Isolation, Characterization and Evaluation of Soil Microorganisms for Bioremediation of Chlorpyrifos

by

KAROLIN K. P

(2012-11-165)

THESIS

Submitted in partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE IN AGRICULTURE

Faculty of Agriculture
Kerala Agricultural University

DEPARTMENT OF AGRICULTURAL MICROBIOLOGY
COLLEGE OF AGRICULTURE
VELLAYANI, THIRUVANANTHAPURAM- 695 522
KERALA, INDIA

2014

DECLARATION

I, hereby declare that this thesis entitled "**Isolation, characterization and evaluation of soil microorganisms for bioremediation of chlorpyrifos**" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

Vellayani
Date : 25/10/14

KAROLIN K.P.
(2012 -11-165)

CERTIFICATE

Certified that this thesis, entitled "**Isolation, characterization and evaluation of soil microorganisms for bioremediation of chlorpyrifos**" is a record of research work done independently by Ms. Karolin, K.P. under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

Meenakumari

Vellayani,
Date : 25/10/14

Dr. K.S. Meenakumari
(Major Advisor, Advisory Committee)
Professor and Head
(Agricultural Microbiology)
College of Agriculture
Vellayani

CERTIFICATE

We, the undersigned members of the advisory committee of Ms. Karolin, K.P., a candidate for the degree of **Master of Science in Agriculture** with major in Agricultural Microbiology, agree that the thesis entitled "**Isolation, characterization and evaluation of soil microorganisms for bioremediation of chlorpyrifos**" may be submitted by Ms. Karolin, K.P., in partial fulfilment of the requirement for the degree.

Dr. K.S. Meenakumari
(Chairman, Advisory Committee)
Professor and Head
Department of Agrl.Microbiology
College of Agriculture, Vellayani

Dr.K. N. Anith
(Member, Advisory Committee)
Associate Professor
Department of Agrl. Microbiology
College of Agriculture, Vellayani

Dr. Thomas Biju Mathew
(Member, Advisory Committee)
Professor
Department of Agrl.Entomology
AINP on Pesticide Residues
College of Agriculture, Vellayani

Dr. K.B. Soni
(Member, Advisory Committee)
Associate Professor
Department of Plant Biotechnology
College of Agriculture, Vellayani

EXTERNAL EXAMINER
(Name and Designation)

Dr. Jisha. M. S.
Associate Professor
School of Biosciences
Mahatma Gandhi University

ACKNOWLEDGEMENT

I am heartily thankful to my Advisor, Professor K.S. Meenakumari, Department of Agricultural Microbiology, College of Agriculture, Vellayani for the continuous support, patience, motivation and enthusiasm from the initial to the final level of my research which made her a backbone of this research and so to this thesis. Through her valuable questioning about this study Dr. Meenakumari brought me closer to the reality and enabling me to grasp its rich complexity. Her capacity to combine critique with an immediate empathy and commitment always inspire me.

I am grateful to Dr. K.N. Anith, Associate Professor of Microbiology Department for providing timely support, advice, encouragement and favorable environment in the department to achieve this task.

I would like to show my gratitude to Professor Thomas Biju Mathew, Professor, Dept. of Agricultural Entomology for his advice on interpreting the results and productive comments on an earlier draft.

Special thanks to Professor Dr. Thomas George, Associate Professor, Dept. of Soil science and Agricultural chemistry for his stimulating discussions and concern. I am thankful to Dr. (Mrs.) K.B. Soni, Associate Professor, Dept. of Biotechnology and Dr. (Mrs.) Aparna Associate Professor, Dept. of Soil science and Agricultural chemistry for their advice & suggestions whenever needed. I extend my thanks to Dr. Ambly Paul as well.

I am deeply indebted to Dr. P. Sivaprasad Professor and Head, Dept of Agricultural Microbiology (Retired) whose constant guidance and constructive criticism during the initial stages of my research work helped me to pursue smoothly.

I am also thankful to Sri. C.E Ajithkumar, Junior Programmer, Department of Agricultural Statistics for the assistance rendered during the analysis of data.

I convey my heartfelt thanks to Dr. S. Naseema Beevi, Professor and Head, Pesticide Residue Laboratory for providing necessary facilities during my study.

My special thanks to Dr. Haseena (CEPC, Kollam) and Mr. Suresh kumar (RGCB, Trivandrum) for their whole hearted co-operation and help during my study.

Looking back, I am surprised and at the same time very grateful for all I have received throughout these years. I would like to thank all my lab buddies. My special thanks to Mrs. Subha, Mrs. Viji, Mrs. Sheeba, Mrs. Sangeetha , Mr. Sooraj, Mrs. Saparya , Mrs. Soumya Mrs. Unnimol, Mrs. Greeshma, Ms. Ancy, Mr. Santhosh, Bindhu chechi and Pushpa chechi for their caring attitude and spending precious time in utile discussions required to smoothly fulfill this task. It is a pleasure to thank them for being a great company and all the fun we have had in the last two years.

I would like to extend my thanks to Miss. Anjana, Mrs. Anooba, Mr. Rajith, Mr. Pradeep and Mr. Pradeesh, to make my surrounding a convivial place to work. Their support in this effort is, however, greatly appreciated.

Words fail to express my sincere thanks to my friends Priya, Annie, Revoo, Nimmi, Anjali, Anupama, Reshma, Lakshmi chechi, Hemanth, Lokesh, Jayanth, Jayasheela, Darshan and my junior Vysakhi for their sincere love and emotional support and affection during these days without which my work wouldn't have been completed.

I owe my deepest gratitude to my dear parents. I am grateful to my dad whose foresight, encouragement and support paved the way for a privileged education. I can't express my feeling of thankfulness and appreciation for my mom in words, whose unconditional love has been my greatest strength.

Last but not least, my oodles of thanks to God as I know whatever he has done so far was the best for me and I strongly believe that whatever comes in near future will be the best.

Karolin K.P.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDIX

LIST OF ABBREVIATIONS

a.i	-	Active ingredient
CD (0.05)	-	Critical difference at 5 % level
cfu	-	Colony forming units
cfu/ml	-	Colony forming units per milli litre
cm	-	Centimetre
DAI	-	Days after inoculation
day^{-1}	-	Per day
dia.	-	Diameter
et al.	-	Co-workers/ Co-authors
Fig.	-	Figure
g	-	Gram
g^{-1}	-	Per gram
$g\ m^{-2}$	-	Gram per square metre
ha	-	Hectare
i.e.	-	that is
kg	-	Kilogram
$kg\ ha^{-1}$	-	Kilogram per hectare
l	-	litre
l^{-1}	-	Per litre
m^{-2}	-	per square metre
mg	-	Milli gram
ml	-	Milli litre
min	-	minutes
MSM	-	Mineral salts medium
Max.	-	maximum

nm	-	Nano meter
NS	-	Not significant
OD	-	Optical density
PDB	-	Potato dextrose broth
plant $^{-1}$	-	per plant
pH	-	Negative logarithm of hydrogen ion concentration
ppm	-	Parts per million
sp.	-	Species
viz.	-	Namely

LIST OF SYMBOLS

%	-	per cent
°C	-	Degree Celsius
@	-	at the rate of

Introduction

1. INTRODUCTION

Pesticides constitute the key control strategy for crop pest and disease management. Continuous application of these pesticides to the soil and aquatic system resulted in health hazards and environmental pollution which has triggered much public concern. The wide spread use of these pesticides over the years has resulted in problems caused by their interaction with the biological systems in the environment. Notwithstanding the hazards, pesticides will continue to be an indispensable tool for the management of pests in the years to come, as there is no suitable alternative to totally replace them. Considering the toxic effect of these pesticides it is essential to remove them from the environment employing suitable remedial measures. Bioremediation exploiting microbial technology is one of the recent techniques for environmental clean-up. In the process, heterotrophic microorganisms breakdown hazardous compounds to obtain carbon and energy.

Currently among the various groups of pesticides, organophosphates form the major, accounting for more than 36 per cent of the total world market (Kanekar *et al.*, 2004). Among the insecticides, monocrotophos, quinalphos and chlorpyrifos top the list of organophosphorus insecticides in Indian market. In India the estimated consumption of technical grade chlorpyrifos during 2002-03 was 5000 MT (Singhal, 2003). In the light of restricted or banned use of organochlorine compounds, chlorpyrifos is gaining importance in Agriculture. It is a broad spectrum pesticide displaying insecticidal activity against a wide range of insects and pests. The most commonly available formulations include emulsifiable concentrates (EC), granules (GR) and wettable powders (WP). Aerial application of chlorpyrifos is a common method followed against surface feeding insects of cotton, rice, mustard, bengal gram etc. (Dhawan and Simwat, 1996; Gupta *et al.*, 2001). Soil applications are used for control of root damaging insect larvae attacking crops such as vegetables, cardamom, tobacco, cole crops, groundnut and onion (Rouchaud *et al.*, 1991; Bhatnagar and Gupta, 1992). The practice of application of 1-2% concentration of chlorpyrifos to soil surrounding

building structures against termite invasion has aggravated the residue problems (Sundararaj *et al.,* 2003).

In soil, chlorpyrifos may remain biologically active for periods ranging from days to months. It is moderately persistent in nature as its residues were detected in soil even after 3 months and hence causes potential environmental hazards (Chapman *et al.*, 1984). Extensive use of chlorpyrifos contaminates air, groundwater, rivers, lakes, rainwater and fog water. The contamination has been found up to about 24 kilometers from the site of application. Considerable residues of chlorpyrifos were found in tomatoes (Aysal *et al.,* 1999), cotton seed (Blossom *et al.,* 2004) and oil of oil seed crops like groundnut, safflower and mustard (Bhatnagar and Gupta, 1998; Gupta *et al.,* 2001). It is speculated that the bioaccumulation ability of chlorpyrifos and other organophosphorus pesticides in living tissues may spell a potential environmental risk to marine organisms and humans as well (Serrano *et al.,* 1997; Tilak *et al.,* 2004).

When organophoshates are released in to the environment, their fate is decided by various environmental conditions and microbial degradation is the key factor for the disappearance of these pesticides, since they possess the unique ability to completely mineralize many aliphatic, aromatic and heterocyclic compounds.

Even though extensive work has been conducted on microbial degradation of chlorpyrifos under laboratory conditions, no attempt has been made so far to exploit this technology for field level application. Hence the present programme has been designed to isolate microorganisms capable of degradation of chlorpyrifos and to develop a consortium for its field level application with the following objectives:

1. Isolation, characterization and evaluation of microorganisms for chlorpyrifos degradation.
2. Development of consortium.
3. Evaluation of bioremediation potential of consortium against chlorpyrifos *in vivo*.

Review of literature

2. REVIEW OF LITERATURE

2.1. PESTICIDE SCENARIO IN INDIA

Pesticides constitute the key control strategy for crop pests and disease management contributing significantly towards enhancing crop yields. Considering the world consumption of pesticides, 85 percent of pesticides are used in Agriculture. In India the average pesticide consumption is 0.5 kg per ha and is ranked tenth in the world in pesticide consumption (Hundal, 2006). Pesticides are often persistent in environment to achieve effective control of pests over long period of time.

Aktar *et al.* (2009) reported that among the total consumption of pesticides in India, the major share is of insecticides (76 per cent) followed by fungicides (13 per cent), herbicides (10 per cent) and others (1 per cent). The average world consumption comprises 25 per cent insecticides, 49 per cent herbicides and 22 per cent fungicides. As far as the chemical nature of products is concerned, the market comprises 16 per cent organochlorines, 50 per cent organophosphates, 4 per cent carbamates, 19 per cent synthetic pyrethroids, 1 per cent biopesticides and 10 per cent others. The higher consumption of insecticides may be attributed to higher hatching rate of insects in warm humid and tropical climate which provides favorable breeding environment. The production of pesticides started in India in 1952 with the establishment of a plant for the production of BHC near Calcutta; the Indian pesticide industry is the fourth largest in the world and second in the Asia Pacific region after China and ranks twelfth globally. There has been a steady growth in the production of technical grade pesticides in India, from 5,000 metric tons in 1958 to 102,240 metric tons in 1998. The pattern of pesticide usage in India is different from that for the world in general. At present, a total of 248 technical pesticides have been registered in the country, of which 93 technical grade pesticides are being manufactured indigenously. According to the Ministry of Chemicals and Fertilizers, the production of technical grade pesticides in the country was 41,822 tonnes in 2009-2010.

The consumption of chemical pesticides, in India was 2,10,600 tonnes in 2009-2010. Of the total chemical pesticides consumed, cotton accounts for the maximum consumption of 45 per cent, rice 22 per cent, vegetables 9 per cent, plantations 7 per cent, pulses 4 per cent, wheat 4 per cent and other crops 9 per cent (Singhal, 2003).

However, over anxiety of farmers and lack of scientific knowledge has led to indiscriminate use of these pesticides, causing short and long-term health effects. The wide spread use of these pesticides over the years has resulted in problems caused by their interaction with the biological systems in the environment. Notwithstanding the hazards, pesticides will continue to be an indispensable tool for the management of pests in the years to come, as there is no suitable alternative to totally replace them.

2.2. IMPORTANCE OF BIOREMEDIATION OF ORGANOPHOSPHORUS COMPOUNDS

Organophoshorus compounds constitute an important versatile group of highly active molecules widely used for pest control. More than one lakh different organophosphorus compounds have been synthesized and evaluated as pesticides of which nearly one hundred and fifty are widely used in agriculture. Substances with wide variety of biological properties like insecticides, acaricides, nematicides, herbicides, defoliants and fumigants are a few among the organophosphorus compounds. Most of the compounds undergo rapid degradation from the environment and disappear after the period of pesticidal action and will not accumulate in the body of animals. Though many of the earlier compounds of this group were highly toxic to vertebrates, a large number of organophoshorus compounds with moderate to low mammalian toxicity have been synthesized recently (Rekha, 2005).

Organophoshorus compounds are the most widely used insecticides, accounting for 36 per cent of world-wide insecticide sales (Kanekar *et al.*, 2004). The persistent organochlorine pesticides were widely used around the world before 1970s. Initially OPs were considered as safe alternative to organochlorines but over the years due to their inordinate use their accumulation and exposure lead to acute toxicity to non-target organisms. There are reports of high mammalian toxicity due to OPs which resulted in three million poisonings and 200,000 deaths annually (Karalliedde and Senanayak 1999; Sogorb *et al.*, 2004). OP's have been cited as potential cause of many diseases in mammals.

Considering the toxic effect of these pesticides it is essential to remove them from the environment employing suitable remedial measures. Organophosphorus insecticides are esters of phosphoric acid which include aliphatic, phenyl and heterocyclic derivatives and have one of the basic building blocks as a part of their complex chemical structure. Some of the main agricultural products are parathion, methyl parathion, chlorpyrifos, malathion, monocrotophos and quinalphos (Anuja George, 2005). Although organophosphates are biodegradable in nature, their residues are found in environment. Considering their toxicity, research on biodegradation of organophosphates is being carried out all over the world.

Bioremediation exploiting microbial technology is one of the recent techniques for environmental clean-up. In this process, heterotrophic microorganisms breakdown hazardous compounds to obtain carbon and energy. The first bacteria, that could degrade organophoshorus compounds was isolated in 1973 (Dragun *et al.*, 1984) and identified as *Flavobacterium* sp. Since then several bacterial and a few fungal species have been isolated which can degrade a wide range of organophoshorus compounds in liquid cultures and soil systems. The wide variety of microorganisms has been found to possess the enzyme catalyzing hydrolysis of organophoshates (Singh *et al.*, 2006). This enhances the feasibility of using bioremediation to treat organophosphate compounds.

Moreover application of molecular biology to genetically engineer microorganisms containing appropriate genes can potentially contribute to efficiency of bioremediation.

2.3. CHLORPYRIFOS

Among the organophosphorus insecticides, monocrotophos, quinalphos, chlorpyrifos, malathion and methylparathion top the list of insecticides in Indian market (Anuja George, 2005). Chlorpyrifos was first discovered by the Dow chemical company in 1962, initially designated as DOWCO 179 and later as chlorpyrifos (Rekha, 2005). In India the estimated consumption of technical grade chlorpyrifos during 2002-03 was 5000 MT (Singhal, 2003). In the light of restricted or banned use of organochlorine compounds, chlorpyrifos is gaining importance in Agriculture. It is a broad spectrum pesticide displaying insecticidal activity against a wide range of insects and pests. The most commonly available formulations include emulsifiable concentrates (EC), granules (GR) and wettable powders (WP). Aerial application of chlorpyrifos is a common method followed against surface feeding insects of cotton, rice, mustard, bengal gram etc. (Dhawan and Simwat, 1996; Gupta *et al.,* 2001). Chlorpyrifos is one of the dominated broad spectrum organophosphorus insecticides inhibiting the neuron function of sucking, chewing and boring insects both in crop and soil (Racke *et al.*, 1994). Soil applications are used for control of root damaging insect larvae attacking crops such as vegetables, cardamom, tobacco, cole crops, groundnut and onion (Rouchaud *et al.,* 1991; Bhatnagar and Gupta, 1992). The practice of application of 1-2% concentration of chlorpyrifos to soil surrounding building structures against termite invasion has aggravated the residue problems (Sundararaj *et al.,* 2003).

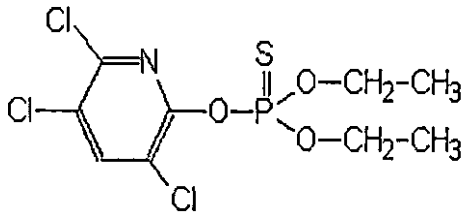
In soil, chlorpyrifos may remain biologically active for periods ranging from days to months. It is moderately persistent in nature as its residues were detected in soil even after 3 months and hence causes potential environmental hazards (Chapman *et al.*, 1984). Extensive use of chlorpyrifos contaminates air,

groundwater, rivers, lakes, rainwater and fog water. The contamination has been found up to about 24 kilometers from the site of application. Considerable residues of chlorpyrifos were found in tomatoes (Aysal *et al.,* 1999), cotton seed (Blossom *et al.,* 2004) and oil of oil seed crops like groundnut, safflower and mustard (Bhatnagar and Gupta, 1998; Gupta *et al.,* 2001). It is speculated that the bioaccumulation ability of chlorpyrifos and other organophosphorus pesticides in living tissues may spell a potential environmental risk to marine organisms and humans as well (Serrano *et al.,* 1997; Tilak *et al.,* 2004 ; Sumit Kumar, 2011).

When organophoshates are released in to the environment, their fate is decided by various environmental conditions and microbial degradation is the key factor for the disappearance of these pesticides, since they possess the unique ability to completely mineralize many aliphatic, aromatic and heterocyclic compounds.

2.3.1. Structure and Chemistry of Chlorpyrifos

The physical and chemical properties of a pesticide plays significant role in determining its environmental fate and transport, the basic pattern of persistence and partitioning is fundamentally derived from the chemical character of the compound. Many researchers have studied the physical and chemical properties of chlorpyrifos. (Brust, 1966; Ringterink and Kenaga, 1966; Neely *et al.*, 1974; Chiou *et al.*, 1977). The chemical structure of chlorpyrifos is shown below:



2.4. RESIDUES OF CHLORPYRIFOS

Maini *et al.* (1972) monitored the residues of chlorpyrifos in various crops after field treatment with emulsifiable concentrate at dosages from 0.5 to 6 kg ai

ha^{-1}. The average residues in sugarbeet leaves were 0.046 to 0.070 ppm and <0.005 to 0.038 ppm in roots. Iwata *et al.* (1985) detected the residues of acephate, amitraz, chlorpyrifos and formetanate hydrochloride on and in fruits after low volume application to orange trees. They reported that the residues of chlorpyrifos dissipated rapidly with a half-life of 2.5 days during the initial two weeks after spray application and slowly thereafter with a half life of 64 days. An investigation of the non-solvent extractable residues of (14C) chlorpyrifos-methyl in stored wheat was conducted by Matthews (1991). The residues remaining in wheat after solvent extraction accounted for 28 per cent of the applied dose after an extended period of storage. Analysis of the solubilized residue revealed that 59 per cent was present as the pyridinol metabolite of the chemical and 26 per cent was a polar material.

Bhatnagar and Gupta (1992) studied the persistence of chlorpyrifos residues in soil and groundnut seed with soil application of the chemical at 800 and 1200 g ai/ha. The average initial deposit of 0.83 ppm in soil at a lower dose of 800 g ai ha^{-1} dissipated to below detectable levels in 30 days, while at the higher dose of initial deposit of 1.48 ppm took 40 days to fall below the detectable limit. Gupta *et al.* (2001) monitored the residues of lindane, chlorpyrifos and quinalphos in mustard seed and oil. However, it was shown that only lindane persisted in seed and oil at the time of crop maturity, while chlorpyrifos and quinalphos did not persist in mustard seed or oil. But, Blossom *et al.* (2004) reported that 26 per cent of cotton seed samples analysed was contaminated with the residues of chlorpyrifos. Some samples had chlorpyrifos above the maximum residue limit of 0.05 mg kg^{-1}.

Putnam *et al.* (2003), in a study on the persistence and degradation of chlorpyrifos in a cranberry bog, detected chlorpyrifos in the fruits at harvest even after 62 days post chlorpyrifos application. Chlorpyrifos oxon and 3, 5, 6-trichloro-2-pyridinol were detected in earlier fruit samples. The dissipation of

dislodgeable foliar residues followed first order kinetics with half lives of 3.5 days.

Safi *et al.* (2002) analysed residues of chlorpyriphos, carbofuran and other pesticides on tomatoes, cucumber and strawberries by GC-MS technique. Tomatoes showed the least number and level of pesticide residues while strawberries showed greater number and level of pesticide residues. Montemurro *et al.* (2002) investigated the residue levels and degradation rates of chlorpyrifos in orange fruits, leaves and soil with emulsifiable concentrate, wettable granules and micro encapsulates. For the first two formulations, the dissipation of chlorpyrifos in orange fruits was faster during the first phase and much slower during the later period. However, residue levels of chlorpyrifos for micro encapsulates remained almost constant for approximately 65 days and resulted in prolonged persistence.

Fenske *et al.* (2002), in an assessment of organophosphorus pesticide exposures in the diets of pre-school children in Washington State noticed detectable levels of chlorpyrifos in some diets. Karasali *et al.* (2002) detected residues of chlorpyrifos ethyl in thermal mineral water in Greece. Salas *et al.* (2003) reported that 39.6 per cent of the commercial pasteurized milk samples in Mexico contained detectable levels of organophosphorus pesticide residues and in some samples; chlorpyrifos exceeded the maximum residue levels.

Sanghi *et al.* (2003) reported residues of organochlorine and organophosphorus pesticides in breast milk samples from Bhopal. They observed that through breast milk infants consumed 8.6 times more endosulfan and 4.1 times more malathion. Organochlorine and organophosphorus insecticide residues in market samples of meat were monitored by Suganathy and Kuttalam (2003). Chlorpyrifos residues were present in both chicken and mutton samples. About 0.455 $\mu g\ g^{-1}$ of residues of chlorpyrifos were present in 99 per cent of the samples.

The maximum contamination in cardamom plantations was found with organophosphorus insecticides like phorate, chlorpyrifos, quinalphos, profenphos and methyl parathion (Siji, 2011).

Beevi *et al.* (2014) reported 32 and 42 per cent residue of chlorpyrifos in first year and second year respectively in cardamom plantations of Idukki district.

2.5. FATE OF CHLORPYRIPHOS PESTICIDE IN THE ENVIRONMENT

Pesticides undergo various changes in environment including their adsorption transmission and degradation, depending on the physicochemical nature of the pesticide and the soil (Redondo *et al.*, 1997). The predominant processes involved in transformation of such molecules is often mediated by microbes (Vink and Van der Zee, 1997) followed by photolysis or photodegradation and chemical transformations (Stangroom *et al.*, 2000). Thus, generally fate of pesticide involves both biological and non-biological agents. Extensive studies have been carried out on the abiotic degradation of chlorpyrifos in soil, plants and animals. Smith *et al.*, (1967) studied the fate of chlorpyrifos in animals and plants found that the parent compound disappears via excretion, volatility, catabolism and photodecomposition.

2.5.1. Abiotic Transformation

The abiotic processes involved either their transport where parent compound remains unchanged and simply transferred from one matrix to another depends on the physicochemical properties of pesticides itself (Stangroom *et al.*, 2000) e.g. volatilization, leaching (Laabs *et al.*, 2000), runoff (Moore *et al.*, 2002), absorption and adsorption of pesticides (Yu *et al.*, 2006) or by abiotic transformations by photodegradation (Walia *et al.*, 1988) and chemical hydrolysis (Liu *et al.*, 2001).

2.5.2. Biotic Transformation

Xenobiotic compounds like organophosphate pesticides are manmade compounds and were not previously present in nature. Consequently the natural microflora does not have potential to metabolize these pesticides due to lack of enzyme and proper transport processes. But over the years due to excessive use of xenobiotic compounds microbes have evolved the new degradation pathways resulting in accelerated degradation of such compounds (Seffernick and Wackett, 2001; Johnson and Spain, 2003). The accelerated bioremediation under natural conditions has also helped by transfer of genes among different microbial cultures by transformation, transduction and conjugation (Ghigo, 2001 and Fux, 2005).

2.5.2.1. Microbial Transformation of Chlorpyrifos

When organophosphates are released in to the environment, their fate is decided by various environmental conditions and microbial degradation. Microbial degradation is the key factor for the disappearance of these pesticides. Micro organisms possess the unique ability to completely mineralize many aliphatic, aromatic and heterocyclic compounds. Several studies conducted in soil indicated significantly longer dissipation half-lives under sterilized versus natural conditions, and led to the conclusion that microbial activities are important in degradation of chlorpyrifos (Getzin, 1981; Miles *et al.*, 1983). Schmimmel *et al.*, (1983), based on laboratory degradation studies with aqueous solution and sediments, concluded that microorganisms play an important role. Cleavage and mineralization of heterocyclic ring occur in soil due to activities of microorganisms (Somasundaram *et al.*, 1987; Racke *et al.*, 1988).

In literature there are many reports regarding screening and isolation of microbes capable of degrading pollutants under laboratory conditions. However, their use at the contaminated sites under field scale application has not been successful (Pilon-Smits 2005; Dua *et al.*, 2002; Kuiper *et al.*, 2004). The reasons behind this include the competition faced from the natural microflora and

microfauna of the soil, suboptimal nutrition or nutritional deficiency leading to low microbial growth, non availability or less bioavailability of the pollutant desired to be degraded and the growth inhibitory concentration of pollutant itself (Kuiper *et al.*, 2004). Shan *et al.* (2006) reported the suppressed growth of bacterial, fungal, and actinomycete populations in the presence of chlorpyrifos (10 mg kg^{-1}). Inspite of these limitations, microbial degradation of organophosphate pesticides is an important process responsible for their biotic degradation in environment (Felsot, 1979). There are reports establishing ability of microbes to degrade pesticides co-metabolically or as source of carbon, nitrogen and phosphorous (Sethunathan and Yoshida, 1973; Serdar *et al.*, 1982; Oshiro *et al.*, 1996; Singh *et al.*, 2004; Singh, 2006; Wang *et al.*, 2006; Rani *et al.*, 2008; Li *et al.*, 2008; Kulshrestha and Kumari, 2011).

The microbial diversity at the contaminated site is constituted by diverse group of microscopic organisms like bacteria, fungi, viruses, protozoa and algae. Among them bacteria, fungi and to some extent algae are the main contributors to degradation of pesticides. Microbial transformation can be mainly achieved by three different mechanisms such as biodegradation or catabolism, co-metabolism and bioaccumulation (Weber, 1972; Racke, 1993; Boonsaner *et al.*, 2002).

Catabolism is a type of degradation in which the organic chemical or a portion there of is completely degraded (e.g. mineralized) and the energy or nutrient gained contributes to cell growth. The second, incidental metabolism or cometabolism, involves the partial degradation of an organic chemical with no net benefit to the organism, the compound being merely caught up in some metabolic pathway during the normal metabolic activities of the microorganisms (Racke, 1993).

Several studies conducted in soil indicated significantly longer dissipation half-lives under sterilized versus natural conditions, and led to the conclusion that microbial activities are important in degradation of chlorpyrifos (Getzin, 1981;

Miles *et al.*, 1983). Schmimmel *et al.*, (1983), based on laboratory studies with aqueous solution and sediments, concluded that microorganisms play an important role. Cleavage and mineralization of heterocyclic ring occur in soil due to activities of microorganisms (Somasundaram *et al.*, 1987; Racke *et al.*, 1988). Many of the scientists and research workers have isolated microorganisms from natural ecosystem which have the capacity to degrade chlorpyrifos (Mukherjee and Gopal, 1996; Mallick *et al.*, 1999; Singh *et al.*, 2003). The possible use of individual microorganisms (Singh *et al.*, 2004; Khanna and Vidyalakshmi, 2004) and microbial consortia for bioremediation of chlorpyrifos in contaminated soil has already been reported (Vidya Lakshmi *et al.*, 2008; Sasikala *et al.*, 2012; Barathidasan and Reetha, 2013; Hindumathy and Gayathri, 2013). Release of chloride due to cleavage and mineralization of heterocyclic ring due to activities of microorganisms has already been reported (Somasundaram *et al.*, 1987; Racke *et al.*, 1988; Yucheng Feng, 2003; Anuja George, 2005).

2.5.2.1.1. Bacterial Degradation of Chlorpyrifos

Microbial degradation of organophosphate pesticides is an important process responsible for their biotic degradation in environment (Felsot, 1979). There are reports regarding ability of microbes to degrade pesticides co-metabolically or as source of carbon, nitrogen and phosphorous. Sethunathan and Yoshida (1973) reported *Flavobacterium* sp. having the ability to degrade chlorpyrifos in liquid medium by cometabolism. Similarly, Serdar *et al.* in 1982 isolated *Pseudomonas diminuta* degrading chlorpyrifos co-metabolically rather than as a source of carbon. The possible metabolism by two lactic acid bacteria (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*) was reported by Shaker *et al.* (1988) who observed 72-83 per cent loss in chlorpyrifos after 96 h. Havens and Rase (1991) circulated a 0.25 per cent aqueous (EC) solution of chlorpyrifos through a packed column containing immobilized parathion hydrolase enzyme obtained from *P. diminuta.* Approximately 25 per cent of the initial dose was degraded after 3 h of constant recirculation through the column. On the other hand; Ohshiro *et al.* (1996) reported that *Arthrobacter* sp. strain B- 5 can use chlorpyrifos as a

substrate rather than a co metabolite. There are many chlorpyrifos degraders reported but very few organisms are known to degrade the compound at higher concentrations. Mallick *et al.* (1999) reported complete degradation of 10 mgl^{-1} of chlorpyrifos in the mineral salts medium by *Flavobacterium* sp. ATCC 27551 and *Arthrobacter* sp. within 24 h and 48 h respectively. Singh *et al.* (2003) isolated six chlorpyrifos degrading bacteria capable of degrading chlorpyrifos in both liquid medium and soil. Singh *et al.*, (2004) also reported degradation of chlorpyrifos by pure culture *Enterobacter* sp. B-14 in liquid as well as in soils. Dutta *et al.* (2006) observed increase in net microbial biomass carbon (MBC) in chlorpyrifos treated soils as compared to the control containing no chlorpyrifos. Wang *et al.*, (2006) observed degradation of chlorpyrifos by pure culture of *Bacillus laterosporus* DSP. *Pseudomonas aeruginosa* NCIM 2074 in scale up process followed by bioremediation of chlorpyrifos in shake flask bioreactor under controlled enrichmental condition was reported by Geetha and Fulekar (2008). Li *et al.* (2008) isolated chlorpyrifos degrading bacterial strains Dsp-2, Dsp-4, Dsp-6 and Dsp-7 identified as *Sphingomonas* sp., *Stenotrophomonas* sp., *Bacillus* sp. and *Brevundimonas* sp. respectively and few other strains distinguished as members of Pseudomonas sp. from chlorpyrifos-contaminated samples. Rani *et al.* (2008) observed that the bacterium, *P. stuartii* MS09, utilized chlorpyrifos as carbon source and able to grow in medium in the presence of added chlorpyrifos (50-700 mg l^{-1}).

Awad *et al.*, (2011) isolated a potent bacterium *P. stuzeri* (B-CP5) which could degrade chlorpyrifos. He also reported that the isolates exhibited substantial growth in mineral salts medium supplemented with 100-300 ppm chlorpyrifos. Studies by Latifi *et al.* (2012) reported that bacterial isolate coded as IRLM.1 was able to grow at concentrations of chlorpyrifos up to 2000 ppm. Bacterial degradation of chlorpyrifos by *Bacillus cereus* in liquid medium degraded chlorpyrifos under the condition of 30^0C, pH 7 concentrations below 150 mgl^{-1}, with degradation rate up to 78.85 percent was reported by Liu *et al.*, (2012). Bacterial strain *Bacillus subtilis* Y242 used chlorpyrifos as a carbon source and

grown in media containing concentrations up to 150 mg/L (Ehab *et al.*, 2013). Farhan *et al.* (2013) observed that a *Kelbsiella* sp. could degrade 90% chlorpyrifos biodegradtion 200 mgl^{-1} at pH 8 and 10^5 cfu ml^{-1} with addition of glucose in 18 days.

The major limitation in the process of chlorpyrifos degradation is the formation of an anti-microbial compound 3, 5, 6-trichloro-2-pyridinol (TCP) which may also affect the growth of chlorpyrifos-transforming microorganisms (Racke *et al.*, 1990). A report by Racke and Coats (1990) indicated that transformation of 30 mg kg-1 of chlorpyrifos in the soil resulted in production of TCP which repress the proliferation of microbes introduced into the soil. The accelerated degradation of chlorpyrifos was observed either due to the ability of degraders to tolerate TCP or their potential to mineralize TCP efficiently at a rate higher rapidly than the rate of its formation in the medium. There are reports regarding degradation of both chlorpyrifos and TCP in aqueous phase (Feng *et al.*, 1998; Mallick *et al.*, 1999; Horne *et al.* 2002; Bondarenko *et al.*, 2004). A *Stenotrophomonas* sp. isolated by Yang *et al.*, (2006) was found to be a degrader of both chlorpyrifos and TCP. On the other hand, Singh *et al.*, (2004) isolated *Enterobacter* sp. capable of degrading chlorpyrifos was not able to degrade TCP but utilize diethylthiophosphate as carbon and phosphorus source. The *Enterobacter* species in this case showed tolerance against TCP even at higher concentrations (150 mg l^{-1}), which might be the reason of effective chlorpyrifos degradation. Bhagobaty and Malik, (2008) isolated four bacteria belonging to *Pseudomonas* sp. that were able to grow and tolerate even up to 1600 ppm chlorpyrifos.

2.5.2.1.2. Fungal Degradation of Chlorpyrifos

There are not many reports of OP degradation by fungal species as compared to those by their bacterial counterparts. Furthermore the organophosphates degradation rate by fungal isolates was found to be slower. Jones and Hastings (1981) reported 95 per cent to 98 per cent degradation of 50

ppm chlorpyrifos by a group of forest fungi namely *Trichoderma harzianum, Penicillium vermiculatum*, and *Mucor* sp. after 28 days of incubation along with accumulation of its metabolite TCP. Bumpus *et al.*, (1993) reported a fungal strain *Phanerochaete chrysosporium* able to mineralize only 26.6 per cent of added chlorpyrifos after 18 days of incubation. Mukherjee and Gopal (1996) reported that chlorpyrifos was effectively degraded by two soil fungi, *T. viridae* and *A. niger*. Omar (1998) studied the availability of phosphorus and sulfur of insecticides origin by 13 fungal species isolated from pesticides treated soil (10, 50 and 100 ppm). P mineralization paralleled to mineralize organic P followed by *A. tamari*, *A.niger*, *T. harzianum* and *Penicillium brevicompactum*. Bending *et al.*, (2002) reported *Hypholoma fasciculare* and *Coriolus versicolor* degraded chlorpyrifos in soil bio-bed after 42 days. Studies had also reported the chlorpyrifos degradation in soil by *Fusarium* sp. (Wang et al. 2005) and *Aspergillus* sp., *Trichoderma* sp. (Liu *et al.*, 2003). A pure fungal strain, *Acremonium* sp. utilized 83.9 per cent chlorpyrifos as a source of carbon and nitrogen (Kulshrestha and Kumari, 2011). Maya *et al.*, (2011) isolated 5 chlorpyrifos degrading fungal isolates such as *Aspergillus* sp., two *Pencillium* sp., *Eurotium* sp., *Emericella* sp. coming under class Ascomycotina. Enrichment procedure allowed to isolate a novel fungal strain named JAS4 belonging to *Ganoderma* (Silambarasasan and Abraham, 2012) obtained 50 per cent reduction in an aqueous medium and *A. terreus* with capacity to degrade the chlorpyrifos and TCP (Silambarasasan and Abraham, 2013).

Bhalerao and Puranik, (2009) noticed inhibition of mycelial growth of *A. oryzae* in flask with increasing concentration of chlorpyrifos. Similarly, mycelial mat formation was found to be reduced in *Ganoderma* sp. (Silambarasan and Abraham, 2012) and in *A. terreus* (Silambarasan and Abraham, 2013) in chlorpyrifos amended medium. Assessment of growth of fungal isolates based on population build up in chlorpyrifos degradation studies has been reported by Abd El-Mongy and Abd El-Ghany (2009).

2.5.2.1.3. Degradation of Chlorpyrifos by Microbial consortia

Chlorpyrifos is normally degraded to a preliminary metabolite 3,5,6 – trichloro-2-pyridinol (TCP) (Macalady and wolfe,1983) which is further degraded to a secondary metabolite 3,5,6 – trichloro- 2-methoxy pyridine (TMP) along with O,O-diethyl phosphorothionic acid (DETP) which subsequently undergoes decomposition to diols and triols and ultimately cleavage of the ring to fragmentary products (Smith,1968). Microorganisms play important role not only in the degradation of the parent compound but also in the subsequent metabolism of breakdown products. The different pesticide pathways for degradation of parent compound and breakdown products or metabolites may not be present in a single species. In this context the concept of the microbial consortia becomes relevant. Different individual organisms of the microbial consortium can work in a concerted manner to achieve an effective degradation of parent compound as well as the metabolites (Macek *et al.* 2000; Kuiper *et al.* 2004; Chaudhry *et al.* 2005).

The effectiveness of consortium compared to individual isolates in the degradation of chlorpyrifos has been reported by earlier workers (Singh *et al.*, 2004, Pino and Penuela 2011, Sasikala *et al.*, 2012). The rhizosphere soil contains 10–100 times more microbes than un-vegetated soil due to presence of plant exudates such as sugars, organic acids, and larger organic compounds in the soil (Lynch and whipps, 1990). However, there are certain factors those can interfere with microbial degradation including (a) the complex molecular and structural features of the degrading compound that may limit its degradability e.g. polyhalogenated compounds (Wackett *et al.*, 1994), (b) natural dominance of a non-productive metabolic pathway (Oh and Bartha 1997), (c) low frequency of an essential degradative gene (Shapir *et al.*, 1998), (d) poor bioavailability e.g. polycyclic aromatic hydrocarbons (Bastiaens *et al.*, 2000) and (c) production of recalcitrant intermediates (Van Hylckama Vlieg and Janssen 2001). The problem may be overcome by developing genetically engineered microbial strains or by developing an efficient consortium from natural degraders. The metabolic

synergism between different microbial species encourages the biodegradation of recalcitrant molecules via aerobic and anaerobic reactions.

Immobilization of the pure cultures and consortium may help to improve bioremediation potential as immobilized cells have prolonged microbial cell viability ranging from weeks to months and improved capacity to tolerate higher concentrations of pollutants (Richins *et al.*, 2000; Chen and Georgiou 2002).

Khanna and Vidyalakshmi (2004) developed microbial consortia, Q1 and Q2 from chlorpyriphos contaminated sites by selective enrichment with degradation efficiency of 72 and 70 per cent respectively. Mixed population of fungi, such as *Alternaria alternata*, *Cephalosporium* sp., *Cladosporium cladosporioides*, *Cladorrhinum brunnescens*, *Fusarium* sp., *Rhizoctonia solani*, and *T. viride*, reveal the degradation of chlorpyrifos in liquid culture more efficiently (Singh, 2006). Vidya Lakshmi *et al.*, 2008 developed a microbial consortium consisting of *Pseudomonas fluorescence*, *Brucella melitensis*, *Bacillus subtilis*, *Bacillus cereus*, *Klebsiella* sp., *Serratia marcescens* and *Pseudomonas aeruginosa* which supported 75–87 per cent degradation of chlorpyrifos after 20 days of incubation.

Pino and Penuela, 2011 obtained simultaneous degradation of the pesticides methyl parathion and chlorpyrifos by bacterial consortium consisting of *Acinetobacter* sp, *Pseudomonas putida*, *Bacillus* sp, *Pseudomonas aeruginosa*, *Citrobacter freundii*, *Stenotrophomonas* sp, *Flavobacterium* sp., *Proteus vulgaris sp.*, *Pseudomonas* sp., *Acinetobacter* sp., *Klebsiella* sp. and *Proteus* sp. In culture medium enriched with each of the pesticides, the consortium was able to degrade 150 mg l^{-1} of methyl parathion and chlorpyrifos in 120 h. When a mixture of 150 mg l^{-1} of both pesticides was used the percentage decreased to 72 per cent for methyl parathion and 39 per cent for chlorpyrifos. Bacterial mixed cultures could be relatively effective in bioremediation of chlorpyrifos contaminated soil and water. Bacterial mixed culture GCC134 was more effective for degradation of

chlorpyrifos compared to individual culture of RCC -2 (*Pseudomonas* sp.) Sumit Kumar (2011). Four bacterial isolates namely *P. putida* (NII 1117), *Klebsiella* sp., (NII 1118), *P. stutzeri* (NII 1119), *P. aeruginosa* (NII 1120) which were more efficient were developed as consortium. The intracellular fractions of the consortium exhibited more organophosphorus hydrolase activity with chlorpyrifos concentration 500 mg l^{-1} (Sasikala *et al.*, 2012). The bacterial consortium of *Pseudomonas* sp. and *Brevibacillus* sp. could degrade 81 per cent of chlorpyrifos in the medium so that it can be potentially utilized for the bioremediation of contaminated soil (Barathidasan and Reetha, 2013).

2.6. BIOREMEDIATION OF CHLORPYRIFOS IN CONTAMINATED SOILS

Bioremediation which includes the effective utilization of microorganisms for the metabolism or biodegradation of target pollutants into safer and innocuous products is amongst the potent technologies that are being used globally for the restoration of contaminated sites. The introduction of microorganisms capable of *in situ* bioremediation into a competitive unsterile environment requires that the microorganism be able to survive in large numbers and also express the desired catabolic phenotype. (Anuja George, 2005)

Singh *et al.* (2004) in his work on biodegradation of chlorpyrifos by *Enterobacter* strain B-14 and its use in bioremediation of contaminated soils, noticed that the addition of strain B-14 (10^6 cells g^{-1}) to soil with a low indigenous population of chlorpyrifos degrading bacteria treated with 35 mg of chlorpyrifos kg^{-1} resulted in a higher degradation rate. The estimated half life of chlorpyrifos was less than 2 days for the first application which was further reduced to less than a day for the second and third treatments (6 and 10 days after first application) in both fumigated and non-fumigated soils. A new research direction for exploiting microorganisms and its development for bioremediation has emerged as a novel process. The chlorinated pyridinyl ring of chlorpyrifos undergoes cleavage during biodegradation by *P. chrysosporium.* But the degradation of chlorpyrifos proves more efficient by mixed populations than by

pure cultures of fungi (Yu *et. al.*, 2006). Fungal degradation of chlorpyrifos was reported in *Verticillium sp.* DSP in pure cultures and its use in bioremediation of contaminated soil (Fang *et al.*, 2008). A fungal strain capable of utilizing chlorpyrifos as sole carbon and energy sources from soil and degradation of chlorpyrifos in pure cultures and on vegetables by this fungal strain and its cell-free extract was also reported.

Prabakaran and Ramaswamy (1990) showed that seed treatment with seven pesticides including chlorpyrifos in green gram and blackgram reduced Rhizobium population to 67-99 per cent in green gram and 95-99 per cent in black gram. Similar studies wherein significant inhibition of nodulation due to chlorpyrifos application has also been reported (Dawson *et al.*, 2001). Rekha, (2005) studied the effect of chlorpyrifos on nodulation in cowpea under different soil conditions and observed that there was decline in nodule count on second and fourth week after application as compared to control. A significant inhibition of nodulation was observed. This could be due to higher persistence and slow rate of dissipation of chlorpyrifos resulting in an inhibition of nodulating bacteria in soil.

The study conducted by Parween *et al.* (2011) in *Vigna radiata* L. also found that application of chlorpyrifos at higher concentrations caused a negative impact on plant biometric characters, pigment and yield parameters. At higher concentrations, all the growth parameters such as plant height, number of branches, number of leaves per plant, total leaf area and plant biomass were remarkably reduced in all the growth phases under study. Similar suppression of biometric characters was noted by Warabi *et al.* (2001) who observed retarded cell growth and division in soy bean roots, cell elongation and conversion of indole-3 acetic acid (IAA) into various photooxidative products under higher concentration of applied insecticide. Action of OP pesticides as strong auxin antagonists to crops has been reported in terrestrial plants (Tevini and Teramura, 1989), maize and sugarcane (Luscombe *et al.*, 1995) and cucumber (Mishra *et al.* 2008). Retardation of yield attributing characters under high concentration of

pesticide was observed by other researchers also (Lagana *et al.*, 2000; Nakamura *et al.*, 2000).

Anuja George (2005) observed that, bioaugmentation of the promising chlorpyrifos degrading bacteria JA-8 and JA-15 individually and in combination improved the germination of seeds. In the treatment where chlorpyrifos spiked soil was inoculated with both the strains, the germination percentage of the cowpea seeds on 3 DAS was found to be higher (59.43 per cent) than the treatments with individual inoculations. It was found that the inoculation of JA-8 and JA-15 either individually or in combination improved the plant height of the seedlings when compared to the uninoculated control on all days of observation. There was a significant reduction in shoot dry weight, root dry weight and total biomass (dry weight) of the seedlings at 30 DAS in the pesticide spiked treatment when compared to the control treatment *i.e.* without any pesticide. However, these negative effects were nullified by the bioaugmentation of JA-8 and JA-15 either singly or dually. The dual inoculation resulted in the shoot dry weight of 1.35 g $plant^{-1}$, root dry weight of 0.41 g $plant^{-1}$ and a total biomass (dry weight) of 1.76 g per plant. The uninoculated control treatment resulted in 0.76, 0.17 and 0.93 g per plant respectively. She also noticed that the population of different groups of microorganisms *i.e.*, bacteria, fungi and actinomycetes in chlorpyrifos spiked soil were severely declined plant growth period. However, they were increased due to the bioaugmentation with the degrading strains singly or dually. The population of chlorpyrifos degrading bacteria in the polluted soil was also found to increase throughout the period of investigation due to breakdown of the pesticide. The biodegradation of the pesticide by the introduced strains either singly or dually was two times higher than that of the environmental degradation by the native strains.

Materials and methods

3. MATERIALS AND METHODS

A study was undertaken to isolate, screen and evaluate microorganisms capable of degradation of organophosphorus pesticide - chlorpyrifos for the bioremediation of contaminated soils. The experiments were carried out during 2012-2014 in the Department of Agricultural Microbiology at College of Agriculture Vellayani. Details of the materials used and the methods followed for the study are furnished below:

3.1. SOIL SAMPLING

Soil samples were collected from the following locations of Idukki district where chlorpyrifos application was a routine practice to control various pests of cardamom and high residue level has been reported (Beevi *et al.*, 2014). The locations for collection of soil samples were selected in consultation with the Pesticide Residue Lab at College of Agriculture, Vellayani.

1. Pampadumpara
2. Puliyanmala
3. Thenamakkal

3.2. CHLORPYRIFOS

Technical formulation of chlorpyrifos insecticide of 99 percent purity was purchased from Sigma Aldrich- chemicals, Bangalore and was used uniformly for laboratory studies. For field experiment, commercial formulation of chlorpyrifos, Radar 20EC was used.

3.3. ISOLATION OF MICROORGANISMS CAPABLE OF DEGRADATION OF ORGANOPHOSPHORUS PESTICIDE- CHLORPYRIFOS

Microorganisms capable of degradation of chlorpyrifos were isolated from soil samples by enrichment culture technique. Rhizosphere soil was collected from cardamom plantations of Idukki district, where high residue level of chlorpyrifos has been reported. The soil samples collected from different locations

were filled into earthen flower pots of size 25 cm diameter after sealing the hole with cement. The pots were enriched by weekly addition of chlorpyrifos (100 ppm) upto 8-10 weeks. After enrichment, one gram of enriched soil was added to mineral salts medium (Karpouzas and Walker, 2000; Singh *et al.*, 2004), supplemented with 100 ppm chlorpyrifos and incubated at room temperature. After one week, one ml from the above medium was transferred to fresh MS medium containing chlorpyrifos and this was repeated 2-3 times for further purification. Finally one ml was plated on MS media containing 100 ppm chlorpyrifos.

As many as nineteen isolates comprising eleven bacteria, seven fungi and one actinomycete capable of utilizing chlorpyrifos as carbon source were isolated by enrichment culture technique, these isolates were allotted code numbers starting from M1-M19.

3.4. PRELIMINARY SCREENING OF THE ISOLATES

The bacterial isolates which showed significant growth on the plates were purified following streak plate method. Single colonies were transferred to MSM slants for further studies. Similarly the purified fungal and actinomycetes colonies were also transferred to MSM slants for further studies. All the 19 isolates obtained were subjected to a preliminary screening to assess the ability of the isolates to utilize chlorpyrifos as carbon source in five different concentrations – 50, 100, 200, 400 and 800 ppm at intervals of 7,15,20,25 and 30 days after inoculation with two replications each. The growth of bacterial isolates was assessed based on optical density (OD value) at 660 nm and for fungi and Actinomycetes, total viable count was assessed at different intervals of 7, 15, 20, 25 and 30 days after inoculation. The isolates which showed significant growth in 50, 100, 200, 400 and 800 ppm concentrations at different intervals were selected for further studies.

3.5. *IN VITRO* EVALUATION OF THE ISOLATES

The isolates which showed significant growth in 100,200,400 and 800 ppm concentrations of chlorpyrifos at different intervals were once again evaluated by secondary screening to assess the ability of the isolates to utilize chlorpyrifos as sole carbon source. Mineral salts medium amended with 100, 200, 400 and 800 ppm concentrations of chlorpyrifos was prepared and the selected isolates were inoculated to MSM containing 100,200,400 and 800 ppm concentrations of chlorpyrifos and incubated at room temperature. Two replications were maintained for each treatment. At regular intervals of 7, 15, 20, 25 and 30 days after inoculation, population build up was assessed based on total viable count. The degradation potential of the selected isolates was evaluated by analyzing the residue of chlorpyrifos in the medium in GC-MS. The release of chloride into the medium was also analyzed by Argentometric method. The isolates which recorded maximum population buildup in different concentrations of chlorpyrifos and maximum degradation of chlorpyrifos were selected for further studies.

