S-METOLACHLOR DEGRADATION EFFICIENCY USING INDIGENOUS SOIL MICRO-ORGANISM

By

Sahaab Farooq

(2011-NUST-MSPhD-EnvS-10)

A thesis submitted in partial fulfilment of requirements for the degree of
Master of Science

in

Environmental Science

Institute of Environmental Sciences and Engineering (IESE)
School of Civil and Environmental Engineering (SCEE)
National University of Sciences and Technology (NUST)
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It is certified that the contents and forms of the thesis entitled

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Submitted by

Sahaab Farooq

Has been found satisfactory for the requirements of the degree of
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This thesis is dedicated to my Parents

For their love, encouragement and prayers

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LIST OF ABBREVIATIONS

µg/ml	Microgram per milliliter
API 20E	Analytical profile index 20E
bp	Base pair
CFU	Colony forming unit
EC	Emulsified concentration
FAO	Food and Agriculture Organization
GC	Gas chromatography
MSM	Minimal salt medium
PCR	Polymerase chain reaction
TOC	Total organic carbon
UNDP	United Nations Development Programme
UNEP	United Nations Environment Programme
WHO	World Health Organization
WRI	World Resources Institute

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ABSTRACT

S-metolachlor is a pre-emergent persistent acetanilide herbicide used to control annual grasses and broadleaf weeds. Health advisory level (HAL) of S-metolachlor by US EPA is 0.525 mg/L in drinking water. The objective of the study was to isolate indigenous soil micro-organism having potential to degrade S-metolachlor. The bacterial culture capable of degrading S-metolachlor was isolated from soil by using enrichment technique. Isolated bacterial culture was identified as *Pseudomonas* sp. by using morphological and biochemical characteristics. Molecular identification of *Pseudomonas* sp. was done with the help of Polymerase Chain Reaction (PCR). The isolated soil micro-organism was ultimately identified as *Pseudomonas aeruginosa*. For biodegradation studies a bench scale reactor system was established. Set-up was run on ambient conditions at different S-metolachlor concentrations (10, 20 and 50µg/ml). Minimal Salt medium (MSM) having S-metolachlor as the sole carbon source was used to determine degradation potential of isolated bacterial strain. Optical density and total organic carbon content were used to determine degradation efficiency by increase in absorbance (at 600nm) and decrease in total organic carbon with time. Gas Chromatography was used to determine the concentration of S-metolachlor at varied time intervals. By inoculating *Pseudomonas aeruginosa* in the medium maximum degradation that is 62.5% was achieved within 120 hours. Optimized concentration and time for the bench scale bioreactor was found to be 20µg/ml at 72 hours respectively at ambient conditions.

Chapter 1

INTRODUCTION

1.1. BACKGROUND

By any measure volume used, hectares treated, or market value, global pesticide consumption is enormous and still rising. In the year 2006-2007 world pesticide consumption was nearly 5.2 billion pounds. Herbicides accounted for the largest portion of total use, followed by other pesticides, insecticides, and fungicides. Among the top 10 pesticides used in terms of pounds applied in the agricultural market were the herbicides glyphosate, atrazine, S-metolachlor, acetochlor, 2,4-D (2,4-Dichlorophenoxyacetic acid), and pendimethalin, and the fumigants meta sodium, dichloropropene, methyl bromide, and chloropicrin (Grube, *et al.,* 2011).

Use of pesticides has improved agricultural production and quality. Though, negative externalities from their use have amplified too. Damage to agricultural land, fisheries, fauna and flora are the major drawbacks of extensive and abusive consumption of pesticides. Unintended destruction of beneficial predators of pests due to pesticides results into increased virulence of many species of agricultural pests. Moreover, increased mortality and morbidity of humans due to exposure to pesticides has also been recorded especially in developing countries. The costs from these externalities are large and affect farmers' returns. Despite these high costs, farmers continue to use pesticides and in most countries in ever increasing quantities (Wilson and Tisdell,

2001). According to a World Health Organization (1992) report approximately three million pesticide poisoning cases take place annually leading to 220,000 deaths globally. Both economically and in terms of human life, these incidents present an enormous cost for society (WHO, 1992). In developed countries less than half of all pesticide-induced deaths occur (Pimentel and Greiner, 1996) despite of the fact, world's approximately 80% of the pesticides produced annually are consumed in these countries (WRI/UNEP/UNDP, 1994). This may be due to the fact that in developed countries farmers are more aware about the hazards of pesticides and health safety measures.

A higher proportion of pesticide poisonings and deaths occur in developing countries most likely due to inadequate occupational safety standards, protective clothing and washing facilities; insufficient enforcement of non-usage of banned pesticides; poor labeling of pesticides; illiteracy and insufficient knowledge of pesticide hazards (Pimentel and Greiner, 1996).

The elimination of a wide range of pollutants from the environment is a sheer requirement to encourage sustainable development of our society. Most common pollution treatment methods that are in practice include land-filling, recycling, pyrolysis and incineration for the remediation of contaminated sites. But these also have adverse effects on the environment, which can result in the formation of toxic

intermediates (Debarati *et al.,* 2005). Additionally, these methods are more expensive and sometimes difficult to implement, especially where the application area is large, as in case of pesticides where large agriculture lands are involved (Jain *et al.,* 2005).

One promising treatment method is to exploit the ability of microorganisms to remove pollutants from contaminated sites, an alternative treatment strategy that is effective, minimally hazardous, economical, versatile and environment-friendly. This process is known as bioremediation (Finley *et al.,* 2010). *Exxon Valdez* spill, the largest and most thoroughly studied application of bioremediation, the application of fertilizer increased rates of biodegradation 3–5 times (Atlas, 1995).

Biodegradation of pesticides is often complex and involves a series of biochemical reactions. Many enzymes efficiently catalyse the biodegradation of pesticides. It is accomplished by adding exogenous microbial populations or stimulating indigenous ones, attempts have been made to raise the rates of degradation found naturally to significantly higher rates. Isolation of indigenous bacteria capable of metabolizing organic pollutants has received considerable attention as these bacteria provide an environmental friendly method of in-situ detoxification (Richins *et al.,* 1997; Mulchaldani *et al.,* 1999). In some contaminated environments, microbial populations have evolved over time to adapt to these contaminants (Pahm and Alexander, 1993). These sites are therefore the most appropriate ecological niches to find and isolate

strains capable of degrading organic pollutants (Ortiz-Hernández *et al.,* 2001; Horne *et al.,* 2002). The isolated bacterial strains *Pseudomonas aeruginosa* (MTCC 9236), *Bacillus sp.* (MTCC 9235) and *Chryseobacterium joostei* (MTCC 9237) had shown high degradation potential for mixed (lindane, methyl parathion and carbofuran) pesticides. Pesticide enriched cultures were able to degrade 72% lindane, 95% carbofuran and 100% of methyl parathion in facultative co-metabolic conditions. The cultures enriched in a particular pollutant always showed high growth rate and low inhibition in that particular pollutant as compared to mixed pesticides (Krishna and Philip, 2008).

S-metolachlor herbicide (2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-metoxi-1-methylethyl) acetamide) is one of the three most commonly used herbicides in the world in the chloroacetanilide class. This herbicide has a high toxicity and can leach down, becoming a powerful source of groundwater pollution (Scribner *et al*., 2000; Liu *et al.,* 2001). Generally, acetanilide residues and their metabolites are commonly found in aquifers in close vicinity to agricultural soils where these herbicides have been applied. Acetanilide biodegradation is the most important factor for its elimination in aerobic and anaerobic environments. Hydrolysis is not as important in soil and water pH conditions, while adsorption in organic matter probably retards the bio-disposability (Stamper and Tuovinen, 1998). These herbicides are somewhat resistant to photodecomposition (Humburg *et al.,* 1989). In several organisms, the greatest factor of acetanilide transformation is detoxification by glutation-S-

transferase (GST) (Stamper and Tuovinen, 1998; Zablotowicz *et al.,* 1995). Despite this, microorganisms do not easily metabolize aromatic fragment (Liu *et al.,* 1987), raising a serious environmental concern.

1.2. OBJECTIVES

Environment preservation is one of the aims of sustainable development. The widespread use of pesticides and their high contamination potential have become serious concerns in the field of environmental engineering and science. Studies of microbial biodegradation are useful in the development of strategies for detoxification of pesticides by microorganisms (Qiu *et al.,* 2006). Microbes transform the applied xenobiotic by utilizing these as energy sources. Otherwise, microbes develop new metabolic strategies and adapt to changing environment through mutation, induction or by selective enrichment technique (Raymond *et al.,* 2003).

Most of these microbes work in natural environment but some modifications may be brought about to encourage the organisms to degrade the pesticide at a faster rate in a limited time frame. This capability of microbes is sometimes utilized as a technique for removal of contaminants from the actual site (Singh, 2008). The present study was aimed at investigating the ability and efficiency of an isolated bacterial strain to

degrade S-metolachlor in Minimal Salt Medium, using S-metolachlor as a sole carbon source. The following were, the objectives of the study:

- Isolation of S-Metolachlor degrading bacterial strain from agricultural soil
- Identification and characterization through:
  - Morphological and Cultural Characteristics
  - Biochemical Tests
  - Polymerase Chain Reaction (PCR)
- Determination of biodegradation efficiency of isolated bacteria at different concentrations of S-metolachlor using:
  - Optical Density
  - Total Organic Carbon (TOC)
  - Gas Chromatography (GC)

Chapter 2

LITERATURE REVIEW

In Pakistan agriculture contributes about 25% to the national economy, and employs over 44 percent of the labour force. It is also a main source of income in rural areas, which accounts for 70 percent of total population. Fluctuating trends can be observed in agriculture sector primarily because of pest attacks on crops, weather conditions and pesticides, thus, play a vital role in the economy of the country.

Presently use of large quantity of pesticides is a key plant protection measure. Its use has increased from about 915 tons (230 tons active ingredient) in 1981 to 129000 tons (28500 tons active ingredient) in 2004 (FAO, 2005). Though extensive usage of pesticides in Pakistan has remarkably controlled the pests, but like other countries, it has started causing environmental problems in the area (Tariq *et al.,* 2007). Cotton alone accounts for about 70% of the total consumption of active ingredient of pesticides. This has resulted in the remarkable increase in cotton production in the country. Paddy, sugarcane, fruits and vegetables come next in pesticide usage. Application of pesticides was commenced in Pakistan in 1952. It initiated with the introduction of an aerial spraying program on the key crops such as, cotton, rice and sugarcane. Simultaneously, pesticides were also used for locust control (FAO, 2005).

In croplands weed infestation remains the most devastating factor as it adversely influence crop productivity. Weeds are the most ubiquitous class of pests. Weeds interfere with crop plants through allelopathy and competition. This results in direct loss to quantity and quality of the product (Gupta, 2004), and indirectly increases production costs. In Pakistan a mix stand of grassy and broad-leaved weed is reported to cause 48% yield loss of wheat (*Triticum aestivum L.*) (Khan and Haq, 2002).

For the management of weeds several approaches have been adopted. Chemical weed control seems vital and indispensable as it has proved efficient in controlling weeds (Kahramanoglu and Uygur, 2010), and hence currently herbicide consumption by accounts for two-third by volume of the pesticides used globally in agriculture production. In Pakistan, herbicide consumption accounts for 14% of the total pesticide usage (Khan, 1998). The indiscriminate use of herbicides during the past few decades has led to serious environmental and ecological problems. For instance resistance, shifts in weed populations (that are more closely related to the crops that they infest), minor weeds becoming dominant (Heap, 2007), and greater environmental and health hazards (Rao, 2000).

