DEVELOPMENT OF DEFINED MICROBIAL CONSORTIUM FOR BIOREMEDIATION OF SYNTHETIC PESTICIDE CONTAMINATED SOIL AND WATER

THESIS SUBMITTED TO
DELHI TECHNOLOGICAL UNIVERSITY
FOR THE AWARD OF THE DEGREE OF

DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY

MARCH 2018

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Dedicated To my family

Especially, My Father

Late Sh. Harpal Singh

&

My Father-in-Law

Sh. Shri Ram Azad

CERTIFICATE

This is to certify that the Ph.D. thesis entitled "**Development of defined microbial consortium for bioremediation of synthetic pesticide contaminated soil and water**" submitted to Delhi Technological University, Delhi, for the award of Doctor of Philosophy is based on the original research work carried out by me under the supervision of **Prof. Jaigopal Sharma,** Department of Biotechnology, Delhi Technological University, Delhi, India. It is further certified that the work embodied in this thesis has neither partially nor fully submitted to any other university or institution for the award of any degree or diploma.

Satish Kumar
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This is to certify that the above statement made by the candidate is correct to the best of our knowledge.

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DECLARATION

I, Satish Kumar, certify that the work embodied in this Ph.D. thesis is my own bonafide work carried out under the supervision of **Prof. Jaigopal Sharma,** Department of Biotechnology, Delhi Technological University, Delhi, for a period of December 2012 to March 2018 at the Department of Biotechnology, Delhi Technological University, Delhi. The matter embodied in this Ph.D. thesis has not been submitted for the award of any other degree/diploma.

I declare that I have devotedly acknowledged, given credit and refereed to the research workers wherever their work has been cited in the text and the body of thesis. I further certify that I have not willfully lifted up some other's work, paragraph, text, data, results etc. reported in the journal, books, reports, dissertations, thesis etc., or available at websites and included them in Ph.D. thesis and cited as own my work.

Date
Place

Satish Kumar
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ACKNOWLEDGEMENT

This thesis is the result of five years of work whereby I have been accompanied and supported by many people. It is a pleasant aspect that I have now the opportunity to express my gratitude for all of them. First of all, I want to thank Almighty God for giving me blessings, strength and knowledge.

I would like to express my greatest gratitude to my supervisors Prof. Jai Gopal Sharma, Professor & Head, Department of Biotechnology, Delhi Technological University supervisor for their proficient guidance and continued encouragement for the fulfillment of the research work. With great pleasure I express my heartfelt gratitude for their advice, fruitful discussions, numerous suggestions, constructive criticism, and constant support throughout the period of my research.

I thank UGC - CSIR, New Delhi for giving an opportunity in the form of Junior and Senior Research Fellowships to make my research venture successful.

I would like to express my kind regards to Prof. Yogesh Singh, Hon'able Vice Chancellor and also grateful to the Registrar, Dean- PG and DR- PG from Delhi Technological University, for providing me necessary facilities .

I am thankful to the former heads of the Department, Biotechnology Prof. B. D. Malhotra and Prof. D. Kumar, for their valuable support during this work.

I thank to Prof. Rina Chakarbarti, Department of Zoology, University of Delhi for providing me necessary facilities for molecular Biology work.

I also would like to thank Prof R. S. Walia and Dr. Pushpender Singh for being very much helpful whenever I approached them.

My heartfelt thanks to Dr. RajKumar Bidlan (UGC-PDF) for taking active interest in my work, for his valuable suggestions and helping me in every step for the fulfillment of the research work.

I am thankful to other faculty members of Biotechnology Department, I am also thankful to DRC and SRC members of my Ph.D. course for their comments, valuable suggestions during my Ph.D.

I am indebted to my colleagues Ms. Shilpi, Dr. Saroj Kumar, Dr. Shikha Gupta and Dr. V. K. Goswami, Mr. Abhishek Saini for supporting, stimulating, fun filled and healthy environment in Lab.

I would like to extend my sincere thanks to office staff Mr. Chhail Bihari, Mr. Jitendra Singh, Ms. Saumya Maurice and all other technical staff for their support.

I would like to give whole hearted credit to my mother Shyamwati for the completion of the doctorate thesis. Their blessings like catalyst speed up the whole process of thesis formation. I would like to express my special thanks and gratitude to my elder brothers Sh. Rajpal Singh and Mr. Sushil Kumar for their support and encouragement.

On a personal note, I express my thanks and love to my wife Suman Azad (Sweety) for his continuous encouragement, cooperation, support and sacrifices and my daughters Riwa and Chitra who have played the biggest role of stress reliever.

In the end, I would like to apologize to my friends and all those people whose names are not mentioned here but they have helped me in completion of Ph.D. thesis in one or another way.

Finally, I thank to all those who helped and supported me. Thank you so much!

Date: 15/03/2018 **Satish Kumar**

Place: Delhi

ABSTRACT

Lindane (γ–Hexachlorocyclohexane) is detected in the environment even after three decades of its cessation of use. It is also found in milk, blood and food samples indicating the vastness of biomagnification. Organophosphates and pyrethroids are the other classes of pesticides being used frequently nowadays leading to further risk enhancement. Commercial formulations of various pesticides of different classes find direct applications in agriculture. Farmers are encouraged to apply these pesticides to improve the crop production. We tried to study the effects of a few commercially available pesticides on the crop seed germination. Green gram (dicotyledonous crop) and wheat (monocotyledonous crop) seeds were considered for the study. Commercially available pesticides were mixed and spiked into the sterilised soil at various concentrations. Malathion is commercially formulated synthetic pesticide which belongs to organophosphates class. Nowadays it is widely used as an effective insecticide in agriculture, health projects and industries. This study evaluated the effect of different concentrations (2ppm to 400ppm) of Malathion towards seed germination, growth of seedling and various photosynthetic pigments in *Vigna radiata* L. Two different methods were used, moist sterile filter paper method and soil in cups. Qualitative and quantitative estimation of photosynthetic pigments was performed by using thin layer chromatography and spectrophotometeric methods. The percentage germination as well as growth of seedling in terms of shoots and roots length enhanced at lower concentrations as compared to control on filter paper method, while in soil method morphogenic response significantly declined. A few seedlings turned yellowish green at higher levels (400 ppm) of Malathion but were still viable. Photosynthetic pigments significantly reduced with increasing concentrations of Malathion as compared to control.

Microbial degradation is the cost effective and safe strategy that can be developed and adapted for improving the environmental health by remediating lindane contamination. We enriched a bacterial population consisting of morphologically 47 distinct bacteria using commercial formulations of organophosphates and pyrethroids mixture. This consortium showed 55.6% - 90.45 % degradation of 5-30ppm lindane by 6 days. The temperature and pH optima were found to be 30 ^{o}C and 6 respectively. When this consortium was induced with lindane in broth only four

cultures survived while 24 isolates showed the ability to clear lindane film on a nutrient agar plate. This is the first report with a microbial population enriched completely on mixtures of commercial formulations of organophosphate and pyrethroid classes of pesticides and used for degrading a pure isomer of an organochlorine pesticide i.e. lindane.

The strain LR_4 exhibited 50% degradation of 20 ppm lindane by 6 days, while the combination studies showed that (LR_2 + LR_3 + LR_4) combination was the most efficient and degraded 25% of the supplied 20 ppm lindane by 6 days. Studies with different carbon compounds resulted in the degradation of 80%, 50%, 45%, and 80% lindane when incubated in presence of glucose (by LR_4), peptone (by LR_3), Tween 80 (by LR_4), and Triton X-100 (by LR_4) respectively in 5 days. Degradation model was generated using RSM. The analysis of variance (ANOVA) was determined the factors A, B, C, D, E and interaction of AB, AC, AD, BC, BD, BE, DE have significant effect ($p<0.0001$) on lindane degradation. 16S rDNA analysis revealed the identities of the four strains as LR_1- *Bacillus cereus*; LR_2- *Pseudomonas* sp; LR_3- *Chryseobacterium* sp; and LR_4- *Pseudomonas putida*.

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1. Introduction and background

1.1 Pesticides

Pesticides are natural or synthetic agents used to destroy or control pest, insects and unwanted species of plant or animal. These could be either a single substance or mixture of various substances with intended use for eradicating the pests including the plant pathogens as well as the vectors of mammalian disease. Apart from killing the pests and insects they regulate, plant growth, desiccation, defoliation, thinning of fruit, prevent premature fruit fall and deterioration of commodity during storage and transport (Vargas, 1975). So pesticides are very important in economic development as their application reduces the unnecessary loss of production due to pathogens.

Pesticides could be broadly classified into naturally occurring and synthesized. Naturally occurring compounds or natural extracts have been used as pesticides since ancient times. The earliest pesticides were most likely salt, sulphurous rock, and extracts of tobacco, red pepper, neem etc. Petroleum products, along with heavy metals like arsenic were used unregulated to control unwanted pests and weeds until the 1940s. Later on, such compounds were replaced by organic synthetic pesticides, the most famous pesticides DDT and endosulphan followed by lindane, which are banned presently due to health hazards to humans and animals.

Though the implementation of the synthetic pesticides was a blessing for the world, yet the same has turned into a curse due to the heavy usage by people ultimately leading to their accumulation in the environment and making its way into the food web. Many of the synthetic pesticides especially organochlorines have been shown to persist in nature and their degradation is either very slow (longer half-life) or negligible. In such cases, they are termed as recalcitrant. Even though the natural degradation by photolysis, hydrolysis, microbial degradation, etc. usually takes care of many pollutants, these pesticides are not easily degraded by any of the natural processes. Pesticides can be degraded by plants, animals, and soil microbes, these also degraded by some physical like exposure to ultra-violet radiation as well as many chemical methods. Microbial degradation is most common type of degradation occurring in the environment. There is unknown enigmatic about microbial degradation of pesticides. Most of microbes usually gain sustenance essential for growth and development. In most of the cases microbes derive essential elements for energy from the degraded of pesticides (Bidlan, 2003).

1.2 Classification of pesticides by type of pest

Depending on the type of pest or the pathogen, pesticides are selected for specific use; therefore these have been classified into following categories.

Table 1.1 Classification of pesticides on basis of various pests

S. No.	Type	Uses
1	Algicides	Control algae in various water bodies like lakes, canals, swimming pools, ponds and water tanks etc.
2	Antifouling agents	Used against the organisms that attach to underwater exteriors, such as boat bottoms to kill or repel.
3	Antimicrobials	Used against the microorganisms (actenomycete, bacteria and viruses) to kill.
4	Attractants	Attract pests (for example, to lure an insect or rodent to a trap). (However, food is not considered a pesticide when used as an attractant).
5	Biopesticides	Biopesticides are certain types of pesticides derived from such natural materials as animals, plants, bacteria, and certain minerals.
6	Biocides	Used against microorganisms.
7	Disinfectants and sanitizers	Used to kill or inactivate disease-producing microorganisms on inanimate objects.
8	Fungicides	Used to kill fungi (including blights, mildews, molds, and rusts).
9	Fumigants	Produce gas or vapour planned to repel and destroy the pests in constructed buildings and/or soil.
10	Herbicides	Used against herbs especially weeds and other unwanted plants to destroy and control the weeds growth and production.
11	Insecticides	Used against the insects and other arthropods to repel and /or kill.
12	Miticides	Used to kill mites that nourishment on the plants and animals.
13	Microbial pesticides	Microorganisms that repel, kill, inhibit, or out compete pests, including insects or other microorganisms like lower fungi.

14	Molluscicides	Used against snails and slugs for repel and kill.
15	Nematicides	Used to kill or control the growth of nematodes like microscopic, worm-like organisms that feed on plant roots.
16	Ovicides	Used against the eggs stage of insects and mites to destroy.
17	Pheromones	Biochemicals used to disrupt the mating behavior of insects.
18	Repellents	Repel pests, including insects (such as mosquitoes) and birds.
19	Rodenticides	Control mice and other rodents.

1.3 Classes of pesticide by hazard

The World Health Organization (1990) has proposed a classification of pesticides based on their health risk, toxic behavior in laboratory animals and estimating median lethal dose (LD_{50}) of exposed animals. The ranks are classified on the basis of toxicity, is extremely toxic, highly toxic, moderately toxic, and slightly toxic.

Table 1.2 The WHO recommended classes of pesticides based on hazards

WHO Class		LD_{50} for the rat (mg/kg body weight)	
		Oral	Dermal
Ia	Extremely hazardous	< 5	< 50
Ib	Highly hazardous	5–50	50–200
II	Moderately hazardous	50–2000	200–2000
III	Slightly hazardous	Over 2000	Over 2000
U	Unlikely to present acute hazard	5000 or higher	

1.4 Classes of pesticide based on chemical nature

Most of the pesticides are chemical in nature, based on source of pasticides, these may be natural substances or derived from natural materials. Pesticides are broadly classified into Fig. 1.1.

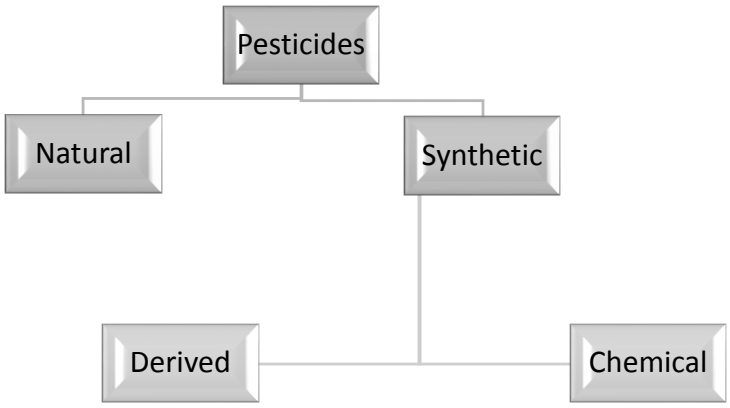


Fig. 1.1 Classes of pesticide based on chemical nature

1.4.1 Natural pesticides

Natural pesticides are the pesticides obtained from natural sources e.g. mineral salts, some metal and raw materials of animals and plants are also use as natural pesticides. Natural pesticides are also derived from natural orgenisms such as fauna, flora and microorganisms. Canola oil and baking soda are well known natural pesticides. 195 bio pesticide and 780 bio products were registered till 2001. Natural pesticides are following type based on source, where they are obtain.

1.4.1.1 Plant pesticide

Plant incorporated protectants (PIPs) are pesticidal substances that plants produce from genetic material that has been added to the plant. Natural plant pesticides are the pesticides obtained from plants e.g. azadirachtin, an extract from the *Azadirachta indica* tree and *Nicotiana tabacum* plant.

1.4.1.2 Biochemical pesticide

Biochemical pesticides occurs naturally and control the growth and development of many pests by non-hazardous way. Some biochemical pesticides such as insect pheromones and steroids, which not only interfere in mating of pests, but also act as trap for them. For example, scientists can take the gene for the *Bacillus thuringiensis* (Bt) protein, and introduce the gene into the plant's own genetic material. Then the plant, instead of the Bt bacterium, manufactures the substance that destroys the pest. The protein and its genetic material from the living orgenism, but not plant or animals, because it is sometimes challenging to determine the classification of biochemical pesticide. EPA has established and controlled a special committee to make such decisions.

1.4.1.3 Microbial pesticide

Microbial pesticides are microorganisms mostly bacterial strains, fungus or products of microorganism used against the pests. *Bacillus thuringiensis* is the most widely used as microbial pesticides, strains of *Bacillus* are also used as growth promoter. Many strains of *Bacillus* are produces a mixture of proteins and specifically attacks and kill's one or few species of insect in larvae stage. While some Bt's control moth larvae found on plants, other Bt's are specific for larvae of flies and mosquitoes. The target insect species are determined by whether the particular Bt produces a protein that can bind to a larval gut receptor, thereby causing the insect larvae to starve.

1.4.2 Classes of synthetic pesticide

Synthetic pesticides are the pesticides produced by synthetically or by chemical methods. These are further classified into four classes are following.

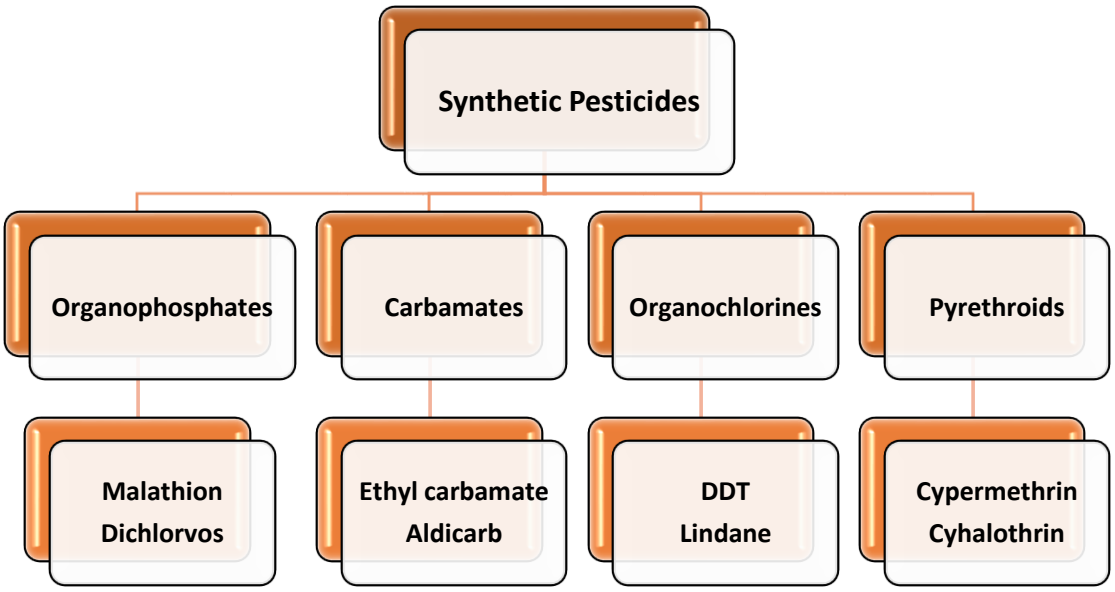


Fig. 1.2 Classification of Synthetic Pesticide

1.4.2.1 Organophosphate pesticides

Organophosphates like malathion and dichlorvos affect the nervous system by disrupting the enzyme (inhibiting acetylcholinesterase) that regulates levels of acetylcholine, a neurogenic transmitter. Most organophosphates are used as insecticides in agriculture and health programme. They were developed during the early 19th century, but still use till date. Their effects on major pest like insects, which are similar to their effects on humans, were discovered in 1932. The

harmful effect of organophosphates in human, the symptoms include a headache, loss of reflexes, nausea, sometime unconsciousness or even death at higher dose. They were used during II World war as nerve agents in human kind. However, they usually are not persistent in the environment because they easily degradable. Organophosphates are most commonly used in agriculture, in the cultivation of cotton, sugarcane, vegetable crops, fruit trees, grains and cosmetic industry etc.

1.4.2.2 Carbamate pesticides

Carbamates are the esters derived from acids or dimethyl N-methyl carbamic acid. Carbonates are less persistent than organophosphates and mainly affect the nervous system by disrupting an enzyme that regulates acetylcholine and the enzyme effects are usually reversible. The action of carbamate is fast and kinetics of blocking is by the covalent attachment of electrophilic groups of the enzyme. Carbamates are divide into several subgroups based on chemical composition, some examples of carbamates are ethyl carbamate and aldicarb.

1.4.2.3 Organochlorine pesticides

Organochlorines are most stable compounds which are too persistent in the environmental sample. Organochlorines or their metabolites act primarily in the central nervous system causing modifications in the flow of Na+ and K+ ions through the membrane of the nerve cell causing seizures and acute poisoning death from respiratory arrest. They were commonly used in the past, but many have been removed from the market due to their health, because they have the tendency to accumulate and store in fatty tissues. Organochlorines have been used in the eradication of disease vectors such as malaria and dengue examples DDT and lindane.

1.4.2.4 Pyrethroid pesticides

They were developed as a synthetic version of the naturally occurring pesticide pyrethrin, which is found in *Chrysanthemums*. They have been modified to increase their stability in the environment. Some synthetic pyrethroids are toxic to the nervous system. Most of the pyrethroid insecticides are fast acting, low in toxicity to mammals/birds and require very low doses to kill insects. But they are poorly soluble in water and bind tightly to the soil which makes them

ineffective in killing underground pests. Some common examples of pyrethroids are cypermethrin and cyhalothrin.

1.5 Uses and consumption pattern of pesticides

Pesticides are used to control organisms that are considered to be harmful. For example, they are used to kill mosquitoes that can transmit potentially deadly diseases like West Nile virus, yellow fever, and malaria. They can also kill bees, wasps or ants that can cause allergic reactions. Insecticides can protect animals from illnesses that can be caused by parasites such as fleas. Pesticides can prevent sickness in humans that could be caused by moldy food or diseased produce.

Herbicides can be used to clear roadside weeds, trees, and brush. They can also kill invasive weeds that may cause environmental damage. Herbicides are commonly applied in ponds and lakes to control algae and plants such as water grasses that can interfere with activities like swimming and fishing and cause the water to look or smell unpleasant. Uncontrolled pests such as termites and mold can damage structures such as houses. Pesticides are used in grocery stores and food storage facilities to manage rodents and insects that infest food such as grain. Each use of a pesticide carries some associated risk. Proper pesticide use decreases these associated risks to a level deemed acceptable by pesticide regulatory agencies such as the United States Environmental Protection Agency (Aktar *et al.*, 2009).

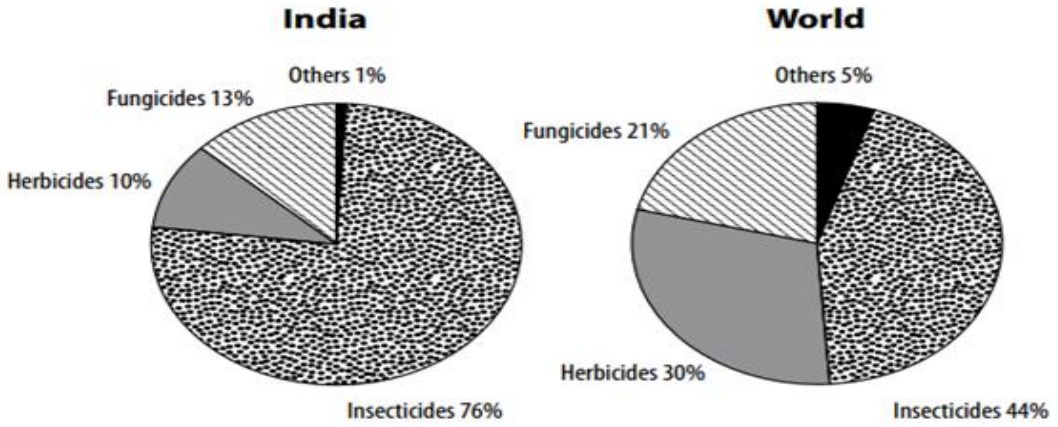


Fig. 1.3 Consumption pattern of pesticide

1.6 Fate of pesticides

The fate of pesticides in soil is controlled by chemical, biological and physical dynamics of this matrix (Sparks, 2003). These processes can be grouped into those that affect persistence, including

chemical and microbial degradation, and those that affect mobility, involving adsorption, plant uptake, volatilization, wind erosion, run-off and leaching (Fig. 1.2).

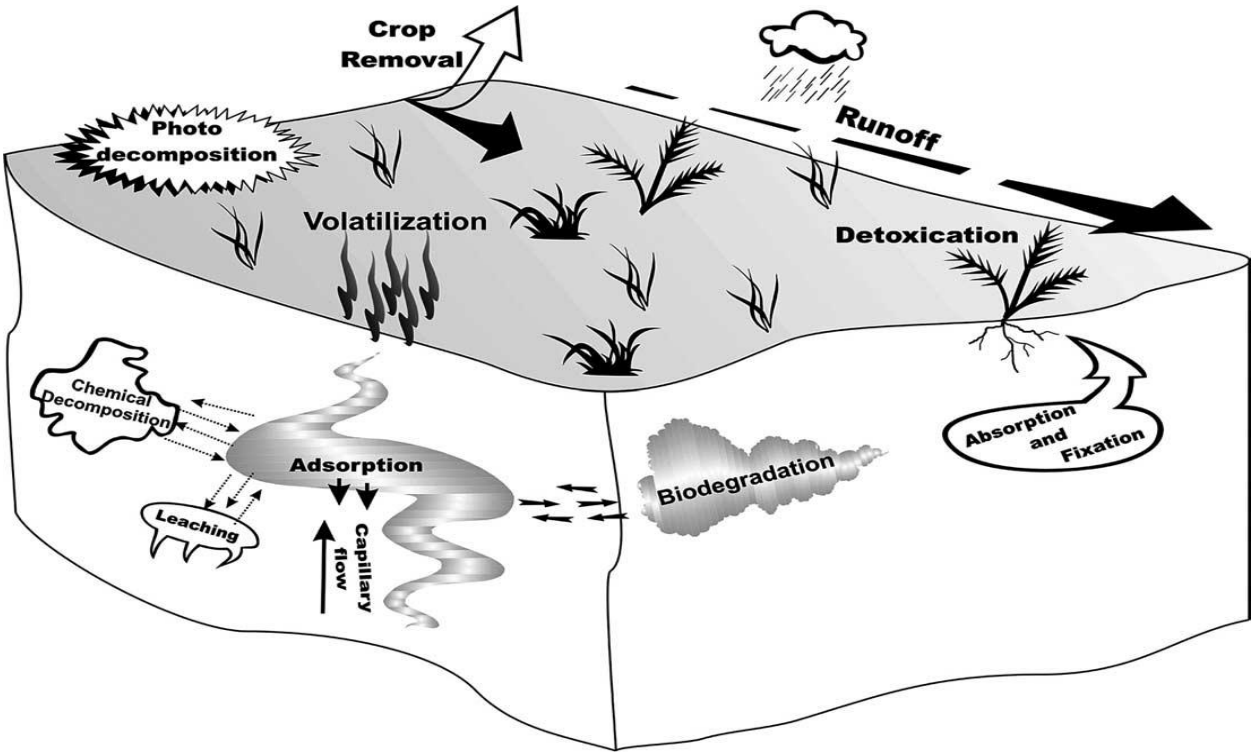


Fig. 1.4 Fate of pesticide residues in soil (source: Sparks, 2003).

1.7 Effect of pesticides

Pesticide exposure can cause a range of neurological health effects such as loss of memory and coordination, reduced speed of response to stimuli, impaired visual ability, and motor skills, altered or uncontrollable mood as well as general behavior. These symptoms are often very subtle and may not be recognized by the medical community as a clinical effect. Many other possible health effects include asthma, allergies, and hypersensitivity, and pesticide exposure is also linked to cancer, hormone disruption, and problems with infertility and fetal development.

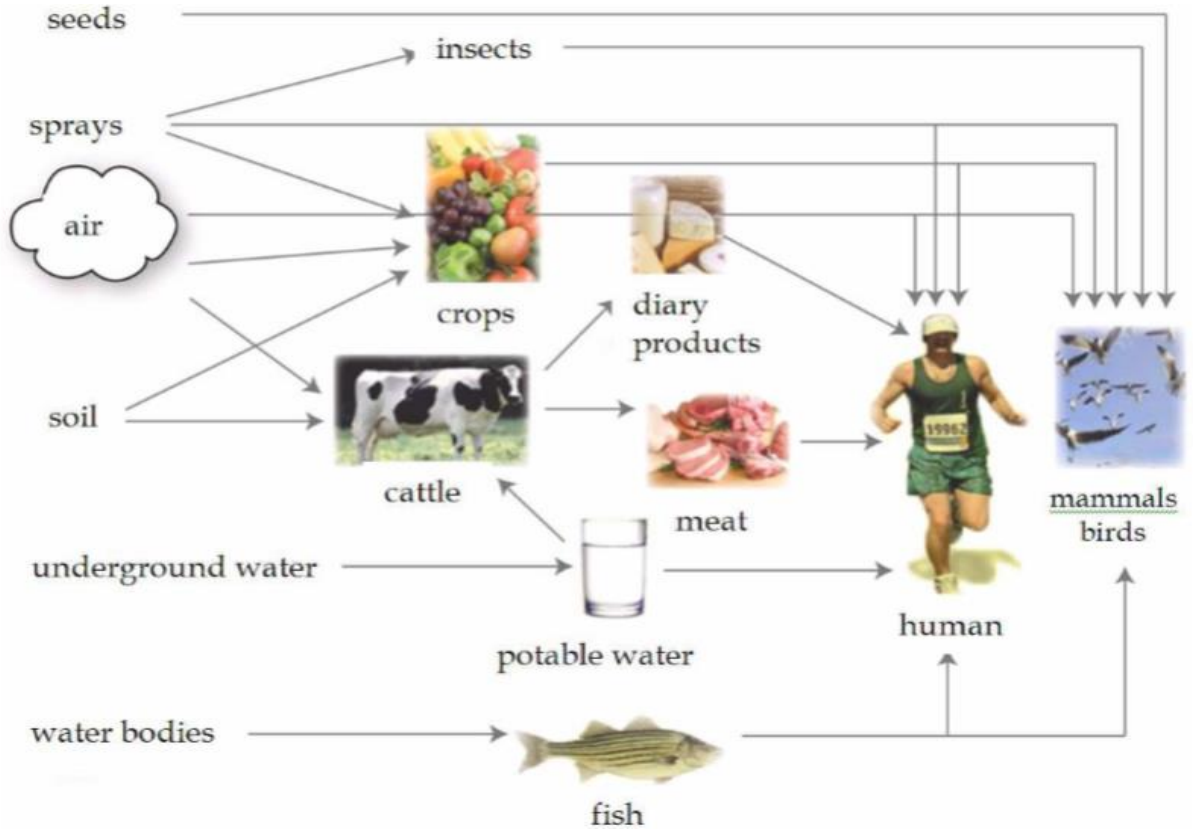


Fig. 1.5 Poison chain of pesticide (source: The Hindu, 2005).

1.8 Lindane

Gamma-hexachlorocyclohexane (γ-HCH) commonly known as lindane has been broadly used in agriculture in the last decades. Lindane belongs to the organochlorine (OC) pesticide class. This is one of the oldest classes of pesticides, and few OC's are still in use today in the health program. Lindane was first synthesized in 1825 The chemical was originally synthesized in 1825 by Michael Faraday It is named after the Dutch chemist Teunis van der Linden (1884–1965), the first to isolate and describe γ-hexachlorocyclohexane in 1912. Its pesticidal action was discovered only in 1942, after which lindane production is continued by Imperial Chemical Industries Ltd, United Kingdom. According to Safe (1993), HCH consists of eight isomers. Only α-, β-, and δ-isomers, as well as technical-grade HCH, are not synonymous with γ-HCH (Farm Chemicals Handbook 1993). The HCH formulation consists of γ-(10-12%), α-(60-70%), β- (5-10%), δ-(6-10%) and ϵ (3-6%) isomers (Vijgen J. 2006) and out of these only γ-HCH possesses insecticidal activity.

1.8.1 Physical and chemical properties of lindane

Few of the important properties of lindane are:

CAS ID	58-89-9
Form	colorless to white colored crystalline solid
Odor	slight musty odor
Molecular weight	290.85
Vapour pressure	9.4×10^{-6} mmHg at 20°C
Enthalpy of Vaporization	50.6 ± 3.0 Kj / mol
Boiling point	288 °C at 760 mmHg
Melting point	112.5°C
Density	1.85

1.8.2 Structure of lindane

Hexachlorocyclohexane (HCH) is one of the several polyhalogenated organic compounds consisting of a six-carbon ring with one chlorine and one hydrogen attached to each carbon. There are many isomers of this structure, differing by the stereochemistry of the individual chlorine substituents on the cyclohexane.

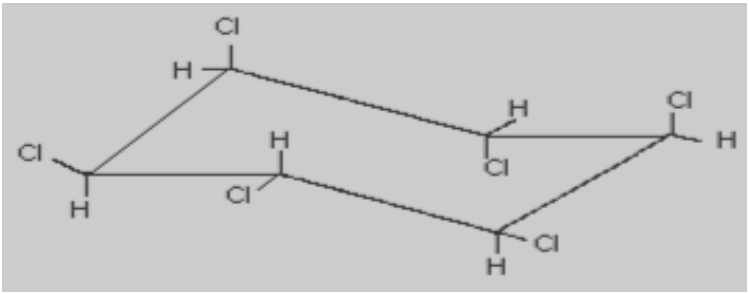


Fig. 1.6 Chemical structure of Lindane

1.8.3 Solubility of lindane

Insoluble in water at 20°C (10 mg/L), moderately soluble in ethanol, ether, benzene acetone, slightly soluble in mineral oils.

1.8.4 Stability of lindane

Stable to light, air, heat, carbon dioxide and strong acids. Dechlorination occurs in the presence of alkali or on prolonged exposure to heat. Trichlorobenzenes and hydrochloric acid are formed.

1.8.5 Synonyms of lindane

- Gamma-hexachlorobenzene,
- gamma-hexachlorocyclohexane,

- 1, 2, 3, 4, 5, 6,-hexachlorocyclohexane,
- 1-alpha,2-alpha,3-beta,4-alpha,5-alpha,6-beta-hexachlorocyclohexane,
- gamma-isomer.
- Gamma-benzene hexachloride,
- gamma-BHC,
- ENT 7, 796,
- Gammahexane,
- Hexachloran,
- Gamma-hexacloran,
- Gamma-hexachlorane.

1.8.6 Trade of lindane

Agrocide, Ambrocide, Aparasin, Aphatiria, Benesan, Benexane, BoreKil, BorerTox, Exagama, Gallogama, Gamaphex, Gammalin, Gamma-Col, Gamene, Gamiso, Gammex, Gammexane, Gamasan, Gexane, Isotox, Jacutin, Kwell, Lindafor, Lindaterra, Lindatox, Lorexane, New Kotol, Noviagam, Quellada, Steward, Streunex, Tri-6, Viton.

1.8.7 Application of lindane

1.8.7.1 Human exposure

The most important route of human exposure to lindane is ingestion of food contaminated with this pesticide. Infants are exposed to lindane through ingestion of their mothers' milk. Higher concentrations of lindane and other HCH isomers have been found in human tissues and mothers' milk in developing countries, as compared with levels found in Canada and the US. In general, most Canadians are exposed to lindane at concentrations considered to be "acceptable". However, Inuit populations may be exposed to higher lindane concentrations. Farmers, pesticide applicators and individuals living in the vicinity of hazardous waste disposal sites contaminated with lindane may receive additional exposure through dermal contact and inhalation. Compared with the general population, higher concentrations are found in serum and adipose tissue of people occupationally exposed to lindane and other HCH isomers.

1.8.7.2 Acute toxicity

Lindane is considered to be highly toxic to aquatic organisms, and moderately toxic to birds and mammals. People who are occupationally exposed to it are advised to avoid its contact with eyes, skin and via inhalation. Lindane has adverse effects on the central nervous system. Symptoms of acute toxicity in humans include a headache, dizziness, seizures as well as effects on the gastrointestinal tract, cardio-vascular and musculo-skeletal systems.

1.8.7.3 Chronic toxicity

A variety of sublethal effects have been observed in aquatic organisms, birds and mammals exposed to lindane in long-term studies. These include biochemical changes, which may affect growth and reproduction in aquatic organisms. Effects in mammals are mostly on the liver and kidney, although other organs may be affected, as well as the immune system and central nervous system. Lindane is considered to be a possible human carcinogen, and HCH is considered a probable human carcinogen by the US Environmental Protection Agency.

1.8.8 Banned for manufacture and use of lindane

It was banned for manufacture, import or formulates w.e.f. 25th March 2011. However, it is allowed for use up to 24th March 2013 for termite control in Building including wood, and termite control in Agriculture as per approved label claims by the Registration Committee and for exports. Finally banned vide Gazette Notification No. S.O. 637(E) Dated 25th March 2011 after the recommendation of Notification No S.O.1472 (E) dated 29th Aug 2007

1.9 Malathion

Malathion is a pesticide and belongs to the chemical family of organophosphates (OPs). Malathion is a wide-spectrum insecticide and has been manufactured in the United States since 1950. Malathion has found its applications in agriculture, health pest control programs, residential gardens, public recreation areas etc. (Srinivas and Damani, 2016). Malathion is an active ingredient in some of the cosmetic items especially shampoos made for treating lice. Malathion has also been to eradicate mosquitoes without affecting human health or the environment when applied in accordance with the instructions and safety precautions specified on the label. However, higher doses of malathion can over stimulate the nervous system causing symptoms like nausea, dizziness, or confusion. High-dose poisoning with any organophosphate has been shown to cause convulsions, respiratory paralysis, and even death (Singh *et al*., 2006).

People who apply products containing malathion are therefore advised to take proper protection. Consumption of food treated with malathion may have detrimental effects (Mersal, 2011). Therefore, the Food and Drug Administration (FDA) and EPA have set a maximum limit of 8 parts per million (ppm) of malathion to be present as a residue on specific crops used as foods. Higher concentration (300 ppm) of malathion has toxic effects on seed germination, starch, amino acid and protein content. Exposure to high amounts of malathion in human may cause many health problems like vomiting, weakness, loss of consciousness, shortness of breath, slow heart rate, headache and diarrhea (Mostafa, 1972). Pets may also be exposed to malathion if they come into contact accidentally or by eating plants that have just been sprayed. The nervous system of humans and animals is very similar, so animals poisoned by malathion may show similar signs to those observed in human beings (Singh, 2013).

