To Study the Effect of Organic Matter on Biodegradation of Thiophanate-Methyl

DISSERTATION -- REPORT

Submitted in Partial Fulfillment of the Requirement for the Award of the Degree of Master of Science in Zoology

BY

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CERTIFICATE

This is to certify that Premlata Rai (11511203), have completed dissertation report entitled "The effect Organic matter on Microbial Degradation of Thiophanate –Methyl " under my guidance and supervision .To the best of my knowledge, the present work is the result of my original investigation and study. No part of their report has ever been submitted for any other degree at any university.

The dissertation is fit for submission and the partial fulfillment of the conditions for the award of Master of Science in Zoology.

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DECLARATION

I hereby declare that the project work entitled "The effect of Organic matter on Microbial Degradation of Thiophanate –Methyl" is an authentication record of my work. The work has been carried out at School of Bioengineering and Biosciences, Lovely Professional University, Phagwara, Punjab, India under the guidance of Dr. Joginder Singh Panwar (Associate Professor), Department of Microbiology, Lovely Professional University for the award of the degree Master of Science in Zoology.

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LISTS OF ABBREVIATIONS

1	FAO	Food and Agricultural Organization of United Nations
2	UNEP	United Nations Environment Programme
3	DDT	Dichlorodiphenyltrichloroethane
4	ECG	Electrocardiogram
5	LDH	Lactate Dehydrogenase
6	ChE	Choline Esterase
7	MFO	Mixed Function Oxidases
8	GST	Glutathion S- Transferase
9	PGPR	Plant Growth Promoting Rhizobacteria
10	NO_2	Nitrogen oxide
11	NADPH	Nicotinamide Adenine Dinucleotide Phosphate
12	AchE	Acetylcholine Esterase
13	GC	Gas Chromatography
14	MS	Mass spectrometer
15	TDS	Thiophante-methyl Degrading Strain
16	MP	Methyl-Parathion
17	rRNA	Ribosomal Ribonucleic Acid
18	MBC	Methyl-2 benzimidazole carbamate
19	HPLC	High Performance Liquid Chromatography
20	TM	Thiophanate- methyl
21.	ARD	Acute Reference Dose
22	PPM	Parts Per Million
23	ISO	International Organization of Standardization
24	NOAEL	No Observed Effect Level
25	ADI	Acceptable Daily Intake
26	LD	Lethal Dose
27	LC	Lethal Concentration
28	EPA	Environmental Protection Agency

29	TSH	Thyroid Stimulating Hormone
30	OECD	Organization of Economic Cooperation and Development
31	mg	Milligram
32	Kg	Kilogram
33	µg	Microgram
34	µl	Microlitre
35	bw	Body Weight
36	Hrs.	Hours
37	Conc.	Concentration
38	ml	Millilitre
39	l	Litre
40	LB	Luria Broth
41	g	Gram
42	OD	Optical Density
42	IAA	Indole Acetic Acid
43	nm	Nanometer

CHAPTER-1

INTRODUCTION

INTRODUCTION:

Pesticide is a substance which is used to poison weeds, insects, molds and rodents. Pesticides involve the use of highly toxic compounds. It is grouped into two categories i.e. inorganic and organic. Inorganic pesticides include arsenic, copper, mercury compound and they are highly neurotoxic and have longer persistence in environment. Natural organic pesticides are mainly the plants extracts including nicotine, nicotinoid alkaloids and rotenone and they are also toxic to humans and various other life forms. The world population increasing rapidly and to fulfill the food demand of such a huge population, synthetic pesticides have been popular among the farmers because of their widespread availability, efficacy and economic returns but at a huge environmental costs and health. In recent years, Pesticides are toxic to living organisms as it can accumulate in soil, ground water and can also pollute the air. It not only harm the agricultural lands but also to beneficial microbes which naturally maintain the health of soil and also to plants by reducing the concentration of essential nutrients like nitrogen and phosphorous.

Pesticides have both acute and chronic impact on human health; it damages skin, eyes, nerves, endocrine system, and reproductive system and causes many types of cancer. A pesticide has the properties like solubility, volatilization, adsorption and degradation. Pesticides can easily dissolve in water and move downward to soil and then to ground water and it can also lost easily in the atmosphere due to its high vapour pressure. It also has adsorption property due to which it has high affinity towards soil and it depends on chemical properties of pesticides, type of soil and organic matters present in the soil and it has also the degradation or breakdown property which involves different processes like photolysis, hydrolysis and oxidation. The effect of pesticides can be reduce by using various methods which includes physical treatments like adsorption and percolators filters, chemical treatments which involve the use of hydroxyl radical, photo catalysis with TiO2 and alkaline hydrolysis. All these methods require expensive technology, proper conditions for the experiments to be performed and it also involves the production of secondary pollutants.

To reduce the environmental pesticides concentration there is another developing approach which is cost effective as well as eco-friendly that is bioremediation. It is a process in which pesticides are biologically broken down into less toxic forms. This involves the use of microorganisms such as bacteria, algae and fungi for the detoxification process. It depends on various factors like the existence of pesticides degrading microbial population and the environmental factors like the presence of oxygen, temperature and pH. The availability of pesticides, physiological status and survival of microbes also determines the rate of degradation. This technology involves various approaches like identification and analysis of problem, nature and its degree of effects on environment and health. Due to expensive and ineffective waste treatment technology the accumulation of pesticides leading to increase in soil and water toxicity. Bioremediation involves the use of various microbes for the detoxification purposes. Degradation process varies among different species and the targeted pesticides.

These microbes are able to produce various enzymes like oxygenases, hydroxylases, hydrolases and isomerases which play important role in the degradation processes. These enzymes have the potential to degrade and detoxify the accumulated pollutants in the environment. Biodegradation is the most effective technique to eliminate the soil and water pollution because it involves fewer expenses, site disruption is minimum, and it eliminates the pollutants permanently without producing any secondary pollutants. Pesticides are used for controlling unwanted plants, insects, and rodents etc. which cause damage and economic loss by interfering with the agricultural production, its processing

and storage. It is also defined by FAO in collaboration with UNEP (1990) as chemicals which are used to minimize the attacks of several pests on crops, animals and human beings.

Pesticides include compounds like insecticides, fungicides, rodenticides, nematicides, herbicides etc. Various synthetic pesticides like organophosphate (1960s), carbamates (1970s), pyrethroids (1980s), herbicides and fungicides (1970-1980s) were introduced to control the pests and increase the productions.

It not only affect to targeted species but also to non-targeted species. In 1952 in Calcutta the manufacturing of pesticides started and now in Asia, India has 2nd rank after the china in pesticides production. No doubt the use of pesticides has enhance economic potential by increasing the agricultural outputs but it has also resulted in contamination of soil and water which adversely affects the health of various life forms . It easily contaminates the air, water and soil if it is not treated properly.

PESTICIDES IMPACT ON:

SOIL:

The properties like solubility, soil adsorption constant, partition coefficient and its half-life in soil determines the persistivity , mobility and the production of transformation products of pesticides.

Pesticides can be categorized into polar which includes herbicides, carbamates, fungicides and some organophosphorous and non-polar which includes Organochlorine, DDT, Endosulfan, aldrin, heptachlor, lindane etc.

Properties like the organic contents and pH of soil and the presence of charged ions in pesticides play important role in accumulation, more the organic content in soil and the presence of positively charged ions in pesticides more is the adsorption and the adsorption also increases with the decrease in pH of soil for ionizable pesticides. Heavy accumulation of pesticides in soil leads to depletion of beneficial microorganisms which hold the nutrient and in turn soil fertility degradation.

WATER:

Pesticides can easily reach to ground water through run off. More than 90 percent of water and fish samples have been found contaminated with more than one pesticide. During one survey in India, 58% of drinking water samples in Bhopal were found to be contaminated with different pesticides. If the ground water once gets contaminated, it becomes very expensive and difficult to treat.

Contaminated water may affect the aquatic life forms. Accumulation of herbicides in water can cause the death of aquatic plants. Increased concentration of pesticides may decrease the oxygen concentration which may affect the population of fishes. Several cases of Dolphin poisoning has been reported throughout the world. It is also toxic to marine invertebrates. It also reduces the growth of algae which is an important organism of food chain.

PLANTS:

Heavy use of pesticides affects the beneficial microorganisms which play important role in retaining soil fertility. Microorganisms play important role in fixing atmospheric nitrogen into nitrite which plants uses. The growth and activity of free-living nitrogen-fixing bacteria in soil gets reduces by use of glyphosate and 2, 4-D also lead to decrease of nitrogen fixation by the bacteria and thus reduces the growth and activity of nitrogen-fixing blue-green algae and thus leads to inhibition of the conversion of ammonia into nitrates by soil bacteria.

Mycorrhiza can also be affected by the herbicides and thus the nutrient uptakes by plants root are also affected and this can lead to decline of crop yields. Pesticides can also kill the pollinator insects e.g. honeybees and thus decreases plants pollination and its reproduction rate.

BIRDS:

Pesticides can also affects insects, rodents, earthworms and seeds present in soil and thus affect the population of birds because of its dependence on these organisms and seeds for feeding. A very common rodenticide named Brodifacoum which is highly toxic to birds, as it possesses a secondary poisoning which affects rodents feeding birds.

The multiple times exposure to herbicides lead to decrease of reproductive rates in birds by causing cracks in eggs as well as sterility. It also affects the population of birds by poisoning their habitats.

HUMAN:

Due to pesticides poisoning worldwide death and various chronic diseases have been observed. Workers are at higher risks which are involved in formulation or production of pesticides. The persons who are involved in pesticides industries, indicating cardio toxic effects of pesticides, and change in the level of their ECG, LDH, and cholinesterase have been noticed.

Pesticides are potent endocrine disruptors, mimics the natural hormones, lead to immune suppression, mental retardation, hormonal disruptors, cause sterility and cancers. Malathion, parathion, DDT, lindane lead to headache, nausea, tiredness, skin and eyes irritation and also leads to cardiorespiratory, gastrointestinal, neurological symptoms with low ChE activity.

To determine the pesticide residues in the food samples, programs 'Monitoring of Pesticide Residues in Products of Plant Origin in the European Union' was established in the European Union in1996 under which several different pesticides were analyzed in fruits and vegetables samples.

In India in 1958, the Kerala was the first state where pesticide poisoning was reported. The best analysis of human exposure and the risk is to measure the chemical contents in total diet and then the level of risk can be analyze by comparing with toxicologically acceptable intake levels of chemicals.

CLASSIFICATION OF PESTICIDES:

Pesticides can be classified based on:

- Targeted pest's species.
- Chemical composition.
- Mode of action.
- Activity spectrum.
- Toxicity level.
- Mode of formulation.

1. TARGETED SPECIES:

In this classification of pesticides are done on the basis of their targeted species.