2.1. CONTAMINATION OF PESTICIDES

The presence of pesticide residues in the soil, water, and air has created potential risks from both human and natural environment perspectives (DeLorenzo *et al.,* 2001;

Barron *et al.,* 2003; Otto *et al.,* 2007). There is considerable evidence that farmers have misused pesticides especially in cotton-growing areas of Pakistan. Groundwater has been found contaminated and is constantly being under the process of contamination due to pesticide use. It is obvious from the biological monitoring studies that due to occupational exposure farmers are at higher risk of acute and chronic health effects associated with pesticides. Also, the intensive use of pesticides (higher sprays more than the recommended dose) in cotton areas pose a special risk for the pickers, field workers, and of an unacceptable residue concentration in cottonseed oil and cakes (Tariq *et al.,* 2007).

The ultimate sink of pesticides applied in agriculture is soil. The longer persistence of pesticides in soil results in more adsorption of such toxic and poisonous chemicals by plants. This leads to accumulation of these compounds in plants to a level that it becomes harmful and hazardous to human being as well as livestock. These pesticides in soil are degraded by indigenous soil microorganism but at a slower rate (Alexander, 1994; Vandevivere and Verstraete, 2002). Therefore, this natural biodegradation may be enhanced by adapted microorganisms that convert these harmful pollutants into simpler non-toxic compounds. For environmental restoration, biodegradation and bioremediation have become the most rapidly developing and growing field (Dua *et al*., 2002).

Herbicides are the main kind of pesticides causing water contamination. Among herbicides chloroacetanilide group is the most commonly detected in surface and ground water at concentration that is exceeding European threshold for drinking water, i.e. 0.1 μg L^{-1} (Silva *et al.,* 2012). S-metolachlor has high water solubility (530 mg L^{-1} at $20^{o}C$) and very low sorption capacity due to this reasons it is frequently detected in ground water and surface waters, representing a potential source of water pollution (Martins *et al.,* 2007).

2.2. S-METOLACHLOR

S-metolachlor (trade name: Dual Gold; manufactured by: Syngenta) was developed by Switzerland Syngenta, which is a clear extremely pale-yellow liquid with a weak odor. It belongs to the chloroacetanilide group of pesticides. This is extensively used to control many broad-leaved weeds and annual grasses in cotton, brassicas, maize, soybean and sugarcane. S-metolachlor is a racemic mixture comprised of 88% S-enantiomer and 12% R-enantiomer. However, only S-enantiomer is the biologically active ingredient (Dale *et al.,* 2006).

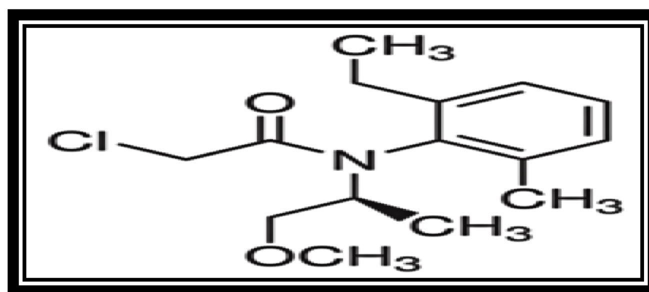


Figure 2.1: S-metolachlor Structure

In 1999, Syngenta Crop Protection, had discontinued sales of metolachlor (Racemic) and replaced it with the reduced risk compound S-metolachlor. This enantiomer is more effective in weed control than racemic metolachlor, providing the same weed control but requiring 35% less applied chemical (O'Connell *et al.,* 1998) Enantiomers of compounds are identical in physical and chemical properties as well as ordinary chemical reactions, but they differ in toxicological and ecological behaviours (Liu *et al.,* 2005; Garrison, 2006; Wen *et al.,* 2011).

Table 2.1: Physiochemical Properties of S-metolachlor

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

S-metolachlor is classified as an inhibitor of synthesis of very long chain fatty acid. It interferes with normal cell development and inhibits both cell division and cell enlargement. The S-metolachlor among the other herbicide has known complicated biodegradation (Tomlin, 2002).

Table 2.2: S-Metolachlor Usage

Target Pests	Broadleaf weed species, and annual grassy weeds (yellow nutsedge (*Cyperus esculentus*), barnyard grass (*Echinochloa crusgalli*), crabgrass (*Digitaria* sp.), fall panicum (*Panicum dichotomiflorum*), and foxtails (*Setaria* sp.).
Target Crops	Cotton, corn, sugarcane, soybean, peanuts, sorghum, potatoes, safflower, and woody ornamentals
Areas of Pakistan	Multan, Sahiwal, Arifwala, Pakpattan, Dipalpur, Okara, Lahore, Karachi

2.3. TOXICITY OF S-METOLACHLOR

Pesticide exposure leads to chronic and acute threats to human health. For example, long term low dose exposure to pesticide causes carcinema, hormonal disruption, diminished intelligence, reproductive abnormalities and immune suppression (Gupta, 2004). Pesticide Program's Carcinogenicity Peer Review Committee classified metolachlor as possible human carcinogen under EPA's Cancer Assessment Guidelines (EPA, 1995). The exposure of metolachlor promotes endocrine problems in reproductive parameters and these changes are reflected by altering the serum concentrations of testosterone, DHT (Dihydrotestosterone), estradiol, and FSH (Follicle-stimulating hormone) as well as by causing morphological alterations in androgen-targeted tissues, at the lowest dose, which may indicate the potential risk of occupational exposure to this herbicide (Mathias *et al.,* 2012).

Both the racemic mixture and the S-enantiomer have the potential to cause adverse effects on microorganisms (Moorman *et al.,* 2001; Papaefthimiou *et al.,* 2004) and on higher terrestrial and aquatic organisms (Papaefthimiou *et al.,* 2004; Liu *et al.,* 2006 ;

Xu *et al.,* 2010). Freshwater microalgae and macrophytes are however the most vulnerable ones (Junghans *et al.,* 2003; Pe´rez *et al.,* 2011). Algal growth inhibition assays were undertaken to investigate S-metolachlor and Rac-metolachlor enantioselective toxicity to *Scenedesmus obliquus*. The results showed that the aquatic toxicity of S-metolachlor was higher than Rac-metolachlor (Liu *et al.,* 2012*)*

2.4. TREATMENT TECHNIQUES

Conventional methods of treatment for contaminated site and wastewater have been largely chemical or physical such as chemical oxidation, solvent extraction and adsorption, but these processes result in secondary effluent problems and such treatment options usually are not available in developing countries (Karstensen *et al.,* 2006). Moreover, such, chemical and physical processes can be a source of more toxic byproducts. Photolysis products are at least as toxic as the parent compounds, whereas in case of metolachlor, byproducts of photolysis are more toxic as compared to the parent compound (Souissi *et al.,* 2013).

Due to these reasons biological treatment i.e biodegradation is preferred. Biodegradation is versatile, inexpensive and can potentially turn a toxic material into harmless products. When a biological process is properly designed and operated, it can result in complete oxidation of organic matter. In this way there can be no sludge that needs to be eradicated after treatment (Annaduarai *et al.,* 2008). Moreover the use of

pure cultures of microorganisms, especially adapted to metabolize the contaminant, can be an effective alternative (Wang and Loh, 1999; Jiang *et al.,* 2007; Yang and Lee, 2007; Wei *et al.,* 2007). Microorganisms have the capability to interact, both physically and chemically, with the pollutant leading to structural changes. This can also result in complete degradation of the target molecule (Raymond *et al.,* 2001; Wiren-Lehr *et al.,* 2001). Biological processes have been used for treatment of wastes and pesticides polluted sites (Araya and Lakhi, 2004).

Complete biodegradation of a pesticide involves the oxidation of parent compound to form carbon dioxide and water. This process provides both carbon and energy for the growth and reproduction of microbes. Each degradation step is catalysed by specific enzyme produced by a degrading cell or enzyme found external to the cell. Degradation of pesticide by either external or internal enzyme will stop at any step if a suitable enzyme is not present. Absence of an appropriate enzyme is one of the common reasons for persistence of any pesticide. If potential degrading microorganism is absent in soil or if biodegrading microbial population has been reduced due to toxicity of pesticide, a specific microorganism can be added or introduced in soil to enhance the biodegradation activity of the existing population (Singh, 2008).

Sequence batch reactors was also used in a study to investigate the biodegradability of herbicides (isoproturon and 2, 4-dichlorophenoxyacetic acid (2,4-D). The aerobic SBR

was run at an ambient temperature, whereas the anaerobic SBR was operated in the lower mesophilic range. Although isoproturon was not degraded in either system during the study, but complete 2,4-D removal was achieved after an acclimation period of approximately 30 d (aerobic SBR) and 70 d (anaerobic SBR) (Celis *et al.,* 2008).

2.5. S-METOLACHLOR DEGRADATION

Metolachlor is found to be relatively more persistent in soils as compared to other widely used chloroacetanilide herbicides, such as alachlor and propachlor. Metolachlor half-lives ranging from 15 to 132 days have been observed in different soils (US. EPA, 1987a; WSSA, 1994). The herbicide is highly persistent in water, over a wide range of pH values, with reported half-life values of ≥200 and 97 days in highly acidic and basic conditions, respectively.

Metolachlor is also relatively stable in water, and under natural sunlight, only about 6.6% was degraded in 30 days (U.S. EPA, 1987b). The half-life of metolachlor in sterile soil was reduced from 97 to 12 days after the addition of an active microbial community (Barra *et al.,* 2005), indicating that other biotic factors influence metolachlor degradation in soils. In several organisms, the greatest factor of acetanilide transformation is detoxification by glutation-S-transferase (GST) (Stamper and Tuovinen, 1998; Zablotowicz *et al.,* 1995; Hammond *et al.,* 1983). It is reported

that microorganisms do not easily metabolize aromatic fragment (Liu *et al.,* 1987), raising a serious environmental concern.

Although the degradation of metolachlor in soils is thought to occur primarily by microbial activity, little is known about the microorganisms that carry out the process and the mechanisms by which this occurs. Pure cultures of *Candida xestobii* and *Bacillus simplex* have been isolated which have the ability to use metolachlor as a sole carbon source for growth. *Candida xestobii* degraded 60% of the added metolachlor after 4 days of growth and converted up to 25% of the compound into CO_2 after 10 days, whereas *Bacillus simplex* was able to degrade 30% of metolachlor in 5 days using minimal medium. Microorganisms comprising two main branches of the tree of life have acquired the capability to degrade the novel chlorinated herbicide that has been recently added to the biosphere (Munoz *et al.,* 2011).

Bacterial strains have also been isolated from metolachlor spiked soil *Bacillus silvestris, Bacillus niacini, Bacillus pseudomycoides, Bacillus cereus, Bacillus thuringiensis, Bacillus simplex, Bacillus megaterium,* and two other *Bacillus sp*. (Met1 and Met2). The isolated metolachlor degrading bacteria showed optimal performance at 30°C. Isolates showed highest efficiency at concentration 10 to 50 µg/ml of the herbicide. After incubation for 119 days, *Bacillus thuringiensis* has highest activity to degrade metolachlor (Wang *et al.,* 2008).