3.6. BIODEGRADATION STUDIES

3.6.1. Chlorpyrifos Residue Analysis in MSM

Chlorpyrifos residue was analyzed by the method of Mukherjee *et al.* (2007). In this assay, at defined intervals, 750 ml distilled water was taken in one liter separating funnel. One ml of sample was transferred in to the flask and added 150g NaCl and 75 ml DCM and shaken in a mechanical shaker at 250 rpm for 5 minutes. Collected organic layer (DCM) containing chlorpyrifos repeatedly partitioned with 40 ml DCM X 2 and 50 ml Hexane. Combined the organic layer and concentrated to 5 ml and added 2 X 20 ml n-Hexane, concentrated and made up to one ml. GC-ECD analysis was done and confirmed by GC-MS. The chlorpyrifos concentration was estimated by referring to the standard curve. The standard curve was prepared by using gradient concentrations of chlorpyrifos (50, 100, 200, 400, and 800 ppm). The residue was calculated as follows.

$$\text{Residue} = \frac{\text{Peak area of sample x Concentration of Std x Final volume of extract}}{\text{Peak area of Standard x Weight of sample (g) x Volume injected in (µl)}}$$

3.6.2. Chloride Analysis in MSM and Soil

The chloride released in to the medium was analyzed following the Argentometric method (Greenberg *et al.*, 1992). This is an indirect method of measuring chlorpyrifos degradation. The method has two steps.

a) Sample preparation

One ml sample was diluted to 10 ml and three ml of Al $(OH)_3$ suspension and one ml H_2O_2 were added and stirred for a minute.

b) Titration

The prepared sample was directly titrated with $AgNO_3$ (0.0141 N) in presence of one ml of K_2CrO_4 indicator solution in the pH range 7.0 to10.0, until a pinkish yellow colour end point was obtained. The same procedure was repeated for blank. Chloride released was calculated as follows.

$$\text{Chloride released } (mgl^{-1}) = \frac{(A-B) \times N \times 35.450}{\text{ml of sample}}$$

Where,

A = ml of sample

B = ml titration for blank

N = Normality of $AgNO_3$

3.7 CHARACTERIZATION OF ISOLATES

The selected efficient isolates capable of degrading chlorpyrifos were characterized based on morphological and molecular studies as detailed below. The isolates were identified up to generic level.

3.7.1 Morphological Characterization of Isolates

The colony morphology, texture and appearance of the isolates on petriplates were studied.

3.7.2 Molecular Characterization of Selected Isolates by DNA Sequencing Using Universal Primers of 18SrRNA

The isolates obtained from different locations were characterized on molecular basis by comparison of the rDNA sequences of the isolates. The procedure for molecular characterization was as follows:

3.7.2.1 *DNA Isolation Using Nucleo Spin Plant II Kit (Macherey-Nagel)*

About 25 mg of the mycelium was homogenized in four hundred microlitres of buffer PL1 and vortexed for 1 minute. Ten microlitres of RNase A solution was added and inverted to mix. The homogenate was incubated at 65°C for 10 minutes. The lysate was transferred to a Nucleospin filter and centrifuged at 11000 x g for 2 minutes. The flow through liquid was collected and the filter was discarded. Four hundred and fifty microlitres of buffer PC was added and mixed well. The solution was transferred to a Nucleospin Plant II column, centrifuged for 1 minute and the flow through liquid was discarded. Four hundred microlitre buffer PW1 was added to the column, centrifuged at 11000 x g for 1 minute and flow though liquid was discarded. Then 700 µl PW2 was added, centrifuged at 11000 x g and flow through liquid was discarded. Finally 200 µl of PW2 was added and centrifuged at 11000 x g for 2 minutes to dry the silica membrane. The column was transferred to a new 1.7 ml tube and 50 µl of buffer PE was added and incubated at 65°C for 5 minutes. The column was then centrifuged at 11000 x g for 1 minute to elute the DNA. The eluted DNA was stored at 4°C.

3.7.2.2 *Agarose Gel Electrophoresis for DNA Quality Check*

The quality of the DNA isolated was checked using agarose gel electrophoresis. 1µl of 6X gel-loading buffer (0.25% bromophenol blue, 30% sucrose in TE buffer pH-8.0) was added to 5µl of DNA. The samples were loaded

to 0.8% agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 µg/ml ethidium bromide. Electrophoresis was performed with 0.5X TBE as electrophoresis buffer at 75 V until bromophenol dye front has migrated to the bottom of the gel. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

3.7.2.3 *PCR Analysis*

PCR amplification reactions were carried out in a 20 µl reaction volume which contained 1X Phire PCR buffer (contains 1.5 mM $MgCl_2$), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 1 µl DNA, 0.2 µl Phire Hotstart II DNA polymerase enzyme, 0.1 mg/ml BSA and 3% DMSO, 0.5M Betaine, 5pM of forward and reverse primers.

Primers used

Target	Primer Name	Direction	Sequence (5' → 3')
18S	NS1	Forward	GTAGTCATATGCTTGTCTC
	NS4	Reverse	CTTCCGTCAATTCCTTTAAG
	NS3	Reverse	CCTTGTTACGACTTCACCTTCCTCT

The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems).

PCR amplification profile

18S

98 °C	-	30 sec	
98 °C	-	5 sec	} 40 cycles
60 °C	-	10 sec	
72 °C	-	15 sec	
72 °C	-	60 sec	
4 °C	-	∞	

3.7.2.4 *Agarose Gel Electrophoresis of PCR Products*

The PCR products were checked in 1.2% agarose gel prepared in 0.5X TBE buffer containing 0.5 µg/ml ethidium bromide. 1 µl of 6X loading dye was mixed with 5 µl of PCR products and was loaded and electrophoresis was performed at 75V power supply with 0.5X TBE as electrophoresis buffer for about 1-2 hours, until the bromophenol blue front had migrated to almost the bottom of the gel. The molecular standard used was 2-log DNA ladder (NEB). The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

3.7.2.5 *ExoSAP-IT Treatment*

ExoSAP-IT (GE Healthcare) consists of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP), in a specially formulated buffer for the removal of unwanted primers and dNTPs from a PCR product mixture with no interference in downstream applications. Five micro litres of PCR product was mixed with 2 µl of ExoSAP-IT and incubated at 37°C for 15 minutes followed by enzyme inactivation at 80°C for 15 minutes.

3.7.2.6 *Sequencing Using BigDye Terminator v3.1*

Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems , USA) following manufactures protocol.
The PCR mix consisted of the following components:

PCR Product (ExoSAP treated)	-	10-20 ng
Primer	-	3.2 pM (either Forward or Reverse)
Sequencing Mix	-	0.28 µl
5x Reaction buffer	-	1.86 µl
Sterile distilled water	-	make up to 10µl

The sequencing PCR temperature profile consisted of a 1st cycle at 96°C for 2 minutes followed by 30 cycles at 96°C for 30 sec, 50°C for 40 sec and 60°C for 4 minutes for all the primers.

3.7.2.7 *Post Sequencing PCR Clean up*

1. Make master mix I of 10µl milli Q and 2 µl 125mM EDTA per reaction
2. Add 12µl of master mix I to each reaction containing 10µl of reaction contents and are properly mixed.
3. Make master mix II of 2 µl of 3M sodium acetate pH 4.6 and 50 µl of ethanol per reaction.
4. Add 52 µl of master mix II to each reaction.
5. Contents are mixed by inverting.
6. Incubate at room temperature for 30 minutes
7. Spin at 14,000 rpm for 30 minutes
8. Decant the supernatant and add 100 µl of 70% ethanol
9. Spin at 14,000 rpm for 20 minutes.
10. Decant the supernatant and repeat 70% ethanol wash
11. Decant the supernatant and air dry the pellet.

The cleaned up air dried product was sequenced in ABI 3500 DNA Analyzer (Applied Biosystems).

3.7.2.8 *Sequence Analysis*

The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1 (Drummond *et al.*, 2010).

3.7.2.9 *Bioinformatics*

The obtained rDNA sequences of all the selected isolates were searched in the NCBI (National centre for Biotechnology Information) database using BLAST (N) (Basic Local Alignment Search Tool (Nucleotide)) and tBLASTx. Based on

genetic similarity, the organisms were identified. The sequences were put into Clustal omega database for obtaining the phylogenetic tree using neighbor joining method.

3.8 DEVELOPMENT OF CONSORTIUM OF ISOLATES

With the objective of developing a consortium, the selected fungal isolates were co-cultured in Mineral salts medium and Potato dextrose broth and their compatibility was studied by assessing the population build up of each of the individual isolates of consortium, recorded as viable count on 7 DAI. The compatibility of the isolates was further confirmed by dual culture technique.

3.8.1 Dual culture technique

The dual culture technique outlined by Skidmore and Dickinson (1967) was followed for studying the antagonistic activity among the selected isolates. Agar discs of five mm diameter of each isolate were placed five cm apart on opposite sides of the petriplates containing sterilized PDA. The plates were then incubated at 28 ± 2^0C for observing the suppression. Control consisted of petridishes inoculated with five mm culture disc of individual isolates alone were maintained. The plates were observed from the third day of inoculation at 24 h interval for about seven days.

3.9 EVALUATION OF THE CONSORTIUM UNDER *IN VITRO* CONDITIONS

The ability of the developed consortium to degrade 50, 100, 200, 400 and 800 ppm concentrations of chlorpyrifos was evaluated under *in vitro* conditions, at intervals of 7, 15, 20, 25 and 30 day after inoculation. Two replications were maintained for each treatment. Growth of isolates was measured by enumerating the total viable count expressed per ml of media. The degradation potential of the consortium was assessed by analyzing the residual chlorpyrifos in the medium in GC-MS. The release of end product, chloride was also measured by Argentometric method at intervals of 7, 15, 20, 25 and 30 days after inoculation.

3.10 EVALUATION OF THE BIOREMEDIATION EFFICIENCY OF THE DEVELOPED CONSORTIUM IN CONTAMINATED SOIL UNDER POT CULTURE CONDITIONS

3.10.1 Location

The experiment was conducted in the green house of the Department of Agricultural Microbiology, Vellayani, Thiruvananthapuram.

Uniform sized earthern pots of 25 cm diameter were used for the experiment after cementing the holes to avoid seepage of chlorpyrifos and filled with 5 kg each of sterilized soil. The formulated liquid consortium of efficient chlorpyrifos degraders containing M5 (80.50 $x10^3$ cfu ml^{-1}), M6 (141.50 $x10^3$ cfu ml^{-1}), M7 (239.0 $x10^3$ cfu ml^{-1}) and M17 (186.50 $x10^3$ cfu ml^{-1}) in MSM was evaluated in chlorpyrifos spiked soil at two different concentrations viz., 100 and 400 ppm with cowpea as the test crop.

Design : CRD

Treatments : 5

Replication : 4

3.10.2 Treatments

The commercial formulation of chlorpyrifos (Radar 20EC) manufactured by Isagro Asia was purchased from local market and applied at two different levels *viz.* 100 ppm and 400 ppm.

T1 : Unspiked soil + cowpea

T2 : Soil spiked with chlorpyrifos (100 ppm) + cowpea

T3 : Soil spiked with chlorpyrifos (100 ppm) +consortium+ cowpea

T4 : Soil spiked with chlorpyrifos (400 ppm) +cowpea

T5 : Soil spiked with chlorpyrifos (400 ppm) +consortium +cowpea

The biometric characters of plants such as plant height, fresh weight and dry weight of plant and roots, nodulation parameter such as nodule number, nodule fresh weight and dry weight and yield were recorded to study the effect of

spiking of chlorpyrifos and application of consortium. The residue of chlorpyrifos in soil was estimated by GC-MS and the chloride released in to the soil was measured by Argentometric method. The population of total soil micro flora and colonization of chlorpyrifos degraders in rhizosphere of cowpea were enumerated by estimating the total viable count per gram of soil.

3.10.3 Chlorpyrifos residue analysis in soil

Extraction and analysis: Twenty five grams of treated soil samples were air dried for extraction and homogenized with 0.5 g charcoal (activated) for 4 hrs at 120°C. Then added 1.0 g Florisil (activated for 4 hours at 650°C) and 5 drops of 25% NH_4OH solution, placed over a 2.5 cm layer of anhydrous sodium sulphate (analytical grade) in a glass column with 34 cm length and 2.5 cm dia. Extraction was done by using a solution of n-hexane (distilled) and acetone (distilled) in a ratio of 2:1 by the method as described by Mumtaz *et al.*, (1983). Eluted material was collected in a 250 ml conical flask (Pyrex) and later evaporated on rotary evaporator to almost dryness, dissolved in 2-5 ml quantity n-hexane in small glass vials for GC-MS determination.

3.11 STATISTICAL ANALYSIS

The data generated from the experiments were statistically analyzed using analysis of variance techniques (ANOVA) as applied to Completely Randomized Design described by Panse and Sukhatme, 1985.

Results

4. RESULTS

The present study on "Isolation, characterization and evaluation of soil microorganisms for bioremediation of chlorpyrifos' was conducted during the period from 2012-2014 in the Department of Agricultural Microbiology, College of Agriculture, Vellayani, Thiruvananthapuram, Kerala. Investigations were carried out to isolate, screen and evaluate microorganisms capable of degrading chlorpyrifos, belonging to the organophoshorus group of pesticides. Efficient microorganisms capable of degradation were formulated as a consortium. Further, the consortium developed was used for *in vivo* bioremediation of chlorpyrifos spiked soil. The results obtained on these studies are presented below:

4.1. ISOLATION OF MICROORGANISMS CAPABLE OF DEGRADATION OF ORGANOPHOSPHORUS PESTICIDE- CHLORPYRIFOS

Microorganisms capable of degrading chlorpyrifos were isolated by enrichment culture technique from identified locations having high toxic level of chlorpyrifos residue. The locations were selected in consultation with the Pesticide Residue Laboratory at College of Agriculture, Vellayani. Soil samples were collected from Cardamom fields of Idukki district where chlorpyrifos was applied to control various pests.

Nineteen different microorganisms comprising eleven bacteria, seven fungi, one actinomycete were isolated by enrichment culture technique. These isolates were allotted code numbers from M1-M19 as shown in Table 1.

4.2. PRELIMINARY SCREENING OF THE ISOLATES

All the nineteen isolates obtained were subjected to a preliminary screening to assess the ability of the isolates to utilize chlorpyrifos as carbon source in five different concentrations- 50, 100, 200, 400 and 800 ppm based on OD value (optical density) for bacteria and total viable count for fungi and

Table 1. List of isolated microorganisms

Sl.No	Isolates	Type of Microorganism
1	M1	Bacteria
2	M2	Bacteria
3	M3	Bacteria
4	M4	Bacteria
5	M5	Fungus
6	M6	Fungus
7	M7	Fungus
8	M8	Bacteria
9	M9	Bacteria
10	M10	Bacteria
11	M11	Fungus
12	M12	Actinomycete
13	M13	Bacteria
14	M14	Bacteria
15	M15	Bacteria
16	M16	Bacteria
17	M17	Fungus
18	M18	Fungus
19	M19	Fungus

actinomycete at different intervals of 7, 15, 20, 25 and 30 days after inoculation (Plate 1).

4.2.1. Preliminary Screening to Assess the Growth of Bacterial Isolates in 50 ppm Concentration of Chlorpyrifos in MSM

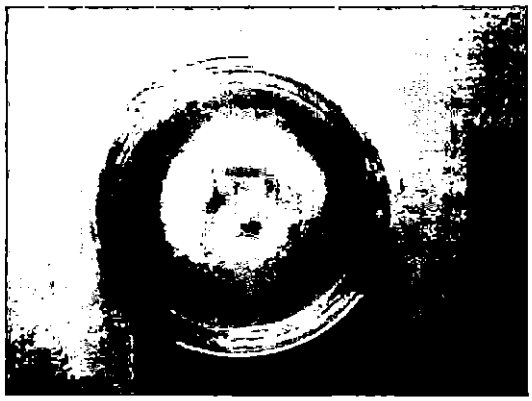
The eleven bacterial isolates obtained were subjected to preliminary screening to assess their growth in 50, 100, 200, 400 and 800 ppm concentrations of chlorpyrifos at different intervals of 7, 15, 20, 25 and 30 days after inoculation and the results are presented in Table 2-6.

On 7th day, maximum growth of 0.280 was recorded with isolate M10 which was significantly superior to all other isolates followed by M3 (0.129), M4 (0.078), M15 (0.070), M16 (0.070), M1 (0.067), M9 (0.062), M8 (0.060), M13 (0.044), M14 (0.030) and M2 (0.017).

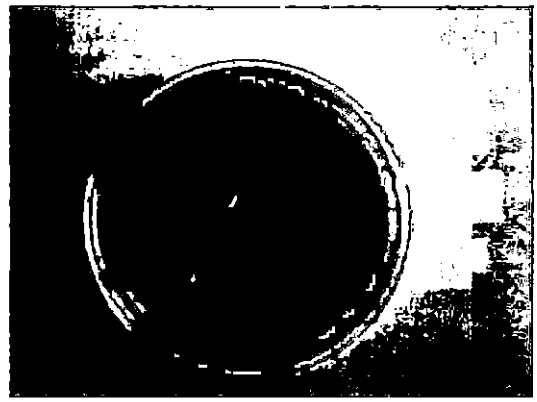
The maximum growth of 0.279 was recorded with isolate M10 on 15th day of inoculation which was significantly superior to all other isolates followed by M3 (0.153), M4 (0.079), M16 (0.077), M15 (0.073), M9 (0.072), M8 (0.071), M1 (0.065), M13 (0.043), M14 (0.032) and M2 (0.026).

After 20 days of inoculation, maximum growth of 0.30 was recorded with isolate M10 which was significantly superior to all other isolates followed by M3 (0.140), M4 (0.081), M16 (0.078), M15 (0.074), M9 (0.073), M8 (0.073), M13(0.058), M1 (0.052), M14 (0.041) and M2 (0.015).

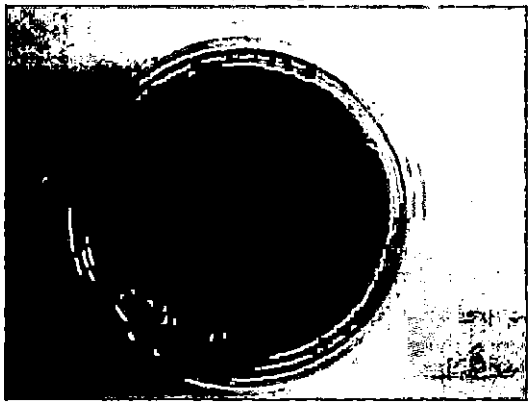
Isolate M10 showed maximum growth of 0.30 on 25th day of inoculation which was significantly superior to all other isolates followed by M3 (0.157), M9 (0.091), M16 (0.078), M15 (0.077), M4 (0.074), M8 (0.071), M1 (0.066), M13 (0.059), M14 (0.041) and M2 (0.016).



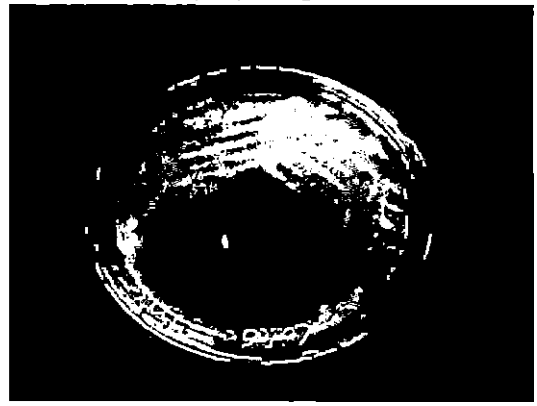
(M5) Fungus



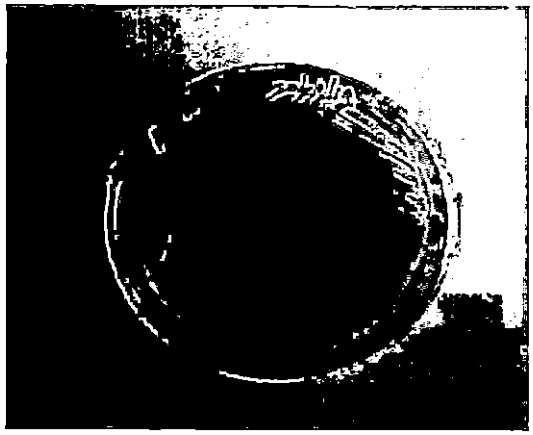
(M6) Fungus



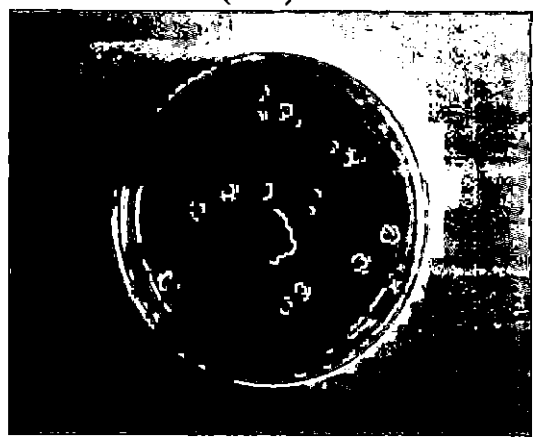
(M7) Fungus



(M10) Bacteria



(M12) Actinomycete



(M17) Fungus

Plate1. Selected isolates after preliminary screening

After 30 days of inoculation, maximum growth of 0.299 was recorded with isolate M10 which was significantly superior to all other bacterial isolates followed by M3 (0.159), M9 (0.105), M15 (0.084), M16 (0.076), M4 (0.075), M8 (0.074), M13 (0.049), M14 (0.049), M1 (0.045) and M2 (0.016).

Thus in 50 ppm concentration, maximum growth was recorded by isolate M10 followed by M3 on 7^{th} ,15^{th} ,20^{th} ,25^{th} and 30^{th} day after inoculation.

4.2.2. Preliminary Screening to Assess the Growth of Bacterial Isolates in 100 ppm Concentration of Chlorpyrifos in MSM

After 7 days of inoculation, maximum growth of 0.292 was recorded with isolate M10 which was significantly superior to all other isolates followed by M9 (0.166), M3 (0.132), M4 (0.089), M15 (0.084), M16 (0.079), M8 (0.069), M1 (0.068), M13 (0.050), M14 (0.039) and M2 (0.017).

After 15 days of inoculation, maximum growth of 0.292 was recorded with isolate M10 which was significantly superior to all other isolates followed by M9 (0.166), M3 (0.154), M4 (0.089), M15 (0.086), M16 (0.079), M8 (0.075), M1(0.069), M13 (0.052), M14 (0.041) and M2 (0.028).

On 20^{th} day, maximum growth of 0.301 was recorded with isolate M10 which was significantly superior to all other isolates followed by M9 (0.173), M3 (0.157), M4 (0.091), M15 (0.089), M16 (0.079), M8 (0.077), M13 (0.055), M1 (0.054), M2 (0.054), and M14 (0.043).

The isolate M10 showed maximum growth of 0.302 on 25^{th} day of inoculation which was significantly superior to all other isolates followed by M9 (0.178), M3 (0.159), M4 (0.094), M15 (0.090), M16 (0.079), M8 (0.075), M1 (0.069), M2 (0.068), M13 (0.059) and M14 (0.045).

Table 2. Preliminary screening to assess the growth of bacterial isolates in 50 ppm concentration of chlorpyrifos in MSM

*Optical Density (absorbance at 660 nm)					
Isolates	7th DAI	15th DAI	20th DAI	25th DAI	30th DAI
M1	0.067	0.065	0.052	0.066	0.045
M2	0.017	0.026	0.015	0.016	0.016
M3	0.129	0.153	0.140	0.157	0.159
M4	0.078	0.079	0.081	0.074	0.075
M8	0.060	0.071	0.073	0.071	0.074
M9	0.062	0.072	0.073	0.091	0.105
M10	0.280	0.279	0.300	0.300	0.299
M13	0.044	0.043	0.058	0.059	0.049
M14	0.030	0.032	0.041	0.041	0.049
M15	0.070	0.073	0.074	0.077	0.084
M16	0.070	0.077	0.078	0.078	0.076
CD (0.05)	0.016	0.011	0.013	0.016	0.018

*Mean of two replications

Table 3. Preliminary screening to assess the growth of bacterial isolates in 100 ppm concentration of chlorpyrifos in MSM

*Optical Density (absorbance at 660 nm)					
Isolates	7th DAI	15th DAI	20th DAI	25th DAI	30th DAI
M1	0.068	0.069	0.054	0.069	0.051
M2	0.017	0.028	0.054	0.068	0.066
M3	0.132	0.154	0.157	0.159	0.157
M4	0.089	0.089	0.091	0.094	0.095
M8	0.069	0.075	0.077	0.075	0.077
M9	0.166	0.166	0.173	0.178	0.180
M10	0.292	0.292	0.301	0.302	0.302
M13	0.050	0.052	0.055	0.059	0.060
M14	0.039	0.041	0.043	0.045	0.047
M15	0.084	0.086	0.089	0.090	0.090
M16	0.079	0.079	0.079	0.079	0.080
CD (0.05)	0.021	0.018	0.020	0.015	0.015

*Mean of two replications

On 30th day, maximum growth of 0.302 was recorded with isolate M10 which was significantly superior to all other isolates followed by M9 (0.180), M3 (0.157), M4 (0.095), M15 (0.090), M16 (0.080), M8 (0.077), M2 (0.066), M13 (0.060), M1 (0.051) and M14 (0.047).

In 100 ppm concentration, the isolate M10 showed maximum growth which was significantly superior to all other isolates followed by M9 on 7th, 15th, 20th, 25th and 30th day after inoculation.

4.2.3. Preliminary Screening to Assess the Growth of Bacterial Isolates in 200 ppm Concentration of Chlorpyrifos in MSM

On 7th day, maximum growth of 0.305 was recorded with isolate M10 which was significantly superior to all other isolates followed by M9 (0.174), M3 (0.149), M4 (0.097), M8 (0.086), M15 (0.086), M16 (0.082), M1 (0.081), M13 (0.063), M2 (0.041) and M14 (0.057).

On 15th day, maximum growth of 0.313 was recorded with isolate M10 which was significantly superior to all other isolates followed by M9 (0.175), M3 (0.149), M4 (0.098), M1 (0.090), M8 (0.087), M15 (0.087), M16 (0.082), M13 (0.064), M14 (0.060) and M2 (0.042).

The isolate M10 showed maximum growth of 0.316 on 20th day after inoculation and was significantly superior to all other isolates followed by M9 (0.177), M3 (0.150), M4 (0.098), M1 (0.094), M8 (0.088), M15 (0.087), M16 (0.082), M13 (0.065), M14 (0.062) and M2 (0.038).

On 25th day, maximum growth of 0.318 was recorded with isolate M10 which was significantly superior to all other bacterial isolates followed by M9 (0.180), M3 (0.155), M4 (0.099), M1 (0.094), M8 (0.089), M15 (0.088), M16 (0.084), M13 (0.066), M14 (0.064) M2 (0.039).

After 30 days of inoculation the maximum growth of 0.318 was recorded with isolate M10 which was significantly superior to all other isolates followed by M9 (0.181), M3 (0.156), M4 (0.099), M1 (0.095), M8 (0.090), M15 (0.088), M16 (0.084), M13 (0.067), M14 (0.061) and M2 (0.039).

Thus in 200 ppm concentration, maximum growth was recorded by isolate M10 followed by M9 on 7^{th}, 15^{th}, 20^{th}, 25^{th} and 30^{th} day after inoculation.

4.2.4. Preliminary Screening to Assess the Growth of Bacterial Isolates in 400 ppm Concentration of Chlorpyrifos in MSM

After 7 days of inoculation, maximum growth of 0.284 was recorded with isolate M10 which was significantly superior to all other isolates followed by M3 (0.135), M9 (0.103), M4 (0.099), M1 (0.087), M8 (0.074), M15 (0.076), M16 (0.063), M13 (0.057), M14 (0.045) and M2 (0.024).

On 15^{th} day, maximum growth of 0.287 was recorded with isolate M10 which was significantly superior to all other isolates followed by M3 (0.135), M9 (0.106), M4 (0.105), M1 (0.088), M15 (0.076), M8 (0.074), M16 (0.064), M13 (0.060), M14 (0.045) and M2 (0.026).

On 20^{th} day, maximum growth of 0.289 was recorded with isolate M10 which was significantly superior to all other isolates followed by M4 (0.130), M3 (0.128), M9 (0.107), M1 (0.088), M15 (0.077), M8 (0.074), M16 (0.064), M13 (0.062), M14 (0.046) and M2 (0.025).

After 25 days of incubation, the maximum growth of 0.295 was recorded with isolate M10 which was significantly superior to all other isolates followed by M3 (0.130), M4 (0.120), M9 (0.109), M1 (0.088), M15 (0.077), M8 (0.075), M16 (0.064), M13 (0.064), M14 (0.048) and M2 (0.024).

Table 4. Preliminary screening to assess the growth of bacterial isolates in 200 ppm concentration of chlorpyrifos in MSM

*Optical Density (absorbance at 660 nm)					
Isolates	7th DAI	15th DAI	20th DAI	25th DAI	30th DAI
M1	0.081	0.090	0.094	0.094	0.095
M2	0.041	0.042	0.038	0.039	0.039
M3	0.149	0.149	0.150	0.155	0.156
M4	0.097	0.098	0.098	0.099	0.099
M8	0.086	0.087	0.088	0.089	0.090
M9	0.174	0.175	0.177	0.180	0.181
M10	0.305	0.313	0.316	0.318	0.318
M13	0.063	0.064	0.065	0.066	0.067
M14	0.057	0.060	0.062	0.064	0.061
M15	0.086	0.087	0.087	0.088	0.088
M16	0.082	0.082	0.082	0.084	0.084
CD (0.05)	0.012	0.012	0.014	0.016	0.016

*Mean of two replications

Table 5. Preliminary screening to assess the growth of bacterial isolates in 400 ppm concentration of chlorpyrifos in MSM

*Optical Density (absorbance at 660 nm)					
Isolates	7th DAI	15th DAI	20th DAI	25th DAI	30th DAI
M1	0.087	0.088	0.088	0.088	0.089
M2	0.024	0.026	0.025	0.024	0.024
M3	0.135	0.135	0.128	0.130	0.130
M4	0.099	0.105	0.130	0.120	0.135
M8	0.074	0.074	0.074	0.075	0.075
M9	0.103	0.106	0.107	0.109	0.111
M10	0.284	0.287	0.289	0.295	0.296
M13	0.057	0.060	0.062	0.064	0.065
M14	0.045	0.045	0.046	0.048	0.049
M15	0.076	0.076	0.077	0.077	0.074
M16	0.063	0.064	0.064	0.064	0.065
CD (0.05)	0.025	0.025	0.021	0.025	0.025

*Mean of two replications

After 30 days of inoculation, maximum growth of 0.296 was recorded with isolate M10 which was significantly superior to all other isolates followed by M4 (0.135), M3 (0.130), M9 (0.111), M1 (0.089), M8 (0.075), M15 (0.074), M16 (0.065), M13 (0.065), M14 (0.049) and M2 (0.024).

Among the eleven isolates tested, isolate M10 recorded the maximum growth in 400 ppm concentration followed by M3 on 7^{th}, 15^{th}, 20^{th} and 25^{th} day and M4 on 20^{th} and 30^{th} days after inoculation.

4.2.5. Preliminary Screening to Assess the Growth of Bacterial Isolates in 800 ppm Concentration of Chlorpyrifos in MSM

On 7^{th} day, maximum growth of 0.132 was recorded with isolate M10 which was significantly superior to all other isolates followed by M16 (0.031), M15 (0.020), M8 (0.016), M13 (0.016), M4 (0.015), M3 (0.015), M14 (0.015), M9 (0.015), M1 (0.014) and M2 (0.013).

On 15^{th} day, maximum growth of 0.133 was recorded with isolate M10 which was significantly superior to all other isolates followed by M16 (0.033), M15 (0.021), M8 (0.017), M4 (0.016), M3 (0.016), M13 (0.016), M9 (0.015), M14 (0.015), M1 (0.014) and M2 (0.014).

After 20 days of inoculation, maximum growth of 0.134 was recorded with isolate M10 which was significantly superior to all other isolates followed by M16 (0.034), M15 (0.022), M8 (0.018), M4 (0.018), M9 (0.018), M3 (0.017), M13 (0.017), M14 (0.016),M1 (0.015) and M2 (0.015).

After 25 days of inoculation, maximum growth of 0.134 was recorded with isolate M10 which was significantly superior to all other isolates followed by M16 (0.035), M15 (0.022), M4 (0.019), M8 (0.019), M13 (0.018), M2 (0.018), M3 (0.018), M9 (0.018), M1 (0.016) and M14 (0.016).

Table 6. Preliminary screening to assess the growth of bacterial isolates in 800 ppm concentration of chlorpyrifos in MSM

*Optical Density (absorbance at 660 nm)					
Isolates	7^{th} DAI	15^{th} DAI	20^{th} DAI	25^{th} DAI	30^{th} DAI
M1	0.014	0.014	0.015	0.016	0.014
M2	0.013	0.014	0.015	0.018	0.016
M3	0.015	0.016	0.017	0.018	0.016
M4	0.015	0.016	0.018	0.019	0.018
M8	0.016	0.017	0.018	0.019	0.018
M9	0.015	0.017	0.018	0.018	0.018
M10	0.132	0.133	0.134	0.134	0.133
M13	0.016	0.016	0.017	0.018	0.017
M14	0.015	0.015	0.016	0.016	0.016
M15	0.020	0.021	0.022	0.022	0.022
M16	0.031	0.033	0.034	0.035	0.036
CD (0.05)	0.006	0.005	0.005	0.005	0.004

*Mean of two replications

On 30th day, maximum growth of 0.133 was recorded with isolate M10 which was significantly superior to all other isolates followed by M16 (0.036), M15 (0.022), M9 (0.018), M4 (0.018), M8 (0.018), M13 (0.017), M3 (0.016), M14 (0.016), M2 (0.016) and M1 (0.014).

In 800 ppm concentration maximum growth was recorded by isolate M10 followed by M16 on 7th, 15th, 20th, 25th and 30th day after inoculation.

The results of the preliminary screening showed that the growth of M10 was significantly superior to all the other isolates in 50,100,200,400 and 800 ppm concentrations at 7th, 15th, 20th, 25th and 30th days after inoculation.

The remaining seven fungal and one actinomycete isolate were screened based on the population build up in 50,100,200,400 and 800 ppm concentrations at different intervals of 7,15,20,25 and 30 days after inoculation and the results are presented in (Tables 7-11).

4.2.6. Preliminary Screening to Assess the Population Build up of Fungal and Actinomycetes Isolates in 50 ppm Concentration of Chlorpyrifos in MSM

After 7 days of inoculation, maximum colony count of 100 x 10^3 cfu ml^{-1} was recorded with isolate M7 which was statistically on par with M17 which recorded a colony count of 92 x 10^3 followed by M6 (80.50 x10^3 cfu ml^{-1}), M5 (65 x10^3 cfu ml^{-1}), M12 (59 x10^3 cfu ml^{-1}), M11 (48.50 x10^3 cfu ml^{-1}), M18 (35.50 x 10^3 cfu ml^{-1}) and M19 (29.50 x 10^3 cfu ml^{-1}).

On 15th day the maximum colony count of 112 x 10^3 cfu ml^{-1} was recorded with isolate M7 which was statistically on par with M17 which recorded a colony count of 108 x10^3 followed by isolate M6 (89.50 x10^3 cfu ml^{-1}), M5 (81.50 x10^3 cfu ml^{-1}), M12 (71 x10^3 cfu ml^{-1}), M11 (52 x10^3 cfu ml^{-1}), M18 (38.50 x 10^3 cfu ml^{-1}) and M19 (26.50 x 10^3 cfu ml^{-1}).

After 20 days of inoculation, maximum colony count of 119.50 x 10^3 cfu ml^{-1} was recorded with isolate M17 which was statistically on par with M7 which recorded a colony count of 118.50 x10^3 cfu ml^{-1} followed by isolate M6 (106 x10^3 cfu ml^{-1}), M5 (104.50 x10^3 cfu ml^{-1}), M12 (96.50 x10^3 cfu ml^{-1}), M18 (49.50 x 10^3 cfu ml^{-1}), M11 (43 x10^3 cfu ml^{-1}) and M19 (36.50 x 10^3 cfu ml^{-1}).

On 25th day, the maximum colony count of 123.50 x 10^3 cfu ml^{-1} was recorded with isolate M17 which was statistically on par with M7 which recorded a colony count of 122 x10^3 cfu ml^{-1} followed by isolate M6 (106x10^3 cfu ml^{-1}), M5 (98.50 x10^3 cfu ml^{-1}), M12 (89.50 x10^3 cfu ml^{-1}), M18 (40 x 10^3 cfu ml^{-1}), M11 (39.50 x10^3 cfu ml^{-1}) and M19 (28 x 10^3 cfu ml^{-1}).

On 30th day, the maximum colony count of 124.50 x 10^3 cfu ml^{-1} was recorded with isolate M7 which was significantly superior to all other isolates followed by M17(110 x10^3 cfu ml^{-1}), M6(90x10^3 cfu ml^{-1}), M12(89 x10^3 cfu ml^{-1}), M5(82 x10^3 cfu ml^{-1}), M18(30.50 x 10^3 cfu ml^{-1}), M11(22 x10^3 cfu ml^{-1}) and M19(20.50 x 10^3 cfu ml^{-1}).

In 50 ppm concentration, maximum colony count was recorded by isolate M7 which was statistically on par with M17 followed by M6 on 7th, 15th and 30th day after inoculation. However, on 20th and 25th day after inoculation, maximum colony count was recorded by isolate M17 which was statistically on par with M7 followed by M6.

4.2.7. Preliminary Screening to Assess the Population Build up of Fungal and Actinomycete Isolates in 100 ppm Concentration of Chlorpyrifos in MSM

On 7th day, the maximum colony count of 111 x 10^3 cfu ml^{-1} was recorded with isolate M7 which was significantly superior to all other isolates followed by M17 (101 x10^3 cfu ml^{-1}), M6 (90 x10^3 cfu ml^{-1}), M5 (71.5 x10^3 cfu ml^{-1}), M12 (65

x10^3 cfu ml^{-1}), M11 (34.50 x10^3 cfu ml^{-1}), M18 (20.50 x 10^3 cfu ml^{-1}) and M19 (19 x 10^3 cfu ml^{-1}).

After 15 days of inoculation the maximum colony count of 118.50 x 10^3 cfu ml^{-1} was recorded with isolate M7 which was significantly superior to all other isolates followed by M17 (110 x10^3 cfu ml^{-1}), M5 (101 x10^3 cfu ml^{-1}), M6 (100x10^3 cfu ml^{-1}), M12 (76.50 x10^3 cfu ml^{-1}), M11 (30.50 x10^3 cfu ml^{-1}), M18 (29.50 x 10^3 cfu ml^{-1}) and M19 (21.50 x 10^3 cfu ml^{-1}).

On 20th day, the maximum colony count of 123.50 x 10^3 cfu ml^{-1} was recorded with isolate M7 which was statistically on par with M6 and M17which recorded a colony count of 121.50x10^3 cfu ml^{-1} and 117.50 x10^3 cfu ml^{-1} respectively followed by M5 (111.50 x10^3 cfu ml^{-1}), M12 (100.50 x10^3 cfu ml^{-1}), M18 (32.50 x 10^3 cfu ml^{-1}), M11 (30.50 x10^3 cfu ml^{-1}) and M19 (28 x 10^3 cfu ml^{-1}).

On 25th day, the maximum colony count of 127 x 10^3 cfu ml^{-1} was recorded with isolate M7 which was statistically on par with M17which recorded a colony count of 121 x10^3 cfu ml^{-1} followed by M5 (109.50 x10^3 cfu ml^{-1}), M6 (109x10^3 cfu ml^{-1}), M12 (90 x 10^3 cfu ml^{-1}), M11 (28.50 x10^3 cfu ml^{-1}), M18 (27.50 x 10^3 cfu ml^{-1}) and M19 (27.50 x 10^3 cfu ml^{-1}).

After 30 days of inoculation, the maximum colony count of 127.50 x 10^3 cfu ml^{-1} was recorded with isolate M7 which was significantly superior to all other isolates followed by M17 (111 x10^3 cfu ml^{-1}), M6 (99x10^3 cfu ml^{-1}), M12 (94.50 x10^3 cfuml^{-1}), M5 (70 x10^3 cfu ml^{-1}), M18 (19 x 10^3 cfu ml^{-1}), M11 (15.50 x10^3 cfu ml^{-1}) and M19 (15 x 10^3 cfu ml^{-1}).

In 100 ppm concentration, the isolate M7 showed significant colony count on 7th, 15th, 20th, 25th and 30th day after inoculation which was statistically on par with M17 on 7th, 15th, 25th and 30th days after inoculation. However, M7 was statistically on par with M6 and M17 on 20 DAI.

Table 7. Preliminary screening to assess the population build up of fungal and actinomycete isolates in 50 ppm concentration of chlorpyrifos in MSM

Isolates	Total viable count (cfux10^3 ml^{-1})*				
	7th DAI	15th DAI	20th DAI	25th DAI	30th DAI
M5	65.00	81.50	104.50	98.50	82.50
M6	80.50	89.50	106.00	106.00	90.00
M7	100.00	112.00	118.50	122.00	124.50
M11	48.50	52.00	43.00	39.50	22.00
M12	59.00	71.00	96.50	89.50	89.00
M17	92.00	108.00	119.50	123.50	110.00
M18	35.50	38.50	49.50	40.00	30.50
M19	29.50	26.50	36.50	28.00	20.50
CD (0.05)	8.31	8.15	9.59	7.98	6.92

*Mean of two replications

Table 8. Preliminary screening to assess the population build up of fungal and actinomycete isolates in 100 ppm concentration of chlorpyrifos in MSM

Isolates	Total viable count (cfux10^3 ml^{-1})*				
	7th DAI	15th DAI	20th DAI	25th DAI	30th DAI
M5	71.50	101.00	111.50	109.50	70.00
M6	90.00	100.50	121.50	109.00	99.50
M7	111.00	118.50	123.50	127.00	127.50
M11	34.50	30.50	30.50	28.50	15.50
M12	65.00	76.50	100.50	90.00	94.50
M17	101.00	110.00	117.50	121.00	111.00
M18	20.50	29.50	32.50	27.50	19.00
M19	19.00	21.50	28.00	27.50	15.00
CD (0.05)	7.36	6.67	8.76	11.17	6.42

*Mean of two replications

4.2.8. Preliminary Screening to Assess the Population Build up of Fungal and Actinomycete Isolates in 200 ppm Concentration of Chlorpyrifos in MSM

On 7th day, maximum colony count of 121.50 x 10^3 cfu ml^{-1} was recorded with isolate M17 which was statistically on par with M7 which recorded a colony count of 120.50 x10^3 cfu ml^{-1} followed by M5 (110.50 x10^3 cfu ml^{-1}), M6 (101.50x10^3 cfu ml^{-1}), M12 (78 x10^3 cfu ml^{-1}), M11 (28.50 x10^3 cfu ml^{-1}), M18 (15.50 x 10^3 cfu ml^{-1}) and M19 (14 x 10^3 cfu ml^{-1}).

After 15 days of inoculation, maximum colony count of 127.50 x 10^3 cfu ml^{-1} was recorded with isolate M7 which was statistically on par with M17 and M6 which recorded a colony count of 126.50 x10^3 cfu ml^{-1}, 117.50x10^3 cfu ml^{-1} respectively followed by M5 (112.50 x10^3 cfu ml^{-1}), M12 (87 x10^3 cfu ml^{-1}), M11 (28.50 x10^3 cfu ml^{-1}), M18 (24 x 10^3 cfu ml^{-1}) and M19 (18 x 10^3 cfu ml^{-1}).

On 20th day, the maximum colony count of 134.50 x 10^3 cfu ml^{-1} was recorded with isolate M17 which was statistically on par with M7 and M6 which recorded a colony count of 133.50 x10^3 cfu ml^{-1}, 126x10^3 cfu ml^{-1} respectively followed by M5 (121 x10^3 cfu ml^{-1}), M12 (108.50 x10^3 cfu ml^{-1}), M18 (27 x 10^3 cfu ml^{-1}), M11 (27 x10^3 cfu ml^{-1}) and M19 (22 x 10^3 cfu ml^{-1}).

After 25 days of inoculation, maximum colony count of 146.50 x 10^3 cfu ml^{-1} was recorded with isolate M7 which was statistically on par with M17 which recorded a colony count of 138.50 x10^3 cfu ml^{-1} followed by M6 (121.50x10^3 cfu ml^{-1}), M5 (117.50 x10^3 cfu ml^{-1}), M12 (101.50 x10^3 cfu ml^{-1}), M18 (25 x 10^3 cfu ml^{-1}), M11 (21 x10^3 cfu ml^{-1}) and M19 (20.50 x 10^3 cfu ml^{-1}).

On 30th day, the maximum colony count of 109.50 x 10^3 cfu ml^{-1} was recorded with isolate M7 which was significantly superior to all other isolates followed by M17 (99.50 x10^3 cfu ml^{-1}), M12 (97.50 x10^3 cfu ml^{-1}), M6

(79.50x10^3 cfu ml^{-1}), M5 (68.50 x10^3 cfu ml^{-1}), M11 (12.50 x10^3 cfu ml^{-1}), M18 (11.50 x 10^3 cfu ml^{-1}) and M19 (10.50 x 10^3 cfu ml^{-1}).

In 200 ppm concentration, significant colony count was recorded by M17 on 7th DAI which was statistically on par with isolate M7. However, on 15 DAI isolate M7 recorded maximum colony count which was statistically on par with M17 and M6. On 20 DAI, the isolate M17 recorded maximum colony count which was statistically on par with M7 and M6. On 25th DAI, isolate M7 which recorded maximum colony count was statistically on par with M17. On 30th DAI, isolate M7 was significantly superior to all other isolates.

4.2.9. Preliminary Screening to Assess the Population Build up of Fungal and Actinomycete Isolates in 400 ppm Concentration of Chlorpyrifos in MSM

On 7th day, maximum colony count of 146.50 x 10^3 cfu ml^{-1} was recorded with isolate M17 which was statistically on par with M7 and M6 which recorded a colony count of 142.50 x10^3 cfu ml^{-1} and 137.50x10^3 cfu ml^{-1} respectively followed by M5 (126.50 x10^3 cfu ml^{-1}), M12 (85 x10^3 cfu ml^{-1}), M11 (21 x10^3 cfu ml^{-1}), M18 (11.50 x 10^3 cfu ml^{-1}) and M19 (10.50 x 10^3 cfu ml^{-1}).

On 15th day, the maximum colony count of 156.50 x 10^3 was recorded with isolate M7 which was statistically on par with M17 which recorded a colony count of 147.50x10^3 cfu ml^{-1} followed by M6 (145.50 x10^3 cfu ml^{-1}), M5 (128.50 x10^3 cfu ml^{-1}), M12 (94.50 x10^3 cfu ml^{-1}), M11 (19.50 x10^3 cfu ml^{-1}), M18 (17 x 10^3 cfu ml^{-1}) and M19 (15 x 10^3).

After 20 days of inoculation, maximum colony count of 167.50 x 10^3 cfu ml^{-1} was recorded with isolate M7 which was statistically on par with M17 which recorded a colony count of 165.50 x10^3 cfu ml^{-1} followed by M6 (153.50x10^3 cfu ml^{-1}), M5 (144.50 x10^3 cfu ml^{-1}), M12 (115 x10^3 cfu ml^{-1}), M19 (16 x 10^3 cfu ml^{-1}), M18 (14.50 x 10^3 cfu ml^{-1}) and M11 (14 x10^3 cfu ml^{-1}).

Table 9. Preliminary screening to assess the population build up of fungal and actinomycete isolates in 200 ppm concentration of chlorpyrifos in MSM

Isolates	Total viable count (cfux10^3ml^{-1})*				
	7th DAI	15th DAI	20th DAI	25th DAI	30th DAI
M5	110.50	112.50	121.00	117.50	68.50
M6	101.50	117.50	126.00	121.50	79.50
M7	120.50	127.50	133.50	146.50	109.50
M11	28.50	28.50	27.00	21.00	12.50
M12	78.00	87.00	108.50	101.50	97.50
M17	121.50	126.50	134.50	138.50	99.50
M18	15.50	24.00	27.00	25.00	11.50
M19	14.00	18.00	22.00	20.50	10.50
CD (0.05)	10.53	10.54	10.42	9.26	9.78

*Mean of two replications

Table 10. Preliminary screening to assess the population build up of fungal and actinomycete isolates in 400 ppm concentration of chlorpyrifos in MSM

Isolates	Total viable count (cfux10^3 ml^{-1})*				
	7th DAI	15th DAI	20th DAI	25th DAI	30th DAI
M5	126.50	128.50	144.50	142.50	75.50
M6	137.50	145.50	153.50	150.50	71.50
M7	142.50	156.50	167.50	172.50	96.50
M11	21.00	19.50	14.00	10.00	5.50
M12	85.00	94.50	115.00	112.50	105.00
M17	146.50	147.50	165.50	170.50	71.00
M18	11.50	17.00	14.50	11.00	9.00
M19	10.50	15.00	16.00	15.00	6.50
CD (0.05)	10.66	10.59	13.70	8.49	9.93

*Mean of two replications

On 25th day, maximum colony count of 172.50 x 10^3 cfu ml^{-1} was recorded with isolate M7 which was statistically on par with M17 which recorded a colony count of 170.50 x10^3 cfu ml^{-1} followed by M6 (150.50x10^3 cfu ml^{-1}), M5 (142.50 x10^3 cfu ml^{-1}), M12 (112.50 x10^3 cfu ml^{-1}), M19 (15 x 10^3 cfu ml^{-1}), M18 (11 x 10^3 cfu ml^{-1}) and M11(10 x10^3 cfu ml^{-1}).

After 30 days of inoculation, maximum colony count of 105 x 10^3 cfu ml^{-1} was recorded with isolate M12 which was statistically on par with M7 which recorded a colony count of 96.50 x10^3 cfu ml^{-1} followed by M5 (75.50 x10^3 cfu ml^{-1}), M6 (71.50x10^3 cfu ml^{-1}), M17 (71 X10^3 cfu ml^{-1}), M18 (9 x 10^3 cfu ml^{-1}), M19 (6.50 x 10^3 cfu ml^{-1}) and M11 (5.50 x10^3 cfu ml^{-1}).

In 400 ppm concentration, the isolate M17 recorded maximum colony count on 7th DAI which was statistically on par with isolates M7 and M6. On 15th, 20th and 25th DAI, isolate M7 recorded maximum colony count which was statistically on par with isolate M17. On 30 days after inoculation, the isolate M12 recorded maximum colony count which was statistically on par with isolate M7.

4.2.10. Preliminary Screening to Assess the Population Buildup of Fungal and Actinomycete Isolates in 800 ppm Concentration of Chlorpyrifos in MSM

On 7th day, maximum colony count of 46.50 x 10^3 cfu ml^{-1} was recorded with isolate M7 which was statistically on par with M17 and M6 which recorded a colony count of 42.50 x10^3 cfu ml^{-1}, 38.50x10^3 cfu ml^{-1} respectively followed by M12 (37X10^3 cfu ml^{-1}), M5 (34.50 x10^3 cfu ml^{-1}), M18 (10 x 10^3 cfu ml^{-1}), M11 (9 x10^3 cfu ml^{-1}) and M19 (5 x 10^3 cfu ml^{-1}).