- Insects-Insecticides
- Rodents-Rodenticides
- Herbs-herbicides
- Fungi-Fungicides
- Bacteria-Bactericides
- Mites-Miticides
- Virus-virucides
- Algae -Algaecides
- Mollusca-Molluscides
- Birds-Avicides
- Arachinids- Acricides.

2. CHEMICAL COMPOSITION:

These are also classified based on their chemical nature and it is very important in the research work because it provide the information of its application, rate of application and about the precautions which are necessary to be taken into account.

Table-1: Groups of Pesticide

GROUPS	CHARACTERISTICS
1.Organochlorines	• Organic compounds with 4-5 chlorine atoms. • 1st synthetic pesticides to be used. • Widely used as an insecticides. • High persistivity in environment. • Resistance to chemical and microbial degradation. • Act as nervous system disruptors. Examples- DDT, lindane, Endosulfan, aldrin, dieldrin and chlordane.
2.Organophosphorus	• Contain phosphate group. • More toxic to vertebrates and invertebrates. • Inhibitor of Acetylcholinesterase. • Cause failure to nerve impulses. • Persistence is low in environment. • Degradable. Example- parathion, Malathion, diaznon and glyphosate.
3.Carbamates	• Derivatives of carbonic acid. • Inhibitor of cholinesterase • Specific and reversible Example- carbaryl, carbofuran and aminocarb.
4.Pyrethroids	• Analogue of pyrethrins. • Low mammalian toxicity. • It is formed by modifying the structure of pyrethrin by the addition of a biphenoxy moiety and replacing some hydrogen by halogen. Example- permethrin, cypermethrin and deltamethrin.

3. MODE OF ACTION:

These are classified based on their way actions to eliminate the pests. It is divided into classes i.e. Systemic and non-systemic.

Table-2: Classification of pesticide based on mode of action

PESTICIDES GROUPS	CHARACTERISTICS
1.Systemic	• Penetrate through the tissues. • Can move through vascular system. Examples- 2, 4-D and glyphosate.
2.Non –systemic	• Do not penetrate through the tissues. • Can't move through the vascular system. • Make contact with the targeted the pests. Examples- paraquat and diquat dibromide.

4. ACTIVITY SPECTRUM:

Classification is done mainly into two groups i.e. broad and selective.

Table -3: Classification of pesticide based on activity spectrum

PESTICIDE GROUPS	CHARACTERISTICS
1. Broad	• Kills wide range of pests. • Non-selective. • Lethal to various non-targeted organisms. Examples- chlorpyrifos and chlordane
2. Selective	• Kills specific pests. • Selective in nature. • Non-lethal to other organisms Examples- herbicide 2,4-D.

5. TOXICITY LEVEL:

In this classification is done on the basis of their potential to harm various life forms and it is divided into five groups.

Table-4: Classification of pesticide based on toxicity level

PESTICIDES GROUPS	CHARACTERISTICS
1.Class Ia	Extremely toxic
2.Class Ib	Highly toxic
3.Class II	Moderately toxic.
4 Class III	Slightly Toxic.
5. Class IV	Non-toxic in normal use.

6. MODE OF FORMULATION:

Table-5: Classification of pesticide based on formulation

PESTICIDES GROUPS	CHARACTERISTICS
Dusts	Applied in dry form not by mixing in water.
Granules	Made by adding active ingredient with clay.
Fumigants	Gaseous, packed under pressure and stored in liquid forms.
Baits	Mixture of active ingredient with food bases.
Wettable powder	Suspension of fine particles with water.
Emulifiable concentrates	Fine suspension of oil droplets in water.

PROPERTIES:

SOLUBILITY: Pesticides which are readily dissolve in water they move downward to soil and then to groundwater.

VOLATILIZATION: Pesticides which have higher vapor pressure they easily lost in the atmosphere.

ADSORPTION: It refers to the attraction between the soil and pesticides and it is determined by chemical nature of pesticides, soil and organic matters present in soil.

DEGRADATION: It refers to the breakdown of pesticides. It involves different processes photolysis, hydrolysis and oxidation. Half-life estimates the measure of its degradation and it depends on physical, chemical and biological properties of soil being tested.

HARMFUL EFFECTS OF PESTICIDES:

Cause various health and environmental problems like

1. It causes various problems such as cardiopulmonary diseases, neurological disorders, dermal diseases, abnormality in fetus, miscarriages and decrease in sperm production.

2. It adversely harms various important organisms and it may also lead to their extinction

CHAPTER -2
SCOPE OF THE STUDY

SCOPE OF THE STUDY

The goal of the study is to collect soil sample from pesticides contaminated sites and to get the most efficient strains of Thiophante-methyl degrading bacteria. Single bacterium may not degrade all the pesticides present in soil. Bacterial consortium has the most powerful biodegradative potential. By introduction of these pesticides degrading bacteria we can minimize or control the environmental pollution naturally without causing any harm to other species or producing any secondary pollutant products. Bacteria are able to degrade the pesticides because it has enzymes which cause the degradation of organic compounds and thus helps in metabolic processes. The end product of the biodegradation is carbon dioxide which is used as an energy source by the bacteria and this process of conversion is known as mineralization.

The aim of the study is not only to get the most efficient stains of Thiophanate-methyl degrading bacteria but also to check its degrading efficacy in the presence of organic matter and its functional attributes. Soil has organic matter which consists of plants and animal residues which act as carbon source of soil. Some organic matter which is not mineralized and change into stable organic matter humus and it act as an adsorbent of pesticides in soil. Organic matter can either interfere with microbial degradation of pesticides by increasing its adsorption or it can enhance its degradation activity by Cometabolism. Several strains of soil bacteria which are found in association with the rhizosphere of plants have the ability to stimulate plant growth. Rhizosphere bacteria promote plant growth directly or indirectly by synthesizing certain substances which are secondary metabolites or by enhancing the uptake of nutrients from the soil .This processes are like solubilization of phosphorous, sequestering of iron and production of IAA. Bacteria that are associated with the plant rhizosphere and stimulate the plants growth by any process are referred to as plant growth promoting rhizobacteria (PGPR). Inoculation of most efficient TM degrading bacteria in agricultural field will not only control or eliminate the environmental pollution but also promote the production of crops because PGPR,s are known to control the plant disease by producing secondary compounds or enhance the nutrient uptake from the environment.

CHAPTER-3
OBJECTIVE

- OBJECTIVE

The aim of the study is to obtain the most efficient strains of pesticide degrading bacteria and to use the culture for the degradation of Thiophanate methyl. The study includes following objectives:

1. To isolate, screen and identify the most efficient Thiophanate methyl degrading bacteria from collected soil sample by enrichment culture technique.

2. To check the degradation efficacy of isolates strains with respect to thiophanate methyl supplemented with organic matter.

3. To check the comparative effect of organic matter and thiophanate methyl on functional attributes of the isolated strains.

CHAPTER -4
REVIEW
OF
LITERATURE

REVIEW OF LITERATURE

Most pesticides have adverse effect on various flora fauna when they accumulate in the environment and their intensity of toxicity varies according to the characteristics of soil, pesticides, dose of pesticides, presence of degrading microbes in the soil and its persistence in the environment, longer will be its persistence ,more will be the environmental impact. Retention of a pesticide in the environment depends on the several physico- chemical properties of soil or the lack of biodegrading microbes. Light, heat and humidity can reduce of some pesticides by either volatilization or degradation. Degradation of pesticides by various biodegrading microbes can be helpful in reducing the environmental pollution and this type of approach is known as bioremediation.

For the bioremediation purposes bacteria, fungi. Algae, plants etc. can be used. Bioremediation is referring to a naturally occurring technique which is used for the elimination of environmental pollutants and the organisms which are capable of degrading the pollutants are known as bioremediators. The concept of biotransformation and bioremediation are closely related. In the process of biodegradation there is an involvement of microbes capable of degrading these pollutants by modifying their chemical structure and thus decreasing the toxicity level and biotransformation involves the reduction of pollutants either by modification or by translocation, it is generally done when biodegradation couldn't occur. Microorganisms which can tolerate the pesticides toxicity are the most suitable bio degrader because pesticides exerts evolutionary pressure on them and only those organisms will survive which are tolerant and this evolutionary pressure selects only few of them as a bio mediator .

It is the biotransformation process in which complex compounds are broken down into simpler or smaller ones by the microorganism and when this process is known as mineralization. The bio degraders perform this activity by the enzymatic action and it is based on two processes growth and metabolism. In growth, an organic pollutant supplements are used as a main source of carbon and energy. In the process of Cometabolism the breakdown organic compounds are done in the presence additional supplements which are used as a primary source of energy.

Biodegradation process varies but their final product is carbon dioxide. Biodegradation processes could take place both aerobically and anaerobically. There are number of examples of pesticides degrading microbes out of them Pseudomonas is the most efficient bacterial genus for degradation and its activity depends on the attachment time with the compound. Bacterium Rhodococcus species degrade triazines to nitrate. Microorganisms have enzymes which involved in hydrolysis.

Bioremediation processes divided into three categories:

- Natural attenuation- Native microbes in the absence of any human augmentation are used to reduce the pollutants.
- Bio stimulation- Additional nutrients and oxygen are given to the systems to increase the biodegradation processes.
- Bio- augmentation- The additional microbes are supplemented in the system which are more efficient than native flora in degrading the target contaminant. Along with the genetic efficiency various factors like temperature, pH, and available nitrogen and phosphorus sources also determine the rate and the level of degradation. Single bacterium may not have the enzymatic ability to degrade all the organic compounds in a polluted soil. Mixed microbes have the most powerful biodegradative potential than a single microbe. Mainly three enzymes which are

involve in pesticides degradation i.e. Hydrolases, estrases, the mixed function (MFO) in the first metabolism step and the glutathione S-Transferases (GST) in the second metabolism step. Different enzymes catalyze different reactions which includes hydrolysis, oxidation, addition of an oxygen to a double bound, oxidation of an amino group (NH_2) to a nitro group, addition of a hydroxyl group to a benzene ring, dehalogenation, reduction of a nitro group (NO_2) to an amino group, replacement of a sulfur with an oxygen, metabolism of side chains, ring cleavage. Biodegradation depends on microbial metabolic capability to detoxify or to change the pollutant molecule. Metabolism of pesticides involves three steps:

Initial properties of a parent compound are

1. Conversion by hydrolysis, reduction or oxidation processes and giving rise to a compound which is less harmful.

2. Joining of a pesticide to amino acid to increase solubility or to make it less toxic.

3. Transformation of step -2 to metabolites into secondary conjugates(less toxic).

NATURAL ATTENUATION

It is the process of reduction of pollutants from the environment through biological processes and it includes processes like biotransformation and biodegradation which is carried out by microorganisms. Under suitable conditions they perform the chemical reactions that lead to conversion of toxic substances into less toxic and it occurs in most of the polluted areas. If the suitable conditions are not present this process will not occur and if it is slow then it is enhanced by using some other processes like bio augmentation and bio stimulation.