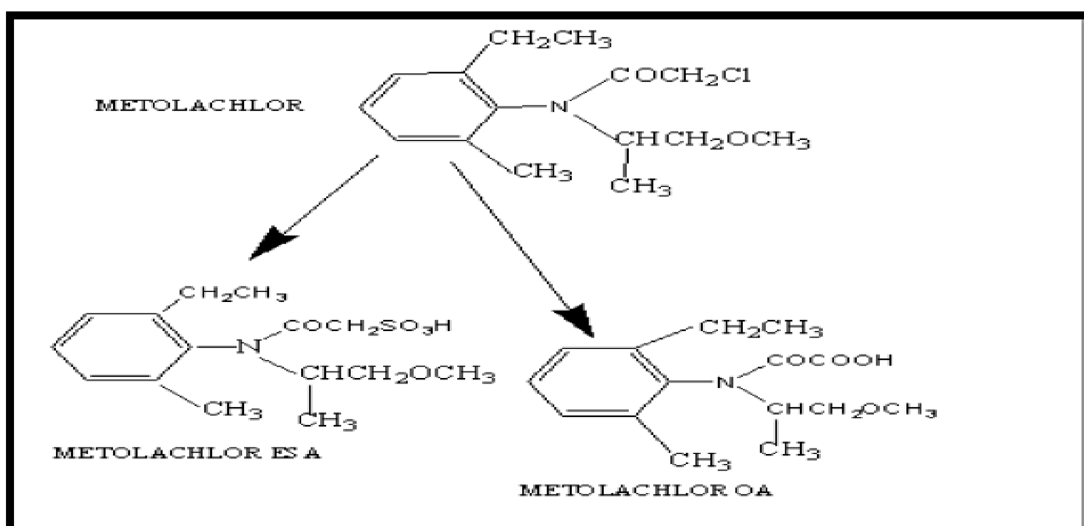


Figure 2.2: Primary Degradation Products

Metolachlor ethane sulfonic acid (ESA) and metolachlor oxanilic acid (OA) are the two most common degradation products of metolachlor. Genus *Pseudomonas* is considered to be very versatile in utilization of carbon resources. Different species of *Pseudomonas* have been reported to degrade environmental pollutants. The *Pseudomonas sp*. (MB1) isolated from sewage sludge degrade up to 85.6% metolachlor when incubated for 20 d at 10 µg mL^{-1} level in a minimal salt medium supplemented with 0.05% mannitol as additional carbon source. *Pseudomonas sp*. MB2 and MB3 degraded metolachlor up to 70 and 70.7%, respectively, when incubated at 20 µg mL^{-1} level for 20 d in the minimal salt medium. The MB3 isolate from field soil degraded Metolachlor and produced four metabolites, two of which are new metabolites being reported (Sanyal and Kulshrestha, 2003).

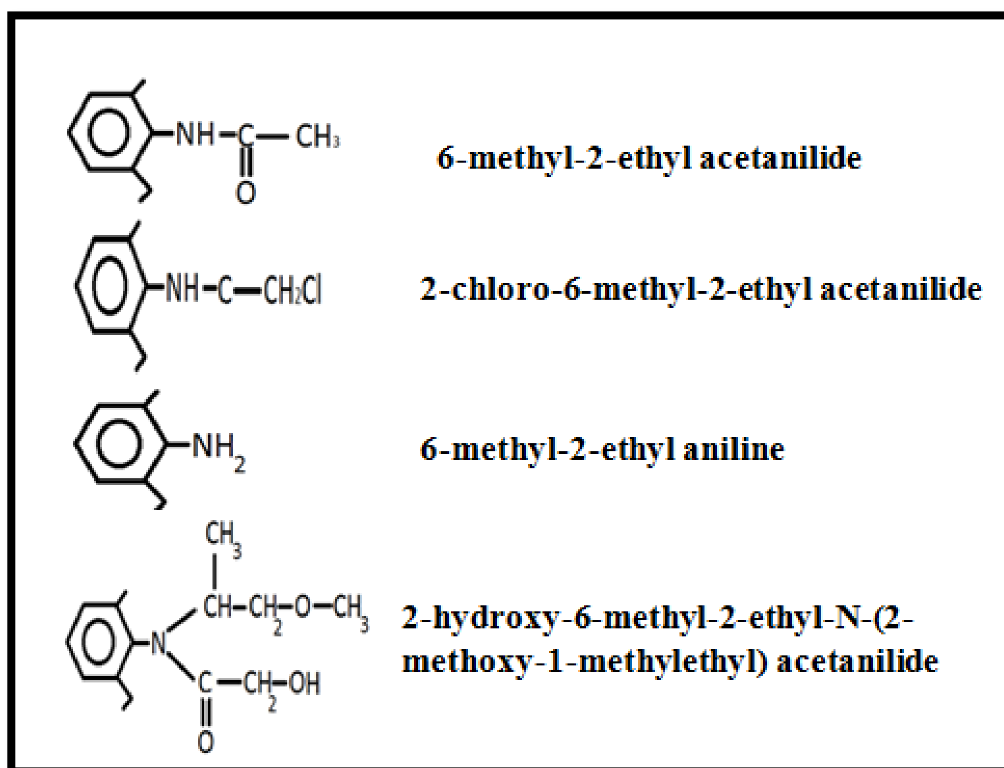


Figure 2.3: Secondary Metabolites of S-metolachlor Biodegradation

Chapter 3

MATERIALS AND METHODS

Bench scale biodegradation study of S-metolachlor was carried out in the Environmental Microbiology and Biotechnology Research Lab, Institute of Environmental Sciences and Engineering, at National University of Sciences and Technology.

Pre-emergent herbicide S-metolachlor was degraded using indigenous soil micro-organism. The experiment was conducted in a bench scale bioreactor system. The growth of micro-organism in Minimal Salt Medium (MSM) and reduction of total organic carbon with time was observed using UV spectrophotometer and TOC analyser respectively. The degradation rate was analysed quantitatively with gas chromatography technique.

3.1. CHEMICALS AND PESTICIDE

The commercial grade S-metolachlor (Dual Gold 960 EC Syngenta) was purchased from local market. The purpose of using commercial grade product was to represent an actual situation to which micro-organisms are likely to be exposed. FLUKA S-metolachlor PESTANAL® (33859), analytical standard was used for gas chromatographic analysis. For liquid-liquid extraction of GC samples was done with

GC grade Ethyl acetate (solvent). API 20E kit (BioMerieux, Canada) was used for biochemical identification.

3.2. CONCENTRATIONS OF S-METOLACHLOR

In MSM different concentrations of S-metolachlor was introduced as sole carbon source to determine the potential of indigenous soil microorganism for degradation of selected pesticide. Concentrations used were 10, 20, 50 µg/ml (Sanyal and Kulshrestha, 2003; Ismail and Shamsuddin, 2005; Wang *et al.,* 2008). These concentrations were too high as compared to agricultural dose of herbicide (Wu *et al.,* 2011).

3.3. PREPARATION OF CULTURE MEDIA

3.3.1. Washing and sterilization of glassware

All glassware that was used in experiment was thoroughly cleaned by using 95% chromic acid. For sterilization, all the glassware was autoclaved at $121^{o}C$ and 15 lb/cm^2 pressure for 15 minutes. The sterilized glassware was then dried in hot air oven at $171^{o}C$ for 2 hours (Prescott *et al.,* 2003).

3.3.2. Preparation of Media

3.3.2.1. Minimum Salt Medium (MSM)

MSM was prepared for the isolation and experimental purpose. The composition of minimal salt media is shown in Table 3.1:

Table 3.1: Composition of MSM

Chemicals	Quantity (gms/L)
$(NH_4)_2HPO_4$	0.5
$MgSO_4.7H_2O$	0.2
K_2HPO_4	0.1
$FeSO_4.7H_2O$	0.001
$Ca(NO_3)_2$	0.01

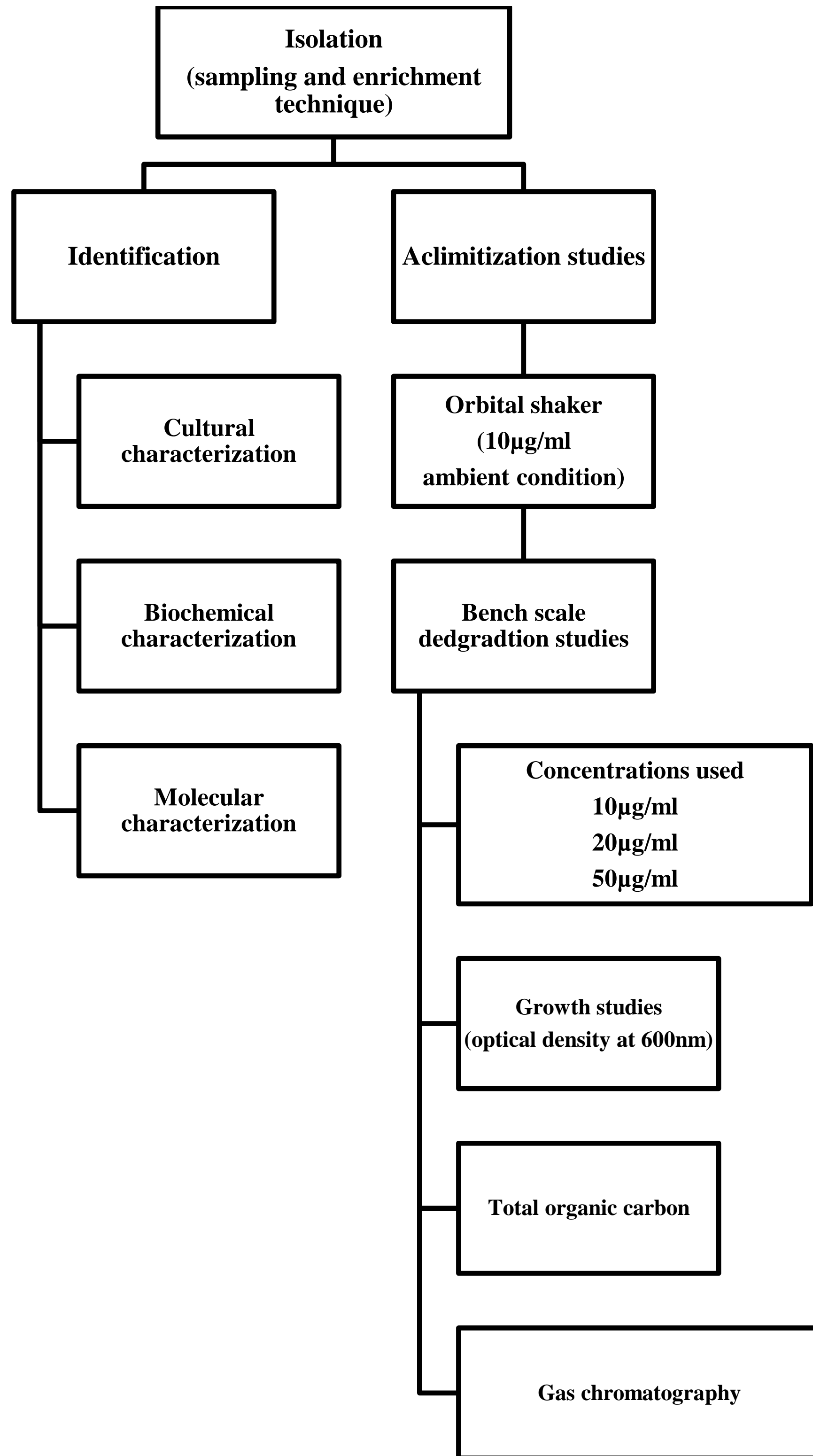
The above mentioned chemicals are mixed in 1 L autoclaved distilled water. The pH of media was 6.2 (Hashmi and Kim, 2003). S-metolachlor was added in MSM to acquire different concentrations i.e. 10, 20, 50 µg/ml.