Bacteria have the capability to break down malathion in soil and in the air, sunlight breaks down malathion into a more toxic product known as malaoxon. Very little amount of malathion mixes with water and has the ability to move quickly through the soil. Because of these properties, malathion can be found in surface waters such as streams, and unlikely found in underground water as it is broken by bacteria in the soil itself. In soil, the time taken by malathion to break down to half of its original amount is around17 days, depending on the soil type. This length of time is known as the half-life. In water, malathion has a half-life of 2 to 18 days, depending on conditions like temperature and pH. Malathion vapour may also transportable long distances in the environmental air or fog (Iriyama *et al*., 1980).

1.9.1 Chemical properties of malathion

Common names of Malathion includes carbophos, maldison, mercaptothion and chemically known as [S-(1, 2-dicarbethoxyethyl)-O, O-dimethyl dithio-phosphate]. Malathion {diethyl (dimethoxyphosphinothioylthio) succinate}, in an organophosphate insecticide. Pure malathion is a clear liquid, however, the technical-grade solution, which contains impurities is an amber liquid.

Few of the important properties of malathion are following.

CAS No.: 121-75-5

Empirical formula: $C_{10}H1_9O_6PS_2$

Molecular weight: 330.36

Water solubility at 20 ^{o}C: 148 mg/L

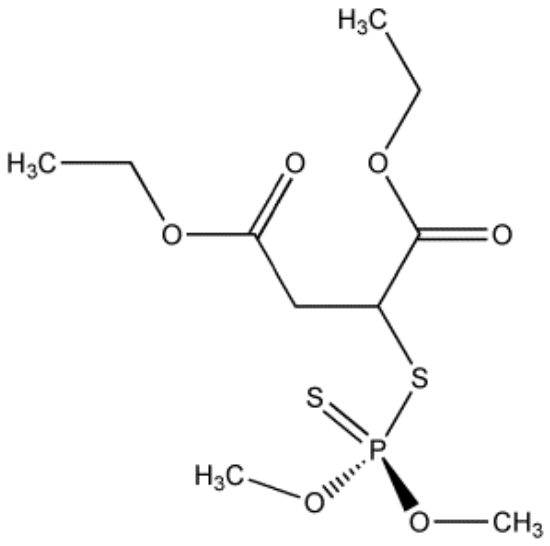


Fig. 1.7 Chemical structure of malathion

1.9.2 Use of malathion

Malathion is used to control a broad spectrum of insects including ants, aphids, fleas, fruit flies, hornets, mites, mosquitoes, moths, spiders, thrips, ticks, wasps, and weevil. Malathion is used for pest control on a wide range of agricultural food and feed crops, the most common of which are blueberries, raspberries, strawberries, limes, cotton, cherries, garlic, greens, dates, and celery. For agricultural sites, application amount ranges from 0.175 - 6.25 lbs active ingredient (a.i.) per acre. It is also approved for residential use, but at a rate of 0.000085 - 0.0003 lb a.i. per square foot. Beyond application to plants, malathion is a component of personal hygiene products used for lice control (Singh *et al*., 2006).

Malathion can be applied by aircraft, duster, fogger, ground boom, irrigation, sprayer or spreader. Malathion is available in many different formulations, including ready-to-use liquid, dust, pressurized liquid and emulsifiable concentrate.

1.9.3 History of use of malathion

Malathion has been a registered pesticide since 1956 and is still in use Malathion is the primary pesticide used in the USDA boll weevil eradication program, which began in the late 1970's to protect cotton crops in the southern United States continued until 2008

In 1998 in Florida, an outbreak of Mediterranean fruit flies (Medflies) threatened to significantly decrease agricultural yields. In an effort to minimize damage, the Medfly Eradication Program was implemented by federal and state authorities. Malathion and another organophosphate, diazinon, were applied to areas of concern. Within 5 months of application, 123 people reported symptoms consistent with pesticide exposure, such as respiratory distress, gastrointestinal distress, neurological problems, skin reaction, and eye distress.

During 2005, malathion was one of many insecticides used to control mosquitoes potentially carrying West Nile Virus in the United States.

1.9.4 Routes of exposure and metabolism

Exposure to malathion can occur in several different ways: oral, dermal, inhalation, or eye contact. Oral exposures typically involve consumption of contaminated food or water, while dermal exposure occurs during the handling of treated crops or during residential use. Inhalation exposure commonly occurs near agricultural fields where malathion is applied and often is the result of spray drift. Inhalation of malathion is also a risk when insecticides are used in homes where ventilation is poor. Malathion is quickly metabolized by the body and does not accumulate. Metabolites are excreted in the urine within several days. One of the metabolites, malaoxon, is more toxic than malathion.

1.9.5 Human health effects

1.9.5.1 Acute

In acute oral doses, symptoms are similar to other cholinesterase (ChE) inhibitors and include: numbness, decreased coordination, dizziness, tremor, nausea, blurred vision, difficulty in breathing, slow heartbeat, headache and tingling sensations. Accidental death in humans from malathion has been documented. Toxicity may vary based on gender, as male and female rats displayed different LD50 values for an oral dose (5400 and 5700 mg/kg, respectively).

1.9.5.2 Chronic

In a chronic toxicity study, human volunteers ingested a low dose for 1.5 months without detrimental effects on blood ChE activity.

1.9.5.3 Carcinogenicity

According to the EPA, malathion has, "suggestive evidence of carcinogenicity".

1.9.6 Environmental effects

1.9.6.1 Persistence

Malathion has low soil and water persistence. The half-life of malathion in soil is 1-25 days, however, it is mobile. Malathion can leach out of the soil into groundwater and has been detected in groundwater in CA, MS, and VA. Malathion has low persistence in water as well, with a half-life of <1 week in river water.

1.9.6.2 Ecological effects

Malathion is slightly toxic to mammals, moderately toxic to birds, and highly toxic to aquatic organisms, both freshwater and estuarine, as well as bees.

Acute toxicity

Rat acute oral LD50: 1178 mg/kg

Rat inhalation LC50: > 5mg/L

Rat inhalation LC50: >5mg/L

Birds acute oral LD50: 359 mg/kg

Fish 96 hr LC50: 0.018 mg/L

Aquatic invertebrates 48hr EC50: 0.0007 mg/L

Honeybees 48 hr LD50: 0.16 µg/ bee

1.10 Wheat (*Triticum astevium*)

1.10.1 Taxonomic position and distribution

Triticum astevium is a member of the family Poaceae (Gramineae). As per Vavilov (1935) wheat (hexaploid) originated from Central Asia. Almost all varieties of wheat are grown in the countries situated in the temperate zone. China is the largest producer of wheat in the world followed by India 94.3 with Million Metric Tonnes. The maximum producing states in India are Uttar Pradesh, Punjab, Madhya Pradesh, Haryana, and Rajasthan.

1.10.2 Morphology

The wheat plant is an herbaceous annual growing to a height of about 2.5 to 5 feet

1.10.2.1 Roots

There are two sets of roots, 3-6 seminal or seedling roots developing from the embryo as the adventitious roots (coronal) arising from the basal underground nodes of the axis representing permanent root system. Secondary roots or tillers arise from the auxiliary buds present on the underground portion of the stem and bear a similar series of coronal roots.

1.10.2.2 Stem

The stem or culms of the wheat plant is erect, cylindrical and differentiated into node and internode. The nodes are solid whereas the internodes are hollow.

1.10.2.3 Leaf

The wheat leaf consists of four parts; (i) the blade or lamina is narrowly linear to linear-lanceolate 20-27cm long and nearly 1-2cm wide. (ii) The sheath, the part which clasps the stem tightly. (iii) The ligule or rain- guard, a thin membrane growing at the junction of the blade and the sheath. (iv) Auricles are curved claw-like appendages attached to the base of the blade and generally clasping the stem from the opposite side.

1.10.2.4 Inflorescence and flower

A spike inflorescence of wheat consists of spikelets which are borne on the alternate side of rachis, giving it a zigzag appearance. Spike may be awned or awnless, an awn is a tapering projection of the flowering flumes of a spikelet. A wheat flower consists of three stamens with threadlike filaments and rather large anthers. The single ovary has two feathery stigmas.

1.10.2.5 Fruit and seed

The wheat grain is a one-seeded dry fruit (caryopsis) with the seed coat fused with pericarp. It has a large starchy endosperm and an embryo.

1.10.3 Economic importance of wheat

1. Wheat is the staple food of north Indian people as well as over the entire world.
2. Wheat grains are grounded into flour (atta) and consumed in the form of chapati.
3. Soft wheat is used for making bread, cake, biscuits, pastry and other bakery products.
4. Hard wheat is used for manufacturing rawa, suji, and sewaya.
5. It is also used for making flakes and sweetmeats like kheer, shira, etc.
6. Wheat grain is used for preparing starch.
7. Wheat straw is used as fodder, padding material, and mulching material.
8. Wheat straw is also used as livestock, animals budding and compost.

1.11 Green Gram (*Vigna radiate* L)

1.11.1 Taxonomic position & distribution

Green gram or mung bean (*Vigna radiate* L.) is an important pulse crop belongs to family Leguminosae and sub-family Papilionaceae, which grown under tropical and subtropical conditions. As per Vavilov, (1935) green gram originated from Indian Centre. It is an excellent source of protein with a perfect mixture of essential amino acids. The seeds of green gram contain a higher proportion of lysine than any other legumes. Green gram is one of the most traditional forms of protein and minerals such as potassium, magnesium and iron intake amongst the world population.

1.11.2 Morphology

Green gram is a small herbaceous annual growing to a height of 1 to 3 feet.

1.11.2.1 Stem

The main stem is more or less erect. The lateral branches are sub-erect.

1.11.2.2 Leaves

The leaves are trifoliate, alternate and compound. The leaflet being large, ovate and entire and size up to 12x10 cm.

1.11.2.3 Flowers

Flowers are in axillary racemes, peduncle up to 13 cm in length with clusters of 10-12 flowers, corolla yellow in color sometimes curved, 5-10 cm long.

1.11.2.4 Pods

The pods are slender and about 7-10 cm long

1.11.2.5 Seeds

Number of seeds per pod ranges 10-15. The seeds are globular, green or olive green in color; sometimes yellow, brown or blackish also.

1.11.3 Economic importance of green gram

It is mostly consumed as a main dish though sometimes it is also used to make sweet dishes. It is either cooked like a dish by boiling and adding spices, or can be made into a paste. Green gram sprouts are consumed directly with lemon and salt as a healthy snack or added to the salad. This to make green gram pulse form of green gram is obtained by germinating them in water. In some parts, its cover is removed. Green gram batter is used to make a certain kind of crepes

named *pesarattu* in Andhra Pradesh. In China green gram is used to make a certain kind of dessert. In some countries, green gram paste is used to make ice creams or frozen ice pops.

1.11.4 Nutritional value of green gram

1. It is a rich source of low-fat protein, has a wide amino acid profile and contains no trans or saturated fats.
2. It has both insoluble and water-soluble fibers that help maintain a healthy appetite
3. Water-soluble fiber helps lower LDL cholesterol and protects from cardiovascular diseases.
4. It digests slowly and gradually releasing sugar into the blood and helps maintain the blood sugar level.
5. It is said that the water in which green gram was soaked is a good medicine for cholera, measles and chicken pox.

1.12 Objectives

Investigations were carried out with respect to the following objectives:

1. Effect of pesticides on the seed germination, growth and development of valuable crops.

2. Isolation and screening of bacterial strains for degradation of lindane from enrichment on mix commercial formulations of pesticides.

3. Optimisation of conditions for lindane degradation by the consortium and individual strains of the consortium.

4. Biochemical and molecular characterization of lindane degrading bacterial strains.

Review of Literature

2. Review of Literature

2.1 Various mechanisms for pesticide degradation

Detoxification of pesticides is mainly done by abiotic and biotic transformations. These two are the most important mechanisms for detoxifying the soil. In the transport mechanism, the total chemical composition doesn't necessarily decrease but is being translocate from one soil compartment to another. Abiotic transformation mechanism makes partial degradation of pesticides due to which the metabolites are accumulated in soil. Biological degradation, on the other hand, makes complete detoxification i.e., mineralization completely to CO_2, H_2O and inorganic ions (Schnoor *et al*, 1995). This concludes that soil is a biologically diverse and dynamic ecosystem. The microbes present in soil i.e., bacteria, fungi etc. continually recycle the major nutrients from organic part of the soil. In particular, bacteria are physiologically versatile metabolite which performs various functions on various different naturally occurring or synthetic compounds, like pesticides, in soil (Newman *et al*, 1997).

Lindane is a persistent organic pollutant (POP) which causes health and environmental hazards and consequently, its use has been reduced and banned in several countries. Due to recalcitrant nature of lindane and its harmful effects, a wide range of physical, chemical and biological methods and their combinations have been devised by the scientists all over the world to degrade it or to reduce its concentrations or to make it less harmful to nature as well as human civilizations. Several methods are employed in order to degrade or to remove the lindane from soil and water sources (Hughes *et al*, 1997; Burken and Schnoor, 1998). Some of the important and well-established methods are:

a) absorption by activated carbon
b) subjection to photo-catalysis
c) bio-catalytic dechlorination
d) phytoremediation
e) bio-sorption
f) microbial degradation
g) remediation by nanoparticles.

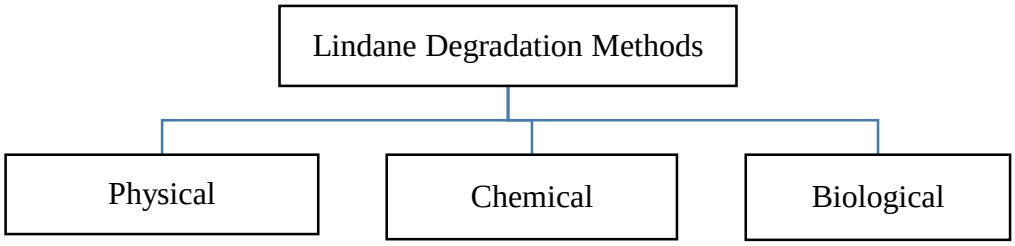


Fig. 2.1 Various methods for lindane degradation

2.1.1 Physical methods

Physical treatments like thermal desorption and incinerations provide efficient degradation but they are very expensive, need huge infrastructure and generate highly toxic gases. Lindane adsorption on porous inorganic and organic material causes the problem of disposal of adsorbed lindane.

2.1.1.1 Remediation by nanoparticles

Singh *et al* 2011, explored the role of iron nanoparticles to remediate lindane contaminated soil and found that 10 µg/g of lindane was completely disappeared within 24 hours when nanoparticles of zero-valent iron were used at a concentration of 1.6g/L in an aqueous solution. They also reported that lindane degradation increased with the increased reaction time. The author reported the scope of development of nanoscale bimetallic particles for the lindane remediation from the environment. Novel synthetic methods should be explored to synthesize nanoparticles of desired size, enhanced activity, and surface transport characteristics. Surface modification of zero-valent iron nanoparticles by the method of adsorption polymers provides an effective coating, which protects the reactive centres against oxidation improving also the determination of the nZVI until its response with lindane to promote its degradation. Bio-catalytic dechlorination of lindane by nano-scale particles of Pd deposited on *Shewanella oneidensis* showed degradation of HCH into less toxic Benzene (Thompson *et al*, 1998; Raskin and Ensley, 2000).

2.1.2 Chemical methods

Chemical treatment includes harsh chemicals like degradation induced by microwave with sodium hydroxide i.e. modified sepiolite and addition of hydrogen peroxide.

2.1.2.1 Photo-catalysis

Photo-catalysis is a catalyst based light-reaction exploited to remediate the wastewater, semisolid waste and pesticides present in the environmental simples. The catalyst is used to enhance the reaction rate. Photo-catalysis depends upon the various factors like type of pollutant, insecticides,

adsorption ability, and temperature of the reaction, light intensity, pH, and catalyst type. Many authors worked on photo-catalytic and reported degradation of lindane and DDT using Sun radiations by various methods like response surface methodology and found it to be environment-friendly and economical to degrade lindane. Photocatalysis of lindane (36 - 68%) degraded or converted into intermediate compounds.

Table 2.1 Various mode of pesticide degradation

S. No.	Methods	Mechanism
1.	Hydrolysis	It involves the reaction of water molecule involving specific catalysis by proton, OH group or inorganic ions such as phosphate ions
2.	Oxidation of sulfur in a molecule	Chemicals containing sulfur molecule are oxidized to sulfoxide and further to sulfone.
3.	Addition of oxygen to a double bond	The oxygen molecule is added to the double bond producing the epoxide.
4.	Oxidation of amino group to a nitro group	RNH2->RNO2
5.	Addition of hydroxyl group to the benzene ring	Oxidation reaction involving a dioxygenase or monooxygenase as a catalyst.
6.	Dehalogenation	In this reaction, a halogen is replaced by an H molecule.
7.	Reduction of the nitro group to an amino group	NO2 is being reduced to NH2. NO2->NH2
8.	Replacement of sulfur with an oxygen	P=S bonds are being modified to P=O bonds which increases the toxicity of molecule.
9.	Metabolism of side chains	In this reaction, the hydrocarbon chain attached to benzene rings of pesticide is removed and the ring is broken down.
10.	Ring cleavage	In this reaction hydroxylation of ring takes place by monooxygenase or dioxygenase to form catechol compound, resulting in ring cleavage via ortho or meta fission to form common biochemical intermediates.

(Source**:** Keun-Yook Chung, 1999)

2.1.3 Biological degradation of lindane

It includes the use of microbial communities such as bacteria, fungi, algae and actinomycetes and also their consortium for lindane degradation. The rate of biological degradation of lindane depends upon the presence of oxygen, bioavailability, initial lindane concentration, temperature, pH, and microbial biomass concentration. Salam and Das (2012) investigated the role of various

microbes for the remediation of lindane from the environment. Removal or degradation of a recalcitrant halogenated compound involves dehalogenation i.e. the removal of halogen atom. Halogen bound with carbon has a greater stability and thus facilitates the greater persistence and toxicity to the lindane. Microorganisms like fungi, bacteria, and algae utilize the lindane as a nutrient source for their growth and thus have a potential for bioremediation of it.

2.1.3.1 Phytoremediation

As the name suggests phytoremediation is the application of plants in remediation of pollutants and other undesired contaminating agents. Plants absorb, assimilate, metabolize, detoxify and degrade pesticides, insecticides, and herbicides etc. from the contaminated soil. Various pesticides like lindane, DDT etc present in water or soil gets metabolized into non-toxic or less toxic products through several processes. Phytoremediation concludes in various modes: phytotransformation, phytodegradation, phytovolatilization, phytoextraction, phytostimulation, phytofilteration or rhizoremediation. Soil characteristics like moisture content, clay content, particle size etc and nature of plants as well as the nature of contaminants, altogether govern the phytoremediation.

Table 2.2 Pesticides uptake by different plants

Pesticides	**Plant species**	**Comments**	**Reference**
Malathion, demeton S-methyl, crufomate	*Myriophyllum aqua-ticum, Spirodela oligorrhiza* L., *Elodea Canadensis*	*S. oligorrhiza* L. and *E. canadensis* exhibited a lag phase of first few days and then started extracting all the pesticides rapidly except crufomate	Gao *et al.* (2000)
Chlorpyrifos, atrazine	*Juncus effusus* L.	Chlorpyrifos was metabolized faster than atrazine.	Lytle and Lytle (2000)
Sulfentrazone	*Nicotiana tobaccum* L., *Gossypium hirsutum*	Herbicide uptake rate increased with a decrease in soil pH due to change in solubility.	Ferrell *et al. (*2003)
DDT, DDD, DDE	*Zucchini,* pumpkin, *alfalfa,* tall fescue, ryegrass	Cucurbita pepo species (pumpkin and zucchini) extracted highest amounts of pesticides.	Lunney *et al.* (2004)
Atrazine	*Populusdeltoides x nigra*	Atrazine was degraded in plant tissues.	Chang *et al.* (2005)

Atrazine, cycloxidim, thanterbutryn, and trifluralin	*Myriophyllum aquaticum*	Atrazine, xidim, and cyclo were taken up more by the plant & terbutryn, trifluralin from solution.	Turgut (2005)
Chlordane	*Cucurbita pepo* L. *Cucumis sativus* L.	Chlordane was detected in plant tissues (root, stem, leaf, and fruit).	Mattina *et al.* (2005)
HCH, DDT	*Cauliflower, spinach*	Both the plants extracted these pesticides from the soil.	Tao *et al.* (2005)
Chlorinated pesticides (OCPs)	*Solanum tuberosum, Daucuscarota* L.	Peels of carrots and potatoes were found to remove 52–100% of the residues of OCPs from the soil.	Zohair *et al. (*2006)

Source: Hussain *et al. (*2009)

Phytoremediation is the use of vegetation for *in situ* treatment of contaminated soils, sediments, and water. It is applicable at sites containing organic, nutrient, or metal pollutants that can be accessed by the roots of plants and sequestered, degraded, immobilized or metabolized in place. In the last few years, a great understanding has been achieved regarding the uptake and metabolism of xenobiotic chemicals by plants especially chlorinated solvents, petrochemicals, some pesticides and explosives (Anderson *et al*, 1993; Terry and Banuelos, 2000). Inorganics and metals (nutrients, selenium, arsenic, lead, cadmium, nickel and zinc) have been successfully remediated using plants. Plants have become popular because of their cost-effectiveness, aesthetic advantages and long-term applicability (Schnoor *et al*, 1995). Applications include hazardous waste sites where other methods of treatments are expensive or impracticable, low-level contaminated sites only. Limitations of phytoremediation include the presence of hazardous metabolites in the food chain. The time required to clean up below action-level is long and toxicity encountered in establishing and maintaining vegetation at the contaminated sites are among few other limitations here. A few plants have been shown to have the capacity to withdraw high concentrations of organic chemicals without showing toxicity effects (Briggs *et* al, 1982; Burken and Schnoor, 1998). These plants have the capacity to take up and transform the contaminants to less toxic metabolites (Newman *et al*, 1997; Schalk *et al*, 1997; Ohkawa *et al*, 1999; Werck-Reichart *et al*, 2000). Plants can also stimulate the degradation of organic chemicals in the rhizosphere by the release of root exudates,

enzymes, and the build-up organic carbon in the soil (Shimp *et al*, 1993; Burken and Schnoor, 1996).

2.1.3.2 Microbial degradation

Microbial degradation is one of the most studied and practiced ways to degrade the soil contaminating agents such as lindane to purify and remediate the soil from contaminating agents. Microbial flora, several algae, fungi, and bacteria, utilize these as a nutrient source for their growth and degrade or reduce their concentration in the environment. It is the most environment friendly and cost-effective method for the remediation. The ability of bacterial strain *Rhodococcus* to degrade several isomers of Hexachlorocyclohexane (HCH) and chlorobenzenes has been demonstrated by Apajalahti and Salonen (1987). Genetic and metabolic engineering mediated approaches in association with nanotechnology to treat lindane have been under exploration for a long time.

Microbial degradation involves the use of microbes to detoxify and degrade environmental contaminants. This has received increased attention as an effective biotechnological approach to clean up the polluted environment (Iwamoto and Nasu, 2001). Microbial remediation of soils and groundwater can be done by excavating the materials or pumping the groundwater and treating them on-site or off-site. When the contamination of the subsurface is limited to the soil in the unsaturated zone, the need for oxygen and water restricts the use of *in-situ* bioremediation to near-surface contamination (Ritter and Scarborough, 1995). Two basic forms of bioremediation had been practiced: microbiological approach and the microbial ecology approach (Protrowski, 1991). The second method involves selection, culture, and application of site-specific strains that exhibit desirable degradative qualities. This approach is to sample the contaminated soil or water at a site and analyze the samples for microbial strains. The strains that exhibit desirable traits for degrading specific contaminant are cultured and applied to the contaminated sites in high densities along with nutrients identified as being important for high activity rates of the microbe.

***Ex-situ* bioremediation** is the treatment that removes contaminants at a separate treatment facility. The treatment is undertaken away from the contaminated site. After remediation, the remediated soil is brought back to the site and refilled. Carberry *et al* (1991) described a controlled land farming technique to bio-remediate petroleum contaminated soil. The contaminated soil was placed in a greenhouse on a plastic sheet to a depth of 18 inches. The soil was periodically stirred

for aeration and nutrients and microorganisms were also added periodically. pH could also be controlled in such a case with a lot of ease. Water can also be added to maintain the moisture content of the soil.

Though there are different strategies adopted worldwide to get rid of these contaminants, the best strategy appears to be only bioremediation. This is the best way to treat pollution because it leaves less or no toxic metabolites in the environment compared to other strategies wherein the intermediary metabolites may remain in the environment that might pose more danger than the parent compound. Moreover, all the other strategies are more expensive and energy consuming. Bioremediation, on the other hand, works out to be cheaper and more effective strategy to eliminate these toxic chemicals from the environment. Phytoremediation involves the cultivation of plants having an affinity towards the contaminant and that take up the maximum amount of the contaminant from the surroundings and then this flora can be taken for incineration as further treatment of the accumulated chemical. Another strategy here is that the plant itself might take the charge to mineralize the chemical into simpler forms that can be used elsewhere in the metabolism. Phytodegradation of DDT by aquatic plant *Elodea Canadensis* and a terrestrial plant *Pueraria thunbergiana* was reported by Garrison *et al* (2000). The major disadvantage here appears to be the length of treatment: the site needs to be left only for such plants till the plant absorbs the complete or maximum quantity of the toxicant. This depends upon the ability of the plants to take up the chemical. Moreover, the plant needs to grow up to the maximum state to absorb the chemicals from the site through roots that may take a longer time factor. With this scenario, microbial remediation appears to be the only way out for treatment of contaminated resources in short time and with more efficiency and safety, with less investment. Many reports are available on the microbial degradation (microbial bioremediation) of environmental pollutants.

Table 2.3 Microorganisms responsible for organic compounds degradation

Organic compounds	***Microorganism***	**Reference**
3- chlorobenzoic acid	*Pseudomonad*	Johnston *et al* (1972)
Cholin-o-sulfate	*Neurospora crassa*	Marzluf (1972)
Benzoate	*Pseudomonas pudita (arvilla) mt-2*	Ahmed and Yokota (1973)
Polychlorinated biphenyls	*Achromobacter*	Nakazawa and Focht (1973)

Bromobenzene	*Bacillium polymyxa*	Shetal and Patel (1973)
3- chlorobenzoate	*Pseudomonas*	Dorn *et al* (1974)
(+)-cis-naphthalene	*Pseudomonas pudita*	Patel and Gibson (1974)
Benzene	*Pseudomonas*	Axell and Geary (1975)
4- chlorobenzoic acid	*Arthrobacter sp.*	Rusinger *et al* (1976)
Lignin	*Phanerochaete chrysosporium*	Kirk *et al* (1976)
Halogenated ACs (2-Catechol)	*Pseudomonad*	Dorn and Knackmuss (1978)
Polychlorinated biphenyls	*Acinetobacter* and *Alcaligenes*	Furukawa *et al* (1978)
3,5- dichlorobenzoate	*Pseudomonad*	Hartmann *et al* (1979)
Ligninolytic	*Phanerochaete chrysosporium*	Fenn and Kirk (1979)
4-chlorobenzoic acid	*Pseudomonas sp.*	Klages and Lingens (1980)
Chlorierten Aromaten	*Pseudomonas*	Ballschmiter and Scholz (1980)
Naphthalenesulfonic acid	*Pseudomonads*	Brilon *et al* (1981)
2,4,5-tricholophenoxy acetic acid	*Pseudomonas ceciapa*	Kilbane *et al* (1982)
2,4,5-tricholophenoxy acetic acid	*Pseudomonas ceciapa*	Kilbane *et al* (1982)
Naphthalene	*Pseudomonas* sp. strain NCIB9816	Ensley *et al* (1982)
Kanellos (Polychlorobiphenyls)	*Acinetobacter*	Furukawa *et al* (1983)
1,2,4- trichlorobenzene	*Staphylococcus epidermidis*	Tsuchiya and Yamaha (1984)
4- chlorophenyl acetate	*Pseudomonas* strain CBS3	Markus *et al* (1984)
Polychorinated biphenyls	*Phanerochaete chrysosporium*	Eaton (1985)
1,4- dichlorobenzene	*Alcaligenes* strain A175	Schraa *et al* (1986)
Polycyclic aromatic hydrocarbons	*Phanerochaete chrysosporium*	Hammel *et al* ((1986)

Chlorinated PCBs	*Pseudomonas* strain LB400	Bopp (1986)
Polychorinated phenols	*Rhodococcus chlorophenolicus*	Apajalahti and Salonen (1987)
3- chlorobenzoate	*Pseudomonas alcaligenes*	Focht and Shelton (1987)
Dichlorobenzene	*Pseudomonas* strain P51	Meer *et al* (1987)
1,4- dichlorobenzene	*Pseudomonas*	Spain and Nishino (1987)
Glycine betaine	*Rhizobium meliloti*	Smith *et al* (1988)
Naphthalene-2,6- &1,6-disulfonic acid	*Moraxella sp.*	Wittich *et al* (1988)
1,2- dichlorobenzene	*Pseudomonas sp.*	Ha*igier et al* (1988)
Crystal violet	*Phanerochaete chrysosporium*	Bumupus and Brock (1988)
Chlorinated biphenyl and benzoic acids	*Pseudomonas sp.*	Parsons *et al* (1988)
Pantachorophenol	*Phanerochaete chrysosporium*	Mileski *et al* (1988)
2-chlorobenzoate	*Pseudomonas sp.*	Sylvestre *et al* (1989)
Dibenzofuran	*Pseudomonas* strain HH69	Fortnagel *et al* (1990)
Polycyclic Aromatic Hydrocarbons	*Mycobacterium*	Cerniglia and Heitkamp (1990)
Mono-, di- and tri-halogenated benzoic acids	*Pseudomonas aeroginosa JB2*	Hickey and Focht (1990)
Acetonitrile and biphenyl	*Pseudomonas aeroginosa*	Mokross *et al* (1991)
1,2&1,4,-dicholo- and 1,2,4,-tricholobenzene	*Pseudomonas strains* P51	Vos and Zehnder (1991)
Chlorobenzoate	*Pseudomonas pudita P111*	Hernandez *et al* (1991)
1,2,3-Tricholo- and 1,2,4,5-Tetracholobenzene	*Pseudomonas aeroginosa JB2*	Hickey and Focht (1991)
2,4,6-tricholophenol	*Pseudomonas pickettii*	Kiyohara *et al* (1992)
Fluoranthene	*Mycobacterium* sp. Strain PYR-1	Kelley *et al* (1993)
Chlorobenzoate	*Pseudomonas aeroginosa JB2* *Pseudomonas pudita P111*	Hickey *et al* (1993)
Aromatic hydrocarbons	*Pseudomonas pudita*	Ramos *et al* (1995)

Choline	*Penicillium fellutanum*	Park and Gander (1998)
Pentachlorophenol	*Pseudomonas sp.*	Lee *et al* (1998)
Polycyclic Aromatic Hydrocarbons	*Coriolopsis gallica* UAMH 8260	Pickard *et al* (1999)
Cholin-o-sulfate	*Bacillus subtilis*	Nau-Wagner *et al* (1999)
Hexadecane	*Pseudomonas aeroginosa*	Beal and Betts (2000)
Polycyclic Aromatic Hydrocarbons	*Defined fungal bacterial ciculture*	Boonchan *et al* (2000)
Alkanes	Sulfate-reducing bacterium strain Hxd3	So *et al* (2003)
Aromatic hydrocarbons	*Cyclothyrium*	De Silva *et al* (2004)
Polycyclic Aromatic Hydrocarbons	*Phytoremediation*	Cofield *et al* (2007)
Polycyclic Aromatic Hydrocarbons (Total 21)	*60 genera of bacteria & 80 genera of fungi*	Al-Turki (2009)

2.2 Pesticides degradation by microorganisms

The microbiological approach involves augmentation of a contaminated site with one or more species of contaminant-specific degrading microorganisms. The idea behind this approach is that the rate of degradation of contaminant would be appreciably enhanced because the density of the contaminant-specific degraders is increased artificially. Two methods have been used to achieve augmentation of the contaminated site with species of contaminant-specific degrading microorganisms. The first one involves the use of pre-packed, contaminant-specific degraders that have been selected because of their ability to degrade that contaminant. Such microorganisms are usually obtained from the contaminated sites by subjecting the native microflora to stress conditions of elevated concentrations of the contaminant for a long time. Various micro-flora able to degrade the pesticides represented in Table 2.4.

Table 2.4 Microorganisms responsible for pesticides degradation

Pesticide	***Microorganism***	**Reference**

Aldrin	*Soil microbial*	Tu *et al* (1968)
Benzene hexachloride	*Soil bacterium*	Sethuran *et al* (1969)
γ-BHC	*Clostridium retum*	Ohisa and Yamaguchi (1978)
2,4-D	*Alcaligenes eutrophus*	Don and Pemberton (1981)
	Alcaligenes xylosoxidans	Kilbane *et al* (1982)
	Flavobacterium sp. 50001	Bulinski and Nakatsu (1998)
	Pseudomonas putida	Gunulan and Fournieer (1993)
	Pseudomonas cepacia	Chaudhry and Huang(1988)
	Comamonas sp.	Lillis *et al* (1983)
2,4,5-T	*Pseudomonas cepacia*	Karns *et al* (1982)
DPA	*Flavobacterium* sp.	Horvath *et al* (1990)
Mecoprop	*Sphingomonas herbicidivorans MH*	Zipper *et al* (1966)
Fluoranthene	*Mycobacterium sp.* Strain PYR-1	Kelley *et al* (1993)
Atrazine	*Pseudomonas sp.*	Mandlbaume *et al* (1995)
Mecocarp	*Alcaligenes denitrificans*	Tett *et al* (1997)
DDT	*Aerobacter aerogenes*	Wedemeyer (1966)
	Alcaligenes eutrophus A5 *Agrobacterium tumefaciens*	Nadeau *et al* (1994)
	Serratia marcescens DT 1P	Bidlan and Manonmani (2002)
	Pseudomonas aeroginosa DT ct 1 and DT ct2	Bidlan (2003)
Methyl parathion	*Pseudomonas* Strain A_3	Ramanathan and Lilithakumari (1999)
Propachlor	*Pseudomonas* Strain GCH 1	Martin *et al.* (2000)
Gramoxone & Matancha	*Pseudomonas putida*	Maria *et al.* (2002)
Cypermethrin	*Sheepdip*	Grant and Betts (2003)
	Pseudomonas	Jilani and Khan (2006)
Dichlorvos	*Xanthomonas*	Ning *et al.* (2010)
Cypermethrin	*Bacillus cereus ZH-3*	Chen *et al.* (2012)

	Streptomyces aureus HP-S-01	
Triazophos	*Canna indica Linn.*	Xiao *et al.* (2010)
Chlorpyrifos	*Pseudomonas putida* (N II- 1117) & *Klebsiella sp.* (N II- 1118)	Sasikala *et al* (2012)
Endosulfan	*Phanerochaete chrysosporium*	Kullman *et al* (1996)
Endosulfan	*Phanerochaete chrysosporium*	Kim *et al* (2001)
Endosulfan	*Mucor thermo-hyalospora MTCC*	Shetty *et al* (2000)
Endosulfan	*Trichoderma harzianum*	Katayama *et al* (1993)
Endosulfan	*Anabaena sp. HB 1071*	Yan *et al* (1998)
Methyl parathion	*Microalgae and cyanobacteria*	Megharaj *et al* (1994)
2,4,6-trinitrotoluene	*Anabaena* sp.	Pavlostathis and Jackson (1999)
Atrazine	*Anabaena flos-aquae*	Abuo-Waly *et al* (1991)
Hexazinone	*Selenastrum capricornutum*	Abuo-Waly *et al* (1991)
Naphthalene	*Oscllatoria* sp. strain JCM	Cerniglia *et al* (1980)

Aldrin	*Bacillus* sp. & *Exiguobacterium Aurantiacum*	Okeke *et al*. (2002)
Lindane	*Pandoraea* sp.	Lopez *et al*. (2005)
Endosulfan	*Mycobacterium* sp. ESD	Sutherland *et al*. (2002)
Endosulfan	*Bacillus* sp.	Sethunathan *et al*. (2004)
Endosulfan	*Klebsiella pneumoniae* KE-1& KE-8	Kwon *et al*. (2005)
Endosulfan	*Aspergillus terreus* & *Cladosporium oxysporum*	Mukherjee and Mittal (2005)
Endosulfan	*Cheatosartorea stromatoides*	Siddique *et al*. (2003a)

Endosulfan	*Pseudomonas cepacia*	Hussain *et al*. (2007a)
Endosulfan	*Fusarium ventricosum*	Hussain *et al*. (2007b)
Cadusafos	*Arthrobacter* sp. & *Sphingomonas paucimobilis*	Karpouzas *et al*. (2005)
Methyl parathion	*Acinetobacter radioresistens*	Liu *et al*. (2007)
Dichlorovos,	*Nostoc muscorum* ARM221	Subramanian *et al*. (1994)
Malathion	*Fusarium oxysporum*	Kim *et al*. (2005)
Parathion	*Flavobacterium* sp.	Sprenger *et al*. (2003)
Chlorpyrifos	*Klebsiella* sp.	Ghanum *et al*. (2007)
Chlorpyrifos	*Enterobacter* sp.	Singh *et al*. (2004)
Chlorpyrifos	*Alcaligenes faecalis*	Yang *et al*. (2005)
Carbofuran	*Novosphingobium* sp.	Yan *et al*. (2007)
Carbamate Aldicarb	*Rotylenchulus reniformis*	McLean and Lawrence (2003)
Flumethrin, Permethrin	*Pseudomonas* sp.& *Serratia* sp.	Grant and Betts (2004)
Cyper-methrin	*Aspergillus niger*	Liang *et al*. (2005)
Beta-cyfluthrin	*Pseudomon asstutzeri*	Saikia *et al*. (2005)
Allethrin	*Acidomonas* sp.	Paingankar *et al*. (2005)
Pentachlorophenol	*Pseudomonas* sp.	Wolski *et al*. (2006)

Vinclozolin	*Cunninghamella elegans*	Pothuluri *et al*. (2000)
2,4-D	*Pseudomonas* sp.	Musarrat *et al*. (2000)
Alachlor	*Streptomyces capoamus, Streptomyces galbus, Streptomyces, Bikiniensis* & *Streptomyces taxa*	Sette *et al*. (2005)
Simazine	*Nocardioides* sp.	Satsuma (2006)
Atrazine	*Alcaligenes xylosoxydans* & *Providencia rustigianii*	Topp (2001)
Atrazine and Alachlor	*Nocardioides* sp.	Chirnside *et al*. (2007)
Triazine	*Rhodococcus erythropolis*TA57	Andersen *et al*. (2001)
Atrazine	*Agrobacterium tumefaciens*	Devers *et al*. (2005)
s-triazine	*Pseudomonas* sp. ADP & *Pseudamino bacter* sp.	Shapir *et al*. (2005)
s-triazine	*Ralstonia basilensis* M91-3,	Stamper *et al*. (2002)
Chlorsulfuron,	*Aspergillus niger*	Boschin *et al*. (2003)
Metsul furon-methyl	*Pseudomonas fluorescens*	Zanardini *et al*. (2002)
Dicamba	*Pseudomonas maltophilia* DI-6	Chakraborty *et al*.(2005)
Diuron	*Pseudomonas* sp.	El-Deeb, (2001)
Isoproturon	*Methylopila* sp.	El Sebai *et al*. (2004)
Isoproturon	*Aspergillus niger*	Ronhede *et al*. (2005)
Isoproturon	*Sphingomonas* sp.	Shi and Bending (2007)

Gramoxone and matancha	*Pseudomonas putida*	Kopytko *et al*. (2002)
Mefenacet	*Sphingobacterium* sp.	Ye *et al*. (2004)
Oxadiazon	*Pseudomonas fluorescens*	Garbi *et al*. (2006)
Phenylurea	*Arthrobacter globiformis* D47	Turnbull *et al*. (2001)
Trifluralin	*Bacillus* sp., *Herbaspirillum* sp., *Klebsiella* sp. & *Pseudomonas* sp.	Bellinaso *et al*., (2003)

2.3 Lindane degrading microorganisms

Lindane can be degraded by certain microorganism in particular environmental conditions. The list of such microorganisms predominantly consist of bacteria followed by fungal species. Although there are very few chances of obtaining optimal condition of these microorganisms in environment, we could deploy these organisms deliberately in the affected areas. The list of microorganisms responsible for lindane degradation are following.