BIOSTIMULATION

It is the process in which biotransformation of soil contaminants are enhanced by supplementing nutrients, minerals, electron acceptor/ donors. There are many examples of bio stimulation in which Trichloroethene and perchloroethene was completely converted into ethane by microorganisms by adding lactate.

BIOAUGMENTATION

It involves the addition of specific strains of pollutants degrading microbes which improve the capacity of soil to remove its contaminants. Bio augmentation approach can be applied when the bio stimulation and bio attenuation have failed .The combination of bio augmentation and bio stimulation might be a useful technique to enhance the process of bioremediation

In these processes microbes produces intracellular as well as extracellular enzymes such as hydrolytic enzymes, peroxidases, oxidases etc.

MAJOR ENZYMES IN BIODEGRADATION:

Table-6: Major enzymes involved in biodegradation

ENZYMES	CHARACTERISTICS
Hydrolases	Wide group of enzymes. Causes hydrolysis of pesticides. Function in the absence of redox cofactors. Example- degradation pathway of carbofuran.
Phosphotriestrases	Most important group of enzymes. Hydrolyze organophosphorus pesticides. Reduces the OPs ability to inhibit AchE. Encoded by the opd (Organophosphate degrading) gene. Hydrolyze phosphoesters bonds. Homodimeric protein.
Esterases	Hydrolysis of carboxylic esters, amides, phosphate esters etc. Example- degradation pathway of carbamates, organophosphates,(parathion, paraoxon and pyrethroids. Highly variable. Protect the acetylcholinesterase inhibition.
Oxidoreductases	Broad group of enzymes. Transfer one electron molecule to another. Require cofactors as an electron donor/acceptor. Reduction of oxygen to water or hydrogen peroxide. Example- endosulfan degradation pathway.
Mixed function oxidase	An atom of one molecule of oxygen is incorporated into the substrate, while the other is reduced to water. Require NADPH and oxygen. Combination of cytochrome P450 and NADPH-cytochrome P450 reductase. Non-specific. Metabolize organophosphates, carbamates, pyrethroids, DDT.
Glutathione S-Transferases (GST)	Consists of different group of enzymes. Cause the joining of hydrophobic components with the tripeptide glutathione.

FACTORS AFFECTING BIODEGRADATION PROCESS

Microorganisms play important role in this process and their degrading capabilities depends on factor which includes both the chemical nature and the concentration of pollutants, their availability to microorganisms, and the physico-chemical properties of the environment. The rate of degradation is influenced by several factors which are related to degrading microbes, their nutrition requirements and also related to the environment. Biological factors are associated with the metabolic processes of microorganisms. Biotic factors include:

- Enzymatic activities.
- Proliferation processes.

Enzymatic activities of the degrading microbes can be directly inhibited due to limited carbon sources and predation of microbes can affect its proliferation rate. The rate of degradation also depends on the population of organisms present to degrade the pollutants as well as the quantity of microbe's enzymes also affect the degradation process. Function of specific enzymes and its affinity towards the pollutants also affects the rate of degradation processes. Appropriate quantities of nutrients and oxygen must be available for the unrestricted growth of microbes and also various other factors which include pH, temperature, moisture and organic matter also affect the degradation processes. The biological enzymes which are involved in the degradation processes, they perform their functions only at an optimum temperature and decrease in the rate of biodegradation is observed at 10°C decrease in temperature. The pH of 6.5 to 8.5 is generally suitable for biodegradation and the rate of contaminant metabolism is also affected by the moisture. Environmental factors include type of soil and pollutants which affect its adsorption. Sedimentation of grains and more water content in soil lead to decrease in the transmission of gases in soil. The Soil organic matters are crop and microbial residues which contribute few percentage of dried soil mass and it leads to increase rate of adsorption of pesticides.

Various studies have been done on the microbial degradation of different group of pesticides and many different bacterial strains having the degrading efficiency have been identified.

Chlorpyrifos is one of the most widely used pesticide which was developed by the U.S. chemical company Dow Agro Sciences (1965) and is widely used in cultivation of various crops like rice, wheat , sugarcane vegetables ,flowers etc. It acts by the mode of contact and stomach poison of pests. From Chinese soil eleven chlorpyrifos degrading bacteria were isolated, studied by both microbiologically and molecular methods and it was found that different strains of bacteria had different degrading efficiency. Out of those 11 isolated bacterial strains only one strain (Bacillus cereus) was selected for further analysis in different conditions which included temperature, pH and concentration (Liu, Z.*et.al. 2011*).

Malathion which is an organophosphorous, itself is of low toxicity but when it enter into the body through ingestion, inhalation or absorption metabolize into malaoxan which is highly toxic. It kills insects by inhibiting AChE which play important role in transmitting nerve impulses which lead to impairment of coordination, paralysis and finally death. (Kumari, A. *et.al.2012*) isolated unknown bacteria from Malathion contaminated soils after the morphological and biochemical analysis it was found that the bacteria belong to Bacillus group and further they studied the degrading efficiency of isolated bacteria. During analysis increased level of phosphorous content was observed due to degradation of Malathion.

Endosulfan an organochlorine is a mixture of two isomers alpha and beta –Endosulfan also acts by contact and stomach poison used to control various pests of cereals, vegetables and fruits. Its residues have been found in water, soil and food samples. Microbial degradation has been studied and Bacillus species of bacteria capable of degrading Endosulfan have been identified by analysis. (Shivaramaiah, H. M., & Kennedy, I. R, 2006) isolated Endosulfan degrading bacteria from cotton growing soil which was able to degrade Endosulfan into Endosulfate which was analyzed by GC. Microbial degradation analysis was done for 6 days, 50% degradation was observed in with an increase in bacterial growth. This degradation process occurred through oxidation process.

Methyl-Parathion degrading 14 bacterial strain were isolated from agricultural soil of Visakhapatnam by enrichment processes in media containing MP and then isolates were purified .Bacteria utilized MP as a carbon source and growth was observed and maximum growth was observed after 48hrs. The most three efficient isolates were selected and their growth was observed by optical density at 600nm. These isolates Pseudomonas were found to tolerate concentration of 3800micro gram /ml and another isolate Pseudomonas aeruginosa species was able to degrade 1920 micro gram/ml MP after 48 hrs. of incubation (Begum, S. & Arundhati, A. 2016).

Carbofuran is a neurotoxic pesticide and a powerful endocrine disruptor lead to reproductive problems. It is an insecticide of N-methyl carbamates family and is extensively used which causes the environmental pollution. Soil microbes are also able to degrade the Carbofuran. (Nisha P. *et.al.2016*) isolated bacteria from the pretreated plantain field which included Bacillus species, Micrococci species and Klebsiella species and degradation of Carbofuran was studied at different concentrations and they showed degradation at all concentrations.

Bifenthrin is a pyrethroid which is widely used as an insecticide has a harmful effect on various life forms. (Pandey *et.al*. 2014) studied about the microbial degradation of Bifenthrin and their aim of the research was to isolate and characterize the efficient Bifenthrin degrading bacterial strains.

PGS-4, a bacterial strain was isolated from the pesticide industrial sewage and analysis of growth was done at the higher concentration of Bifenthrin (800mg/litre) at the wide range pH and temperature. This isolated strain utilized the Bifenthrin as a sole source of carbon and 16s rRNA gene sequencing method showed 99% similarity with that *Bacillus cibi.*

Cypermethrin is also a pyrethroid which is also harmful for the environment. (Muradov.*et.al*.2012) isolated five bacterial isolates *Pseudomonas aeruginosa, P.fluorescens, Bacillus licheniformis, Alcaligenes sp. and Corynebacterium sp.*by enrichment technique using different concentration of Cypermethrin in the medium. Isolates were inoculated with and without Cypermethrin, concentration and its intermediate of degradation product was analyzed by GC-MS and it was observed that *P.aeruginosa* culture degraded to greater extent while others degraded to lesser extent. This research suggested that *Psedomonas sp.*and *Bacillus licheniformis* were able to utilize Cypermethrin.

(Gupta .S. *et.al*.2014) isolated rhizobacteria and selected plant growth bacteria by performing biochemical tests like Nitrogen fixation, phosphate solubilization and Indole acetic acid production. They isolated 30 bacteria out of which 10 isolates showed positive growth promoting activity for nitrogen fixation, one showed positive result for phosphate solubilization and one showed IAA production test.

Fungicides are also used continuously and extensively, its use has created environmental problems because of its toxicity towards the non-target organisms. One of the most widely used fungicides is thiophanate methyl which is a broad-spectrum fungicide which was first introduced in the United States in 1973. It is labeled and is mostly used to control soil borne diseases of plants because it has therapeutic properties in plant tissues. It gets converted into methyl 2-benzimidazolecarbamate on surface and tissues of plants. It is a systemic fungicide therefore it is used as longer interval and is effective at low rates which help in reducing environmental, applicator exposer, saves energy and labor costs. It is one of the cheapest and effective controlling methods of damaging diseases of plants. It is produced and sold under different trade names like Topsin, Cercobin, Fungo and Senator.

Table-7: Properties of Thiophanate -methyl

Common name	Thiophanate-methyl
Chemical name	dimethyl 4, 4- (o – phenylene) bis (3-thioallophante)
Molecular weight	342
Formula	$C_{12}H_{14}N_4O_4S_2$
Melting point	178degree Celsius
Mode of action	Inhibition of respiratory activities
Solubility	Dissolve less in water and n-hexane but moderately dissolve in methanol, acetone and cyclohexane.
Stability	Highly stable in acidic condition but unstable in alkaline conditions.
Transformation pH	Negligible at pH 5.0 Appreciable at pH 8.3 Rapid at pH 8.0
Physical state	Crystalline powder
Color	White
Odor	Faint sulfur odor

In conversion of Thiophanate-methyl into MBC both light and biological activities play important role. It is actively converted by fungi into 2- aminobezimidazole and 5-hydroxy –MBC. It undergoes rapid transformation in soil at pH 7.4 and rate reduces by steam treatment of soil. Its transformation product MBC is stable in soil for 3-12 months.