3.3.2.2. Other Media

For preparing fresh cultures and bacterial inoculum nutrient agar slants and plates were prepared. For this 20 g of nutrient agar was mixed in 1 litre distilled water and then it was autoclaved at 121^oC and 15 lb/cm^2 pressure for 15 minute. Then nutrient agar was poured in autoclaved petri plates and test tubes. Sterility test was performed by placing, petri plates and test tubes in incubator at 37^oC for 48 hours.

Nutrient broth was prepared by adding 8 g of media in 1L distilled water. Then it was autoclaved at 121°C and 15 lb. /cm^{2} pressure for 15 minute. For sterility test nutrient broth was placed, in incubator at 37°C for 48 hours.

Figure 3.1. EXPERIMENTAL DESIGN



3.4. ISOLATION

3.4.1. Sampling

Soil samples was collected from cotton field (Cotton Research Centre Multan) that was previously sprayed with Dual Gold (S-metolachlor 82.1 % W/W). Soil samples were taken from the top 0-15 cm of soil from three different sites. Composite sample was made by mixing soils from each site. Soil was kept at 4°C in sterile polythene bags before bacterial isolation. The pH of soil sample was measured using portable pH meter (Nannipieri, 1994).

3.4.2. Enrichment technique

S-metolachlor degrading species was isolated using enrichment technique. 1g of soil sample was added to 20 ml of autoclaved MSM containing S-metolachlor (6 µg/ml) as a sole carbon and energy source. 6 µg/ml of active constituent of herbicide was selected as it corresponds to the agricultural dose (Wu *et al.,* 2011). Cultures were incubated in an orbital shaker at 30°C at 150 rpm for 5 days. 1 ml of each enrich culture was then transferred to 20 ml of fresh autoclaved MSM and incubated for additional 5 days under similar conditions. After enrichment 1 ml of sample was taken and serially diluted and spread on nutrient agar plates. Single colony from each isolate was then used to inoculate 20 ml of MSM containing S-metolachlor (6µg/ml) and incubated for a week in an orbital shaker under identical conditions in order to confirm the biodegradability. The isolate that showed notable growth under this condition was

selected as possible S-metolachlor degrader and designated as SM1 (Mohammad, 2009). Isolated strain was cultured on nutrient agar slants having S-metolachlor (6µg/ml). Strain was kept at $4^{o}C$ and sub-cultured after every 3 months.

3.5. IDENTIFICATION

Identification of isolated strain was performed with the help of colony morphology, gram-staining, biochemical test (Collins and Lyne, 1985) and growth on selective media. Species level identification was done through polymerase chain reaction.

3.5.1. Morphological Characteristics

Biochemical and physiological characteristics were determined according to the procedures outlined in Bergey's Manual of Determinative Bacteriology (Holt *et al.,* 1994). Colony morphological characteristics of isolated strain grown on nutrient agar plates were observed to identify and classify the strain. Following morphological characteristics were observed;

Table 3.2: Colony Morphology

Morphological Characteristics	Description
Colony Size	Punctiform, Small, Large
Margins	Entire, Undulate, Filiform, Curled, Lobate
Forms	Circular, Irregular, Filamentous, Rhizoid
Texture	Creamy, Mucoid, Dry
Elevation	Raised, Convex, Flat, Umbonate
Colour	Yellow, Orange, Pale Yellow, Off-White

(Pelczar, 1957)

Cell morphology was observed through Gram staining method under oil immersion at 100X resolution using light microscope.

3.5.2. Biochemical Characterization

Biochemical characterization and identification was conducted using different standard biochemical tests and API20E identification kit as given below.

3.5.2.1. *Pseudomonas* Cetrimide Agar

Pseudomonas Cetrimide Agar is used for the selective isolation and identification of *Pseudomonas aeruginosa*. Isolated pure culture of SM1 was picked and streaked on cetrimide *Pseudomonas* agar. Then streaked petri plates were kept in an incubator at 37°C. Growth at 42°C was also observed. After 24 hours plates were examined for the growth and characteristic of green/ yellow green pigment.

3.5.2.2. Catalase Test

Bacteria able to live in oxygenated environments produce enzymes which neutralize toxic forms of oxygen. One such enzyme is catalase, which breaks hydrogen peroxide into water and molecular oxygen. Colony was picked with sterilized inoculating loop and transferred on clean slide. A drop of 3% hydrogen peroxide was then added on the slide. Bubbling showed positive result for catalase test.

3.5.2.3. Citrate Utilization Test

This test is performed for the ability of bacteria to convert citrate into oxaloacetate. In this media, citrate is the only carbon source available to the bacteria. If it cannot use citrate then it will not grow. These organisms also convert ammonium dihydrogen phosphate to ammonia and ammonium hydroxide which creates an alkaline environment in the medium. At pH 7.5 or above bromothymol blue turns royal blue. At neutral pH bromothymol blue is green. Simmon's citrate agar plates were prepared and inoculated with 24 hour fresh culture. After inoculation agar plates were incubated for 24 hours at 37°C.

3.5.2.4. Oxidase Test

A loop full of inoculum was taken from 24 hour fresh culture and placed on a piece of filter paper. One drop of 1% N, N-dimethyl-p-phenylenediamine dihydrochloride solution was added. The appearance of blue or purple colour formation within few seconds is indicative of oxidase positive showing the presence of enzyme cytochrome oxidase.

3.5.2.5. Analytical Profile Index-20E

This API-20E test strip (BioMerieux, Canada) is used to identify the enteric gram negative rods. There are 20 separate small capsules on the strip in dehydrated form

each responding to a biochemical test. A bacterial suspension is used to rehydrate each of the wells. Single colony of the SM1 was inoculated into the 0.85% NaCl solution, ensuring that the suspension was homogenous and without clumps of floating bacteria. Capsules of LDC, ODC, ADH, H_2S, and URE are half filled. These capsules were then filled up to the top with sterile mineral oil. CIT, VP, and GEL capsule were filled up-to the top of the well. Strip was labelled and kept in an incubator at 37°C for 24 hours. After 24 hours reagents were added in IND, VP and TDA chambers.

Table 3.3: API E20 Interpretation

Tests	Substrate	Reaction Tested	- Results	+ Results
ONPG	ONPG	Beta-galactosidase	Colorless	Yellow
ADH	Arginine	Arginine dihydrolase	Yellow	Red/orange
LDC	Lysine	Lysine decarboxylase	Yellow	Red/orange
ODC	Ornithine	Ornithine decarboxylase	Yellow	Red/orange
CIT	Citrate	Citrate utilization	Pale green/yellow	Blue-green/blue
H2S	Na thiosulfate	H_2S production	Colorless/gray	Black deposit
URE	Urea	Urea hydrolysis	Yellow	Red/orange
TDA	Tryptophan	Deaminase	Yellow	Brown-red
IND	Tryptophan	Indole production	Yellow	Red (2 min.)
VP	Na pyruvate	Acetoin production	Colorless	Pink/red (10 min.)
GEL	Charcoal gelatin	Gelatinase	No diffusion of black	Black diffuse
GLU	Glucose	Fermentation/oxidation	Blue/blue-green	Yellow
MAN	Mannitol	Fermentation/oxidation	Blue/blue-green	Yellow
INO	Inositol	Fermentation/oxidation	Blue/blue-green	Yellow
SOR	Sorbitol	Fermentation/oxidation	Blue/blue-green	Yellow
RHA	Rhamnose	Fermentation/oxidation	Blue/blue-green	Yellow
SAC	Sucrose	Fermentation/oxidation	Blue/blue-green	Yellow
MEL	Melibiose	Fermentation/oxidation	Blue/blue-green	Yellow
AMY	Amygdalin	Fermentation/oxidation	Blue/blue-green	Yellow
ARA	Arabinose	Fermentation/oxidation	Blue/blue-green	Yellow
OX	Oxidase	Oxidase	Colorless/ Yellow	Violet

The positive and negative results of API E20 translated into numerical profiles (as shown in Figure 3.2) and interpreted with the API web software.

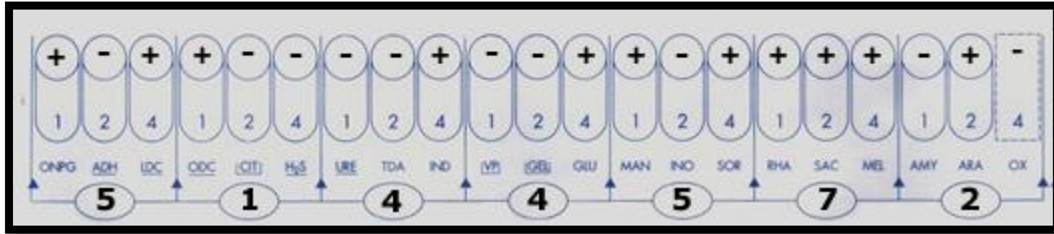


Figure 3.2: API E20 Numerical Code

3.5.3. Polymerase Chain Reaction

3.5.3.1. DNA Extraction

DNA from the isolate was extracted with the help of DNA extraction kit (Norgen Biotek Corporation). 1 ml of bacterial suspension was transferred to eppendorf and centrifuge at 14,000 rpm for 30 sec. to form cell pellet. Supernatant was poured off. Then 250 µL of re-suspension solution was added to the cell pellet. The cells were re-suspended by gentle vortexing. Then 250 µL of the Lysis Solution and 12 µL of Proteinase K to the cell suspension were added. After this it was mixed well and then was placed in incubator at 55°C for 30 minutes. 50 µL of Binding Solution was added to the lysate and mixed well with gentle vortexing using vortex. Then mixture was applied to spin column assembly and centrifuged the unit for 4 min. at 8000 rpm. After centrifugation, the flow through was discarded, and the spin column reassembled with its collection tube. 500 µL of Wash Solution was applied to the column, and the unit was centrifuge for 1 minute at 14,000 rpm. The spin column (with DNA bound to the

resin) was assembled with a provided 1.7 mL Elution tube. Then 200 μL of Elution Buffer was added to the center of the resin bed. It was centrifuged for 1 minute at 6,000 rpm. The assembly was centrifuged at 14,000 rpm for an additional 2 minutes to collect the total elution volume. The purified genomic DNA was stored at 2-8°C for a few days. For longer term storage, -20°C is recommended.

3.5.3.2. Molecular Identification:

The Polymerase Chain Reaction (PCR) is an enzyme catalyzed biochemical reaction in which small amounts of a specific DNA sequences are amplified into large amounts of linear double stranded DNA (Mullis, 1990). It comprises of numbers of cycles and each cycle consists of denaturation, annealing and extension step (Figure 3.3).