Table 2.5 Microorganisms responsible for lindane degradation

S. No.	***Microorganism***	**Reference**
1.	*Anabaena* sp. Strain PCC7120	Kuritz *et al* (1997)
2.	*Rhodococcus rhodochrous*	Deeb *et al* (1999)
3.	*Sphingomonas paucimobilis UT26*	Nagata *et al* (1999)
4.	*Novosphingobium sp. strain MT1*	Tiirola *et al.* (2002)
5.	*Pseudomonas aeruginosa ITRC-5*	Chaudhary *et al* (2006)
6.	*Microbacterium sp. ITRC1*	Manickam *et al* (2006)
7.	*Sphingobium sp. MI1205*	Ito *et al* (2007)
8.	*Streptomyces sp. M7*	Benimeli *et al* (2008)
9.	*Sphingobium indicum B90A*	Raina *et al* (2008)
10.	*Pseudomonas sp. & Sphingobium indicum*	Lal *et al* (2008)
11.	*Sphingobium ummariense sp.*	Sing and Lal (2009)

12.	*Actinomycetes*	Fuentes *et al* (2010)
13.	*Sphingobium strains*	Zheng, *et al.* (2011)
14.	*Daphnia magna*	Pereira Miranda *et al* (2011)
15.	*Chlorella vulgaris*	Borodulina *et al* (2011)
16.	*Chlorella vulgaris*	Stravinskene *et al.* (2012)
17.	*Cytisus striatus*	Becerra-Castro *et al* (2013)
18.	*Cuperus sp.*	Ortega-González *et al* (2013)
19.	*Actinobacteria*	Polti *et al.* (2014)
20.	*Streptomyces strains*	Álvarez *et al* (2015)
21.	*Actinobacteria*	Álvarez *et al* (2017)

2.4 Various genes and plasmids involve in lindane degradation

Lindane can be degraded by certain microorganism due to present some genes are mention in Table 2.6 and some bacterial plasmid represented in Table 2.7.

Table 2.6 Genes responsible for lindane degradation

S. No.	**Gene**	***Microorganism***	**Accession No.**
1	lin A	*Sphingomonas*	AB549720.1
2	lin B	*Sphingobium*	FJ966202.1
3	lin B	*Xanthomonas*	DQ910544.1
4	lin B	*Sphingomonas*	DQ767899.1
5	lin B	*Sphingomonas*	GQ915275.1
6	lin B	*Sphingobium*	D14594.2
7	lin B	*Microbacterium*	DQ143989.1
8	lin B	*Sphingobium*	AB304078.1
9	lin C	*Sphingomonas*	GQ915276.1
10	lin C	*Sphingomonas*	DQ767900.1
11	lin C	*Sphingobium*	D89733.1
12	lin C	*Sphingobium*	D14595.1
13	lin C,F,B	*Sphingomonas*	AB549722.2

14	lin R	*Sphingomonas*	AB021863.1
15	lin R2	*Sphingomonas*	DQ399711.1
16	LinX	*Sphingomonas*	AY150579.1
17	LinX	*Sphingobium*	AY150579.1

Table 2.7 Bacterial plasmids responsible for lindane degradation

Plasmid	**Organism**	**Length (nt)**	**ORF**	**Accession Number**
R721	*Escherichia coli*	75582	91	NC_002525
p9123	*Escherichia coli*	6222	8	NC_005324
pC15-1a	*Escherichia coli*	92353	100	NC_005327
pCol-let	*Escherichia coli*	5847	7	NC_002487
pAPEC-O2-R	*Escherichia coli*	101375	119	NC_006671
pColK-K235	*Escherichia coli*	8318	7	NC_006881
pRK2	*Escherichia coli*	5360	6	NC__005970
pECO29	*Escherichia coli*	3895	2	NC_001537
CloDF13	*Escherichia coli*	9957	8	NC_002119
pBHRK18	*Escherichia coli*	5721	4	NC_005568
pBHRK19	*Escherichia coli*	5721	4	NC_005569
pFL129	*Escherichia coli*	6464	4	NC_005923
pAPEC-O2-ColV	*Escherichia coli*	184501	209	NC_007675
pCoo	*Escherichia coli*	98396	94	NC_007635
pB171	*Escherichia coli*	68817	80	NC_002142
pO113	*Escherichia coli*	165548	155	NC_007365
pLG13	*Escherichia coli*	6293	7	NC_005019
pIGAL1	*Escherichia coli*	8145	3	NC_005248
p1658/97	*Escherichia coli*	125491	141	NC_004998
pKL1	*Escherichia coli* KL4	1549	1	NC_002145
pO157	*Escherichia coli* O157:H7 str. Sakai	92077	184	NC_002128
pOSAK1	*Escherichia coli* O157:H7 str. Sakai	3306	3	NC_002127
pSFD10	*Salmonella choleraesuis*	4091	6	NC_003079
pOU1113	*Salmonella enterica*	80156	89	NC_007208
pC	*Salmonella enterica* serovar Enteritidis	5269	4	NC_003457
pBERT	*Salmonella enterica* subsp.*enterica* serovar Berta	4656	9	NC_001848
pKDSC50	*Salmonella enterica* subsp. *enterica* serovar Choleraesuis	49503	48	NC_002638

cryptic_plasmid	*Salmonella enterica* subsp.*enterica* serovar Choleraesuis	6066	7	NC_005862

Materials and Methods

3 Materials and Methods

3.1 Materials

3.1.1 Chemicals

Lindane (97% purity) was purchased from Sigma-Aldrich Company, USA. Commercial formulations of mixed pesticides (organophosphates and pyrethroids) viz. chloropyriphos, profenofos, cypermethrin, deltamethrin, triazophos, lambda- cyhalothrin, dichlorvos, cypermethrin were procured from Hindustan Pulverising Mills (HPM) Chemicals & Fertilizers Ltd. India, Insecticide India Ltd. and Bharat Insecticides Ltd., India. Nutrient agar, nutrient broth and antibiotic disks were purchased from HiMedia Lab Pvt. Ltd., India and Titan Biotech Ltd., India respectively. Malathion (technical grade) was obtained from Davani Crop Science, Hapur, India. Sodium dihydrogen orthophosphate, disodium hydrogen phosphate, yeast extract; beef extract, potassium chloride, calcium chloride, hydrogen peroxide, starch, tryptone, *p*-dimethyl amino benzaldehyde, iodine, gelatine, mercuric chloride, magnesium sulfate, peptone, NaCl, glucose, K_2HPO_4, EDTA, bromophenol blue, bromothymol blue, agar, nutrient agar, phenol red, crystal violet, safranin, agarose, ethidium bromide, tris base, were purchased from Siska Research Laboratory, India DNA gel loading dye (6X), 1 kb DNA ladder, and Taq polymerases were procured from HiMedia Lab Pvt. Ltd., India. Other chemicals used in this study were of standard manufacturers and purchased from authorized vendors.

3.1.2 Solvents

Acetone, cyclohexane, n-hexane, dichloromethane, N, N'- dimethylformamide, ethanol, hydrochloric acid, amyl alcohol, glacial acetic acid, immersion oil, Glycerol. All other reagents were of analytical grade and were purchased from authorized vendors.

3.1.3 Seeds

Wheat (*Triticum aestivum*) and green gram (*Vigna radiata*) forms the staple food for a large part of the population in India. Wheat being a monocot while green gram is a dicot, the seeds of these two major food crops were considered for the present studies. Seeds of wheat WR544 (Pusa Gold) and green gram (Pusa 329) were purchased from Agricultural Seed Store, Pusa complex, Delhi, India.

3.1.4 Soil and filter papers

The soil was collected from Delhi Technological University ground; most of the organic matter was first removed and then dug around one feet deep. Whatman No. 1 filter papers were used in the present study.

3.1.5 Media for growth and biochemical tests

3.1.5.1 Nutrient Agar (NA) Medium (pH 7.2 ± 0.2)

Constituents	Quantity
Beef extract	30.0 g
Peptone	5.0 g
NaCl	5.0 g
Agar-agar	20.0 g
Distilled water	1000 ml

3.1.5.2 Nutrient Broth (NB) Medium (pH 7.2 ± 0.2)

Constituents	Quantity
Beef extract	30.0 g
Peptone	5.0 g
NaCl	5.0 g
Distilled water	1000 ml

3.1.5.3 Luria Bertani (LB) Agar Medium (pH 7.2 ± 0.2)

Constituents	Quantity
Casein hydrolysate	10.0 g
Yeast extract	5.0 g
NaCl	10.0 g
Agar-agar	15.0 g
Distilled water	1000 ml

3.1.5.4 Luria Bertani (LB) Broth (pH 7.2 ± 0.2)

Constituents	Quantity
Casein hydrolysate	10.0 g
Yeast extract	5.0 g
NaCl	10.0 g
Distilled water	1000 ml

3.1.5.5 Minimal (M_4) Medium (pH 7 ± 0.2)

Constituents	Quantity
KH_2PO_4	0.675 g
Na_2HPO_4	5.455 g
NH_4PO_3	0.25 g
Distilled water	1000 ml

3.1.5.6 Czapek Dox Agar (CDA) Medium (pH 7.3 ± 0.2)

Constituents	Quantity
Sucrose	30.0 g
$NaNO_3$	2.0 g
K_2HPO_4	1.0 g
$MgSO_4$	0.5 g
KCl	0.5 g
$FeSO_4$	0.01 g
Agar-agar	15.0 g

Distilled water	1000 ml

3.1.5.7 Bacterial Motility Medium (pH 7.0)

Constituents	**Quantity**
Beef extract	30.0 g
Peptone	5.0 g
NaCl	5.0 g
Agar-agar	4.0 g
2,3,5-Triphenyl tetrazolium Chloride	0.5 g
Distilled water	1000 ml

3.1.5.8 Nitrate Reduction Medium (pH 7.0 ± 0.2)

Constituents	**Quantity**
Beef extract	30.0 g
Peptone	5.0 g
NaCl	5.0 g
KNO_3	1.0 g
Distilled water	1000 ml

3.1.5.9 Gelatin Hydrolysis Medium (pH 7.0 ± 0.2)

Constituents	**Quantity**
Gelatin	120.0 g
Beef extract	30.0 g
Peptone	5.0 g
NaCl	5.0 g
Distilled water	1000 ml

3.1.5.10 Starch Agar Medium (pH 6.9 ± 0.2)

Constituents	**Quantity**
Soluble starch	10.0 g

Beef extract	30.0 g
Peptone	5.0 g
NaCl	5.0 g
Agar-agar	20.0 g
Distilled water	1000 ml

3.1.5.11 Casein Hydrolysis Medium (pH 7.0 ± 0.2)

Constituents	**Quantity**
Pancreatic digest of casein	5.0 g
Yeast extract	2.5.0 g
Glucose	1.0 g
Agar-agar	20.0 g
Skim milk	70.0 ml
Distilled water	1000 ml

3.2 Methods

3.2.1 Sterilization

1 kg soil was sterilized by autoclaving at 121^{o}C for 40 min at 1.05 Kg/cm^{2} (115 lb psi) pressure for three times every alternate day. Distilled water, glass wares, and filter papers were sterilized by autoclaving at 121^{o}C for 20 min at 115 lb psi. Forceps, needle, petri-plates and plastic ice-cream cups were cleaned with 70% ethanol before use. The seeds were surface sterilized using liquid detergent Teepol (Ricket & Colman, India Limited Calcutta) and they were thoroughly washed under running tap water for 10 min. The seeds were treated with 1% citric acid solution, an antioxidant kept in a flask and placed on a rotary shaker (Eppendorf) for 10 min at 100 rpm and washed with distilled water. Subsequently, these seeds were surface sterilized with 0.1% (w/v) freshly prepared $HgCl_2$ solution for 5 min with constant shaking and were finally washed 4-5 times with autoclaved distilled water. All materials like autoclaved distilled water, tissue paper, lamp, *etc.* except seeds, were exposed to ultraviolet light in a laminar flow cabinet for 30 min prior to their use. The surface of laminar flow and working platform were cleaned with 70% ethanol before the start of seeds germination experiment.

3.2.2 Sample collection and preparation

Sewage and sludge were collected from the surrounding areas of the Delhi Technological University, Delhi, India and streams in Saharanpur and Lucknow Districts of India, considering the fact that the wastes/effluents are dumped into these water bodies by almost every sector. The samples were kept in cold until further processing. Samples were mixed thoroughly just before use and filtered through Whatman No.1 filter paper. The filtrate was used as the source of native microbial population for the enrichment of pesticide tolerant microbes.

Fig. 3.1 Environmental sample and enrichment of consortium

3.2.3 Enrichment of the microbial consortium

The enrichment technique was developed according to Bidlan (2003) with slight modifications. The sewage sludge filtrate was incubated at room temperature = 25 - 30 ^{o}C for 9 months with the addition of gradual increasing pesticide concentrations to it at regular intervals. This was done by initially adding peptone (1%) and 2 ppm each of commercial formulations of various pesticides (organophosphates and pyrethroids but not organochlorine) as mention in table 3.1, and then

increasing the pesticide concentrations gradually to 500 ppm by the end of 9th month. The cultures thus enriched were then isolated and screened for their potential to degrade lindane.

Table 3.1 Pesticides used for enrichment of consortium.

S. No.	Pesticide	% (EC)	Manufacturer	Volume Diluted in water	Volume added to 100 ml sample
1	Chloropyriphos	20%	Hindustan Pulverising Mills (HPM) Chemicals & Fertilizers Ltd.	100ml->1000ml	10 ml
2	Profenofos & Cypermethrin	40% 4%	-do-	50ml--> 1000ml	40 ml
3	Deltamethrin & Triazophos	1% 35%	-do-	50ml--> 1000ml	30 ml
4	Lambda-Cyhalothrin	5%	-do-	100ml--> 1000ml	10 ml
5	Dichlorvos	76%	Insecticide India Ltd.	10ml--> 1000ml	8 ml
6	Cypermethrin	25%	Bharat Insecticides Ltd.	100ml--> 1000ml	12 ml

3.2.4 Screening for lindane degrader strains

The pure cultures isolated from the enriched consortium by repeated streaking were further streaked for dense growth onto nutrient agar plates and allowed to grow for 24 h. These plates were then sprayed with 0.5% lindane (in acetone) under aseptic conditions. The acetone was allowed to evaporate leaving a thin lindane film behind. The plates were incubated for another 48 hour (h) at RT and observed for the zone of clearance in the lindane film due to degradation by the respective cultures. The positive strains were further screened and investigated for lindane degradation individual or in various combinations.

3.2.5 Inoculum

The consortium was inoculated with nutrient broth and incubated for 24 h at RT. 5 ppm of lindane was added to the growing culture and was incubated further for 72 h at RT. This was harvested by centrifugation, washed twice in minimal medium and further induced with 5 ppm lindane in minimal medium for 72 h at RT. This induced culture was harvested by centrifugation, washed twice in minimal medium and was used as inoculum for degradation studies.

3.2.6 Lindane degradation by the consortium

The mixed population obtained as an enriched culture from the 9 month enrichment was screened for lindane degradation in broth. 5 ml minimal medium was placed in test tubes and autoClaved for 20 minutes at 121 ^{o}C. After cooling to RT, lindane (as dimethylformamide solution) was added to the medium to a final concentration of 5 ppm through 30 ppm, separately in triplicate sets. The induced consortium was inoculated at 0.01 OD600, mixed well and incubated at RT for various time periods. Whole tube samples (in triplicates) were collected at the predetermined periods for estimating growth and residual lindane. Growth was estimated by reading OD_{600} against the minimal medium and residual lindane was estimated after extraction and performing thin layer chromatography (TLC) (sec. 2.11). Tubes with minimal medium and lindane were taken as controls for each concentration and each incubation period.

3.2.7 Effect of temperature on the degradation of lindane

The induced consortium was inoculated to 20 ppm lindane on minimal medium (in triplicates) maintained and incubated at different temperatures 10 ^{o}C to 50 ^{o}C for 0, 3 and 6 days. The growth and degradation of the added 20 ppm lindane were analyzed for optimum temperature.

3.2.8 Effect of pH on degradation of lindane

Different pH was used for studying the degradation of lindane by the induced consortium. 0.1M phosphate buffers with pH 5, 6, 7 and 8 were prepared. 5 ml of each buffer was placed separately in triplicate test tubes and 10 ppm lindane (in DMF) was added. The induced consortium was inoculated to these tubes and incubated at RT (25 - 30 ^{o}C) on a rotary shaker with 110 rpm. Controls for each pH and sampling periods were also placed without inoculating the consortium. The residual lindane and the growth were analyzed for optimum pH.

3.2.9 Parameters and their levels for RSM

Response surface method (RSM) is an example of central composite rotatory design (CCRD) experiment. A central composite rotatory design was used to investigate the effect of five independent variables on the degradation of lindane and growth of the bacterial consortium. Total 32 experiments were performed with 6 centre and 10 axial points, represented in Fig 3.2.

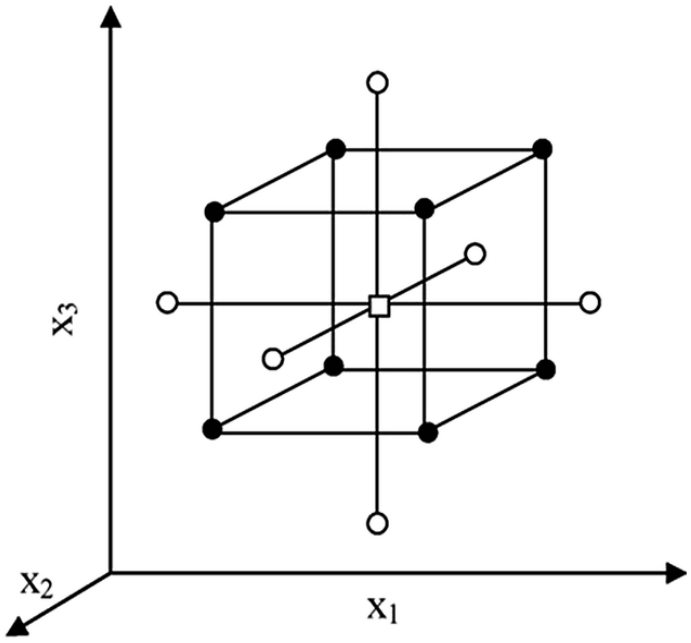


Fig. 3.2 Centre and axial points of CCRD experiment

Equation – Modelling:

$$Y = \beta_o + \sum_{i=1}^{5} \beta_i X_i + \sum_{i=1} \beta_{ii} X_i^2 + \sum \sum_{\substack{i=1 \\ j=i+1}} \beta_{ij} X_i X_j$$

Y = Response (Degradation)
β_0= Constant co-efficient (intercept)
β_i= Linear Co-efficient
β_{ii}= Quadratic co-efficient
β_{ij}= Second order interaction co-efficient
X = Variable investigated

Table 3.2 Parameters and their levels for RSM.

Factor	Variable Name	Units	Minimum (-2)	Maximum (2)	Coded Values		Mean (0)
					-1	1	
A	Ini Lin Conc	ppm	5	30	11.25	23.75	17.5
B	Ini Inoculum	OD_{600}	0.01	0.1	0.0325	0.0775	0.055
C	pH		4	8	5	7	6
D	Temp	deg C	10	50	20	40	30
E	Incubation Time	Days	0	40	10	30	20

3.2.10 Extraction of lindane

Residual lindane was extracted using dichloromethane as solvent. The samples were first acidified by adding 2-3 drops of concentrated HCl immediately after the sampling. The acidified sample was transferred into a glass separating funnel and extracted twice with equal volume of dichloromethane. The organic layer, carrying lindane, was passed through anhydrous sodium sulfate after standing for 5 minutes to separate the two layers. The organic layers for each sample were pooled and allowed to evaporate at RT, followed by dissolving in acetone before transferring to a microcentrifuge tube. The acetone was evaporated. The residual lindane was redissolved in a known volume of acetone for analysis by TLC.

3.2.11 Germination test on soil

First of all, 100 g of sterilized soil was filled in all the plastic cups with 8.0 cm diameter. Different concentrations from 2 ppm to 100 ppm from the stock of pesticide were mixed thoroughly on the

plastic sheet along with control. Pesticide stock was prepared in organic solvent i.e. acetone. The sides and bottom of plastic ice-cream cups were punched with a needle to facilitate air and also help in removing extra water if inside. 10 seeds per cups were propagated at 1 cm depth at equal distance with help of forceps. After the seeds propagated, 10 ml of sterile distilled water was dispensed with a pipette for moisture. 10 plastic cups of each concentration were set aside at room temperature (28 ± 2 ^{0}C) in an air-conditioned room under natural light. 2 ml sterile distilled water was added with pipette in each cup every day at evening time to maintain water content. After five days, the experiments were terminated and various growth parameters of seedling such as morphogenesis, rhizogenesis and leaf surface area etc. were measured and calculated.

3.2.12 Germination test on filter paper

Sterile Whatman No. 1 filter papers, 10 cm round shape were placed on sterile petri-discs, 9 cm in diameter and covered by sterile petri-plate (10 cm diameter). The filter papers were moistened with sterile distilled water and then different concentrations (2 ppm to 100 ppm) from the stock of pesticide along with control (without pesticide) were added. 10 seeds were placed on filter paper at equal distances. 1ml sterile distilled water was added to each petri-disc every day in the morning to maintain moisture. Most of the germination conditions were same to the soil experiment. The seedling was observed and measured after five days of the experiment.

3.3 Analytical

3.3.1 Growth of seedling

Observations were recorded every day. The final data was recorded after 6 days of sowing of seeds. After recording the final data, the *in vivo* raised shoots were excised and preserved at 4 ^{0}C for further work like photosynthesis pigments and enzyme study. The data were recorded in terms of (i) Percentage of seed germination, (ii) Average shoot length, (iii) Average root length, (iv) Average number of roots per shoot, and (v) Average number of leavse per shoot.

3.3.2 Photosynthetic pigments

Chlorophyll pigments were estimated by using slightly modified Holden protocol (1960). 0.2g of plant samples from each concentration were weighed and homogenized individually using mortar-

pestle in the presence of an excess of acetone: ethanol: water (60 %: 20 %: 20%) until all the color was released from the tissue. Few amounts of $CaCO_3$ was added to prevent pheophytin formation and this was then centrifuged at 5000 rpm for 10 minutes at RT. The clear supernatant was collected and then made up to a known volume (10 ml). The test tubes were wrapped with black paper to protect chlorophyll degradation. The Bio-spectrophotometer (Eppendorf) was adjusted at a wavelength of 663 nm, 645 nm, 665 nm, 434 nm, 480 nm, 486 nm for chlorophyll a, chlorophyll b, pheophytin 'a', pheophytin 'b', carotenoid, and xanthophyll, respectively. The optical density was measured and the pigment contents in the original extract were estimated using the standard formula.

3.3.3 Thin layer chromatography

The residual lindane was estimated by using thin layer chromatography on silica gel G coated plates. The 20 x 20 cm 2 plates were prepared by spreading a uniform layer of 0.25 mm thickness silica gel G using a spreader. The plates were allowed to dry at RT and activated at 105 ^{o}C for one hour. A predetermined volume of the extracted lindane (in acetone) was spotted and the plates were developed in cyclohexane: n-hexane (4:1) solvent system as mobile phase till the solvent front reached 2/3 height of the plate. The plates were removed, and the solvent allowed to evaporate at RT. Lindane spots were detected by spraying 2% o-tolidine (in acetone) and exposing the plates to bright sunlight. The spots, peacock green in color, were delineated using a needle and the intensity of spots were observed and the spot area was calculated with help of standard graph. The square root of the area under each spot was linearly related to the logarithm of lindane concentration. The known amounts of lindane were spotted as standards for generating the reference curve.

3.3.4 Gas chromatography

The residual lindane was confirmed and quantified by injecting known volume of appropriately diluted sample- extract dissolved in acetone into the gas chromatography (Shimadzu AQC - 2010) equipped with flame ionization detector and SS column RXi-5 Sil MS with internal diameter of 0.25 mm. Helium was used as carrier gas. The conditions were spilt injection mode with temperature 260 C, pressure 15.6 kPa with total flow rate 16.3 mL/min. The detection limit of lindane by GC is 2 pg and above under the particular set of condition. The retention time of lindane was 13.097 min.

3.3.5 Statistical analysis

All the experiments have been repeated thrice with either triplicates (degradation) or replicates on nine (germination). The results were tabulated by taking the average of the replicates by considering the standard deviation. For response surface methodology, ANOVA was performed using Design of Experiments ver. 10.

Table 3.3 Retention time with area of lindane.

Peak#	Ret.Time	Area	Area%
1	13.097	241665	100.0000
Total		241665	100.0000

Fig. 3.3 GC (Shimadzu AQC - 2010)

3.4 Identification of bacterial strains

3.4.1 Morphological identification of bacterial strains

3.4.1.1 Gram staining of bacterial strains

The Gram stain procedure was originally developed by the Danish physician Hans Christian Gram to differentiate eubacteria into two fundamental groups according to their stainability, ie Gram-positive bacteria and Gram-negative bacteria. Gram staining consists of four components:

• Primary stain (crystal violet)

- Mordant (Gram's iodine)
- Decolourizer (ethyl alcohol)
- Counter stain (safranin)

3.4.1.2 Motility test of bacterial strains

The motility of bacterial strains were studied using motility medium in culture vials supplemented with 0.5% of 2,3,5-triphenyl tetrazolium chloride. These culture vials were inoculated by stabbing a loopful of culture in the center at greater than half of the depth of tube and incubated at 25°C for 48 h. The occurrence of the red turbid area extending from the line of inoculation indicated the motility of bacterial cells.

3.4.2 Biochemical characterization

The biochemical characterizations of isolated antagonistic bacterial strain were carried out using the following parameters/tests.

3.4.2.1 Catalase test

The catalase activity of bacterial strains were determined by flooding the 24 h old broth culture on 1.0 ml of 10% hydrogen peroxide. In this reaction, the formation of gas bubble indicated the presence of catalase activity and non-formation of gas bubble indicated the absence of catalase activity.

3.4.2.2 Oxidase test

To determine the oxidase activity of four bacterial strains, its loopful sample (24 h old bacterial culture) was rubbed on a tiny piece of filter paper treated with Kova'cs oxidase reagent (1% tetramethyl-phenylenediamine) was observed the changing in color.

3.4.2.3 Nitrate reduction test

The nitrate reduction activity of bacterial strains were determined by a loopful of bacterial culture was inoculated in nutrient broth added with 0.1% potassium phosphate and incubated at 25°C for 48 h at 120 rpm. After incubation, 10 ml of broth culture was flooded with 1.0 ml of reagent A and reagent B and after that observed the appearance of pink color, indicating positive nitrate reduction test. If it remained colorless, then a small amount of zinc powder was added and mixed. After 10-15 minute at room temperature, turning of broth culture to pink indicated a negative result.

Reagent A: 1% Sulphanilamide in 3N HCl

Sulphanilamide	1.0 g
3 N HCl	100 ml

Reagent B: 0.02% N (1-Naphthyl) ethylene-daimine dihydrochloride (NEDD)

NEDD	0.02 g
Distilled water	100 ml

3.4.2.4 Gelatin hydrolysis test

Nutrient broth (0.8%) with 12% gelatin solution and 10 ml of the medium was poured into culture vials and then autoclaved. After autoclaving, these culture vials were inoculated with loopful bacterial culture and incubated at RT for 48 h. After the complete incubation period, the culture vials were kept at 4°C along with control vial and examined on the basis of solidification and liquefaction of medium. If culture vials remained liquid, it indicates that bacterial cells hydrolyzed gelatin.

3.4.2.5 Starch hydrolysis test

The bacterial culture was point inoculated in the growth medium containing nutrient agar added with 1% soluble starch and incubated at RT for 48 h. After the complete incubation period, the plates were flooded with Lugol's iodine solution to observe zone of hydrolysis.

Lugol's iodine solution

Iodine	1.0 g
Potassium iodine	3.0 g
Distilled water	300 ml

The Lugol's iodine solution was filtered and stored in a brown bottle.

3.4.2.6 Casein hydrolysis test

Protease activity was checked in the medium using 1% casein as a substrate and 7% skim milk as an inducer. The plate was point inoculated by antagonistic bacterial strain and incubated at 28°C for 96 h and checked for the formation of halo zone around the bacterial colony.

3.4.2.7 Phosphate solubilization test

For the phosphate solubilization test, Pikovskaya's agar medium containing tricalcium phosphate (Pikovskaya, 1948) as a phosphate source was used. The antagonistic bacterium was point inoculated and incubated at 28°C for 96 h and checked for the formation of halo zone around the bacterial colony.

A primary stain (malachite green) was used to stain the endospores because endospores have a keratin covering and resist staining, the malachite green will be forced into the endospores by heating. In this technique, heating acts as a mordant. Water was used to decolorize the cells. As the endospores are resistant to staining, the endospores are equally resistant to de-staining and will retain the primary dye while the vegetative cells will lose the stain. A counterstain or secondary stain (safranin) was used to stain the decolorized vegetative cells. When visualized under the microscope, the cell should have three characteristics: the vegetative cells should appear pink, the vegetative cells that contain endospores should stain pink, while the spore should be seen as green ellipses within the cells. Mature, free endospore should not be associated with the vegetative bacteria and should be seen as green ellipses.

3.4.3 Antibiotic sensitivity of bacterial strains

Seven antibiotics were used Viz. Kanamycin (5 μg/disc; K-5), Norfloxacin (5 μg/disc; Nx-5), Streptomycin (10 μg/disc; S-10), Rifampicin (5 μg/disc; Rif-5), Penicillin-G (10 μg/disc; P-10), Cefixime (5 μg/disc; Cfm-5) and Nalidixic acid (30 μg/disc; Na-30). These antibiotic disks were placed on the surface of nutrient agar which has already been inoculated overnight with pure bacterial strain

3.4.4 Effect of different carbon sources on bacterial growth and degradation of lindane

Four different strains were cultured in different carbon sources (glucose, peptone, tween-80 and Triton X-100 to observe growth and degradation of the initial concentration of lindane (20 ppm) on minimal medium (3.1.4.5). After incubating at 30°C for 0 h to 120 h, optical density and residual lindane (%) were measured and calculated at 600 nm and by TLC & GC, respectively.

3.4.4.1 Stock solution of glucose

Glucose solution was prepared in 100 ml distilled water by 2 gram of glucose; the solution was autoclaved at 15 lbs, 121 °C for 20 minutes.

3.4.4.2 Stock solution of peptone

Peptone solution was prepared in 100 ml distilled water by 2 gram of peptone; the solution was autoclaved at 15 lbs, 121 °C for 20 minutes.

3.4.4.3 Stock solution of Tween-80

Tween-80 solution was prepared in 100 ml distilled water with 0.1 ml (v/v) of Tween-80. The solution was autoclaved at 15 lbs, 121 °C for 20 minutes.

3.4.4.4 Stock solution of Triton X-100

Triton X-100 solution was prepared in 100 ml distilled water with 0.1 ml (v/v) of Triton X-100. The solution was autoclaved at 15 lbs, 121 °C for 20 minutes.

3.5 Molecular characterization of bacterial strains

3.5.1 Genomic DNA isolation of bacterial strains

1. The bacterial cultures that were screened positive for degradation of lindane was designated and streaked in nutrient agar plates to obtain single colonies.
2. Plates were incubated for 24 hours at room temperature (30°C). Later, in the sterile conditions of laminar airflow hood, 180 µl of buffer ATL from the QIAamp DNA mini kit

was taken in 1.5 ml microcentrifuge tubes and used to suspend single bacterial colonies picked from culture plates using a sterile loop.

3. 20 µl of proteinase-K was added to the tubes and the mixture was vortexes thoroughly for 4-5 minutes followed by incubation at 56°C in water bath for 2 hours with the occasional vortex. Now, add 4 µl RNase A into the tubes, mix by pulse-overtaxing for 15 seconds and incubate for 2 min at room temperature to cleave RNA into smaller fragments.
4. Add 200 µl Buffer AL to the sample and again mix by pulse-vortexing for 15 seconds followed by incubation at 70°C for 10 min. Add 200 µl of ethanol to the tubes, and mix by pulse-vortexing for 15 seconds.
5. Mixture from the tubes was transferred to the QIAamp mini spin column and centrifuged at 6000 x g (8000 rpm) for 2 min.
6. The filtrate was discarded with the collection tubes and the columns were placed in clean collection tubes. Again, centrifugation at 6000 x g (8000 rpm) for 2 min after addition of 500 µl Buffer AW1 into the column.
7. Filtrate was discarded with the collection tubes and the columns were placed in clean collection tubes. 500 µl of Buffer AW2 was added into the column without wetting the rim and centrifuged at 20,000 x g; 14,000 rpm for 4 min. This time collection tubes with filtrate were discarded but the QIAamp Mini spin columns were placed in clean 1.5 ml microcentrifuge tube.
8. QIAamp Mini spin column was carefully opened to 50 µl Buffer AE followed by incubation at room temperature for 1 min. Later, columns were again centrifuged at 6000 x g (8000 rpm) for 2 min to elute DNA into the microcentrifuge tube. DNA samples were stored at -20°C in the deep freezer.