After 15 days of inoculation, maximum colony count of 55.50 x 10^3 cfu ml^{-1} was recorded with isolate M7 which was followed by M17 (45.50 x10^3 cfu ml^{-1}), M12 (45 X10^3 cfu ml^{-1}), M6 (44.50x10^3 cfu ml^{-1}), M5 (40.50 x10^3 cfu ml^{-}

^{1}), M18 (7.50 x 10^3 cfu ml^{-1}), M11(5.50 x10^3 cfu ml^{-1}) and M19 (4 x 10^3 cfu ml^{-1}).

On 20th day, maximum colony count of 57.50 x 10^3 cfu ml^{-1} was recorded with isolate M7 which was statistically on par with M5, M6 and M17 which recorded a colony count of 52.50 x 10^3 cfu ml^{-1}, 52.50 x 10^3 cfu ml^{-1} and 51.50 x10^3 cfu ml^{-1} followed by M12 (43.50X10^3 cfu ml^{-1}), M11 (0 x10^3 cfu ml^{-1}), M19 (0 x 10^3 cfu ml^{-1}) and M18 (0 x 10^3 cfu ml^{-1}).

After 25 days of inoculation, maximum colony count of 60.50 x 10^3 cfu ml^{-1} was recorded with isolate M7 which was significantly superior to all other isolates followed by M17 (53 x10^3 cfu ml^{-1}), M6 (47.50x10^3 cfu ml^{-1}), M5 (47.50 x10^3 cfu ml^{-1}), M12 (35 X10^3 cfu ml^{-1}), M11 (0 x10^3 cfu ml^{-1}), M18 (0 x 10^3 cfu ml^{-1}) and M19 (0 x 10^3 cfu ml^{-1})

On 30th day, the maximum colony count of 54.50 x 10^3 cfu ml^{-1} was recorded with isolate M7 which was significantly superior to all other isolates followed by M5 (48.50 x10^3 cfu ml^{-1}), M6 (47.50 x 10^3 cfu ml^{-1}), M17 (43.50 x10^3 cfu ml^{-1}), M12 (28 X10^3 cfu ml^{-1}), M11 (1.50 x10^3 cfu ml^{-1}), M18 (0 x 10^3 cfu ml^{-1}) and M19 (0 x 10^3 cfu ml^{-1}).

In 800 ppm concentration, isolate M7 showed significant colony count on 7th and 20th DAI which was statistically on par with M17 and M6 on 7DAI and statistically on par with M5, M6 and M17 on 20th DAI. On 15th, 25th and 30th isolate M7 recorded maximum significant growth.

The isolates M5, M6, M7, M12 and M17 which showed significant growth in 50,100,200,400 and 800 ppm concentrations compared to other isolates were selected for secondary screening studies. However, isolates M11, M18 and M19 which showed less population buildup in all the concentrations tested was rejected.

Table 11. Preliminary screening to assess the population build up of fungal and actinomycete isolates in 800 ppm concentration of chlorpyrifos in MSM

Isolates	Total viable count (cfux10^3 ml^{-1})*				
	7^{th} DAI	15^{th} DAI	20^{th} DAI	25^{th} DAI	30^{th} DAI
M5	34.50	40.50	52.50	47.50	48.50
M6	38.50	44.50	52.50	47.50	47.50
M7	46.50	55.50	57.50	60.50	54.50
M11	9.00	5.50	1.50	0.00	0.00
M12	37.00	45.00	43.50	35.00	28.00
M17	42.50	45.50	51.50	53.00	43.50
M18	10.00	7.50	0.00	0.00	0.00
M19	5.00	4.00	0.00	0.00	0.00
CD (0.05)	8.78	7.24	6.67	5.85	5.15

*Mean of two replications

4.3. *IN VITRO* EVALUATION OF THE ISOLATES

The bacterial isolate M10 and fungal isolates M5, M6, M7 and M17 and the actinomycete isolate M12, which recorded maximum growth in 50,100,200,400 and 800 ppm concentrations were selected for secondary screening studies. The total viable count, residue of chlorpyrifos and release of chloride was measured in 100,200,400 and 800 ppm concentrations on 7th, 15th, 20th, 25th and 30th days after inoculation and the data are presented in Tables 12-15.

4.3.1. Secondary Screening to Assess the Population Buildup of Selected Isolates in 100 ppm Concentration of Chlorpyrifos in MSM

On 7th day of inoculation, the maximum colony count of 112.50 x 10^3 cfu ml^{-1} was recorded with isolate M7 which was statistically on par with M17 which recorded a colony count of 103 x10^3 followed by M6 (92.50x10^3 cfu ml^{-1}), M5 (72.50 x10^3 cfu ml^{-1}), M10 (9 x 10^3 cfu ml^{-1}) and M12 (0 X10^3 cfu ml^{-1}).

Isolate M7 recorded maximum colony count of 119.50 x 10^3 cfu ml^{-1} on 15th day which was significantly superior to all other isolates followed by M17 (113 x10^3 cfu ml^{-1}), M6 (102.50x10^3 cfu ml^{-1}), M5 (100 x10^3 cfu ml^{-1}), M10 (12 x 10^3 cfu ml^{-1}) and M12 (0 X10^3 cfu ml^{-1}).

On 20th day, the maximum colony count of 125.50 x 10^3 cfu ml^{-1} was recorded with isolate M7 which was statistically on par with M6 and M17 which recorded a colony count of 124.50x10^3 cfu ml^{-1} and 119 x10^3 cfu ml^{-1} respectively followed by M5 (112.50 x10^3 cfu ml^{-1}), M10 (20 x 10^3 cfu ml^{-1}) and M12 (0 X10^3 cfu ml^{-1}).

After 25 days of inoculation, maximum colony count of 128.50 x 10^3 cfu ml^{-1} was recorded with isolate M7 which was statistically on par with M17 which recorded a colony count of 122.50 x10^3 cfu ml^{-1} followed by M6 (113.00x10^3 cfu ml^{-1}), M5 (110 x10^3 cfu ml^{-1}), M10 (14 x 10^3 cfu ml^{-1}) and M12 (0 X10^3 cfu ml^{-1}).

On 30th day of inoculation, maximum colony count of 127.50 x 10^3 cfu ml^{-1} was recorded with isolate M7 which was significantly superior to all other isolates followed by M17 (112.50 x10^3 cfu ml^{-1}), M6 (103.00x10^3 cfu ml^{-1}), M5 (72.50 x10^3 cfu ml^{-1}), M10 (9 x 10^3 cfu ml^{-1}) and M12 (0 X10^3 cfu ml^{-1}).

In 100 ppm concentration, the isolates M7, M17, M6 and M5 showed significant growth compared to M10. The actinomycete isolate M12 did not grow in 100 ppm concentration.

4.3.2. Secondary Screening to Assess the Population Buildup of Selected Isolates in 200 ppm Concentration of Chlorpyrifos in MSM

After 7 days of inoculation, maximum colony count of 123.00 x 10^3 cfu ml^{-1} was recorded with isolate M17 which was statistically on par with M7 which recorded a colony count of 122.50 x10^3 cfu ml^{-1} followed by M5 (112.50 x10^3 cfu ml^{-1}), M6 (103.x10^3 cfu ml^{-1}), M10 (12.50 x 10^3 cfu ml^{-1}) and M12 (0 X10^3 cfu ml^{-1}).

On 15th day, the maximum colony count of 129.50 x 10^3 cfu ml^{-1} was recorded with isolate M7 which was significantly superior to all other isolates followed by M17 (128.00 x10^3 cfu ml^{-1}), M6 (119.50x10^3 cfu ml^{-1}), M5 (113.50 x10^3 cfu ml^{-1}), M10 (14 x 10^3 cfu ml^{-1}) and M12 (0 X10^3 cfu ml^{-1}).

Twenty days after inoculation, maximum colony count of 136 x 10^3 cfu ml^{-1} was recorded with isolate M7 and M17 which was significantly superior to all other isolates followed by M6 (128.50.x10^3 cfu ml^{-1}), M5 (122.50 x10^3 cfu ml^{-1}), M10(22 x 10^3 cfu ml^{-1}) and M12 (0 X10^3 cfu ml^{-1}).

On 25th day, the maximum colony count of 148 x 10^3 cfu ml^{-1} was recorded with isolate M7 which was significantly superior to all other isolates followed by M17 (140 x10^3 cfu ml^{-1}), M6 (125.50.x10^3 cfu ml^{-1}), M5 (119 x10^3 cfu ml^{-1}), M10 (23.50 x 10^3 cfu ml^{-1}) and M12 (0 X10^3 cfu ml^{-1}).

Table 12. Secondary screening to assess the population build up of selected isolates in 100 ppm concentration of chlorpyrifos in MSM

Isolates	Viable count (cfu x10^3 ml^{-1}) *				
	7th DAI	15th DAI	20th DAI	25th DAI	30th DAI
M_5	72.50	100.00	112.50	110.00	72.50
M_6	92.50	102.50	124.50	113.00	103.00
M_7	112.50	119.50	125.50	128.50	127.50
M_{10}	9.00	12.00	20.00	14.00	9.00
M_{12}	0	0	0	0	0
M_{17}	103.00	113.50	119.00	122.50	112.50
CD(0.05)	13.31	2.74	8.45	13.81	5.78

*Mean of two replications

Table 13. Secondary screening to assess the population build up of selected isolates in 200 ppm concentration of chlorpyrifos in MSM

Isolates	Viable count (cfu x10^3 ml^{-1}) *				
	7th DAI	15th DAI	20th DAI	25th DAI	30th DAI
M_5	112.50	113.50	122.50	119.00	70.00
M_6	103.00	119.50	128.50	125.50	81.00
M_7	122.50	129.50	136.00	148.00	145.00
M_{10}	12.50	14.00	22.00	23.50	12.50
M_{12}	0	0	0	0	0
M_{17}	123.00	128.00	136.00	140.00	101.50
CD (0.05)	5.78	1.22	0.99	3.31	1.73

* Mean of two replications

After 30 days of inoculation, maximum colony count of 145 x 10^3 cfu ml^{-1} was recorded with isolate M7 which was significantly superior to all other isolates followed by M17 (101.50 x10^3 cfu ml^{-1}), M6 (81x10^3 cfu ml^{-1}), M5 (70 x10^3 cfu ml^{-1}), M10 (12.50 x 10^3 cfu ml^{-1}), and M12 (0 X10^3 cfu ml^{-1}).

In 200 ppm, isolates M7, M17, M6 and M5 showed maximum significant growth compared to M10 and M12. No viable count was recorded with isolate M12.

4.3.3. Secondary Screening to Assess the Population Buildup of Selected Isolates in 400 ppm Concentration of Chlorpyrifos in MSM

After 7 days of inoculation, maximum colony count of 147 x 10^3 cfu ml^{-1} was recorded with isolate M17 which was significantly superior to all other isolates followed by M7 (144 x10^3 cfu ml^{-1}), M6 (139x10^3 cfu ml^{-1}), M5 (128.50 x10^3 cfu ml^{-1}), M10 (23.50 x 10^3 cfu ml^{-1}) and M12 (0 X10^3 cfu ml^{-1}).

On 15th day, maximum colony count of 158 x 10^3 cfu ml^{-1} was recorded with isolate M7 which was significantly superior to all other isolates followed by M17 (148 x10^3 cfu ml^{-1}), M6 (146x10^3 cfu ml^{-1}), M5 (130 x10^3 cfu ml^{-1}), M10 (28 x 10^3 cfu ml^{-1} and M12 (0 X10^3 cfu ml^{-1}).

After 20 days of inoculation, maximum colony count of 169 x 10^3 cfu ml^{-1} was recorded with isolate M7 which was statistically on par with M17 which recorded a colony count of 168 x10^3 cfu ml^{-1} followed by M6 (155x10^3 cfu ml^{-1}), M5 (146 x10^3 cfu ml^{-1}), M10 (32.50 x 10^3 cfu ml^{-1}), and M12 (0 X10^3 cfu ml^{-1}).

On 25th day, the maximum colony count of 175 x 10^3 cfu ml^{-1} was recorded with isolate M7 which was significantly superior to all other isolates followed by M17 (172.50 x10^3 cfu ml^{-1}), M6 (151x10^3 cfu ml^{-1}), M5 (144 x10^3 cfu ml^{-1}), M10 (30.50 x 10^3 cfu ml^{-1}) and M12 (0 X10^3 cfu ml^{-1}).

After 30 days of inoculation, maximum colony count of 175 x 10^3 cfu ml^{-1} was recorded with isolate M7 which was significantly superior to all other isolates followed by M5 (77.50 x10^3 cfu ml^{-1}), M6 (73x10^3 cfu ml^{-1}), M17 (71.50 x10^3 cfu ml^{-1}), M10 (23.50 x 10^3 cfu ml^{-1}) and M12 (0 X10^3 cfu ml^{-1}).

In 400 ppm concentration, isolates M7, M17, M5 and M6 showed maximum significant growth compared to M10 and M12. No viable count was recorded with isolate M12.

4.3.4. Secondary screening to Assess the Population Buildup of Selected Isolates in 800 ppm Concentration of Chlorpyrifos in MSM

On 7th day, the maximum colony count of 48 x 10^3 cfu ml^{-1} was recorded with isolate M7 which was significantly superior to all other isolates followed by M17 (43 x10^3 cfu ml^{-1}), M6 (39x10^3 cfu ml^{-1}), M5 (36 x10^3 cfu ml^{-1}), M10 (8 x 10^3 cfu ml^{-1}) and M12 (0 X10^3 cfu ml^{-1}).

After 15 days of inoculation, maximum colony count of 57 x 10^3 cfu ml^{-1} was recorded with isolate M7 which was significantly superior to all other isolates followed by M17 (47.50 x10^3 cfu ml^{-1}), M6 (45.50x10^3 cfu ml^{-1}), M5 (41.50 x10^3 cfu ml^{-1}), M10 (6 x 10^3 cfu ml^{-1}) and M12 (0 X10^3 cfu ml^{-1}).

On 20th day, the maximum colony count of 59 x 10^3 cfu ml^{-1} was recorded with isolate M7 which was significantly superior to all other isolates followed by M17 (52.50 x10^3 cfu ml^{-1}), M5 (50 x10^3 cfu ml^{-1}), M6 (49.50x10^3 cfu ml^{-1}), M10 (14 x 10^3 cfu ml^{-1}), and M12 (0 X10^3 cfu ml^{-1}).

After 25 days of inoculation, maximum colony count of 61.50 x 10^3 cfu ml^{-1} was recorded with isolate M7 which was significantly superior to all other isolates followed by M17 (55 x10^3 cfu ml^{-1}), M6 (51.50x10^3 cfu ml^{-1}), M5 (48.50 x10^3 cfu ml^{-1}), M10 (12 x 10^3 cfu ml^{-1}), and M12 (0 X10^3 cfu ml^{-1}).

Table 14. Secondary screening to assess the population build up of selected isolates in 400 ppm concentration of chlorpyrifos in MSM

Isolates	Viable count (cfu $x10^3$ ml^{-1}) *				
	7^{th} DAI	15^{th} DAI	20^{th} DAI	25^{th} DAI	30^{th} DAI
M_5	128.50	130.00	146.00	144.00	77.50
M_6	139.00	146.00	155.00	151.00	73.00
M_7	144.00	158.00	169.00	175.00	175.00
M_{10}	23.50	28.00	32.50	30.50	23.50
M_{12}	0	0	0	0	0
M_{17}	147.00	148.00	168.00	172.50	71.50
CD(0.05)	0.999	3.159	2.913	2.643	2.34

* Mean of two replications

Table 15. Secondary screening to assess the population build up of selected isolates in 800 ppm concentration of chlorpyrifos in MSM

Isolates	Viable count (cfu $x10^3ml^{-1}$) *				
	7^{th} DAI	15^{th} DAI	20^{th} DAI	25^{th} DAI	30^{th} DAI
M_5	36.00	41.50	50.00	48.50	49.50
M_6	39.00	45.50	49.50	51.50	47.00
M_7	48.00	57.00	59.00	61.50	55.00
M_{10}	8.00	6.00	14.00	12.0	10.00
M_{12}	0	0	0	0	0
M_{17}	43.00	47.50	52.50	55.0	45.00
CD(0.05)	2.447	1.869	1.730	1.224	1.580

* Mean of two replications

On 30th day, the maximum colony count of 55 x 10^3 cfu ml^{-1} was recorded with isolate M7 which was significantly superior to all other isolates followed by M5 (49.50 x10^3 cfu ml^{-1}), M6 (47 x10^3 cfu ml^{-1}), M17 (45 x10^3 cfu ml^{-1}), M10 (10 x 10^3 cfu ml^{-1}) and M12 (0 X10^3 cfu ml^{-1}).

In 800 ppm concentration, isolates M7, M17, M5 and M6 showed maximum significant growth compared to M10 and M12. No viable count was recorded with isolate M12.

4.3.5. Degradation of 100 ppm Chlorpyrifos by Selected Isolates at different Intervals

The efficiency of the isolates to degrade chlorpyrifos was tested by analyzing the residue of chlorpyrifos in Mineral salts medium and the results are presented in Tables 16-19.

After 7th day of inoculation, least residue of 28.27 ppm was recorded by isolate M17 followed M7 (35.030 ppm), M5 (42.29 ppm), M6 (65.01 ppm), M12 (86.150 ppm) and M10 (89.050 ppm). A residue of 93.00 ppm was observed in the control treatment.

Fifteenth day after inoculation, lowest level of residue of 22.450 ppm was recorded by isolate M17, followed by M7 (32.295 ppm), M5 (37.100 ppm), M6 (60.100 ppm), M12 (84.200ppm) and M10 (88.300 ppm). The control treatment recorded a residue of 92.40 ppm.

After 20th day of inoculation, least residue of 20.030 ppm was detected in culture media with isolate M17, followed by M7 (24.050 ppm), M5 (37.050 ppm), M6 (56.205 ppm), M12 (82.300ppm) and M10 (87.150 ppm). The control recorded a residue of 91.70 ppm.

On 25th day after inoculation, least residue of 19.00 ppm was detected in culture media with isolate M7 followed by M17 (19.400 ppm), M5 (34.050 ppm) M6 (48.100 ppm), M12 (80.900ppm) and M10 (82.200 ppm). A residue of 90.80 ppm was observed in the control treatment.

After 30th day of inoculation, lowest level of residue of 18.150 ppm was recorded by isolate M7 followed by M17 (19.00 ppm), M5 (32.100 ppm), M6

Tables 16. Degradation of 100 ppm chlorpyrifos in MSM by selected isolates at different intervals

*Isolates	7 DAI		15 DAI		20 DAS		25 DAI		30 DAI	
	Residue of chlorpyrifos (ppm)	% Reduction of chlorpyrifos	Residue of chlorpyrifos (ppm)	% Reduction of chlorpyrifos	Residue of chlorpyrifos (ppm)	% Reduction of chlorpyrifos	Residue of chlorpyrifos (ppm)	% Reduction of chlorpyrifos	Residue of chlorpyrifos (ppm)	% Reduction of chlorpyrifos
M_5	42.29	53.7	37.10	59.7	37.05	59.5	34.05	62.4	32.10	64.2
M_6	65.00	30.1	60.10	34.8	56.21	38.5	48.10	46.9	36.10	59.8
M_7	35.03	62.3	32.30	64.7	24.05	73.7	19.00	79.0	18.15	79.6
M_{10}	89.05	4.2	88.30	4.1	87.15	4.7	82.20	7.0	83.10	7.6
M_{12}	86.15	7.2	84.20	8.6	82.30	9.9	80.90	9.9	79.45	11.3
M_{17}	28.27	69.3	22.45	75.2	20.03	78.1	19.40	78.2	19.00	78.9
control	93.00		92.40		91.70		90.80		90.10	
CD (0.05)	0.537		0.838		0.506		3.050		0.638	

* Mean value of two replications

(36.100 ppm), M12 (79.450ppm) and M10 (83.100 ppm). Residue of 90.10 ppm was detected in the control treatment.

Least residue of chlorpyrifos was recorded by isolates M7, M17, M5 and M6, whereas isolates M10 and M12 recorded maximum residue in 100 ppm chlorpyrifos amended media.

4.3.6. Degradation of 200 ppm Chlorpyrifos by Selected Isolates at different Intervals

7th day after inoculation, lowest level of residue of 50.400 ppm was recorded by isolate M17 followed by M7 (63.250 ppm), M6 (66.0 ppm), M5 (130.0 ppm), M10 (193.250) and M12 (196.200 ppm). Control recorded 198.00 ppm residue.

After 15th day of inoculation, least residue of 44.350 ppm was detected in culture media with isolate M17 followed by M7 (47.300 ppm), M6 (60.250 ppm), M5 (104.00ppm), M10 (188.200 ppm) and M12 (192.150 ppm). Control recorded 194.00 ppm residue.

On 20th day after inoculation, least residue of 36.150 ppm ppm was recorded by isolate M7 followed by M17 (40.150), M5 (79.350 ppm), M6 (59.150 ppm), M10 (188.200 ppm) and M12 (191.400 ppm). A residue of 192.00 ppm was observed in the control treatment.

After 25th day of inoculation, lowest residue level of 30.600 ppm was recorded by isolate M17 followed by M7 (32.450 ppm), M5 (50.200 ppm), M6 (57.200 ppm), M10 (186.200 ppm) and M12 (187.150 ppm). The control treatment recorded a residue of 191.00 ppm.

On 30th day after inoculation, least residue of 29.300 ppm was detected in isolate M17 followed by M7 (32.10 ppm), M5 (43.00 ppm), M6 (55.10 ppm), M10 (183.100 ppm) and M12 (185.20 ppm). A residue of 190.00 ppm was observed in the control treatment.

Lowest residue level of chlorpyrifos was recorded by isolates M17, M7, M5 and M6 whereas isolates M10 and M12 recorded maximum residue in 200 ppm chlorpyrifos.

Table 17. Degradation of 200 ppm chlorpyrifos in MSM by selected isolates at different intervals

*Isolates	7 DAI		15 DAI		20 DAI		25 DAI		30 DAI	
	Residue of chlorpyrifos (ppm)	% Reduction of chlorpyrifos	Residue of chlorpyrifos (ppm)	% Reduction of chlorpyrifos	Residue of chlorpyrifos (ppm)	% Reduction of chlorpyrifos	Residue of chlorpyrifos (ppm)	% Reduction of chlorpyrifos	Residue of chlorpyrifos (ppm)	% Reduction of chlorpyrifos
M_5	130.00	34.3	104.00	46.3	79.35	58.4	50.20	73.6	43.00	77.3
M_6	66.00	66.6	60.25	68.8	59.15	69.2	57.20	69.9	54.10	71.4
M_7	63.25	67.9	47.30	75.4	36.15	81.0	32.45	82.7	32.10	83.0
M_{10}	193.25	2.4	188.20	2.8	188.00	2.0	186.20	2.5	183.10	3.5
M_{12}	196.20	0.8	192.15	0.9	191.40	0.3	187.15	1.9	185.20	2.4
M_{17}	50.40	74.3	44.35	76.9	40.15	79.0	30.60	83.9	29.30	84.4
control	198.00		194.00		192.00		191.00		190.00	
CD (0.05)	0.660		0.734		0.748		0.743		0.506	

*Mean value of two replications

4.3.7. Degradation of 400 ppm Chlorpyrifos by Selected Isolates at different Intervals

After 7th day of inoculation, lowest level of residue of 137.100 ppm was recorded by isolate M5 followed by M17 (186.350 ppm), M6 (201.00 ppm), M7 (240.00 ppm), M12 (349.00 ppm) and M10 (354.00 ppm). Control recorded 365.00 ppm residue.

Fifteenth day after of inoculation, least residue of 135.2 ppm was recorded by isolate M5 followed by M7 (140.0 ppm), M17 (166.00 ppm), M6 (201.00 ppm), M12 (341.30 ppm) and M10 (350.35 ppm) and. A residue of 364.00 ppm was observed in the control treatment.

After 20th day of inoculation, least residue of 123.00 ppm was detected by isolates M6 and M7 followed by M17 (131.40 ppm), M5 (135.10 ppm), M12 (336.72 ppm) and M10 (344.40 ppm). Control treatment recorded a residue of 363.00 ppm.

After 25th day of inoculation, least residue of 75.150 ppm was recorded by isolate M6 followed by M7 (110.00 ppm), M17 (120.40 ppm), M5 (132.10 ppm), M12 (325.20 ppm) and M10 (342.45 ppm). A residue of 362.00 ppm was observed in the control treatment.

Thirtieth day after inoculation, lowest level of residue of 65.300 ppm was recorded isolate M6 followed by M5 (69.250 ppm), M7 (99.250 ppm), M17 (110.25 ppm), M10 (336.20 ppm) and M12 (391.40 ppm). A residue of 361.00 ppm was observed in the control treatment.

Least residue of chlorpyrifos was detected by isolates M6, M5, M7 and M17, whereas isolates M10 and M12 recorded maximum residue in 400 ppm chlorpyrifos.

4.3.8. Degradation of 800 ppm Chlorpyrifos by Selected Isolates at different Intervals

After 7th day after inoculation, lowest level of residue 279.20 ppm was recorded isolate M17 followed by M6 (284.15 ppm), M5 (293.10 ppm),

Tables 18. Degradation of 400 ppm chlorpyrifos in MSM by selected isolates at different intervals

*Isolates	7 DAI		15 DAI		20 DAI		25 DAI		30 DAI	
	Residue of chlorpyrifos (ppm)	% Reduction of chlorpyrifos	Residue of chlorpyrifos (ppm)	% Reduction of chlorpyrifos	Residue of chlorpyrifos (ppm)	% Reduction of chlorpyrifos	Residue of chlorpyrifos (ppm)	% Reduction of chlorpyrifos	Residue of chlorpyrifos (ppm)	% Reduction of chlorpyrifos
M_5	137.10	62.4	135.20	62.9	135.10	62.8	132.10	63.5	69.25	80.8
M_6	201.00	44.9	201.00	44.7	123.00	66.1	75.15	79.1	65.30	81.9
M_7	240.00	34.2	140.00	61.5	123.00	66.1	110.00	69.6	99.25	72.5
M_{10}	354.00	3.0	350.35	3.6	344.40	5.0	342.45	5.3	336.20	6.8
M_{12}	349.00	4.3	341.30	6.15	336.35	7.2	325.20	0.1	391.40	14.4
M_{17}	186.35	49.0	166.00	54.3	131.40	63.6	120.40	66.6	110.25	69.5
control	365.00		364.00		363.00		362.00		361.00	
CD (0.05)	0.460		0.635		0.850		0.834		0.827	

* Mean value of two replications

M7 (296.20 ppm), M12 (726.20 ppm) and M10 (730.25 ppm). Control treatment recorded a residue of 741.40 ppm.

On 15th day, after inoculation, lowest level of residue of 274.05 ppm was detected in M17 followed by isolate M6 (279.10 ppm), M7 (283.15 ppm), M5 (287.30 ppm), M12 (719.40 ppm) and M10 (729.10 ppm). Control treatment recorded a residue of 740.20 ppm.

After 20th day of inoculation, least residue of 272.35 ppm was recorded by M17 followed by M6 (273.10 ppm), M7 (280.45 ppm), M5 (284.25 ppm), M12 (712.45ppm) and M10 (724.45 ppm). Control treatment recorded a residue of 737.40 ppm.

After 25th day of inoculation, lowest residue of 269.00 ppm was detected in M17 followed by M6 (273.00 ppm), M7 (278.20 ppm), M5 (282.30 ppm), M12 (708.20 ppm) and M10 (716.35 ppm). Control treatment recorded a residue of 729.60 ppm.

Thirtieth day after inoculation, least residue of 265.10 ppm was recorded by M17 followed by M6 (270.20 ppm), M7 (276.45 ppm), M5 (279.00 ppm), M12 (698.80 ppm) and M10 (702.20 ppm).Control treatment recorded a residue of 724.30 ppm.

Lowest level of residue of chlorpyrifos was detected by isolates M17, M6, M7 and M5 whereas isolates M10 and M12 recorded maximum residue in 800 ppm chlorpyrifos.

4.3.9. Release of Chloride in to the Medium by Selected Isolates at Different Concentrations of Chlorpyrifos in MSM

The efficiency of the isolates to release chloride in to the medium was also analyzed. The chloride released into the MSM in 100, 200, 400 and 800 ppm concentrations on 7th, 15th, 20th, 25th and 30th day after inoculation was not statistically significant (Table 20-23).

Tables 19. Degradation of 800 ppm chlorpyrifos in MSM by selected isolates at different intervals

*Isolates	7 DAI		15 DAI		20 DAI		25 DAI		30 DAI	
	Residue of chlorpyrifos (ppm)	% Reduction of chlorpyrifos	Residue of chlorpyrifos (ppm)	% Reduction of chlorpyrifos	Residue of chlorpyrifos (ppm)	% Reduction of chlorpyrifos	Residue of chlorpyrifos (ppm)	% Reduction of chlorpyrifos	Residue of chlorpyrifos (ppm)	% Reduction of chlorpyrifos
M_5	293.10	60.4	287.30	61.2	284.25	61.5	282.30	61.3	279.00	61.0
M_6	284.15	61.6	279.10	62.3	273.10	62.9	273.00	62.7	270.20	62.0
M_7	296.20	60.0	283.15	61.7	280.45	62.0	278.20	61.8	276.45	61.8
M_{10}	730.25	1.5	729.10	1.48	724.45	1.78	716.35	1.76	702.20	3.03
M_{12}	726.20	2.0	719.40	2.75	712.45	3.4	708.20	2.9	698.80	3.5
M_{17}	279.20	62.3	274.05	62.9	272.35	63.1	269.00	63.1	265.10	63.0
control	741.40		740.20		737.40		729.60		724.30	
CD (0.05)	0.586		0.687		0.740		1.437		0.610	

* Mean value of two replications

Table 20. Release of chloride by selected isolates in 100 ppm concentration of chlorpyrifos in MSM

Chloride release (mg l^{-1}) *					
Isolates	7^{th} DAI	15^{th} DAI	20^{th} DAI	25^{th} DAI	30^{th} DAI
M_5	0.124	0.149	0.199	0.224	0.099
M_6	0.124	0.149	0.199	0.224	0.099
M_7	0.099	0.199	0.224	0.249	0.149
M_{10}	0.024	0.024	0.049	0.049	0.049
M_{12}	0.024	0.024	0.099	0.049	0.049
M_{17}	0.099	0.199	0.199	0.224	0.149
Control	0	0	0	0	0
CD(0.05)	NS	NS	NS	NS	NS

* Mean of two replications

Table 21. Release of chloride by selected isolates in 200 ppm concentration of chlorpyrifos in MSM

Chloride release (mg l^{-1}) *					
Isolates	7^{th} DAI	15^{th} DAI	20^{th} DAI	25^{th} DAI	30^{th} DAI
M_5	0.049	0.149	0.274	0.299	0.149
M_6	0.049	0.249	0.274	0.299	0.199
M_7	0.099	0.124	0.274	0.349	0.149
M_{10}	0.049	0.049	0.074	0.049	0.099
M_{12}	0.049	0.049	0.049	0.049	0.049
M_{17}	0.149	0.249	0.274	0.299	0.199
Control	0	0	0	0	0
CD (0.05)	NS	NS	NS	NS	NS

* Mean of two replications

Table 22. Release of chloride by selected isolates in 400 ppm concentration of chlorpyrifos in MSM

Chloride release (mg l^{-1}) *					
Isolates	7th DAI	15th DAI	20th DAI	25th DAI	30th DAI
M_5	0.099	0.274	0.499	0.449	0.174
M_6	0.099	0.299	0.349	0.373	0.124
M_7	0.224	0.249	0.249	1.290	0.074
M_{10}	0.049	0.024	0.099	0.099	0.024
M_{12}	0.049	0.049	0.024	0.049	0.024
M_{17}	0.074	0.373	0.398	0.39	0.124
Control	0	0	0	0	0
CD(0.05)	NS	NS	NS	NS	NS

* Mean of two replications

Table 23. Release of chloride by selected isolates in 800 ppm concentration of chlorpyrifos in MSM

Chloride release (mg l^{-1}) *					
Isolates	7th DAI	15th DAI	20th DAI	25th DAI	30th DAI
M_5	0.099	0.224	0.249	0.249	0.149
M_6	0.099	0.099	0.249	0.249	0.149
M_7	0.249	0.249	0.249	0.298	0.249
M_{10}	0.099	0.049	0.099	0.049	0.074
M_{12}	0.049	0.049	0.099	0.049	0.049
M_{17}	0.099	0.199	0.199	0.249	0.199
Control	0	0	0	0	0
CD(0.05)	NS	NS	NS	NS	NS

* Mean of two replications

4.4 CHARACTERIZATION OF ISOLATES

After secondary screening, isolates M5, M6, M7 and M17 which recorded maximum population build up and chlorpyrifos degradation were selected for characterization studies.

4.4.1. Morphological Characterization of the Isolates

The morphological characteristics comprising colony colour, texture and appearance of selected isolates was studied and data are presented in Table -24 and Plate 2.

4.4.2. Molecular Characterization of the Isolates

The molecular characterization of the selected fungal isolates was done in collaboration with Rajiv Gandhi Centre for Biotechnology, Trivandrum. The genomic DNA was extracted from the four selected fungal isolates namely M5, M6, M7 and M17. The agarose gel electrophoresis (0.8%) of the extracted genomic DNA showed presence of good quality, unshared DNA bands on the gel. (Plate 3)

4.4.2.1. *rDNA Amplification*

The 18S rRNA universal primers were used for amplification of the genomic DNA of fungal isolates. The amplicons of size between 1 KB and 1.5 KB were observed (Plate 4).

4.4.2.2. *Sequencing of 18Sr DNA Fragment*

The rDNA regions of the isolates were sequenced for the identification and molecular characterization and for studying the variability of the isolates. Sequencing was done by using the Big-Dye Terminator v3.1 Cycle sequencing Kit. The sequence quality was checked using Sequence scanner Software v1 (Applied Biosystems). Sequence alignment and editing of the obtained sequences were carried out using Geneious Pro v5.1. Sequencing of four isolates of the

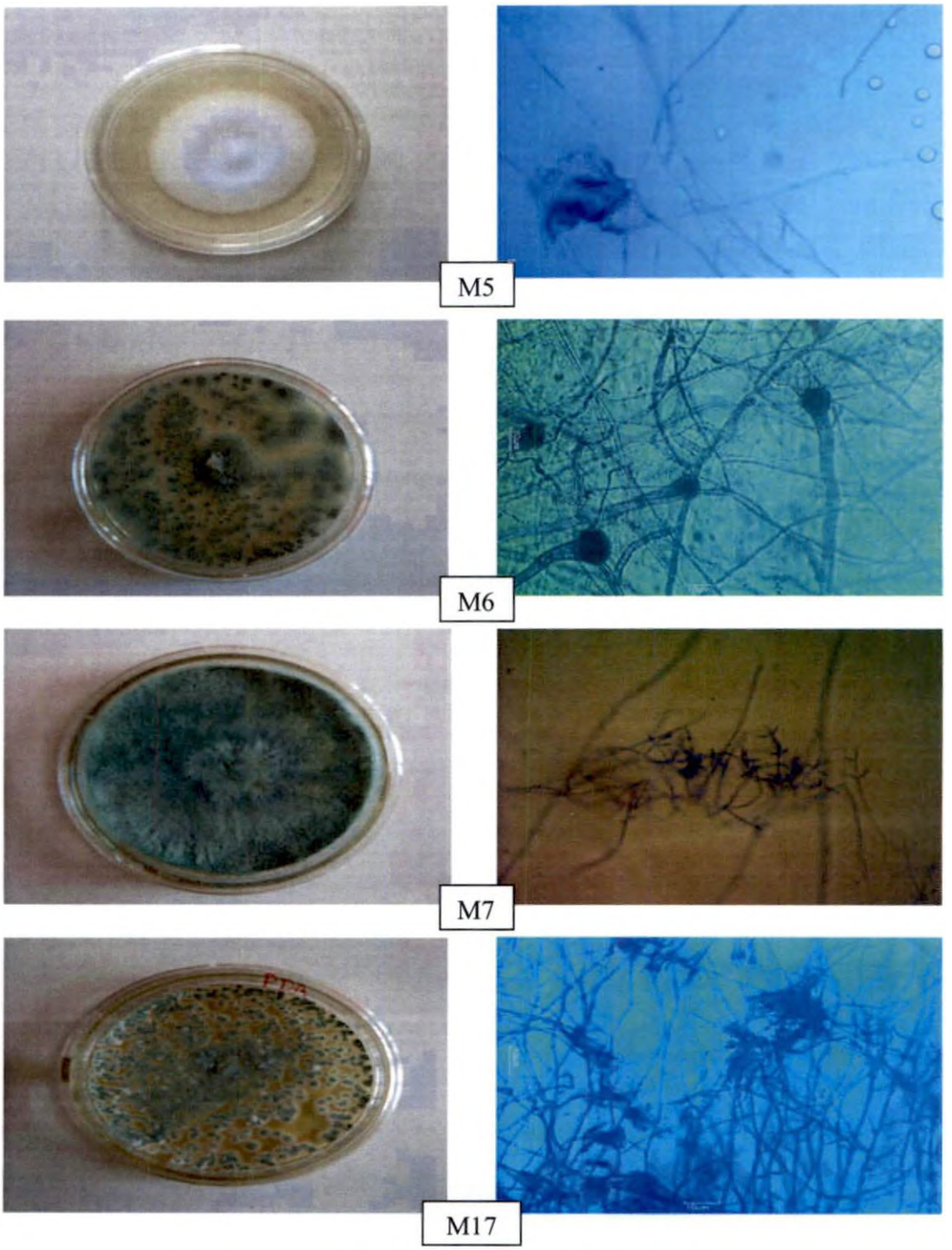


Plate 2. Morphological characters of selected fungal isolates

Table 24. Morphological characteristics of selected fungal isolates

Isolates	Type of growth	Colony colour		Conidial characters
		Upper side	Lower side	
M5	White flat cottony slow growth 7cm (dia) in 14 days	Pure white	Pale	Lemon shaped, transparent
M6	Powdery appearance	Blue green	Pale yellowish grey	Globose and green colour
M7	Green flat growth	Green to dark green	Pale	Globose, light green colour
M17	Rapid growing, flat and velvety, woolly or cottony in texture	Initially white and become blue green or grey green	Pale to yellowish	Flask shaped, green colour

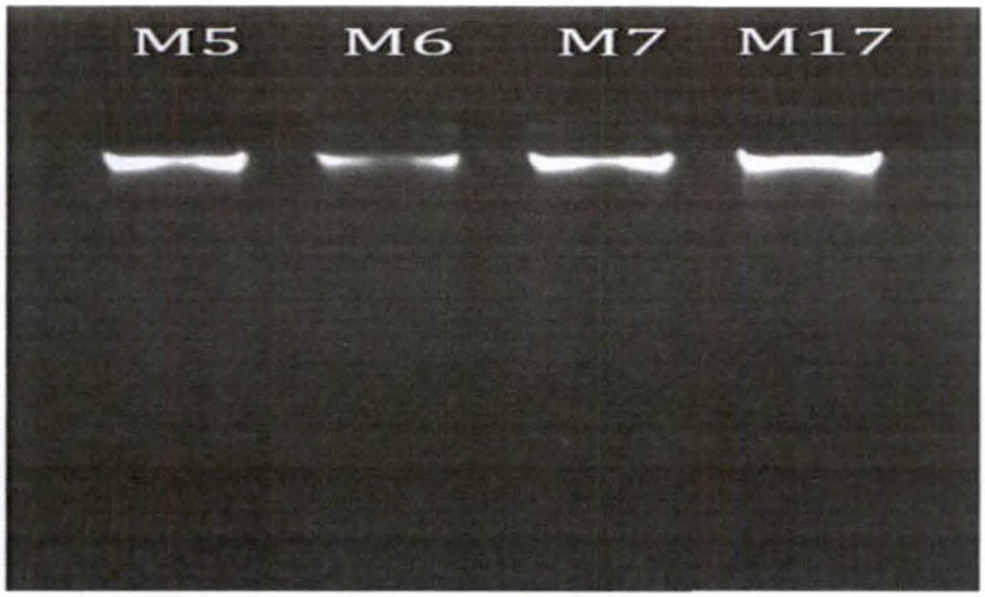


Plate 3. Extracted DNA

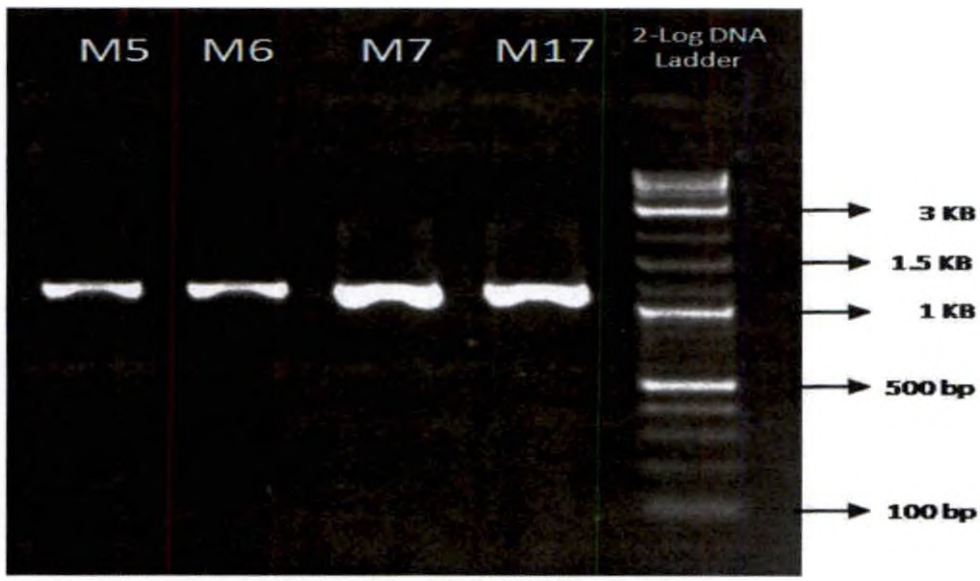


Plate 4. PCR products

microorganisms was done using universal primers of rDNA (18 SrRNA). The sequences obtained for different isolates were as follows:

>M5-18S

TTATACAGCGAAACTGCGAATGGCTCATTATATAAGTTATCGTT
TATTTGATAGTACCTTACTACTTGGATAACCGTGGTAATTCTAGAGCTA
ATACATGCTGAAAATCCCGACTTCGGAAGGGAGGTATTTATTAGATTA
AAAACCAATGCCCTCTGGGCTCCTTGGTGATTCATGATAACTGTTCGA
ATCGCACGGCCTTGCGCCGGCGATGGTTCATTCAAATTTCTTCCCTATC
AACTTTCGATGTTTGGGTATTGGCCAAACATGGTCGCAACGGGTAACG
GAGGGTTAGGGCTCGACCCCGGAGAAGGAGCCTGAGAAACGGCTACT
ACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCCGATTCG
GGGAGGTAGTGACAATAAATACTGATACAGGGCTCTTTTGGGTCTTGT
AATTGGAATGAGTACAATTTAAATCTCTTAACGAGGAACAATTGGAGG
GCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTAT
ATTAAAGTTGTTGTGGTTAAAAAGCTCGTAGTTGAACCTTGGGCCTGG
CTGGCCGGTCCGCCTCACCGCGTGTACTGGTCCGGCCGGGCCTTTCCCT
CTGTGGAACCTCATGCCCTTCACTGGGTGTGGCGGGGAAACAGGACTT
TTACTTTGAAAAAATTAGAGTGCTCCAGGCAGGCCTATGCTCGAATAC
ATTAGCATGGAATAATAAAATAGGACGCGTGGTTCTATTTTGTTGGTTT
CTAGGACCGCCGTAATGATTAATAGGGACAGTCGGGGGCATCAGTATT
CAATTGTCAGAGGTGAAATTCTTAGATTTATTGAAGACTAACTACTGC
GAAAGCATTTGCCAAGGATGTTTTCATTAATCAGGAACGAAAGTTAGG
GGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACTATGC
CGACTAGGGATCGGACGATGTTATTTTTTGACGCGTTCGGCACCTTAC
GAGAAATCAAAGTGCTT

>M6-18S

GGCTCATTAAATCAGTTATCGTTTATTTGATAGTACCTTACTACA
TGGATACCTGTGGTAATTCTAGAGCTAATACATGCTAAAAACCTCGAC
TTCGGAAGGGGTGTATTTATTAGATAAAAAACCAATGCCCTTCGGGGC

TCCTTGGTGAATCATAATAACTTAACGAATCGCATGGCCTTGCGCCGG
CGATGGTTCATTCAAATTTCTGCCCTATCAACTTTCGATGGTAGGATAG
TGGCCTACCATGGTGGCAACGGGTAACGGGGAATTAGGGTTCGATTCC
GGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGGAAGGCAGCAG
GCGCGCAAATTACCCAATCCCGACACGGGGAGGTAGTGACAATAAAT
ACTGATACGGGGCTCTTTTGGGTCTCGTAATTGGAATGAGTACAATCT
AAATCCCTTAACGAGGAACAATTGGAGGGCAAGTCTGGTGCCAGCAG
CCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCAGTTA
AAAAGCTCGTAGTTGAACCTTGGGTCTGGCTGGCCGGTCCGCCTCACC
GCGAGTACTGGTCCGGCTGGACCTTTCCTTCTGGGGAACCTCATGGCC
TTCACTGGCTGTGGGGGGAACCAGGACTTTTACTGTGAAAAAATTAGA
GTGTTCAAAGCAGGCCTTTGCTCGAATACATTAGCATGGAATAATAGA
ATAGGACGTGCGGTTCTATTTTGTTGGTTTCTAGGACCGCCGTAATGAT
TAATAGGGATAGTCGGGGGCGTCAGTATTCAGCTGTCAGAGGTGAAAT
TCTTGGATTTGCTGAAGACTAACTACTGCGAAAGCATTCGCCAAGGAT
GTTTTCATTAATCAGGGAACGAAAGTTAGGGGATCGAAGACGATCAG
ATACCGTCGTAGTCTTAACCATAAACTATGCCGACTAGGGATCGGGCG
GTGTTTCTATGATGACCCGCTCGGCACCTTACGAGAAATCAAAGTTTTT
GGGTTCTGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGAAATTG
ACGGAAGGGCACCACAAGGCGTGGAGCCTGCGGCTTAATTTGACTCA
ACACGGGGAAACTCACCAGGTCCAGACAAAATAAGGATTGACAGATT
GAGAGCTCTTTCTTGATCTTTTGGATGGTGGTGCATGGCCGTTCTTAGT
TGGTGGAGTGATTTGTCTGCTTAATTGCGATAACGAACGAGACCTCGG
CCCTTAAATAGCCCGGTCCGCATTTGCGGGCCGCTGGCTTCTTAGGGG
GACTATCGGCTCAAGCCGATGGAAGTGCGCGGCAATAACAGGTCTGT
GATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACTGACAGGGCC
AGCGAGTACATCACCTTGGCCGAGAGGTCTGGGTAATCTTGTTAAACC
CTGTCGTGCTGGGGATAGAGCATTGCAATTATTGCTCTTCAACGAGGA
ATGCCTAGTAGGCACGAGTCATCAGCTCGTGCCGATTACGTCCCTGCC
CTTTGTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCGGTGAG
GCCTTCGGACTGGCTCAGGGGAGT

>M7-18S

TAAGCAATTATACCGCGAAACTGCGAATGGCTCATTATATAAGT
TATCGTTTATTTGATAATACTTTACTACTTGGATAACCGTGGTAATTCT
AGAGCTAATACATGCTAAAAATCCCGACTTCGGAAGGGTTGTATTTAT
TAGATTAAAAACCAATGCCCCTCGGGGCTCTCTGGTGAATCATGATAA
CTAGTCGAATCGACAGGCCTTGTGCCGGCGATGGCTCATTCAAATTTC
TTCCCTATCAACTTTCGATGTTTGGGTCTTGTCCAAACATGGTGGCAAC
GGGTAACGGAGGGTTAGGGCTCGACCCCGGAGAAGGAGCCTGAGAAA
CGGCTACTACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATC
CCGACACGGGGAGGTAGTGACAATAAATACTGATACAGGGCTCTTTTG
GGTCTTGTAATCGGAATGAGTACAATTTAAATCCCTTAACGAGGAACA
ATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAA
TAGCGTATATTAAAGTTGTTGTGGTTAAAAAGCTCGTAGTTGAACCTT
GGGCCTGGCTGGCCGGTCCGCCTCACCGCGTGCACTGGTCCGGCCGGG
CCTTTCCCTCTGCGGAACCCCATGCCCTTCACTGGGTGTGGCGGGGAA
ACAGGACTTTTACTTTGAAAAAATTAGAGTGCTCAAGGCAGGCCTATG
CTCGAATACATTAGCATGGAATAATAGAATAGGACGTGTGGTTCTATT
TTGTTGGTTTCTAGGACCGCCGTAATGATTAATAGGGACAGTCGGGGG
CATCAGTATTCAATTGTCAGAGGTGAAATTCTTGGATTTATTGAAGACT
AACTACTGCGAAAGCATTTGCCAAGGATGTTTTCATTAATCAGGAACG
AAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCAT
AAACTATGCCGACTAGGGATCGGACGATGTTACATTTTTGACGCGTTC
GGCACCTTACGAGAAATCAAAGTG

>M17-18S

CACTTTATACTGTGAAACTGCGAATGGCTCATTAAATCAGTTAT
CGTTTATTTGATAGTACCTTACTACATGGATACCTGTGGTAATTCTAGA
GCTAATACATGCTACAAACCCCGACTTCAGGAAGGGGTGTATTTATTA
GATAAAAAACCAACGCCCTTCGGGGCTCCTTGGTGAATCATAATAACT
TAACGAATCGCATGGCCTTGCGCCGGCGATGGTTCATTCAAATTTCTG
CCCTATCAACTTTCGATGGTAGGATAGTGGCCTACCATGGTGGCAACG

GGTAACGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAAC
GGCTACCACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCC
CGATACGGGGAGGTAGTGACAATAAATACTGATACGGGGCTCTTTCGG
GTCTCGTAATTGGAATGAGAACAATTTAAATCCCTTAACGAGGAACAA
TTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAAT
AGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGAACCTTG
GGCCTGGCTGGCCGGTCCGCCTCACCGCGAGTACTGGTCCGGCTGGGC
CTTTCCTTCTGGGGAACCTCATGGCCTTCACTGGCTGTGGGGGGAACC
AGGACTTTTACTGTGAAAAAATTAGAGTGTTCAAAGCAGGCCTTTGCT
CGAATACATTAGCATGGAATAATAGAATAGGACGTGCGGTTCTATTTT
GTTGGTTTCTAGGACCGCCGTAATGATTAATAGGGATAGTCGGGGGCG
TCAGTATTCAGCTGTCAGAGGTGAAATTCTTGGATTTGCTGAAGACTA
ACTACTGCGAAAGCATTCGCCAAGGATGTTTTCATTAATCAGGGAACG
AAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCAT
AAACTATGCCGACTAGGGATCGGACGGGATTCTATGATGACCCGTTCG
GCACCTTACGAGAAATCAAAGTTTTTG