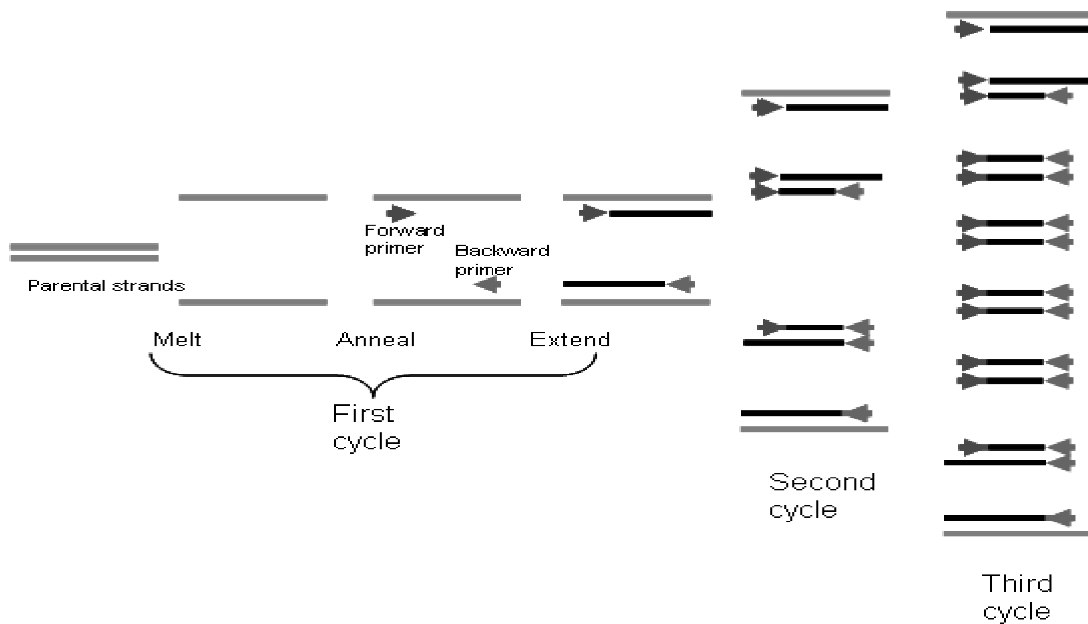


Figure 3.3: Simplified Mechanism of PCR

The 16S rDNA gene was amplified by Polymerase Chain Reaction using PA-SS-F (GGGGGATCTTCGGACCTCA) and PA-SS-R (TCCTTAGAGTGCCCACCCG) targeting 16S rDNA, (Spilker *et al.,* 2004). PCR reactions were carried out using TE Thermocycler (Extragene). 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 58°C and 10 min at 72°C. Then PCR product and 100 bp DNA ladder was run on Gel Electrophoresis. For gel electrophoresis, 1% agrose gel was prepared in TBE buffer. Ethidium bromide (3 µl) was added in agrose gel as this is a chemical that intercalates DNA and makes it visible under UV light (Parveen and Khan, 2012).

Table 3.4: Composition of PCR Master Mix

Ingredients	Concentration	Volume (µl)
$MgCl_2$	20 mM	2.5
dNTPs	2.5 mM	2
Taq Buffer (BioBasic, Canada)	10 X	2.5
Forward primer	10 µM	1
Riverse primer	10 µM	1
DNA template	1-100 ng/µl	3
Taq polymerase	500 U/ µl	0.3
PCR water	---	12.7

3.6. DEGRADATION STUDIES

3.6.1. Preparation of Inoculum

The inoculum for the experiment was prepared by growing acclimatized SM1 on nutrient agar slants. The culture slants were washed with 10 ml sterilized phosphate

buffer having pH 7. The turbidity of the culture was assessed against McFarland's Turbidometric Index ($3x10^9$ CFU/ml) (Qiu *et al.,* 2007).

3.6.2. Acclimatization

The concentrations of herbicide used in this research work were higher than normal rates applied in the field, which are less than 10 µg/g of herbicide for the top 5 cm of soil (Ismail and Shamsuddin, 2005). The strain was first acclimatized in MSM having S-metolachlor (10µg/ml). The conical flask containing S-metolachlor and inoculum was kept in orbital shaker at ambient for 5 days. Optical density at 0, 24, 48, 72, 96 and 120 hour was measured using spectrophotometer.

Table 3.5: Technical Data for Orbital Shaker

Orbital Shaker	Labcon Spo-MP 8
Speed	120 rpm
Operating temperature	Ambient
Volumetric load	250 ml
Retention time	120 hours
Size of inoculum	10ml ($3x10^9$ CFU/ml)
Sample collection	24 hours

3.6.3. Bench Scale Bioreactor System

For this study a bench scale reactor system was fabricated. The system consists of four rectangular reactors (having 8 liters working volume), Aluminum hot plate magnetic stirrer, DO meter, pH meter and digital thermometer (TPM10) (Figure 3.4). This

system was experimentally run at ambient conditions. MSM having different concentration of S-metolachlor (10, 20 and 50 μg/ml) was used to determine degradation efficiency of isolated bacterium. These concentrations were far high from the agricultural dose. Control of the study was minimal media having bacterial culture but no carbon source. The sample was collected at 0, 24, 48, 72, 96 and 120 hrs. The experiments were run in triplicates to validate the results.

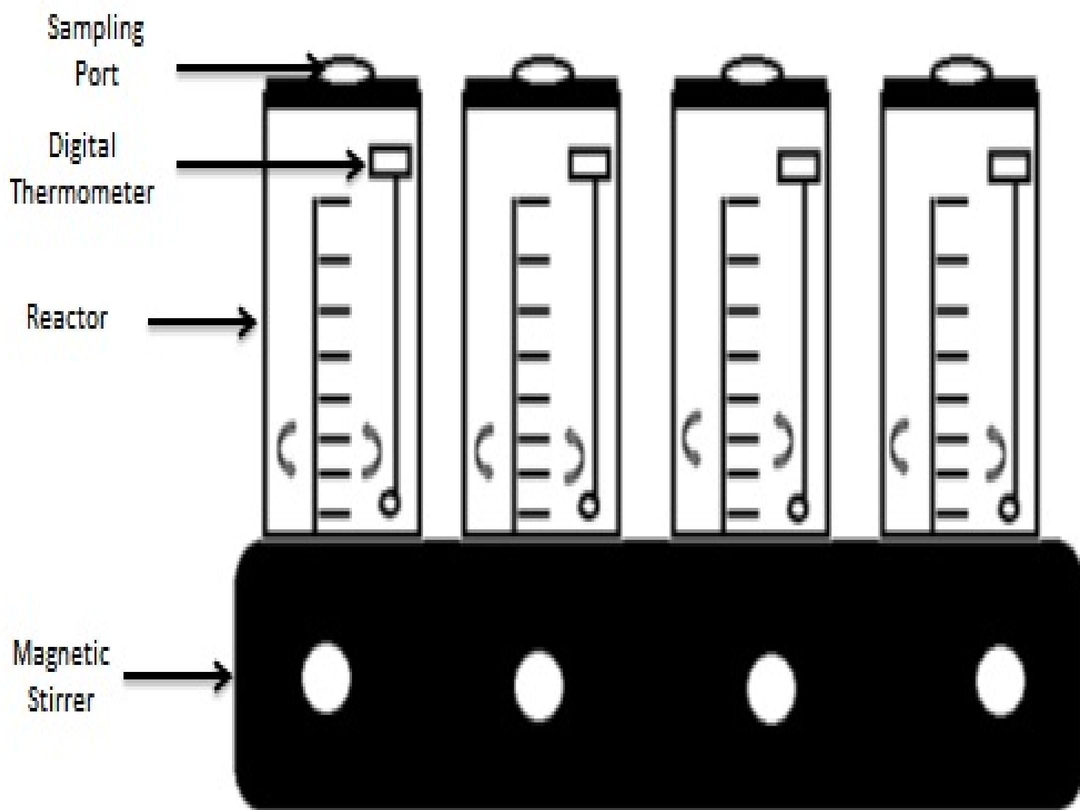


Figure 3.4: Bench Scale Setup for Biodegradation Studies

3.7. ANALYTICAL PROCEDURES

The degradation potential of the isolated micro-organism for S-metolachlor was determined using spectrophotometer, Total organic carbon (TOC) analyzer and gas chromatography (GC).

3.7.1. Growth Studies

Optical density, measured in a spectrophotometer, can be used as a measure of the concentration of bacteria in a suspension (Figure 3.5). Optical density of samples taken at different time interval was determined at 600nm using spectrophotometer. This wavelength is specific for bacteria (Munoz *et al.,* 2011).

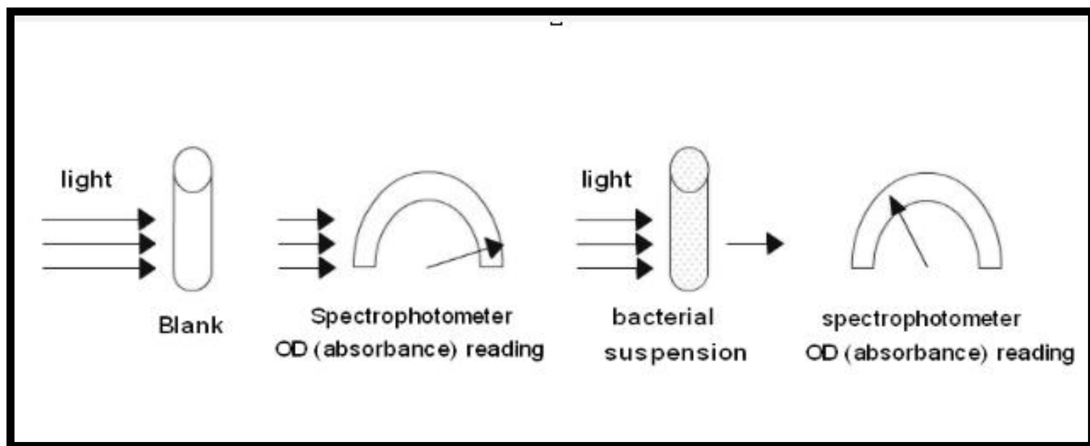


Figure 3.5: Simplified Mechanism of Determining Optical Density (OD)

3.7.2. Total Organic Carbon (TOC)

Estimation of total organic carbon reduction with time was performed by taking 15 ml samples at 0, 24, 48, 72, 96, 120 hrs. Sample was filtered in a test tube. Few drops of 3% H_2SO_4 were added for preservation of sample. Each sample was then analyzed by TOC analyzer (Multi N/C 3100, Analytikjena).

3.7.3. Gas Chromatography

3.7.3.1. Sample Extraction

For gas chromatography sample was prepared by adding few drops of 3% H_2SO_4 to kill bacteria present in the sample. The sample was stored at 4^oC. Liquid-liquid extraction technique was used for sample extraction. In this technique 5 ml of organic solvent ethyl acetate was added to 20 ml of sample in a test tube. The sample and ethyl acetate was mixed vigorously using vortex. After this it was sonicated for 2 min. After sonication the sample was left undisturbed for 15-20 minutes. After that separate organic and inorganic layers were formed. Upper layer (organic) was collected. This step was repeated for two times more. The extracted sample was filtered and kept in refrigerator at 4^oC.

3.7.3.2. Gas Chromatography analysis

Shimadzu GC 2010 with Electron Capture Detector (ECD) was used for analysis. The separation column used was fused silica capillary column with 30 m length (Figure 3.6). Liquid samples are rapidly vaporized then transported through column by mobile phase.