Fig. 3.4 QIAmp DNA mini kit used to isolate bacterial DNA

3.5.2 DNA quantification

For quantification of DNA in the samples extracted from the bacterial culture using Synergy H1 hybrid multi-mode microplate reader 2 µl sample of Buffer AE was loaded on the microplate to assign Blanks that illustrates the background contributed by the instrument and labware. Later, 2 µl of DNA samples were loaded in separate wells on the microplate and plate was placed on the microplate carrier. Wells with the samples were selected in Gen 5 (Microplate Data Collection & Analysis Software) and samples were analyzed. The ratio of absorbance was (260 and 280).

For quantification of DNA using Thermo Scientific Nanodrop 1000 spectrophotometer, 2 µl of MQ water was loaded onto the lower sample pedestal and OD was measured for blank. Then, DNA samples were loaded on the lower pedestal after wiping both, the upper and the lower, pedestal with clean tissue paper. The ratio of absorbance was at 260 and 280, to determine the concentration of DNA in the samples. On completion of sample measurement, OD of MQ water was measured to ensure that the sample pedestals are clean.

3.5.3 Electrophoresis

1. 10 ml of 10X TAE buffer containing {4.84 gm of Tris base, 1.14 of glacial acetic acid (17.4 M), 0.37 gm of EDTA, disodium salt} was diluted with 90 ml of distilled water to make

1X TAE buffer.100ml of 1% agarose gel solution was prepared by adding 1gm agarose in 80ml of 1X TAE buffer and making up the volume to 100ml.

2. Agarose gel solution was heated up to 80 °C to dissolve agarose. The solution was allowed to cool up to 50-55°C and 2 µl of ethidium bromide (10 mg/ml) was added.
3. The solution was poured slowly into the gel tray with the well comb fitted in place and left undisturbed to polymerize. 30 ml of 10X TAE buffer containing {1.452gm of Tris base, 0.342 of glacial acetic acid (17.4 M), 0.111 gm of EDTA, disodium salt} was diluted with 270ml of distilled water to make 1X TAE buffer.
4. 300 ml of 1X TAE buffer was poured into the chamber to fill it after carefully removing well comb. 10µl of DNA samples were mixed with 2µl of loading dye in the ratio of 5:1 and loaded into the wells.
5. 1 kb DNA ladder was also loaded in one of the wells. Electrophoresis chamber was connected to the power pack with a negative terminal connected to the end closer to the wells and positive terminal to the opposite side at and voltage of 150V was applied across the ends.
6. After 2 hours when tracking dye traveled 3 quarter distance through the gel, power pack was disconnected.
7. The gel was removed carefully from the electrophoresis chamber and place under UV transilluminator to visualize it, lower, pedestal with clean tissue paper.

Fig. 3.5 Electrophoresis unit with agarose gel

3.5.4 Polymerase chain reaction (PCR)

To perform several parallel reactions, prepare a master mix containing water, buffer, dNTPs, primers and Taq DNA Polymerase in a single tube, which can then be aliquoted into individual tubes. $MgCl_2$ and template DNA solutions are then added. This method of setting reactions minimizes the possibility of pipetting errors and saves time by reducing the number of reagent transfers.

- Gently vortex and briefly centrifuge all solutions after thawing.
- Add, in a thin-walled PCR tube, on ice. Gently vortex the sample and briefly centrifuge to collect all drops from walls of tube.
- Overlay the sample with half volume of mineral oil or add an appropriate amount of wax. This step is omitted if the thermal cycler is equipped with a heated lid.
- Place samples in a thermocycler and start PCR.

PCR for each DNA sample was performed in triplicate in 0.5 mL microfuge ml PCR tubes and 5 µl DNA was used as template. Forward primer 8F with sequence 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer 928R with sequence 5'-CCCTCAATTCCTTTGAGTT-3' was used to amplify the 16S rDNA region.

PCR master mix was made by adding the following.

17.5 µl (2.5 µl X 7) of 10X buffer {160 mM $(NH_4)_2SO_4$, 500 mM Tris HCl (pH 9.2 at 22°C) 17.5 mM $MgCl_2$ and 0.1% triton X-100}, 14 µl (2 µl X 7) of 50 mM $MgCl_2$, 3.5 µl (0.5 µl X 7) of dNTP mix, 3.5 µl (0.5 µl X 7) of forward primer, 3.5 µl (0.5 µl X 7) of reverse primer, 2 µl (~ 0.17 X 7) of taq polymerase (3 U/µl), 98 µl (14 µl X 7) of distilled water. 25µl of master mix is distributed in each PCR tube.

Applied Biosystems® Veriti® 96-well thermal cycler was used to maintain PCR cycle with a denaturation temperature of 95°C for 45 sec, the annealing temperature of 55°C for 45 sec and elongation temperature of 72°C for 1 min. Total 30 cycles were performed by thermal cycler and samples were stored at 4°C after completion of cycles. PCR product was again amplified by PCR to attain higher DNA concentration and stored at -20° C.

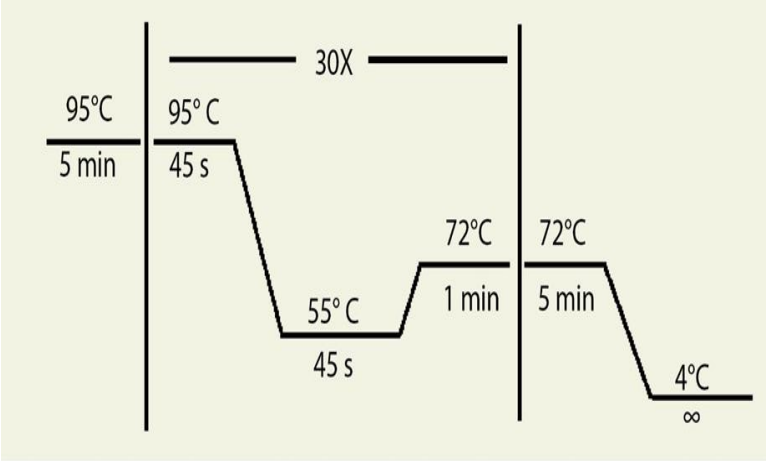


Fig. 3.6 PCR program to amplify 16S rDNA region

Fig. 3.7 Applied Biosystems Veriti 96-well thermal cycler

3.5.5 PCR product purification

1 volume of DNA samples obtained after PCR was mixed with 2 volumes of Buffer NT from NucleoSpin® gel extraction and PCR cleanup kit in the microcentrifuge tubes. The mixture was added into a NucleoSpin® Extract II Columns were placed into the collection tubes and each sample was added to a different column. Columns were centrifuged for 2 min at 11,000 x g and the filtrate was discarded. Then, silica membrane was washed with 700 µl of buffer NT3 by centrifuging for 2 minutes. The filtrate was again discarded and silica membrane was dried by centrifuging at for 3 minutes. Later, DNA was eluted from the columns into a new 1.5 ml microcentrifuge tubes by placing column into the tube, adding 50 µl of buffer NE and centrifuging at 11,000 x g for 2 minutes.

Fig. 3.8 Nucleo-spin gel and PCR clean-up kit used to purification of PCR product

3.5.6 Sequencing and analysis of amplified 16s rDNA

Purified PCR product of DNA samples was sent to Central Instrumentation Facilities (CIF), South Campus, University of Delhi, Delhi, India for sequencing. Sequencing was performed by Sanger's chain termination method using Applied Biosystems® Genetic Analyzers 3130. Each sample was sequenced by two reactions i.e. forward and reverse. Obtained result of 16S rDNA sequence was aligned against NCBI database using BLAST-N.

4 Results and Discussion

4.1 Effect of malathion on seeds germination

4.1.1 Effect of malathion on seeds germination of wheat

4.1.1.1 Effect of malathion on seeds germination of rheat in soil

The effect of malathion at different levels (2 ppm to 100 ppm) on the germination and growth of wheat seeds in soil was observed. The inhibitory effects towards seed germination and growth of seedling gradually increased with increase in the concentration of malathion. A maximum of 97.5% seeds was germinated with an average of 24.06 cm seedling length on 2 ppm (Table 4.1). However, on control, the percentage germination was less compared to the seeds grown at 2 ppm. Interestingly, the low toxicity towards seed germination was observed in the higher concentration of malathion (50 &100 ppm). A low percentage of seed germination and survival was also observed at 100 ppm (Table 4.1, Fig 4.1). We observed that the malathion is less toxic in higher concentration as compared to organ-chlorine pesticides i.e. Lindane and DDT, because at lower concentrations most of seedlings survived and germination was around 64%.

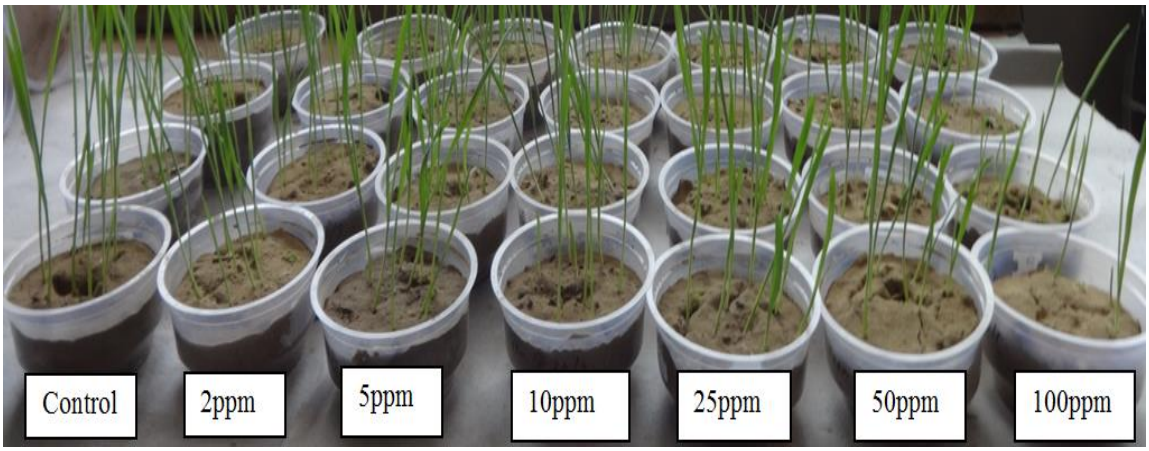


Fig. 4.1 Seeds germinated at different concentrations of malathion after 6 days

Table 4.1 Average values of the effect of malathion on the germination of wheat seeds in the soil

Sample	Germination (%)	Shoot length (cm)	Root length (cm)	Roots per shoot
Control	97	13.41±0.55	11.98±0.60	5.74±0.084
2ppm	97.5	12.81±0.40	11.25±0.40	5.18±0.65
5ppm	96	11.88±0.70	11.11±0.80	4.83±0.55
10ppm	96	9.15±0.30	9.70±0.74	4.66±0.63
25ppm	96.5	9.00±0.20	9.45±0.90	4.35±0.37
50ppm	90.8	6.87±0.35	6.85±0.28	3.85±0.45
100ppm	64	5.08±0.60	0575±0.82	3.75±0.58

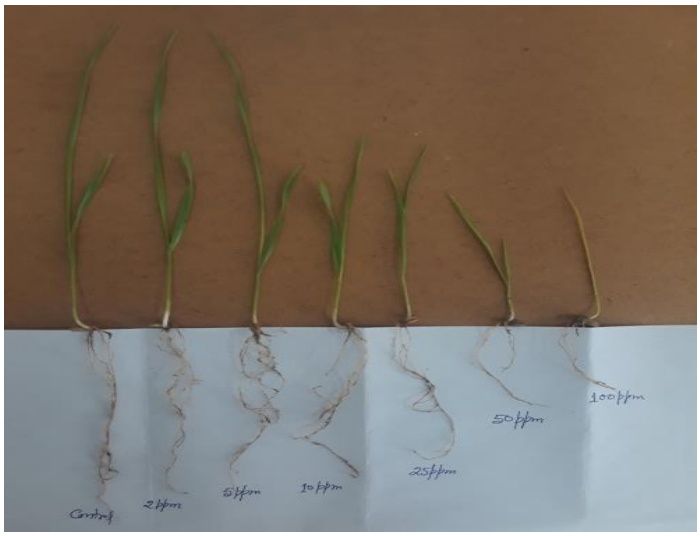


Fig. 4.2 Seedling at different concentrations (2 ppm to 100 ppm) of malathion after 6 days

4.1.1.2 Effect of malathion on seeds germination of wheat on filter paper

The wheat seeds were grown on filter paper supplemented with 2, 5, 25, 50 and 100 ppm malathion. 100% germination was observed with an average shoots length 10.41±0.7 per seedling with maximum roots per shoot. It was also observed that the percentage germination and shoot lengths gradually increased at lower concentrations (5 ppm & 10 ppm) as compared to the controls (Fig. 4.3, Table 4.2). The morphogenic response in term of shoot length and percentage germination decreased with an increasing concentration (25 ppm to 100 ppm) of malathion. At a

higher level of malathion (100 ppm) proved toxic as seen having a single leaf seedling with small and thick roots. However, seedling could survive on this concentration (100 ppm). The morphogenic response was variable at different concentrations (Table 4.2; Fig. 4.3).

Table 4.2 Average values of the effect of malathion on the germination of wheat seeds on filter paper

Sample	Germination (%)	Shoot length (cm)	Root length (cm)	Roots per shoot
Control	98	10.39±0.60	6.67±0.44	5.21±0.80
2ppm	100	10.41±0.70	6.25±0.40	5.40±0.65
5ppm	98.5	11.80±0.40	5.81±0.66	5.20±0.50
10ppm	99	12.68±0.55	4.70±0.70	4.66±0.60
25ppm	96	8.87±0.20	4.25±0.90	4.35±0.30
50ppm	88.2	7.87±0.30	4.85±0.48	4.25±0.40
100ppm	80	5.40±0.85	3.75±0.22	4.75±0.50

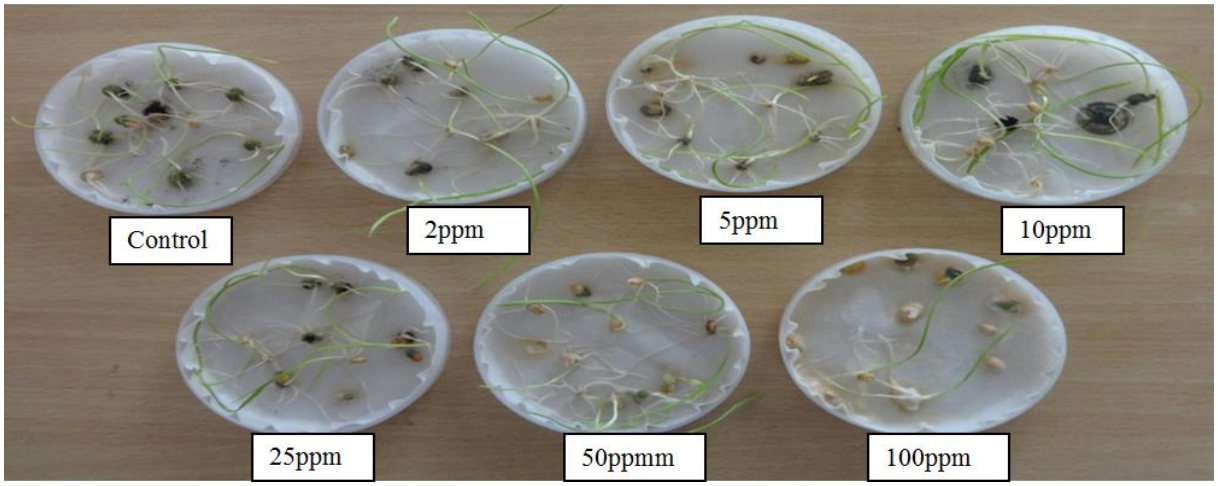


Fig. 4.3 Seed germination on filter paper observed after 6 days at different concentrations of malathion

4.1.1.3 Estimation of photosynthetic pigments in wheat leaf

In the present investigation, 6 days old seedlings, which were cultivated by soil method was evaluated qualitatively and quantitatively for the analysis of various photosynthetic pigments,

total soluble chlorophyll and carotenoid. The observed values of various photosynthetic pigments are presented in Table 4.3. As we know chlorophyll is the most valuable primary metabolite of the plant that is the center point for capturing the sunlight and responsible for photosynthesis. Amount of various photosynthetic pigments were estimated, the maximum amount of total chlorophyll was found in control (11.58 mg/g), and a minimum of 100 ppm sample (2.16 mg/g). The effects of malathion on the amount of total chlorophyll and carotenoid gradually decrease with increase in concentration (Table 4.3).

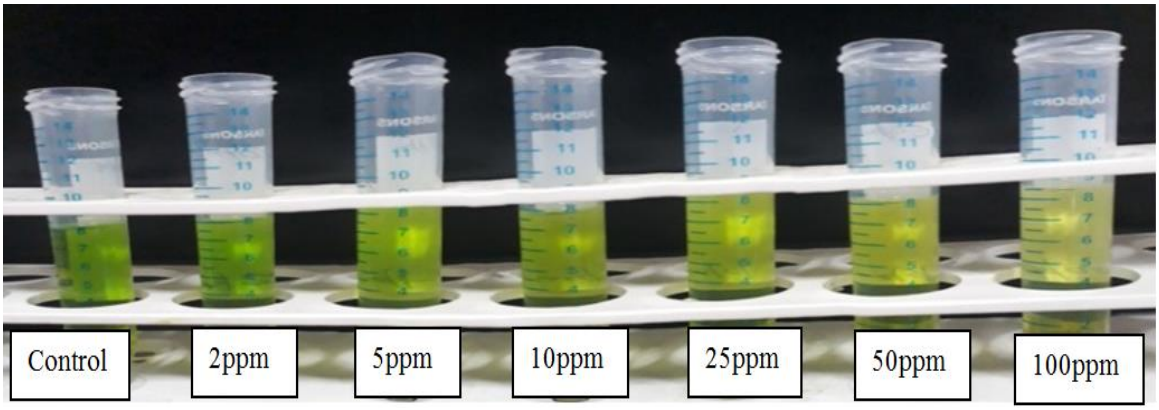


Fig. 4.4 Intensity of photosynthetic pigments decreases with increasing concentration

Table 4.3 Average absorbance values of wheat leaf extract for photosynthetic pigments

Sample	663 nm	645 nm	665 nm	434 nm	480 nm	486 nm
Control	0.724	0.286	0.734	1.318	0.529	0.353
2ppm	0.648	0.226	0.660	1.221	0.464	0.290
5ppm	0.531	0.209	0.540	0.967	0.386	0.265
10ppm	0.475	0.167	0.479	0.875	0.312	0.188
25ppm	0.297	0.111	0.302	0.632	0.232	0.138
50ppm	0.216	0. 075	0.202	0.510	0.209	0.124
100ppm	0.126	0. 057	0.123	0.300	0.149	0.096

Table 4.4 Average content of various photosynthetic pigments in wheat leaf.

Sample	Chlorophyll a (mg/l)	Chlorophyll b (mg/l)	T. Chlorophyll (mg/l)	Carotenoid (mg/l)
Control	8.420	3.160	11.580	0.211
2ppm	7.620	2.140	9.760	0.186

5ppm	6.180	2.300	8.480	0.154
10ppm	5.580	1.610	7.190	0.125
25ppm	3.470	1.150	4.620	0.093
50ppm	2.540	0.670	3.210	0.840
100ppm	1.450	0.710	2.160	0.600

The TLC for photosynthetic pigments was studied by using fresh and frozen leaf samples. The analysis of the various photosynthetic pigments on TLC plates showed the presence of xanthophyll, chlorophyll a chlorophyll b and carotenoid (Fig. 4.5 & 4.6) but no spot of pheophytin was observed on TLC plate for frozen sample (Fig. 4.6), while pheophytin indicating very poor in samples were prepared by fresh leaf (Fig. 4.5), Pigments pheophytin a and b are observed at R_f values consistent with literature reports in addition to the corresponding chlorophyll a and b when fresh or frozen leaves were used to prepare extract. The demetalation of chlorophyll using strong acids has been previously described (Talreja 2011). The elution order (R_f) using the elution solvent system was carotenoid (R_f = 0.96), chlorophyll a (R_f = 0.89), chlorophyll b (R_f = 0.83), and xanthophyll (R_f = 0.17).

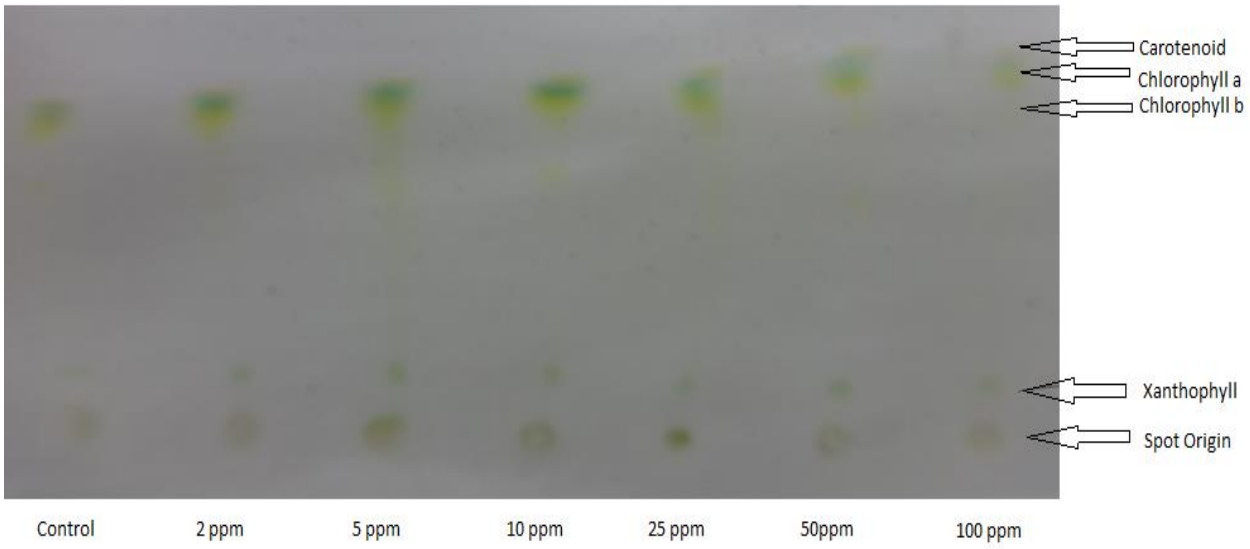


Fig. 4.5 TLC analysis of photosynthetic pigments using the fresh green leaf of wheat

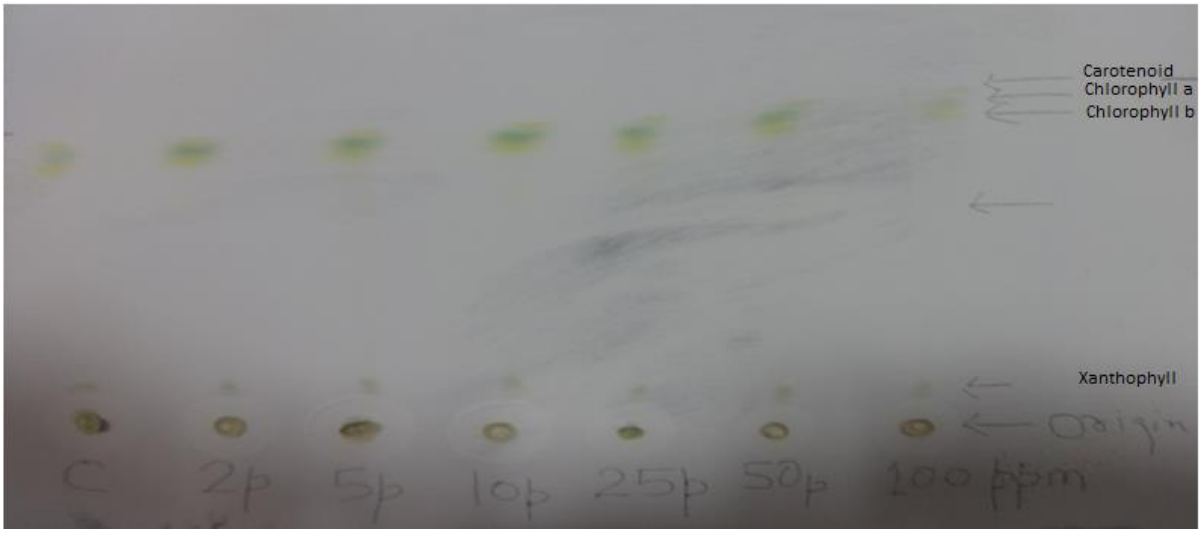


Fig. 4.6 TLC analysis of photosynthetic pigments using the frozen green leaf of wheat

On the basis of our result, we found that the low concentrations of malathion promoted the germination and growth of seedling, while photosynthetic pigments were adversely affected. Similarly low concentration (1 ppm) has been reported by reduce chlorophyll content (Singh *et al*., 2006, Mersal, 2011). Malathion inhibits the production of adenosine triphosphate (ATP) due to their effect on the photophosphorylation in the light reaction. Similar results were obtained by Gafer *et al.* (2013) on some other vegetables using the same pesticide and doses. Also, pesticides affect crop growth and reduce its yield and quality due to their phytotoxicity and also due to their effects on soil fertility and salinity (Mostafa, 1972 & Lan, *et al*., 2006).

4.1.2 Effect of malathion on seeds germination of green gram

4.1.2.1 Effect of malathion on seed germination of green gram in soil

The effect of malathion was observed at different concentrations (5 ppm to 400 ppm) on the germination and growth of seedling in soil. A maximum of 99.8% seeds germinated with an average shoot length 22.66±0.38 cm and root length 12.00±0.52 cm on 5 ppm (Table 4.4, Figs 4.7 & .8). However, in control, the percentage germination of seeds was less compared to the seeds grown on 5 ppm (Table 4.4, Fig 4.7). The inhibitory effects towards seed germination and growth of seedling were seen at higher concentration (400 ppm) of malathion Interestingly, the low toxicity towards seed germination and growth of seedlings was observed in higher

concentrations (200 & 400 ppm) of malathion as compared to organochlorine pesticides i.e. Lindane and DDT. We also observed that toxicity of malathion a varied from crop to crop, in case of Wheat 100 ppm was toxic concentration (Kumar and Sharma, 2017 & Bidlan *et al.*, 2017)

Table 4.5 Average values of seedling of green gram seeds tested in the soil

Sample	Germination (%)	Shoot length (cm)	Root length (cm)	Roots per shoot
Control	99.20	20.44±0.62	11.33±0.54	18.22±0.055
5 ppm	99.80	22.66±0.38	12.00±0.52	23.77±0.56
25 ppm	98.55	16.80±0.65	7.70±0.78	22.30±0.46
50 ppm	97.20	20.50±0.43	8.60±0.64	27.20±0.75
100 ppm	96.80	18.70±0.54	6.00±0.89	13.30±0.73
200 ppm	94.42	16.77±0.82	3.44±0.92	5.22±0.88
400 ppm	90.50	12.12±0.78	2.19±0.80	3.00±0.96

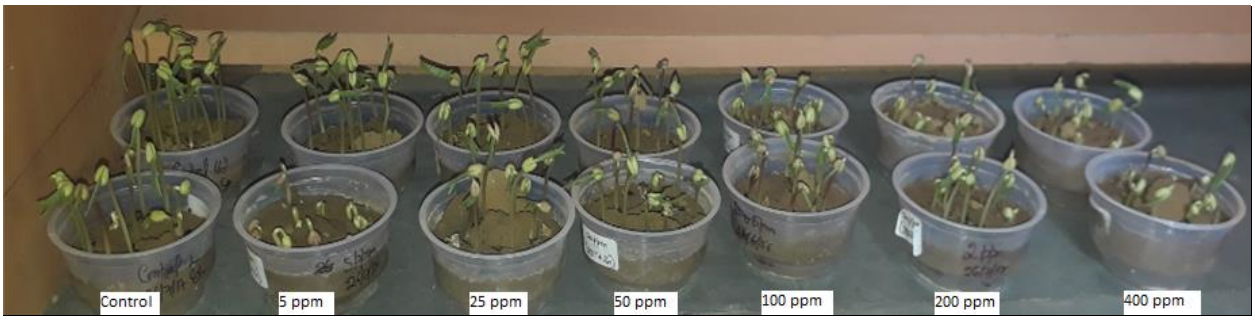


Fig. 4.7 Germination at different concentrations of malathion after 3 days

Fig. 4.8 Shoots at different concentrations of malathion after 6 days

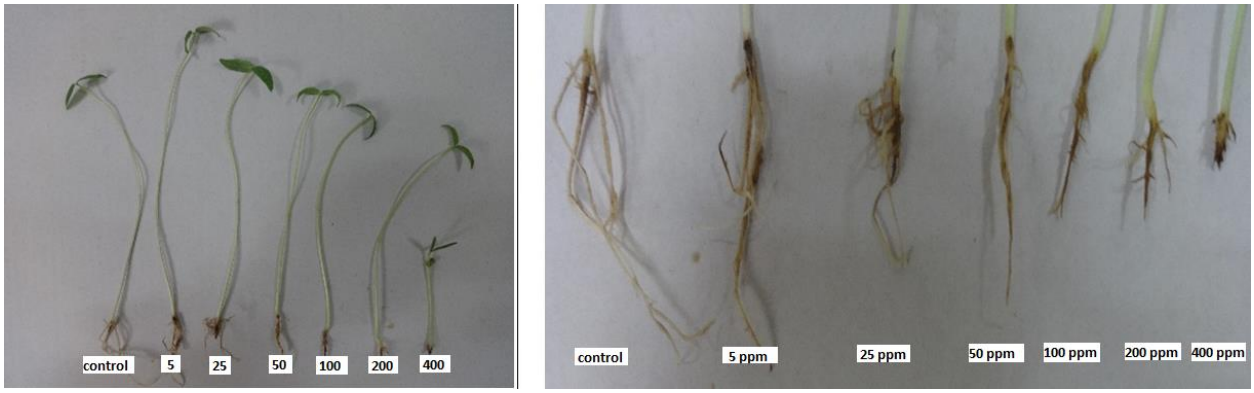


Fig. 4.9 Seedling and roots at different concentrations of malathion after 6 days

4.1.2.2 Effect of malathion on seed sermination of green gram on filter paper

The green gram seeds were grown on the filter paper augmented with different concentrations (5 ppm to 400 ppm) of malathion. The maximum germination of seeds (100%) was observed with an average shoots length of 4.81±0.65 and average root length 1.65±0.48 at 5 ppm. It was also observed that the percentage germination shoots length and roots length bettered at lower concentration (5 ppm) as compared to the controls (Fig.4.9), whereas in the rest of concentrations, the percentage germination and growth of seedling in term of shoot's length and root's length gradually declined with an increasing concentration. A higher level of malathion (400 ppm) showed inhibitory effect on shoot and root lengths as seen in Table 4.5 & Fig. 4.10.

Table 4.6 Average values of seedling of green gram seeds tested on filter paper

Sample	Germination (%)	Shoot length (cm)	Root length (cm)	Roots per shoot
Control	99.20	4.39±045	1.27±0.42	4.21±0.56
5 ppm	100	4.81±0.65	1.65±0.48	4.40±0.45
25 ppm	99.10	4.30±0.48	1.81±0.63	4.20±0.23
50 ppm	98.85	3.68±0.52	1.70±0.65	3.66±0.34
100 ppm	98.25	3.87±0.32	1.25±0.45	3.35±0.32
200 ppm	94.20	2.87±0.28	1.10±0.43	2.25±0.25
400 ppm	92.50	2.40±0.15	0.75±0.28	1.75±0.20

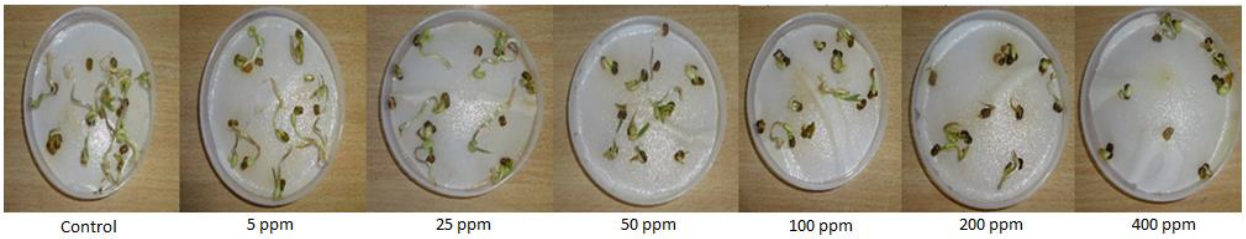


Fig 4.10 Seed germination of a green gram on filter paper observed after 6 days

4.1.2.3 Estimation of photosynthetic pigments in green gram leaf

In the investigation of photosynthetic pigment, 6 days old seedlings, which were cultivated by soil method, were evaluated qualitatively by TLC and quantitatively by spectrophotometry using Bio-spectrophotometer for the analysis of various photosynthetic pigments. As we know chlorophyll is the most valuable pigment of a plant for photosynthesis process. It is the center point for capturing the sunlight and is responsible for primary metabolism. The maximum amount of total chlorophyll was found in control i.e. 36.72 mg/g and minimum in 400 ppm sample i.e. 23.09 mg/g, while carotenoid was higher in 5 ppm as compared to control (Table 4.6). The amount of total chlorophyll and carotenoid gradually decreased thereafter with an increase in concentration (25 ppm to 400 ppm).

Table 4.7 Average absorbance values of green gram leaf extract for photosynthetic pigments

Sample	663 nm	645 nm	665 nm	480 nm	486 nm
Control	2.362	0.892	2.707	1.104	0.779
2ppm	2.026	0.884	2.305	1.123	0.764
5ppm	2.182	0.842	2.225	1.095	0.739
10ppm	2.107	0.805	2.212	1.037	0.712
25ppm	2.103	0.796	2.157	0.967	0.678
50ppm	1.535	0.583	1.525	0.772	0.510
100ppm	1.450	0.570	1.482	0.705	0.479

Table 4.8 Average content of photosynthetic pigments in green gram leaf

Sample	Chlorophyll a (mg/l)	Chlorophyll b (mg/l)	T. Chlorophyll (mg/l)	Carotenoid (mg/l)

Control	27.620	9.100	36.720	0.442
5 ppm	24.310	10.490	34.810	0.449
25 ppm	25.450	8.980	34.430	0.438
50 ppm	24.620	8.290	32.840	0.412
100 ppm	23.980	5.170	29.150	0.387
200 ppm	17.930	6.110	24.030	0.309
400 ppm	16.880	6.210	23.090	0.282

The TLC for photosynthetic pigments was studied by using fresh leaf samples. The analysis of the various photosynthetic pigments on TLC plates showed the presence of phaeophytin, xanthophyll, chlorophyll a, chlorophyll b and carotenoid (Fig. 4.6). The demetalation of chlorophyll using strong acids has been previously described.The elution order using this elution solvent system was carotenoid (*R*f= 0.99), phaeophytin (*R*f= 0.82) chlorophyll a (*R*f= 0.8), chlorophyll b (*R*f= 0.78), and xanthophyll (*R*f= 0.22).

Fig. 4.11 Intensity of fresh leaf samples in different concentration (0- 400 ppm) of malathion

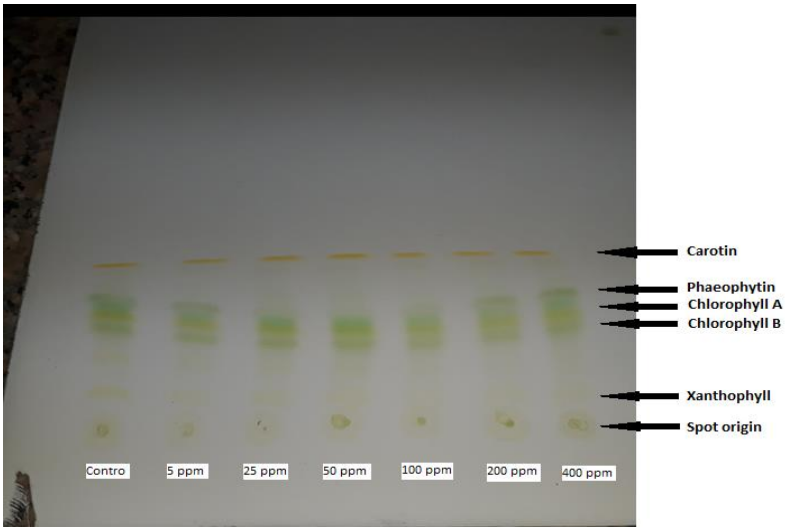


Fig. 4.12 Spots of photosynthetic pigments on TLC plate from green gram fresh leaf

On the basis of observations, we found that the low concentration of malathion may be used to promote the seeds germination and growth of seedling. However, it showed an adverse effect on photosynthetic pigments. Even a low concentration of 1&2 ppm has been reported in reducing chlorophyll content (Mostafa, 1972 & Srinivas and Damani, 2016). Malathion inhibits the production of primary metabolites such as adenosine triphosphate (ATP) due to their effect on the photophosphorylation in the light reaction (Talreja, 2011). A similar study was done by Gafer *et al.* (2006) on some other vegetables using the same pesticides and doses. Also, pesticides affect crop growth and reduce its yield and quality due to their phytotoxicity. It even has detrimental effects on soil fertility and salinity (Iriyama *et al*., 1980 & Mersal, 2011).