The activity of TM against fungi is mainly based on the inhibition of nuclear division while undergoing mitosis and it also causes the destruction of cell structure of fungi and thus it inhibit the formation of germ tube of fungi ,development of appressoria and mycelia growth are also blocked *(Roberts.et.al.1998).*

About 90% degradation of TM by various processes occur in 6-18 weeks depending on the different properties of the soil *(Fleeker et.al.1974; Roberts's et.al.1998).*

Many bacterial species capable of degrading benzimidazole groups have been isolated from the soil contaminated with fungicides and their degrading efficiency have been studied in media as well as soil *(Motonaga et.al.1996; Sahin et.al.2000; Nagase.et.al.2006).*

(Cycoń, M., *et.al. 2011*) isolated the bacterial species which are capable of degrading TM and their efficacy was also analyzed. Two isolates Enterobacter species TDS-1and Bacillus species TDS-2 were isolated from pretreated sandy soil and they were able to grow in MSM supplemented with TM

as the main carbon source. Addition of glucose stimulated the bacterial growth but degradation of TM was decreased by 21 % and 27% for TDS1and TDS-2 respectively and this indicated that additional carbon sources lead to decrease in degradation process. A concentration of degradation product was measured by HPLC.

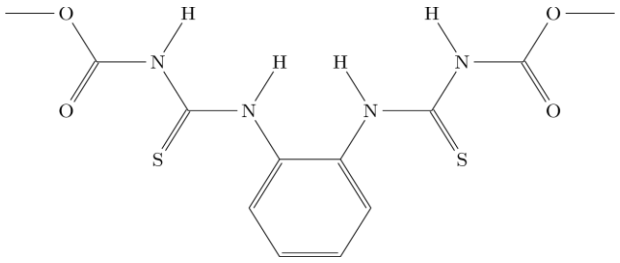


Figure-1 .Chemical structure of thiophanate- methyl

TOXICITY:

Thiophante-methyl is the common name used for dimethyl 4, 4'- (o-phenylene)-bis (3-thioallophante) benzimidazole fungicide which inhibits the synthesis of ß- tubulin approved by International Organization of Standardization (ISO). Because of low acute toxicity on administrating TM orally or dermally, in 1998 JMPR concluded that an acute reference dose (ARD) is not required. Based on the no observed adverse effect level (NOAEL) of 8mg/kg bw per day in the study of three generation of reproductive toxicity in rats and one year study in dogs, an acceptable daily intake (ADI) was established in 1998.

Oral toxicity – Low (LD_{50} >5000mg/kg bw)

Dermal toxicity in rats – Low (LD_{50} > 2000mg/kg bw)

Inhalation toxicity – Moderate (LC_{50} > 1900mg/m^3)

A short term study of toxicity was done in compliance with the test guidelines of the United States Environmental Protection Agency (EPA), 4 male and 4 female beagle dogs were given gelatin capsules of TM (96-55%) of 0,8,40 and 200mg/kg bw/day after 1-2 hrs. of feed of 400g for 1 yr. Several observations which included physical, ophthalmic, body weight, hematology, biochemistry and urine test. No death of rat was observed. It was observed that the body weight of animals was reduced at higher doses of TM as compared to control, decrease in erythrocyte and hemoglobin count was also noticed. There was no effect of TM ophthalmological and urine analysis. Decrease in albumin aminotransferases activity, increase cholesterol level and decrease in albumin globulin ratio, calcium, potassium and phosphorus conc. decrease thyroxin conc.in males but no effect on TSH was observed. Increased in liver weight and thyroid weight was also observed at high dose.

To study the genotoxicity of TM micronucleus formation was done in compliance with the guidelines of Organization for Economic Cooperation and Development (OECD) and United State EPA. To study the effect of TM on genotoxicity five males and five females mice were given or TM

(97.28%) orally in 1% aqueous methylcellulose of doses 0, 500, 1000/2000mg/kg bw/day and control was given (98%) carbendazamin at a dose of 1000mg/kg. The bone marrow smear was taken from all mice at the interval of 24hrs and 48hrs after the treatment of TM and control was taken only at 24hrs. The presence of micronucleus in 2000 immature erythrocytes was observed from one smear of each mouse. The increase in number of micro nucleated immature erythrocytes at both the sampling time was observed in treated mice and decrease in immature erythrocyte was observed in mice sampled at 24hrs.In control the increase of micro nucleated immature erythrocytes and decrease in the proportion of immature erythrocyte was observed. The centromeric staining and size analysis of micronuclei revealed that in control 68% micronuclei had centromere and high number of large micronuclei of size 40.1 units but in TM treated mice an intermediate proportion of centromere having micronuclei 34% and size of micronuclei was 31.9 units. TM can cause aneuploidy but do not cause mutation in genes or aberrations in structure of chromosome.

In a recent two generation study of reproductive toxicity at dose of 2000ppm no effect on fertility or on reproductive performance was observed. The toxicity of TM on development has been found in mice and rabbits. The 500mg/kg bw/day is the NOAEL for developmental toxicity which is based on decreased number of fetuses at 1000mg/kg bw/day but no maternal toxicity at this dose in mice whereas in rats 1000mg/kg bw/day was the developmental toxicity and maternal toxicity was also observed. The 20mg/kg bw/day is the NOAEL for the developmental toxicity was observed based on the increased supernumery thoracic ribs and decreased fetus weight at 50mg/kg bw/day and 10mg/kg bw/day based on reduced feeding consumption and decreased body weight at 20mg/kg bw/day is for maternal toxicity in rabbits.

To study the neurotoxicity of TM which was performed in compliance of EPA and OECD, 10 male and 10 females rats were given TM (99.9%) at a dose of 0,500,1000/2000mg/kg bw/day dissolved in methylcellulose. The viabilities, clinical observance, body weight, feed consumption measure, analysis of functional observational battery and motor activities were noticed. Neurohistological analysis was performed and it was observed that the NOAEL for general toxicity is 500ppm that is 30.3 mg/kg bw /day in males and 34.9 mg/kg bw /day in females which was based on reduced body weight and feed consumption values in female and gain in liver and thyroid weight in both male and female 2500ppm which is the NOAEL for neurotoxicity (149.6 in males and 166.3mg/kg bw/day in females).

CHAPTER-5
MATERIALS
AND
METHODOLOGY

5.1. MATERIALS:

5.1.1. CHEMICALS:

Luria broth, agar, Pikosvaya's agar, kings Medium B base, $FeCl_3$, Per chloric acid, Humic acid, L-tryptophan , Thiophanate –methyl, acetone, Ethyl acetate

5.1.2. INSTRUMENTS:

Autoclave, Orbital shaker, Bacteriological incubator, Spectrophotometer, HPLC/GC system, laminar air flow.

1.1.3. APPARATUS:

Test tubes, petri plates**,** conical flasks, beakers, micropipettes.

5.2METHODOLOGY:

5.2.1. SOIL SAMPLING:

Soil sample was collected from the two different pesticides contaminated agricultural field.

5.2.2 ISOLATION OF BACTERIA:

5.2.2.1 ENRICHMENT PROCEDURE:

4 flasks of 100ml were taken. Each flask was filled with 100 ml of distilled water and 2g of LB was added.1g of four different soil samples and 0.4 g of pesticide was added in each flask. Four flasks containing sample was incubated at 28˚ for 72 hr. on a rotary shaker. After that 1 ml of soil suspension was added to freshly prepared medium and 0.4 g pesticide was also added to it, again it was kept on shaker for 24 hrs.

5.2.2.2 PLATING PROCEDURE:

LB agar media was prepared by adding 3g of LB agar in 150ml of distilled water and along with 7 petriplates were autoclaved for 20 min at 121˚C. After autoclaving 0.4g of thiophanate-methyl was added in medium and then mixed properly. LB agar medium supplemented with thiophanate-methyl was poured on Petri plates and allowed to solidify.100ul of soil suspension was added in 6 plates and then spreaded evenly with the help of spreader and 1 plate was taken as control without soil suspension. Plates were kept in incubator for overnight at 30˚C and then bacterial colonies were observed.

5.2.2.3. SUBCULTURING:

The LB agar media was prepared and along with 7 petriplates was autoclaved. After autoclaving 0.4g of TM was added and mixed. Media 15-20 ml was poured into plates and allowed to solidify. After solidification, single colony was picked up from the primary culture plates and streaked on petriplates and was marked as TM-1, TM-2, TM-3, TM-4, TM-5, TM-6 and 1 plate was taken as control. Plates were incubated for 24 hrs. at 30˚C.

5.2.3. PREPARATION OF BROTH/ INOCULUM:

Six 100ml flasks were taken and filled with 50ml of distilled water and then 1g of LB was added in each conical flask. Flasks were subjected to autoclave. After autoclaving 0.4g of TM was added in each flask. Each flask was inoculated with different isolates with the help of loop. Flasks were kept on shake incubator at 120 rpm and 29˚C for 48hrs for bacterial growth to occur. Control was also kept which was not inoculated with isolate

.TABLE-8: Composition of LB agar

Component	Quantity g/L
Peptone	10g
Yeast extract	5g
Sodium chloride	5g
Agar	12g

5.2.4. DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION OF TM:

For the determination of MIC of TM, 6 petriplates were taken .200ml of flask was taken which filled with 100ml of distilled water and then 2 g of LB broth and 2g of agar was taken and heated . Flask containing LB media and petriplates were subjected to autoclave for 20 min.at 121˚C.After autoclaving media was poured into plates and allowed to solidify. After solidification 100µl of all the 6 isolates were inoculated in 6 different petriplates and uniformly spreaded on plates. Different concentration of TM solutions was prepared which were of 1000ppm, 500ppm and 250ppm. Disk diffusion method was used to determine the MIC by placing disk on inoculated plates dipped in the three different Conc.of TM. Plates were incubated for overnight. Antimicrobial activity of the TM was evaluated by observing the zone of inhibition and isolates were selected for further study.

5.2.5. ANALYSIS OF BACTERIAL GROWTH IN TM WITH/ WITHOUT ORGANIC MATTER

For the analysis of TM degradation 12 flasks of 100 ml were taken. In each flask 50 ml of distilled water and LB media was taken. The solution of TM was prepared which was of 50ppm and 100 ppm concentrations. Two isolates were selected for the analysis TM-1 and TM-4. Four flasks were taken as control without inoculum and 8 flasks were inoculated with selected isolates (1ml).

Table-9: Components of biodegradation analysis of isolates.

FLASKS	COMPONENTS	FLASKS	COMPONENTS
1.	LB+TM(50ppm) control	7.	LB+TM(100ppm) control
2.	LB+TM(50ppm) +TM1 (1ml)	8.	LB+TM(100ppm) +TM1 (1ml)
3.	LB+TM(50ppm) + TM-4 (1ml)	9.	LB+TM(100ppm) + TM-4 (1ml
4.	LB+TM(50ppm) + Humic acid-control	10.	LB+TM(100ppm) + Humic acid-control
5.	LB+TM(50ppm) +TM-1 (1ml) + Humic acid	11.	LB+TM(100ppm) +TM-1 (1ml) + Humic acid
6.	LB+TM(50ppm) +TM-4 (1ml) + Humic acid	12.	LB+TM(100ppm) +TM-4 (1ml) + Humic acid

5.2.6. FUNCTIONAL CHARACTERIZATION:

5.2.6.1. PHOSPHATE SOLUBILIZATION:

The ability of isolates TM-1 and TM-4 to solubilize phosphate was determined by using Pikovskaya's agar.