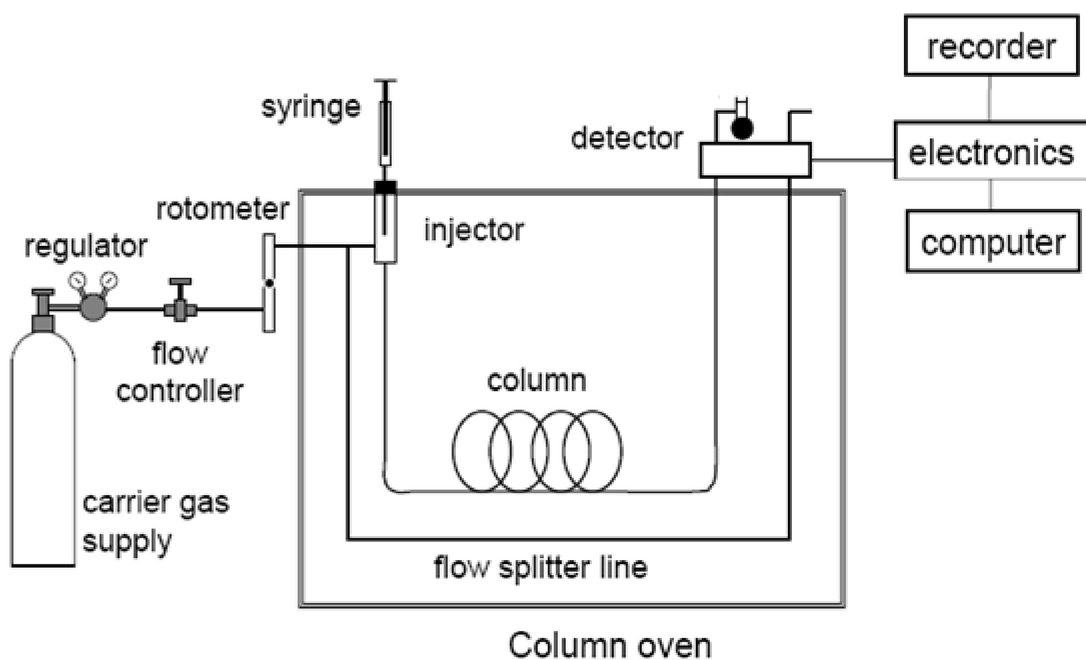


Figure 3.6: Schematic Diagram of Gas Chromatography

Stock solution of 200 ppm was prepared by dissolving standard FLUKA S-metolachlor PESTANAL® (33859) in GC grade Ethyl Acetate. From stock solution four dilutions (25, 50, 75, 100 ppm) were prepared to formulate calibration curve and line equation. This line equation was used to determine unknown concentration in the sample.

Before running sample, the instrument was optimized by changing various conditions such as injector temperature, column oven temperature, and detector temperature, split ratio and flow rate. When all the parameters were optimized the retention time of S-metolachlor and solvent were identified. 1 µl of each dilution was injected to check the signal and retention time of target herbicide. Table 3.6 shows the optimized parameters of GC:

Table 3.6: Gas Chromatographic Conditions

Parameters	**Conditions**
Column	95% dimethyl polysiloxane,5% diphenyl (TRB-1), 30 m, 0.32mm, 0.5µm
Carrier gas	Helium
Make-up gas	Nitrogen
Flow rate	112.2 ml/min
Split ratio	90
Program	120°C for 1 min 15°C/min to 250°C 250°C for 2 min
Program run time	13.67 min
Injection temperature	250 ^{o}C
Detector temperature	280 ^{o}C

Chapter 4

RESULTS AND DISCUSSION

Biodegradation of the relatively new herbicide S-Metolachlor was studied using indigenous micro-organism isolated from soil obtained from Cotton Research Institute Multan. Degradation potential of isolated strain was determined at different concentration of the herbicides.

4.1. ISOLATION AND IDENTIFICATION

Agriculture soil that was sprayed with S-Metolachlor was used for isolation of the bacterial strain. The pH of the sampled soil was 6.8. Neutral soils have more bacterial diversity (Fierer and Jackson, 2006). Isolation of indigenous bacteria capable of metabolizing organophosphate compounds has received considerable attention because these bacteria provide an environmental friendly method of in situ detoxification (Richins *et al.,* 1997; Mulchaldani *et al*., 1999). In some contaminated environments, indigenous microbial populations have evolved over time to adapt to these contaminants (Pahm and Alexander, 1993). These sites are therefore the most appropriate ecological niches to find and isolate strains capable of degrading xenobiotics (Ramos and Rojo 1990; Oshiro *et al.,* 1996; Ortiz-Hernández *et al.,* 2001; Horne *et al.,* 2002).

S-Metolachlor degrading specie was isolated from the agriculture soil with the help of enrichment technique. Colony Morphology, Gram staining and different biochemical test (including API E20) was conducted to identify the bacterial specie. On the basis of observations of above results the isolated strain was identified as *Pseudomonas sp*.

Table 4.1: Characterization of Isolated Specie SM1

<table>
<tr><th colspan="2">Characteristics</th><th>Observations</th></tr>
<tr><td colspan="2">Colony Morphology</td><td>Irregular, Undulate, Raised, Yellowish Green Pigment , Smooth And Shiny</td></tr>
<tr><td colspan="2">Gram Staining</td><td>Gram Negative (Pink Short Rods)</td></tr>
<tr><td rowspan="2">Pseudomonas Citrimide Agar</td><td>37°c</td><td>Growth, Green Pigment Colonies</td></tr>
<tr><td>42°c</td><td>Growth, Green Pigment Colonies</td></tr>
<tr><td colspan="2">Citrate Test</td><td>Positive</td></tr>
<tr><td colspan="2">Catalase Test</td><td>Positive</td></tr>
<tr><td colspan="2">Oxidase Test</td><td>Positive</td></tr>
</table>

The results of API E20 were also noted and converted into numerical code which is then run on API web software. The results of APIE20 identified the strain tentatively as *Pseudomonas aeruginosa.* For further confirmation of identification of SM1, Polymerase Chain reaction (PCR) was used. In this specific *Pseudomonas aeruginosa* forward and reverse primers were used. The size of the expected product was 956 bp (Spiker *et al.,* 2004). When the PCR product was run on Gel electrophoresis with 100 bp DNA ladder, the product size was observed 956 bp.

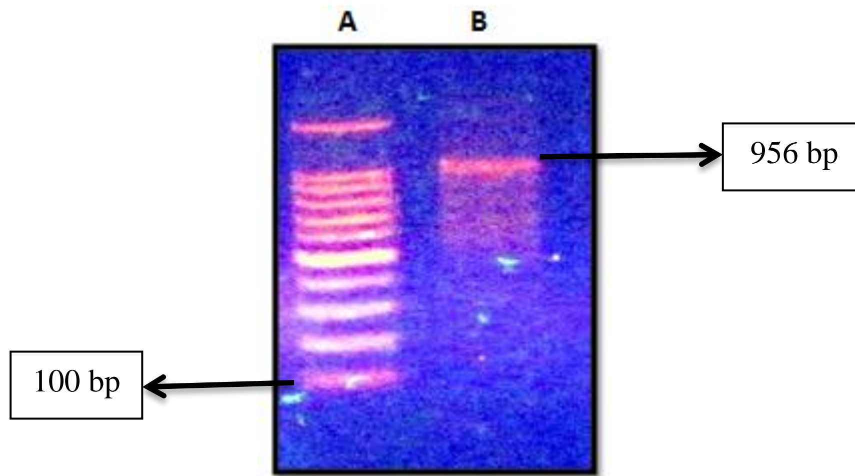


Figure 4.1: (A) DNA ladder 100 bp; (B) PCR Product

Pseudomonas aeruginosa is a Gram-negative bacterium commonly found in soil and ground water. It rarely affects healthy people and most community acquired infections are associated with prolonged contact with contaminated water. *Pseudomonas* species is a vast heterogeneous group of bacteria that occur in substantial numbers in the soil where these are active agents for mineralization of organic matter. Most species can grow well in simple minimal medium with a single organic compound as carbon and energy source (Palleroni, 1986).

4.2. ACCLIMITIZATION

When the microorganisms can survive in an environment where the pollutant is prevalent, it has the potential to degrade that harmful substance. *Pseudomonas aeruginosa* was, thus, acclimatized in MSM at 10 µg/ml concentration of S-

Metolachlor. Normal rates applied in the field were less than 10 µg/ml of herbicide for the top 5 cm of soil (Ismail and Shamsuddin, 2005). *Pseudomonas aeruginosa* showed positive growth (Figure: 4.2). As S-metolachlor was the only carbon source available to SM1, so its growth is an indication of degradation of S-metolachlor.

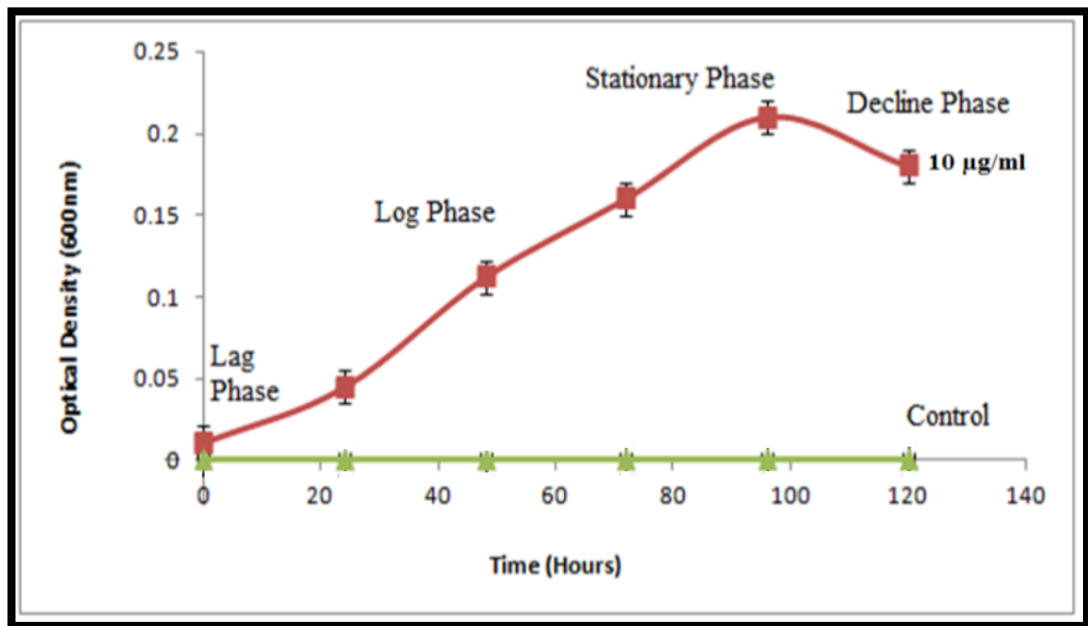


Figure 4.2: Growth Curve of *Pseudomonas aeruginosa*

The lag phase of the *Pseudomonas aeruginosa* was 24 hours. After 24 *hours* *Pseudomonas aeruginosa* entered into log phase which lasted for 44 hours, leading to the stationary phase and then decline in growth.