4.2 Effect of lindane on seeds germination

4.2.1 Effect of lindane on seeds germination of wheat in soil

The effect of lindane at different concentrations (5 ppm to 50 ppm) on the germination and growth of wheat seeds in soil was observed. The inhibitory effects towards seed germination, the growth of seedling and roots per shoot rapidly increased with increase in the concentration of lindane. We observed that the lower concentration (5 ppm) shows a decline in the growth and development of seedling as compare to control (Table 4.8). This is contrary to the response with

malathion. The toxicity of lindane towards seed germination and survival of seedling was also observed beyond 20 ppm (Fig 4.15).

Table 4.9 Average values of the effect of lindane on the germination of wheat seeds in the soil

Sample	Vigous index	Shoot length (cm)	Root length (cm)	Roots per shoot
Control	194.00	10.88±0.42	10.44±0.87	6.44±0.024
5ppm	94.88	7.78±0.58	3.11±0.80	5.85±0.35
10ppm	72.35	5.35±0.36	1.50±0.34	4.28±0.48
20ppm	46.58	3.85±0.78	0.75±0.92	3.15±0.67
50ppm	34.98	1.98±0.65	0.82±0.68	1.85±0.85

4.2.1.1 The seedling vigour

The seedling vigour index is an indicator of plant's vigor in term of percentage germination and length of the seedling. In the present study on wheat, seedling reflected the harmful effects on the vigour indices with increasing concentrations of lindane. At 20 ppm of lindane, the vigour index was reduced by almost 46.58% in wheat (Table 4.8). The vigour index reflects the abnormal condition in both roots and shoots length of seedling along with the germination percentage of seeds. Even though the germination percentage doesn't give much information as to what is happening to the overall health of the plant.

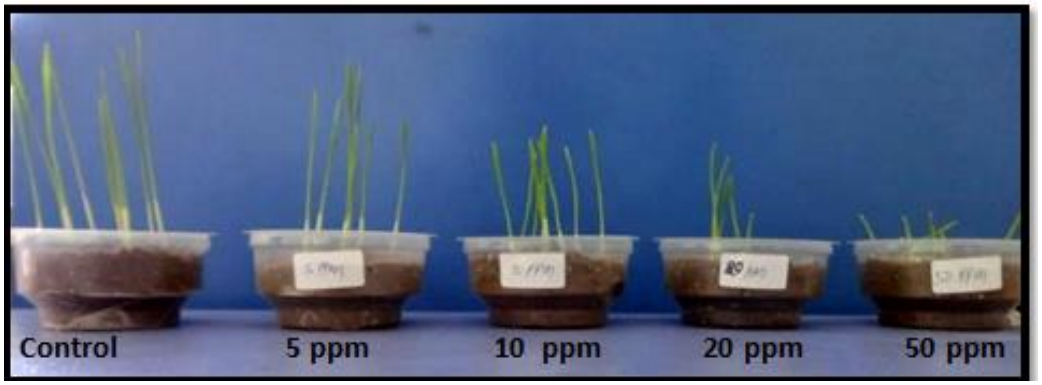


Fig. 4.13 Wheat seeds germinated at different concentrations of lindane after 3 days

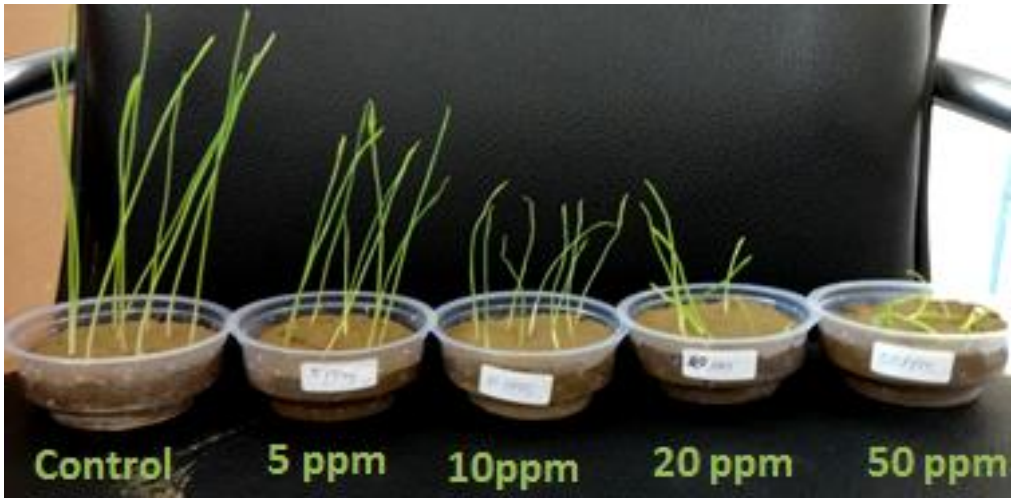


Fig. 4.14 Wheat shoots at different concentrations of lindane after 6 days

4.2.1.2 Effect on roots

The average root length of wheat seedlings reduced with increasing concentrations of the lindane. The average root lengths in wheat decreased from 10.44±0.87cm in control to 2.91 cm, 3.11±0.8 cm and 0.82±0.68 cm in 5 ppm and 50 ppm respectively (Table 4.8 & Fig. 4.15). A remarkable observation in a lower concentration of lindane (10 ppm) was stunted root almost diminished to the extent that it couldn't support the growing seedling. The stunted root with fewer numbers of secondary roots can't hold itself to the soil and there is every possibility for the plant to droop easily and uproot from the soil. Earlier, Bidlan *et al.* (2004) showed that at lower concentrations there was an increase in root lengths of seedling in green gram and radish that reduced significantly at higher concentrations of technical HCH. Our observations with 97% pure isomer of HCH have more or less similar effects at higher concentrations, while at the lower concentrations results were different. In our study at 5 ppm lindane, the length of roots and number of secondary roots declined (Fig. 4.15).

Fig. 4.15 Seedling of wheat at different concentrations (5 ppm to 50 ppm) of lindane after 6 days

4.2.1.3 Effect on the seed viability

The seed viability is a test for how many seeds are alive and could develop into healthy plants when given the appropriate conditions. (Copeland and Mc Donald, 1980). The different concentrations of lindane were used on the viability of the wheat seeds for different periods (0 h to 120 h). The viability of the wheat embryos decreased with the increased concentration of lindane. It was observed that the viability of wheat embryos increased within the first 24 h for all concentrations and then decreased with increasing lindane in concentration and exposure time (48 h onwards, Fig. 4.15). The early rise in viability might be due to the initial and quick stimulatory effect in all concentrations. The decrease in viability count can be assigned to the toxicity due to lindane. According to Dalvi and Salunkhe (1975), plants can be used as indicators in the form of a bioassay to assess the antagonistic effects of pesticides. They also gave a warning signal almost four-decade ago that the toxicological indications of these pesticides in a long-term use can be hazardous not only to the plants' health but also to the consumers, depend on the plants.

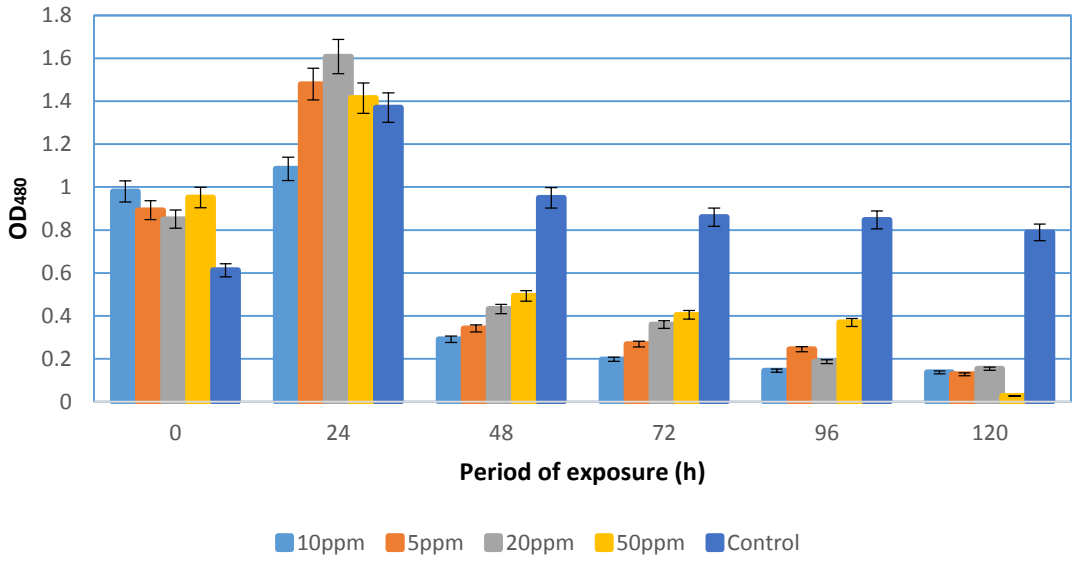


Fig. 4.16 Viability of wheat embryos after various periods of exposure to different concentrations of lindane

4.2.2 Effect of lindane on seeds germination of green gram in soil

The effect of lindane at different concentrations (2 ppm to 50 ppm) was observed on the germination and growth of green gram seeds by soil method. The germination and growth were drastically affected by the higher concentration (20 ppm) of lindane. Fig. 4.17 & 4.18 gives direct evidence of toxicity of the pesticides towards seed germination and growth and development of seedling. The length of roots, shoots, leaves and the number of roots per shoot were measured, and observations are presented in Table 4.9. It is clear from the results, that the overall germination of seeds and growth of the seedling are severely affected as we increased the lindane concentrations. Surprisingly, the lower concentrations (2 ppm & 5 ppm) of lindane promote the number of roots per shoot as compare to control (Fig. 4.18 and Table 4.9). Many investigators and research teams in the past have reported similar findings. According to Zhang *et al.* (2008), the use of 5-aminolevulinic acid and propyl-4-{2-(4, 6-dimethoxypyrimidin-2-yloxy) benzyl amino} benzoate together inhibited the root and shoot in *Brassica* but the lower concentrations enhanced the growth of plantlet.

Table 4.10 Average values of the effect of lindane on the germination of green gram in soil

Sample	Vigour index	Shoot length (cm)	Root length (cm)	Roots per shoot	Leaf length (cm)
Control	210	20.98±0.34	12.18±0.28	12.84±0.024	1.65±0.18
2ppm	198	15.85±0.42	6.25±0.46	15.18±0.45	1.45±0.26
5ppm	192	12.58±0.48	5.82±0.38	14.65±0.52	1.12±0.28
10ppm	158	7.25±0.56	4.20±0.64	4.36±0.58	1.05±0.28
20ppm	132	3.2±0.72	2.15±0.69	1.35±0.77	0.84±0.34
50ppm	98	0.88±0.75	0.88±0.70	0.85±0.62	0.65±0.38

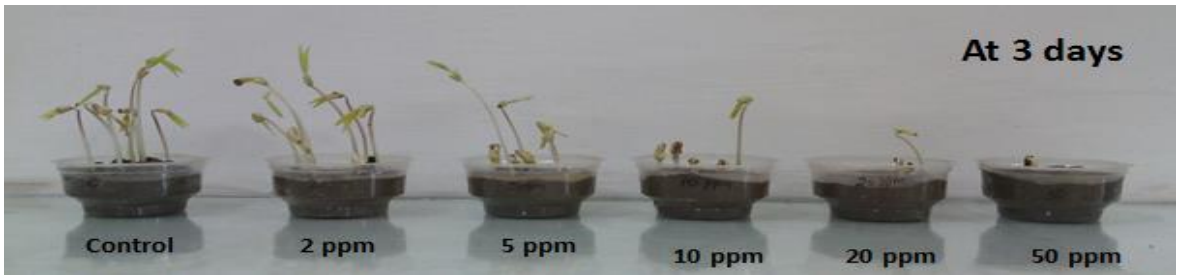


Fig. 4.17 Green gram shoots at different concentrations of lindane after 3 days

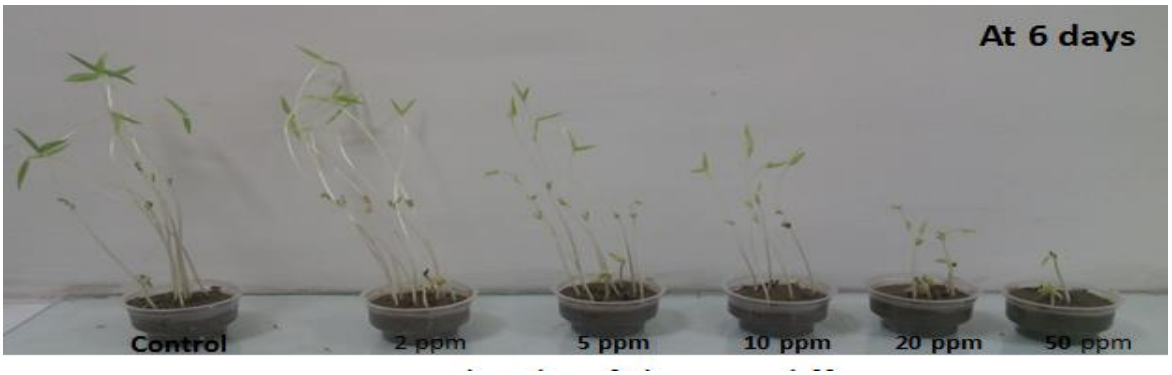


Fig. 4.18 Green gram shoots at different concentrations of lindane after 6 days

4.2.2.1 The seedling vigour

Vigour index is a representation of the quality of the seedling and plant. In this study with green gram reflected the adverse effects of lindane on the vigour indices with increasing pesticide concentration. Table 4.9 gives the vigour index of the green gram at different concentrations (2 ppm to 50 ppm) of lindane. At higher concentrations of lindane (20 ppm & 50 ppm), the vigour index reduced 132 to 98 respectively (Table 4.9).

Fig. 4.19 Seedling of green gram at different concentrations (ppm) of lindane after 6 days

4.2.2.2 Effect on leaves

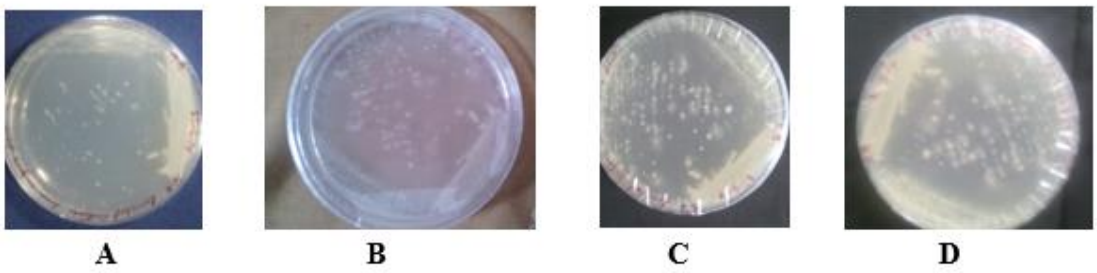
The average length of leaves in green gram reduced from 1.65±0.18 cm in control to 0.65±0.38 cm in 50 ppm of lindane. The reduced size of leaves can later be accountable for the reduced production of primary and secondary metabolites, and finally affecting the farmer's economically and the consumers with food scarcity and other products. This is because less area of leaves will carry small quantities of chlorophyll that in turn result in the lesser process of photosynthesis, hence less crop production.

The delay in germination and reduction in percentage germination can most probably be attributed to the reduced enzyme activities in presence of pesticides. It had been documented that the amylase and protease enzymes are affected negatively when seeds are grown in presence of tech- HCH (Bidlan *et al.*, 2004), DDT (Deepthi *et al.*, 2005) and DDT-Lindane mixture (Saghee *et al.*, 2017). Most probably these pesticides interfere in the enzyme activity and do not allow the stored food in the endosperm of seeds to be hydrolysed. This restricts the food supply to the growing/ germinating embryo, thereby delaying in the germination time and reduced germination percentage.

4.3 Isolation and screening of bacterial strains for degradation of lindane

4.3.1 Enrichment by the mixed commercial formulation of pesticides

At the end of 9 months of enrichment, morphologically 47 distinct bacteria were obtained on nutrient agar plates. These were purified by repeated streaking and maintained on nutrient agar with 5 ppm pesticide- mixture. All the 47 isolates were again mixed in equal quantities to reconstitute the consortium (undefined) and used for screening.



Figs 4.20 Plates during enrichment by mixed pesticides (After: A= 24H; B= 1M; C= 3M; D= 6M)

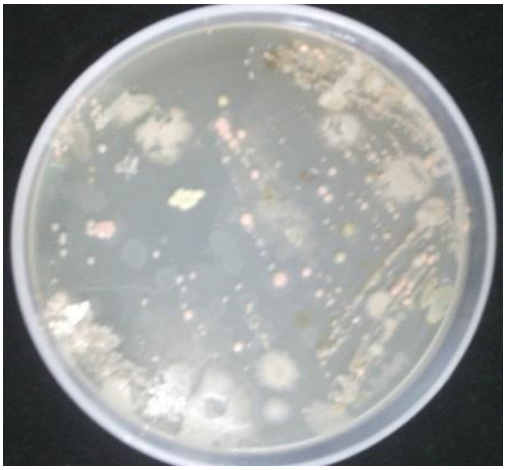
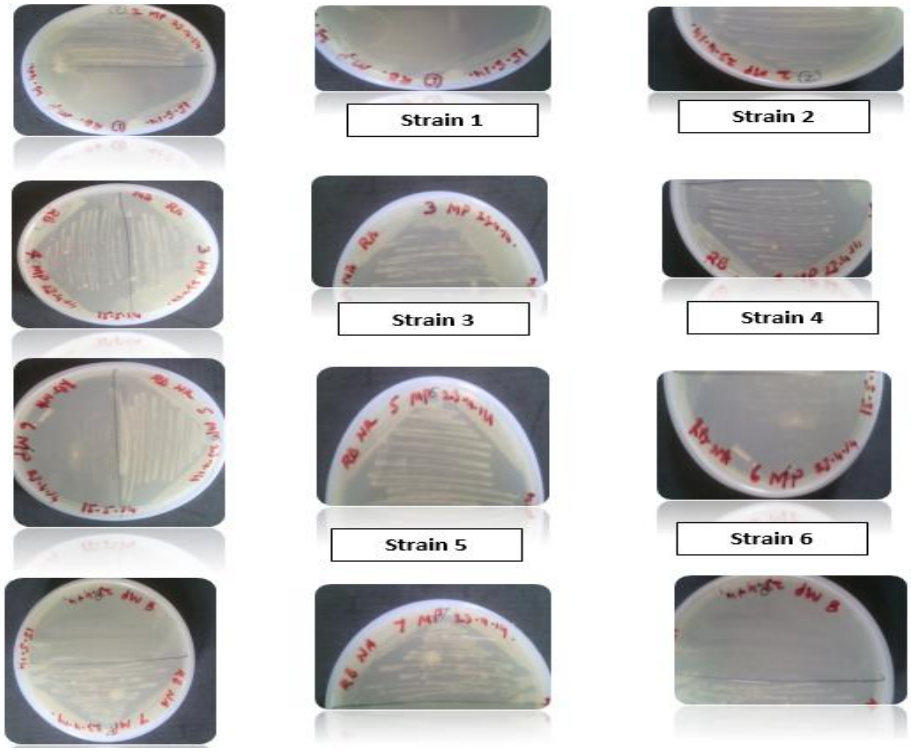


Fig. 4.21 Bacterial population after 9 months enriched by mixed pesticides (500 ppm)

4.3.2 Screening of the individual isolates for lindane degradation on agar

Cultures 3, 5, 6, 7, 9, 10, 11, 14, 15, 18, 19, 20, 22, 23, 26, 28, 29, 30, 33, 35, 36, 38, 44 and 46 showed clearance of lindane film (Fig. 4.23). During screening 0.5% lindane solution sprayed on culture plates and incubated at RT. 24 cultures that have the potential for degrading lindane. This was an indication that the cultures have the potential for degrading lindane.



Figs 4.22 Purification of Bacterial strain from a consortium

Figs 4.23 Screening for the potential strains for degrading lindane from consortium

4.3.3 Screening of the individual isolates for lindane degradation in broth

It was observed that the lindane- induced consortium had dominatingly 4 distinct cultures. These cultures were purified, designated as LR1, LR2, LR3 and LR4 respectively (Fig. 4.23) and maintained on minimal agar supplemented with 1/50 NB and 5 ppm lindane for future studies later.

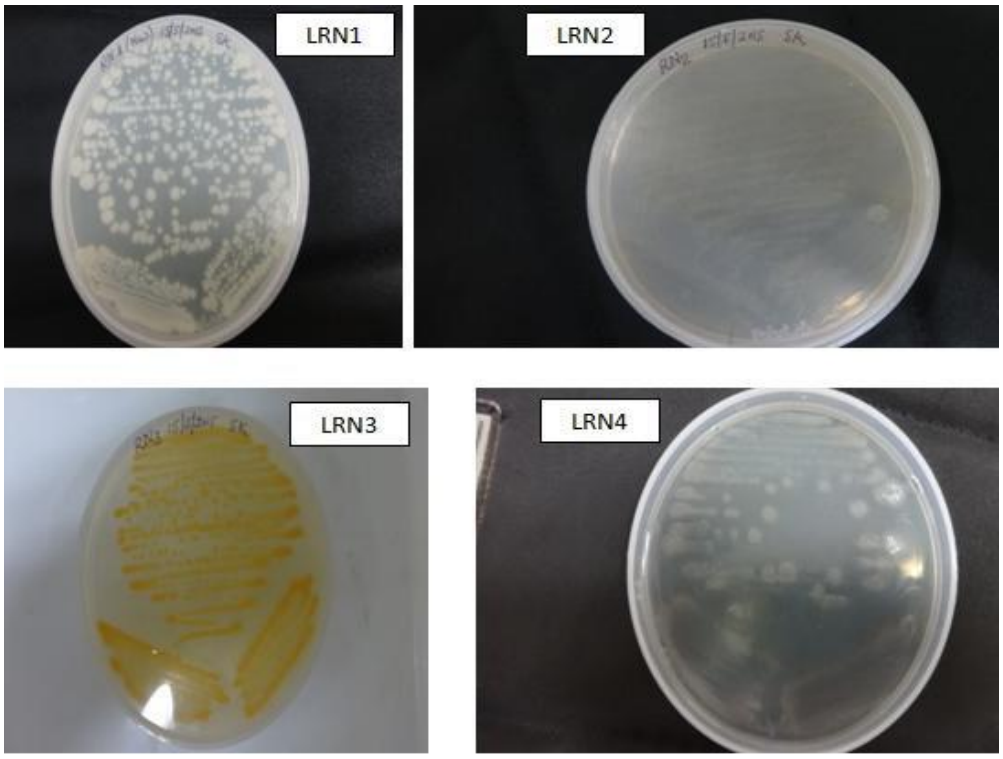
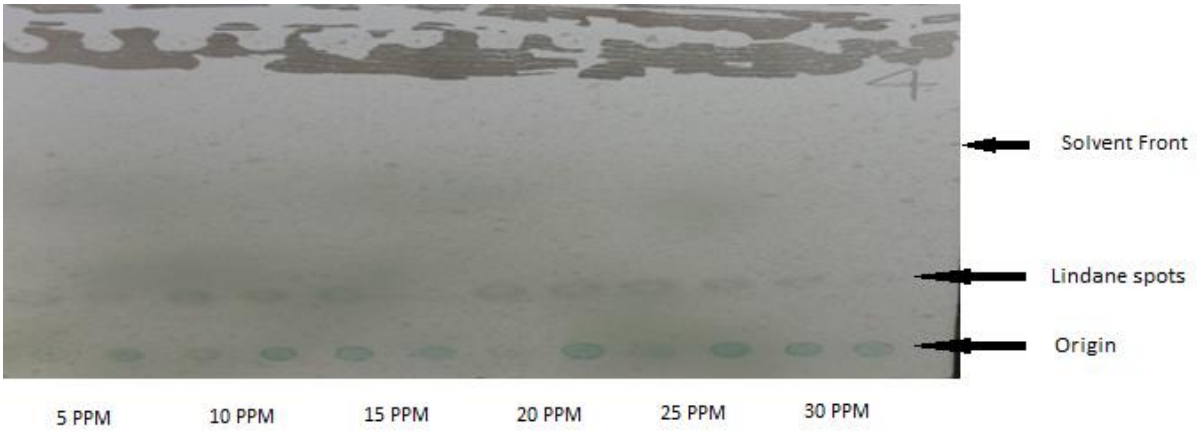


Fig. 4.24 Four bacterial strains isolated for degrading lindane in broth

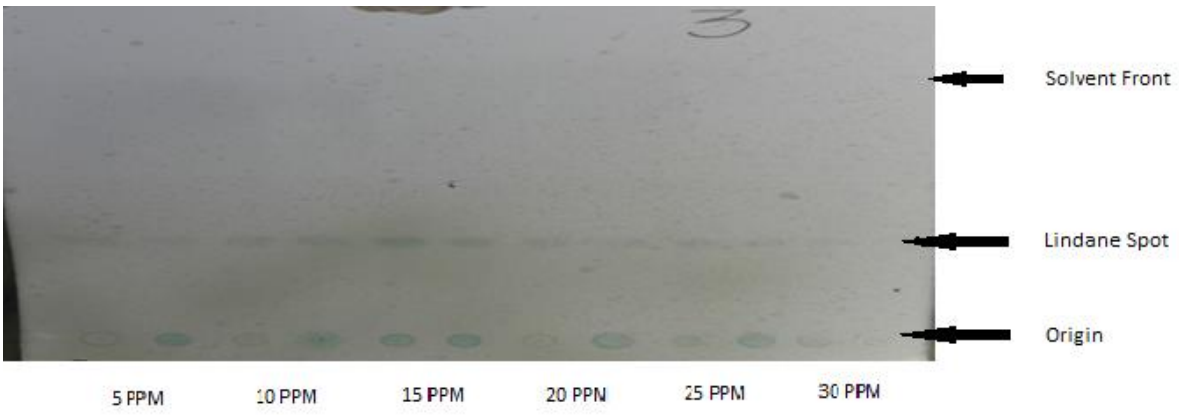
4.3.4 Degradation of lindane by the bacterial consortium

The consortium showed 55.61% - 90.35% degradation of lindane (Fig.4.37). The initial concentration of 5 ppm lindane was reduced to 9.65% at the end of 144 h of incubation while initially added higher concentrations of 10, 15, 20, 25 and 30 ppm were reduced to 10.65, 29.21, 29.94, 39.25 and 44.39% during the same time. The corresponding growth of the microbial consortium is shown in Fig. 4.36. It was observed that the growth of the consortium increased drastically with increasing concentrations of lindane. The six-fold increase in growth on 30ppm lindane by 18 h is a reflection of utilization of lindane as a carbon source by the members of the consortium (Fig. 4.36). It was observed that the growth followed a zig-zag pattern with the initial increase and then decrease and again an increase. At the end of 144 h, the final growth was approximately 2 ½ - 3 ½ folds with respect to the initial

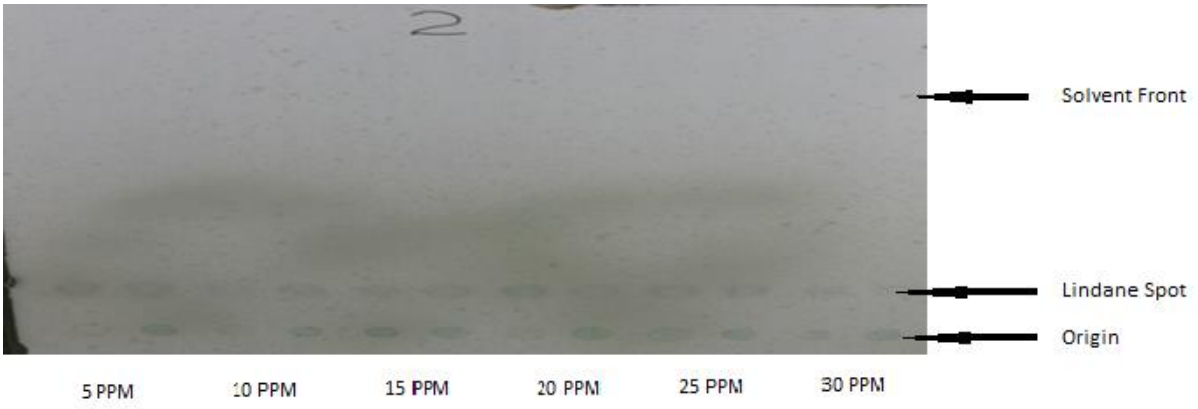
inoculum. There appeared to be a direct correlation between the concentration and the time for degrading the initially supplied lindane. The other common trend observed was that when growth reduced, the degradation also diminished in that phase. This was an indication of the direct relationship between consortium growth and lindane degradation. Similar observations were made by Jilani (2013).



Figs 4.25 TLC plate for estimating of different concentrations of lindane (5 ppm to 30 ppm) at 24 h



Figs 4.26 TLC plate for estimating of different concentrations of lindane (5 ppm to 30 ppm) at 48 h



Figs 4.27 TLC plate for estimating of different concentrations of lindane (5 ppm to 30 ppm) at 96 h

Figs 4.28 TLC plate for estimating of different concentrations of lindane (5 ppm to 30 ppm) at 144 h

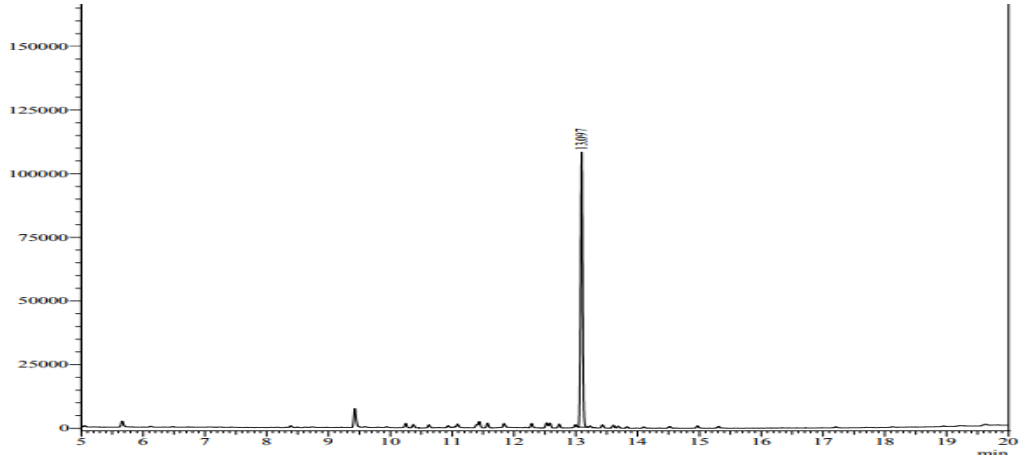


Fig. 4.29 GC chromatogram of standard lindane

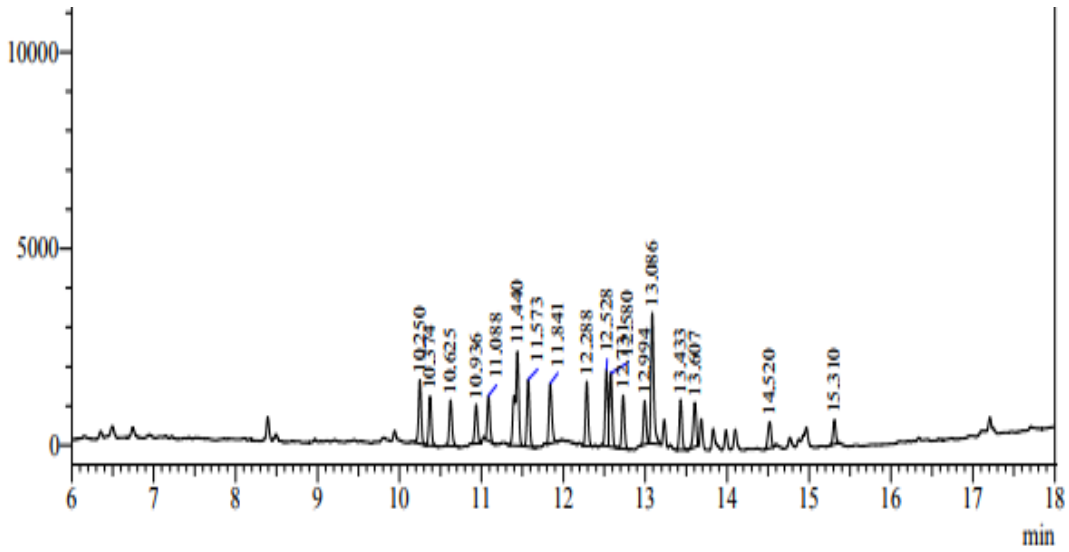


Fig. 4.30 GC chromatogram of 20 ppm lindane at 0 h

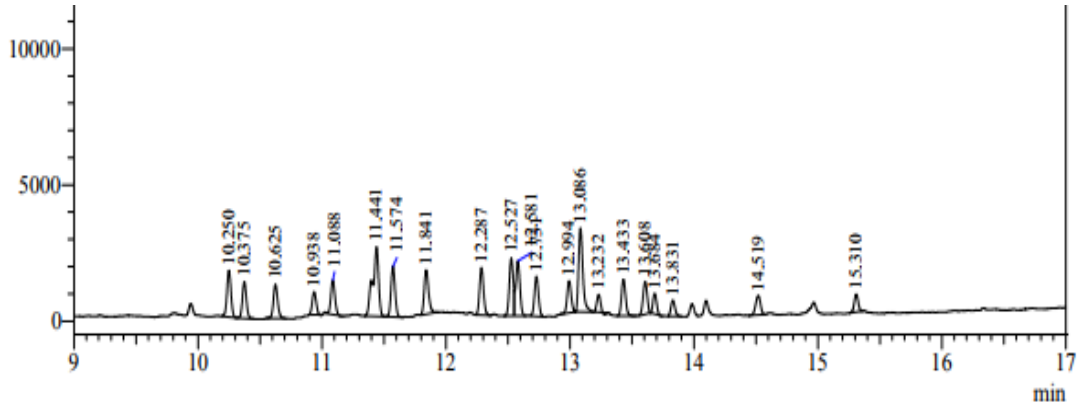


Fig. 4.31 GC chromatogram of 20 ppm lindane at 24 h

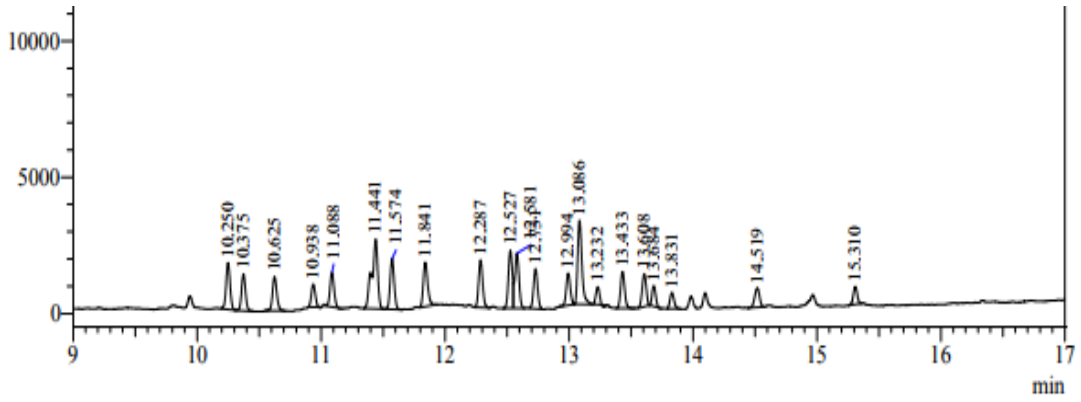


Fig. 4.32 GC chromatogram of 20 ppm lindane at 48 h

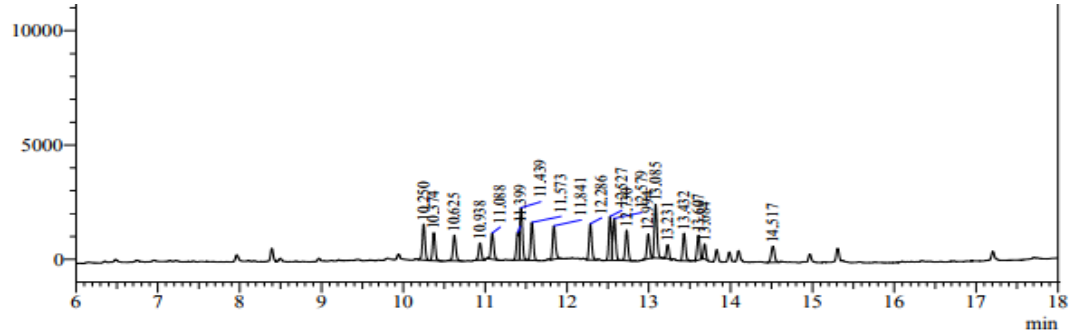


Fig. 4.33 GC chromatogram of 20 ppm lindane at 96 h

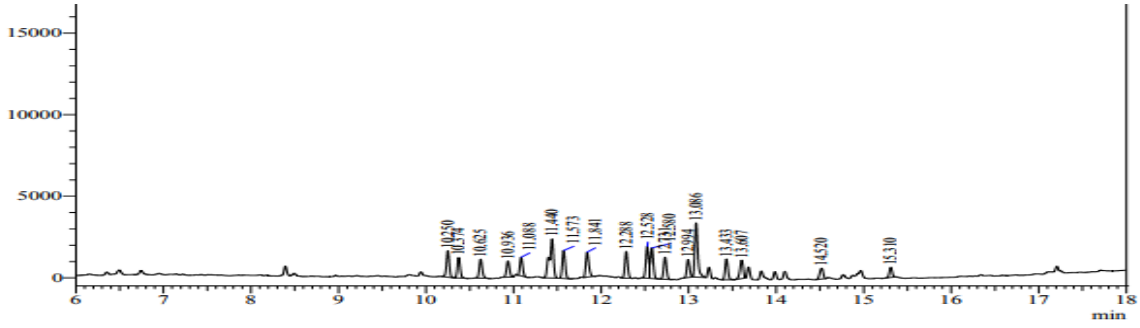


Fig. 4.34 GC chromatogram of 20 ppm lindane at 144 h

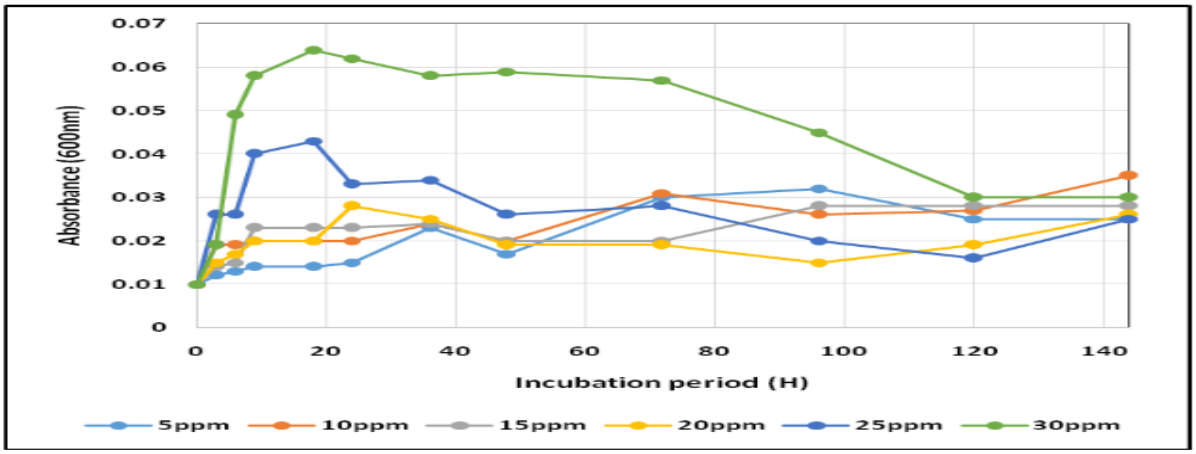


Fig. 4.35 Growth of the bacterial consortium on different concentrations of lindane (5 ppm to 30 ppm)