Material required: Pikovskaya's agar, Bacterial culture,inoculating loop and petriplates.

Procedure: conical flasks of 100 ml were taken. Flasks were filled with 100 ml of distilled water and 3.13g of Pikovskaya's agar was added in each flasks and heated till boiling. Flasks containing media along with 10 petriplates were autoclaved for 20 min. After autoclaving different conc.of TM (0.001,0.025,0.05,0.1ppm) was added in 4 flasks and 1 flask was taken as control without TM. Media from 5 flasks were poured on 10 plates (2 plates from each flask.) and allowed to solidify.After solidification single point inoculation of isolates TM-1 and TM-4 was done (5 plates each of TM-1 and TM-4). Plates were incubated for 1 week on 30˚C. Phosphate solubilization was evaluated by observing zone of clearing and zone of clearence around the colony was scored as positive test for phosphate solubilization

Table-10: Components of phosphate solubilization test

PLATES OF TM-1	CONTENTS	PLATES OF TM-4	CONTENTS
1	Media+TM-1 (Control)	1	Media+TM-1 (Control)
2	Media+TM (0.001)+TM-1	2	Media+TM (0.001)+TM-4
3	Media +TM(0.025)+TM-1	3	Media +TM(0.025)+TM-4
4	Media+TM(0.05)+TM-1	4	Media+TM(0.05)+TM-4
5	Media+TM(0.1)+TM-1	5	Media+TM(0.1)+TM-4

Table-11: Composition of Pikovskaya's Agar

Components	Quantity g/L
Yeast extract	0.500
Dextrose	10.000
Calcium phosphate	5.000
Ammonium sulphate	0.500
Potassium chloride	0.200
Magnesium sulphate	0.100
Manganese sulphate	0.0001
Ferrous sulphate	0.0001

5.2.6.2.SIDEROPHORES PRODUCTION :

The ability of isolates TM-1 and TM-4 was determined by using King'B base media.

Material Required: Bacterial culture, king's B base agar media, $FeCl_3$, inoculation loop.

Procedure: 5 flasks of 100ml were taken. Each flasks were filled with 100ml of distilled water , King Medium B.Base 4.223g and $feCl_3$ (50µg/ml) was added in each flasks. Flasks containing media was then autoclaved for 20mins at 121˚ C. After sterilization of media different conc.(0.001,0025,0.05,0.1) of TM was added in media and one was taken as a control without TM. Media was poured into plates and allowed to solidify.After solidification 100µl culture of isolates TM-1 and TM-4 was spreaded onto plates. Plates were kept on incubator (30˚C) for overnight. Florescence pigment was observed which was formed by isolates indicating siderophores production.

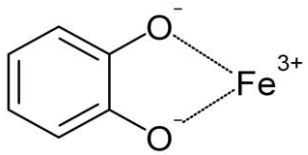


Figure-2. Structure of siderophore

5.2.6.3.INDOLE ACETIC ACID PRODUCTION :

IAA test was performed to determine the ability of isolates TM-1 and TM-4 to produce indole acetic acid. Colorimetric assay was done using salkowski reagent. Appearance of orange colour idicates the production of IAA.

Material Required: LB media, L-tryptophan, Bacteria culture, Salkowsky reagent (0.5M $Fecl_3$ in 35% perchloric acid), spectrophotometer, inoculation loop.

Procedure:

Bacterial isolates TM-1 and TM-4 were cultured in LB medium containing L-tryptophan (50µg/ml) and different concentration of TM (0.001,0.025,0.05,0.1) and control was taken without TM for 72 hrs at 30˚C at 150 rpm on shaker. Tubes containing growth of isolates was centrifuged at 4000 rpm for 12 mint. 2ml of supernatants was taken in another tubes and 1ml of salwoksy reagent was added and kept for 30 min. and then O.D. was measured at 530 nm.

CHAPTER-6
RESULTS
AND
DISCUSSION

RESULTS

A.Isolation of TM degrading bacteria:

After 24hrs of incubation of petriplates a uniform growth of bacterial culture was observed. Bacterial colonies were selected to get the pure culture of bacteria. 6 different types of colonies from the culture were streaked on the LB media plates by quadrant streaking and they were named as TM-1, TM-2, TM-3 TM-4, TM-5 and TM-6.

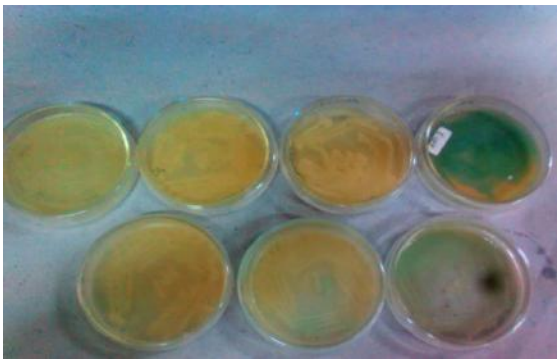


Figure-3: Sub-culture of isolated Bacteria.

Figure-4 Broth of isolated bacteria from the soil

After the 48hrs of incubation of flasks on rotary shaker bacterial growth was observed in flasks by observing change in turbidity of media whereas there was no change in turbidity of control flask.

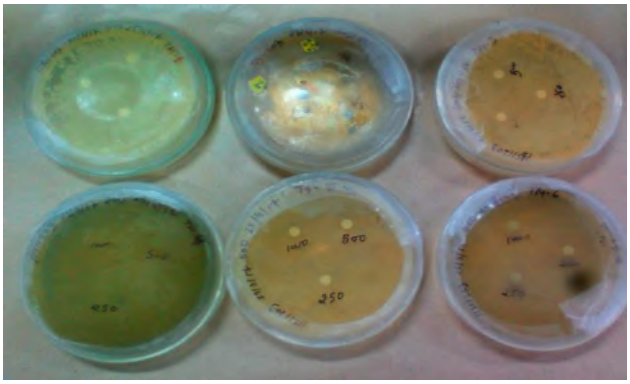


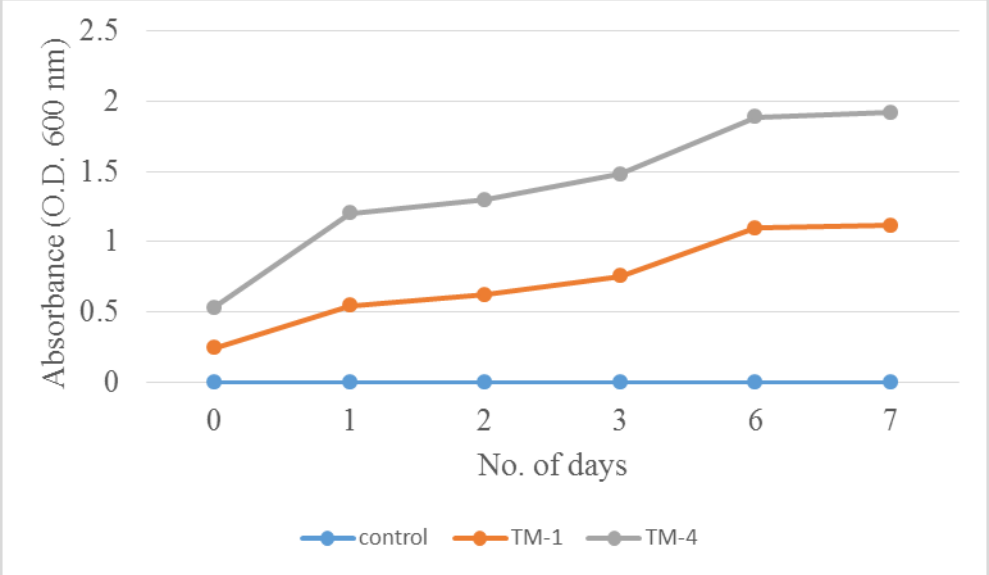
Figure-5 MIC of isolated bacteria

Fig -5 represents the determination of minimum inhibitory concentration of TM. Zone of inhibition of all the isolates were observed at different concentration of TM and two highly resistant to TM isolates were selected for further analysis.

B. Bacterial Growth in TM with and without Humic Acid:

Table-12: O.D of TM-1 and TM-2 growth in 50ppm concentration of TM.

SAMPLES (Conc.50ppm)	Number of days (O.D. 600 nm)					
	0	**1**	**2**	**3**	**6**	**7**
Control MSM+ TM	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
MSM+TM+TM-1	**0.246**	**0.547**	**0.625**	**0.757**	**1.097**	**1.115**
MSM+TM+ TM-4	**0.289**	**0.656**	**0.673**	**0.721**	**0.787**	**0.802**

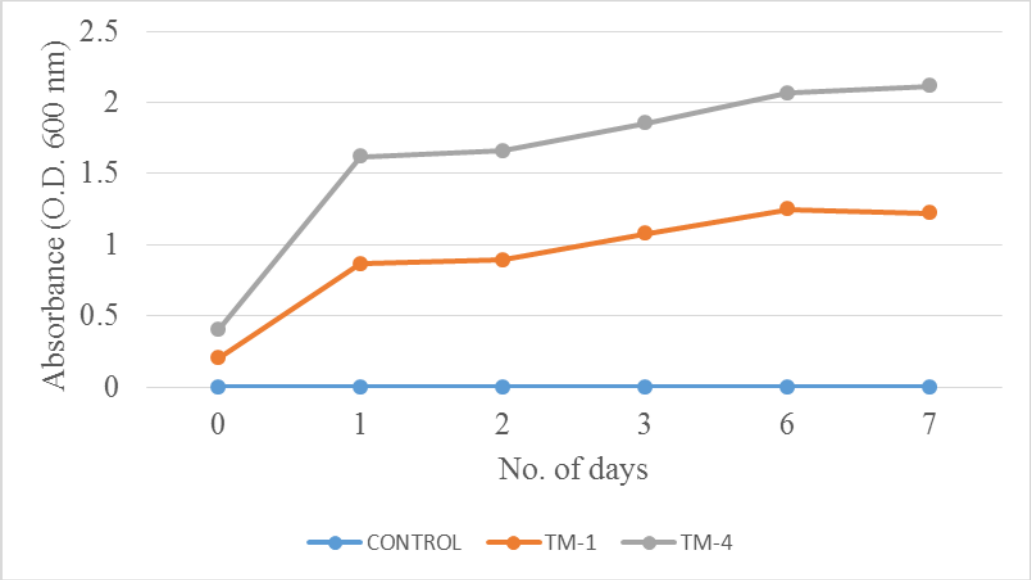


Graph-1 Bacterial growth in 50ppm concentration of TM

Table-12 represents the O.D of isolates TM-1 and TM-4 in 50ppm concentration of TM. It is observed from the value of O.D that both isolates TM-1 and TM-4 growth is continuously increasing from day zero to day 7th. The O.D of TM-1 was observed 0.246 which gradually increased to the value of O.D 1.115 on 7th day whereas the O.D of TM-4 was observed to be 0.289 which reached to the O.D value of 0.802. This analysis suggested the growth of TM-1 was more in 50ppm concentration of TM as compared to TM-4.