4.4.2.3. *Sequence Analysis*

The sequences obtained were analyzed using bioinformatic tool *viz.* BLASTN and tBLASTx i.e., nucleotide query is used to search in amino acid database. The results of isolates are given in M5 (Plate 5a, b), M6 (Plate 6a, b), M7 (Plate 7a, b) and M17 (Plate 8a, b) and (Table 25). From the results, isolate M5 showed 100 per cent identity with *Isaria farinosa* and *Beauveria bassiana.* M6 is 100 per cent identical with *Aspergillus fumigates*. M7 showed 100 per cent identity with *Trichoderma viride* and *Hypocrea sulphurea.* M17 showed 100 per cent identity with *Penicillium griseofulvum.*

Data based on tBLASTx homology search showed that the isolate belong is given in M5 (Plate 5c, d), M6 (Plate 6c, d), M7 (Plate 7c, d), (Plate 8c, d) and (Table 26). From the result of tBLASTx M5 showed 100 per cent identity with *Isaria farinosa* and *Beauveria bassiana.* M6 is 100 per cent identical with *Aspergillus fumigates*. M7 showed 100 per cent identity with *Hypomyces*

Basic Local Alignment Search Tool

NCBI/ BLAST/ blastn suite/ Formatting Results - RPYPK8T401R
Formatting options
Download
Blast report description

Nucleotide Sequence (1023 letters)

RID	RPYPK8T401R (Expires on 05-21 23:54 pm)		
Query ID	lcl|244375	**Database Name**	nr
Description	None	**Description**	Nucleotide collection (nt)
Molecule type	nucleic acid	**Program**	BLASTN 2.2.29+
Query Length	1023		

Graphic Summary

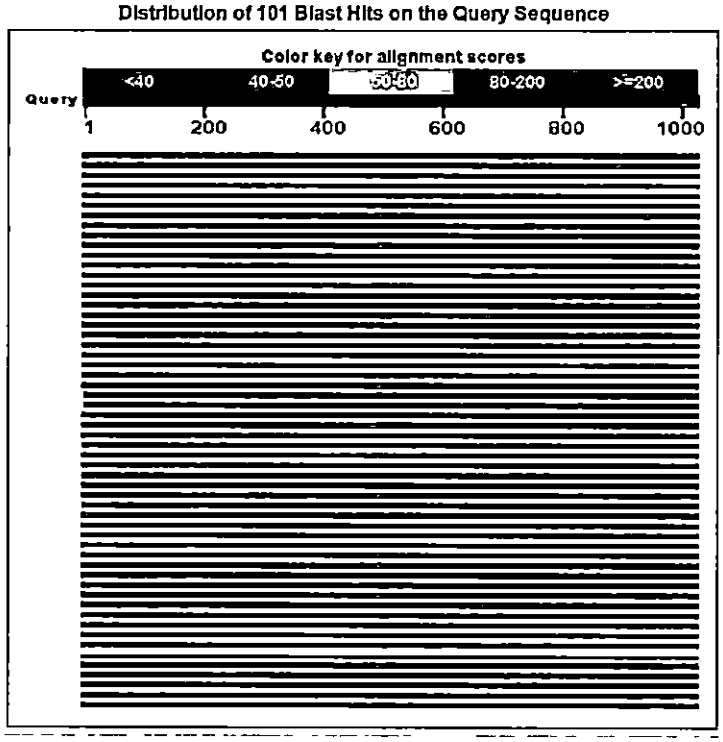


Plate 5 a. 18Sr DNA sequence analysis of M5 using BLASTN (1023 bp)

Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident	Accession
Isaria farinosa strain STH3 28S-18S rRNA intergenic spacer, partial sequence; 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1879	1879	100%	0.0	99%	KC510278.1
Fungal sp. I287 18S ribosomal RNA gene, partial sequence	1879	1879	100%	0.0	99%	KC242721.1
Beauveria bassiana strain CYT5 18S ribosomal RNA gene, partial sequence, internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1879	1879	100%	0.0	99%	HQ259053.1
Beauveria bassiana strain GXSK1011 18S ribosomal RNA gene, partial sequence	1870	1870	100%	0.0	99%	JQ909977.1
Beauveria sp. 419_12 18S ribosomal RNA gene, partial sequence	1879	1879	100%	0.0	99%	JQ977765.1
Beauveria bassiana isolate 1577 18S ribosomal RNA gene, partial sequence	1879	1879	100%	0.0	99%	JQ861945.1
Beauveria bassiana isolate 1570 18S ribosomal RNA gene, partial sequence	1879	1879	100%	0.0	99%	JQ861944.1
Beauveria bassiana strain IHEM3558 18S ribosomal RNA gene, partial sequence	1879	1879	100%	0.0	99%	JF797222.1
Isaria farinosa strain STH3 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1879	1879	100%	0.0	99%	JF429899.1
Isaria farinosa strain STH1 28S-18S rRNA intergenic spacer, partial sequence; 18S ribosomal RNA gene, complete sequence; putative homing endonuclease gene, complete cds, internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1879	1879	100%	0.0	99%	JF429896.1
Beauveria bassiana strain STD 28S-18S rRNA intergenic spacer, partial sequence; and 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 28S ribosomal RNA gene, complete sequence	1879	1879	100%	0.0	99%	JF429894.1
Beauveria bassiana isolate DAOM216540 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1879	1879	100%	0.0	99%	EU334679.1
Beauveria bassiana isolate DAOM195005 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1879	1879	100%	0.0	99%	EU334677.1

Plate 5 b. 18 Sr DNA sequence analysis of M5 using BLASTN (1023 bp)

Basic Local Alignment Search Tool

NCBI BLAST tblastx Formatting Results - RR5BHS9A01R
Formatting options
Download
Blast report description

Nucleotide Sequence (1023 letters)

RID RR5BHS9A01R (Expires on 05-22 01:48 am)

Query ID lcl|162077
Description None
Molecule type nucleic acid
Query Length 1023

Database Name nr
Description Nucleotide collection (nt)
Program TBLASTX 2.2.29+

Graphic Summary

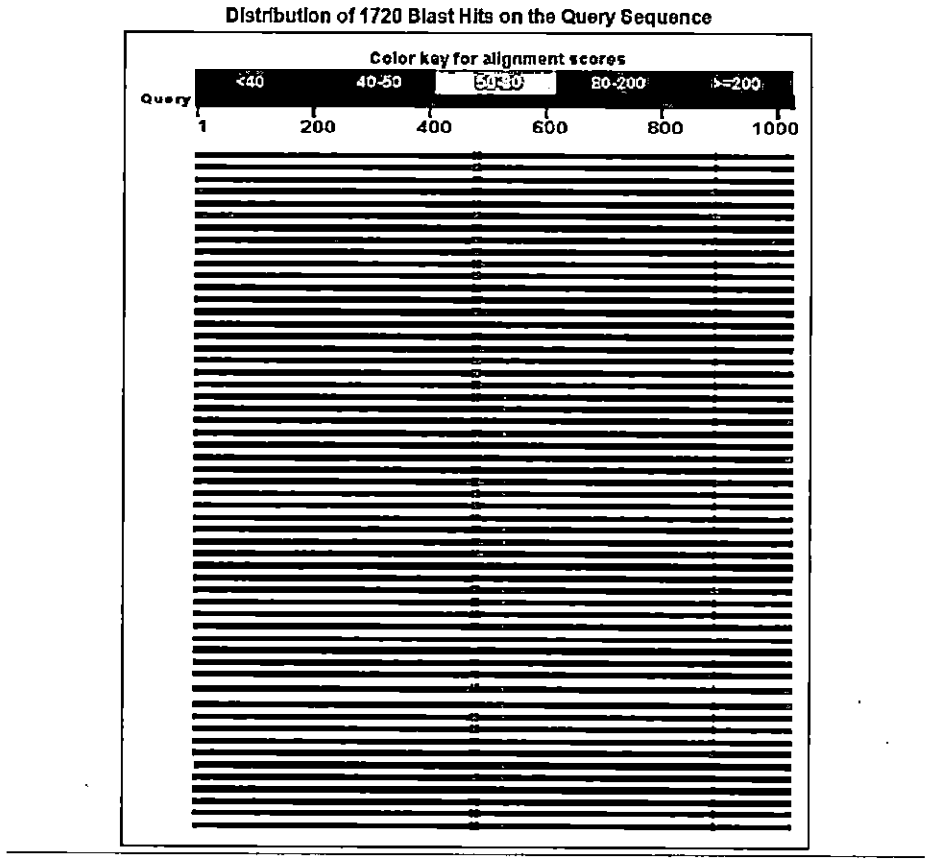


Plate 5 c. 18 Sr DNA sequence analysis of M5 using tBLASTx (1023 bp)

Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	N	Accession
Isaria farinosa strain STH1 28S-18S rRNA intergenic spacer, partial sequence; 18S ribosomal RNA gene, complete sequence; putative homing endonuclease gene, complete cds; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	395	4142	100%	0.0	Sumn3	JF429895.1
Beauveria bassiana strain STB 28S-18S rRNA intergenic spacer, partial sequence; and 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 28S ribosomal RNA gene, complete sequence	395	4142	100%	0.0	Sumn3	JF429894.1
Isaria farinosa strain STH3 28S-18S rRNA intergenic spacer, partial sequence; 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	395	4142	100%	0.0	Sumn3	KC510278.1
Beauveria bassiana isolate DAOM 16540 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	395	4142	100%	0.0	Sumn3	EU334879.1
Beauveria bassiana isolate ARSEF2991 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	395	4142	100%	0.0	Sumn3	EU334876.1
Beauveria bassiana isolate INRS-CFL 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	395	4142	100%	0.0	Sumn3	EU334874.1
Beauveria bassiana isolate DAOM106005 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	395	4142	100%	0.0	Sumn3	EU334877.1
Cordyceps bassiana genes for 18S rRNA, putative homing endonuclease, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence	395	4142	100%	0.0	Sumn3	AB237657.1
Cordyceps bassiana genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequences	395	4142	100%	0.0	Sumn3	AB079125.1
Beauveria bassiana strain CYT5 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	395	4142	100%	0.0	Sumn3	HQ259059.1
Cordyceps bassiana genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, strain: BCMU BB01	395	4142	100%	0.0	Sumn3	AB079609.1
Isaria farinosa strain STH3 18S ribosomal RNA						

Plate 5 d. 18Sr DNA sequence analysis of M5 using tBLASTx (1023 bp)

BLAST ®

Basic Local Alignment Search Tool

NCBI/ BLAST/ blastn suite/ Formatting Results - RR0BPA3801R
Formatting options
Download
Blast report description

Nucleotide Sequence (1601 letters)

RID	RR0BPA3801R (Expires on 05-22 00:23 am)		
Query ID	lcl|158231	**Database Name**	nr
Description	None	**Description**	Nucleotide collection (nt)
Molecule type	nucleic acid	**Program**	BLASTN 2.2.29+
Query Length	1601		

Graphic Summary

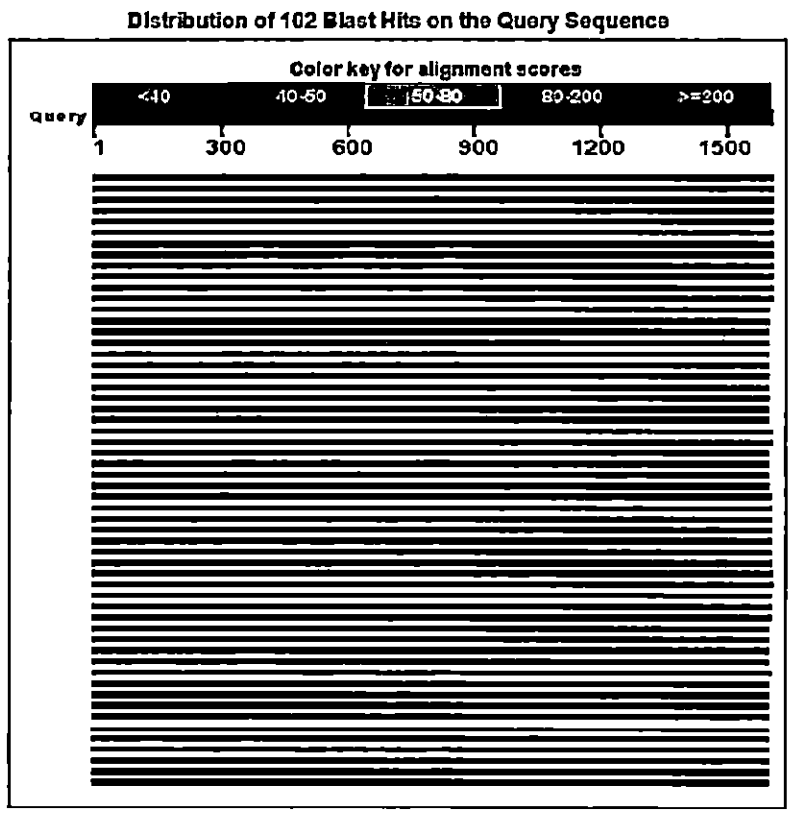


Plate 6 a. 18Sr DNA sequence analysis of M6 using BLASTN (1601 bp)

Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident	Accession
Aspergillus fumigatus strain UPSC 2006 18S ribosomal RNA gene, partial sequence >gb|AF548063.1| Aspergillus fumigatus strain ALI 57 18S ribosomal RNA gene, partial sequence	2957	2957	100%	0.0	100%	AF548062.1
Aspergillus fumigatus strain UPSC 1771 18S ribosomal RNA gene, partial sequence	2957	2957	100%	0.0	100%	AF548061.1
Aspergillus fumigatus gene for 18S rRNA, partial sequence	2957	2957	100%	0.0	100%	AB008401.1
Aspergillus fumigatus small subunit ribosomal RNA	2957	2957	100%	0.0	100%	M60300.1
Aspergillus fumigatus small subunit ribosomal RNA	2957	2957	100%	0.0	100%	M55626.1
Aspergillus fumigatus strain MJ-X6 18S ribosomal RNA gene, complete sequence	2950	2950	100%	0.0	99%	HM590663.1
Neosartorya fischeri 18S small subunit rRNA gene	2950	2950	100%	0.0	99%	U21299.1
Aspergillus fumigatus strain SK1 18S ribosomal RNA gene, partial sequence >gb|KF905648.1| Aspergillus fumigatus strain KIBGE-IB33 18S ribosomal RNA gene, partial sequence; mitochondrial	2946	2946	100%	0.0	99%	JQ665711.1
Aspergillus fumigatus strain FS160 18S ribosomal RNA gene, partial sequence	2946	2946	100%	0.0	99%	FJ840480.1
Aspergillus sp. FE8 gene for 18S rRNA	2946	2946	100%	0.0	99%	AB179824.1
Aspergillus clavatus gene for 18S rRNA, partial sequence	2940	2940	100%	0.0	99%	AB008398.1
Aspergillus fumigatus strain M1 18S ribosomal RNA gene, partial sequence	2935	2935	100%	0.0	99%	KF322139.1
Aspergillus sojae strain JPDA1 18S ribosomal RNA gene, partial sequence	2926	2926	99%	0.0	99%	KF175513.1
Aspergillus sp. MJ8+16 18S ribosomal RNA gene, complete sequence	2926	2926	99%	0.0	99%	HM590656.1
Aspergillus oryzae strain SEMCC-3.248 18S ribosomal RNA gene, partial sequence	2926	2926	99%	0.0	99%	HM064501.1
Aspergillus flavus strain TZ1985 18S ribosomal RNA gene, partial sequence	2926	2926	99%	0.0	99%	GU953210.1
Aspergillus flavus strain UPSC 1768 18S ribosomal RNA gene, partial sequence	2926	2926	99%	0.0	99%	AF548060.1
Aspergillus oryzae RIB40 DNA, rDNA_te13	2926	2926	99%	0.0	99%	AP007173.1
Aspergillus oryzae RIB40 DNA, SC206	2926	2926	99%	0.0	99%	AP007172.1
Aspergillus parasiticus DNA for 18S rRNA, partial sequence	2926	2926	99%	0.0	99%	D63699.1
Aspergillus sojae DNA for 18S rRNA, partial sequence	2926	2926	99%	0.0	99%	D63700.1
Aspergillus flavus DNA for 18S rRNA, partial sequence	2926	2926	99%	0.0	99%	D63698.1
Aspergillus oryzae DNA for 18S rRNA, partial sequence	2926	2926	99%	0.0	99%	D63698.1
Aspergillus clavatus gene for 18S rRNA, partial sequence, strain:NRRL 1	2924	2924	100%	0.0	99%	AB002070.1

Plate 6 b. 18Sr DNA sequence analysis of M6 using BLASTN (1601 bp)

Basic Local Alignment Search Tool

NCBI/ BLAST/ tblastx/ Formatting Results - RR4RNRTY01R
Formatting options
Download
Blast report description

Nucleotide Sequence (1601 letters)

RID	RR4RNRTY01R (Expires on 05-22 01:37 am)		
Query ID	lcl|17545	**Database Name**	nr
Description	None	**Description**	Nucleotide collection (nt)
Molecule type	nucleic acid	**Program**	TBLASTX 2.2.29+
Query Length	1601		

Graphic Summary

Distribution of 200 Blast Hits on the Query Sequence

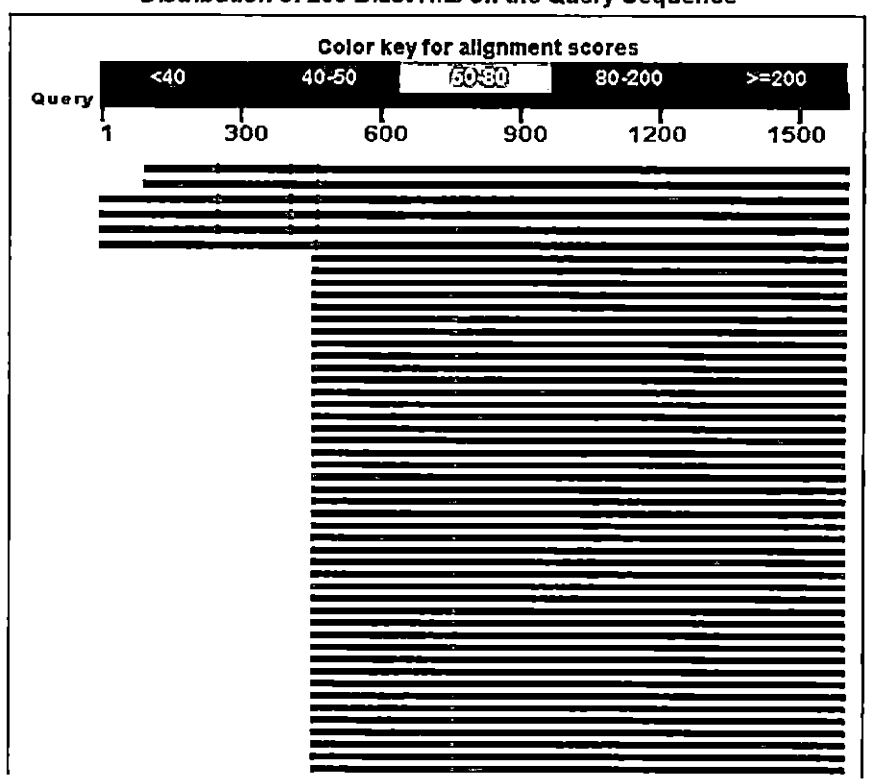


Plate 6 c. 18Sr DNA sequence analysis of M6 by using tBLASTx (1601 bp)

Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	N	Accession
Aspergillus fumigatus strain s1813 18S ribosomal RNA gene, partial sequence	863	6518	94%	0.0	Sumn2	HQ871892.1
Aspergillus fumigatus strain YA-14 18S ribosomal RNA gene, partial sequence	863	6522	94%	0.0	Sumn2	FJ560718.1
Aspergillus fumigatus strain UPSC 2006 18S ribosomal RNA gene, partial sequence >gb|AF548063.1| Aspergillus fumigatus strain ALI57 18S ribosomal RNA gene, partial sequence	863	6944	100%	0.0	Sumn2	AF548062.1
Aspergillus fumigatus strain UPSC 1771 18S ribosomal RNA gene, partial sequence	863	6944	100%	0.0	Sumn2	AF548061.1
Aspergillus sp. FE8 gene for 18S rRNA	863	6925	100%	0.0	Sumn2	AB179824.1
Aspergillus fumigatus gene for 18S rRNA, partial sequence	863	6944	100%	0.0	Sumn2	AB008401.1
Aspergillus clavatus gene for 18S rRNA, partial sequence	863	6912	100%	0.0	Sumn2	AB008398.1
Aspergillus fumigatus small subunit ribosomal RNA	863	6944	100%	0.0	Sumn2	M60300.1

Plate 6 d. 18Sr DNA sequence analysis of M6 using tBLASTx (1601 bp)

Basic Local Alignment Search Tool

NCBI BLAST/ blastn suite/ Formatting Results - RR0F5B6701R
Formatting options
Download
Blast report description

Nucleotide Sequence (1029 letters)

RID RR0F5B6701R (Expires on 05-22 00:25 am)

Query ID kl|40561
Description None
Molecule type nucleic acid
Query Length 1029

Database Name nr
Description Nucleotide collection (nt)
Program BLASTN 2.2.29+

Graphic Summary

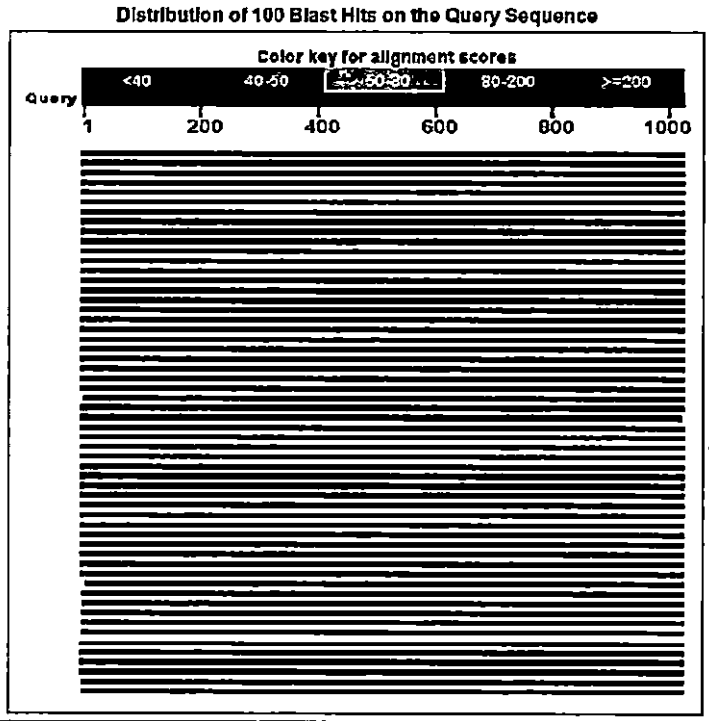


Plate 7 a. 18Sr DNA sequence analysis of M7 using BLASTN (1029 bp)

Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident	Accession
Trichoderma sp. SM-2014-A gene for 18S ribosomal RNA, partial sequence	1901	1901	100%	0.0	100%	AB923813.1
Hypocrea muroiana strain NBRC 31203 18S ribosomal RNA (SSU) gene, partial sequence >gb|JN941683.1| Hypocrea muroiana strain NBRC 31288 18S ribosomal RNA (SSU) gene, partial sequence >gb|JN941094.1| Hypocrea muroiana strain NBRC 31207 18S ribosomal RNA (SSU) gene, partial sequence >gb|JN941691.1| Hypocrea atroviridis strain NBRC 8436 18S ribosomal RNA (SSU) gene, partial sequence	1901	1901	100%	0.0	100%	JN941682.1
Hypocrea sulphurea strain NBRC 8437 18S ribosomal RNA (SSU) gene, partial sequence	1901	1901	100%	0.0	100%	JN941672.1
Hypocrea sulphurea strain NBRC 8438 18S ribosomal RNA (SSU) gene, partial sequence	1901	1901	100%	0.0	100%	JN941671.1
Hypocrea sulphurea strain NBRC 8439 18S ribosomal RNA (SSU) gene, partial sequence	1901	1901	100%	0.0	100%	JN941670.1
Hypocrea koningii strain ET-3 18S ribosomal RNA gene, partial sequence	1901	1901	100%	0.0	100%	HM152770.1
Hypocreales sp. CMCA18 18S ribosomal RNA gene, partial sequence	1901	1901	100%	0.0	100%	EU594372.1
Trichoderma viride 18S ribosomal RNA gene, partial sequence	1901	1901	100%	0.0	100%	AF525230.1
Hypocrea koningii 18S rRNA gene, 5.8S rRNA gene, 28S rRNA gene (partial), internal transcribed spacer 1 (ITS1) and internal transcribed spacer 2 (ITS2), strain ATCC 64767	1901	1901	100%	0.0	100%	AJ301990.1
Hypocrea rufa 18S rRNA gene, 5.8S rRNA gene, 28S rRNA gene (partial), internal transcribed spacer 1 (ITS1) and internal transcribed spacer 2 (ITS2), strain Hy9	1901	1901	100%	0.0	100%	AJ301991.1
Trichoderma koningiopsis strain T 440 18S ribosomal RNA gene, partial sequence	1895	1895	100%	0.0	99%	JQ276020.1
Trichoderma koningiopsis strain T-404 18S ribosomal RNA gene, partial sequence >gb|JQ276021.1| Hypocrea koningii strain T-450 18S ribosomal RNA gene, partial sequence	1895	1895	100%	0.0	99%	JQ276019.1
Trichoderma koningiopsis strain T-403 18S ribosomal RNA gene, partial sequence	1895	1895	100%	0.0	99%	JQ276018.1
Pseudomyces variotii strain DAOM 210375 18S ribosomal RNA (SSU) gene, partial sequence	1895	1895	100%	0.0	99%	JN939029.1
Hypocrea sp. 2F 18S ribosomal RNA gene, partial sequence	1895	1895	100%	0.0	99%	FJ716249.1
Hypocrea koningii strain JH 18S ribosomal RNA gene, partial sequence;						

Plate 7 b. 18Sr DNA sequence analysis of M7 using BLASTN (1029 bp)

Basic Local Alignment Search Tool

NCBI/ BLAST/ blastx/ Formatting Results - RR46VV6C01R
Formatting options
Download
Blast report description

Nucleotide Sequence (1029 letters)

RID RR46VV6C01R (Expires on 05-22 01:28 am)

Query ID lcl|171553
Description None
Molecule type nucleic acid
Query Length 1029

Database Name nr
Description Nucleotide collection (nt)
Program TBLASTX 2.2.29+

Graphic Summary

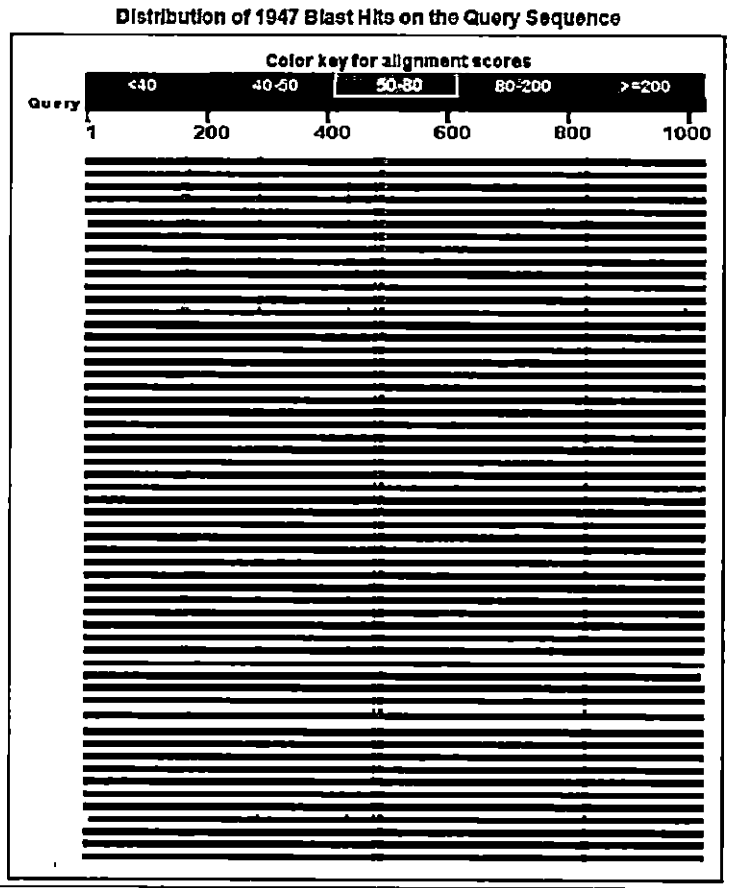


Plate 7 c. 18Sr DNA sequence analysis of M7 using tBLASTx (1029 bp)

Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	N	Accession
T.deformans gene for 18S rRNA	393	4210	100%	0.0	Sumn4	X59852.1
Podostroma cordyceps 18S ribosomal RNA gene, partial sequence	393	4217	100%	0.0	Sumn4	AY245667.1
Hypomyces chrysospermus partial 18S ribosomal RNA sequence	390	4137	100%	0.0	Sumn4	M89993.1
Hypomyces chrysospermus gene for 18S ribosomal RNA	388	4122	100%	0.0	Sumn4	AB027339.1
Hypocrea sp. SP-4 18S ribosomal RNA gene, partial sequence	224	4220	100%	0.0	Sumn5	HM152771.1
Verticillium incurvum strain CBS 460.68 small subunit ribosomal RNA gene, partial sequence	388	4062	99%	0.0	Sumn4	AF339600.1
Trichoderma viride internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; 28S ribosomal RNA gene, partial sequence; and 18S ribosomal RNA gene, complete sequence	391	4184	99%	0.0	Sumn4	AF218788.1
Trichoderma koningii 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA ribosomal RNA gene, partial sequence	390	4157	99%	0.0	Sumn4	AF218790.1
Hypocrea lutea gene for 18S rRNA, strain: IFO 9061	361	4151	100%	0.0	Sumn5	D14407.1
Paecilomyces nipheiodes strain CBS 364.76 18S small subunit ribosomal RNA gene, partial sequence	385	4083	100%	0.0	Sumn4	AY526471.2
Hypocrea rufa 18S rRNA gene, 5.8S rRNA gene, 28S rRNA gene (partial), internal transcribed spacer 1 (ITS1) and internal transcribed spacer 2 (ITS2), strain Hy9	393	4196	100%	0.0	Sumn4	AJ301991.1
Hypocrea koningii 18S rRNA gene, 5.8S rRNA gene, 28S rRNA gene (partial), internal transcribed spacer 1 (ITS1) and internal transcribed spacer 2 (ITS2), strain ATCC 64262	393	4196	100%	0.0	Sumn4	AJ301990.1
Sphaerostilbella berkeleyana small subunit ribosomal RNA gene, partial sequence	365	4183	99%	0.0	Sumn5	AF543770.1
Hypocrea koningii strain SP-3 18S ribosomal RNA gene, partial sequence	393	4196	100%	0.0	Sumn4	HM152770.1
Hypocrea muroiana strain NBRC 31293 18S ribosomal RNA (SSU) gene, partial sequence >gb|JN941683.1| Hypocrea muroiana strain NBRC 31288 18S ribosomal RNA (SSU) gene, partial sequence >gb|JN941684.1| Hypocrea muroiana strain NBRC 31287 18S ribosomal RNA (SSU) gene, partial sequence >gb|JN941691.1| Hypocrea atroviridis strain NBRC 8436 18S ribosomal RNA (SSU) gene, partial sequence	393	4196	100%	0.0	Sumn4	JN941682.1
Hypocrea rufa strain GJS89-127 18S ribosomal RNA gene, partial sequence	393	4182	100%	0.0	Sumn4	AY489694.1

Plate 7 d. 18Sr DNA sequence analysis of M7 using tBLASTx (1029 bp)

Basic Local Alignment Search Tool

NCBI/ BLAST/ blastn suite/ Formatting Results - RR1J5CB301R
Formatting options
Download
Blast report description

Nucleotide Sequence (1030 letters)

RID RR1J5CB301R (Expires on 05-22 00:43 am)

Query ID	lcl|147867	Database Name	nr
Description	None	Description	Nucleotide collection (nt)
Molecule type	nucleic acid	Program	BLASTN 2.2.29+
Query Length	1030		

Graphic Summary

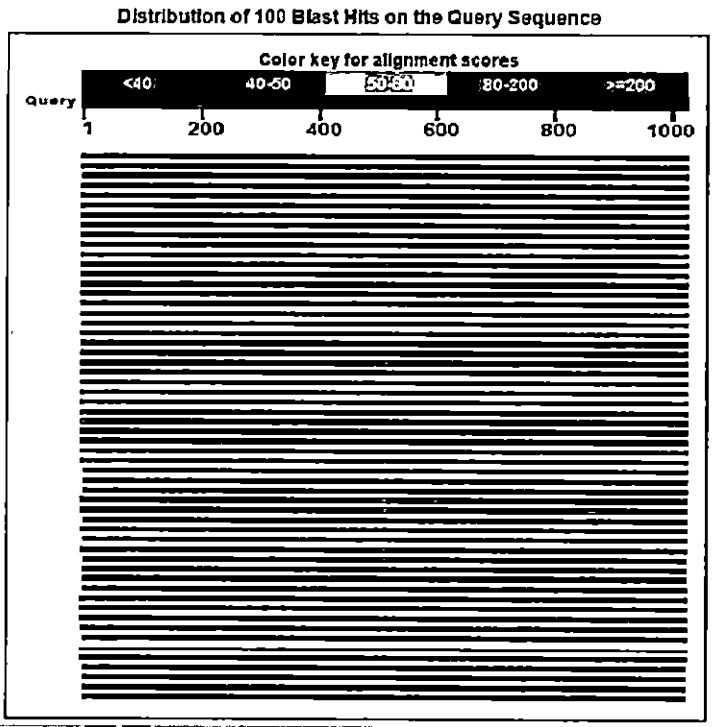


Plate 8 a. 18Sr DNA sequence analysis of M17 using BLASTN (1030 bp)

Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident	Accession
Penicillium sp. WC-29-5 18S ribosomal RNA gene, partial sequence	1903	1903	100%	0.0	100%	KJ138167.1
Penicillium griseofulvum isolate Pen0 18S ribosomal RNA gene, partial sequence	1903	1903	100%	0.0	100%	FJ717697.1
Penicillium sp. B19 18S ribosomal RNA gene, partial sequence	1899	1899	99%	0.0	100%	HQ686091.1
Penicillium citrinum strain Salicorn 46 18S ribosomal RNA gene, partial sequence	1897	1897	100%	0.0	99%	KF768801.1
Penicillium decumbens 16S ribosomal RNA gene, partial sequence	1897	1897	100%	0.0	99%	KC842215.1
Penicillium citrinum strain DAOM 221147 18S ribosomal RNA (SSU) gene, partial sequence	1897	1897	99%	0.0	99%	JN938960.1
Penicillium griseofulvum strain T22-13 18S ribosomal RNA gene, partial sequence	1897	1897	100%	0.0	99%	GU325479.1
Penicillium sp. CPCC 480032 18S ribosomal RNA gene, partial sequence	1897	1897	100%	0.0	99%	EU835656.1
Penicillium sp. M31 genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, partial and complete sequence	1897	1897	100%	0.0	99%	AB245443.1
Penicillium decumbens genomic DNA containing 18S rRNA gene, ITS1, 5.8S rRNA, ITS2, isolate MMH 89-p1	1893	1893	100%	0.0	99%	FR774046.1
Penicillium decumbens genomic DNA containing 18S rRNA gene, ITS1, 5.8S rRNA, ITS2, isolate ZHE 89-p3	1892	1892	100%	0.0	99%	FR774045.1
Penicillium sp. CPCC 480465 18S ribosomal RNA gene, partial sequence	1892	1892	100%	0.0	99%	EU827607.1
Eupenicillium javanicum isolate AFTOL-ID 429 18S ribosomal RNA gene, partial sequence	1881	1881	100%	0.0	99%	EF413520.1
Eupenicillium limosum isolate AFTOL-ID 2014 18S ribosomal RNA gene, partial sequence	1881	1881	100%	0.0	99%	EF411061.1
Uncultured ascomycete gene for 18S rRNA, partial sequence, clone:APB_76	1881	1881	100%	0.0	99%	AB074659.1
Eupenicillium javanicum 18S small subunit rRNA gene	1881	1881	100%	0.0	99%	U21298.1
Penicillium sp. SA29 gene for 18S ribosomal RNA, partial sequence	1879	1879	100%	0.0	99%	AB304918.1
Penicillium sp. 6-16M 18S ribosomal RNA gene, partial sequence	1877	1877	99%	0.0	99%	KC143067.1
Penicillium sp. 6-12c 18S ribosomal RNA gene, partial sequence	1875	1875	100%	0.0	99%	KC790520.1
Eupenicillium sp. SAUFC3-1 18S ribosomal RNA gene, partial sequence	1875	1875	100%	0.0	99%	JN176207.1
Fungal sp. FCAS31a 18S small subunit ribosomal RNA gene, partial sequence	1875	1875	100%	0.0	99%	GQ120164.1
Penicillium sp. CPCC 480008 18S ribosomal RNA gene, partial sequence	1875	1875	100%	0.0	99%	EU881148.1
Penicillium oxalicum strain 114-2 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete	1873	1873	100%	0.0	99%	KF152942.1

Plate 8 b. 18Sr DNA sequence analysis of M17 using BLASTN (1030 bp)

Basic Local Alignment Search Tool

NCBI/ BLAST/ tblastx/ Formatting Results - RR1VZNGY01R
Formatting options
Download
Blast report description

Nucleotide Sequence (1030 letters)

RID RR1VZNGY01R (Expires on 05-22 00:49 am)

Query ID lcl|177295
Description None
Molecule type nucleic acid
Query Length 1030

Database Name nr
Description Nucleotide collection (nt)
Program TBLASTX 2.2.29+

Graphic Summary

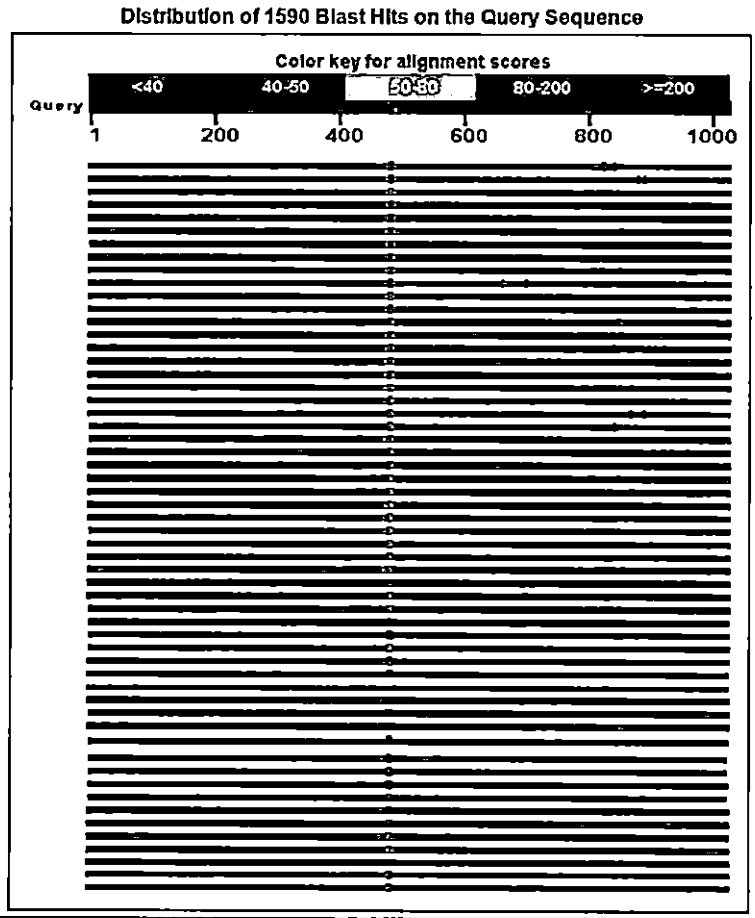


Plate 8 c. 18Sr DNA sequence analysis of M17 using tBLASTx (1030 bp)

Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	N	Accession
Penicillium decumbens 16S ribosomal RNA gene, partial sequence	396	4390	100%	0.0	Sumn3	KC842215.1
Penicillium decumbens genomic DNA containing 18S rRNA gene, ITS1, 5.8S rRNA, ITS2, isolate MMH 89-p1	396	4389	100%	0.0	Sumn3	FR774046.1
Penicillium sp. CPCC 480032 18S ribosomal RNA gene, partial sequence	431	4375	100%	0.0	Sumn2	EU835665.1
Penicillium griseofulvum isolate Pen0 18S ribosomal RNA gene, partial sequence	431	4393	100%	0.0	Sumn2	FJ717697.1
Penicillium sp. WC-29-5 18S ribosomal RNA gene, partial sequence	431	4383	100%	0.0	Sumn2	KJ136167.1
Penicillium sp. M31 genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, partial and complete sequence	431	4373	100%	0.0	Sumn2	AB245443.1
Penicillium sp. B19 18S ribosomal RNA gene, partial sequence	431	4373	99%	0.0	Sumn2	HQ696091.1
Penicillium citrinum strain Selicum 46 18S ribosomal RNA gene, partial sequence	431	4372	100%	0.0	Sumn2	KF758801.1
Penicillium citrinum strain DAOM221147 18S ribosomal RNA (SSU) gene, partial sequence	423	4333	99%	0.0	Sumn2	JN938900.1
Fungal sp. FCAS31a 18S small subunit ribosomal RNA gene, partial sequence	392	4392	100%	0.0	Sumn3	GQ120164.1
Penicillium griseofulvum strain T22-13 16S ribosomal RNA gene, partial sequence	431	4370	100%	0.0	Sumn2	GU325679.1
Penicillium sp. CPCC 480405 18S ribosomal RNA gene, partial sequence	431	4393	100%	0.0	Sumn2	EU827607.1
Penicillium griseofulvum strain 3.5190 18S ribosomal RNA gene, partial sequence	391	4310	100%	0.0	Sumn3	EF606151.1
Uncultured Penicillium clone USN5 18S ribosomal RNA gene, partial sequence	391	4279	100%	0.0	Sumn3	JN597370.1
Chrysocleista sp. SF 18S ribosomal RNA gene, partial sequence	391	4301	100%	0.0	Sumn3	FJ716248.1
Eupenicillium javanicum 18S small subunit rRNA gene	428	4334	100%	0.0	Sumn2	U21298.1
Eupenicillium javanicum isolate AFTOL-ID 429 18S ribosomal RNA gene, partial sequence	428	4334	100%	0.0	Sumn2	EF413620.1
Eupenicillium limosum isolate AFTOL-ID 2014 18S ribosomal RNA gene, partial sequence	428	4334	100%	0.0	Sumn2	EF411061.1
Uncultured ascomycete gene for 18S rRNA, partial sequence, clone APB_76	428	4334	100%	0.0	Sumn2	AB074658.1
Penicillium expansum strain HDJZ-ZWM-17 18S ribosomal RNA gene, partial sequence	391	4324	100%	0.0	Sumn3	GU727344.1
Penicillium chrysogenum 18S ribosomal RNA gene, partial sequence	391	4322	99%	0.0	Sumn3	EU203859.1
Penicillium sp. 6-16M 18S ribosomal RNA gene, partial sequence	428	4324	99%	0.0	Sumn2	KC143067.1
Penicillium sp. CPCC 480008 18S ribosomal RNA gene, partial sequence	428	4324	100%	0.0	Sumn2	EU581148.1
Penicillium sp. 09F-404 18S ribosomal RNA gene, partial sequence	426	4318	100%	0.0	Sumn2	JX134614.1
Fungal sp. FCAS85 18S small subunit ribosomal RNA gene, partial sequence	427	4308	100%	0.0	Sumn2	GQ120166.1

Plate 8 d. 18Sr DNA sequence analysis of M17 using tBLASTx (1030 bp)

Table 25. BLAST N results of the four selected isolates

Sequence identifier	BLAST N hits	Species
(1023bp) M5	101 Blast Hits on the Query Sequence	*Isaria farinosa*
(1601bp) M6	102 Blast Hits on the Query Sequence	*Aspergillus fumigatus*
(1029bp) M7	100 Blast Hits on the Query Sequence	*Trichoderma viride*
(1030bp) M17	100 Blast Hits on the Query Sequence	*Penicillium griseofulvum*

Table 26. tBLASTx results of the four selected isolates

Sequence identifier	tBLASTx hits	Species
(1023bp) M5	1720 Blast Hits on the Query Sequence	*Isaria farinosa*
(1601bp) M6	200 Blast Hits on the Query Sequence	*Aspergillus fumigatus*
(1029bp) M7	1947 Blast Hits on the Query Sequence	*Trichoderma viride*
(1030bp) M17	1590 Blast Hits on the Query Sequence	*Penicillium griseofulvum*

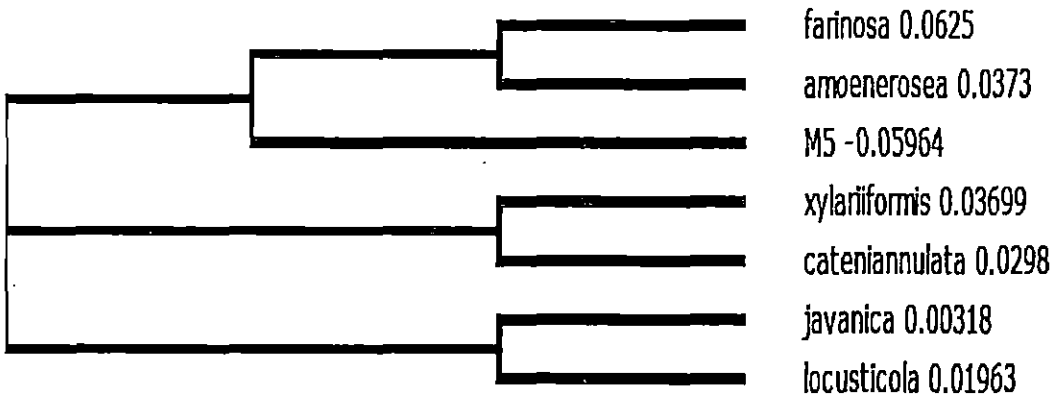


Plate 9. Phylogenetic tree of M5

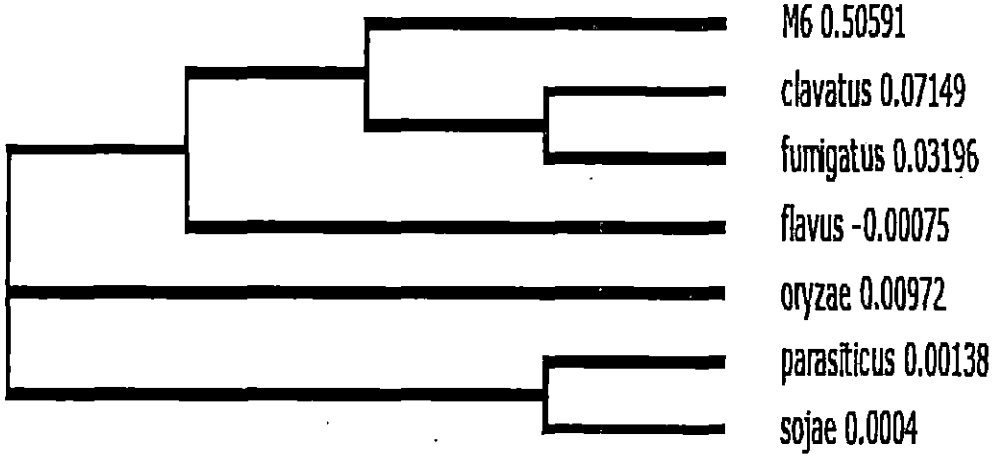


Plate 10. Phylogenetic tree of M6

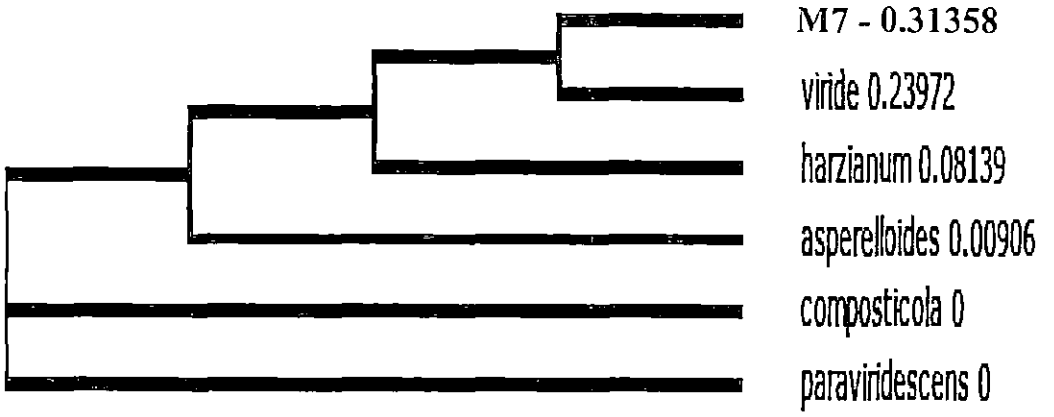


Plate 11. Phylogenetic tree of M7

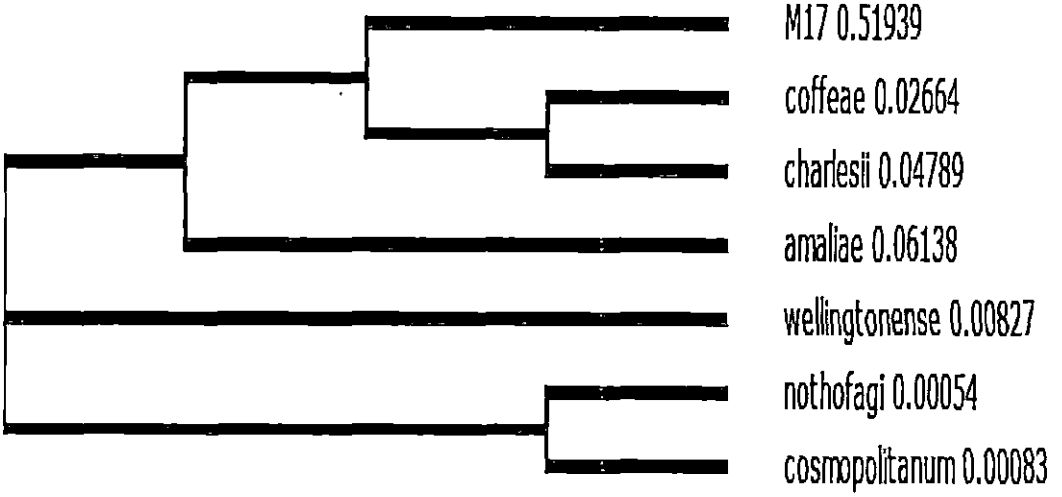


Plate 12. Phylogenetic tree of M17

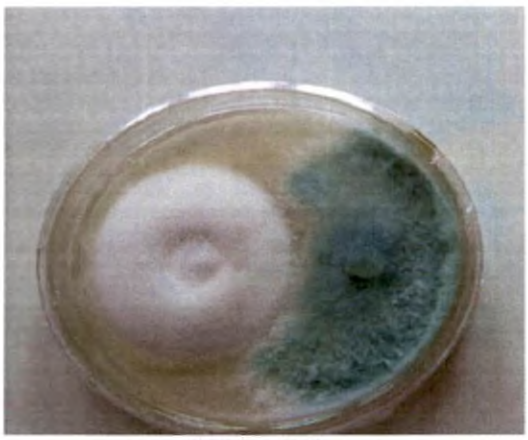
chrysospermus and *Trichoderma deformans*. M17 showed 100 per cent identity with *Penicillium griseofulvum*.