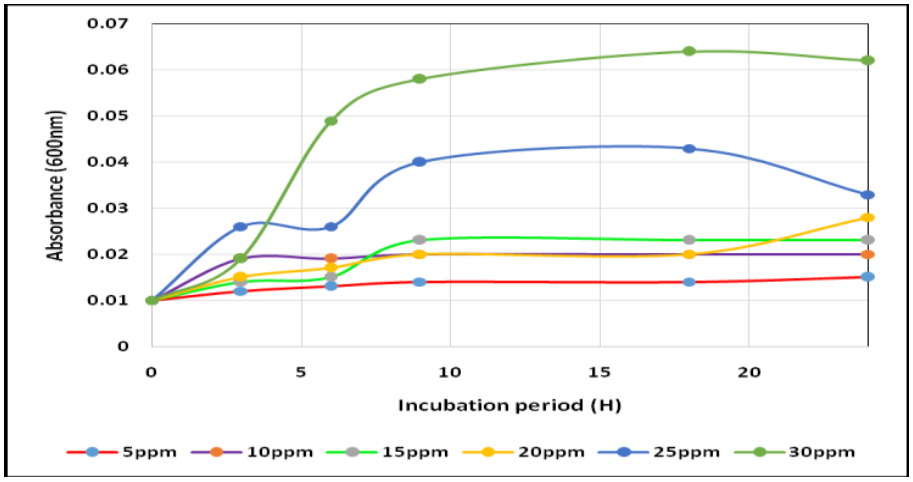


Fig. 4.36 Growth of the bacterial consortium on different concentrations of lindane during the initial 24 h

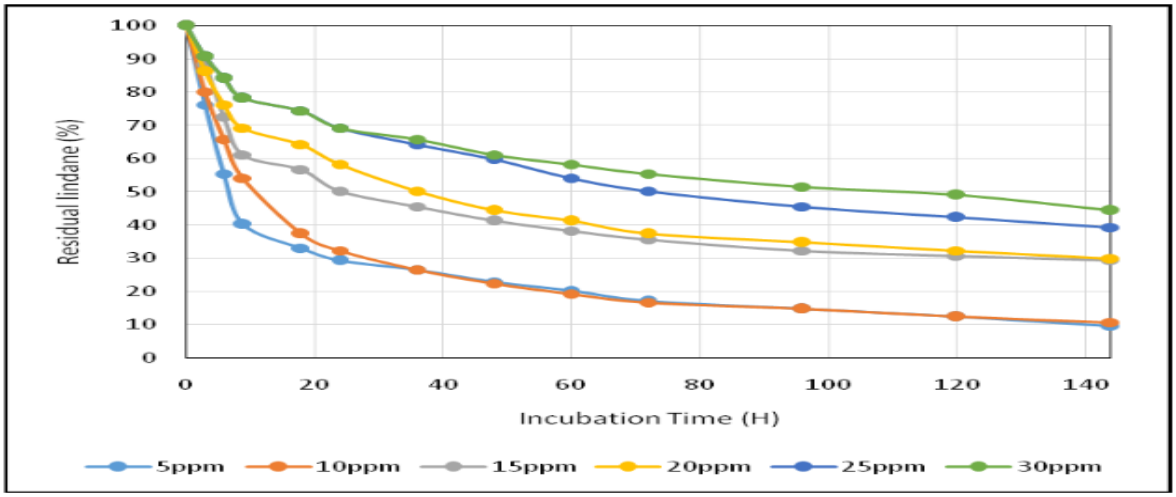


Fig. 4.37 Degradation of lindane (5 ppm to 30 ppm) by the bacterial consortium

4.3.5 Lindane degradation at different temperatures by the bacterial consortium

The initially added 20 ppm of lindane was degraded by 13.89% at both 10 ^{0}C and 20 ^{0}C while 23.6% and 36.2% degradation was observed at 40 ^{0}C and 50 ^{0}C respectively. Maximum degradation of 64.9% was seen at 30 ^{0}C (Fig. 4.38). The corresponding growth is shown in Fig. 4.38. Overall growth was observed only at 30 ^{0}C with 6.67% increase and at 40 ^{0}C with 34.6% increase in 6 days of incubation. The added inoculum decreased at other temperatures. Even though the growth was better at 40 ^{0}C than 30 ^{0}C, degradation was better in the latter case. The reason may be the temperature optima for catabolic enzymes is in favor of the 30 ^{0}C. With 46% reduction in the inoculum at 50 ^{0}C, degradation was more effective than 40 ^{0}C. This may be because of the higher inoculum size provided to 50 ^{0}C and also certain catabolic enzymes (from a certain member of the consortium) might have higher temperature optima.

The consortium comprises of different cultures that may have different behavior at different temperatures and pH. In an earlier study, Bidlan and Manonmani (2002) have earlier detected the degradation of DDT from 40 ^{0}C to 50 ^{0}C but the most efficient degradation was shown to be at 30 ^{0}C while the efficient growth was observed at 37 ^{0}C.

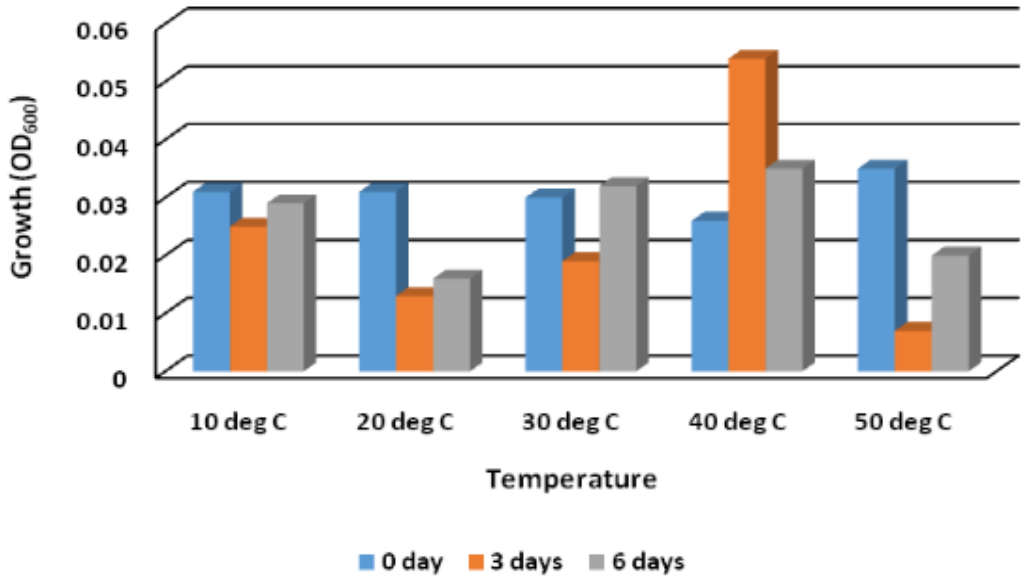


Fig. 4.38 Growth of the bacterial consortium on 20 ppm lindane at different temperatures

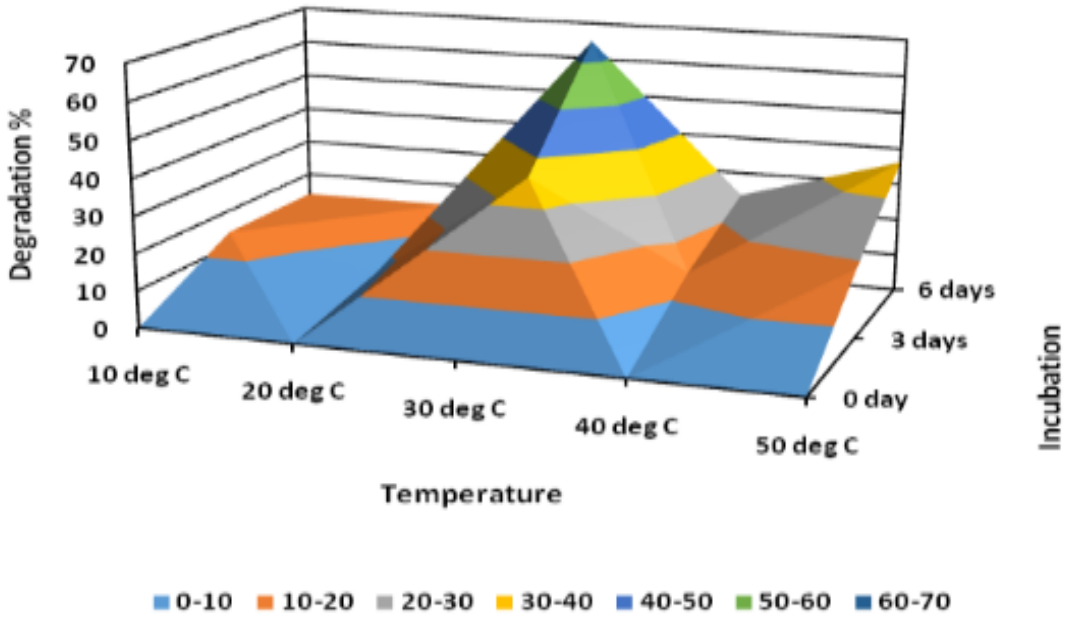


Fig. 4.39 Degradation of 20 ppm lindane by the bacterial consortium at different temperatures

4.3.6 Lindane degradation at different pH by the bacterial consortium

The consortium could degrade the initially provided 10 ppm lindane by 13.9%, 18.9%, 23.6% and 50.5% at pH 5, pH 6, pH 7 and pH 8 respectively by the end of 144 h (6 days) of incubation (Fig. 4.39).

The corresponding growth pattern is shown in Fig. 4.39. It was observed that the growth increased by 42.86%, 52.17%, 39.13% and 86.96% in the first 3 days of incubation at pH 5, pH 6, pH 7 and pH 8 respectively that reduced to 14.29% for pH 5 and to 26.09% for other 3 pH respectively by the end of 6th day of incubation with respect to the initial inoculum level. The maximum growth increment in the first 3 days was at pH 8 followed by pH 6, pH 5 and pH 7 respectively while the reduction in next 3 days was least at pH 7 followed by pH 6, pH 5 and pH 8 with 33.33%, 50%, 66.67% and 70% reduction respectively.

The degradation of lindane was more effective with an increase in pH indicating a direct correlation between studied pH levels and lindane degradation. Even though the growth in pH 8 decreased maximum in last three days, yet the degradation was more efficient suggestive towards the secretory enzymes/enzyme systems involved in lindane degradation (this is our hypothesis and need further studies to prove; though it is believed that the degradative enzymes are non-secretory through experiments with *Pseudomonas paucimobilis*).

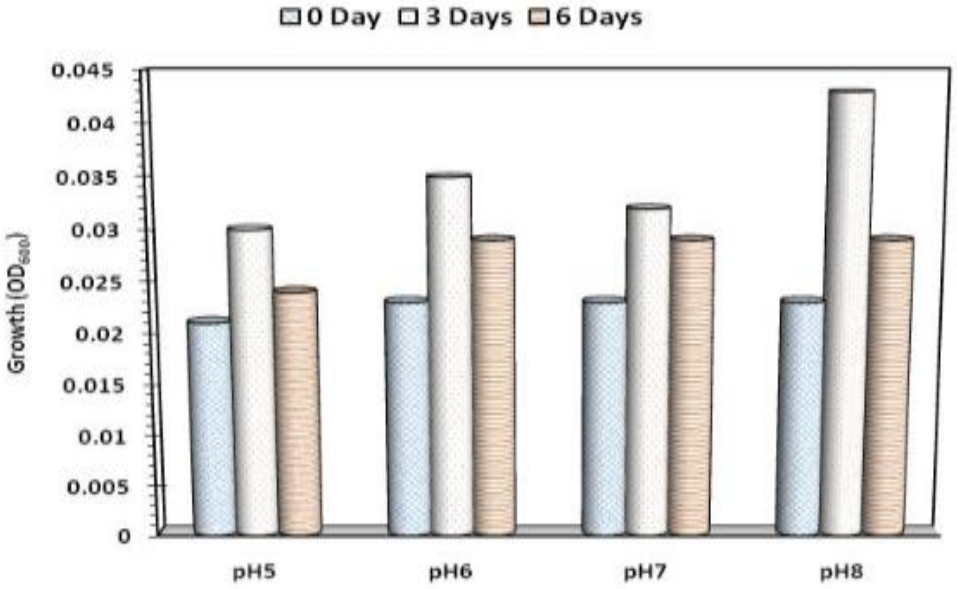


Fig. 4.40 Growth of the bacterial consortium on 20 ppm lindane at different pH

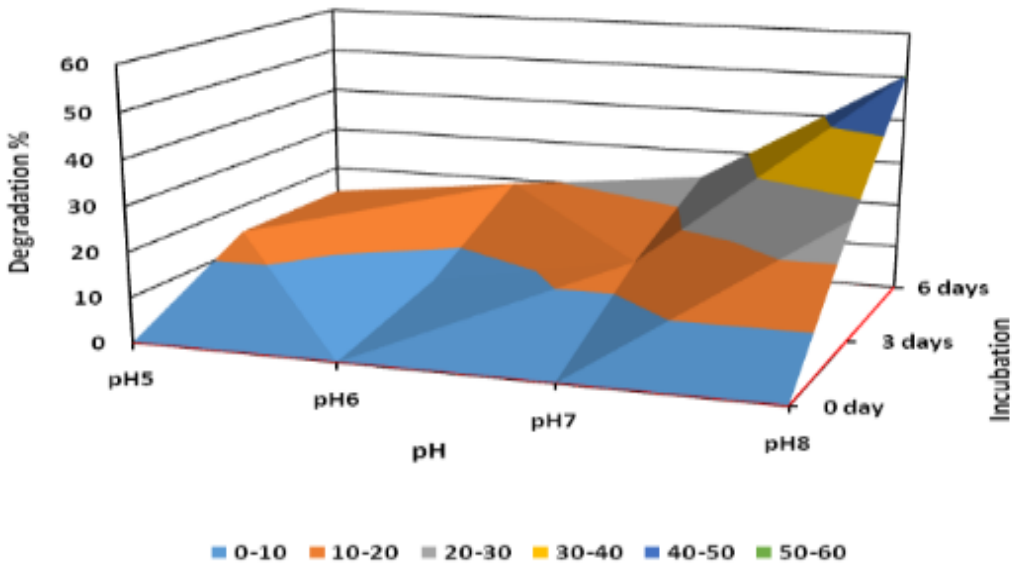


Fig. 4.41 Degradation of 20 ppm lindane by the bacterial consortium at different pH

Bidlan and Manonmani (2002) and Bidlan (2003) have earlier shown that the pH 7- 7.5 was more favorable for bioremediation of DDT by *Serratia marcescens* DT-1P, *Pseudomonas aeruginosa* DT-ct1 and *Pseudomonas aeruginosa* DT- ct2. Sreedharan *et al*. (1999) demonstrated the degradation of HCH in acidic soils with pH above 3 using Pseudomonas paucimobilis. More growth during the first 3 days in pH 8 in our case could have enhanced the enzyme production that continued to act during the subsequent three days of incubation, thereby degrading almost 87.29% of lindane as compared to first three days. At pH 6 the degradation in last three days was 25.32% as compared to the first three days.

Since the growth in pH 6 was second to that of pH 8, the greater degradation percentage in the second half as compared to the first half might be due to the pH optima for the catabolic enzymes. The culture can be grown at pH 8 initially and then the pH 6 can be provided for efficient degradation. The pH 7 showed the least increase in biomass in the first half but it also showed the least decrease in the biomass in the second half; thereby giving the consortium optimum condition to degrade 69.89% lindane in last three days as compared to the first three days. Yet, the most efficient degradation among different pH provided was at pH 8. Earlier, Bidlan and Manonmani (2002) found that the degradation and growth reduced at pH greater than

Most reports show that the enrichment is done with the same (parent) compound (Manonmani *et al*., 2000; Bidlan, 2003; Bidlan *et al*., 2004; Nagpal and Paknikar, 2006; Murthy and Manonmani, 2007; Deepthi *et al*., 2007; Pannu and Kumar, 2014) or the analogues of the compound (Focht and Alexander,

1970; Bartha, 1990) that needs to be degraded by the microbes. In contrast to the basic practice, our approach was to adopt a technique that could be beneficial in the present and future scenario, keeping in view that most parts of the world are now applying the organophosphates and pyrethroids instead of organochlorine pesticides.

Amyotrophic Lateral Sclerosis (ALS), a progressive fatal neurodegenerative human disease has been associated with pesticide exposure especially with organochlorines, pyrethroids, herbicides, and fumigants (Kamel *et al*., 2012) and organophosphates (Chen, 2012). Slotkin and Seidler (2009) have demonstrated the neurological and neurotoxic effects of organophosphates while David *et al*. (2014) discussed the teratogenic effect of paraoxon, an organophosphate, on neurodifferentiation.We wish to establish a population tolerant to the existing concentrations of non- organochlorine pesticides in the environment and probably still higher concentrations in future. Being tolerant to these other classes of pesticides will help the application of our cultures as they would stay active and remediate the organochlorine load already present in nature. Apart from this, the same culture might be able to remediate the loads from other classes of pesticides.

Grung *et al*. (2015) stressed the need for research on organophosphates since they are the dominating group being used at present. Viel *et al*. (2015) discussed the cognitive developmental disabilities in postnatal stages of children due to the exposure of pyrethroids leading to an urgent realization of considering the remediation for this pesticide class as well. Our cultures from the consortium may act upon these pesticides as they were enriched by the mixture of organophosphates and pyrethroids. The probability of the application of our consortium towards simultaneous degradation of different classes of pesticides cannot be ruled out. Selvi *et al*. (2013) worked with DDT- degrading consortium to study the degradation of the pesticide isoprothiolane. Pino and Penuela (2011) developed a microbial consortium with 12 different bacterial strains through enrichment technique that could degrade 150 ppm each of chlorpyrifos and methyl- parathion separately as well as simultaneously. Liu *et al*. (2009) engineered strain LZ1 of Stenotrophomonas species that could produce the enzyme organophosphorus hydrolase (OPH). This enzyme was capable of degrading p-nitrophenol substituted organophosphates and their products. The three organophosphates paraoxon, parathion, and methyl parathion degradation was enhanced in the presence of 4-chlorophenol.

Yang *et al*. (2012) could co-express the lin A and mpd genes in a cloned E. coli for simultaneous degradation of organochlorine (lindane) and organophosphate (methyl parathion). This clone is good to work in a bioreactor as it also expresses the green fluorescent protein for detection. Our consortium on the other hand, with longer acclimatization, may work in-situ. The need of acclimatization (pre-

exposure or induction) in bioremediation was earlier emphasized by many researchers in the past as well (Bidlan and Manonmani, 2002; Bidlan, 2003; Jilani, 2013). Sonkong *et al*. (2008) also demonstrated the effectiveness of 72 h induction of inoculum to DDT in the presence of glucose and yeast extract for enhancing the degradation of DDT. Successive exposure of culture to lindane improved its ability to degrade the pesticide (Wada *et al*., 1989; Bhuyan *et al*., 1992) while the same with DDT did not yield any improvement (Bidlan and Manonmani, 2002).

Microorganisms transform the complex organic compounds to CO_2 or other simple organic compounds through their metabolic enzymes. These reducing equivalents from the oxidation is assimilated and result in the growth of organisms that work out the degradation (Latha, 2012). Our results also reflect the similar view along with the other hypothesis proposed here by us that the degradation during the decline phase of growth may be due to the enzyme/ enzyme systems already synthesized by the microbial cells during their active growth phase (need further studies to prove). Baczynski *et al*. (2010) noted the initial increase in growth with substantial decrease and then the biomass remaining constant until the end of the studies. The zig-zag pattern of growth as in the present study was also observed by Sander *et al*. (1991), Bidlan and Manonmani (2002), Sonkong*et al*. (2008) and Jilani (2013). Jilani (2013) discussed the decline in the viable count due to non- acclimation of the culture to the pesticides while we hypothesize it differently here; further studies might establish the fact.

Acclimation of consortium for DDT degradation by Bidlan and Manonmani (2002) also resulted in the ultimate survival of four strains comprising *Serratia marcescens* DT- 1P and other three Pseudomonas strains. Pannu and Kumar (2014) isolated 78 strains out of which only 9 strains could clear the lindane film after 7 days of incubation and only 3 strains RP-1, RP-3and RP-9 were able to withstand 100 ppm of this insecticide. The other strains were able to tolerate lindane concentrations from 20- 60 ppm (parts per million= 1×10^{-6}). Similar observations with limiting cadmium were described by Kumar *et al*.(2010). The cultures in pure form were able to degrade lindane in agar medium while in the broth where the cultures were in mixed form, the total number of surviving/ actively growing cultures were observed to be only four. This might be due to the interaction between the various types of bacteria present. The two cultures viz. RL2 and RL4 were dominating and appeared indiscriminately, overcasting the other members of the consortium. This might be due to the antibiotic or inhibitory factors produced by the two cultures. The other two cultures (RL1 &RL3) were able to resist these factors along with the lindane; hence survived.

The degradations of pesticides have been achieved in many works in the past 50- 60 years. The time required and the percentage of degradation varied from case to case. The microbe used and the type of

pesticide understudy play a vital role than the other parameters like pH and temperature. Many times the significant parameter described is the inoculum size (Bidlan and Manonmani, 2002; Selvi *et al*., 2013). There might also be the effect of a threshold level of the initial pesticide concentration necessary for the degradation below which the culture induction is not possible. More investigations may further support the findings.

4.3.7 Effect of parameters on residual lindane

Thirty two different runs of experiment were performed and the results were modeled according to a polynomial quadratic equation to identify the variables that affected significantly or non-significantly. Determination coefficient (R^2 = 0.9836) indicates that 98.36% variability of the model can be explained by the model. Adjusted determination coefficient (adjusted R^2=0.9310) is also high, which indicates the model is highly significant. The analysis of variance (ANOVA) was determined and is given in Table 4.12 ANOVA showed that the factors A, B, C, D, E and interaction of AB, AC, AD, BC, BD, BE, DE have significant effect ($p<0.0001$) on lindane degradation. The regression model which represent residual lindane in following equation.

Residual Lindane = – 10.10 + 2.28A + 149.79B + 3.80C + 0.63D + 0.034E + 11.15AD – 1.14AC – 9.43AD – 26.95BC – 1.96BD – 4.94BE + 0.04CD + 0.08CE – 0.01DE – $0.03A^2$ + $0.35C^2$ – $6.52D^2$ +$3.46E^2$.

The F value which is indicates that the model is significant. The coefficients in the equation showed that all the factors significantly affect the degradation the lindane. The degradation the lindane was also depended on load of concentration, environmental factors like temperature, pH and incubation period.

As per CCRD (RSM) various mathematical models developed. Quadratic model was more appropriate in ours sturdy.

Table 4.11 Experimental design matrix with observations

Exp No.	A (Lin)	B (OD)	C (pH)	D (Temp)	E (Days)	Y1 (BM)	Y2 (TLC)
1.	-1	-1	-1	-1	1	0.119	8.375
2.	1	-1	-1	-1	-1	0.107	9.0
3.	-1	1	-1	-1	-1	0.117	9.25
4.	1	1	-1	-1	1	0.185	10.25
5.	-1	-1	1	-1	-1	0.083	9.85
6.	1	-1	1	-1	-1	0.100	9.75
7.	-1	1	1	-1	1	0.187	9.5
8.	1	1	1	-1	-1	0.186	9.62
9.	-1	-1	-1	1	-1	0.107	8.5
10.	1	-1	-1	1	1	0.078	8.37
11.	-1	1	-1	1	-1	0.128	8.45
12.	1	1	-1	1	-1	0.162	10.5
13.	-1	-1	1	1	1	0.092	9.37
14.	1	-1	1	1	-1	0.077	8.62
15.	-1	1	1	1	-1	0.156	8.12
16.	1	1	1	1	1	0.141	9.5
17.	-2	0	0	0	0	0.168	8.12
18.	2	0	0	0	0	0.165	11.25
19.	0	-2	0	0	0	0.145	8.12
20.	0	2	0	0	0	0.196	9.12
21.	0	0	-2	0	0	0.067	9.6
22.	0	0	2	0	0	0.088	**7.75**
23.	0	0	0	-2	0	0.041	9.37
24.	0	0	0	2	0	0.027	8.35
25.	0	0	0	0	-2	0.036	9.5
26.	0	0	0	0	2	0.133	9.75
27.	0	0	0	0	0	0.190	8.2
28.	0	0	0	0	0	0.180	8.5
29.	0	0	0	0	0	0.185	9.25
30.	0	0	0	0	0	0.173	8.62
31.	0	0	0	0	0	0.188	9.25
32.	0	0	0	0	0	0.175	8.25

Table 4.12 Pooled ANOVA showing the variables as a linear, quadratic and interaction terms on residual lindane

Source	Sum of Squares	Df	Mean Square	F Value	P value Pro > F
Model	657.44	19	34.60	88.16	< 0.0001
A- Initial Lindane	324.63	1	324.63	827.1	< 0.0001
B- Initial Inoculum	7.84	1	7.84	19.98	0.0008
C- pH	7.61	1	7.61	19.39	0.0009

D- Temperature	43.81	1	43.81	111.6	< 0.0001
E-Incubation Period	45.94	1	45.94	117.0	< 0.0001
AB	39.34	1	39.34	100.2	< 0.0001
AC	14.00	1	14.00	35.68	< 0.0001
AD	5.56	1	5.56	14.18	0.0027
AE	0.46	1	0.46	1.18	0.3000
BC	5.88	1	5.88	14.99	0.0022
BD	3.12	1	3.12	7.96	0.0154
BE	19.81	1	19.81	50.47	< 0.0001
CD	3.13	1	3.13	7.98	0.0153
CE	12.01	1	12.01	30.60	0.0001
DE	55.60	1	55.60	141.6	< 0.0001
A^2	46.19	1	46.19	117.6	< 0.0001
B^2	9.831	1	9.831	0.025	0.8769
C^2	3.77	1	3.77	9.60	0.0092

Table 4.13 Optimized factors with a unit value of RSM

Optimized Factors	**Unit**
Initial Lindane	10 ppm
Inoculum	0.0325 OD_{600}
pH	6
Temperature	30˚C
Incubation period	20 days

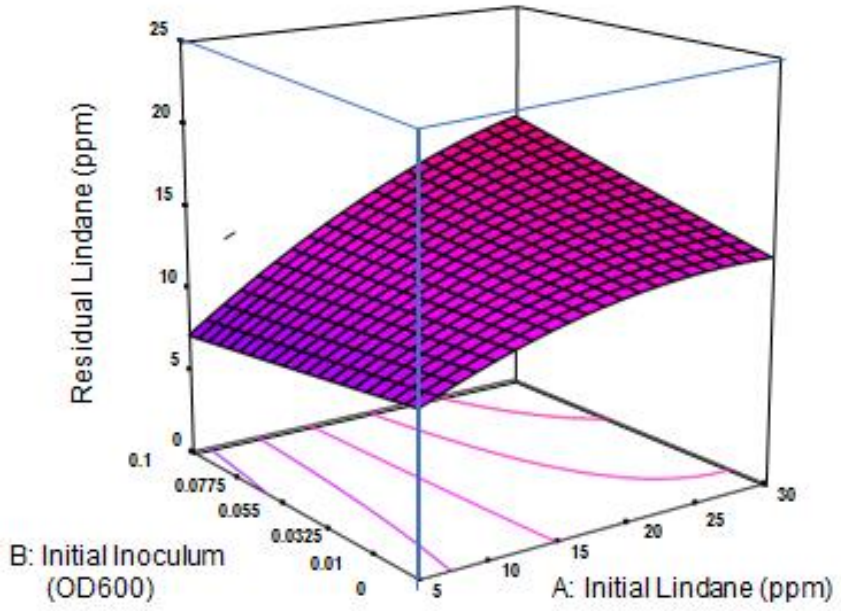


Fig. 4.42 Interaction between initial inoculum and initial lindane concentration during the lindane degradation by the bacterial consortium (pH-6, temperature- 30 ˚C, incubation period- 20 days)

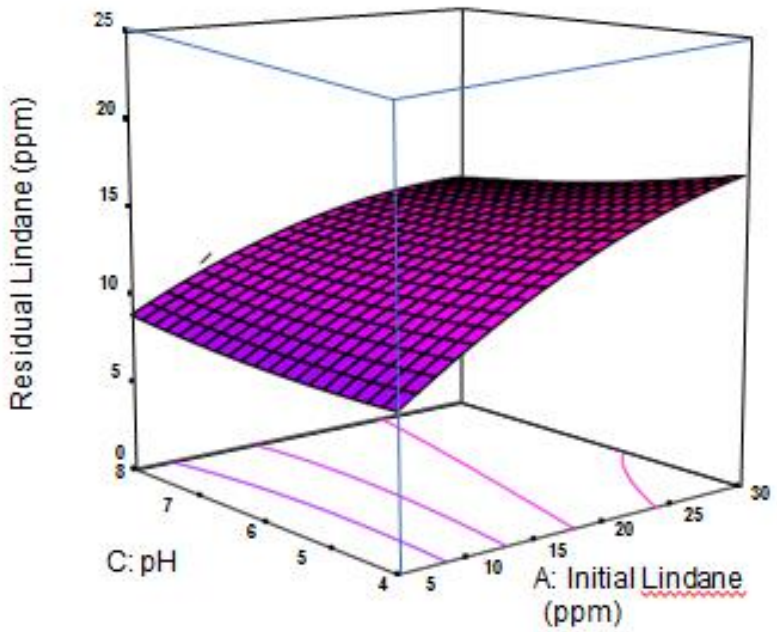


Fig. 4.43 Interaction between pH and initial lindane concentration during the lindane degradation by the bacterial consortium (initial inoculum- 0.0325 OD, temperature- 30 ˚C, incubation period- 20 days)

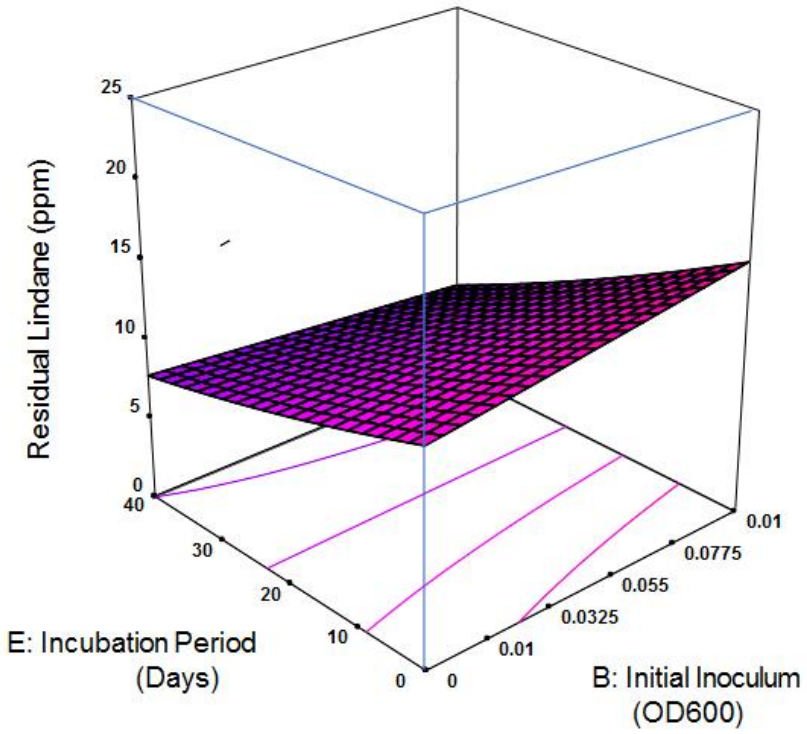


Fig. 4.44 Interaction between incubation period and initial inoculum during the lindane degradation by the bacterial consortium (pH-6, temperature- 30 ˚C, initial lindane concentration- 10 ppm)

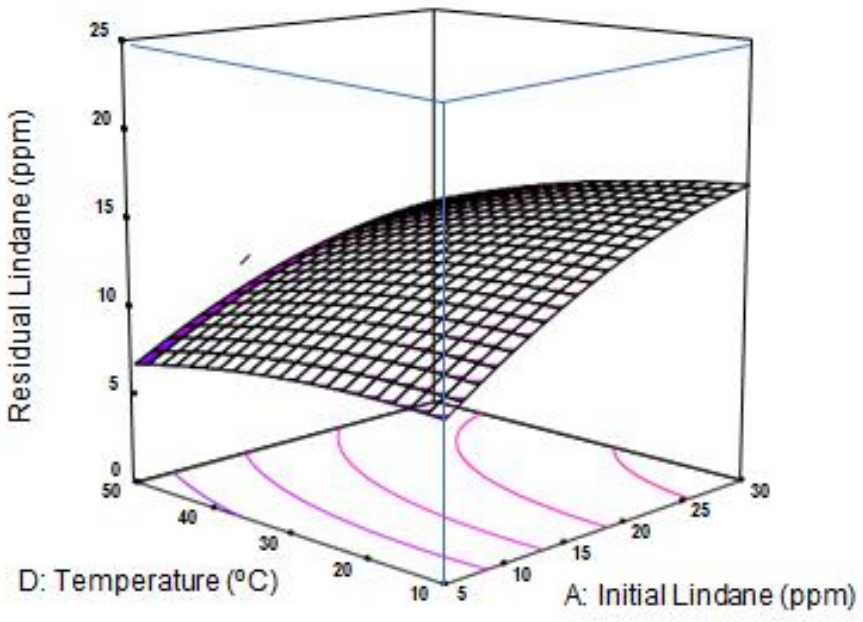


Fig. 4.45 Interaction between temperature and initial lindane concentration during the lindane degradation by the bacterial consortium (pH- 6, initial inoculum- 0.0325 OD, incubation period- 20 days)

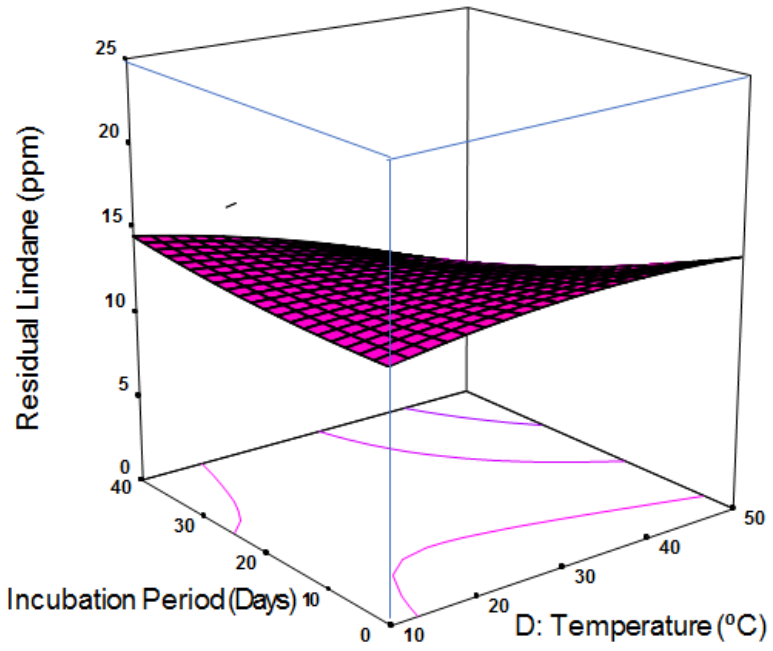


Fig. 4.46 Interaction between incubation period and temperature during the lindane degradation by the bacterial consortium (pH- 6, initial inoculum- 0.0325 OD, initial lindane concentration- 10 ppm)

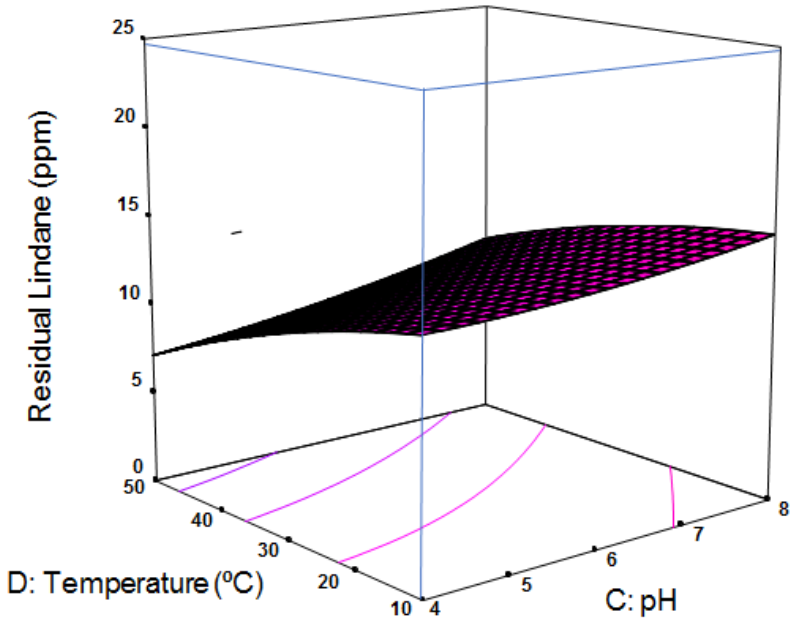


Fig. 4.47 Interaction between temperature and pH during the lindane degradation by the bacterial consortium (initial inoculum- 0.0325 OD, initial lindane concentration- 10 ppm, incubation period- 20 days)

4.4 Study for identification of individual strain

4.4.1 Morphological and biochemical behavior of bacterial strains

Morphology including cell shape, cell arrangement, and Gram staining was done with the help of a light microscope. For biochemical behavior, many tests such as catalase, nitrate reduction, indole test, H_2S production, citrate utilization test, starch hydrolysis, and gelatin hydrolysis were carried out. The response of cultures can be observed in Table 4.14. Gram's nature of bacterial strains was studied using standard Gram staining procedure which has following steps (Popescu and Doyle, 1996). The fresh culture of the strain was used for this study. Those bacteria that hold on to primary dye-iodine complex and remain violet are called Gram-positive and those which get decolorized and subsequently take up counter stain (pink/red) are called Gram-negative.