4.2.1. Generation time

Generation time was calculated using optical density of *Pseudomonas aeruginosa* at 10 µg/ml concentration. The time required for a cell to divide (and its population to double) is called the generation time. It varies considerably among organisms and with environmental conditions, such as temperature, and availability of nutrients etc. Most bacteria have a generation time of 1 to 3 hours; others require more than 24 hours per generation.

Generation Time *= (Time in minutes to obtain the absorbance 0.112) – (Time in minutes to obtain the absorbance 0.045)*

= 48-24

= 24 hours

Let

N_o = the initial population number = 0.045 (absorbance)

N_t = population at time t= 0.112 (absorbance)

N = the number of generations in time t

Therefore,

$$N_t = N_o \times 2^n \text{...} (1)$$

$$\log N_t \quad = \quad \log N_o + n \log 2$$

Therefore,

$$n \;=\; (\log N_t - \log N_o) / \log 2$$

$$n = (\log N_t - \log N_o) / 0.301 \text{..............................} (2)$$

The growth rate can be expressed in terms of mean growth rate constant (k), the number of generations per unit time.

$$k = n / t$$

$$k = (\log N_t - \log N_o) / (0.301 \times t) \text{......................} (3)$$

Mean generation time or mean doubling time (g), is the time taken to double its size.

Therefore,

$$N_t = 2\, N_o \text{..} (4)$$

Substituting equation 4 in equation 3

$$k = (\log N_t - \log N_o) / (0.301 \times t)$$

$$k = (\log 2\, N_o - \log N_o) / (0.301 \times t)$$

$$k = \log 2 + (\log N_o - \log N_o) / 0.301\, g$$

(Since the population doubles t= g)

Therefore,

$$k = 1/g$$

Mean growth rate constant

$$k = 1/g$$

Mean generation time

$$g = 1/k$$

Mean Generation Time= g = 24 hrs. or 1440 min

Generation times differ depending on the growth medium and environmental conditions used. As the *Pseudomonas aeruginosa* was allowed to grow in a media having minimal nutrients due to this elongated generation time is expected.

4.3. BENCH SCALE DEGRADATION STUDIES

The significance of the mode of growth of microorganism has the potential biotechnological application in bioremediation of environmental pollution through bioreactors (Lauwer *et al.,* 1990). Biological decomposition of pesticides is the most important and effective way to remove these compounds from the environment. Microorganisms have the ability to interact, both chemically and physically, with substances leading to structural changes or complete degradation of the target molecule (Raymond *et al.,* 2001; Wiren-Lehr *et al*., 2001).

4.3.1. Growth Studies

In the current study growth of *Pseudomonas aeruginosa* was observed for three different concentrations of S-metolachlor. Wide ranges of carbon sources may be utilized by Genus *Pseudomonas* (Roostan *et al.,* 2012; Ajaz *et al.,* 2012; Tanti and Buragohain, 2013), as this genus is characterized by great metabolic diversity. Optical density using spectrophotometer of *Pseudomonas aeruginosa* was observed at different time intervals for each concentration separately.

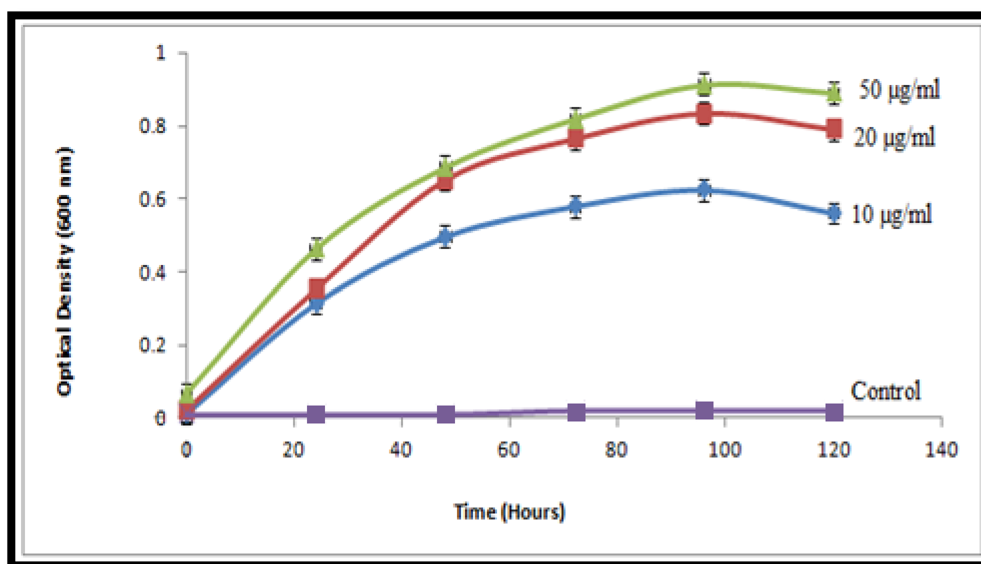


Figure 4.3: Growth of *Pseudomonas aeruginosa* at Different Concentrations of S-Metolachlor

4.3.2. Total Organic Carbon

The significant reduction of total organic carbon was observed as the bacterial growth increased (Figure 4.4).

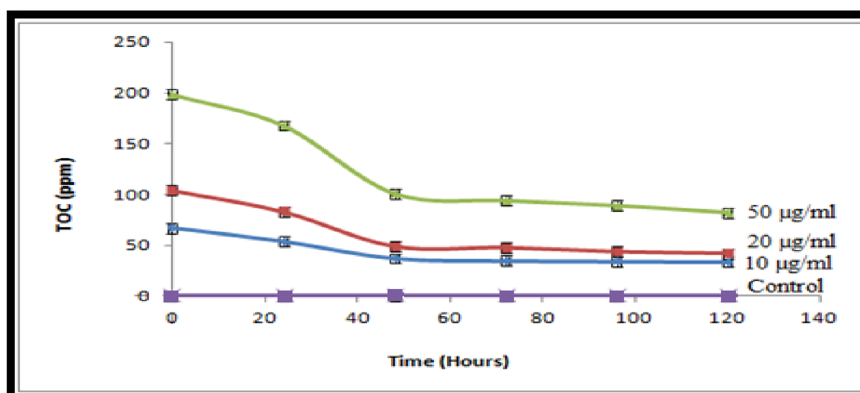


Figure 4.4: TOC Reduction at Different Time Intervals

As the growth increases with time the total organic carbon reduces, as inoculated bacteria consume S-metolachlor as a carbon source for its growth. Significant reduction was observed in first 72 hours as the after that stationary phase of *Pseudomonas aeruginosa* started.

4.3.3. Gas Chromatography

4.3.3.1. Standard Calibration Curve

From standard of S-metolachlor (FLUKA S-metolachlor PESTANAL® (33859)) stock solution was prepared in Ethyl acetate (organic solvent). Four different dilutions (25, 50, 75, 100 ppm) were prepared in ethyl acetate from the stock solution. Signals were observed by injecting 1 µl of each dilution into the injection port. Figure 4.5 shows the calibration curve along with the line equation.

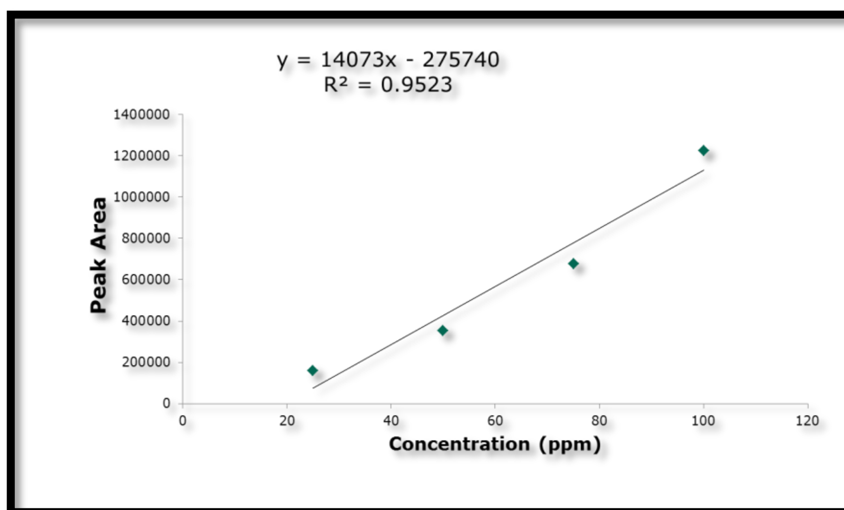


Figure 4.5: Standard Calibration Curve and Line Equation

4.3.3.2. Retention Time

For gas chromatography analysis different parameters (Table 3.6) were optimized after trial and error. Standard dilutions were injected at least three times and the mean was used to calculate the average retention time of S-metolachlor and ethyl acetate. The retention time for S-Metolachlor and the solvent (Ethyl-acetate) was 7.34 min and 2.4 min respectively (Figure 4.6).

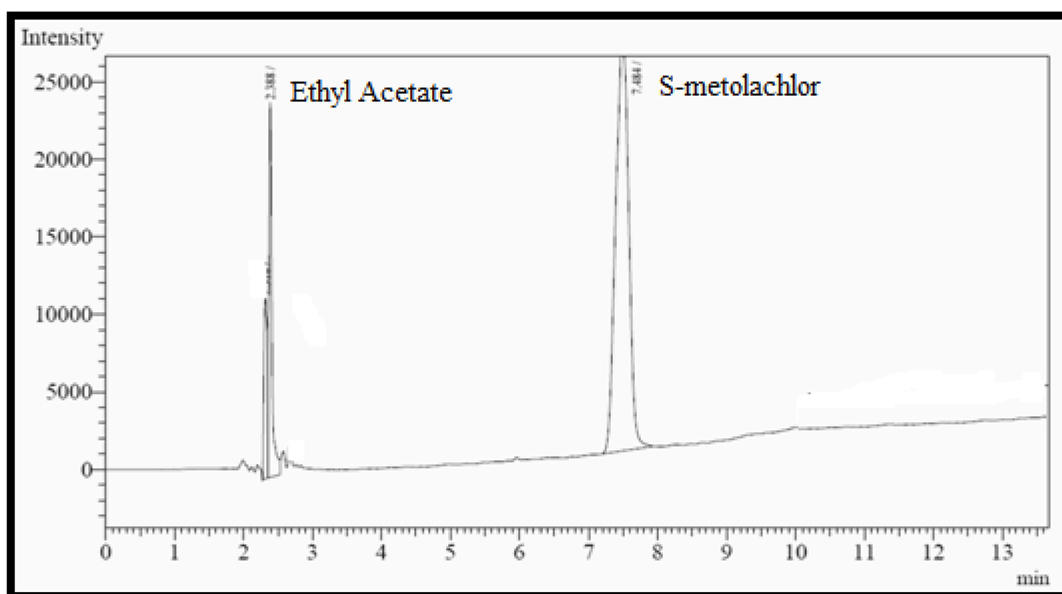


Figure 4.6: Gas Chromatogram Showing S-metolachlor Peak

4.3.3.3. Percentage Degradation

Bench scale bioreactor system runs efficiently at ambient temperature. Continuous stirring with the help of magnetic stirrer maintained dissolve oxygen at 5 mg/l ± 1, providing an aerobic environment to *Pseudomonas aeruginosa*. Quantitative analysis

of S-metolachlor at various concentrations was conducted. Each sample was injected in injection port at least for three times and the average value used. Maximum percentage degradation was obtained at 50 µg/ml concentration (Figure 4.7).