The sequences of isolates M5, M6, M7 and M17 were uploaded Clustal omega database for multiple sequence alignment and phylogenetic tree was drawn using neighbor joining method is shown in Plate 9, Plate 10, Plate 11and Plate 12 respectively. Construction of Phylogenetic tree revealed the relatedness of *Isaria* to *I. farinosa* and *I. amoenerosea* sp., *Aspergillus* to *A. clavatus* and *A. fumigatus*, *Trichoderma* to *T. viride* and *T. harzianum, Penicillium* to *P. coffeae* and *P. charlesii*.

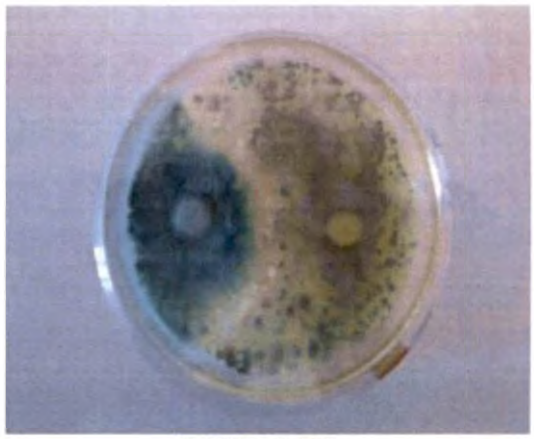
4.5. DEVELOPMENT OF CONSORTIUM OF ISOLATES

For development of liquid consortium of selected isolates, their compatibility was evaluated in Potato dextrose broth and Mineral salts medium (Table 27) and it was confirmed by dual culture technique (Plate 13).

The growth of individual isolates was monitored in both MSM (Mineral Salts Media) and PDB (Potato Dextrose Broth) after 7 days of inoculation. Eventhough the population of each of the isolate was maximum in PDB, all the isolates showed sufficient viable count in MSM also. In PDB, maximum population was recorded by isolate M7 (74.25×10^6 cfu ml^{-1}), followed by M6 (59.75×10^6 cfu ml^{-1}), M17 (45.75×10^6 cfu ml^{-1}) and M5 (32.25×10^6 cfu ml^{-1}). In MSM, maximum population was recorded by isolate M7 (239×10^3 cfu ml^{-1}) followed by M17 (186.50×10^3 cfu ml^{-1}), M6 (141.50×10^3 cfu ml^{-1}) and M5 (80.50×10^3 cfu ml^{-1}). All the isolates were compatible and could grow well in PDB and MSM and sufficient population build up was recorded. The compatibility of the isolates was also tested by dual culture technique. All the four isolates were tested for antagonism and no inhibition was observed between the isolates tested (Table 28). Since all the isolates (M5, M6, M7 and M17) were compatible, they were used to prepare the liquid consortium.

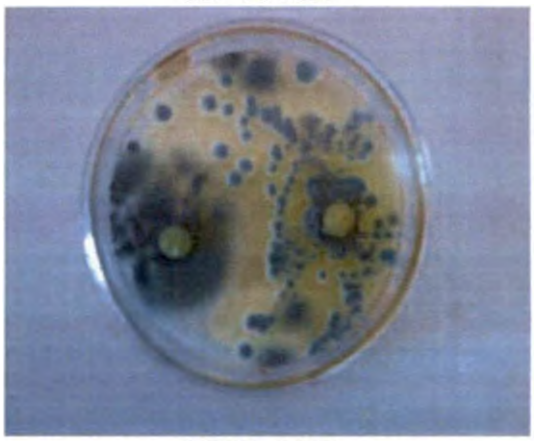


M5 Vs M7

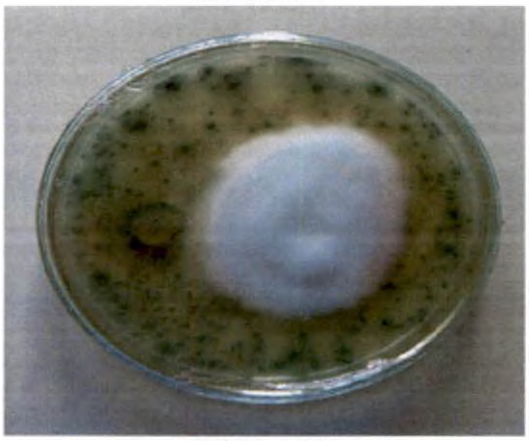


M7 Vs M17

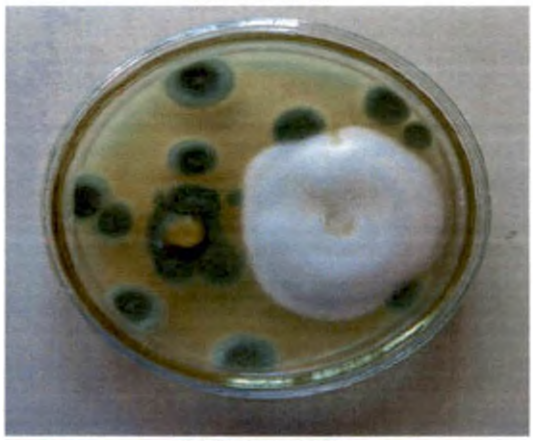
M6 Vs M7



M6 Vs M17



M6 Vs M5



M17 Vs M5

Plate 13. Dual culture studies of selected fungal isolates

Table 27. Compatibility of selected fungal isolates in MSM and PDB

SI.NO	ISOLATES	7 DAI	
		MSM	PDB
		cfu X 10^3 ml^{-1}	cfu X10^6 ml^{-1}
1	M5	80.50	32.25
2	M6	141.50	59.75
3	M7	239.00	74.25
4	M17	186.50	45.75
5	CD (0.05)	3.801	8.705

Table 28. Dual culture studies of selected fungal isolates

Sl.No.	Fungal isolates	Fungal isolates			
		M5	M6	M7	M17
1	M5		+	+	+
2	M6	+		+	+
3	M7	+	+		+
4	M17	+	+	+	

\+ No inhibition

4.6 EVALUATION OF THE CONSORTIUM UNDER *IN VITRO* CONDITIONS

The prepared liquid consortium of M5, M6, M7 and M17 was evaluated under *in vitro* conditions, to assess the ability to degrade 50, 100, 200, 400 and 800 ppm concentrations of chlorpyrifos based on viable count (Table 29-33) and release of chloride (Table 35) at intervals of 7, 15, 20, 25 and 30 days after inoculation. However, the residue of chlorpyrifos was analyzed on 25th day after inoculation (Table 34).

4.6.1. Population Build up of Individual Isolates in the Consortium in 50 ppm Concentration of Chlorpyrifos in MS Media

Isolate M7 recorded 48 x 10^3 cfu ml^{-1} on 7 DAI which increased to 74 x10^3 cfu ml^{-1} on 15th day and to 82.50x10^3 cfu ml^{-1}on 20th day after inoculation and reached a maximum count of 83.50x10^3 cfu ml^{-1} on 25th day after inoculation thereafter declined to 82 x 10^3 cfu ml^{-1} on 30th day after inoculation.

On 7th day of inoculation, isolate M17 recorded a viable count of 42.50 x10^3 cfu ml^{-1}. The population buildup of M17 gradually increased to 82.50 x10^3 cfu ml^{-1} on 15th day of inoculation and to 85.50 x10^3 cfu ml^{-1} on 20th day. Maximum viable count of 96 x10^3 cfu ml^{-1} was recorded by M17 on 25th day after inoculation which showed a decline to 41x10^3 cfu ml^{-1} on 30th day after inoculation.

On 7th day of inoculation, isolate M6 recorded 38.50 x 10 ^{3} cfu ml^{-1} and showed an increase to 78.50 x 10^3 cfu ml^{-1} on 15th day and reached a maximum of 85.50 x10^3 cfu ml^{-1} on 20th day, thereafter a gradual decline to 81 x 10^3 cfu ml^{-1}l and showed a decreased viable count of 37 x 10^3 cfu ml^{-1} on 30th day of inoculation.

Similarly isolate M5 recorded a total viable count of 43 x10^3 cfu ml^{-1} on 7DAI, it gradually increased to 84.5 x 10^3 cfu ml^{-1} on 15th day reaching a maximum population buildup of 88.50 x10^3 cfu ml^{-1} on 20th day and thereafter showed a decline of 87 x10^3 cfu $ml^{-1}$and 39 x10^3 cfu ml^{-1} on 25th and 30 DAI respectively.

4.6.2. Population Build up of Individual Isolates in the Consortium in 100 ppm Concentration of Chlorpyrifos in MS Media

On 7th day of inoculation, maximum viable count of 141 $x10^3$ cfu ml^{-1} was recorded by M17. The population buildup of M17 graduallly increased to 154 $x10^3$ cfu ml^{-1} on 15th day of inoculation and to 172.5 $x10^3$ cfu ml^{-1} on 20th day. Maximum viable count of M17 was recorded on 25th day after inoculation ($194x10^3$ cfu ml^{-1}) which showed a decline to $131x10^3$ cfu ml^{-1} on 30th day after inoculation.

Isolate M7 recorded 120 x 10^3 cfu ml^{-1} on 7 DAI which increased to 125.50 $x10^3$ cfu ml^{-1} on 15 DAI and reached a maximum count of 219.50 $x10^3$ cfu ml^{-1} on 20 DAI thereafter declined to 128.5 and 125 x 10^3 cfu ml^{-1} on 25th and 30th day after inoculation respectively.

On 7th day of inoculation, isolate M6 recorded 119 x 10^3 cfu ml^{-1} showing an increase in growth to 122.5 x 10^3 cfu ml^{-1} on 15th day and reached a maximum of 144 $x10^3$ cfu ml^{-1} on 20th day thereafter gradually declined to 140 x 10^3 cfu ml^{-1} on 25 DAI and showed a decreased viable count of 113.5 x 10^3 cfu ml^{-1} on 30th day of inoculation.

Similarly isolate M5 recorded a total viable count of 110 $x10^3$ cfu ml^{-1} on 7DAI, it gradually increased to 112.5 $x10^3$ cfu ml^{-1} on 15th day reaching a maximum population buildup of 144.5 $x10^3$ cfu ml^{-1} on 20 DAI and thereafter showed a decline of 120 $x10^3$ cfu ml^{-1} and 100 $x10^3$ cfu ml^{-1} on 25th and 30 DAI respectively.

4.6.3. Population Build up of Individual Isolates in the Consortium in 200 ppm Concentration of Chlorpyrifos in MSM

On 7th day of inoculation, maximum viable count of 150 $x10^3$ cfu ml^{-1} was recorded by isolate M17. The population buildup of M17 gradually increased to158 $x10^3$ cfu ml^{-1} on 15th day of inoculation and 161.50 $x10^3$ cfu ml^{-1} on 20th day. Maximum viable count of 196.50 $x10^3$ cfu ml^{-1} was recorded by M17 on 25th day after inoculation which showed a decline to 142.50 $x10^3$ cfu ml^{-1} on 30th day after inoculation.

Table 29. Population build up of individual isolates of consortium in 50 ppm concentration of chlorpyrifos in MSM

Fungal Isolates	Total viable count (cfu x 10^3 ml^{-1}) *				
	7^{th} DAI	15^{th} DAI	20^{th} DAI	25^{th} DAI	30^{th} DAI
M_7	48.00	74.00	82.50	83.50	82.00
M_6	38.50	78.50	85.50	81.00	37.00
M_{17}	42.50	82.50	85.50	96.00	41.00
M_5	43.00	84.50	88.50	87.00	39.00
CD(0.05)	3.104	2.597	1.963	3.539	3.926

* Mean of two replications

Table 30. Population build up of individual isolates of consortium in 100 ppm concentration of chlorpyrifos in MSM

Fungal Isolates	Total viable count (cfu x 10^3 ml^{-1}) *				
	7^{th} DAI	15^{th} DAI	20^{th} DAI	25^{th} DAI	30^{th} DAI
M_7	120.00	125.50	219.50	128.50	125.00
M_6	119.00	122.50	144.00	140.00	113.50
M_{17}	141.00	154.00	172.50	194.00	131.00
M_5	110.00	112.50	144.00	120.00	100.00
CD(0.05)	3.926	2.597	3.104	3.539	3.539

* Mean of two replications

Isolate M7 recorded 123 x 10^3 cfu ml^{-1} on 7 DAI which increased to 127.50 x10^3 cfu ml^{-1} on 15 DAI and to 128.50 x10^3 cfu ml^{-1} on 20 DAI, reached a maximum count of 132.50 x 10^3 cfu ml^{-1} on 25th DAI which showed a decline to 120 x 10^3 cfu ml^{-1} on 30th day after inoculation.

On 7th day of inoculation, isolate M6 recorded 122.50 x 10 ^{3} cfu ml^{-1} showing an increase in growth to 125x 10^3 cfu ml^{-1} on 15th day and reached a maximum of 146.50 x10^3 cfu ml^{-1} on 20th day thereafter gradually declined to 128.50 x 10^3 cfu ml^{-1} on 25 DAI and showed a decreased viable count of 113.5 x 10^3 cfu ml^{-1} on 30th day of inoculation.

Similarly isolate M5 recorded a total viable count of 112 x10^3 cfu ml^{-1} on 7DAI, it gradually increased to 117 x10^3 cfu ml^{-1} on 15th day reaching a maximum population buildup of 148.50 x10^3 cfu ml^{-1} on 20 DAI and thereafter showed a decline of 117.50 x10^3 cfu ml^{-1} and 112 x10^3 cfu ml^{-1} on 25th and 30 DAI respectively.

4.6.4. Population Build up of Individual Isolates in the Consortium in 400 ppm Concentration of Chlorpyrifos in MSM

On 7th day of inoculation, maximum viable count of 125.5 x10^3 cfu ml^{-1} was recorded by M17. The population buildup of M17 gradually increased to128 x10^3 cfu ml^{-1} on 15th day of inoculation and to 129 x10^3 cfu ml^{-1} on 20th day. Maximum viable count of 136 x10^3 cfu ml^{-1} was recorded by M17 on 25th day after inoculation which showed a decline to113x10^3 cfu ml^{-1} on 30th day after inoculation.

Isolate M7 recorded 160.5 x 10^3 cfu ml^{-1} on 7 DAI which increased to 163.50 x10^3 cfu ml^{-1} on 15 DAI and to 169.0 x10^3 cfu ml^{-1} on 20 DAI and reached a maximum count of 172.50 x10^3 cfu ml^{-1} on 25th DAI thereafter showed a decline to 153.50 x 10^3 cfu ml^{-1} on 30th day after inoculation.

On 7th day of inoculation, isolate M6 recorded 130.5 x 10 ^{3} cfu ml^{-1} showing an increase in growth to 138.5 x 10^3 cfu ml^{-1} on 15th day and reached a maximum of 142 x10^3 cfu ml^{-1} on 20th day, thereafter gradually declined to 140.50

Table 31. Population build up of individual isolates of consortium in 200 ppm concentration of chlorpyrifos in MSM

Fungal Isolates	Total viable count (cfu x $10^3 ml^{-1}$) *				
	7th DAI	15th DAI	20th DAI	25th DAI	30th DAI
M_7	123.00	127.50	128.50	132.50	120.00
M_6	122.50	125.00	146.50	128.50	113.50
M_{17}	150.00	158.00	161.50	196.50	142.50
M_5	112.00	117.00	148.50	117.50	112.00
CD(0.05)	3.539	3.539	1.963	1.963	3.104

* Mean of two replications

Table 32. Population build up of individual isolates of consortium in 400 ppm concentration of chlorpyrifos in MSM

Fungal Isolates	Total viable count (cfu x $10^3 ml^{-1}$) *				
	7th DAI	15th DAI	20th DAI	25th DAI	30th DAI
M_7	160.5	163.5	169.0	172.5	153.50
M_6	130.5	138.5	142.00	140.50	129.0
M_{17}	125.5	128.0	129.0	136.0	113.0
M_5	125.5	128.5	133.00	129.50	116.5
CD(0.05)	1.963	2.597	3.926	2.597	3.104

* Mean of two replications

x 10^3 cfu ml^{-1} on 25 DAI and showed a decreased viable count of 116.50 x 10^3 cfu ml^{-1} on 30^{th} day of inoculation.

Similarly isolate M5 recorded a total viable count of 125.50 x10^3 cfu ml^{-1} on 7DAI, it gradually increased to 128.5 x10^3 cfu ml^{-1} on 15^{th} day reaching a maximum population buildup of 133.00 x10^3 cfu ml^{-1} on 20 DAI and thereafter showed a decline of 129.50 x10^3 cfu ml^{-1} and 116.50 x10^3 cfu ml^{-1} on 25^{th} and 30 DAI respectively.

4.6.5. Population Build up of Individual Isolates in the Consortium in 800 ppm Concentration of Chlorpyrifos in MSM

On 7^{th} day of inoculation, maximum viable count of 5.50 x10^3 cfu ml^{-1} was recorded by M17. The population buildup of M17 gradually increased to15.50 x10^3 cfu ml^{-1} on 15^{th} day of inoculation and to 49.50 x10^3 cfu ml^{-1} on 20^{th} day. Maximum viable count of 55.50 x10^3 cfu ml^{-1} was recorded by M17on 25^{th} day after inoculation which showed a decline to 10.50 x10^3 cfu ml^{-1} on 30^{th} day after inoculation.

Isolate M7 recorded 4 x 10^3 cfu/ml on 7 DAI which increased to 21.50 x10^3 cfu ml^{-1} on 15 DAI and to 30 x10^3 cfu ml^{-1} on 20 DAI, reached a maximum count of 67 x10^3 cfu ml^{-1} on 25^{th} DAI thereafter showed a decline to 10 x 10^3 cfu ml^{-1}on 30^{th} day after inoculation.

On 7^{th} day of inoculation, isolate M6 recorded 8 x 10^3 cfu ml^{-1} showing an increase in growth to 18.50 x 10^3 cfu ml^{-1} on 15^{th} day and reached a maximum of 37.50 x10^3 cfu ml^{-1} on 20^{th} day, thereafter gradually declined to 37 x 10^3 cfu ml^{-1} on 25 DAI and showed a decreased viable count of 6 x 10^3 cfu ml^{-1} on 30^{th} day of inoculation.

Similarly isolate M5 recorded a total viable count of 18 x10^3 cfu ml^{-1} on 7DAI, it gradually increased to 34.50 x10^3 cfu ml^{-1} on 15^{th} day reaching a maximum population buildup of 92.50 x10^3 cfu ml^{-1} on 20 DAI and thereafter showed a decline of 55.00 x10^3 cfu ml^{-1} and 25 x10^3 cfu ml^{-1} on 25^{th} and 30 DAI respectively.

Table 33. Population build up of individual isolates of consortium in 800 ppm concentration of chlorpyrifos in MSM

Fungal Isolates	Total viable count (cfu x 10^3 ml^{-1}) *				
	7^{th} DAI	15^{th} DAI	20^{th} DAI	25^{th} DAI	30^{th} DAI
M_7	4.00	21.50	30.00	67.00	10.00
M_6	8.00	18.50	37.50	37.00	6.00
M_{17}	5.50	15.50	49.50	55.50	10.50
M_5	18.00	34.50	92.50	55.00	25.00
CD(0.05)	2.195	3.143	2.597	3.539	2.195

* Mean of two replications

4.6.6. Degradation of Chlorpyrifos by Consortium at Different Concentrations of Chlorpyrifos on 25th Day of Inoculation

The ability of the developed consortium to degrade 50, 100, 200, 400 and 800 ppm concentrations of chlorpyrifos in MSM was analyzed on 25th day of inoculation. Degradation of chlorpyrifos was analysed by using GC-MS. Maximum residue of 260.0 ppm was recorded in 800 ppm concentration, followed by 45.0 ppm(400 ppm), 32.0 ppm (200 ppm), 17.0 ppm (100 ppm) and 17.0 ppm (50 ppm), compared to control which recorded 741.0, 365.0, 194.0, 93.0 and 48.0 ppm in 800, 400, 200,100 and 50 ppm respectively. Maximum degradation of 87.6 per cent was recorded in 400 ppm followed by 83.0 per cent in 200 ppm and 81.7 per cent in 100 ppm and least value of 64.9 per cent in 800 ppm concentration In 50 ppm, only 65.0 per cent degradation was recorded (Table 34).

4.6.7. Release of Chloride in to the Medium by the Consortium at Different Concentrations of Chlorpyrifos at Different Intervals

The release of chloride into the medium by the consortium after 7 DAI was not statistically significant. However, on 15th DAI, maximum chloride release of 0.299 mg^{-1} was observed in 200 ppm followed by 0.249 mg^{-1} in 400 ppm, 0.199 mg^{-1} in 50 and 800 ppm. The chloride release gradually increased from 15th to 20th DAI, reaching a maximum on 25th DAI and then showed a decline on 30th DAI. On 20th DAI maximum release of 0.349 mg^{-1} was observed in 200 ppm followed by 0.274 mg^{-1} in 100ppm, 0.249 in 400 ppm, 0.224 mg^{-1} in 50 ppm and 0.199 mg^{-1} in 800 ppm. The release of chloride on 25th DAI was maximum (0.373 mg^{-1}) in 200 ppm, followed by 0.349 mg^{-1} in 100 ppm, 0.298 mg^{-1} in 400 ppm and 0.249 mg^{-1} in 800 and 50 ppm. On 30th DAI maximum release of 0.249 mg^{-1} was observed in 400 ppm followed by 0.199 mg^{-1} in 800 ppm, 0.149 mg^{-1} in 50 and 100 ppm and 0.124 mg^{-1} in 200 ppm (Table 35).

Table 34. Degradation of different concentrations of chlorpyrifos by consortium on 25th day of inoculation

Concentrations	Residue of chlorpyrifos in consortium (ppm)	Residue of chlorpyrifos in control (ppm)	Percentage degradation of chlorpyrifos
50 PPM	17.0	48.0	65.0
100 PPM	17.0	93.0	81.7
200 PPM	32.0	194.0	83.0
400 PPM	45.0	365.0	87.6
800 PPM	260.0	741.0	64.9
CD (0.05)	10.024	12.086	

Table 35. Release of chloride in to the medium by consortium at different concentrations of chlorpyrifos at different intervals

Chloride release (mg l^{-1})*						
SI.NO	ISOLATES	7 DAI	15 DAI	20 DAI	25 DAI	30 DAI
1	50 ppm	0.099	0.199	0.224	0.249	0.149
2	100 ppm	0.099	0.124	0.274	0.349	0.149
3	200 ppm	0.099	0.299	0.349	0.373	0.124
4	400 ppm	0.099	0.249	0.249	0.298	0.249
5	800 ppm	0.099	0.199	0.199	0.249	0.199
6	CD(0.05)	NS	0.004	0.003	0.004	0.003

* Mean of two replications

4.7. EVALUATION OF THE BIOREMEDIATION EFFICIENCY OF THE DEVELOPED CONSORTIUM IN CONTAMINATED SOIL UNDER POT CULTURE CONDITIONS USING STERILIZED SOIL

A pot culture study was conducted to evaluate the bioremediation efficiency of the developed liquid consortium in soil spiked with chlorpyrifos. The efficiency of consortium was evaluated by monitoring the biometric characters of cowpea such as plant height, fresh and dry weight of plants and roots, nodule number, nodule fresh and dry weight and yield of plant (Plate 14 and 15). Analysis of residue of chlorpyrifos and release of chloride in soil were evaluated. The total soil microflora and the chlorpyrifos degraders in rhizosphere of cowpea were also enumerated (Table 36).

4.8. EFFECT OF DEVELOPED CONSORTIUM ON BIOMETRIC CHARACTERS OF COWPEA IN STERILIZED SOIL

Maximum plant height of 193.25 cm was recorded in control (T1). Significant reduction in plant height was observed due to spiking of soil with 100 and 400 ppm chlorpyrifos which recorded 139.62 cm and 118.75 cm respectively. The application of consortium to 100 ppm (T3) and 400 ppm spiked soil (T5) recorded 191.25cm and 176.25 cm plant height respectively, which were statistically on par with the control treatment (T1).

Maximum yield of 193.25 g was recorded in control (T1) followed by T3 and T5 which recorded 130.75 and 124.50 g respectively. A significant reduction in yield was observed in soil spiked with 100 ppm (T2) and 400 ppm (T4) chlorpyrifos which recorded 93.0 and 73.75 g respectively.

Fresh weight of plants was also maximum (83.33 g) in the control plants (T1). This was statistically on par with T3 and T5 which recorded 81.4 and 77.58 g respectively. Significant reduction in plant fresh weight was observed in treatment T2 (22.83 g) and T4 (24.20 g) where soil was spiked with 100 ppm and 400 ppm chlorpyrifos respectively.

The root fresh weight of 5.25 g was maximum in T3 which received consortium application in 100 ppm spiked soil, which was statistically on par with

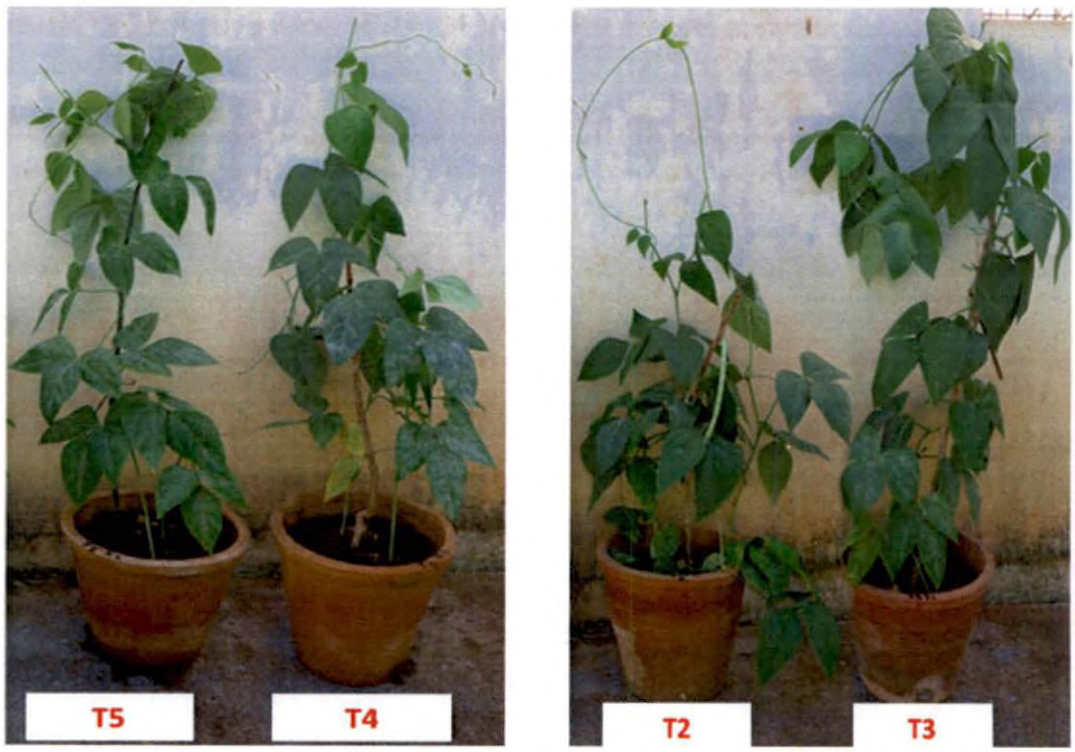


Plate14. Effect of consortium on biometric characters

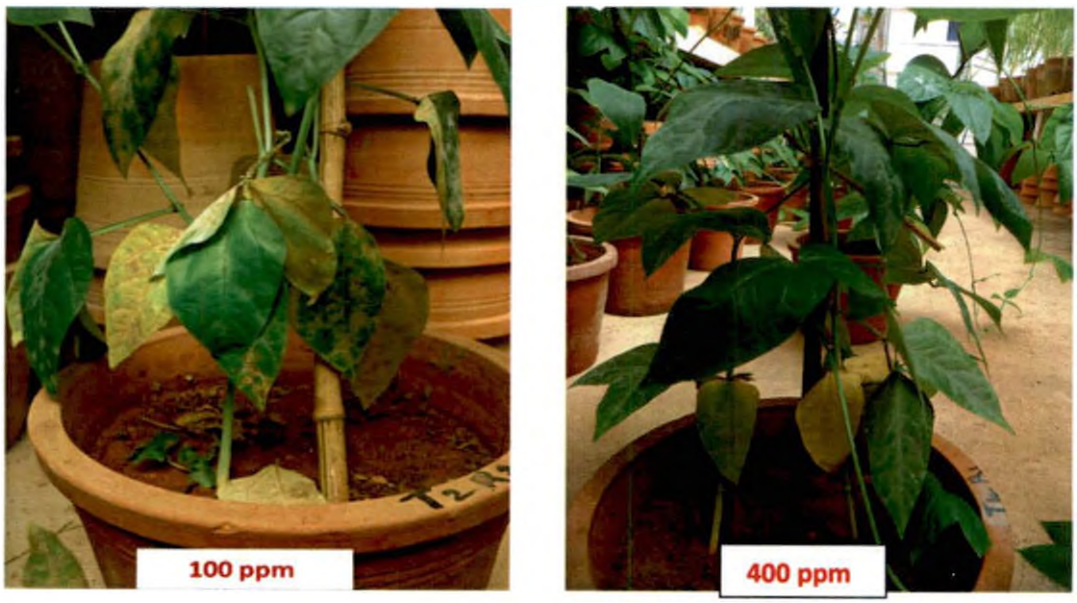


Plate 15. Yellowing symptoms due to chlorpyrifos spiking

control T1 (4.78 g). The treatment T5 recorded 4.17 g which was also statistically on par with control treatment. The root fresh weight was significantly reduced in treatments T4 and T2 which received 400 ppm and 100 ppm spiking without consortium which recorded 1.28 and 1.63 g respectively.

Maximum plant dry weight of 2.83 g was recorded in control treatment T1 followed by T3 (2.57 g) and T5 (2.05 g). Significant reduction in plant height was observed due to soil spiking with 100 ppm (T2) and 400 ppm (T4) chlorpyrifos which recorded 1.07 g.

Maximum root dry weight of 0.96 g was recorded in T3 which received consortium application in soil spiked with 100 ppm chlorpyrifos followed by control T1 (0.76 g) and T5 (0.45 g). Significant reduction in root dry weight was observed in treatments T2 (0.08 g) and T4 (0.02 g) which received 100 ppm and 400 ppm chlorpyrifos application.

Maximum nodule number of 82.25 was recorded in T3 followed by T5 (76.0) compared to control which recorded 56.75. A significant reduction in nodulation was observed due to 100 ppm (T2) and 400 ppm (T4) chlorpyrifos application which recorded 11.00 and 8.50 respectively. The control treatment recorded a nodule number of 56.75.

Maximum nodule fresh weight of 1.53 g was recorded in the treatment (T3) which received consortium application in soil spiked with 100 ppm chlorpyrifos and was statistically on par with treatment T5 (1.52 g) which also received consortium application in soil spiked with 400 ppm chlorpyrifos. The nodule fresh weight was reduced significantly in treatment T2 (0.20 g) and T4 (0.05 g) which received 100 and 400 ppm chlorpyrifos spiking respectively.

Maximum nodule dry weight was recorded in treatment T3 (0.083 g) which received consortium application in 100 ppm spiked soil, which was statistically on par with T5 (0.075 g) which received consortium application in 400 ppm chlorpyrifos spiked soil. The control T1 recorded a nodule dry weight of 0.065g. Significant reduction in nodule dry weight was recorded in T2 and T4 which recorded 0.006 g and 0.001g respectively (Table 36).

Table 36. Effect of consortium on biometric characters of cowpea in sterilized soil

Treatments	Plant height (cm)	Nodule number plant^{-1}	Fresh weight (g plant^{-1})			Dry weight (g plant^{-1})			Yield (g plant^{-1})
			Plant	Root	Nodule	Plant	Root	Nodule	
T_1-Unspiked soil	193.25	56.75	83.33	4.78	1.05	2.83	0.76	0.065	193.25
T_2-soil spiked with chlorpyrifos (100ppm)	139.62	11.00	22.83	1.63	0.20	1.07	0.08	0.006	93.00
T_3-soil spiked with chlorpyrifos (100ppm) +consortium	191.25	82.25	81.40	5.25	1.53	2.57	0.96	0.083	130.75
T_4- soil spiked with chlorpyrifos (400ppm)	118.75	8.500	24.20	1.28	0.05	1.07	0.02	0.001	73.75
T_5- soil spiked with chlorpyrifos (400ppm) +consortium	176.25	76.00	77.58	4.17	1.52	2.05	0.45	0.075	124.50
CD(0.05)	19.63	5.38	6.323	0.627	0.186	0.058	0.088	0.023	11.458

4.9. EFFECT OF DEVELOPED CONSORTIUM ON DEGRADATION OF CHLORPYRIFOS AND CHLORIDE RELEASE UNDER POT CULTURE CONDITIONS

The residue of chlorpyrifos was lowest in the treatment T3 (0.30 ppm) which received consortium application in soil spiked with 100 ppm chlorpyrifos compared to 0.87 in treatment T2 (with 100 ppm chlorpyrifos alone). It was observed that 65.70 per cent reduction in chlorpyrifos residue could be obtained due to application of microbial consortium.Similarly the residue of chlorpyrifos was reduced to 2.02 in 400 ppm spiked soil, which received consortium application compared to the treatment T4 (with 400 ppm chlorpyrifos alone) which recorded 3.72. Thus 45.60 per cent reduction of chlorpyrifos residue could be obtained due to application of consortium (Table 37).

The release of chloride is an indirect method of estimation of degradation of chlorpyrifos. The chloride analysis of soil samples revealed that maximum release of chloride was observed in treatments which received consortium application in soil spiked with 400 ppm chlorpyrifos (1.95 mg l^{-1}) which was statistically on par with T3 (1.80 mgl^{-1}). Significant reduction in chloride release was observed in treatments T4 and T2 which recorded 1.40 and 1.48 mgl^{-1} respectively. The percentage release of chloride was maximum in T5 (39.28 per cent) and T3 (21.62 per cent), which received consortium application in soil spiked with 400 and 100 ppm chlorpyrifos respectively (Table 37).

4.10. EFFECT OF DEVELOPED CONSORTIUM ON POPULATION OF TOTAL SOIL MICROFLORA AND CHLORPYRIFOS DEGRADER IN STERILIZED SOIL

Maximum viable count of bacteria (40 $x10^5$ cfu g^{-1}) was recorded in treatment T5 followed by T3 (32 x 10^5 cfu g^{-1}) which received consortium application in soil spiked with 400 and 100 ppm chlorpyrifos. This was followed by T2 (27 x 10^3 cfu g^{-1}) and the control treatment recorded a population of 15 $x10^5$ cfu g^{-1}.

Table 37. Effect of consortium on degradation of chlorpyrifos and chloride release under pot culture conditions

Treatments	Chlorpyrifos residue (ppm)	Percentage reduction of chlorpyrifos residue	Release of chloride (mg l^{-1})	Percentage release of chloride
T_1-Unspiked soil	0.00		0.40	
T_2-soil spiked with chlorpyrifos(100ppm)	0.87	65.70	1.48	21.62
T_3-soil spiked with chlorpyrifos(100ppm)+consortium	0.30		1.80	
T_4- soil spiked with chlorpyrifos(400ppm)	3.72	45.60	1.40	39.28
T_5- soil spiked with chlorpyrifos(400ppm)+consortium	2.02		1.95	
CD(0.05)	0.140		0.21	

The fungal population was maximum in treatment T5 (230 x 10^3 cfu g^{-1}) followed by T3 (221.75 x 10^3 cfu g^{-1}) which received consortium application in soil spiked with 400 and 100 ppm chlorpyrifos respectively. The fungal population was 18.75 x 10^3 cfu g^{-1} in T4 and 12 x 10^3 cfu g^{-1} in T2. The control treatment recorded a fungal count of 10.00 x 10^3 cfu g^{-1}. The bacterial population was maximum in treatment T5 (40.0 x 10^5), followed by T3 (32.0 x10^5), T2 (27 x10^5) and T4 and T1 (15x10^5). Similarly the Actinomycetes population was maximum in the control treatment T1 (5.00 x 10^3 cfu g^{-1}), followed by T2 (3.5 x 10^3 cfu g^{-1}), T5 (3.25 x 10^3 cfu g^{-1}), T4 (3.00 x10^3 cfu g^{-1}) and T3 (2.00 x10^3 cfu g^{-1}).

The population of chlorpyrifos degraders (M5, M6, M7 and M17) in the rhizosphere of cowpea was estimated. The viable count of M5 (*Isaria farinosa*) was maximum in T5 (99.5 x 10^3 cfu g^{-1}) followed by T3 (91.0 x 10^3 cfu g^{-1}). The population of M6 (*Aspergillus fumigatus*) was maximum in T3 (196. 5 x 10^3 cfu g^{-1}) followed by T5 (152.5 x 10^3 cfu g^{-1}). The population of M7 (*Trichoderma viride*) and M17 (*Penicillium griseofulvum*) were maximum in T3 (182 x 10^3 cfu g^{-1}) and T5 which recorded 118.5 x 10^3 cfu g^{-1} and 162.5 x 10^3 cfu g^{-1} respectively (Table 38).

Table 38. Effect of consortium on population of total soil microflora and chlorpyrifos degraders in sterilized soil

Treatments	Soil microflora cfu g^{-1}			Chlorpyrifos degraders cfu g^{-1}			
	Bacteria ($x10^5$)	Fungi ($x10^3$)	Actinomycetes ($x10^3$)	Viable count($x10^3$)			
				M_5	M_6	M_7	M_{17}
T_1-Unspiked soil	15.0	10.00	5.000	0	0	0	0
T_2-soil spiked with chlorpyryfos(100ppm)	27.0	12.00	3.500	0	0	0	0
T_3-soil spiked with chlorpyrifos(100ppm)+ consortium	32.0	221.75	2.000	91.0	196.5	182.0	182.0
T_4- soil spiked with chlorpyrifos(400ppm)	15.0	18.75	3.000	0	0	0	0
T_5- soil spiked with chlorpyrifos(400ppm)+ consortium	40.0	230.00	3.250	99.5	152.5	118.5	162.5
CD(0.05)	1.97	3.05	0.515	1.869	2.234	3.079	3.079

Discussion

5. DISCUSSION

Pesticides are key components of pest management practices in intensive agriculture which advocates IPM strategy for sustainability. Pesticides will continue to be an indispensable tool for the management of pests in the years to come, as there is no suitable alternative to replace them totally. Currently, among the various groups of pesticides, organophosphates constitute the major group accounting for more than 36 percent of the total world market (Kanekar *et al.*, 2004). Among the insecticides, monocrotophos, quinalphos, chlorpyrifos, malathion and methylparathion top the list of OP insecticides used in India (Anuja George, 2005). Chlorpyrifos is one of the dominated broad spectrum organophosphorus insecticides inhibiting the neuron function of sucking, chewing and boring insects both in crop and soil (Racke *et al.*, 1994). It is reported to have a moderately high persistence in soil. The wide use of these pesticides especially chlorpyrifos over the years has resulted in environmental pollution, human and animal health hazards (Sumit Kumar, 2011). Considering the inherent toxic nature of these pesticides it is essential to remove them from the environment employing suitable remedial measures. Bioremediation exploiting microbial technology is one of the recent techniques for environmental clean-up. In the process, heterotrophic microorganisms breakdown hazardous compounds to obtain carbon and energy (Singh et al., 2004; Jisha and Ambily, 2012). Many of the scientists and research workers have isolated microorganisms from natural ecosystem which have the capacity to degrade chlorpyrifos (Mukherjee and Gopal, 1996; Mallick *et al.*, 1999; Singh *et al.*, 2003). The possible utilization of individual microorganisms (Singh *et al.*, 2004; Khanna and Vidyalakshmi, 2004) and microbial consortia for bioremediation of chlorpyrifos in contaminated soil has already been reported (Vidya Lakshmi *et al.*, 2008; Sasikala *et al.*, 2012; Barathidasan and Reetha, 2013; Hindumathy and Gayathri, 2013).

Eventhough extensive work has been conducted on microbial degradation of chlorpyrifos under laboratory conditions, not much attempt has been made so

far to exploit this technology for large scale field application. Hence the present programme was designed to isolate microorganisms capable of degradation of chlorpyrifos and to develop a consortium for its field level application.

In the present study, microorganisms capable of degradation of chlorpyrifos were isolated by enrichment culture technique from identified locations with known history of chlorpyrifos use and high level of chlorpyrifos residue in soil. Nineteen different isolates comprising eleven bacteria, seven fungi and one actinomycete were obtained. All the nineteen isolates were subjected to a preliminary screening to assess the ability of isolates to utilize chlorpyrifos as the sole source of carbon in 50, 100, 200, 400 and 800 ppm concentrations and the population buildup by the isolates was monitored at 7, 15, 20, 25 and 30 days after inoculation. The growth of 11 bacterial isolates obtained was evaluated by measuring their optical density at 660 nm. Out of the 11 bacterial isolates screened, isolate M10 showed maximum significant growth in 50, 100, 200, 400 and 800 ppm concentrations compared to other isolates (Fig. 1-5). The isolate M10 which recorded maximum growth was selected for subsequent studies. The ability of bacteria to utilize chlorpyrifos as the sole source of carbon has already been reported in *Flavobacterium* sp. (Sethunathan and Yoshida, 1973), *Pseudomonas diminuta* (Serdar *et al.*, 1982), *Arthrobacter* sp. (Oshiro *et al.*, 1996), *Enterobacter* sp. (Singh *et al.*, 2004) *Bacillus laterosporus* (Wang *et al.*, 2006) *Providencia stuartii* (Rani *et al.*, 2008) *Sphingomonas* sp., *Stenotrophomonas* sp., *Bacillus* sp. *Brevundimonas* sp. (Li *et al.*, 2008) and *Bacillus cereus* (Liu *et al.*, 2012).

It was interesting to note that the growth of isolates increased with increasing concentration of chlorpyrifos. Moreover, the ability of bacterial isolates to tolerate and grow at higher concentrations even up to 800 ppm was observed. The growth of isolates was maximum around 20-25 days after inoculation. An increased growth of isolates observed with increase in concentrations may be due to the enhanced carbon level and its utilization. Such

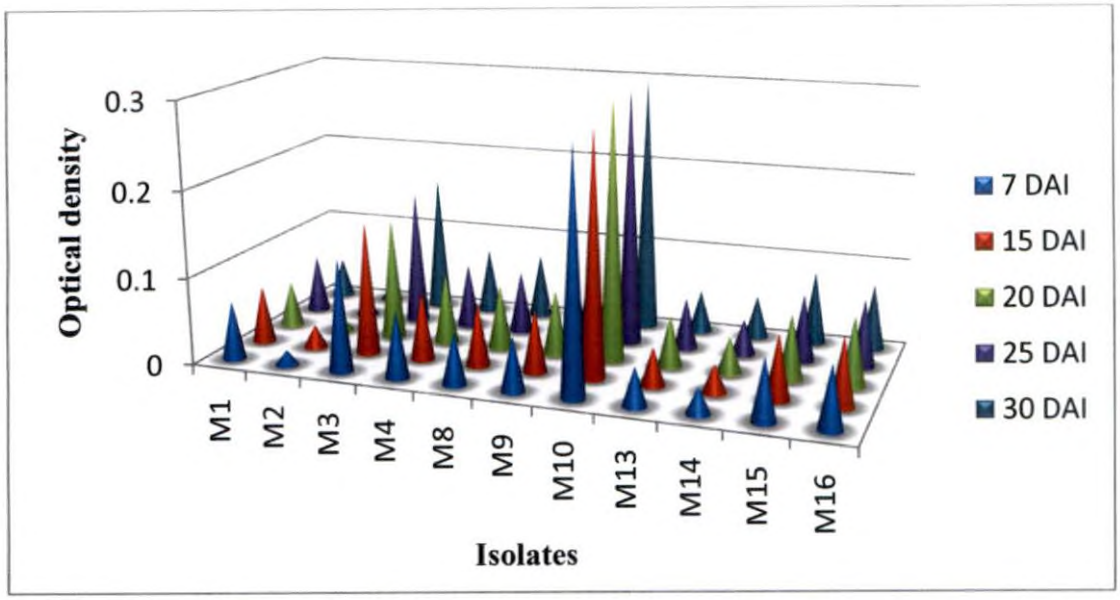


Fig. 1. Preliminary screening to assess the growth of bacterial isolates in 50 ppm concentration of chlorpyrifos in MSM

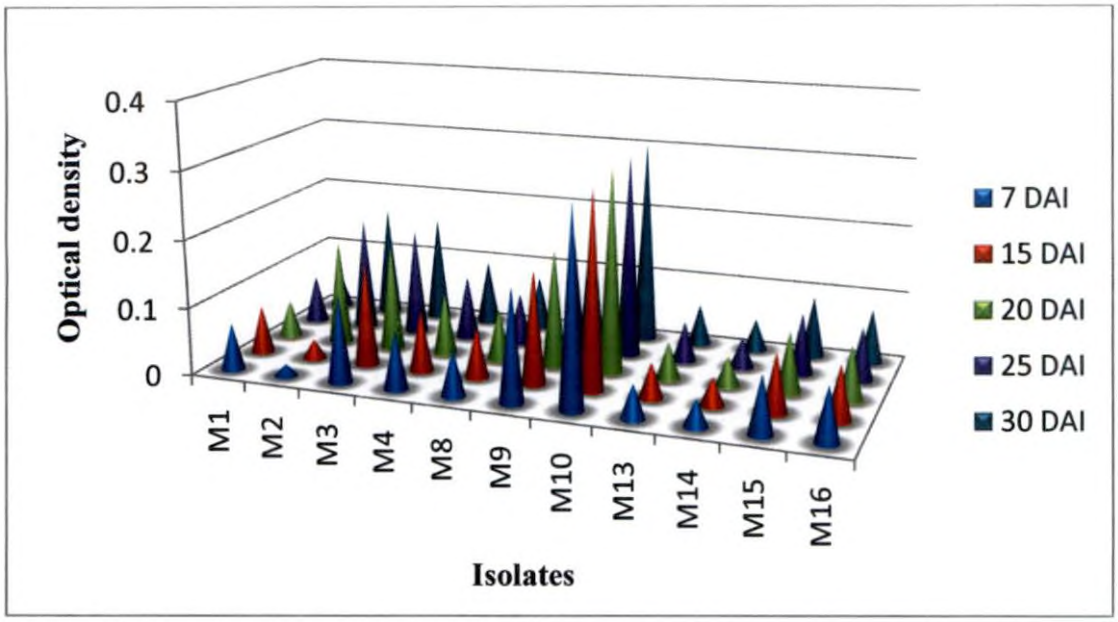


Fig. 2. Preliminary screening to assess the growth of bacterial isolates in 100 ppm concentration of chlorpyrifos in MSM

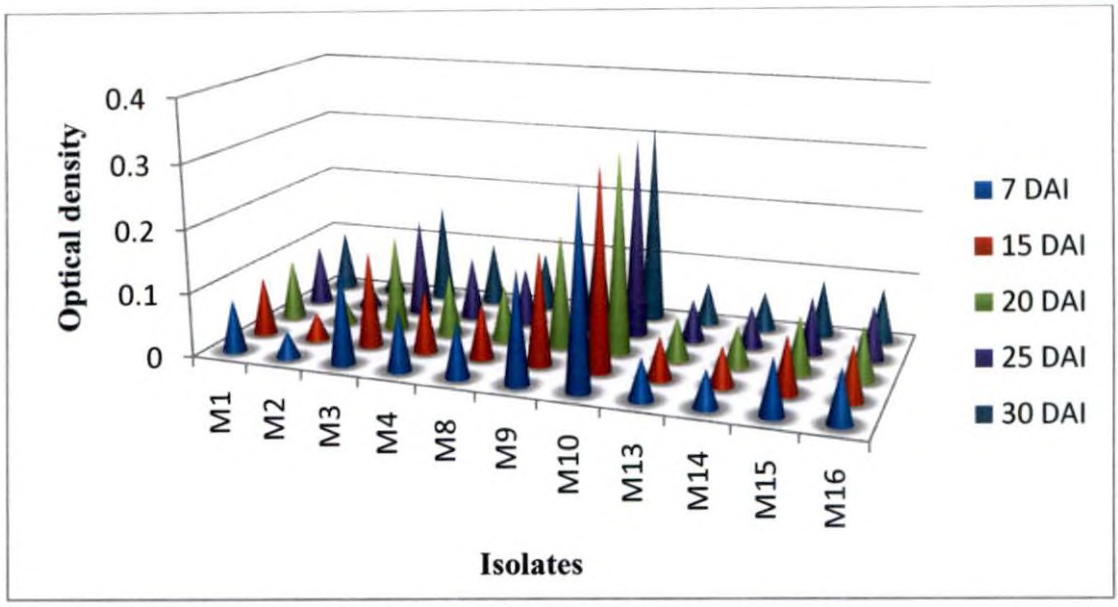


Fig. 3. Preliminary screening to assess the growth of bacterial isolates in 200 ppm concentration of chlorpyrifos in MSM

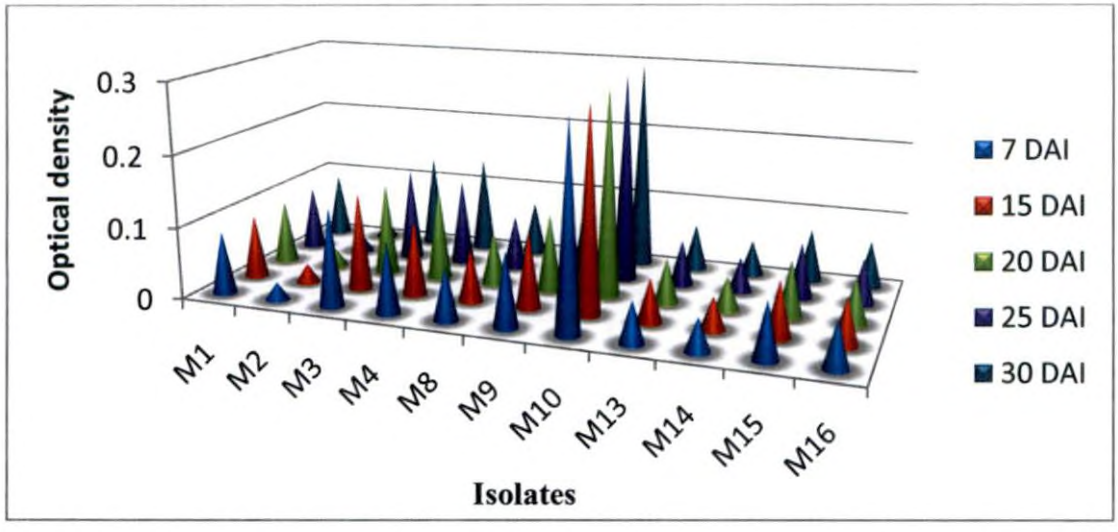


Fig. 4. Preliminary screening to assess the growth of bacterial isolates in 400 ppm concentration of chlorpyrifos in MSM

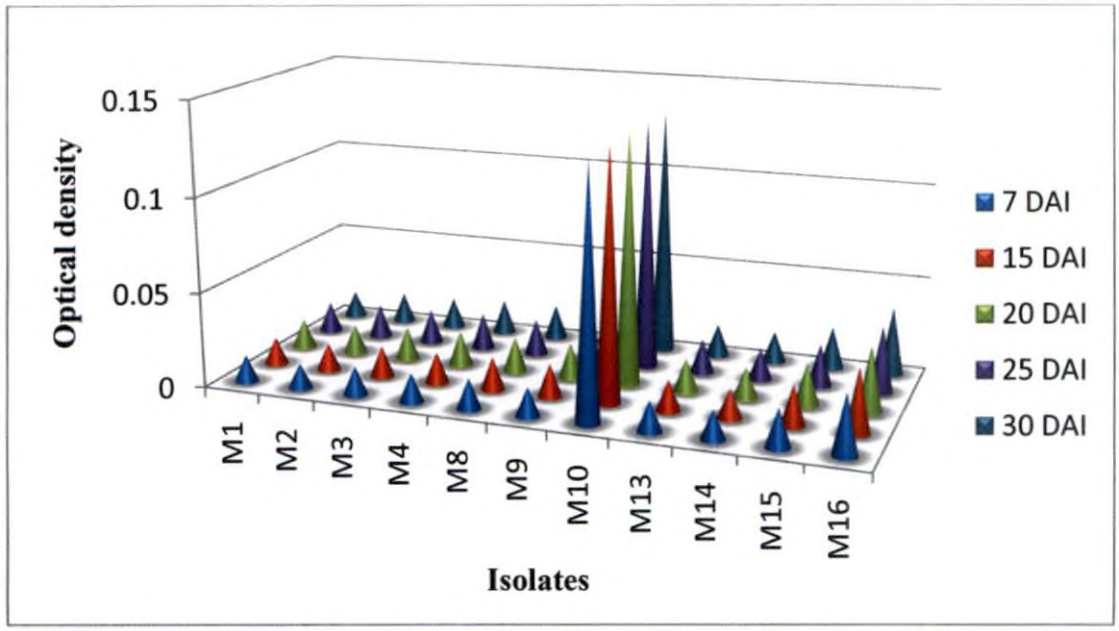


Fig. 5. Preliminary screening to assess the growth of bacterial isolates in 800 ppm concentration of chlorpyrifos in MSM

adaptation of bacteria with increasing concentrations of pesticides has already been reported (Rani *et al.*, 2008; Farhan *et al.*, 2013). Farhan *et al.*, 2013 reported that bacterial isolate *Klebsiella* showed higher degradation capacity in 200 ppm chlorpyrifos enriched medium. Similarly Award *et al.*, (2011) also reported that the isolates exhibited substantial growth in mineral salts medium supplemented with 100-300 ppm chlorpyrifos. Studies conducted by Rani *et al.*, 2008 showed that *P. stuartii* strain MS09 utilized chlorpyrifos to grow in Luria-Bertani broth containing different concentrations of chlorpyrifos at 50 - 700 mg/l. Due to the reduction of substrate utilization; bacterial population with prolonged lag phase was observed during incubation at higher concentrations of chlorpyrifos. They also observed a significant increase in bacterial growth with increase in concentration of chlorpyrifos. Earlier studies have already established that microorganisms possess the ability to tolerate and grow at higher concentrations of chlorpyrifos. Similarly, studies by Latifi *et al.*, (2012) reported that bacterial isolate coded as IRLM.1 was able to grow at concentrations of chlorpyrifos up to 2000 ppm. Bhagobaty and Malik, (2008) could isolate four bacteria belonging to *Pseudomonas* sp. that were able to grow and tolerate even up to 1600 ppm chlorpyrifos. Ehab *et al.*, (2013) also observed the ability of bacterial strain *Bacillus subtilis* Y242 to utilize chlorpyrifos as a carbon source and grow in media containing concentrations up to 150 ppm.