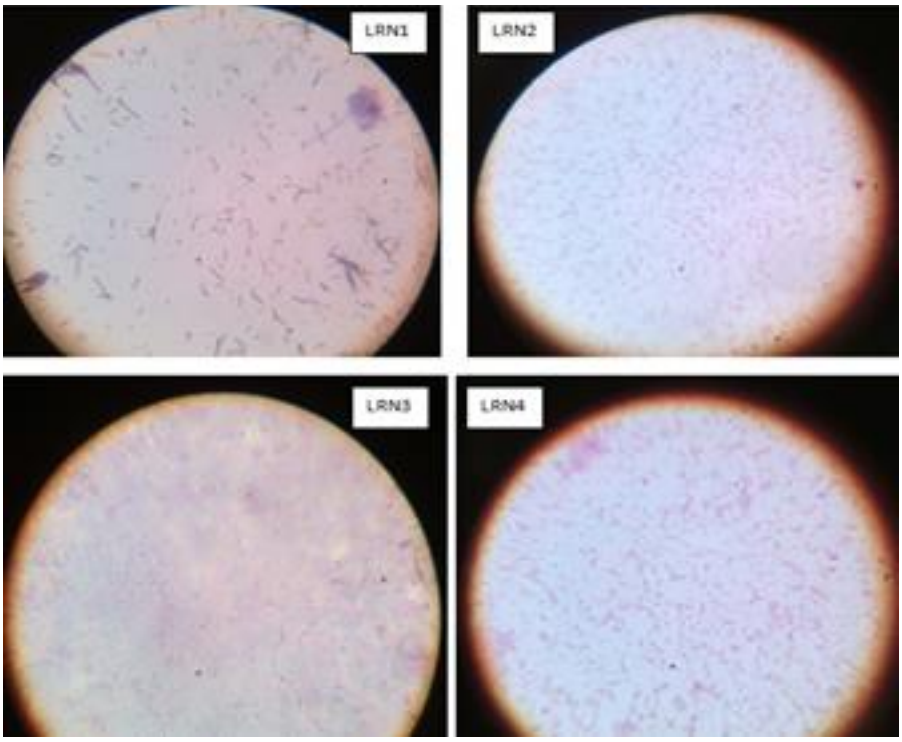


Fig. 4.48 Gram staining of individual bacterial strain

Table 4.14 Biochemical testing (triplicate) of four isolates LR_1, LR_2, LR_3 and LR_4

S. No.	Test	Bacterial strains											
		LR_1			**LR_2**			**LR_3**			**LR_4**		
1	Shape	Rods			Cocci			Short rods			Cocci		
2	Motile (Aerobic)	Non-Motile			Non-Motile			Motile			Non-Motile		
3	Motile (Anabolic)	Non-Motile			Non-Motile			Motile			Non-Motile		
4	Gram staining	+	+	+	+	+	+	+	+	+	-	-	-
5	Catalase	-	-	-	+	+	+	+	+	+	+	+	+
6	Nitrate Reduction	+	+	+	+	+	+	+	+	+	+	+	+
7	Indole Test	-	-	-	-	-	-	-	-	-	-	-	-
8	H_2S Production	-	-	-	-	-	-	-	-	-	+	+	+
9	Citrate utilization Test	-	-	-	-	-	-	-	-	-	-	-	-
10	Starch hydrolysis	-	-	-	-	-	-	+	+	+	-	-	-
11	Gelatin hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+

4.4.2 Antibiotic sensitivity of bacterial strains

The diameters of the zone of inhibition are shown in Fig 4.48. During the incubation period, the antibiotics/chemicals diffuse outward from the disks into the agar. This will create a concentration gradient in the agar which depends on the solubility of the chemical and its molecular size. The absence of growth of the organism around the antibiotic disks indicates that the respected organism is susceptible to that antibiotic and the presence of growth around the antibiotic disk indicates the organism is resistant to that particular antibiotic. This area of no growth around the disk is known as a zone of inhibition, which is uniformly circular with a confluent lawn of growth in the media. The diameters of the zone of inhibition are measured including disk using a scale (Table 4.15).

In our study, it was observed that there was the utilization of organophosphates and pyrethroids as a carbon source during the enrichment process. A similar study was done by many research teams, where 3 bacterial strains exhibited almost equal capability to utilize endosulfan as a sole carbon source. Morphological and biochemical tests are helpful for identification of bacterial strains. From a health point of view, antibiotic resistance/ susceptibility are a very important parameter. Here we used 7 antibiotics with different concentrations like 5 μg/disc, 10 μg/disc and 30 μg/disc, while Naphade *et al* (2011) used 15 different antibiotics and Kumari *et al* (2006) applied two different antibiotics with different levels, like 10 μg/ml to 50 μg/ml.

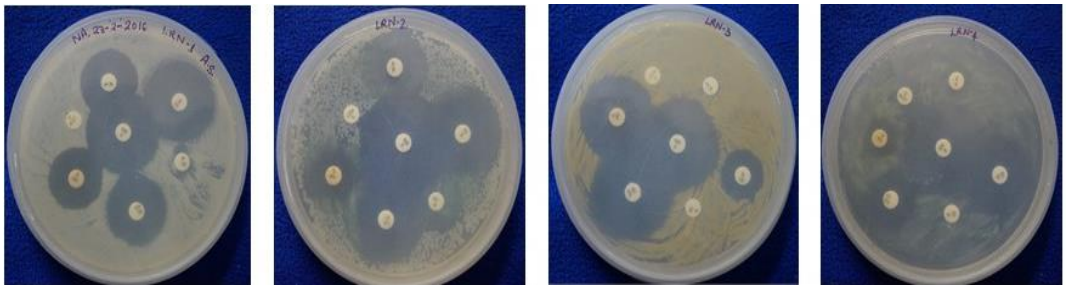


Fig. 4.49 Antibiotic sensitivity, the test was conducted using antibiotic-impregnated discs placed over the culture spread on nutrient agar and incubating for 18 h at R.T.

Table 4.15 Antibiotic sensitivity with radius (mm) of four isolates

Antibiotic	LR_1		LR_2		LR_3		LR_4	
	Sensitivity	Radius	Sensitivity	Radius	Sensitivity	Radius	Sensitivity	Radius
K-5	+	11	+	6	-	-	+	5
Nx-5	+	14	+	17	+	16	+	16
S-10	+	15	+	13	+	8	+	13
Rif-5	+	10	+	9	+	14	+	6
P-10	-	-	-	-	-	-	-	-
Cfm-5	-	-	+	10	-	-	-	-
Na-30	+	11	+	10	+	15	+	9

4.4.3 Study of individual strain for growth and lindane degradation

4.4.3.1 Growth by the individual strain

The individual bacterial strain was inoculated into 20ppm lindane with MM and samples were drawn at 0, 3, 6 and 10 days for analysis of growth. It was observed that the growth of bacterial isolates LR_2, LR_3, and LR_4 were increased at the end of 3 days while LR_1 was little enhanced. At the end of 6 days, the growth of all bacterial isolates was a decline (Fig. 4.49).

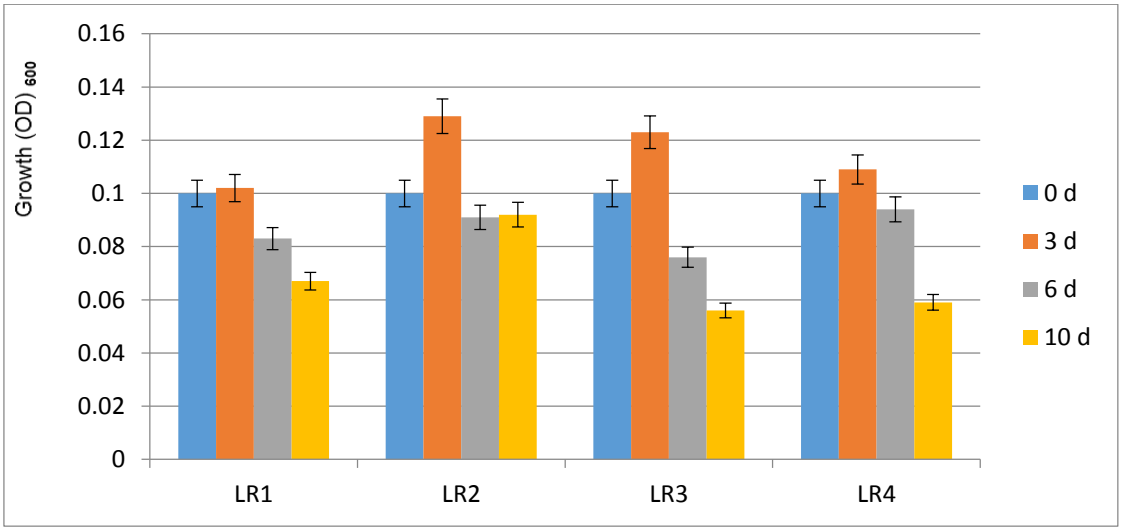


Fig. 4.50 Growth of the individual strain was inoculated to 20ppm lindane in MM

4.4.3.2 Degradation of lindane by the pure bacterial strains

The individual bacterial strain was inoculated into 20ppm lindane with minimal medium (MM) and samples were drawn at 0, 3, 6 and 10 days for analysis of the degradation of lindane. It was detected that the degradation of lindane by bacterial isolates LR_2, LR_3 and LR_4 were similar at the end of 3 days while LR_1 was more significant. Bacterial isolates LR_2, LR_3 indicated similar degradation rate in the 6 and 10 days incubation respectively. It was also observed that maximum degradation of lindane by bacterial isolate LR_4 followed by LR_1 (Fig. 4.50).

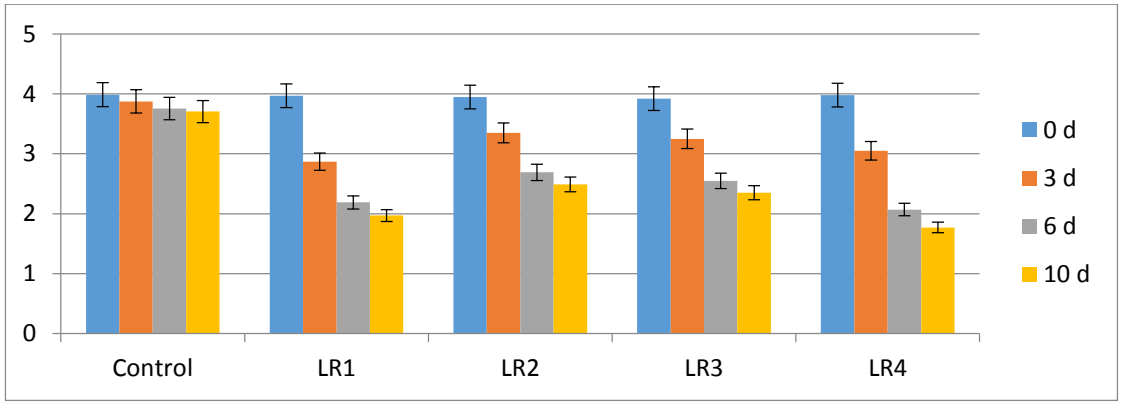


Fig. 4.51 Degradation of lindane by the individual strain

4.5 Study of combinations of bacterial strains for growth and lindane degradation

4.5.1 Growth of lindane by the combinations of bacterial strains

The various combinations by bicultural, triculture and all four of individual bacterial strain were inoculated into 20 ppm lindane with MM and samples were evaluated at 0 and 6 days for growth. Maximum growth was observed by LR_3 + LR_4 and followed by LR_1 + LR_4. The combination of LR_1 + LR_3 showed that growth was maximum decline the binary culture system. The best combination of triculture was LR_1 + LR_2 + LR_4 followed by LR_2 + LR_3 + LR_4 are strained. Maximum decline of growth was observed by LR_1 + LR_3 + LR_4. It was indicated that LR_1 and LR_3 showed the antagonistic effect (Fig. 4.52). Combination of all four bacterial strains was also declined of growth.

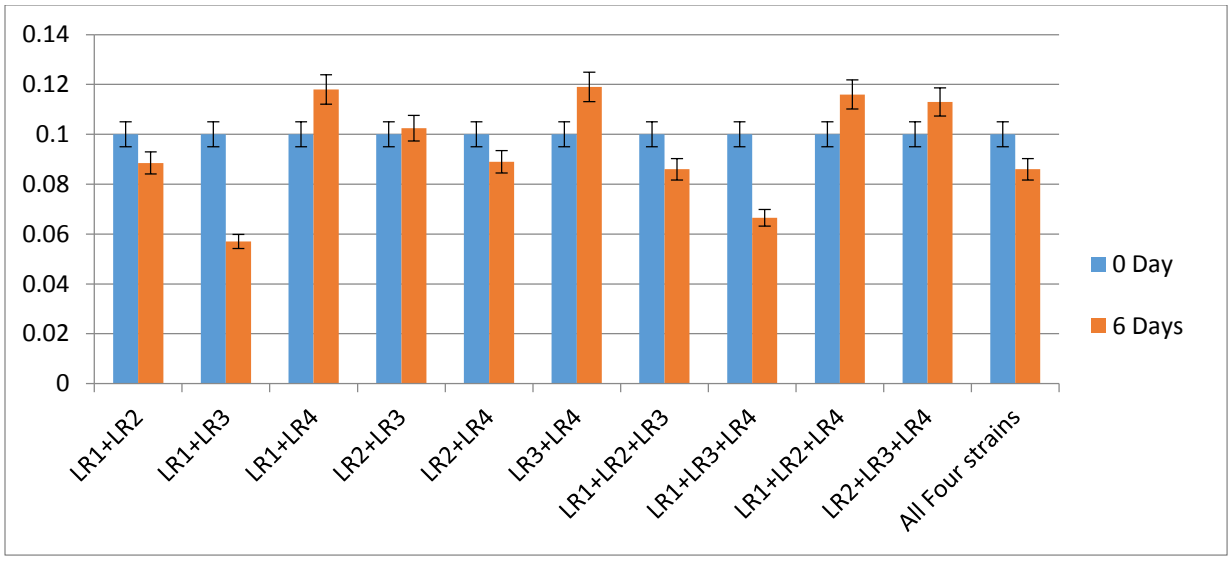


Fig. 4.52 Growth of the various combinations of bacterial strain were inoculated to 20ppm lindane

4.5.2 Degradation of lindane by the combinations of bacterial strains

The various combinations of binary culture system, ternary culture system and all four of individual bacterial strain were inoculated into 20 ppm lindane with MM and samples were evaluated at 0 and 6 days for degradation of lindane. In binary culture system, maximum degradation was observed by $LR_3 + LR_4$ and followed by $LR_1 + LR_4$. In ternary culture system maximum degradation was observed by $LR_2 + LR_3 + LR_4$ followed $LR_1 + LR_2 + LR_3$. These best combinations are applied as a defined consortium having two or three cultures on contaminated site of the similar load for effective treatment. Combination of all four cultures was not significant for degradation of lindane.

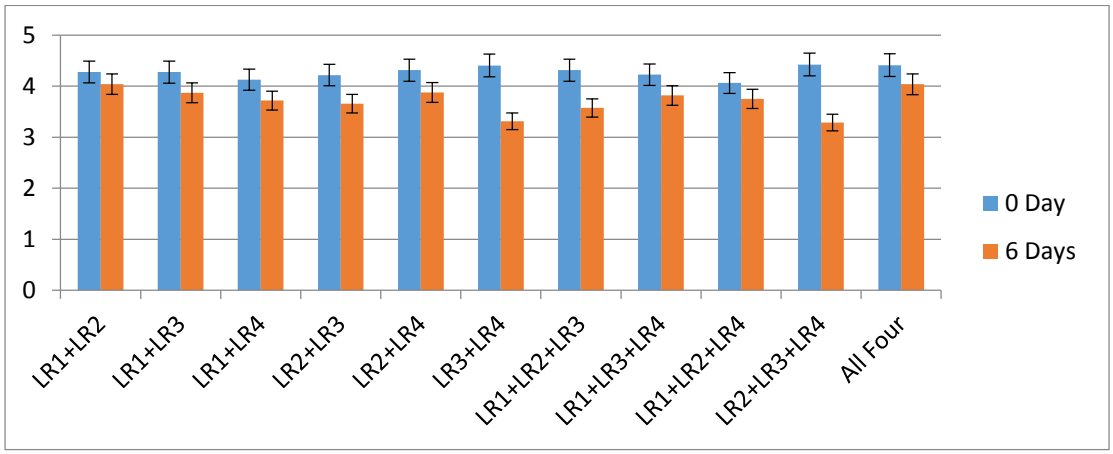


Fig. 4.53 Degradation of lindane by the combination of bacterial strains was inoculated to 20ppm lindane in MM

4.6 Growth and degradation of lindane in presence of different carbon sources

4.6.1 Growth and degradation of lindane in presence of glucose

4.6.1.1 Growth of individual strain in presence of glucose

The individual bacterial strain was inoculated into glucose medium with 20 ppm lindane for 0 to 5 days. Samples were observed every day for analysis of growth (Fig. 4.54). It was observed that the growth of bacterial isolate LR_3 was maximum increased around four times to initial inoculum within 24 h. The growth pattern of all strains was same except LR_4. At the end of 5 days, the maximum growth was observed in LR_3 bacterial strain than other strains. The growth pattern of all four strains was clear indication that glucose was being utilized as a good carbon source for the growth.

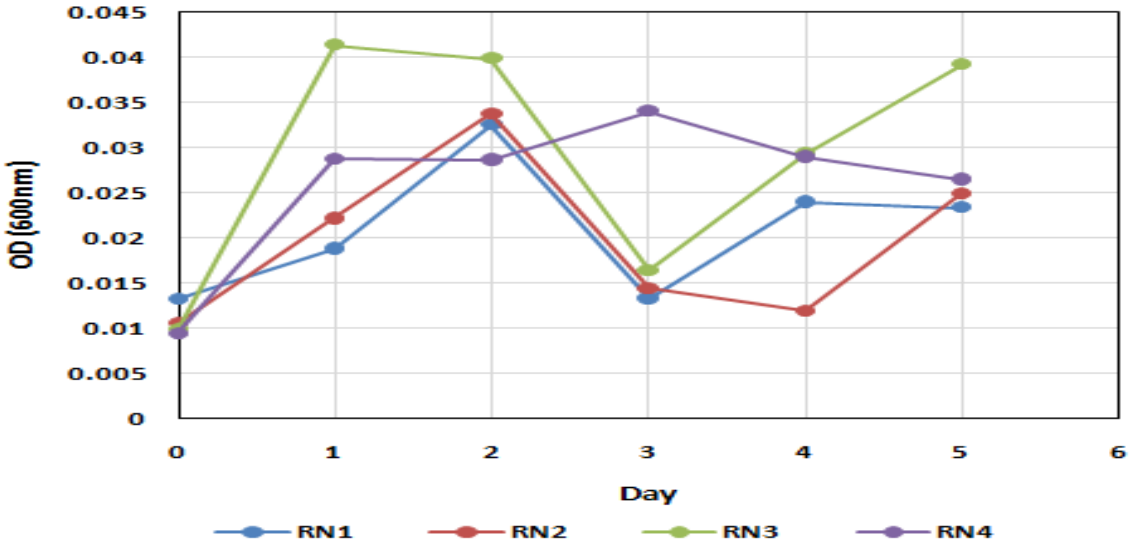


Fig. 4.54 Growth of the individual strain when inoculated to 20ppm lindane in presence of glucose

4.6.1.2 Degradation of lindane in presence of glucose

The individual bacterial strain was inoculated into glucose medium with 20 ppm lindane and sampling duration 0 to 5 days for analysis of the degradation of lindane. It was observed that around 50% lindane was degraded by bacterial isolates LR_3 within 24 h. It was also observed

that maximum (80%) degradation of lindane by three bacterial isolates LR_4 and LR_3 followed by LR_2 (Fig. 4.55).

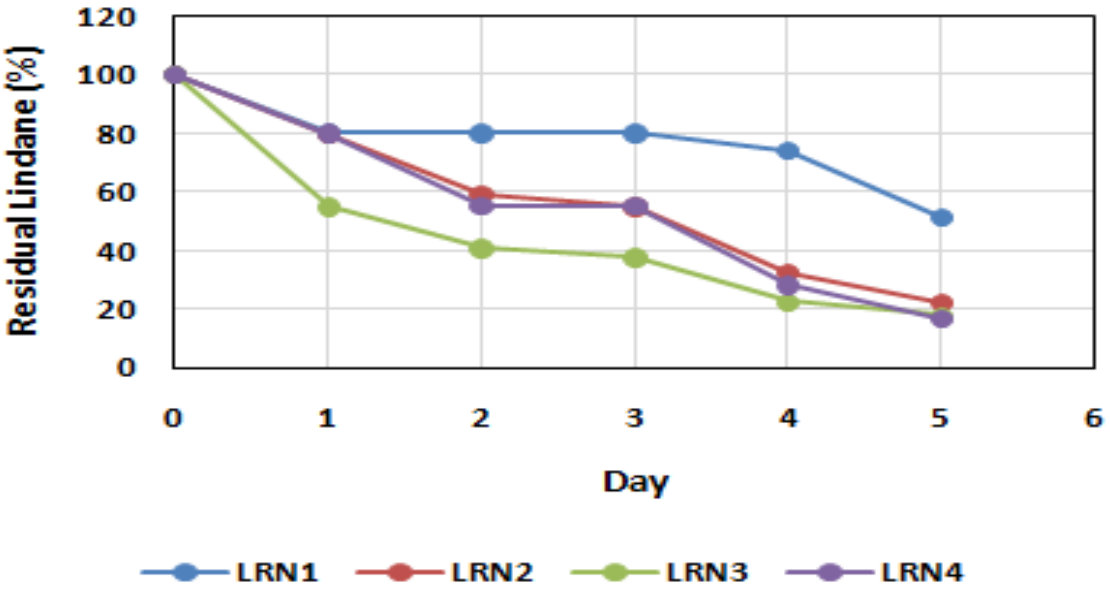


Fig.4.55 Degradation of lindane by the individual strain. The individual strain was inoculated to 20ppm lindane in presence of glucose

4.6.2 Growth and degradation of lindane in presence of peptone

4.6.2.1 Growth of individual strain in presence of peptone

The individual bacterial strain was inoculated in presence of peptone with 20 ppm lindane for 0 to 5 days. Samples were observed every day for analysis of growth (Fig. 4.56). It was observed that the maximum growth by bacterial isolate LR_2 within 48 h. The growth pattern (log phase) of all strains was same except LR_1 i.e. minimum growth at the end of 3 days. At the termination of the experiment, the growth pattern of all four strains was same to each other. Due to such pattern of growth was clear indication that peptone was existence used as a good carbon source for the growth of all four strains.

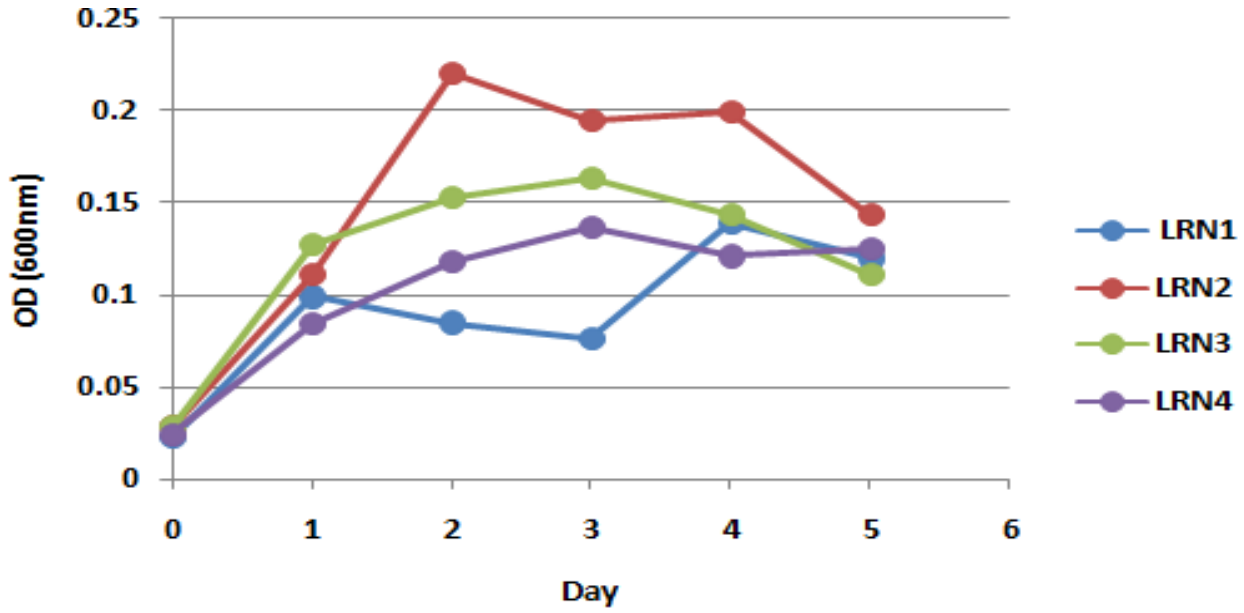


Fig. 4.56 Growth of the individual strain was inoculated to 20ppm lindane in presence of peptone

4.6.2.2 Degradation of lindane in presence of peptone

The individual bacterial strain was inoculated in presence of peptone with 20 ppm lindane and sampling duration 0 to 5 days for analysis of the degradation of lindane. It was detected that initially 24 h, around 30% lindane was degraded by bacterial isolates LR_3. It was also observed that maximum (50%) degradation of lindane by LR_3. The minimum degradation of lindane performed was by bacterial isolates LR_2 andLR_4 (Fig. 4.57).

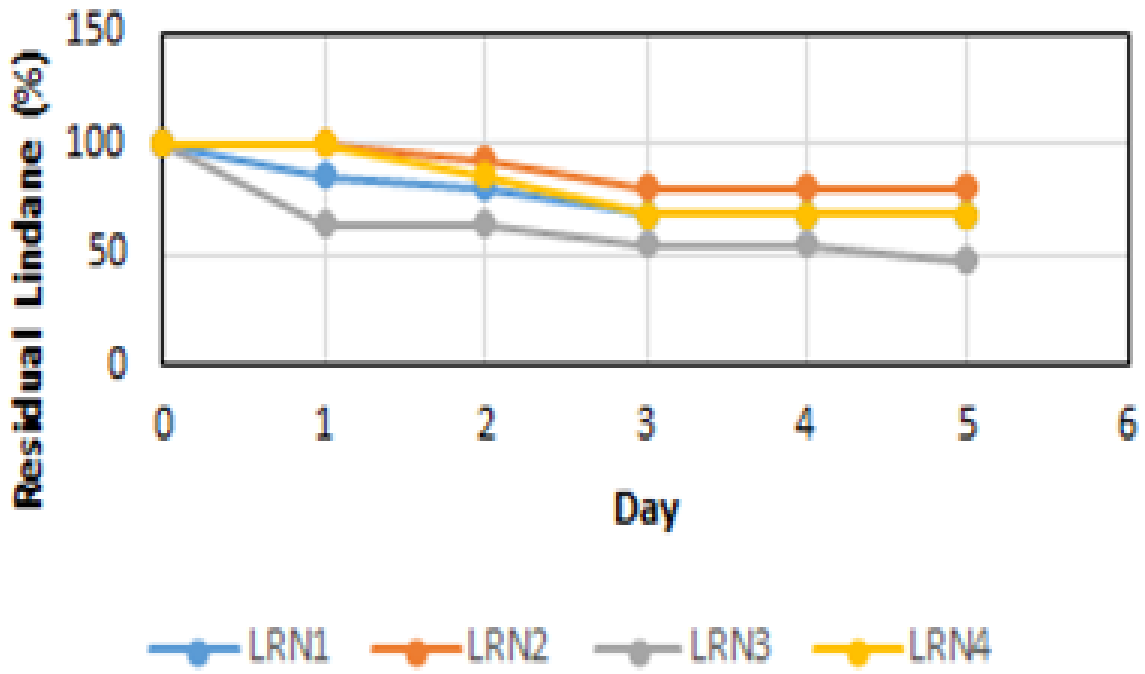


Fig. 4.57 Degradation of lindane by the individual strain. The individual strain was inoculated to 20ppm lindane in presence of peptone

4.6.3 Growth and degradation of lindane in presence of Tween 80

4.6.3.1 Growth of individual strain in presence of Tween 80

Tween 80 to 20 ppm lindane was supplemented for analysis of growth by individual bacterial strain incubated for 0 to 7 days. The log phase of growth was observed by two bacterial strains LR_2 and LR_4, while rest two bacterial strains LR_1 and LR_3 was sustained in lag phase of growth after 7 days (Fig. 4.58). 48 h. It was observed that the maximum growth by bacterial isolate LR_2 within 48 h. It was also observed that the maximum growth by bacterial isolate LR_2 at the end of 7 days. The pattern of growth was indicated that tween 80 was survival for the growth of two bacterial strains LR_2 and LR_4.

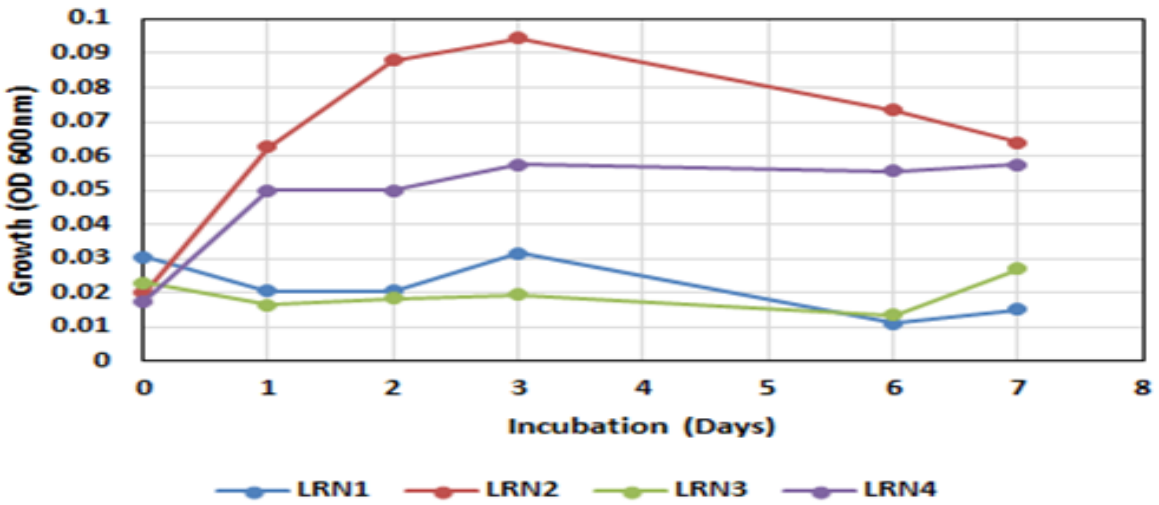


Fig. 4.58 Growth of the individual strain was inoculated to 20ppm lindane in presence of tween 80

4.6.3.2 Degradation of lindane in presence of Tween 80

Fig. 4.59 indicates that tween 80 supplemented medium showed the degradation of lindane by individual bacterial strain. It was observed that around 50% lindane was degraded by bacterial isolates LR_3 at the end of 5 days; the surprise in case of bacterial isolates LR_1 there was nil degradation of lindane after 3 days. It was also observed that maximum degradation of lindane by bacterial isolates LR_3 followed by LR_{24} and LR_2 (Fig. 4.59).

