to avoid any photo-degradation. For extraction of sample, liquid-liquid extraction technique was used. Methanol: water (80:20 v/v) and sample was added in test-tube and was kept in orbital shaker for 1 hour. S-Metolachlor was determined with gas chromatograph. The injection temperature was 250°C and the detector temperature was 280°C. The program for detecting S-Metolachlor was as follows: initial oven temperature 80°C (hold 1min), ramped at 20°C min⁻¹ to 230°C, and then held at 230°C for 2.5 min, with a run time of 11 min. Under these conditions the retention times of Metolachlor was 9.45min. The detection limit was 0.1 μ gml⁻¹ for herbicide.

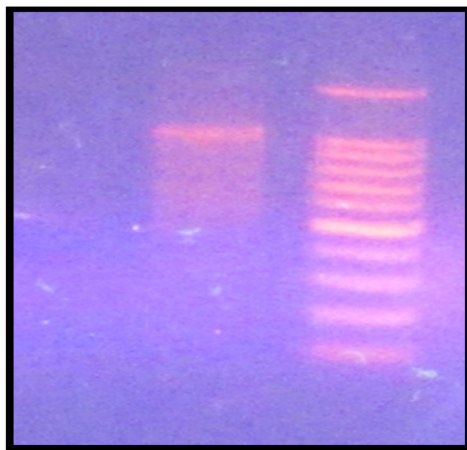


Figure 1: Amplification of 16S DNA genes of (1) *Pseudomonas aurignosa* on Tm= 57°C run with (2) 100 bp ladder

3 Results and discussions

The pH of soil was 7.1. Elements present in soil were Mg (1.61 %), Al (10.99%), Si (57.74%), S (0.22%), K (5.97%), Ca (3.59%) Ti (2.10%), V (0.009%), Cr (0.099%), Mn (0.32%), Fe (16.93%), Zn (0.05%), Sr (0.062%) and Zr (0.23%).

Morphological characteristics of isolate SF was irregular shape, undulate margins, off-white colour, smooth and shiny surface. Isolate also release yellowish green characteristic pigment (production of pyocyanin). According to gram staining results isolate was gram negative rods. API kit E20 results predict that the isolated specie was *Pseudomonas aeruginosa*. Further identification was confirmed through molecular identification. The amplified PCR product was of the 900 base pair which were the characteristics of *Pseudomonas aeruginosa*.

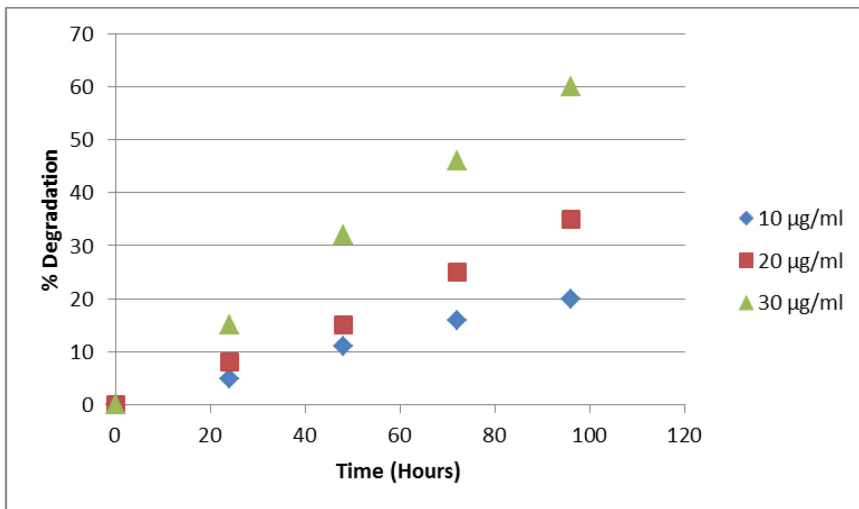


Figure 2: Graph showing % degradation at different time intervals.

A wide range of carbon sources can be utilized as *Pseudomonads* are characterised by great metabolic diversity. *Pseudomonas* sp. that was isolated from soil had degraded 70.7% Metolachlor in 20 days as reported by Sanayal and Kulshrestha [14]. Biodegradation has been depicted to be the primary mechanism of metolachlor dissipation in soil (Frank *et al.* [15]).

Results of total organic carbon and gas chromatography depict a significant reduction of S-Metolachlor at a concentration of 50µg/ml within 96 hrs. Degradation efficiency of isolated *Pseudomonas aeruginosa* makes it promising bacteria for bioremediation.

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