Table-13: O.D of isolates TM-1 and TM-4 in 100ppm concentration of TM

SAMPLES (Conc.100ppm)	Number of days (O.D. 600 nm)					
	0	**1**	**2**	**3**	**6**	**7**
Control MSM+TM	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
MSM+TM+TM-1	**0.203**	**0.866**	**0.893**	**1.077**	**1.247**	**1.221**
MSM+TM+TM-4	**0.196**	**0.752**	**0.767**	**0.778**	**0.818**	**0.893**

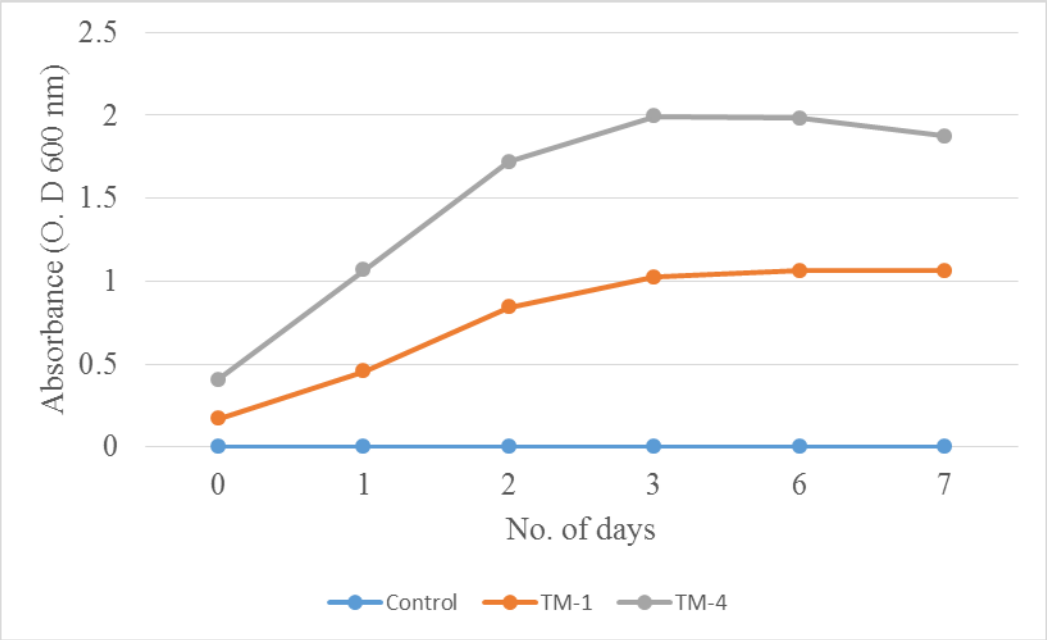


Graph-2 Bacterial growth in 100ppm concentration of TM.

Table-13 represents the O.D of isolates TM-1 and TM-4 in 100ppm concentration of TM. It is observed from the value of O.D that both isolates TM-1 and TM-4 growth is continuously increasing from day zero to day 7th. The O.D of TM-1 was observed 0.203 which gradually increased to the value of O.D 1.221 on 7th day whereas the O.D of TM-4 was observed to be 0.196 which reached to the O.D value of 0.893. This analysis suggested the growth of TM-1 was more also in 100 ppm concentration of TM as compared to TM-4.

Table-14: O.D of isolates TM-1 and TM-4 in 50ppm concentration of tm+ Humic acid

SAMPLES (Conc.50ppm) +Humic Acid	Number of days (O.D.600nm)					
	0	1	2	3	6	7
Control MSM+TM+HA	0.000	0.000	0.000	0.000	0.000	0.000
MSM+TM+HA +TM-1	0.172	0.456	0.844	1.023	1.063	1.063
MSM+TM+HA +TM-4	0.236	0.612	0.876	0.917	0.929	0.811

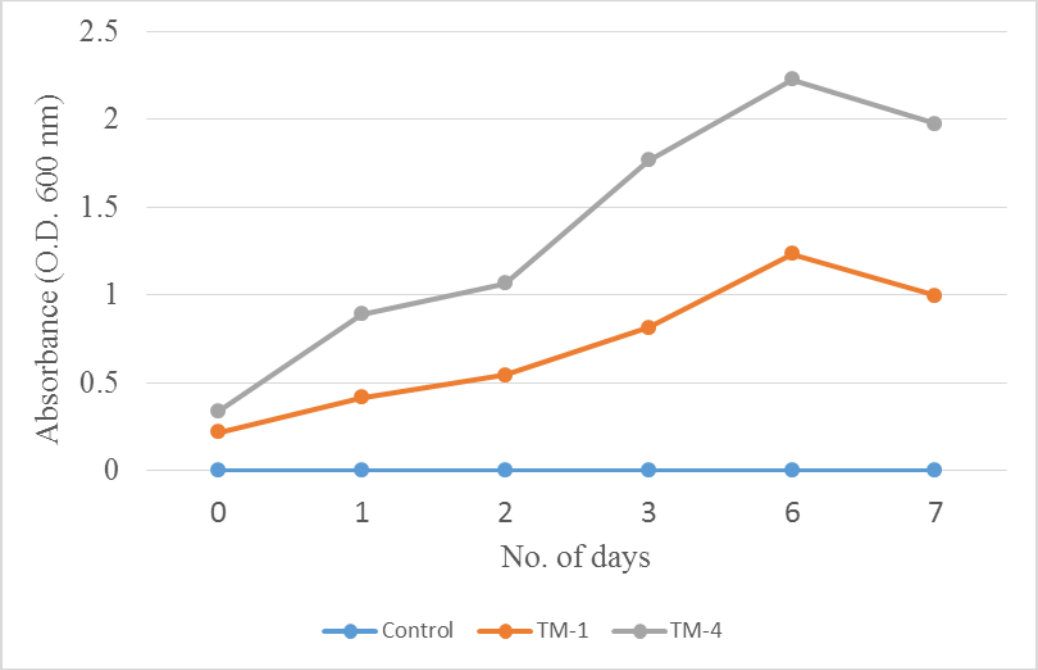


Graph-3 Bacterial growth in 50 ppm concentration of TM+Humic acid

Table-14 represents the O.D of isolates TM-1 and TM-4 in 50ppm concentration of TM and Humic acid. It is observed from the value of O.D that both isolates TM-1 and TM-4 growth is continuously increasing from day zero to day 7th. The O.D of TM-1 was observed 0.172 which gradually increased to the value of O.D 1.063 on 7th day whereas the O.D of TM-4 was observed to be 0.236 which reached to the O.D value of 0.811. This analysis suggested the growth of TM-1 was more in 500 ppm concentration of TM and humic acid as compared to TM-4 and decline in growth of TM-4 was observed on the day 7$^{th.}$

Table-15: O.D of isolates TM-1 and TM-4 in 100ppm concentration of TM+Humic acid.

SAMPLES (Conc.100ppm)+ Humic acid	Number of days (O.D.600nm) DAYS					
	0	1	2	3	6	7
Control MSM+TM+HA	0.000	0.000	0.000	0.000	0.000	0.000
MSM+TM +HA+TM-1	0.218	0.416	0.543	0.813	1.232	0.996
MSM+TM +HA+TM-4	0.213	0.476	0.524	0.952	0.995	0.978



Graph-4 Bacterial growth in 100ppm concentration of TM+Humic acid

Table-15 represents the O.D of isolates TM-1 and TM-4 in 100ppm concentration of TM and Humic acid. It is observed from the value of O.D that both isolates TM-1 and TM-4 growth is continuously increasing from day zero to day 7th. The O.D of TM-1 was observed 0.218 which gradually increased to the value of O.D 1.232 on 6th day and then growth declined to 0.996 whereas the O.D of TM-4 was observed to be 0.213 which reached to the O.D value of 0.995 on 6th day and then it declined to the value of 0.978 on the 7th day of observation.. This analysis suggested the growth of TM-1 was more in 100 ppm concentration of TM and humic acid as compared to TM-4 and declination in growth of both TM-1 and TM-4 was observed on the day 7th.