Out of the remaining eight isolates, seven were fungi and one was actinomycete. These seven fungal and one actinomycete isolates were also subjected to a preliminary screening to assess the population build up in terms of total viable count in 50, 100, 200, 400 and 800 ppm concentrations of chlorpyrifos at 7, 15, 20, 25 and 30 days after inoculation. In the preliminary screening, the fungal isolates, M5, M6, M7 and M17 and the actinomycete isolate, M12 recorded significant population buildup compared to M11, M18 and M19 in all the five concentrations tested at different intervals (Fig. 6-10). It is well known that fungal and actinomycete isolates are highly versatile organisms with ability to metabolize even the most complex polymers (Jones and Hastings, 1981;

De Schrijver and De Mot, 1999). Similar assessment of growth of fungal isolates based on population buildup in chlorpyrifos degradation studies has already been reported (Abd El-Mongy and Abd El-Ghany, 2009). Studies have also shown that fungi including *Trichoderma harzianum*, *Penicillium vermiculatum* and *Mucor* sp. (Jones and Hastings, 1981), *Phenerochaete chyrsosporium* (Bumpus *et al.*, 1993) *Aspergillus* sp., *Trichoderma* sp. (Liu *et al.*, 2003) and *Fusarium* sp. (Wang *et al.*, 2005) have the ability to degrade different concentrations of chlorpyrifos. A pure fungal strain, *Acremonium* sp. utilized 83.9 per cent chlorpyrifos as a source of carbon and nitrogen (Kulshrestha and Kumari, 2011). Maya *et al.*, 2012 isolated 5 chlorpyrifos degrading fungal isolates such as *Aspergillus* sp., two *Pencillium* sp., *Eurotium* sp., *Emericella* sp. coming under phylum Ascomycota. Silambarasan & Abraham, (2013) also obtained an isolate *A. terreus* JAS1 which could degrade 300 mg kg^{-1} chlorpyrifos in soil within 24h. Bumpus *et al.* (1993) reported a fungal strain *Phanerochaete chrysosporium* which was able to mineralize chlorpyrifos. Bending *et al.* (2002) could obtain two fungi *Hypholoma fasciculare* and *Coriolus versicolor* capable of degrading chlorpyrifos in soil bio-bed after 42 days.

An increase in growth with increase in concentration of chlorpyrifos was observed with fungal isolates also. Eventhough the population buildup gradually increased with increase in concentration of chlorpyrifos up to 400 ppm, a decrease was observed at 800 ppm concentration. The population buildup of isolates, M5, M6, M7, M12 and M17 was less in 800 ppm concentration compared to lower concentrations. The growth of isolates M11, M18 and M19 were significantly reduced in 800 ppm concentration of chlorpyrifos (Fig. 10). A very high concentration usually leads to failure of biodegradation because certain microorganisms are not resistant to such high concentrations. The significant number of active microbial population depends on resistant level of microbial strain and also the chemical nature of material to be degraded (Fang *et al.*, 2008)

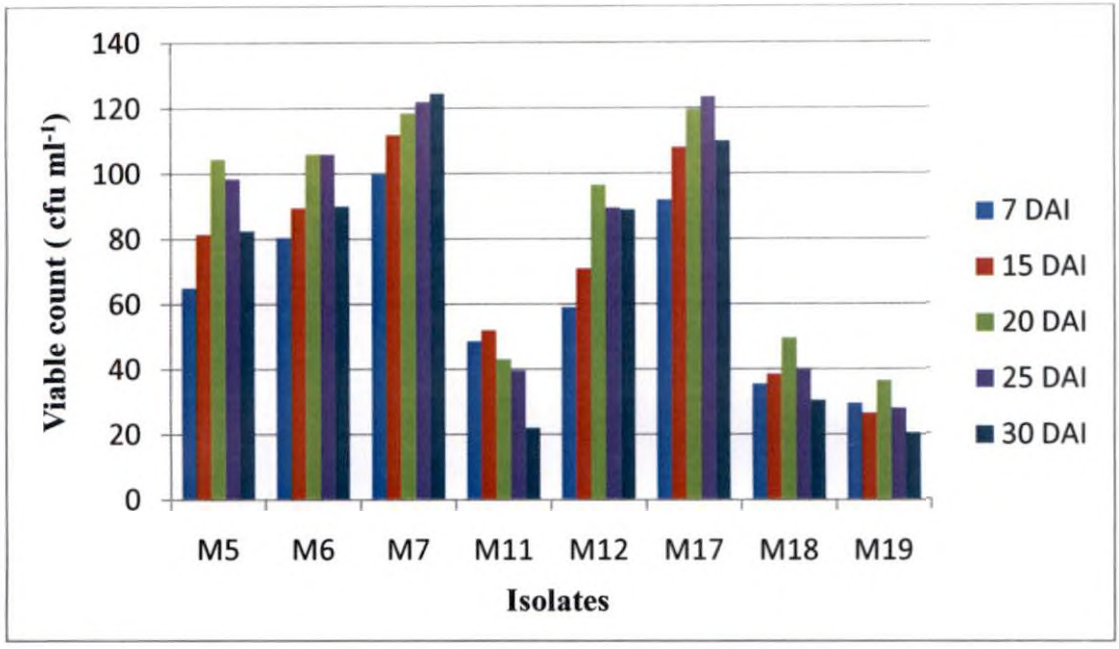


Fig. 6. Preliminary screening to assess the population build up of fungal and actinomycete isolates in 50 ppm concentration of chlorpyrifos in MSM

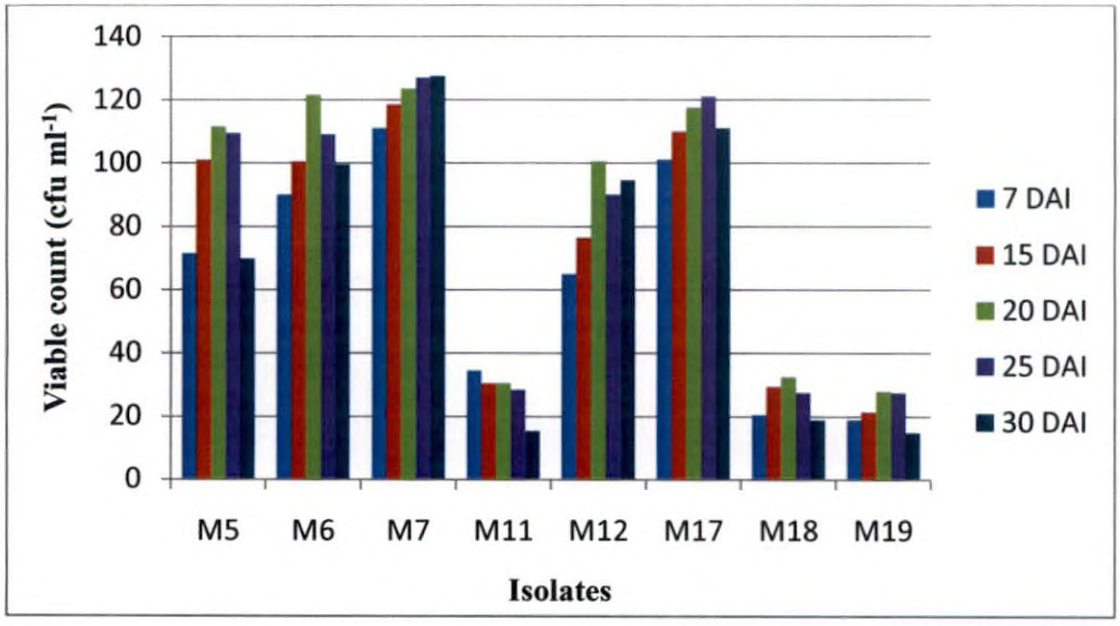


Fig. 7. Preliminary screening to assess the population build up of fungal and actinomycete isolates in 100 ppm concentration of chlorpyrifos in MSM

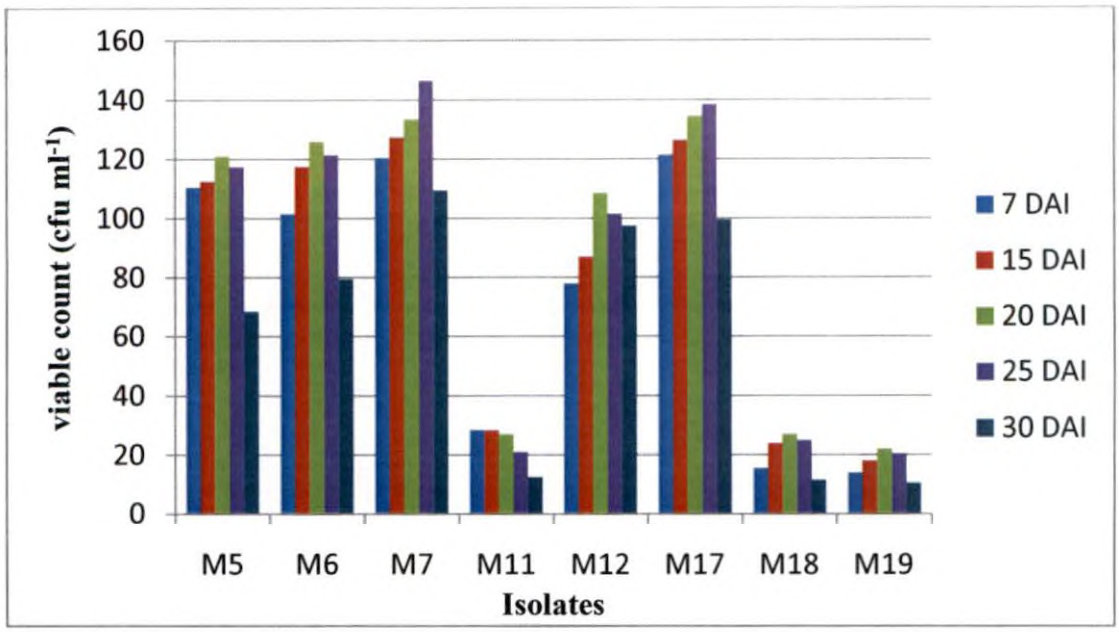


Fig. 8. Preliminary screening to assess the population build up of fungal and actinomycete isolates in 200 ppm concentration of chlorpyrifos in MSM

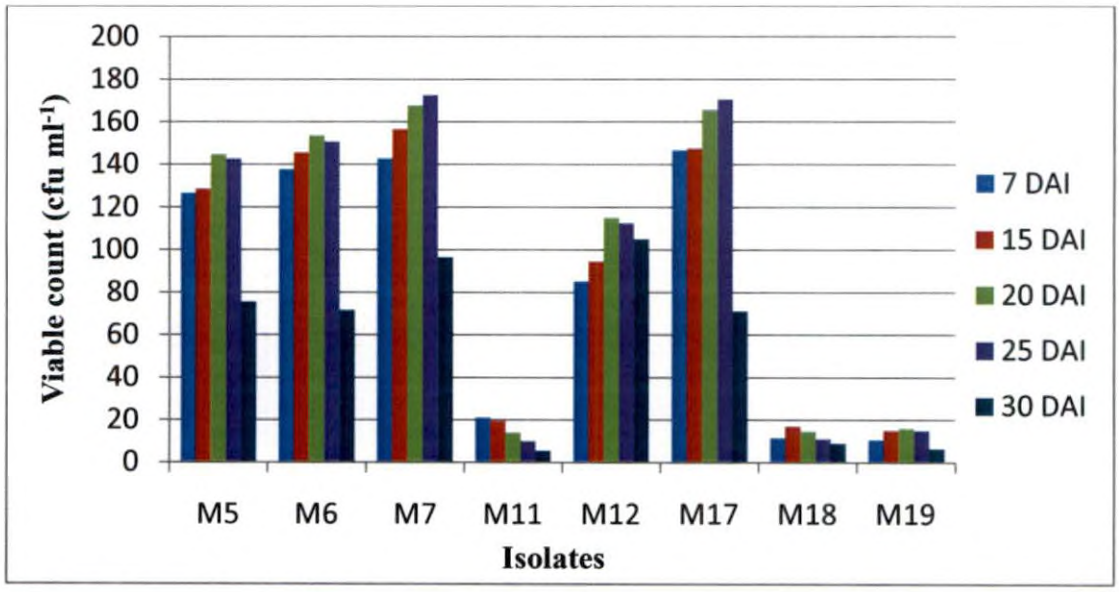


Fig. 9. Preliminary screening to assess the population build up of fungal and actinomycete isolates in 400 ppm concentration of chlorpyrifos in MSM

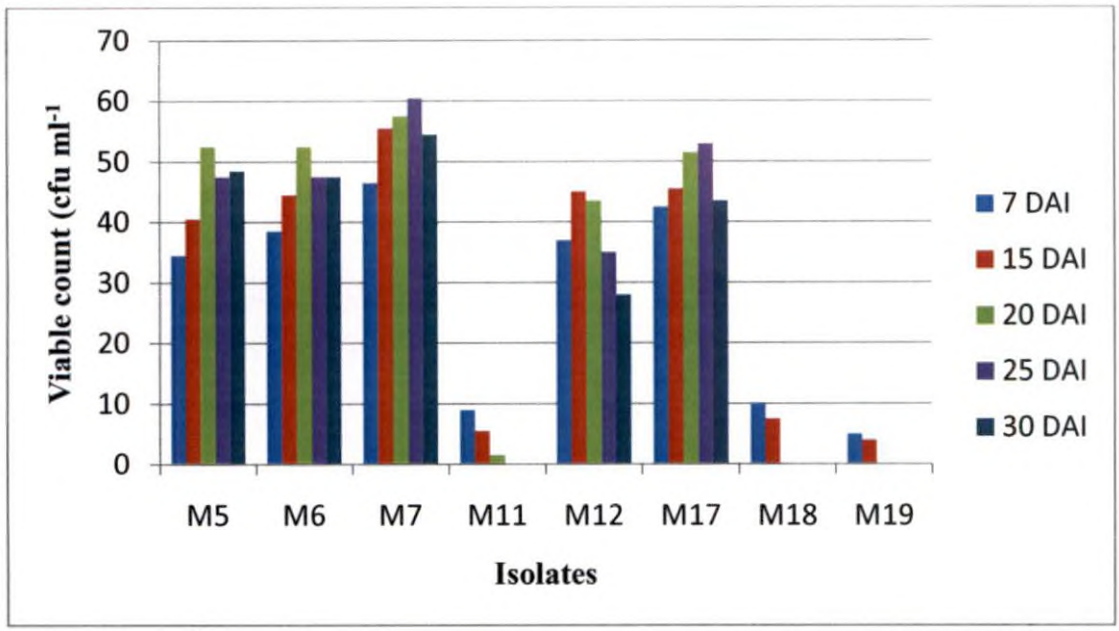


Fig. 10. Preliminary screening to assess the population build up of fungal and actinomycete isolates in 800 ppm concentration of chlorpyrifos in MSM

After preliminary screening, the bacterial isolate, M10 and fungal isolates, M5, M6, M7 and M17 and actinomycete isolate, M12 were selected for secondary screening to confirm their efficiency to grow and degrade 100, 200, 400 and 800 ppm concentrations of chlorpyrifos at 7, 15, 20, 25 and 30 days interval. In the secondary screening, significant population build up was observed with isolates, M5, M6, M7 and M17 in all concentrations tested. The bacterial isolate, M10 and actinomycete isolate, M12 was unable to grow even in 100 ppm concentrations of chlorpyrifos in the secondary screening (Fig. 11-14). This may be due to the loss of ability of the isolates to tolerate and grow in chlorpyrifos amended medium. Another significant observation was that eventhough there was significant colony count, the mycelial mat formation was comparatively less in chlorpyrifos amended medium which is often advantageous for development of liquid formulations. Similar results were obtained by Bhalerao and Puranik, (2009) who noticed inhibition of mycelial growth of *Aspergillus oryzae* in flask with increasing concentration of chlorpyrifos. Similarly, mycelial mat formation was found to be reduced in *Ganoderma* sp. (Silambarasan and Abraham, 2012) and in *Aspergillus terreus* (Silambarasan and Abraham, 2013).

In the secondary screening, the residue of chlorpyrifos remaining in the MSM in 100, 200, 400 and 800 ppm concentrations was also analyzed on 7, 15, 20, 25 and 30 days after inoculation. In 100 ppm concentration, maximum reduction of chlorpyrifos residue of 69.3 per cent was recorded by isolate M17 followed by 62.3 per cent by M7. The percentage degradation of chlorpyrifos by isolate M17 gradually increased from 7^{th} day of inoculation and recorded a maximum of 78.9 per cent on 30^{th} day after inoculation. The isolates M7, M5, M6 also gave significant reduction of chlorpyrifos residue of 79.6, 64.2 and 59.8 per cent respectively compared to 11.3 and 7.6 percent recorded by isolates M12 and M10 respectively on 30 DAI in 100 ppm concentration. A similar trend was also observed in 200, 400 and 800 ppm concentrations (Fig. 15-18). These results are in agreement with the findings of Jones and Hastings (1981) who could obtain 95 to 98 per cent degradation of 50 ppm chlorpyrifos by a group of forest fungi

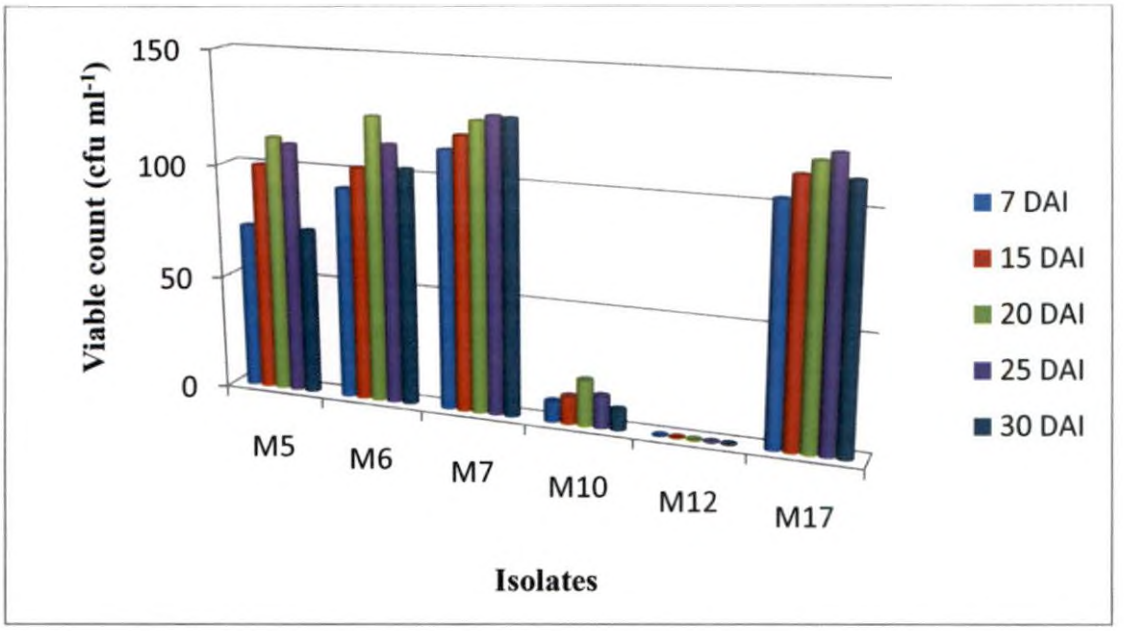


Fig. 11. Secondary screening to assess the population build up of selected isolates in 100 ppm concentration of chlorpyrifos in MSM

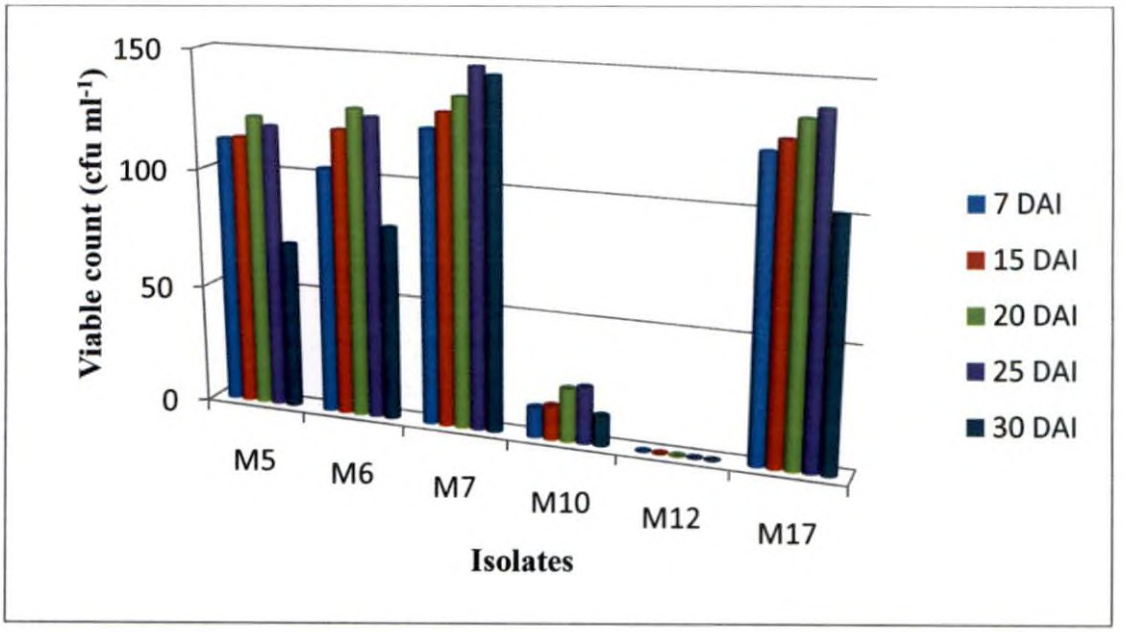


Fig. 12. Secondary screening to assess the population build up of selected isolates in 200 ppm concentration of chlorpyrifos in MSM

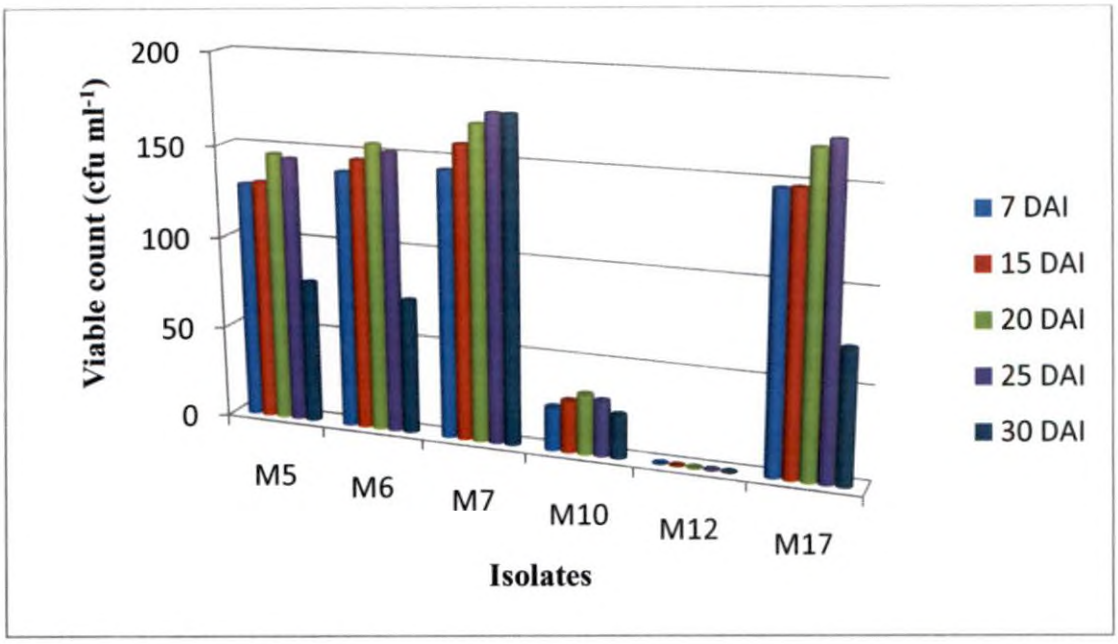


Fig. 13. Secondary screening to assess the population build up of selected isolates in 400 ppm concentration of chlorpyrifos in MSM

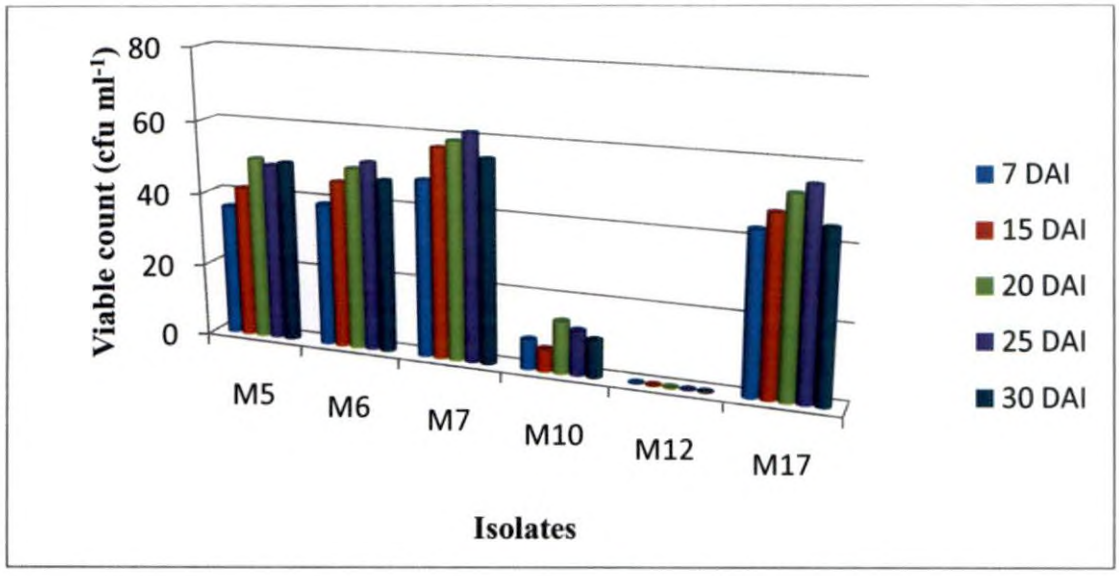


Fig. 14. Secondary screening to assess the population build up of selected isolates in 800 ppm concentration of chlorpyrifos in MSM

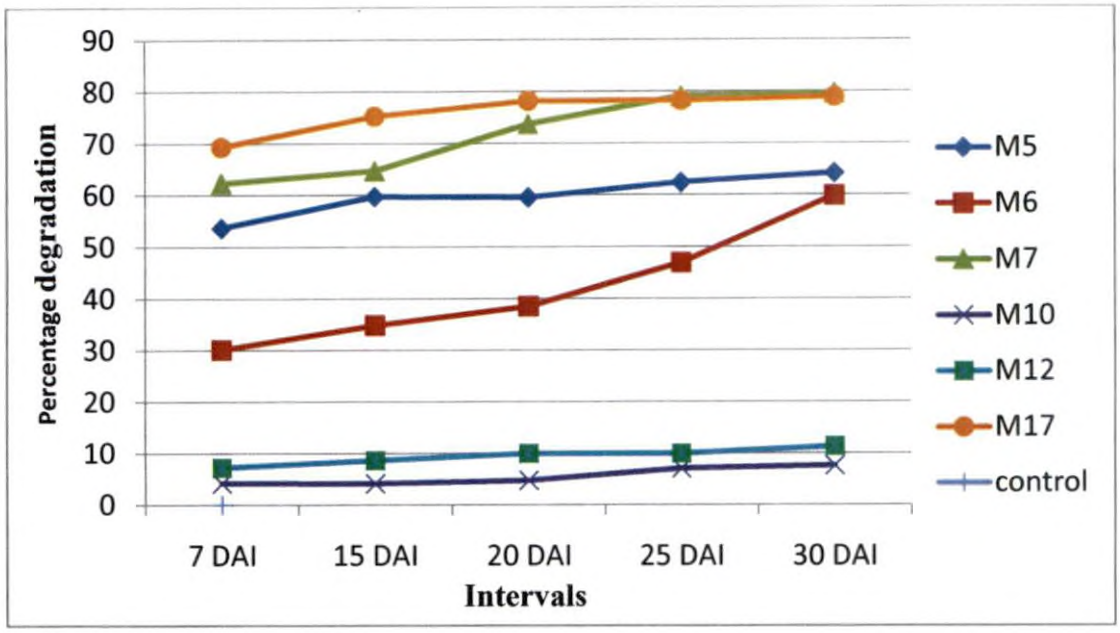


Fig. 15. Degradation of 100 ppm chlorpyrifos by selected isolates at different intervals

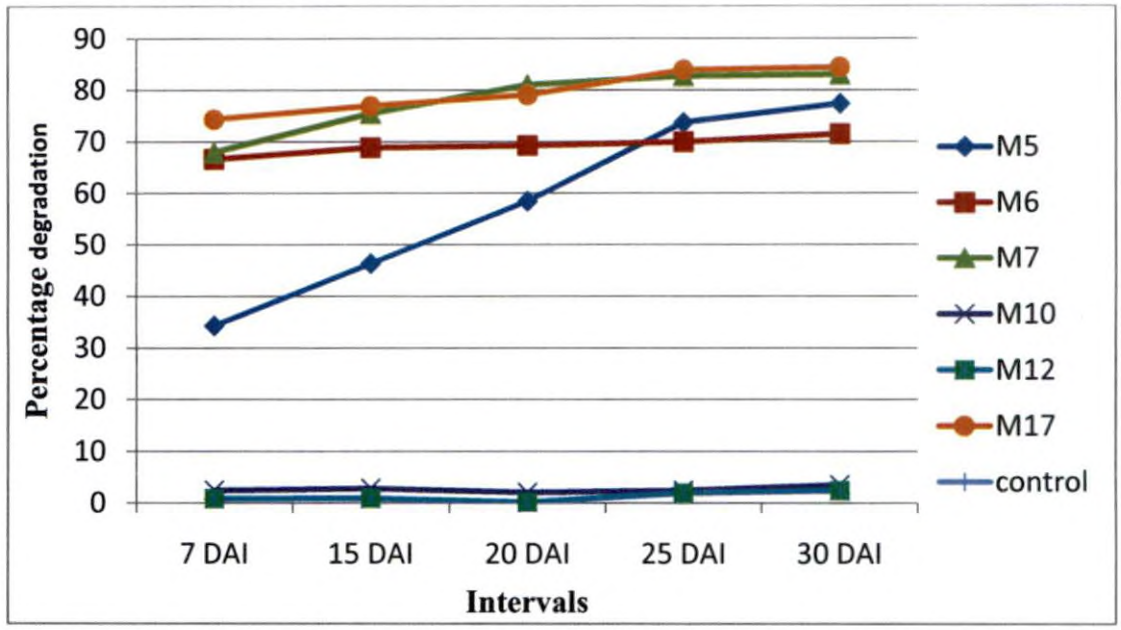


Fig. 16. Degradation of 200 ppm chlorpyrifos by selected isolates at different intervals

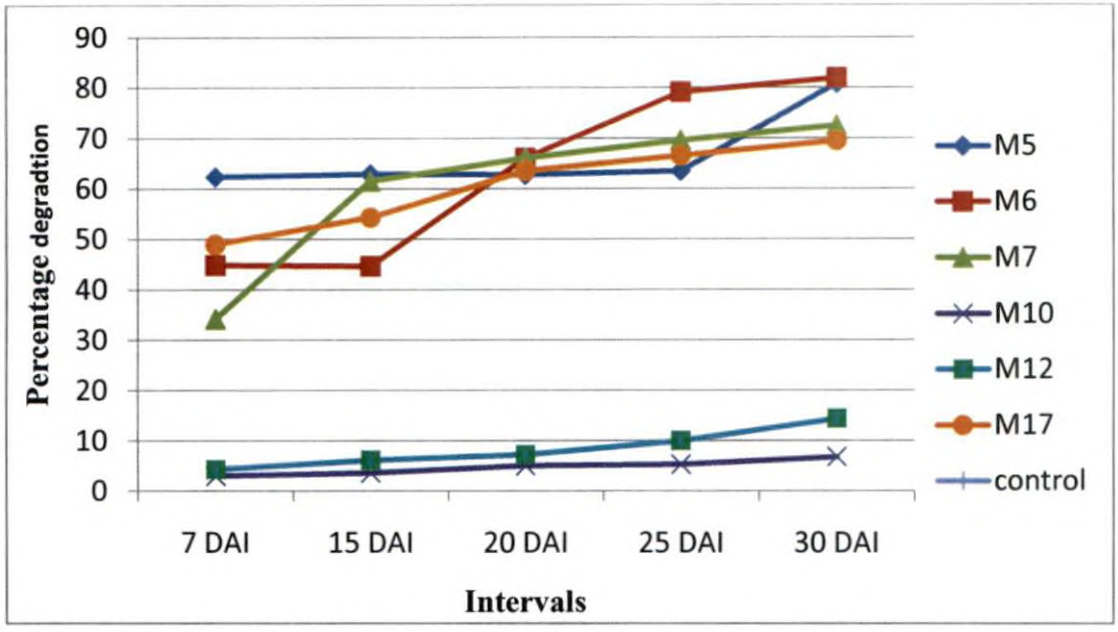


Fig. 17. Degradation of 400 ppm chlorpyrifos by selected isolates at different intervals

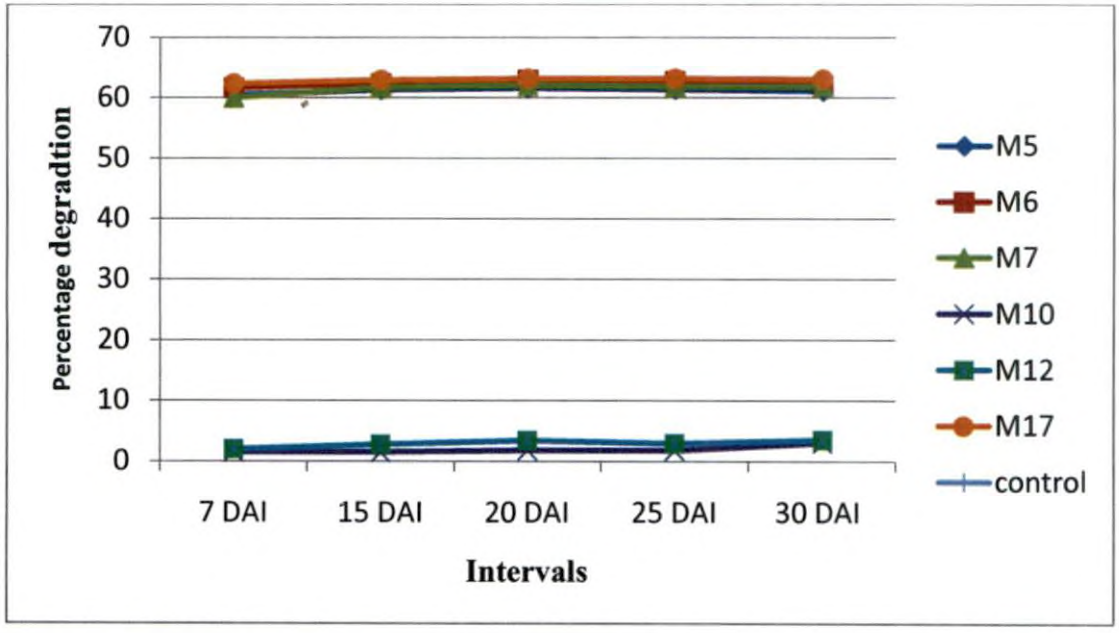


Fig. 18. Degradation of 800 ppm chlorpyrifos by selected isolates at different intervals

namely *Trichoderma harzianum*, *Penicillium vermiculatum*, and *Mucor* sp. after 28 days of incubation. Similar work conducted by Latifi *et al.*, 2012 has shown that when microorganisms were initially exposed to low concentration of pesticides, followed by gradual higher concentrations, increase in degradation ability could be observed. In his study, 140 ppm was the optimum concentration for growth, but with increasing concentration, sufficient growth was observed upto 2000 ppm chlorpyrifos which is the highest OP concentration ever reported to support growth of microbes. However, the degradation of chlorpyrifos by M10 and M12 were significantly reduced in all the higher concentrations at different intervals. This may be due to the loss of ability of the isolates to tolerate and grow at higher concentrations of chlorpyrifos.

Eventhough growth and degradation of chlorpyrifos by isolates M17, M6, M5 and M7 was comparatively less in 800 ppm concentration, about 62.3, 61.6, 60.4 and 60.0 per cent degradation was observed on 7^{th} day after inoculation and this trend continued even up to 30 days after inoculation which recorded 63.0, 62.0, 61.8, and 61.0 per cent by M17, M6, M7 and M5 respectively. In 800 ppm, maximum degradation was observed between 20-30 days. Hua *et al.*, (2009) also obtained similar results wherein maximum degradation of chlorpyrifos was observed on 35^{th} day of inoculation.

Release of chloride into the medium is often taken as an indirect method of measuring chlorpyrifos degradation. Hence in the secondary screening, release of chloride into the MSM was also analyzed. Eventhough a slight increase in the release of chloride by selected isolates was observed in all the concentrations, the data was not statistically significant (Fig.19-22). Microorganisms transform chlorpyrifos co-metabolically in to its metabolite TCP and as TCP contains three chlorine atoms at its pyridinol ring, to break this ring, chlorine atom will be removed ultimately (Feng *et al.*, 1997). Similar reports on release of chloride due to cleavage and mineralization of heterocyclic ring due to activities of

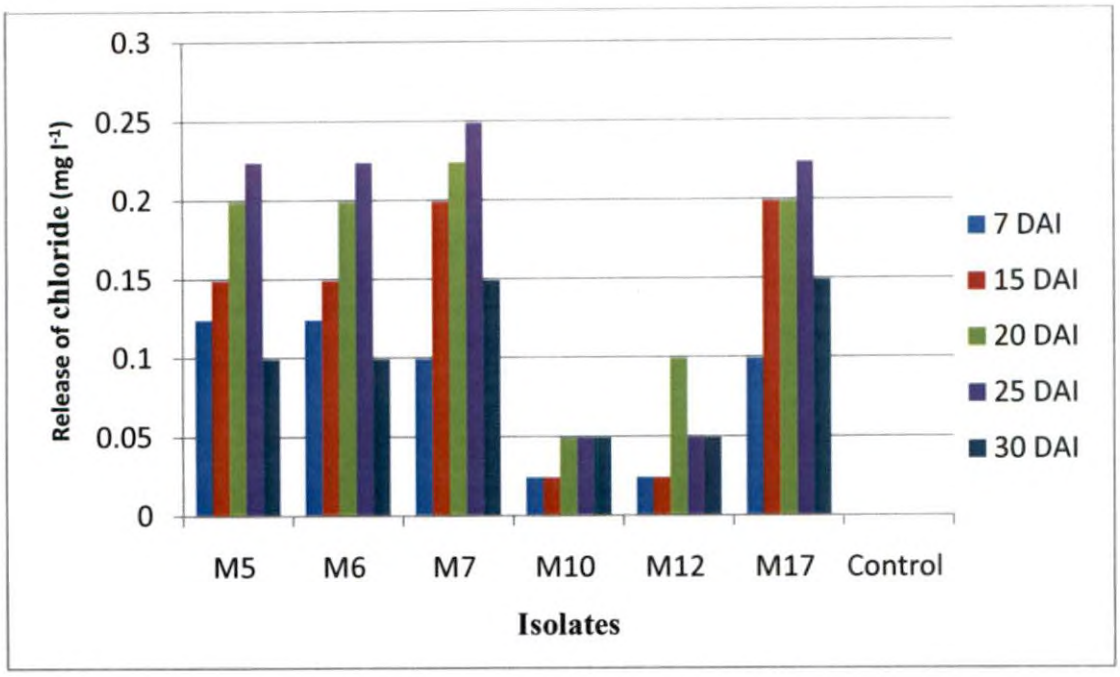


Fig. 19. Release of chloride by selected isolates in 100 ppm concentration of chlorpyrifos in MSM

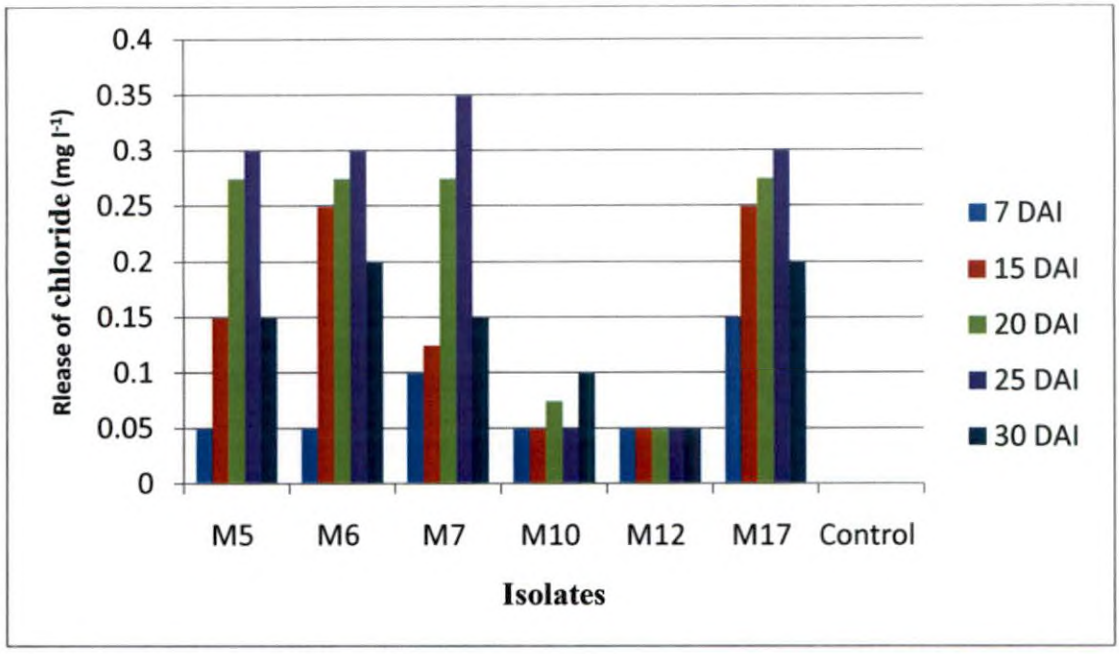


Fig. 20. Release of chloride by selected isolates in 200 ppm concentration of chlorpyrifos in MSM

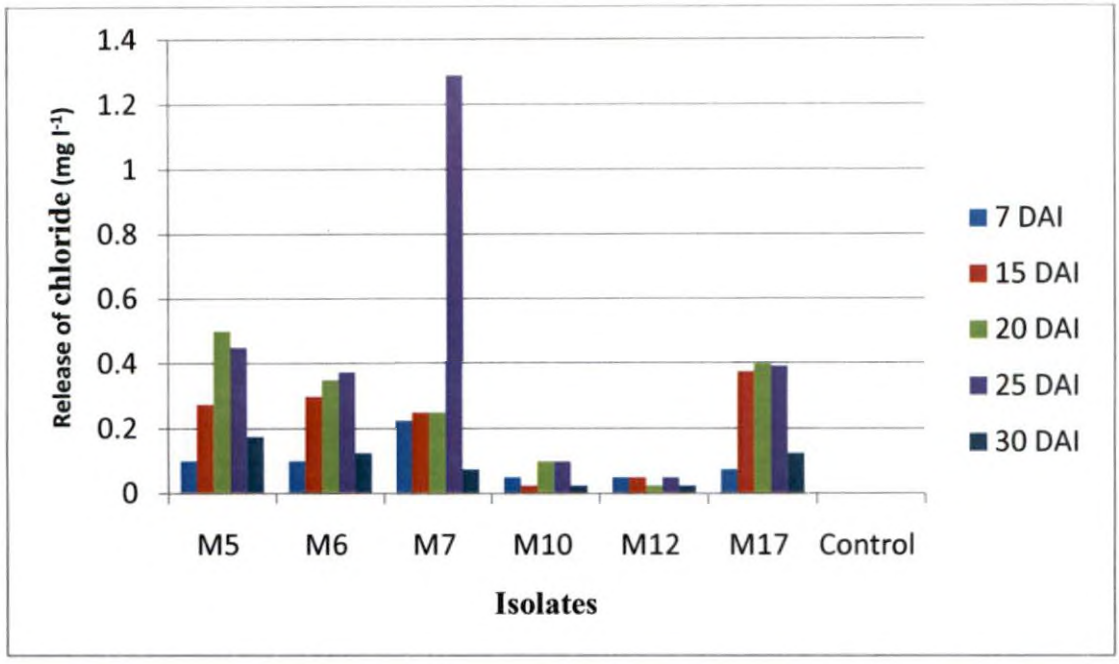


Fig. 21. Release of chloride by selected isolates in 400 ppm concentration of chlorpyrifos in MSM

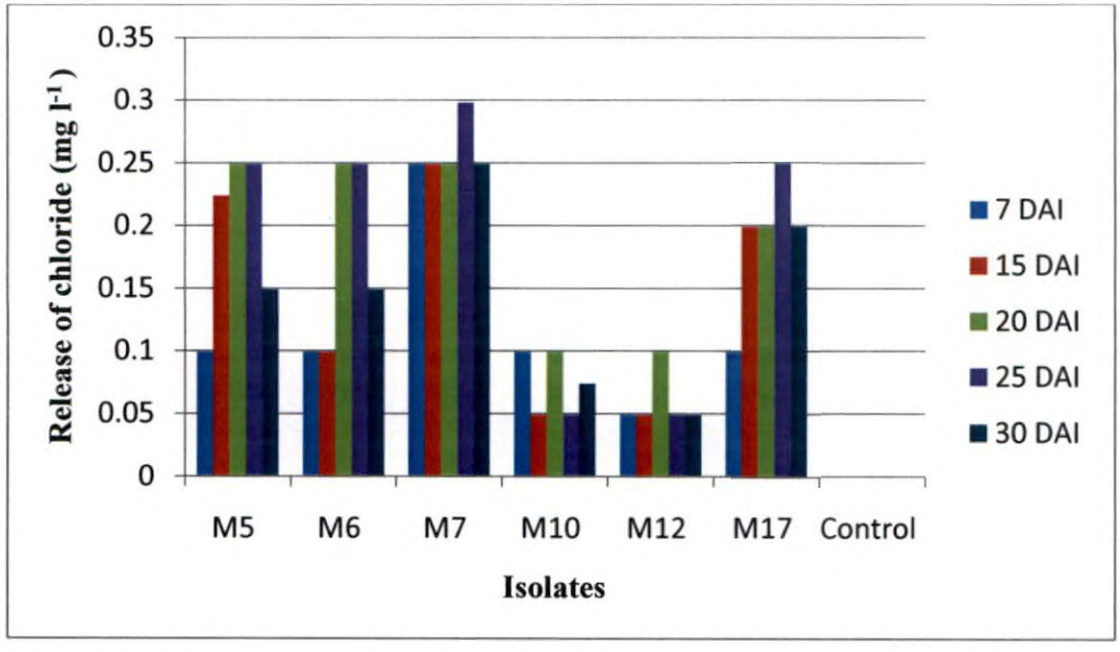


Fig. 22. Release of chloride by selected isolates in 800 ppm concentration of chlorpyrifos in MSM

microorganisms has already been reported (Somasundaram *et al.*, 1987; Racke *et al.*, 1988; Yucheng Feng, 2003; Anuja George, 2005).