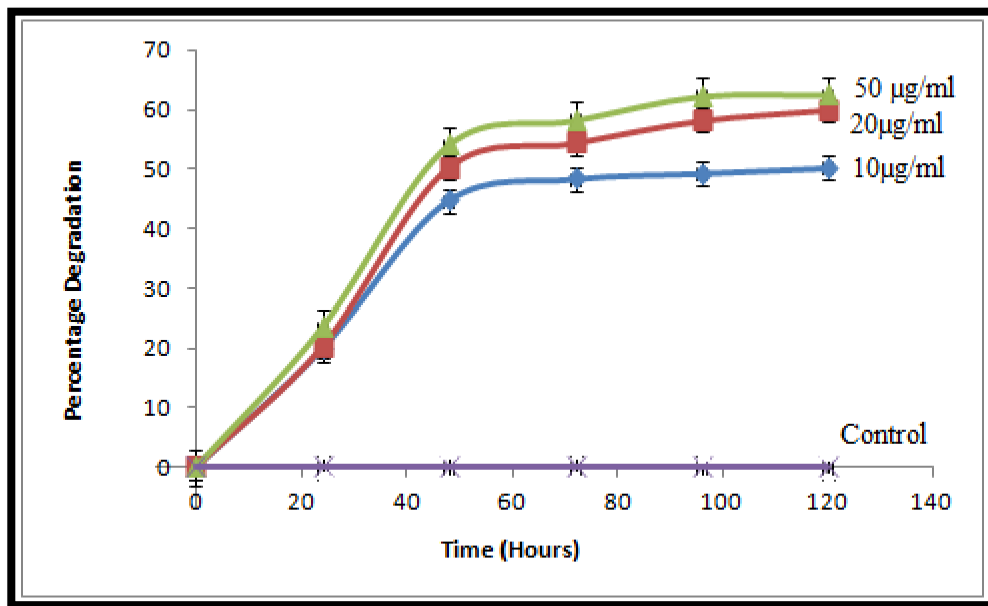


Figure 4.7: Percentage Degradation at Different Time Interval

Optimized dose of S-metolachlor was determined as 20 µg/ml. Beyond this concentration due to the inhibitory effect of S-metolachlor there was no significant increase in persentage degradation. In addition to this, after 72 hours the curve become near to constant as the stationary phase of *Pseudomonas aeruginosa* was started.

It is reported that *Pseudomonas aeruginosa* had been used as indigenous soil microorganism to degrade fungicide (karathane) in bioreactors. To increase the process of biomass production, fully acclimatized strain in optimized conditions was used. The strain was isolated from previously contaminated soil and acclimatized to

grow on karathane (upto 1%) as sole carbon source. The biodegradation capacity of *Pseudomonas aeruginosa* was determined by using contaminated soil (0.2% karathane). The strain utilized 45.8% of the pesticide within 72 hours (Dinu *et al.,* 2011).

Researches had shown that S-metolachlor was a persistent soil pollutant. It takes on average week time to biodegrade (Sanyal and Kulshrestha, 2003; Wang *et al.,* 2008; Vryzas *et al.,* 2012) and in a study it was reported that even after 246 days, only 26% of the S-Metolachlor was mineralized in soil (Baran and Gourcy, 2013).

On contrast to this present study showed that *Pseudomonas aeguginosa* was able to degrade 58% of S-metolachlor within 72 hours. The results exhibited that with the increase in concentration rate of percentage degradation was also increased. But the percentage reduction difference between 20 µg/ml and 50 µg/ml is not very significant and may be due to the inhibitory effect of S-metolachlor on the growth of *Pseudomonas aeruginosa*.

Chapter 5

CONCLUSIONS AND RECOMMENDATIONS

5.1. CONCLUSIONS

The main aim of this study was to study biodegradation of an herbicide belonging to the chloroacetanilide group. The herbicide was selected because of its persistence nature and solubility. Due to these reasons selected herbicide is potent water contaminant. The outcomes of this bench scale biodegradation studies are listed below:

1. S-metolachlor degrading bacterium was isolated from previously contaminated soil through enrichment technique. On the basis of cultural and biochemical characterization bacterial isolate was found to belong to genus *Pseudomonas*. Molecular characterization was done with the help of Polymerase Chain reaction and the specie was identified as *Pseudomonas aeruginosa.*
2. S-metolachlor was effectively removed at 10 and 20 µg/ml concentration in bench scale bioreactor system.
3. Generation time of *Pseudomonas aeruginosa* was found to be 24 hours or 1440 mins.

4. Increase in growth and reduction of total organic carbon with time depicted the ability of *Pseudomonas aeruginosa* to utilize S-metolachlor as a sole carbon source.
5. Quantitative analysis through gas chromatography showed percentage degradation 50%, 60% and 63% at 10, 20 and 50 µg/ml of S-metolachlor respectively.
6. Optimized concentration of S-metolachlor and time were 20 µg/ml at 72 hours as beyond this there was not significant increase in percentage degradation.

5.2. RECOMMENDATIONS

Following research studies may be conducted

1. Bench scale biodegradation studies using a consortium of bacteria and mixture of pesticides via activated sludge bioreactor.
2. Quantitative studies of toxicity of pesticide and its metabolites.
3. Biokenetics characterization for the batch and continuous biodegradation studies.
4. Application of viable treatment technology using highly potential bacterial consortium for pesticide industrial effluents.

Chapter 6

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ANNEXURE-I

Calculations:

Percentage purity = 87.3%

Molecular weight of S-metolachlor = 283.8

Density = 1.1 g/ml

Molarity = (Density of S-metolachlor x % purity x 10)/ Molecular weight

= (1.1 x 87.3 x 10)/ 283.8

= 3.44

Molarity = (Concentration in mg/L)/ Molecular weight x 1000

Concentration in mg/L = Molarity x Molecular weight x 1000

= 3.44 x 283.8 x 1000

= 977760 mg/L

= 977760 µg /ml

For 10 µg/ml:

$$C_1V_1 = C_2V_2$$

$$V_1 = (C_2V_2)/ C_1$$

$$= (10\ \mu g/ml \times 2000\ ml) / 977760\ \mu g/ml$$

$$= 0.02\ ml$$

$$V_1 = 20.45\ \mu l$$

For 20 µg/ml:

$$C_1V_1 = C_2V_2$$

$$V_1 = (C_2V_2)/ C_1$$

$$= (20\ \mu g\ /ml\ x\ 2000\ ml)\ /\ 977760\ \mu g\ /ml$$

$$= 0.04\ ml$$

$$V_1 = 40.9\ \mu l$$

For 50 µg/ml:

$$C_1V_1 = C_2V_2$$

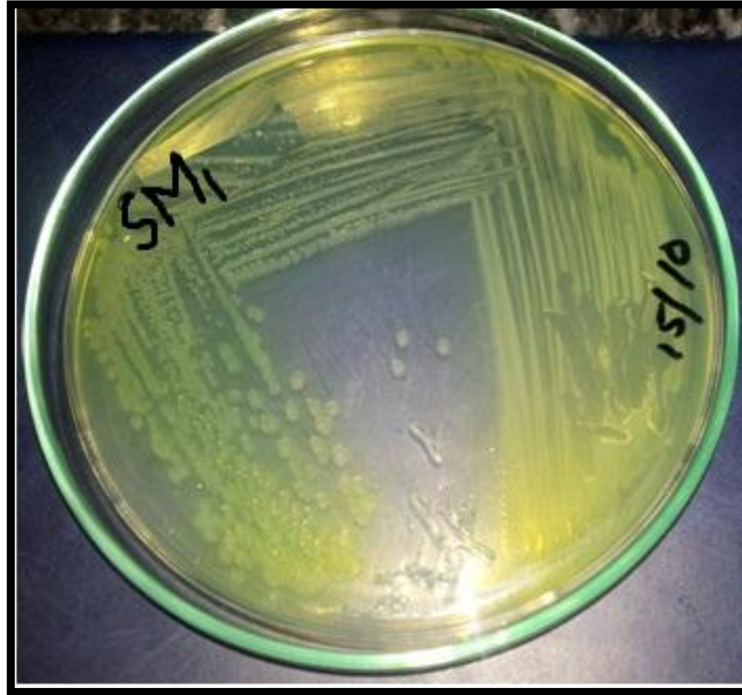
$$V_1 = (C_2V_2)/ C_1$$

$$= (50\ \mu g\ /ml\ x\ 2000\ ml)\ /\ 977760\ \mu g\ /ml$$

$$= 0.1\ ml$$

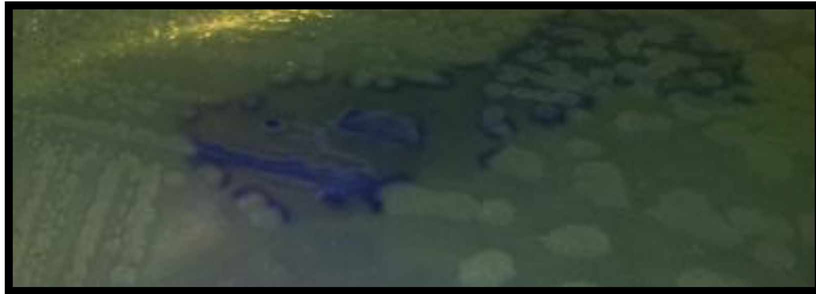
$$V_1 = 102.3\ \mu l$$

ANNEXURE-II



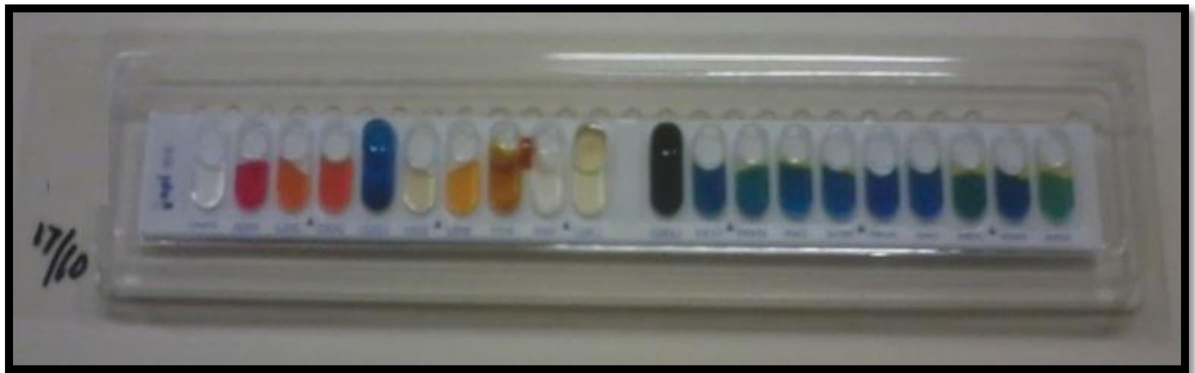
Isolated strain *Pseudomonas aeruginosa*

Catalase test for *Pseudomonas aeruginosa*



Oxidase test for *Pseudomonas aeruginosa*

Citrate Utilization Test for *Pseudomonas aeruginosa*



APIE20 for *Pseudomonas aeruginosa*

Bench Scale Bioreactor System