C. Functional Characterization of TM Degrading Bacteria:

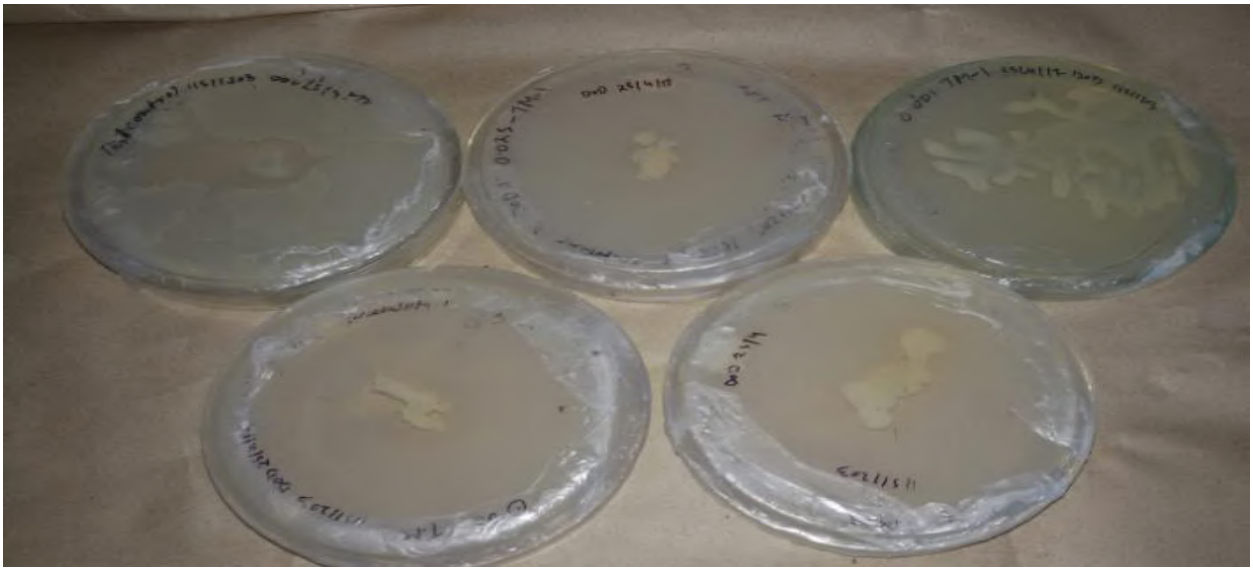


Figure-6: Phosphate solubilization activity by isolate TM-1

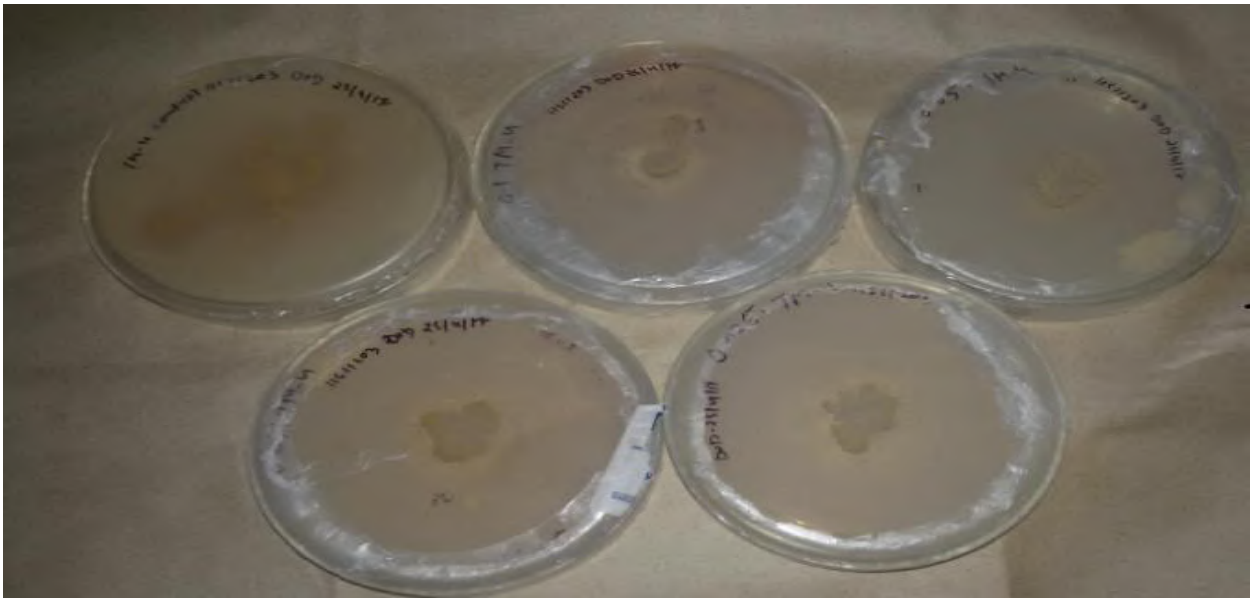


Figure-7: Phosphate solubilization activity by isolate TM-4

Fig-6 and 7 represents the phosphate solubilization activity of isolates TM-1 and TM-4 . Both isolates were found to have the ability of solubilizing phosphate but TM-4 was more efficient in solubilizing phosphate as compared to TM-1 because the zone of clearance was more in case of TM-4.

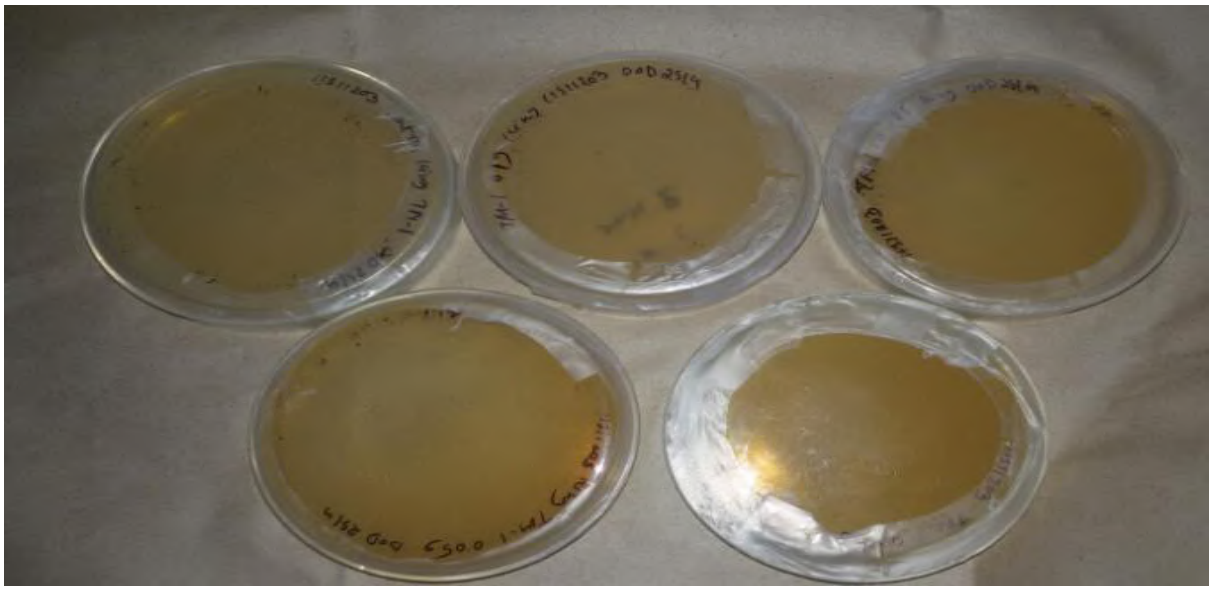


Figure-8 Sidrophore production test of isolate TM-1

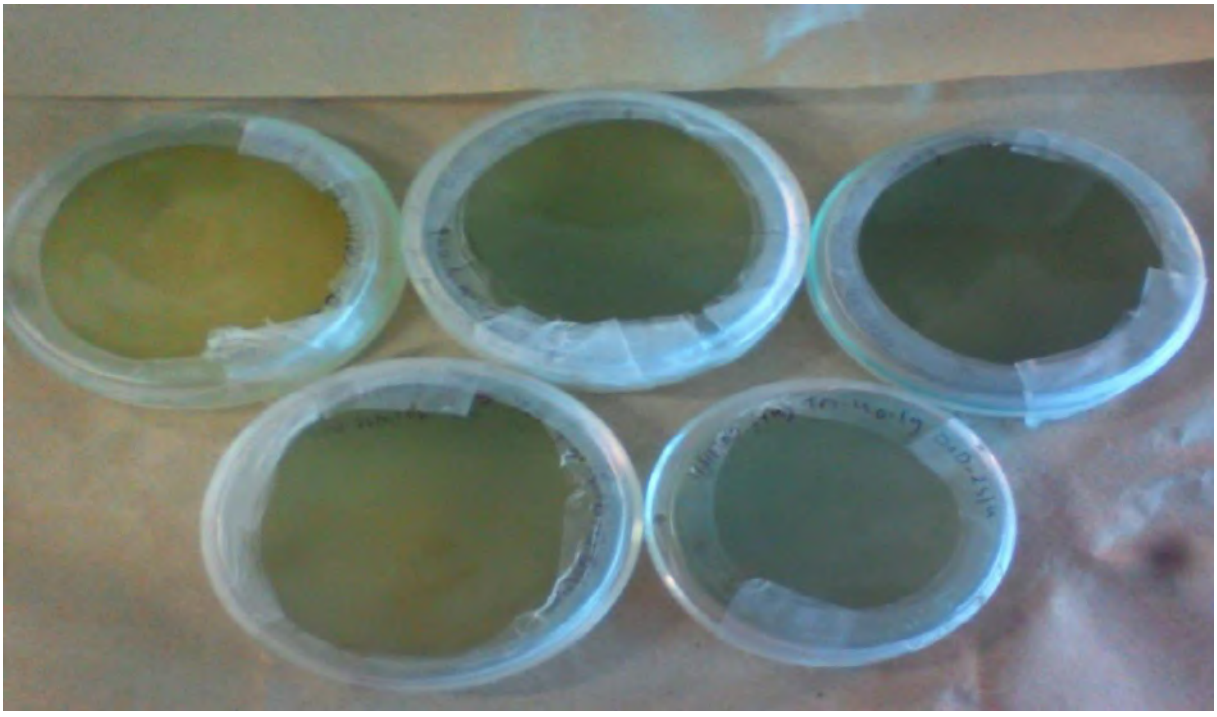


Figure-9 Sidrophore production test of isolate TM-4

Fig.8 and 9 represents the Sidrophore production activity of isolates TM-1 and TM-4. Only TM-4 shown the Sidrophore positive activity whereas TM-1 shown the negative Sidrophore activity**.** Tm-4 produced fluorescent pigment on king's B base media which specify the Sidrophore production.

Figure-9 Culture of isolate TM-1and TM-4 for IAA test .