After secondary screening, the fungal isolates, M5, M6, M7 and M17 which recorded significant population build up in 100,200,400 and 800 ppm concentrations and which gave maximum reduction in chlorpyrifos residue were selected for further studies.

The selected fungal isolates, M5, M6, M7 and M17 were subjected to morphological and molecular characterization. The molecular characterization study was done in collaboration with Rajiv Gandhi Centre for Biotechnology. After BLAST analysis the selected fungal isolates, M5, M6, M7 and M17 were identified as- *Isaria farinosa*, *Aspergillus fumigatus*, *Trichoderma viride* and *Penicillium griseofulvum* respectively. Construction of Phylogenetic tree revealed the relatedness of *Isaria* to *I. farinosa* and *I. amoenerosea* sp., *Aspergillus* to *A. clavatus* and *A. fumigatus*, *Trichoderma* to *T. viride* and *T. harzianum*, *Penicillium* to *P. coffeae* and *P. charlesii*. Several species of the same genera obtained in the present study -*Aspergillus, Trichoderma and Penicillium* have already been reported to degrade organophosphorus pesticides. However, the degradation efficiency of *Isaria* sp. has not been reported earlier. Studies have shown that efficient degradation of chlorpyrifos could be obtained using *Aspergillus* sp. and *Trichoderma* sp. (Liu *et al.*, 2003). Strains of *Aspergillus flavus* and *Aspergillus niger* isolated from soil with previous history of chlorpyrifos use were also reported to biomineralise chlorpyrifos in liquid culture medium (Swati and Singh, 2002).

With the objective of developing a consortium, the compatibility of the selected isolates was studied by co-culturing in Potato dextrose broth as well as in MSM. Significant growth of all the isolates could be obtained in MSM as well as in PDB on 7th day of inoculation confirming that they are highly compatible. The compatibility of the selected isolates was also ascertained by dual culture

technique wherein no inhibition was recorded. After assessing the compatibility of all the fungal isolates, a liquid consortium was prepared in MSM medium. The concept of consortium was investigated by many earlier researchers (Anuja George, 2005; Khanna and Vidyalakshmi 2004; Macek *et al.* 2000; Kuiper *et al.* 2004; Chaudhry *et al.* 2005).

Chlorpyrifos is normally degraded to a preliminary metabolite 3,5,6 – trichloro-2-pyridinol (TCP) (Macalady and wolfe,1983) which is further degraded to a secondary metabolite 3,5,6 – trichloro- 2-methoxy pyridine (TMP) along with O,O-diethyl phosphorothionic acid (DETP) which subsequently undergoes decomposition to diols and triols and ultimately cleavage of the ring to fragmentary products (Smith *et al.*,1967). Microorganisms play important role not only in the degradation of the parent compound but also in the subsequent metabolism of breakdown products. The different pesticide pathways for degradation of parent compound and breakdown products or metabolites may not be present in a single species. In this context the concept of the microbial consortia becomes relevant. Different individual organisms of the microbial consortium can work in a concerted manner to achieve an effective degradation of parent compound as well as the metabolites (Macek *et al.* 2000; Kuiper *et al.* 2004; Chaudhry *et al.* 2005).

The growth of the individual isolates present in the consortium was also enumerated in 50,100,200,400 and 800ppm concentrations on 7^{th}, 15^{th}, 20^{th}, 25^{th} and 30^{th} DAI. The total viable count of all the isolates gradually increased from 7^{th} day of inoculation in 100 ppm with maximum growth on 20^{th} and 25^{th} day of inoculation. The population buildup of all the isolates gradually increased with increase in concentration up to 400 ppm and was reduced considerably at 800 ppm concentration of chlorpyrifos (Fig. 23-27). The analysis of chlorpyrifos residue on 25^{th} day revealed that the percentage degradation of chlorpyrifos by the consortium attained maximum of 87.6 per cent in 400 ppm concentration followed by 83.0 per cent in 200 ppm and 81.7 per cent in 100 ppm concentration (Fig. 28,

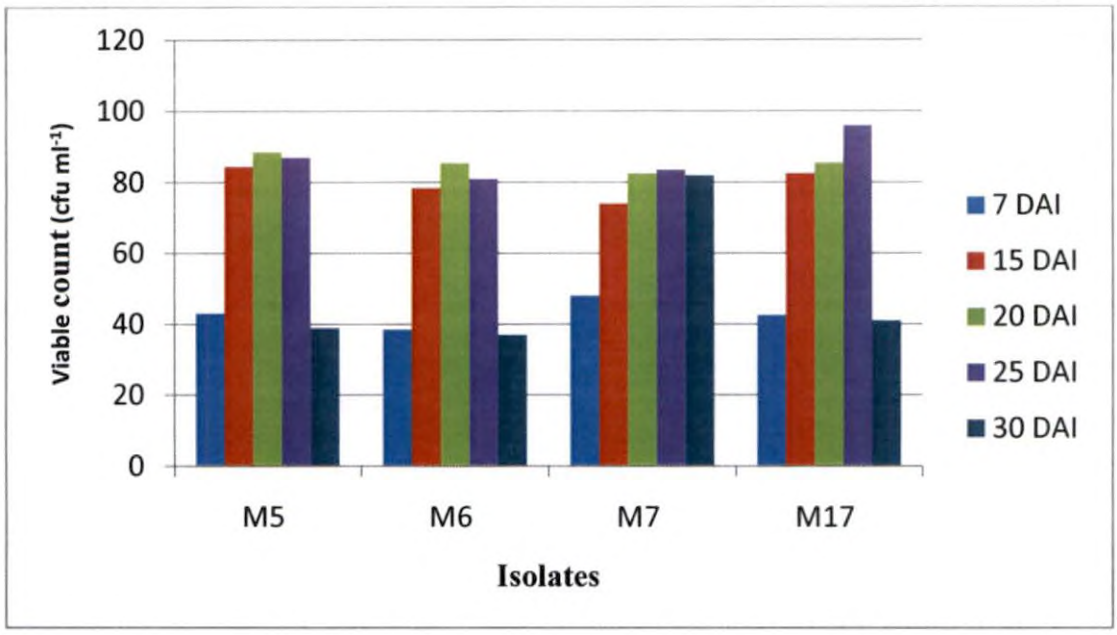


Fig. 23. Population build up of individual isolates of consortium in 50 ppm concentration of chlorpyrifos in MSM

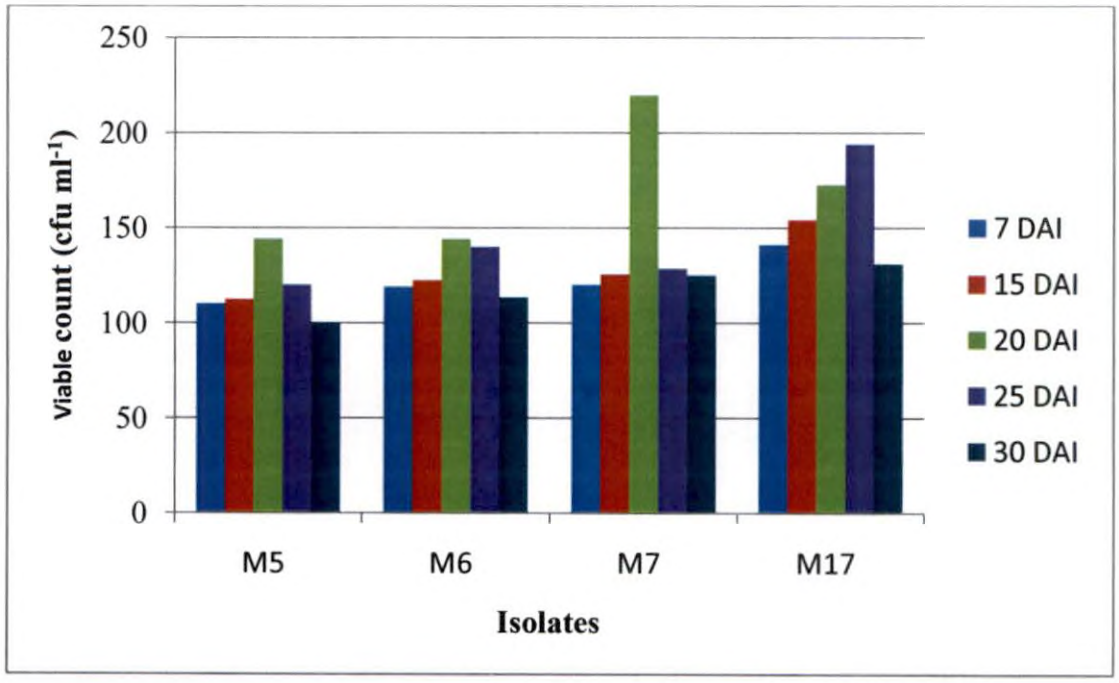


Fig. 24. Population build up of individual isolates of consortium in 100 ppm concentration of chlorpyrifos in MSM

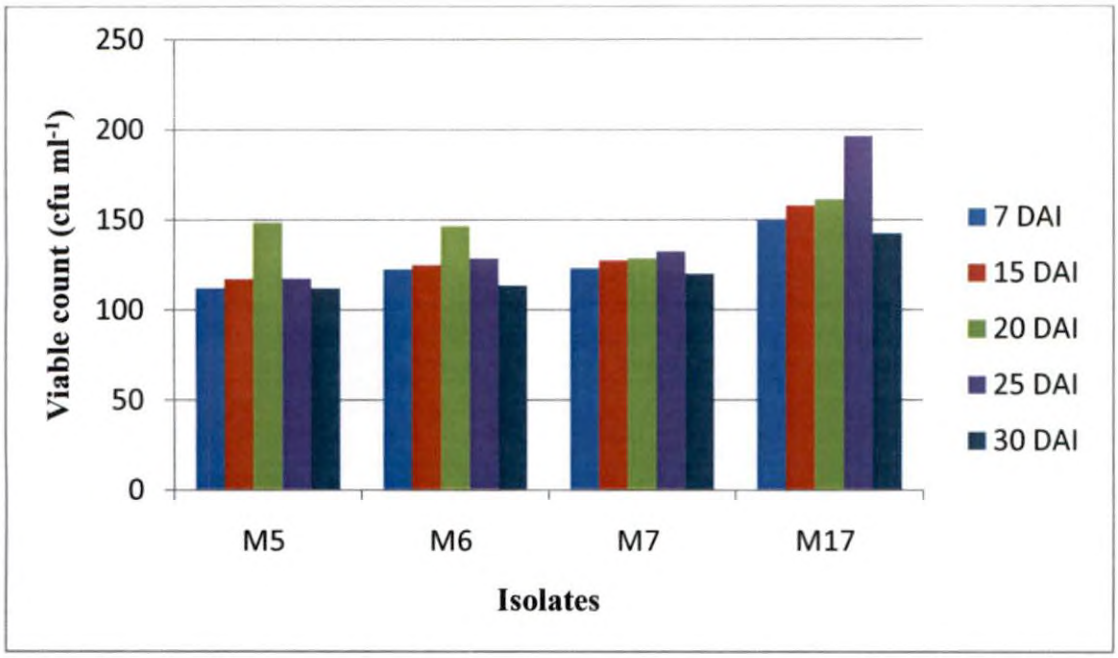


Fig. 25. Population build up of individual isolates of consortium in 200 ppm concentration of chlorpyrifos in MSM

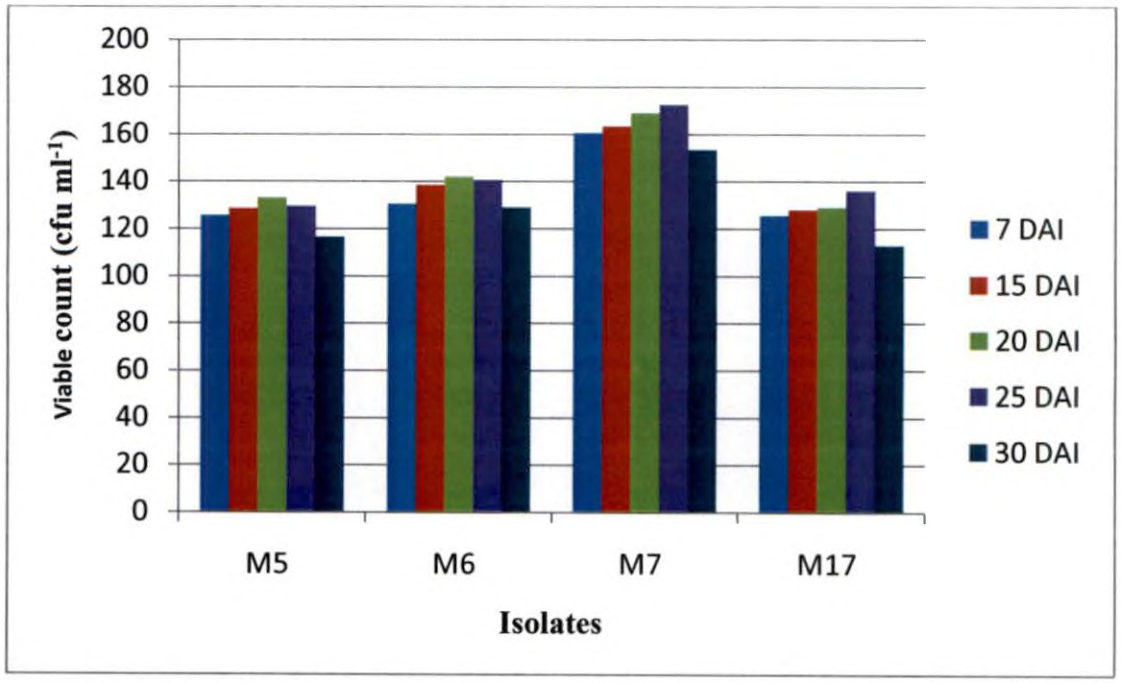


Fig. 26. Population build up of individual isolates of consortium in 400 ppm concentration of chlorpyrifos in MSM

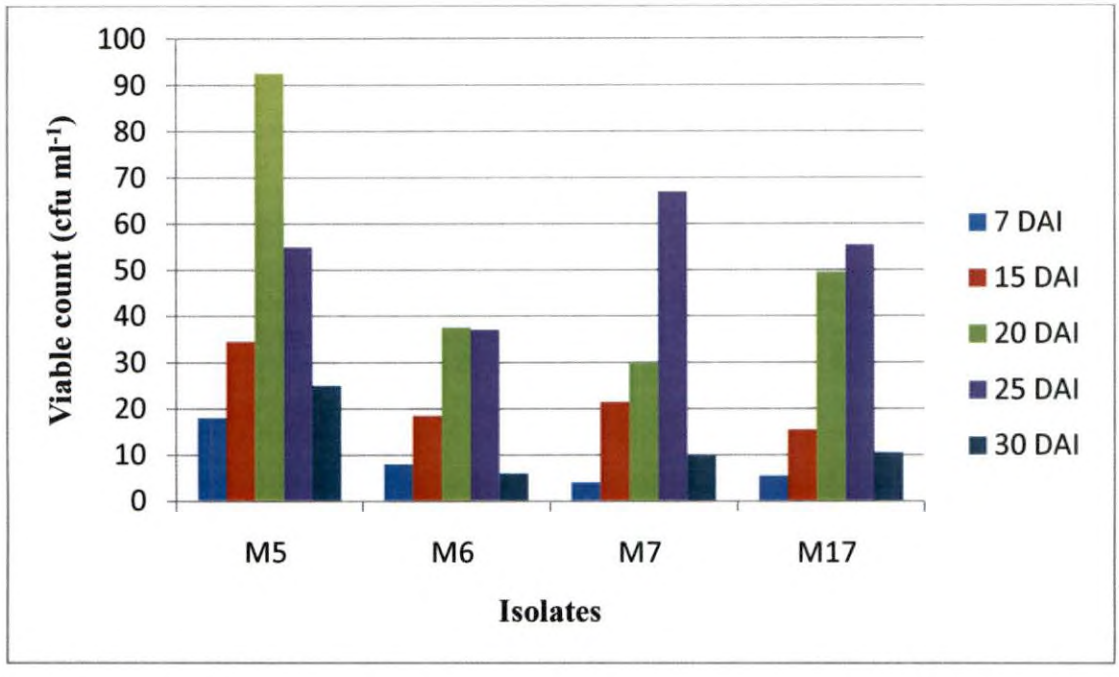


Fig. 27. Population build up of individual isolates of consortium in 800 ppm concentration of chlorpyrifos in MSM

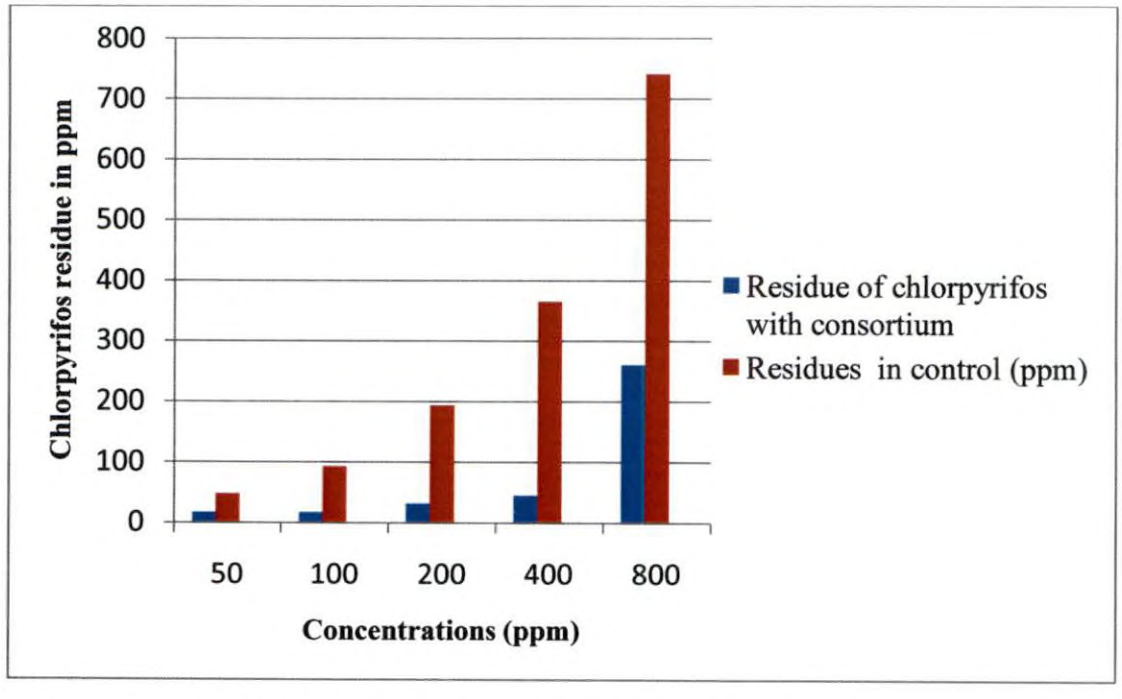


Fig. 28. Degradation of different concentrations of chlorpyrifos by consortium on 25th day of inoculation

29). However, the degradation of chlorpyrifos was reduced to 64.9 per cent in 800 ppm concentration of chlorpyrifos. Eventhough significant reduction of growth of isolates was recorded in 800 ppm, there was sufficient population of individual isolates for degradation of chlorpyrifos and hence around 64.9 per cent degradation could be obtained. Jones and Hastings (1981) reported the metabolism of 50-ppm chlorpyrifos in cultures of several forest fungi (*Trichoderma harzianum, Penicillium vermiculatum, and Mucor* sp.) and obtained 2-5 per cent residue of chlorpyrifos after 28 days. The possible metabolism by two lactic acid bacteria (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*) was reported by Shaker *et al.* (1988) who observed 72-83 per cent loss in chlorpyrifos after 96 h. Havens and Rase (1991) circulated a 0.25 per cent aqueous (EC) solution of chlorpyrifos through a packed column containing immobilized parathion hydrolase enzyme obtained from *Pseudomonas diminuta*. Approximately 25 per cent of the initial dose was degraded after 3 h of constant recirculation through the column. Similar results were also obtained by Silambarasan and Abraham (2012) who obtained 50 per cent reduction in chlorpyrifos residue by *Ganoderma* sp. in an aqueous medium.

Significant quantity of chloride was released in to the medium by the consortium in 50, 100, 200, 400 and 800 ppm on 15th, 20th, 25th and 30th DAI (Fig. 30). Similar results were reported by Anuja George, (2005) who observed significant release of chloride by strain JA15 in chlorpyrifos amended medium.

Another significant observation of the study was that the degradation capacity of the consortium was comparatively higher when compared to individual isolates (Fig. 31). The effectiveness of consortium compared to individual isolates in the degradation of chlorpyrifos has been reported by earlier workers (Singh *et al.*, 2004, Pino and Penuela 2011, Sasikala *et al.*, 2012). Similar results was also reported by Vidya Lakshmi *et al.*, (2008) who developed a microbial consortium consisting of *Pseudomonas fluorescence*, *Brucella melitensis*, *Bacillus subtilis*, *Bacillus cereus*, *Klebsiella* sp., *Serratia*

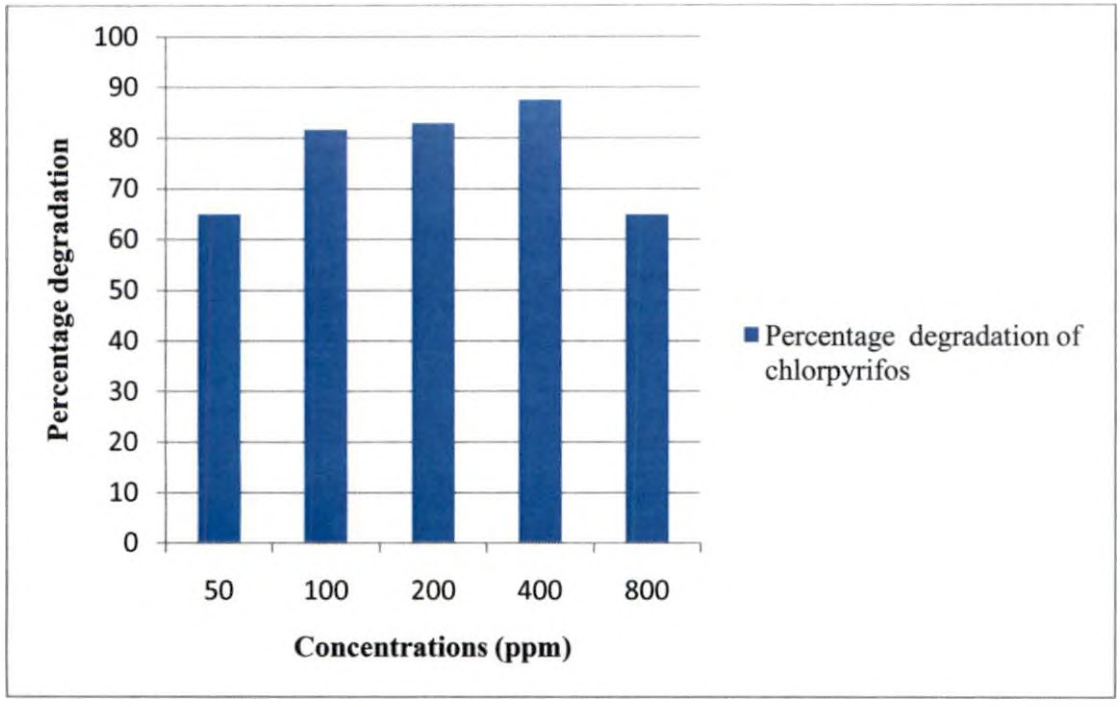


Fig. 29. Percentage degradation of chlorpyrifos by consortium

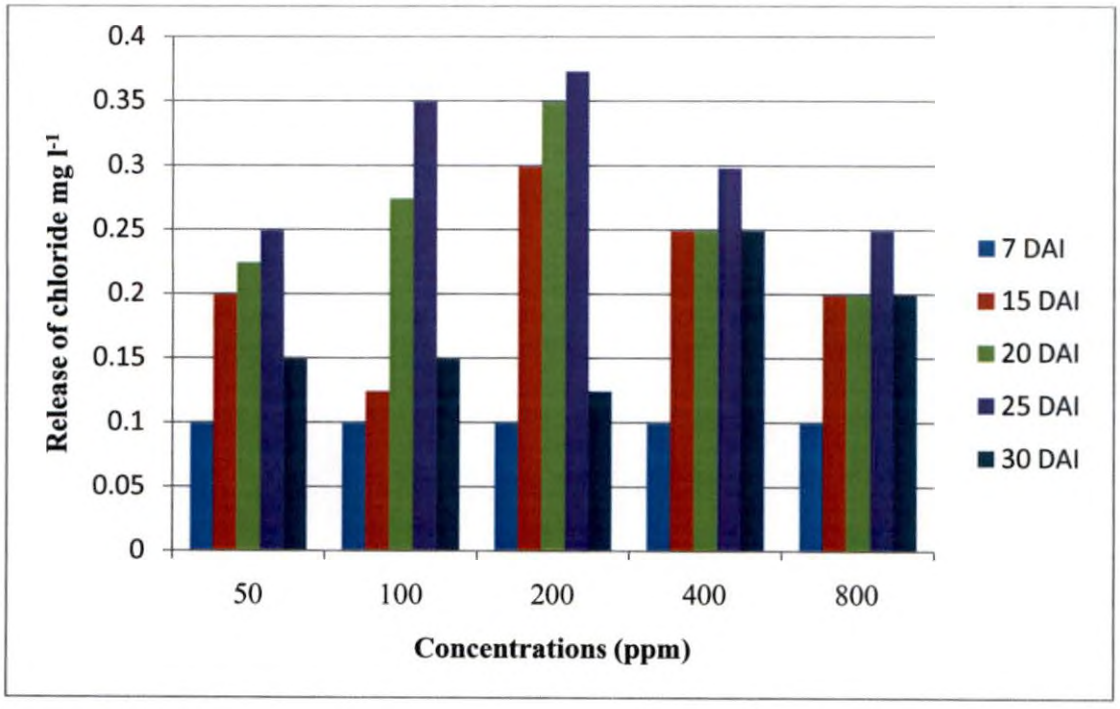


Fig. 30. Release of chloride in to the medium by consortium at different concentrations of chlorpyrifos at different intervals

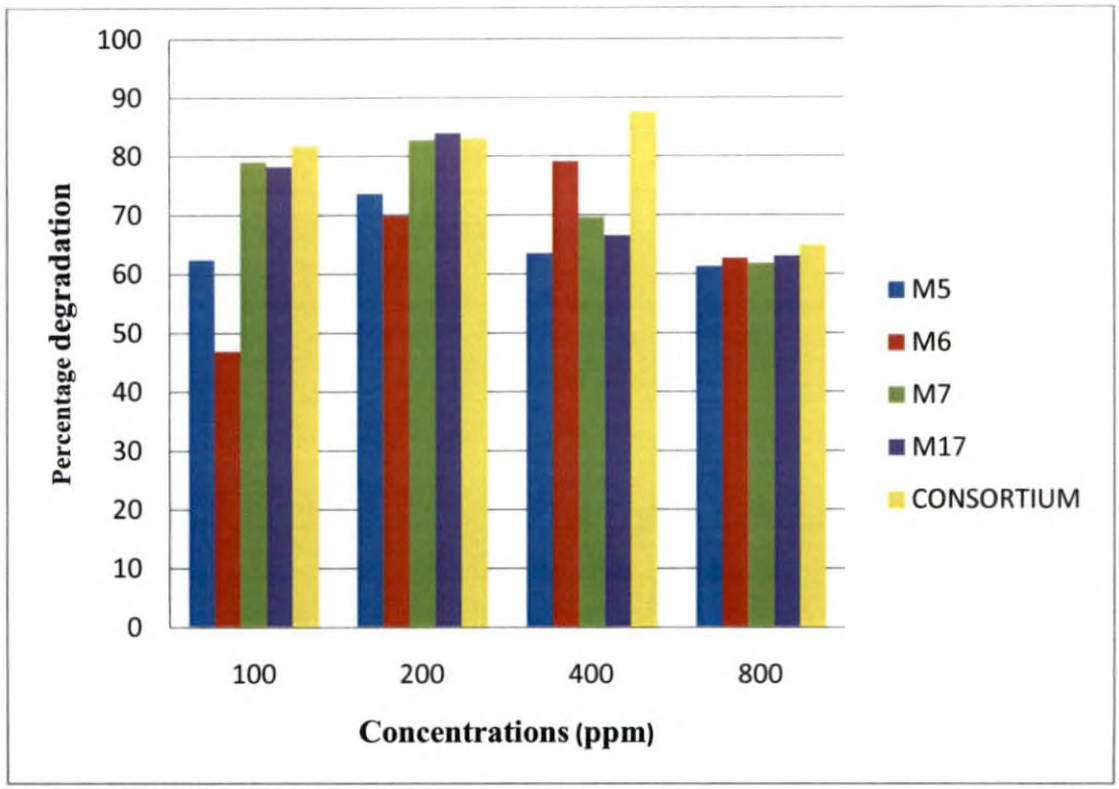


Fig. 31. Comparison of degradation of chlorpyrifos by individual isolates and consortium on 25th DAI

marcescens and *Pseudomonas aeruginosa* which gave 75–87 per cent degradation of chlorpyrifos after 20 days of incubation. Barthidasan and Reetha (2013) also found that the bacterial consortium of *Pseudomonas* sp. and *Brevibacillus* sp. could degrade 81 per cent of chlorpyrifos in the medium. Similarly Khanna and Vidyalakshmi (2004) developed microbial consortia, Q1 and Q2 from chlorpyriphos contaminated sites by selective enrichment with degradation efficiency of 72 and 70 per cent respectively. Singh *et al.* (2006) observed that mixed population of fungi, such as *Alternaria alternata*, *Cephalosporium* sp., *Cladosporium cladosporioides*, *Cladorrhinum brunnescens*, *Fusarium* sp., *Rhizoctonia solani*, and *Trichoderma viride*, could degrade chlorpyrifos in liquid culture more efficiently.

The bioremediation efficiency of the liquid consortium was evaluated in sterilized soil under pot culture conditions with cowpea as the test crop. Significant reduction in biometric characters such as plant height, fresh and dry weight of plants and roots, nodule number, nodule fresh and dry weight and yield were observed due to spiking of soil with 100 and 400 ppm concentration of chlorpyrifos (Fig.32-36). However, application of the consortium to the spiked soil improved all the biometric characters including yield of cowpea at 100 and 400 ppm levels. In a similar study conducted by Anuja George (2005) in cowpea plants, who observed a negative effect on biometric characters due to application of chlorpyrifos and enhancement of biometric characters due to application of consortium. The consortium could also give an increased germination per cent of 97.76 which was statistically on par with the control (99%).

The study conducted by Parween *et al.* (2011), in *Vigna radiata* L. found that application of chlorpyrifos at higher concentrations caused a negative impact on plant biometric characters, pigment and yield parameters. At higher concentrations, all the growth parameters such as plant height, number of branches, number of leaves per plant, total leaf area and plant biomass were remarkably reduced in all the growth phases under study. Similar suppression of

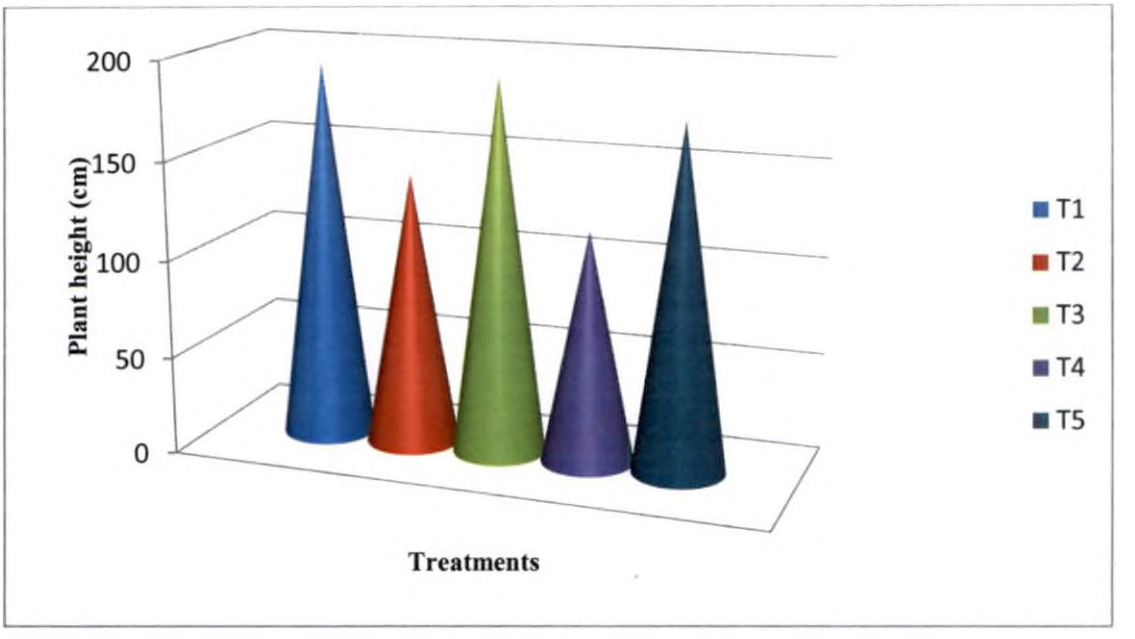


Fig. 32. Effect of consortium on plant height of cowpea in sterilized soil

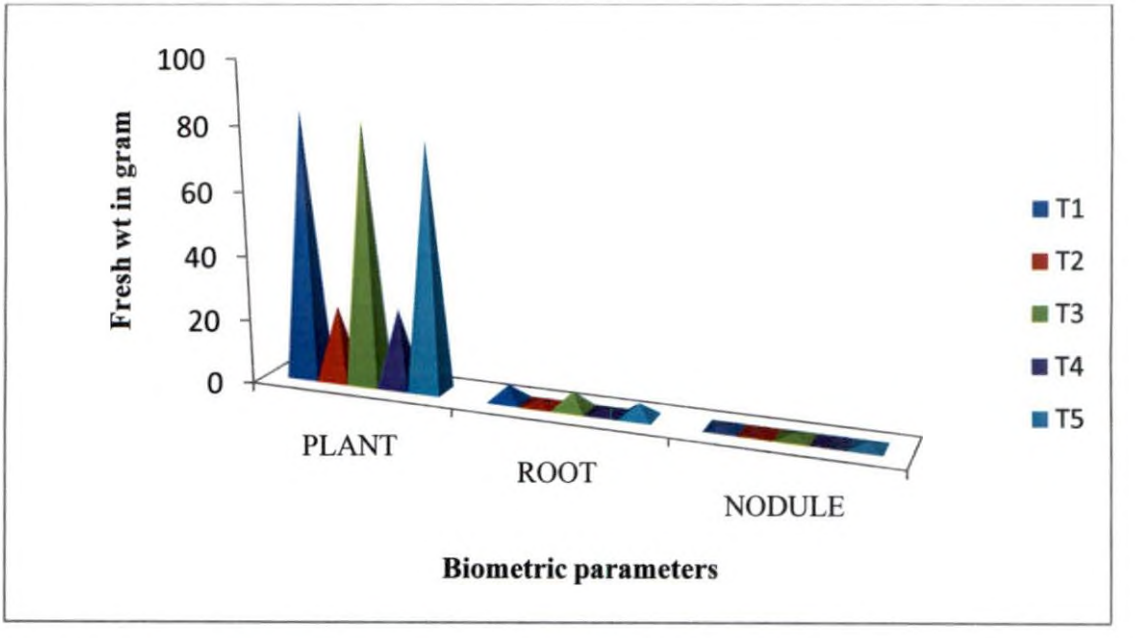


Fig. 33. Effect of consortium on plant, root and nodule fresh weight of cowpea in sterilized soil

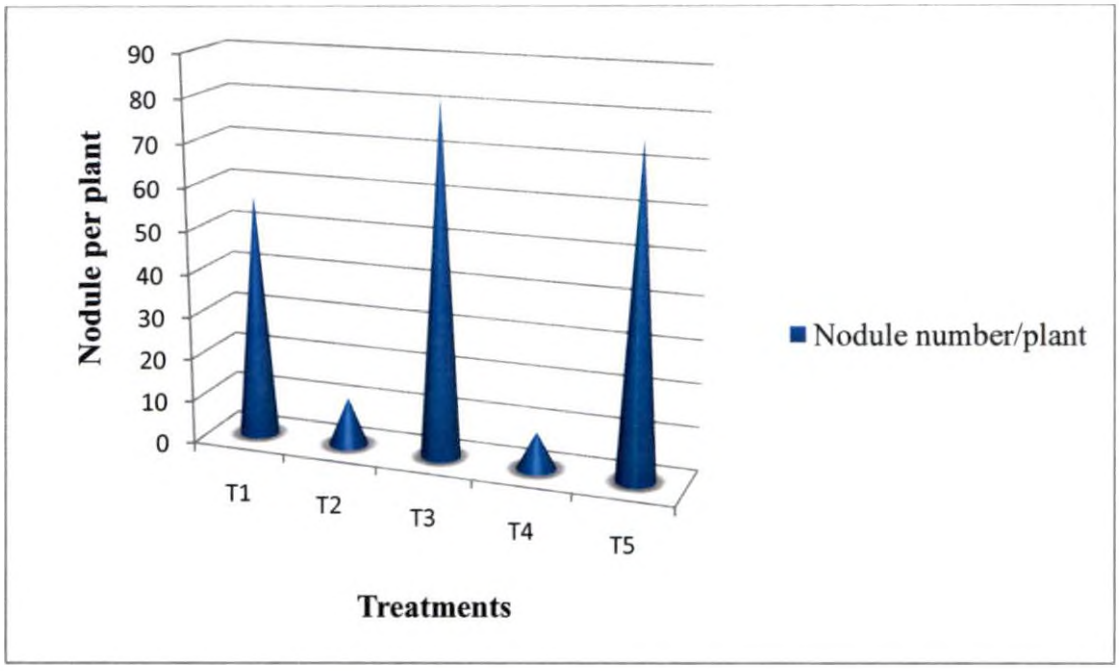


Fig. 34. Effect of consortium on Nodule number of cowpea in sterilized soil

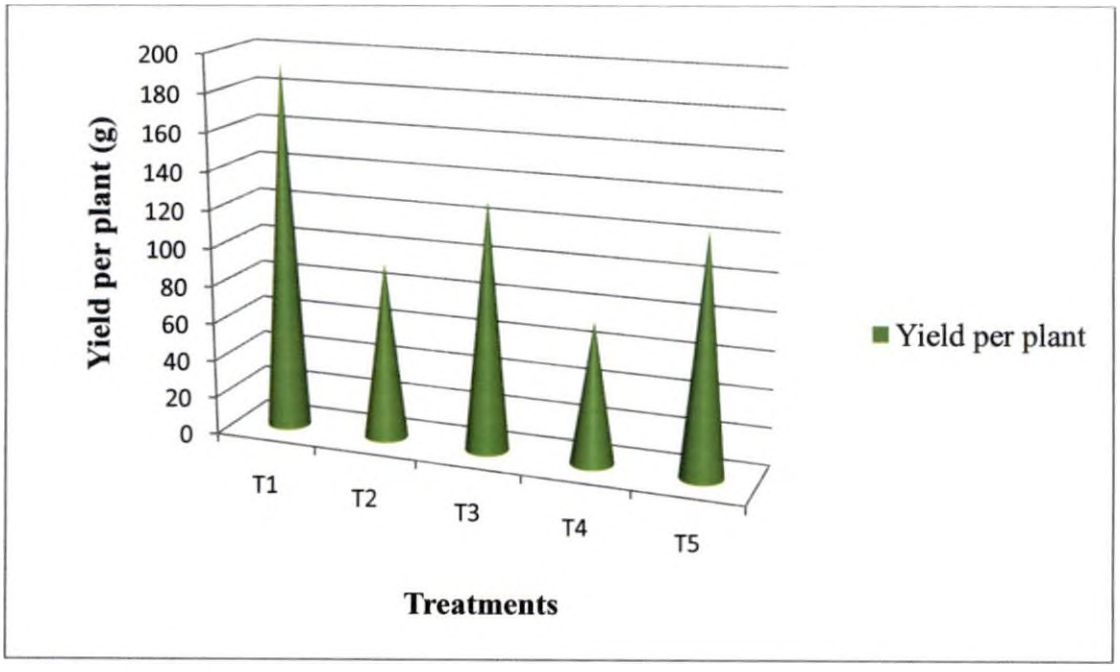


Fig. 35. Effect of consortium on yield of cowpea in sterilized soil

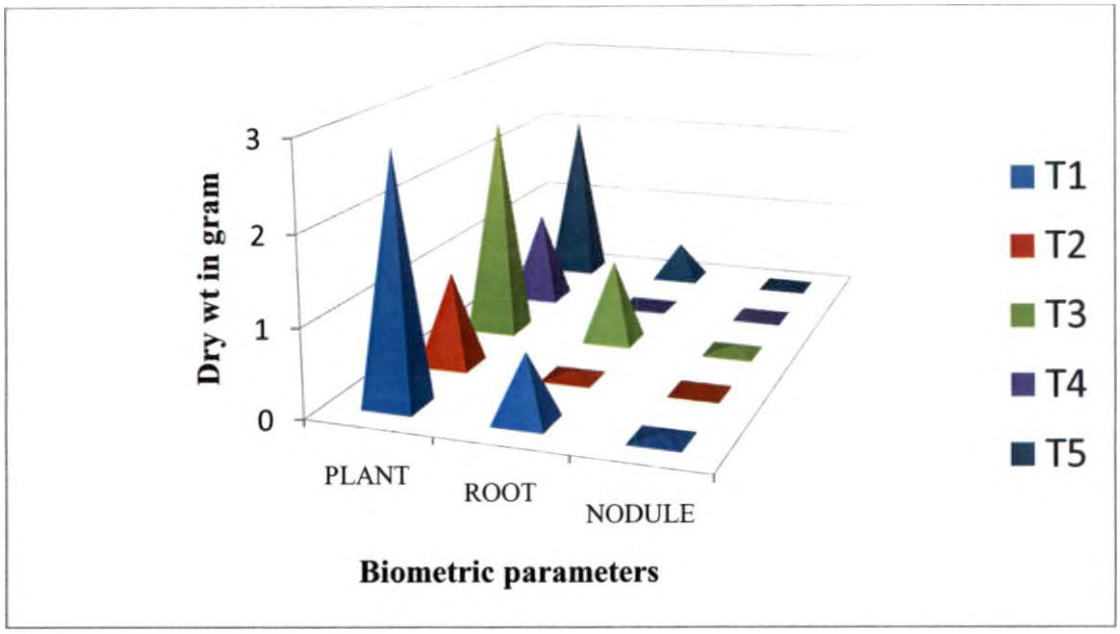


Fig. 36. Effect of consortium on plant, root and nodule dry weight of cowpea in sterilized soil

biometric characters was noted by Warabi *et al.* (2001) who observed retarded cell growth and division in soy bean roots, cell elongation and conversion of indole-3 acetic acid (IAA) into various photooxidative products under higher concentration of applied insecticide. Action of OP pesticides as strong auxin antagonists to crops have been reported in terrestrial plants (Tevini and Teramura, 1989), maize and sugarcane (Luscombe *et al.*, 1993) and cucumber (Mishra *et al.*, 2008). Retardation of yield attributing characters under high concentration of pesticides were observed by other researchers also (Lagana *et al.*, 2000; Nakamura *et al.*, 2000) which are in confirmation with our result.

In the present study the nodulation of cowpea was significantly reduced due to the toxicity of chlorpyrifos application. However, this toxicity could be alleviated by application of consortium, where nodulation was found to be enhanced. Prabakaran and Ramaswamy (1990) reported 67-99 per cent reduction of *Rhizobium* population due to chlorpyrifos application. Similar studies wherein significant inhibition of nodulation due to chlorpyrifos application were reported earlier (Dawson et al., 2001; Rekha, 2005). Higher persistence and slow rate of dissipation of chlorpyrifos may be resulting in the inhibition of nodulating Rhizobia in soil.

In the present study significant reduction of chlorpyrifos residue in soil was recorded in treatments which received consortium application. The consortium could reduce residue level of chlorpyrifos to 65.70 per cent in soil spiked with 100 ppm chlorpyrifos and to 45.60 per cent in soil spiked with 400 ppm chlorpyrifos. (Fig. 37) In a similar study, Anuja George (2005) obtained two times greater degradation of chlorpyrifos in the soil inoculated with bacterial isolates (JA-8 or JA-15) compared to uninoculated control. She also observed that the rate of chlorpyrifos degradation was enhanced to 97 per cent due to application of consortium of the strains JA-8 and JA-15. This may be due to the synergistic interaction between the two strains. Similar efficiency of microbial

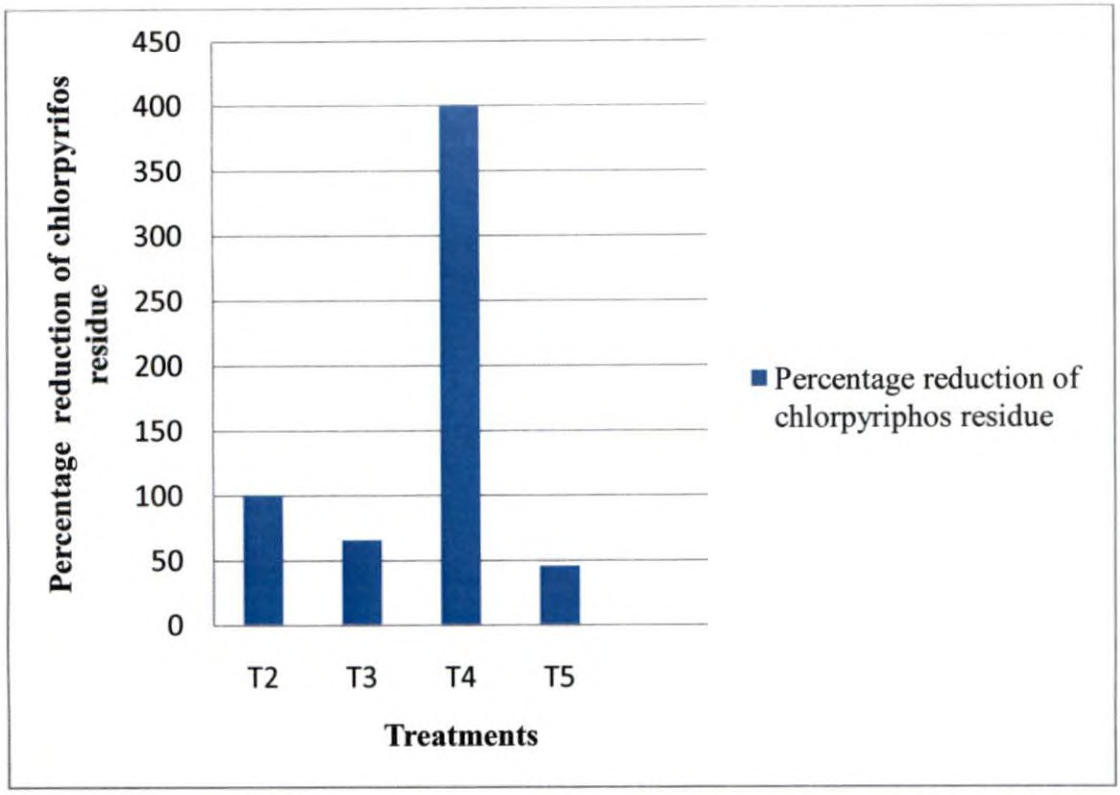


Fig. 37. Percentage reduction of chlorpyrifos residue in soil by consortium

consortia in successful degradation of chlorpyrifos in soil was reported by Khanna and Vidyalakshmi (2004).

The colonization of individual members of consortium in the rhizosphere soil was also monitored. Successful colonization of all the individual members of consortium could be obtained in soil spiked with chlorpyrifos at 100 ppm and 400 ppm concentration along with application of consortium (Fig. 39). Anuja George, (2005) also observed that the population of chlorpyrifos degraders in the polluted soil was found to increase throughout the period of investigation. This is due to the breakdown of the pesticide and root exudates in rhizosphere which supported the multiplication of organisms.

Earlier studies have shown suppressed growth of bacterial, fungal, and actinomycete populations in the presence of chlorpyrifos (Shan *et al.*, 2006; Vischetti *et al.*, 2007). Since the pot culture experiment was undertaken using sterilized soil a correct estimate on the effect of pesticide application on native soil microflora could not be obtained. However, the fungal population was maximum in treatments T5 and T3 which may be due to the application of consortium which comprised of fungal isolates. The bacterial, fungal and actinomycete population observed in other treatments may be due to the contamination from irrigation water (Fig. 38).

The release of chloride is often considered as an indirect method of measuring chlorpyrifos degradation. In the present study, the release of chloride into the soil was also monitored. It was found that 39.28 per cent chloride release was recorded in soil spiked with 400 ppm chlorpyrifos and 21.62 per cent in soil spiked with 100 ppm chlorpyrifos. Biologically mediated dehalogenation of several chlorine-containing pesticides has already been reported earlier (Feng *et al.*, 1997; Struthers *et al.*, 1998; Anuja George, 2005).

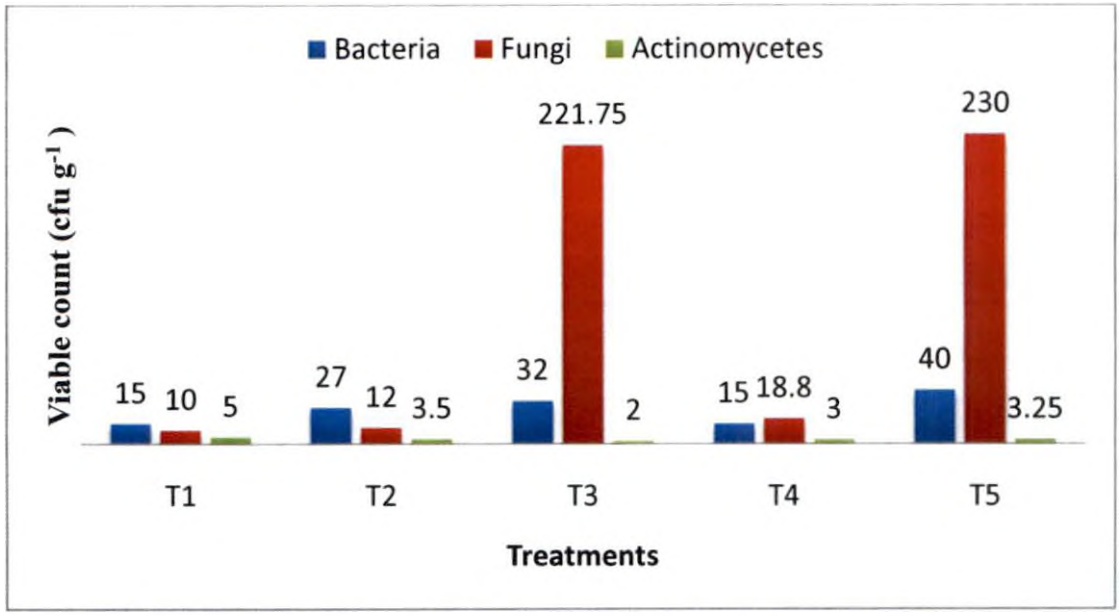


Fig. 38. Effect of consortium on population of total soil microflora in sterilized soil

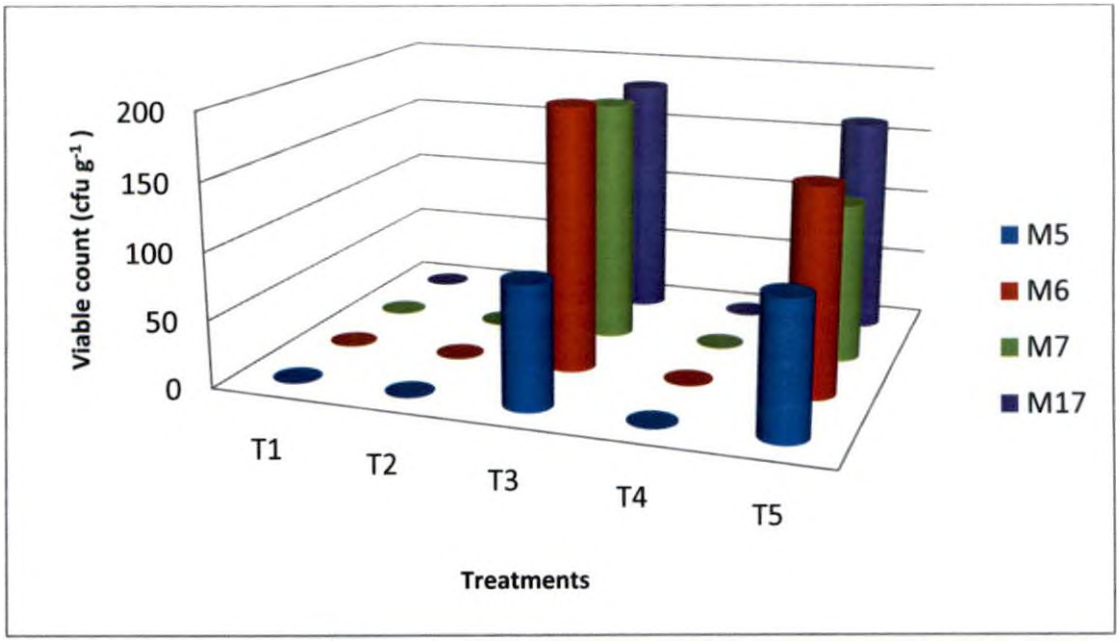


Fig. 39. Effect of consortium on population of chlorpyrifos degraders in sterilized soil

The present investigation facilitated isolation, characterization and development of an efficient microbial consortium for degradation of chlorpyrifos for *in situ* bioremediation. The consortium comprised of four efficient fungal isolates such as *Isaria farinosa, Aspergillus fumigutes, Trichoderma viride* and *Penicillium griseofulvum*. The consortium could degrade 65.70 per cent of 100 ppm and 45.6 per cent of 400 ppm chlorpyrifos in soil. Effective colonization of the organisms present in the consortium could also be obtained in the rhizosphere of cowpea. Earlier reports of efficient degradation of chlorpyrifos by species of *Aspergillus, Penicillium* and *Trichoderma* are available. However, the capacity of *Isaria farinosa* to degrade chlorpyrifos has not been reported earlier. Moreover all the isolates could degrade chlorpyrifos effectively up to a maximum concentration of 800 ppm. Reports of degradation efficiency at such higher concentration is very meager. Hence the present study forms a novel approach to exploit microorganisms that could effectively degrade chlorpyrifos for bioremediation of contaminated soil.

Future Line of Research

- The consortium developed could be further evaluated in cardamom plantations where drenching of soil with chlorpyrifos is a routine practice.
- The efficiency of the developed consortium to degrade intermediate metabolites such as TCP could be evaluated.
- The effectiveness of consortium to degrade other OP pesticides could also be assessed.
- Search for new organisms capable of degradation of intermediary metabolites of chlorpyrifos.

Summary

6. SUMMARY

The present programme was envisaged to isolate, characterize and evaluate soil microorganisms capable of degradation of chlorpyrifos. The efficient microorganisms selected were further used for preparation of consortium and evaluation of bioremediation potential *in vivo*.

As many as nineteen microorganisms comprising eleven bacteria, seven fungi and one actinomycete were isolated from soil samples with high level of chlorpyrifos residue , collected from cardamom plantations of Idukki district by enrichment culture technique. Isolates capable of utilizing chlorpyrifos as the sole source of carbon were selected for subsequent studies.

All the nineteen isolates obtained were subjected to a preliminary screening to assess the ability of the isolates to utilize chlorpyrifos as carbon source in 50, 100, 200, 400 and 800 ppm concentrations at intervals of 7, 15, 20, 25 and 30 days after inoculation. The growth of isolates was assessed based on optical density for bacteria and total viable count for fungi and actinomycetes at intervals of 7, 15, 20, 25 and 30 days after inoculation. The isolates which showed significant growth in 50, 100, 200, 400 and 800 ppm concentrations at different intervals were selected for further studies. Out of the 11 bacterial isolates screened, isolate M10 which showed maximum significant growth in 50, 100, 200, 400 and 800 ppm concentrations was selected for subsequent studies. Out of the remaining eight isolates, seven were fungi and one was actinomycete. These seven fungal and one actinomycete isolates were also subjected to a preliminary screening to assess the population build up in 50, 100, 200, 400 and 800 ppm concentrations of chlorpyrifos at 7, 15, 20, 25 and 30 days after inoculation. In the preliminary screening, the fungal isolates, M5, M6, M7 and M17 and the actinomycete isolate, M12 which recorded significant growth compared to M11, M18 and M19 in all the five concentrations tested at different intervals, were selected for further studies.

The isolates which showed significant growth in preliminary screening were once again evaluated by secondary screening to assess the ability of the isolates to utilize chlorpyrifos as sole carbon source in 100, 200, 400 and 800 ppm concentrations at intervals of 7, 15, 20, 25 and 30 days after inoculation. The population buildup was assessed based on total viable count and the degradation potential of the selected isolates was evaluated by analyzing the residue of chlorpyrifos and release of chloride in to the medium. The fungal isolates M5, M6, M7 and M17 which recorded maximum population buildup in different concentrations of chlorpyrifos and maximum degradation of chlorpyrifos were selected for further studies.

The selected fungal isolates (M5, M6, M7 and M17) capable of degrading chlorpyrifos were characterized based on morphological and molecular studies. The isolates M5, M6, M7 and M17 were identified as *Isaria farinosa*, *Aspergillus fumigatus*, *Trichoderma viride* and *Penicillium griseofulvum* respectively.

With the objective of developing a consortium, the selected fungal isolates were co-cultured in Mineral salts medium and Potato dextrose broth and their compatibility was studied by assessing the population build up of each of the individual isolates of consortium recorded as viable count on 7^{th} DAI. The compatibility of the isolates was further confirmed by dual culture technique. All the four fungal isolates were tested for antagonism and no inhibition was observed between the isolates tested. Since all the isolates (*Isaria farinosa*, *Aspergillus fumigatus*, *Trichoderma viride* and *Penicillium griseofulvum*) were compatible, they were used to prepare the liquid consortium.

The ability of the developed consortium to degrade 50, 100, 200, 400 and 800 ppm concentrations of chlorpyrifos was evaluated under *in vitro* conditions, at intervals of 7, 15, 20, 25 and 30 days after inoculation. Growth of isolates was measured by enumerating the total viable count and the degradation potential of the consortium was assessed by analyzing the residual chlorpyrifos in the medium

in GC-MS on 25th day after inoculation. The release of end product, chloride was also measured at intervals of 7, 15, 20, 25 and 30 days after inoculation. All the fungal isolates present in the consortium - *Isaria farinosa, Aspergillus fumigatus, Trichoderma viride and Penicillium griseofulvum* showed significant colony count and degradation of chlorpyrifos and release of chloride on 20-25 days after inoculation.

. The bioremediation efficiency of the developed liquid consortium was evaluated in sterilized soil under pot culture conditions with cowpea as the test crop. Significant reduction in biometric characters such as plant height, fresh and dry weight of plants and roots, nodule number, nodule fresh and dry weight and yield were observed due to spiking of soil with 100 and 400 ppm of concentration of chlorpyrifos. However, application of the developed consortium to the spiked soil enhanced all the biometric characters including yield of cowpea at 100 and 400 ppm levels. Significant reduction of chlorpyrifos residue in soil was recorded in treatments which received consortium application. The residue level of chlorpyrifos was reduced to 65.70 per cent in soil spiked with 100 ppm chlorpyrifos and 45.60 per cent in soil spiked with 400 ppm chlorpyrifos which received consortium application. Successful colonization of all the individual members of consortium (*Isaria farinosa*, *Aspergillus fumigatus*, *Trichoderma viride* and *Penicillium griseofulvum*) could be obtained in soil spiked with chlorpyrifos at 100 and 400 ppm concentration along with application of consortium.

References

7. REFERENCES

Abd El-Mongy, M. and Abd El-Ghany. T.M. 2009. Field and laboratory studies for evaluating the toxicity of the insecticide Relden on soil fungi. *Int. Biodeterioration and Biodegradation* 63: 383-388.

Aktar, M.W., Sengupa, D., and Chowdhury, A. 2009. Impact of pesticides use in agriculture: their benefits and hazard. *Interdiscp. Toxicol.* 2: 1-12.

Anuja George, 2005. Isolation, screening and selection of efficient chlorpyriphos degrading Microorganisms. MSc.(Ag) thesis, Dharwad Agricultural University, Dharwad, India, 80p.

Awad. N.S, Hussein, H., Sabit, Salah, E. M, Abo-Aba., and Reda, A. Bayoumi. 2011. Isolation, characterization and fingerprinting of some chlorpyrifos-degrading bacterial strains isolated from Egyptian pesticides-polluted soils *Afr. J. Microbiol. Res.* 5 (18): 2855-2862.

Aysal, P., Gozek, K., Artik, N., and Tuncbilek, A.S. 1999. 14C-chlorpyrifos residues in tomatoes and tomato products. *Bull. Environ. Cont. Toxicol.* 62: 122-125.

Barathidasan, K. and Reetha, D. 2013. Biodegradation of chlorpyrifos and their intermediate metabolites identified by liquid chromatography mass spectroscopy (LC-MS). *Int. J. Pharm. Bio. Sci.* July, 4(3): (B) 1277 – 1283.

Bastiaens, L., Springael, D., Wattiau, P., Harms, H., De wachter, R., Verachtert, H., and Diels, L. 2000. Isolation of adherent polycyclic aromatic hydrocarbon (PAH) degrading bacteria using PAH –sorbing carriers. *Appl. Environ. Microbiol.* **66**:1834-1843.

Beevi, N.S., Paul, A., George, T., Mathew, B.T., Pratheesh Kumar, N., Georgae Xavier, Pradeep Kumar, G.T., Rajith, R., Prathibha Ravi, K and Visal Kumar, S. 2014. Pesticide Residues in Soils under Cardamom Cultivation in Kerala *India Pesticide Res. J.* 26(1): 35-41.

Bending, G. D., Friloux, M., and Walker, A. 2002. Degradation of contrasting pesticides by white rot fungi and its relationship with ligninolytic potential. FEMS *Microbiol. Lett.* 212: 59–63.

Bhagobaty, R.K. and Malik, A. 2008. Utilization of chlorpyrifos as a sole source of carbon by bacteria isolated from wastewater irrigated agricultural soils in an industrial area of western Uttar Pradesh, India. *Res. J. Microbiol.* 3: 230-293.

Bhalerao, T. S. and Puranik, P. R. 2009. Biodegradation of organochlorine pesticide, endosulfan, by a fungal soil isolate, Aspergillus niger. *Int.* Biodeterioration and Biodegradation. 59: 315–321.

Bhatnagar, A. and Gupta, A. 1992. Persistence of chlorpyriphos residues in soil and groundnut seed. *Int. Arachis Newsl.* 11: 16-17.

Bhatnagar, A. and Gupta, A. 1998. Lindane, chlorpyrifos and quinalphos residues in safflower seeds and oil. *Pesticide Residue J.* 10: 127-128.

Blossom., Singh, B., and Gaganjyot, 2004. Monitoring of insecticide residues in cotton seed in Punjab, India. *Bull. Environ. Cont. Toxicol.* 73: 707-712.

Bondarenko, S., Gan, J., Haver, D. L. and Kabashima, J.N. 2004. Persistence of Selected Organophosphate and Carbamate Insecticides in Waters from a Coastal Watershed, *Environ. Toxicol. Chem.*, Vol.23, No.11, (November 2004), pp. 2649-2654, ISSN 0730-7268.

Boonsaner, M., Kaewumput, T., and Boonsaner, A. 2002. Bioaccumulation of organophosphate pesticides in vetiver grass (*Vetiveria zizanioides* Nash). *Thammasat Int. J. Sci. Technol.* 7: 30-39.

Brust, H.F. 1966. A summary of chemical and physical properties of Dursban. *Down to Earth.* **22**: 21-22.

Bumpus, J. A., Kakar, S. N., and Coleman, R. D. 1993. Fungal degradation of organophosphorous insecticides. *Appl. Biochem. Biotechnol.* 39(40):715–726.

Chapman, R. A., Harris, C. R., Svec, H. J., and Robinson, J.R. 1984. Resistance and mobility of granular insecticides in an organic soil following furrow application for onion maggot. *J. Environ. Sci. Health.* 19: 259-270.

Chaudhry, Q., Blom-Zandstra, M., Gupta, S., and Joner, E.J. 2005. Utilising the Synergy Between Plants and Rhizosphere Microorganisms to Enhance Breakdown of Organic Pollutants in the Environment. *Environ. Sci. Pollut. Res.* 12: 34-48.

Chen, W. and Georgiou G. 2002. Cell-surface display of heterologous proteins: from high throughput screening to environmental applications, *Biotechnol. Bioeng.* 5: 496–503.

Chiou, C.T., Freed, V.H., Schmedding, D.W. and Kohnert, R.L. 1977, Partitioncoefficient and bioaccumulation of selected organic chemicals. *Environ. Sci. Technol.* 11: 475-478.

Cullington, J.E. and Walker, A., 1999, Rapid biodegradation of diuron and other phenyl urea herbicides by a soil bacterium. *Soil Biol. Biochem.* 31:677-686.

Dawson , I.A., Grayston, S.J., Murray, P.J., and Cook, R. 2001. Special issue: The dynamic interface between plants and the earth. Sixth symp. *Int. Soc. Root Res. Nagoya,* Japan 255 (1): 121-130.

De Schrijver, A. and De Mot, R. 1999. Degradation of pesticides by Actinomycetes. *Crit. Rev. Microbiol.* 25: 85-119.

Dhawan, A. K. and Simwat, G. S. 1996. Pattern of insecticides use on cotton by farmers in Punjab. *Pestology.* 20: 30-34.

Dragun, J., Kuffner, A. C., and Schneiter, R.W. 1984. Ground water contamination. Transport and transformation of organic chemicals. *Chem. Eng.* 91:65-70.

Drummond, A.J., Ashton, B., Buxton, S., Cheung, M., Cooper, A., Heled, J., Kearse, M., Moir, R., Stones-Havas, S., Sturrock, S., Thierer, T., and Wilson, A. 2010. Geneious v5.1, Available: http://www.geneious.com.

Dua, M., Singh A., Sethunathan, N., and Johri, A.K. 2002. Biotechnology and bioremediation successes and limitations. *Appl. Microbiol. Biotechnol.* 58: 143-152.

Dutta, K., Schuur, E.A.G., Neff, J.C., and Zimov, S.A. 2006.Potential carbon release from permafrost soils of northeastern Siberia. *Global Change Biol.* 12: 2336–2351.

Ehab, R., El-Helow., Mohamed, E. I., Badawy., Mona, E. M., Mabrouk., Eman A. H. Mohamed., and Youssef, M. El-Beshlawy. 2013. Biodegradation of Chlorpyrifos by a Newly Isolated Bacillus subtilis Strain, Y242. *Bioremediation J.* 17:2, 113-123.

Fang, H., Xiang, Y. Q., Hao, Y. J., Chu, X. Q., Pan, X. D., Yu, J.Q., and Yu, Y. L. 2008. Fungal degradation of chlorpyrifos by *Verticillium* sp. DSP in pure cultures and its use in bioremediation of contaminated soil and pakchoi. *Int. Biodeterioration and Biodegradation.* 61:294–303.

Farhan. M, Khan A.U, Wahid. A, Ali. A.S, Ahmad.F. 2013. Potential of indigenous *klebsilla* sp. for chlorpyrifos biodegradation. *Pakist. J. Sci.* 65:133-137.

Felsot, A. and Dahm, P.A., 1979. Sorption of organophosphorus and carbamate insecticides by soil. *J. Agric. Food Chem.* 27: 557-563.

Feng, Y., Racke, K.D., and Bollag, J.M. 1997. Isolation and characterization of a chlorinated pyridinol degrading bacterium. *Appl. Environ. Microbiol.*63: 4096-4098.

Feng, Y.E., Minard, R.D., and Bollag, J.M. 1998. Photolytic and microbial degradation of 3, 5, 6-trichloro-2-pyridinol. *Environ. Toxicol and Chem.* 17: 814-819.

Fenske, R.A., Kedan, G., LU, C., Fisker-andersen, J.A., and Curl, C.L. 2002. Assessment of organophosphorus pesticide exposures in the diets of preschool children in Washington state. *J. Exponential Analytical Environ. Epidemiology.* 12: 21-28.

Fux, C. A. 2005. Survival strategies of infectious biofilms. Trends in *Microbiol.* 13: 34–40.

Geetha, M. and Fulekar, M.H., 2008. Bioremediation of pesticides in surface soil treatment unit using microbial consortia. *Afr. J. Environ. Sci. Technol.* 2 (2): 36–45.

Getzin, L.W. 1981. Degradation of chlorpyrifos in soil: influence of autoclaving, soil moisture, and temperature. *J. Econ. Ent.* 74: 158– 162.

Ghigo, J. M. 2001. Natural conjugative plasmids induce bacterial biofilm development. *Nature* 412: 442–445.

Greenberg, A.E., Clesceri, L.S., and Eaton, A.D. 1992. *Standard Methods for the Examination of Water and Waste Water* (18th ed.). APHA, Washington 300p.

Gupta, A., Parihar, N. S., and Bhatnagar, A. 2001. Lindane, chlorpyriphos and quinalphos residues in mustard seed and oil. *Bull. Environ. Cont. Toxicol.* 67: 122-125.

Havens, P.L. and Rase, H.F. 1991. Detoxification of organophosphorus pesticide solutions. In: Tedder, D.W., Pohland, F.G. (eds), *Emerging technologies in Hazardous waste management II.* ACS, Washington, DC, pp.261-281.

Hindumathy, C.K. and Gayathri, V. 2013. Effect of Pesticide (Chlorpyrifos) on Soil Microbial Flora and Pesticide Degradation by Strains Isolated from Contaminated Soil. *J. Bioremedediation and Biodegradation.* 4:178.

Horne, I., Sutherland, T.D., Harcourt, L.R, Russell, R.J., and Oakeshott, J.G. 2002. Identification of an opd (organophosphate degradation) gene in an Agrobacterium isolate. *Appl. Environ. Microbiol.* 68: 3371-3376.

Hua, F., Yunlong, Y., Xiaoqiang, C., Xiuguo, W., Xiaoe, Y., and Jingquan, Y. 2009. Degradation of chlorpyrifos in laboratory soil and its impact on soil microbial functional diversity. *J. Environ. Sci.* 21: 380–386.

Hundal , B.S. and Singh, A.R.2006. Pesticide marketing: The Indian scenario, *Icfai j. Manage.Econ.*4:32-37.

Iwata, Y., Walker, G.P., O'neal, J.R., and Barkley, J.H. 1985. Residues of acephate, amitraz, chlorpyrifos and formetahate hydrochloride on and in fruits after lowvolume applications to orange trees. *Pesticide Sci.*16: 172-178.

Jisha, M.S. and Ambily P.S. 2012. Biodegradation of anionic surfactant, sodium dodecyl sulphate by *Pseudomonas aeruginosa* MTCC 10311. *J. Environ. Biol.* Pp.717-720

Johnson, G. R. and Spain, J. C. 2003. Evolution of catabolic pathways for synthetic compounds: bacterial pathways for degradation of 2,4-dinitrotoluene and nitrobenzene. *Appl. Microbiol. Biotech.* 62: 110–123.

Jones, A.S. and Hastings, F.L. 1981. Soil microbe studies. In: Hastings F.L. and Coster, J.E. (eds), *Field and laboratory evaluations of insecticides for southern pine beetle control.* USDA. Southern Forest Experiment Station, Forest Service, SE 21, pp. 13-14.

Kahlon, R. S. and Saurabh, G. 2003. To study the effect of bioaugmentation on pesticides contaminated soil by fluorescent *Pseudomonas*. 44th AMI Conference; November, 12-14, Dharward. 125p.

Kanekar, P.P., Bhadbhade, B., Deshpande, N.M. and Sarnaik, S.S. 2004. Biodegradation of organophosphorus pesticides. *Proceedings of Indian National Sci. Acadamy*, 70: 57-70.

Karalliedde, L. and Senanayake, N. 1999. Organophosphorus insecticide poisoning. *J. Int. Fed. Clin. Chem.* 11: 4–9.

Karasali, H., Hourdakis, A., Anagnostopoulos, H., and Doulia, D. 2002. Pesticide residues in thermal mineral water in Greece. *J. Environ. Sci. Health* 37: 465-474.

Karpouzas, D.G. and Walker, A. 2000. Factors influencing the ability of *Pseudomonas putida* strains ep I and II to degrade the organophosphate ethoprophos. *J. Appl. Microbiol.* 89: 40-48.

Khanna, S. and Vidyalakshmi. 2004. Chlorpyrifos degrading microbes capable of bioremediating contaminated sites. *Microbial Diversity – A Source of Innovation in Biotechnology*, National Seminar, May 27-29, TBGRI, Thiruvananthapuram. pp.84-85.

Kuiper, I., Lagendijk, E.L., Bloemberg, G.V., and Lugtenberg, B.J.J. 2004. Rhizoremediation: A beneficial plant microbe interaction. *Mol. Plant Microbe. Interact.* 17: 6-15.

Kulshrestha, G. and Anupriya Kumari., 2011. Fungal degradation of chlorpyrifos by *Acremonium* sp. strain (GFRC-1) isolated from a laboratory-enriched red agricultural soil. *Biol. Fertil. soils*. 47:219-225.

Kulshrestha, G. and Kumari, A. 2011. Fungal Degradation of Chlorpyrifos by *Acremonium sp.* Strain (GFRC-1) isolated from a Laboratory-enriched Red Agricultural Soil. *Biol. Fertil. Soils*. 47: 219-225.

Laabs, V., Amelung, W., Pinto, A., Altstaedt, A., and Zech, W. 2000. Leaching and degradation of corn and soybean pesticides in an oxisol of the Brazilian Cerrados. *Chemosphere* 41(9): 1441–1449.

Lagana, A., Fasciani, G.F.L., Marino, A., and Mosse, M. 2000. Determination of diphenyl ether herbicides and metabolites in natural waters using HPLC with diode array tandem mass spectrophotometric detection. *Anal. Chem. Acta*. 414: 74-94.

Lal, S. and Lal, R. 1987. Bioaccumulation, metabolism and effects of DDT, fenitrothion and chlorpyriphos on *Saccharomyces cerevisiae. Arch. Environ. Cont. Toxicol.* 16: 753-757.

Latifi. M. A., Khodi. S., Mirzaei. M, Miresmaeili. M., and Babavalian.H. 2012. Isolation and characterization of five chlorpyrifos degrading bacteria; *Afr. J. Biotechnol.* 11(13):140-3146.

Li, X., Jiang, J. D., Gu, L., Ali, S. W., He, J., and Li, S.P. 2008. Diversity of chlorpyrifos-degrading bacteria isolated from chlorpyrifos-contaminated samples. *Int. Biodeterioration and Biodegradation.* 62: 331–335.

Liu, X., You, M.S., Wei, Y.Z., Liao, J.Y., Ye, L.L., and Chen, J. 2003. Isolation of chlorpyrifos degrading Aspergillus sp.Y and measurement of degradation efficiency.*Chin. J. Appl. Environ. Biol.* 9: 78-80.

Liu, Y. H., Chung, Y.C., and Xiong, Y. 2001. Purification and characterization of a dimethoate-degrading enzyme of *Aspergillus niger* ZHY256, isolated from sewage. *Appl. Environ. Microbiol.* 67: 3746–3749.

Liu, Z., Chen. X., Shi. Y., and ZhenCheng Su. 2012. Bacterial Degradation of Chlorpyrifos by *Bacillus cereus Adv. Mater. Res.* 360 :676-680.

Luscombe, B.M., Pallett, K.E., Loubiere, P., Millet, J., Melgorejo, J., and Vrabel, T.E. 1995. RPA 201772 a novel herbicide for broad leaf and grass weed control in maize and sugarcane. *Proceedings of the Brighton crop Protect, Weeds*, 2: 35-42.

Lynch, J. M. and Whipps, J. M., 1990. Substrate flow in the rhizosphere. *Plant Soil.* 129:1-10.

Macalady, D.L. and Wolfe, N.L. 1983. New perspectives on the hydrolytic degradation of the organo phosphorothioate insecticide chlorpyrifos. *J. Agric. Food Chem.* 31: 1139-1147.

Macek, T., Mackova, M., and Kas, J. 2000. Exploitation of Plants for the Removal of Organics in Environmental Remediation. *Biotechnol. Adv.* 18: 23-34.

Maini, P., Collina, A., and Parassini, I., 1972. Residues of chlorpyriphos in variouscrops II. Residues after field treatments. *Pesticide Sci.* 3: 533-538.

Mallick, K., Bharati, K., Banerji, A., Shakil, N.A. and Sethunathan, N. 1999. Bacterial degradation of chlorpyrifos in pure cultures and in soil. *Bull. Environ. Cont. Toxicol.* 62: 48-54.

Martin, J.P., 1950, Use of acid rose Bengal and streptomycin in plate count for estimating soil fungi. *Soil Sci.* 9: 215-232.

Matthews, W.A. 1991. An investigation of the non-solvent extractable residues of [14C]-chlorpyrifos-methyl in stored wheat. *Pesticide Sci.* 31: 141-149.

Maya, K., Singh, R.S., Upadhyay, S.N., and Dubey, S.K. 2011. Kinetic analysis reveals bacterial efficacy for biodegradation of chlorpyrifos and its hydrolyzing metabolite TCP. Process *Biochem.* 46: 2130–2136.

Miles, J.R.W., Harris, C.R. and Tu, C.M., 1983, Influence of temperature on the persistence of chlorpyriphos and chlorfenvinphos in sterile and natural mineral and organic soils. *J. Environ. Sci. Health* 18: 705-712.

Mishra, V., Srivastava, G., Prasad, S.M., and Abraham, G. 2008. Growth, photosynthetic pigment and photosynthetic activity during seedling stage of cowpea (*Vigna unguiculata*) in response to UV-B and dimethoate. *Pesticide Biochem. Physiol.* 92: 30-37.

Montemurro, N., Grieco, F., Lacertosa, G. and Visconte, A., 2002, Chlorpyrifos decline curves and residue levels from different commercial formulations applied to oranges. *J. Agric. Food Chem.*50: 5975-5980.

Moore, M. T., Schulz, R., Cooper, C. M., Smith, S. and Rodgers, J. H. 2002. Mitigation of chlorpyrifos runoff using constructed wetlands. *Chemosphere* 46: 827–835.

Mukherjee, I. and Gopal, M. 1996. Degradation of chlorpyrifos by two soil fungi *Aspergillus niger* and *Trichoderma viride. Environ. Toxicol Chem.*, 37: 145-151.

Mukherjee, I., Gupta, S., Kulshrestha, A., Pant, S., Singh, A., Kumar, A., and Gajbhiye, V.T. 2007. Simulateneous Estimation of aldicarb, carbofuran and carbaryl in water by HPLC. *Pestic.Res.J.*19 (1): 128-130.

Mumtaz, M., Nasir, N., and Baig, M. M. H. 1983. DDT residues in agricultural soil environment of the Punjab and NWFP. *Proc.UN World Environment Day Seminar on Hazards of ToxicWaste and Water Pollution. Institute of Environmental Studies, University of Karachi,* 29-34.

Nakamura, A., Yohorti, Y., Watanabe, K., Sato, Y., Boeger, P., and Wakabayashim, K. 2000. Peroxidation formation of lipid hydroperoxidase in etiolated leaves. *Pesticide Biochem. Physiol.* 66: 206-212.

Neely, W.B., Branson, D.R. and Blau, G.E. 1974. Partition coefficient to measure bioconcentration potential of organic chemicals in fish. *Environ. Sci. Technol.* 8: 1113-1115.

Oh, Y. S. and Bartha, R. 1997. Construction of a bacterial consortium for the biofiltration of benzene, toluene and xylene emissions. *World J. Microbiol. Biotechnol.* 13:627-632.

Ohshiro, K., Kakuta, T., Sakai, T., Hirota, H., Hoshino, T., and Uchiyama, T. 1996. Biodegradation of organophosphorus insecticides by bacteria isolated from turf green soil. *J. Fermentation Bioeng.* 82: 299–305.

Omar, S.A. 1998. Availability of phosphorus and sulfur of insecticide origin by fungi. *Biodegradation.* **9**: 327-336.

Panse, V.S. and Sukhatme, P.V. 1985. *Statistical Methods for Agricultural Workers*, ICAR, New Delhi, pp.152-155.

Parween. T., Sumira Jan., Mahmooduzzafar., and Tasneem Fatma. 2011. Assessing the impact of chlorpyrifos on growth, photosynthetic pigments

and yield in *Vigna radiata* L. at different phenological stages. *Afr. J Agric. Res.*6(19): 4432-4440.

Pilon-Smits E. 2005. Phytoremediation. *Annu. Rev. Plant. Biol.* 56: 15-39.

Pino, N. and Penuela, G. 2011. Simultaneous degradation of the pesticides methyl parathion and chlorpyrifos by an isolated bacterial consortium from a contaminated site. *Int. Biodeterioration and Biodegradation.* 65: 827–831.

Prabakaran, J. and Ramaswamy, K.1990. Effect of seed dressing chemicals on the survival of Rhizobium sp. and growth, nodulation and grain yield of green gram and black gram. *Madras agric.J.* 77: 285-290.

Putnam, R.A., Nelson, J.D. and Clark, J.M. 2003. The persistence and degradation of chlorothalonil and chlorpyrifos in a cranberry bog. *J. Agric. Food Chem.* 51: 170-176.

Racke, K. D., Coats, J. R. and Titus, K. R. 1988. Degradation of Chlorpyrifos and Its Hydrolysis Products, 3, 5, 6-trichloro-2-pyridinol, in Soil. *J. Environ. Sci. Health B.* 23: 527-539.

Racke, K. D., Fontaine, D. D., Yoder, R. N., and Miller, J. R. 1994. Chlorpyrifos degradations in soil at termiticidal application rates. *Pesticide Sci.* 42: 43–51.

Racke, K.D. 1993. Environmental fate of chlorpyrifos. *Rev. Environ. Cont. Toxicol.* 131: 1-151.

Racke, K.D. and Coats, J.R. 1990. Pesticides in soil microbial ecosystems. *Am. Chem. Soc. Symp. Ser.* 426: 1–12.

Rani, M.S., Lakshmi. K.V., Devi, P.S., Madhuri, R.J, Aruna, S., Jyothi, K., Narasimha, G., and Venkateswarlu, K. 2008. Isolation and characterization of chlorpyrifos degrading bacterium from agricultural soil and its growth response. *Afr J. Microbiol.* Res. 2: 026-031.

Redondo, M.J., Ruiz, M.J., Font, G., and Boluda, R. 1997. Dissipation and distribution of atrazine, simazine, chlorpyrifos and tetradifon residues in citrus orchard soil. *Arch. Environ. Cont. Toxicol.* 32: 346-352.

Rekha, P.R. 2005. Dissipation of chlorpyrifos in redloam soil and its effect on soil organisms. MSc.(Ag) Thesis, Kerala Agricultural University, Thrissur, India.77p.

Richins, R., Mulchandani, A., and Chen, W. 2000. Expression, immobilization, and enzymatic characterization of cellulose-binding domain-organophosphorus hydrolase fusion enzymes, *Biotechnol. Bioeng.* 69: 591–596.

Rigterink, R.H. and Kenaga, E.E. 1966. Synthesis and insecticidal activity of some 0, 0-diakyl 0-(3,5,6-trichloro-2-pyridyl) phosphates and phosphorothioates. *J. Agric. Food Chem.* 14: 304-306.

Rouchaud, J., Gustin, F. and Vanparys, L. 1991. Influence of slurry and ammonium nitrate fertilizations on soil and plant metabolism of chlorpyriphos in field cauliflower. *Bull. Environ. Cont. Toxicol.* 46: 705-712.

Safi, J.M., Abou-foul, N.S., El-nahhal, Y.Z., and El-sebal, A.H. 2002. Monitoring of pesticide residues on cucumber, tomatoes and strawberries in Gaza governorates, Palestine. *Nahrung.* 46: 34-39.

Salas, J.H., Gonzalez, M.M., Noa, M., Perez, N.A., Diaz, G., Guttierrez, R.,Zazueta, H. and Osuna, I. 2003. Organophosphorus pesticide residues in Mexican commercial pasteurized milk. *J. Agric. Food Chem.* 51: 4468-4471.

Sanghi, R., Pillai, M.K., Jayalekshmi, T.R., and Nair, A. 2003. Organochlorine and organophosphorus pesticide residues in breast milk from Bhopal, Madhya Pradesh, India. *Human Experimental Toxicol.* 22: 73-76.

Sasikala, C., Jiwal, S., Rout, P., and Ramya, M., 2012. Biodegradation of chlorpyrifos by bacterial consortium isolated from agriculture soil. *World J. Microbiol. Biotechnol.* 28: 1301-1308.

Schmimmel, S.C., Garnas, R.L., Patrick, J.M., and Moore, J.C. 1983. Acute toxicity, bioconcentration and persistence of benthiocarb, chlorpyrifos, fenvalerate, methyl parathion and permethrin in the estuarine environment. *J. Agric. Food Chem.* 31: 104-113.

Seffernick, J. L. and Wackett, L. P. 2001. Rapid evolution of bacterial catabolic enzymes: a case study with atrazine chlorohydrolase. *Biochem.* 40: 12747–12753.

Serdar, C.M., Gibson, D.T., Munnecke, D.M., and Lancaster, J.H. 1982. Plasmidinvolvement in parathion hydrolysis by *Pseudomonas diminuta. Appl. Environ. Microbiol.* 44: 246-249.

Serrano, R., Lopez, F. J., Hernandez, F., and Pena, J.B. 1997. Bioconcentration of chlorpyrifos, chlorfenvinphos and methidathion in *Mytilus galloprovincialis. Bull. Environ. Cont. Toxicol.* 59: 968-975.

Sethunathan, N. N. and Yoshida, T. 1973. A *Flavobacterium* that degrades diazinon and parathion. *Canadian J. Microbiol.* 19: 873–875.

Shaker, N., Abo-Donia, S., El-Shaheed, Y.A., and Ismail. 1988. Effect of lactic acid bacteria and heat treatments on pesticides contaminated milk. *Egypt J. Diary Sci.* 16:309-317.

Shan, M., Fang, H., Wang, X., Feng, B., Chu, X. Q., and Yu, Y. L. 2006. Effect of chlorpyrifos on soil microbial populations and enzyme activities. *J. Environ. Sci.*18 (1): 4–5.

Shapir, N., Mandelbaum, R. T., and Jacobsen, C.S. 1998. Rapid atrazine mineralization underdenitrifying conditions by Pseudomonas sp. strain ADP in aquifer sediments. *Environ. Sci. Technol.* 32:3789-3792.

Siji, N. Nath. 2011. Dynamics of pesticide residues in cardamom growing soils of Idukki district M.Sc.(Ag) thesis, Kerala Agricultural University, Thrissur, India. 109p.

Silambarasan, S and Abraham. J. 2013. Ecofriendly Method for Bioremediation of Chlorpyrifos from Agricultural Soil by Novel Fungus *Aspergillus terreus* JAS1 *Water Air Soil Pollut.* 224:1369.

Silambarasan, S. and Abraham, J. 2012. Efficacy of *Ganoderma* sp. JAS4 in bioremediation of chlorpyrifos and its hydrolyzing metabolite TCP from agricultural soil *J. Basic Microbiol.* 54:44–55.

Singh, B. K., Walker, A. and Wright, D. J. 2006. Bioremedial potential of fenamiphos and chlorpyrifos degrading isolates: Influence of different environmental conditions. *Soil Biol. Biochem.* 38: 2682–2693.

Singh, B. K., Walker, A., Morgan, J. A. W., and Wright, D. J. 2003. Effects of soil pH on the biodegradation of chlorpyriphos and isolation of a chlorpyrifos-degrading bacterium. *Appl. Environ. Microbiol.* 69: 5198-5206.

Singh, B.K., Allan, S., Walker A., Alun, J., Morgan, W., and Denis, J. 2004. Biodegradation of Chlorpyrifos by Enterobacter Strain B-14 and Its Use in Bioremediation of Contaminated Soils. *Appl. Environ. Microbiol.* 70: 4855-4863.

Singhal, V. 2003. *Indian Agriculture* published by Indian Economic Data Research Centre, New Delhi, pp.85-93.

Skidmore, A.M. and Dickinson,C.M.1976. *Trans.Br. Mycol Soc.*66:57-60.

Smith, G.N., Watson, B.S., and Fischer, F.S. 1967. Investigations on dursban insecticide: metabolism of [36Cl] O,O-diethyl-O-3,5,6-trichloro- 2-pyridyl phosphorothioate in rats. *J. Agric. Food Chem.* 15:132–138.

Sogorb, M.A., Vilanova, E., and Carrera, V. 2004. Future application of phosphotriesterases in the prophylaxis and treatment of organophosphorus insecticide and nerve agent poisoning. *Toxicol. Lett.* 151: 219–233.

Somasundaram, L., Racke, K.D., and Coats, J.R. 1987. Effect of manuring on the persistence and degradation of soil insecticides. *Bull. Environ. Cont. Toxicol.* 39: 579-586.

Stangroom, S. J. C., Collins, C. D., and Lester, J. N. 2000. Abiotic behaviour of organic micropollutants in soils and the aquatic environment. A review: II. Transformations. *Environ. Technol.* 21: 865–882.

Stewart, D. K., Chrisholm, D., and Ragab, M. T. 1971. Long term persistence of parathion in soil. *Nature.* 229: 47-48.

Struthers JK, Jayachandran K, Moorman TB (1998). Biodegradation of atrazine by *Agrobacterium radiobacter* J14a and use of this strain in

bioremediation of contaminated soil. *Appl. Environ. Microbiol.* 64:3368-3375.

Suganathy, M. and Kuttalam, S. 2003. Organochlorine and chlorpyriphos insecticide residues in market samples of meat. *Pestology*. 27: 51-54.

Sumit kumar, 2011. Isolation, characterization and growth response study of chlorpyrifos degrading bacteria from cultivated soil. *Int. J. Adv. Eng. Technol.*2(3):199-203.

Sundararaj, R., Remadevi, O. K. and Muthukrishnan, R. 2003. Comparative efficacy of some insecticides in ground contact against subterranean termites. *Pestology*. 27: 17-20.

Swati. and Singh, D.K. 2002. Utilization of chlorpyrifos by *Aspergillus niger* and *A. flavus* as carbon and phosphorus source. 17th *World Cong. of Soil Sci.* 14 - 21, Bangkok, Thailand.

Tevini, M. and Teramura A.H. 1989. UV-B effects on terrestrial plants. *Photochem. Photobiol.* 50: 479-487.

Tilak, K. S., Veeraiah, K. and Rao, D. K. 2004. Toxicity and bioaccumulation of chlorpyrifos in Indian carp *Catla catla* (Hamilton), *Labeo rohita* (Hamilton), and *Cirrhinus mrigala* (Hamilton). *Bull. Environ. Cont. Toxicol.* 73: 933-941.

Van Hylckama Vlieg J.E.T, Janssen D.B 2001 Formation and detoxification of reactive intermediates in the metabolism of chlorinated ethenes. *J. Biotechnol.* 85:81-102.

Vidya-Lakshmi, C., M. Kumar and S. Khanna, 2008. Biotransformation of chlorpyrifos and bioremediation of contaminated soil. *Int. Biodeterioration and Biodegradation.* 62: 204–209.

Vink, J. P. M. and Van der Zee, S. E. A. T. M. 1997. Pesticide biotransformation in surface waters: multivariate analyses of environmental factors at field sites. *Water Res.* 31: 2858–2868.

Vischetti, C., Coppola, L., Monaci, E., Cardinali. A., and Castillo, M. 2007. Microbial impact of the pesticide chlorpyrifos on Swedish and Italian biobeds. *Agron. Sustain. Dev.* 27: 267-272.

Wackett, L. P., Sandowsky, M. J., Newman, L. M., Hur, H. G., and Li, S. 1994. Metabolism of polyhalogenated compounds by a genetically engineered bacterium. *Nature.* 368:627-629.

Walia, S., Dureja, P. and Mukerjee, S. K. 1988. New photodegradation products of chlorpyrifos and their detection on glass, soil and leaf surface. *Arch. Environ. Cont. Toxicol* 17: 183–188.

Wang, J.H., L.S. Zhu, J. Wang and K. Qin, 2005. Degradation characteristics of three fungi to chlorpyrifos. *Chin. J. Appl. Environ. Biol.*11: 211-4.

Wang, X., Chu, X. Q., Yu, Y. L., Fang, H., Chen, J., and Song, F. M. 2006. Characteristics and function of *Bacillus latersprorus* DSP in degrading chlorpyrifos. *Acta Pedologica Sinica* 43: 648–654.

Warabi, E., Usni, K., Tanaka, Y., and Matsumoto, H. 2001. Resistance of a soybean cell line to oxyfluorfen by overproduction of mitochondrial protoporphyrinogen oxidase. *Pest Manage. Sci.* 57: 743-748.

Weber, J. B. 1972. Interaction of organic pesticides with particulate matter in aquatic and soil systems. *Am. Chem. Soc. Adv. Chem. Ser.* 111: 55-120.

Yang, C., Liu, N., Guo, X., and Qiao, C. 2006. Cloning of *mpd* gene from chlorpyrifos-degrading bacterium and use of this strain in bioremediation of contaminated soil. FEMS *Microbiol. Lett.* 265:118-125.

Yu, Y. L., Wu, X. M., Li, S. N., Fang, H., Zhan, H. Y., and Yu, J. Q. 2006. An exploration of the relationship between adsorption and bioavailability of pesticides in soil to earthworm. *Environ. Pollut.* 141: 428–433.

Yucheng Feng. 2003. Microbial and Photolytic Degradation of 3,5, 6-Trichloro-2-pyridinol Pesticide Decontamination and Detoxification, *ACS Symposium Series*, 863:15–24.

Appendices

APPENDIX I

Composition of media used

1. Mineral salts medium (Cullington and Walker, 1999)

Group I

KH_2PO_4 – 2.27 g

$Na_2HPO_4 . 12H_2O$ – 5.97 g

NaCl – 1.00 g

Group II

$MgSO_4 . 7H_2O$ – 0.5 g

$CaCl_2 . 2H_2O$ – 0.01 g

$MnSO_4 .4 H_2O$ – 0.02 g

Group III

$FeSO_4$ – 0.025 g

Distilled water – 1000 ml

Agar – 15 g

pH – 6.9

The first two groups of compounds were autoclaved separately and combined when cool. The iron sulphate solution was filter sterilized and added to the cool medium.

2. Martin's rose–bengal agar (Martin, 1950)

Glucose – 10.0 g

Peptone – 5.0 g

$MgSO_4 . 7H_2O$ – 0.5 g

Rose bengal – 0.33 g

KH_2PO_4 -1 g

Distilled water – 1000 ml

Streptomycin sulphate – 30 mg

Agar – 20.0 g

pH – 7

Three ml of one per cent solution of streptomycin was added to the medium just before pouring into the petriplates.

3. Potato Dextrose Agar

Potato – 200 g

Dextrose – 20 g

Agar agar -15 g

Distilled water -1000 ml

4. Kenknight's media

Dextrose -1.0 g

KH_2PO_4 -0.10 g

$NaNO_3$ .0.10 g

KCl- 0.10 g

$MgSO_4.7H_2O$ – 0.10g

Agar- 15.0 g

Distilled water – 1000 ml

pH - 7.0

5. Nutrient agar

Beef extract – 3g

Peptone – 5g

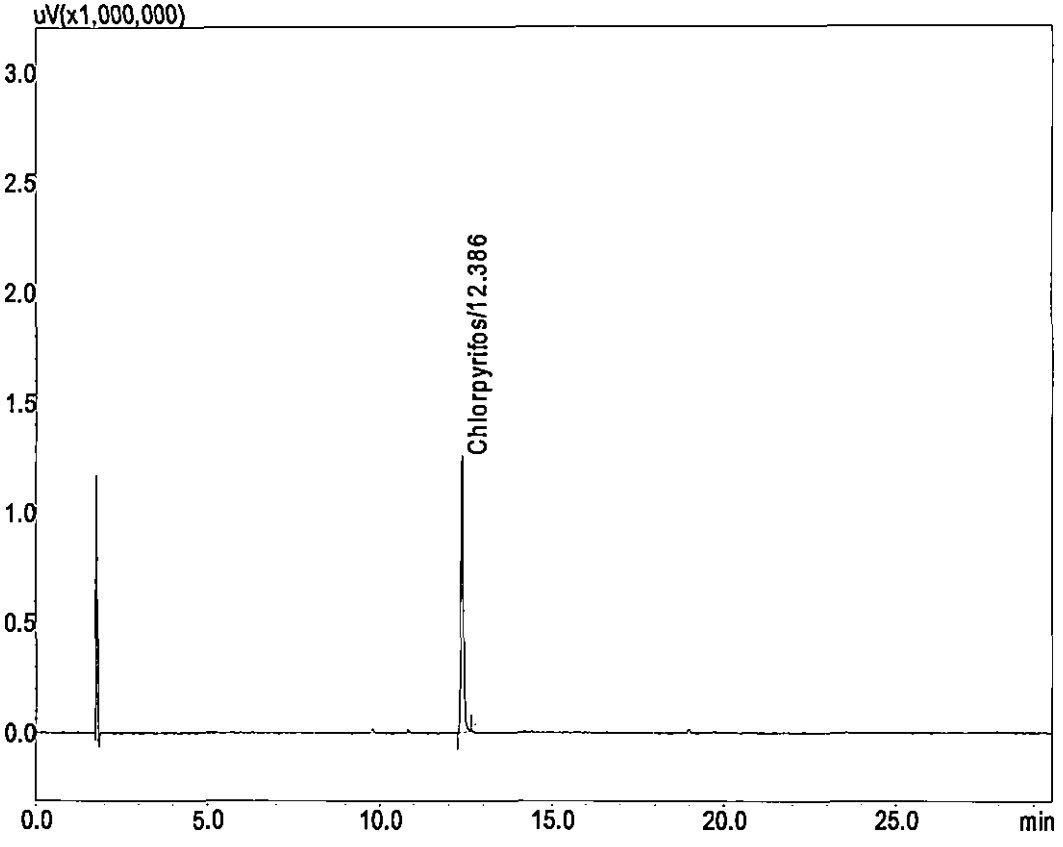
$NaCl_2$- 5g

Agar – 20 g

Distilled water – 1000 ml

pH -7

APPENDIX II



GC-MS Chromatogram of Chlorpyrifos

Abstract

Isolation, Characterization and Evaluation of Soil Microorganisms for Bioremediation of Chlorpyrifos

by

KAROLIN K. P

(2012-11-165)

ABSTRACT

Submitted in partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE IN AGRICULTURE

Faculty of Agriculture
Kerala Agricultural University

DEPARTMENT OF AGRICULTURAL MICROBIOLOGY
COLLEGE OF AGRICULTURE
VELLAYANI, THIRUVANANTHAPURAM- 695 522
KERALA, INDIA

2014

ABSTRACT

The present study on "Isolation, characterization and evaluation of soil microorganisms for bioremediation of chlorpyrifos", was conducted in the Department of Agricultural Microbiology at College of Agriculture, Vellayani during 2012-14, with the objective of isolation, characterization and evaluation of microorganisms for chlorpyrifos degradation, development of consortia and evaluation of bioremediation potential against chlorpyrifos *in vivo*. Microorganisms capable of degradation of chlorpyrifos were isolated by enrichment culture technique from identified locations with high residue levels of chlorpyrifos. In all, nineteen isolates comprising eleven bacteria, seven fungi and one actinomycete obtained were subjected to a preliminary screening based on the ability of isolates to utilize 50,100,200,400 and 800 ppm concentrations of chlorpyrifos at intervals of 7, 15, 20, 25, 30 DAI. The six isolates selected (M5, M6, M7, M10, M12, M17) were further evaluated for their ability to degrade different concentrations of chlorpyrifos based on population build up , analysis of chlorpyrifos residue and chloride released into the medium. The fungal isolates, M5, M6, M7 and M17 which recorded significant growth in terms of viable count, maximum reduction in chlorpyrifos residue and release of chloride were selected and subjected to morphological and molecular characterization. The isolates M5, M6, M7 and M17 were identified as *Isaria farinosa*, *Aspergillus fumigatus*, *Trichoderma viride* and *Penicillium griseofulvum* respectively.

In order to develop a consortium, the compatibility of the selected fungal isolates - M5, M6, M7 and M17 was tested by co-culturing in liquid MSM and by dual culture technique. All the fungal isolates were compatible and no inhibition could be recorded. A consortium of the four fungal isolates was prepared in liquid formulation and its ability to degrade different concentrations of chlorpyrifos was studied under *in vitro* conditions on 25th day of inoculation. The percentage degradation of chlorpyrifos by the isolates increased with increase in

concentrations, but showed a decline at 800 ppm. The percentage degradation of chlorpyrifos was higher in consortium compared to individual isolates under *in vitro* conditions.

The developed liquid consortium was evaluated in sterilized soil spiked with 100 and 400 ppm concentration of chlorpyrifos with cowpea as the test crop. Significant reduction in all biometric characters was observed due to spiking with chlorpyrifos at 100 and 400 ppm concentrations. Application of consortium in soil spiked with chlorpyrifos enhanced all the biometric characters and reduced the residue of chlorpyrifos. The study also established efficient colonization of the chlorpyrifos degraders present in the consortium in the rhizosphere of cowpea.