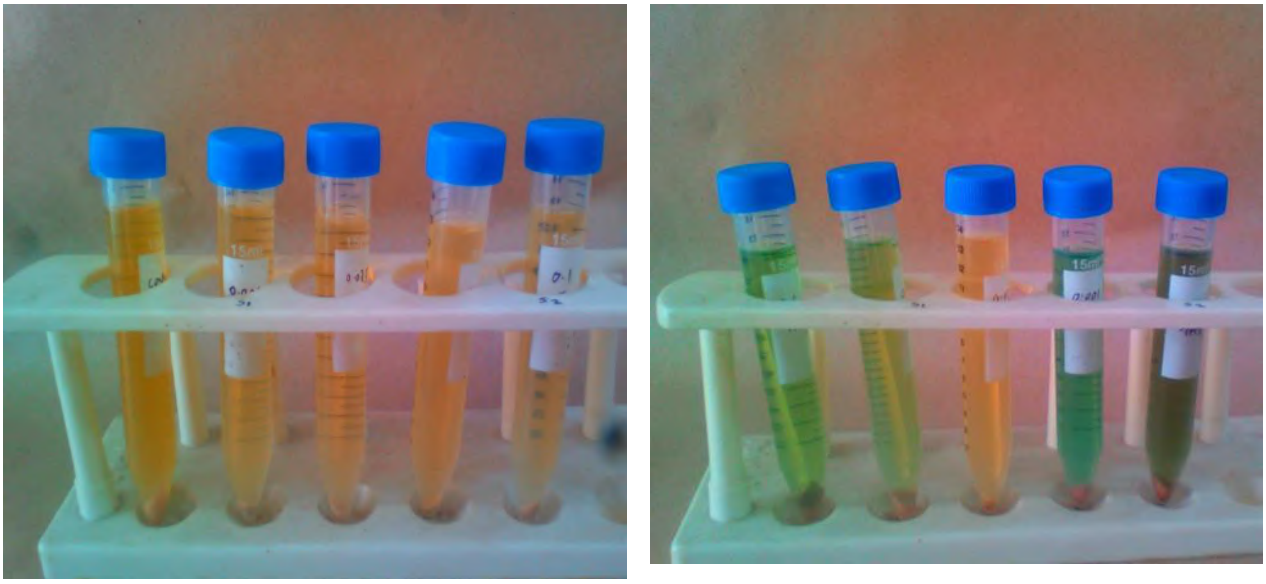


Figure-10 Centrifuged culture of isolate TM-1 and TM-4 for IAA test

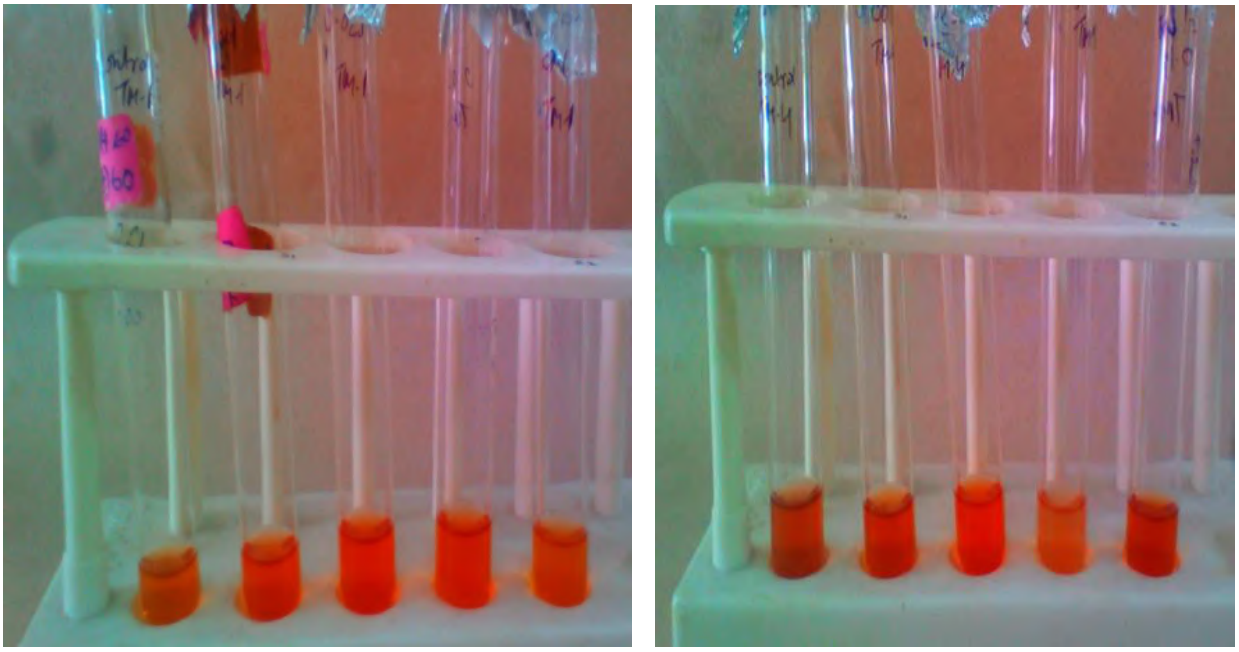


Figure-11: IAA test for isolate TM-1 and TM-4

Table -16 O.D of IAA production of TM-1 and TM-4

Bacterial culture (TM-1) in different Conc. of TM	Conc. of IAA (Absorbance 530nm)	Bacterial culture (TM-4) in different Conc. of TM	Conc.of IAA (Absorbance 530nm)
0ppm	0.804	0ppm	1.011
100ppm	1.505	100ppm	1.067
250ppm	1.473	250ppm	1.035
500ppm	1.396	500ppm	0.895
1000ppm	1.048	1000ppm	0.466

Table-16 represents O.D. of IAA production of TM-1 and TM-4. It was observed that both isolates TM-1 and TM-4 were able to produce IAA. The concentration of IAA was observed more in TM-1 as compared to TM-4. It was observed that the production of IAA by TM-1 was 0.804 in 0ppm conc. of TM which gradually increased to 1.505 in 100ppm and then decreased in IAA concentration was observed which was 1.473 in 250ppm to 1.048 in 1000ppm conc. of TM. It was observed that the production of IAA by TM-4 was 1.011 in 0ppm conc. of TM which gradually increased to 1.067 in 100ppm and then decreased in IAA concentration was observed which was 1.035 in 250ppm to 0.466 in 1000ppm conc. of TM.

DISCUSSION

A. Isolation of TM degrading bacteria:

The enrichment procedure is a common method to isolate pesticide degrading bacteria. It has been applied to isolate soil bacteria which are capable of degrading various kinds of pesticides (Rajgopal *et al*. 1984; Mohapatra and Awasthi 1999; Bhaleraoand Puranik 2007; Kumari.A.*et.al*.2012, Liu.Z.*et.al* 2011; Shivaramaiah and Kennedy, 2006). The two bacterial strains *Enterobacter species* and *Bacillus species* which were capable of degrading Thiophanate-Methyl were isolated by using enrichment procedure (Cycon. M. *et.al* 2011). Using this same technique 6 bacterial culture TM-1, TM-2, TM-3 TM-4, TM-5 and TM-6 were obtained. Inoculum/Broth of all six cultures was prepared by growing them in LB media supplemented with TM. The two highly resistance to TM isolates TM-1 and TM-4 was selected for further analysis by determining maximum inhibitory concentration of isolates.

B. Bacterial Growth in TM with and Without Humic acid:

Bacterial growth of isolates of TM-1 and TM-4 was performed in LB media supplemented with TM of 50ppm and 100 ppm conc. and also with /without humic acid. Humic acid is an organic matter. Organic matter is one of the factor known to affect the microbial degrading activity of pesticide .The number of studies have been conducted on the various factors like pH, temperature, concentration of pesticides affecting the microbial degradation of various pesticides but very few papers have been published on the effect of organic matter on the microbial degradation process of pesticides. Organic matters act as an adsorbent of pesticides in soil and it interfere with the microbial activities.

In a study conducted by (Cycon.M et.al .2011) on the microbial degradation of thiophanate –methyl, the results of the research revealed the differences in growth of individual bacterial isolates in media containing TM. It was observed that the growth was effective during the first 4 days of incubation. There was no change in control which was without inoculum in O.D for 16 days of incubation. During 16 days of incubation it was observed that 60% and 75% of TM disappearance in media inoculated with strains TDS-1 and TDS-2 respectively. They also performed the degradation process in the presence and absence of glucose and it was found that the growth is isolates were three times higher in presence of glucose but the rate of TM degradation was lower after 16 days of incubation. The process degradation of isolates was also observed in soil and the differences in degrading potential of TDS-1 and TDS-2 was observed. The *Enterobacteria species* (TDS-1) was able to degrade complete TM in 24days and *Bacillus Species (*TDS-2) was able to degrade TM completely in 20 days.

In the present study of the effect of organic matter was observed on TM degrading efficiency of isolates TM-1 and TM-4. The two concentration of TM 50 ppm and 100ppm was used to study the degrading efficiency of isolates and two control (LB+TM) and (LB+TM+ Humic acid) was used.

The growth of TM-1 and TM-4 in presence of 50ppm conc. of TM was observed by measuring the O.D. The results showed the continuous increase in values of both the isolates from 0-7 days of incubation. The value of TM-1 was 0.246 on day zero which reached to 1.115 and the value of TM-4 was 0.289 which reached to 0.802 on the 7^{th} day of incubation. The values showed that the growth of TM-1 was more as compared to TM-4 in 50ppm.In 100 ppm concentration of TM also the TM-1 showed higher values as compared to TM-4 but values of both strains were higher as compared to

the values of growth in 50ppm concentration of TM. The growth of both isolates was also observed in media containing 50ppm and100ppm supplemented with 50ppm and 100ppm of humic acid respectively. The values of O.D. of isolates culture containing 50ppm TM and Humic acid showed that there was continuous increase in values till 6^{th} days of incubation but no change in the value of TM-1 was observed on 7^{th} day and there was decrease in the value of TM-4 on the 7^{th} days of incubation. The results of growth of isolates in 100ppm TM and humic acid showed the decrease in values on 7^{th} days of incubation in both TM-1 and TM-4.

C. Functional characterization of TM Degrading Bacteria:

There are several bacteria which are associated with rhizosphere of plants and stimulate plants growth by any mechanism is known as plant growth promoting rhizobacteria (PGPR) (Vessey, 2003; Arnou, 1953). This rhizosphere bacterium enhances either directly or indirectly plant growth and its yield (Kloepper *et.al.* 1989; Glick, 1995). In a study conducted by (Gupta, S.*et.al*.2014) selection of PGPR was done by their biochemical screening like IAA test, Siderophore production test and phosphate solubilizing test. In present study these tests were used to study the functional characterization of isolates TM-1 and TM-4 at different concentration (100,250,500,1000ppm) of TM.

In phosphate solubilizing test both isolates showed positive results but the zone of clearance was observed more in TM-4 as compared to TM-1.

In Siderophore production test only TM-4 showed the positive result.

In Indole acetic acid production test both isolates showed positive results. The production of IAA was comparatively higher than TM-4 in case of TM-1 and it was also observed that the production of IAA was decreasing with the increase in concentration of TM in case of both isolates. For the identification isolates gene sequencing has to be performed.

CHAPTER-7
CONCLUSION

CONCLUSION:

Environmental pollution is one of the most serious problems. It is increasing day by day due to increase in population, industrialization and urbanization. Uses of pesticides have been very popular among the farmers to increase the yields. Due to continuous use of pesticides soil and ground water is getting contaminated which is affecting the environment as well as various other life forms. Considering the toxic effect of pesticides it is very essential to eliminate them. Microbial degradation is the easiest and efficient technique to control the pollution of soil and water and thus protecting various other life forms. This method is economic as well as ecofriendly.

In this study it was found that both isolates TM-1 and TM-4 was able to grow in media supplemented with TM but there growth was decreased slightly in the presence of humic acid. These isolates showed positive results for PGPR activity. Both isolates showed positive result for phosphate solubilization but activity was more in TM-4, Siderophore production was showed only by TM-4 and IAA production test was positive for both TM-1 and TM-4.

By inoculating these isolates we can minimize the environmental pollution by degrading thiophanate-methyl in the soil and these bacteria will also enhance plant growth and thus help in increasing the yield of the crops.

For the biodegradation of pesticides by the use microorganisms it is important to understand the genetic as well as molecular mechanisms of enzymatic catalysis done by microorganisms and this will help to design some other alternative technique to eliminate pesticides from the contaminated soil or water.

CHAPTER-8
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