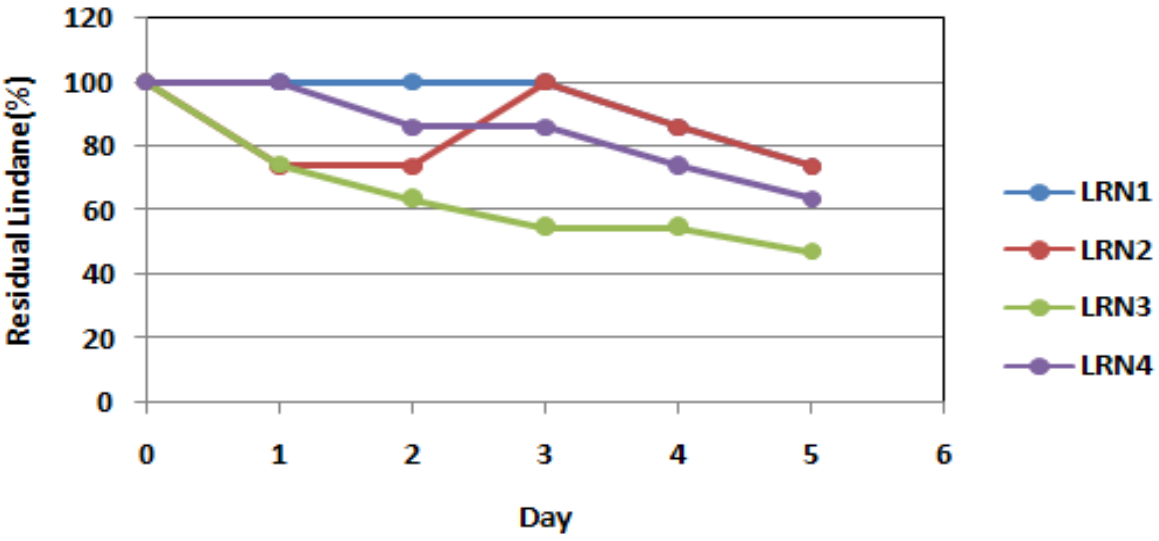


Fig. 4.59 Degradation of Lindane by the individual strain. The individual strain was inoculated to 20ppm lindane in presence of tween 80

4.6.4 Growth and degradation of lindane in presence of Triton X- 100

4.6.4.1 Growth of individual strain in presence of Triton X- 100

In presence of Triton X-100 with 20 ppm lindane, the growth of different strains was improved in first 24 h i.e. maximum growth by all four bacterial strains. It was observed that out of four bacterial strains, the maximum growth by bacterial isolate LR1 within 24 h and minimum after 5 days. At the end of 5 days, the maximum growth was observed in LR_2 bacterial strain, while other strains were similar growing (Fig. 4.60). The overall growth of all four strains was clear indication that Triton X-100 was being utilized as a good carbon source for the growth.

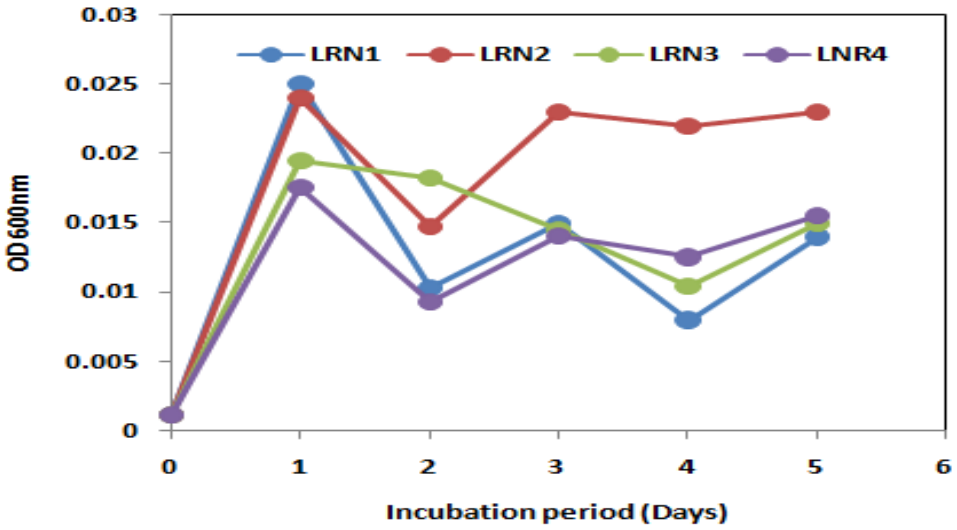


Fig. 4.60 Growth of the individual strain was inoculated to 20ppm lindane in presence of Triton X- 100

4.6.4.2 Degradation of lindane in presence of Triton X- 100

The individual bacterial strain was inoculated in presence of Triton X-100 medium with 20 ppm lindane and sampling period 0 to 5 days for analysis of the degradation of lindane. The degradation rate of lindane was similar to degradation in peptone medium. It showed that maximum (70%) degradation of lindane by bacterial isolates LR_3 than other strains in presence of Triton X-100 surfactant.

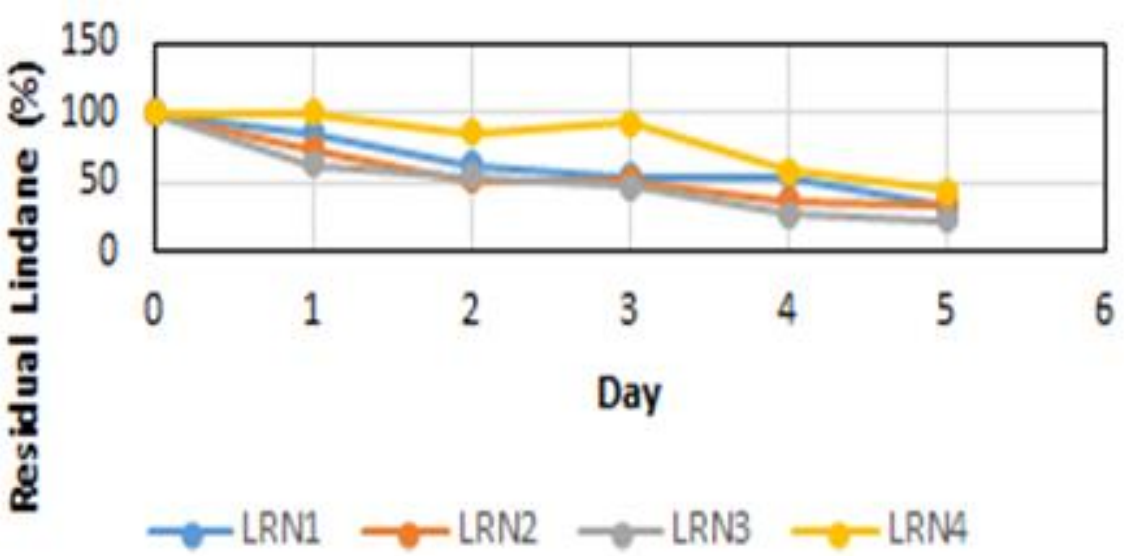


Fig. 4.61 Degradation of Lindane by the individual strain. The individual strain was inoculated to 20ppm lindane in presence of Triton X- 100

4.7 Molecular characterization of lindane degrading bacterial strains

4.7.1 Isolation of genomic DNA

Single bacterial colonies were picked to isolate DNA using QIAamp DNA Mini Kit. Lysis buffer AL and ATL with proteinase K and RNase a helped to cleave protein and RNA in small fragments.

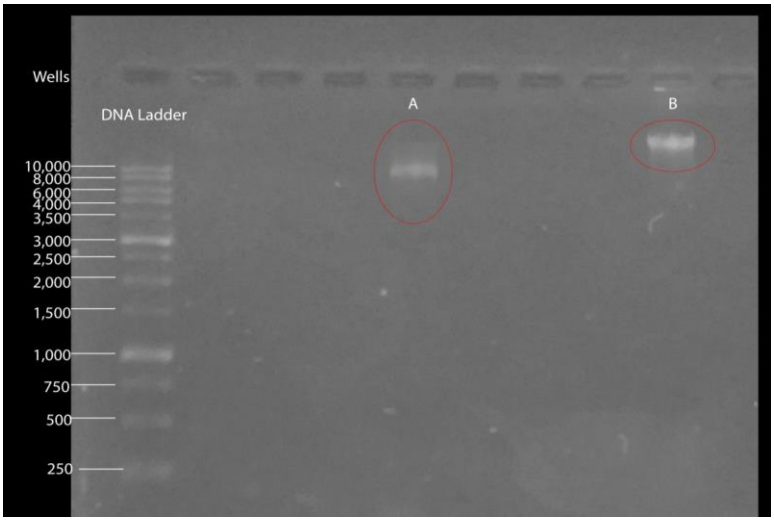


Fig. 4.62 Isolation of genomic DNA on gel

Buffer AW1 and AW2 helped to dissolve protein and RNA fragments Buffer AE was used to elute DNA from the column into microcentrifuge tubes by centrifuging at 11,000 for 3 min. The ratio of absorbance at 260/280 shows contamination level of samples with protein.

4.7.2 PCR of genomic DNA

Pure DNA sample has 260/280 nm of 1.8. Universal forward primer 8F Sequence 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse 928R with Sequence 5'-CCCTCAATTCCTTTGAGTT-3' were used to amplify approximately 900 bp long region of 16S rDNA. PCR program of 30 cycles with a denaturation temperature of 95°C for 45 sec, the annealing temperature of 55°C for 45 sec and elongation temperature of 72°C for 1 min was used.

4.7.3 Purification of PCR product

PCR amplified products were purified by using NucleoSpin gel extraction and PCR cleanup kit to remove contamination of dNTPs, primer dimers, template, buffer and protein enzyme that can interfere with DNA sequencing reactions. DNA binds to a silica membrane in the presence of chaotropic salt added by Binding Buffer NT. Contaminations like salts and soluble macromolecular components are removed by a simple washing step with ethanolic Wash Buffer NT3. Pure DNA was finally eluted under low ionic strength conditions with slightly alkaline Elution Buffer NE in the microcentrifuge tubes.

4.7.4 Quantification of genomic DNA

The absorption spectrum of DNA samples was used to determine the concentration of DNA and protein content. The ratio of absorbance at 260 nm and 280 nm showed the level of contamination with protein molecules. Samples with an approximate ratio of 1.8 are considered free from protein contamination. The ratio at 260/280 nm is sensitive to protein whereas 260/230 nm is insensitive to protein concentration but used to determine RNA contamination.

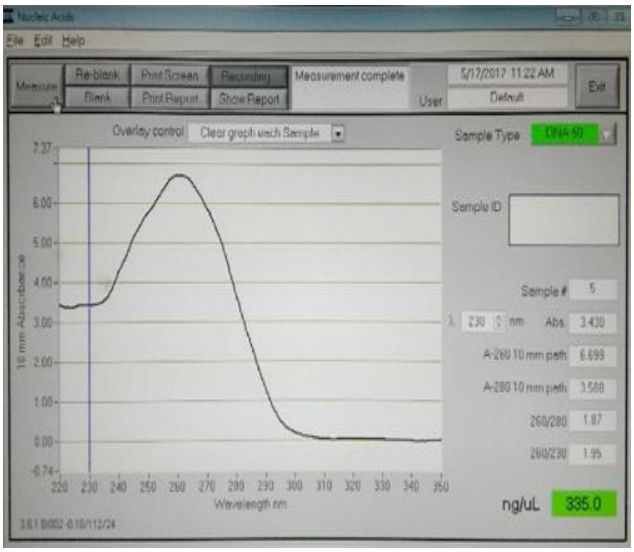


Fig. 4.63 Absorption Spectra of a DNA sample

Table 4.16 Quantification of genomic DNA

S.No.	PCR product before purification		PCR product after purification	
	$A_{260/280}$	Concentration (ng/µl)	$A_{260/280}$	Concentration (ng/µl)
1.	1.85	361.8	1.8	42.3
2.	2	335	1.87	58.4
3.	1.92	334.9	1.84	72.9
4.	1.88	375.4	1.86	46.7

Quantification results obtained from 1000 Nanodrop Spectrophotometer

4.7.5 Electrophoresis of PCR product (16S rDNA)

Electrophoresis in 2% agarose gel and at 150V potential difference was used to separate DNA fragments. 1kb DNA ladder was used to compare the length of amplified fragments. The gel was visualized under UV transilluminator. Visualization of gel showed single bands of DNA fragment. This signifies that the samples were free from contamination and the target region of 16S rDNA

was properly amplified in the PCR. The sample that was amplified twice in PCR showed brighter bands due to a higher concentration of DNA.

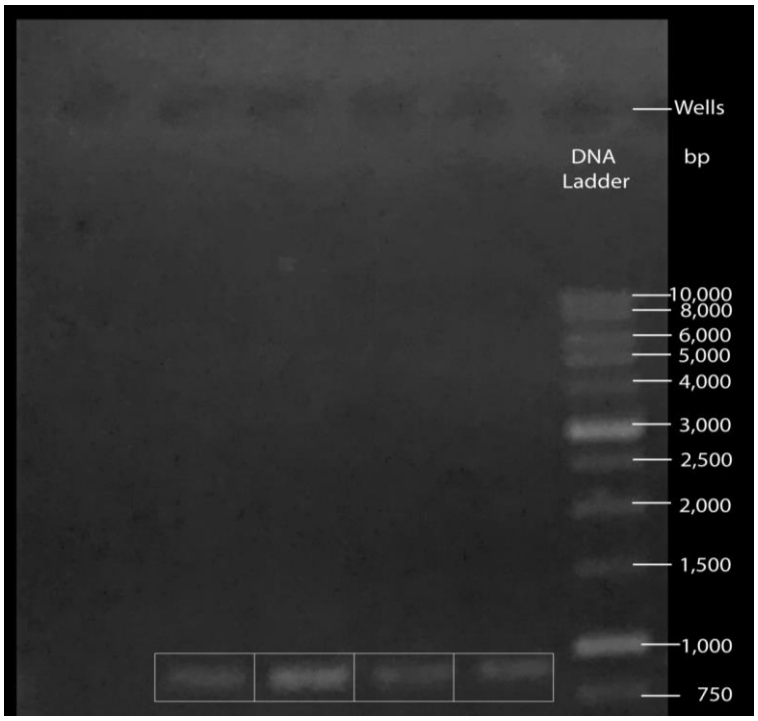


Fig. 4.64 16 S rDNA fragments of four strains under UV transilluminator

4.7.6 Analysis of sequences of 16S rDNA

Sequencing of amplified 16S rDNA fragments using Applied Biosystems® Genetic Analyzers 3130 by Sanger's chain termination method. The analyses of 16S rDNA sequence of four strains are following**.**

4.7.6.1 Strain LR_1

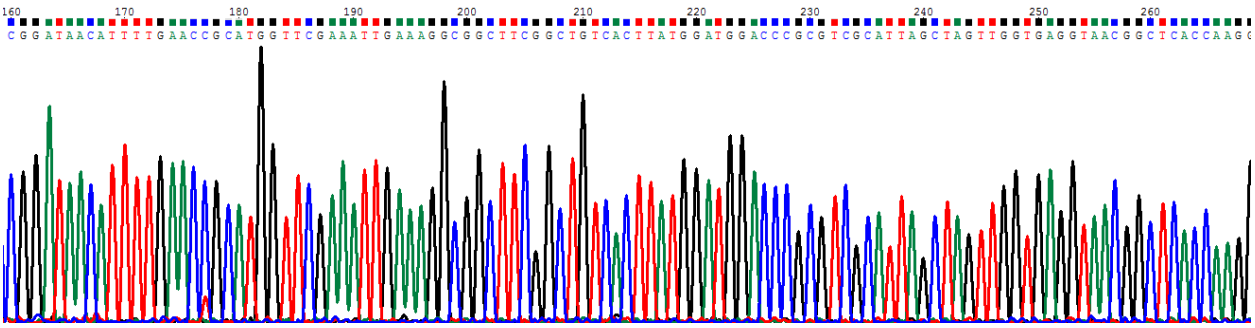


Fig. 4.65 DNA sequence chromatogram of LR_1 strain

DNA Sequence in FASTA format of bacteria strain LR_1

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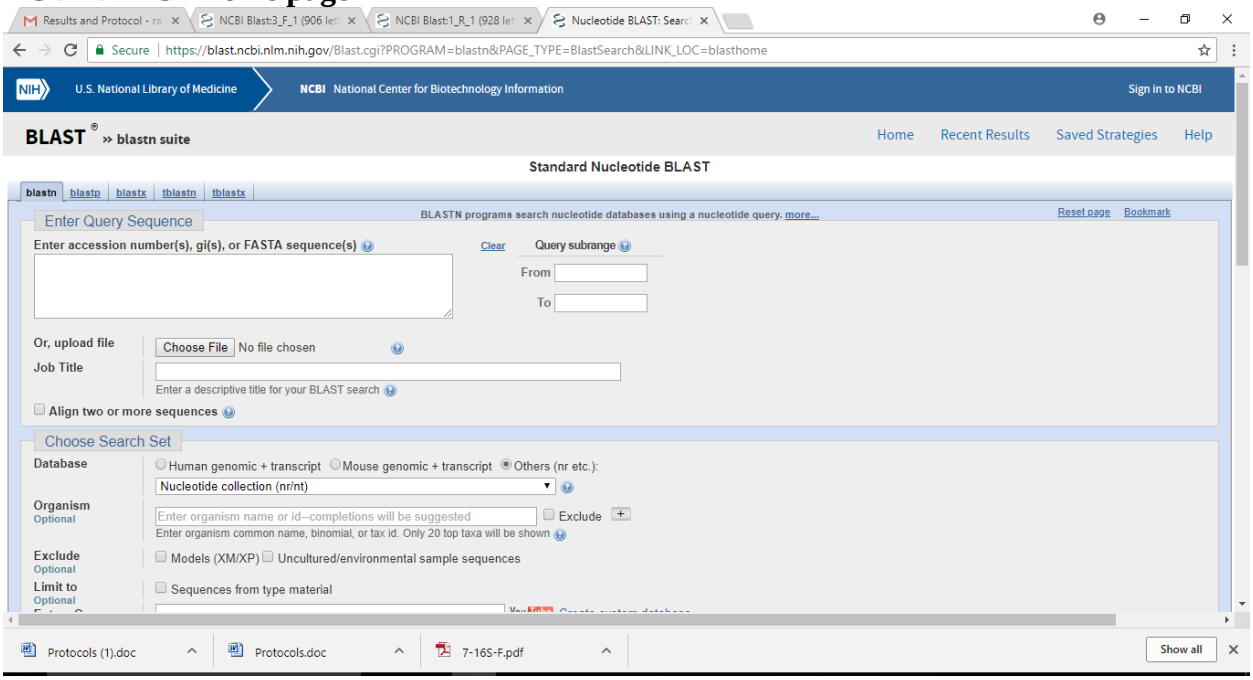
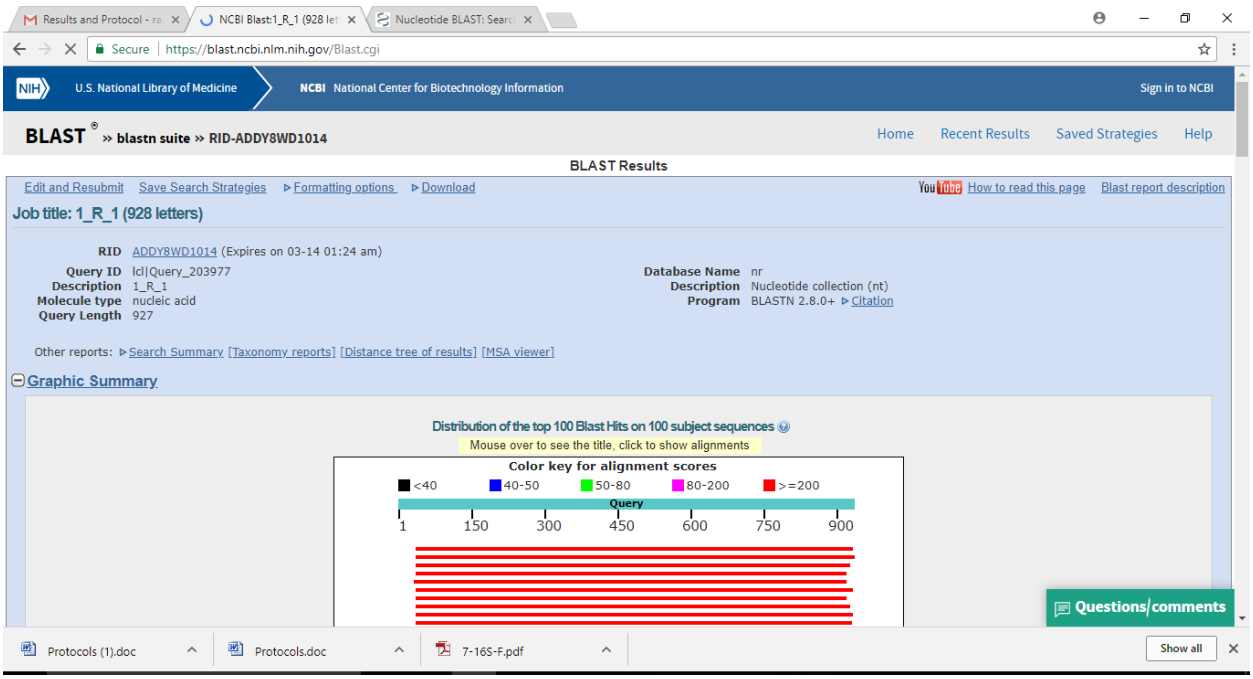
NCBI BLAST home page



BLAST Result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Home
Recent Results
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Edit and Resubmit
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Program BLASTN 2.8.0+ Citation
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Graphic Summary
Distribution of the top 100 Blast Hits on 100 subject sequences
Mouse over to see the title, click to show alignments
Color key for alignment scores
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50-80
80-200
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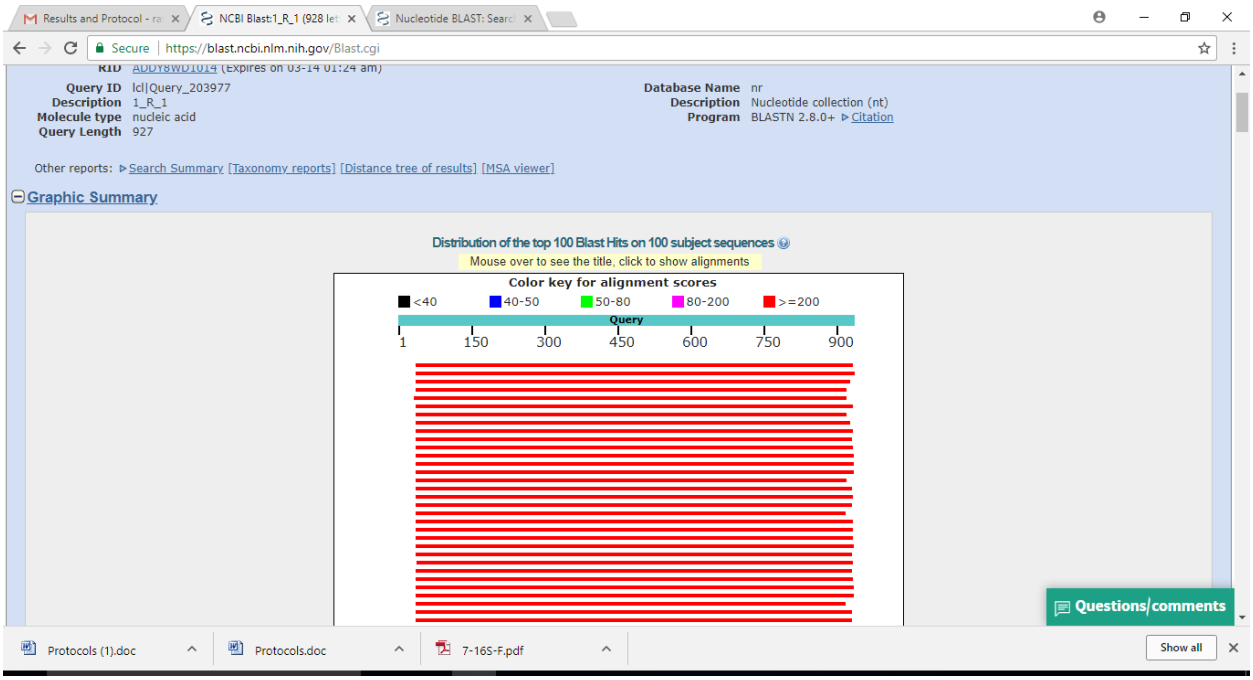
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Graphic Summary
Distribution of the top 100 Blast Hits on 100 subject sequences
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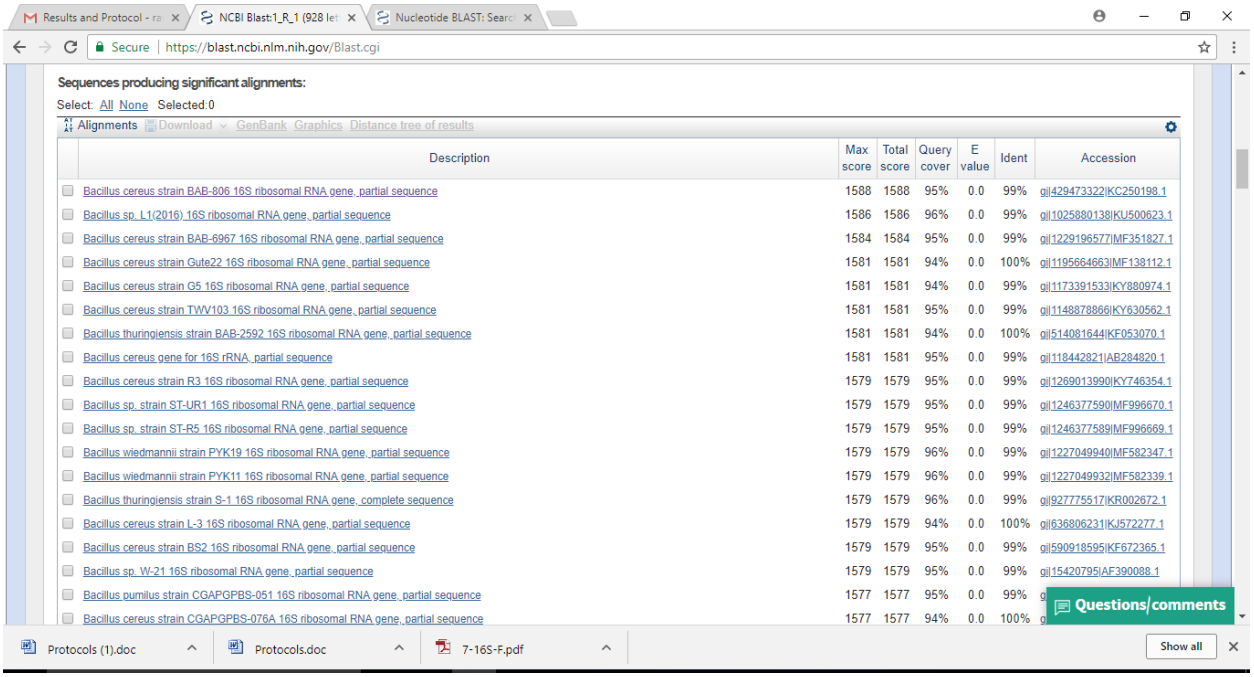
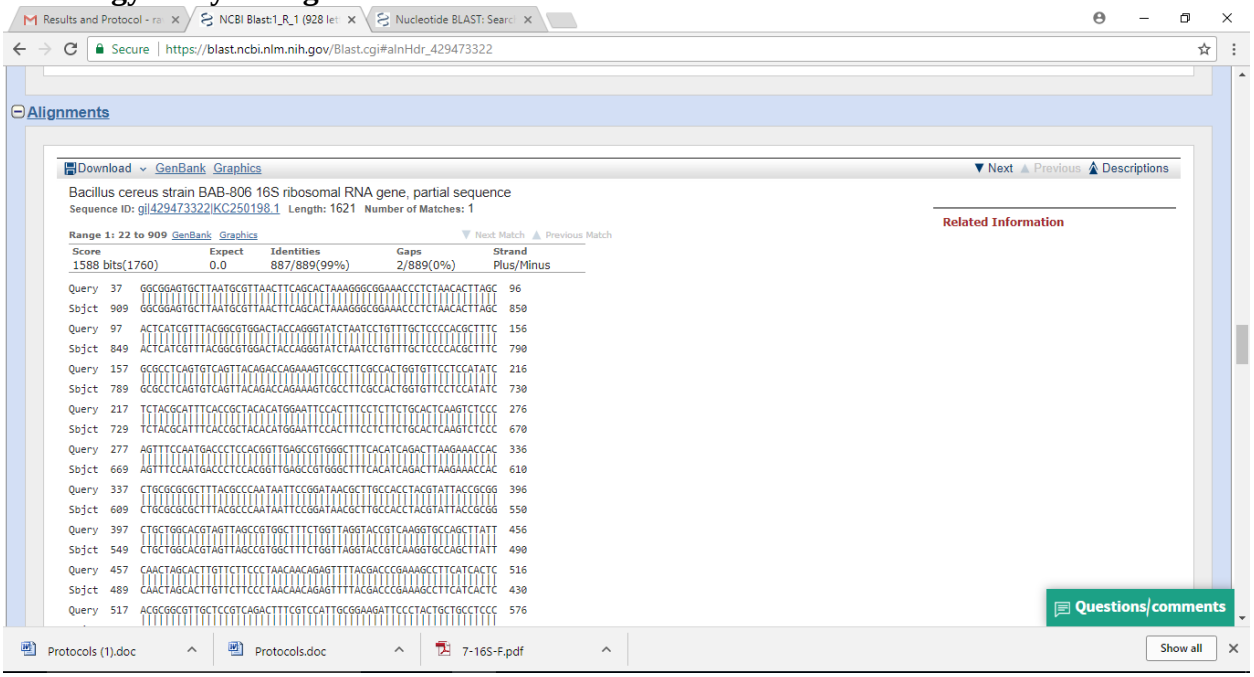


Homology analysis/alignment



Figs. 4.66 Blast results of strain LR_1

The BLAST results indicate a good match with DNA database with over 100% homology and an e-Value of 0.0. The microbe in the present study was found to be from the *Bacillus cereus*.

4.7.6.2 Strain LR_2

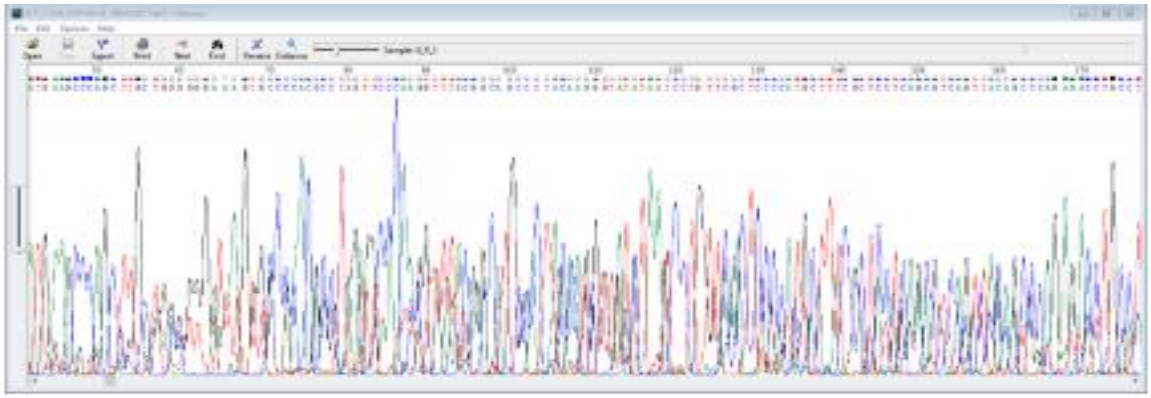


Fig. 4.67 DNA sequence chromatogram of LR_2 strain

DNA Sequence in FASTA format

>CTTAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGCA
GCACGGGTACTTGTACCTGGTGGCGAGCGGCGGACCCGTGAGTAATGCCTAGGAATCTGCCTAGTAGT
GGGGGATTACGCTCGGAAACGGACTGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTT
CGGGCCTTGCGCTATCAGATGAGCGCCCAGGTCGGAATAGCTNGTNNGTGAGCTAATGGCTNCACCAA
GGCGACGATCCNNTAACTGGTCNNAGAGGATGNCAGTCACACTGGAANGAGACACGGTCCANACTCC
TACGGGAGGNAGCNGTGGGGAATATTGGACAATGGGCGAAAGCNNGATCCAGCCATGCCGCGTGTNN
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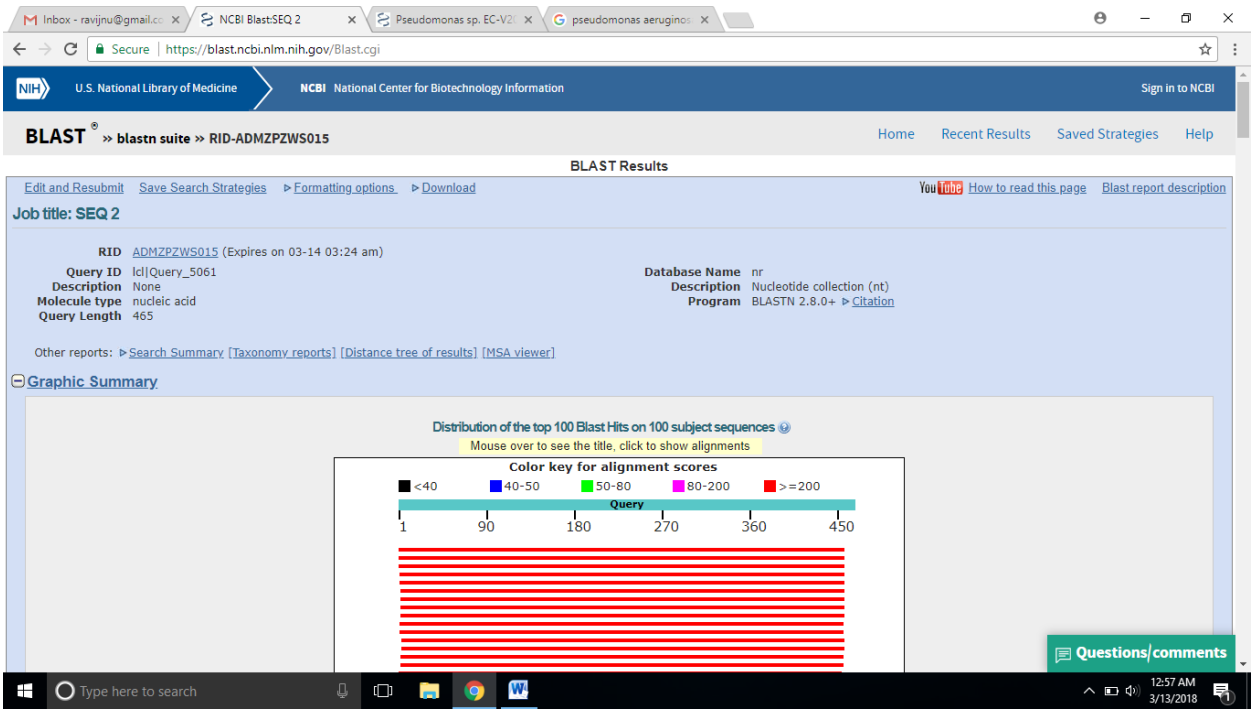
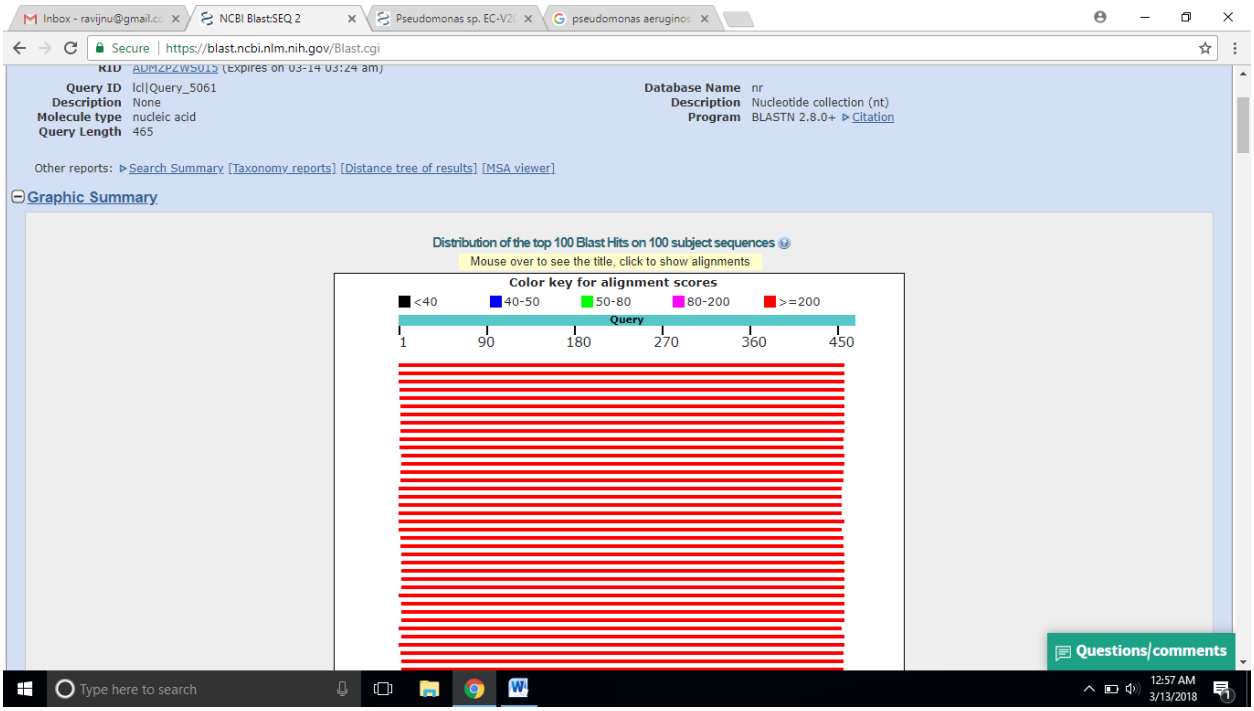
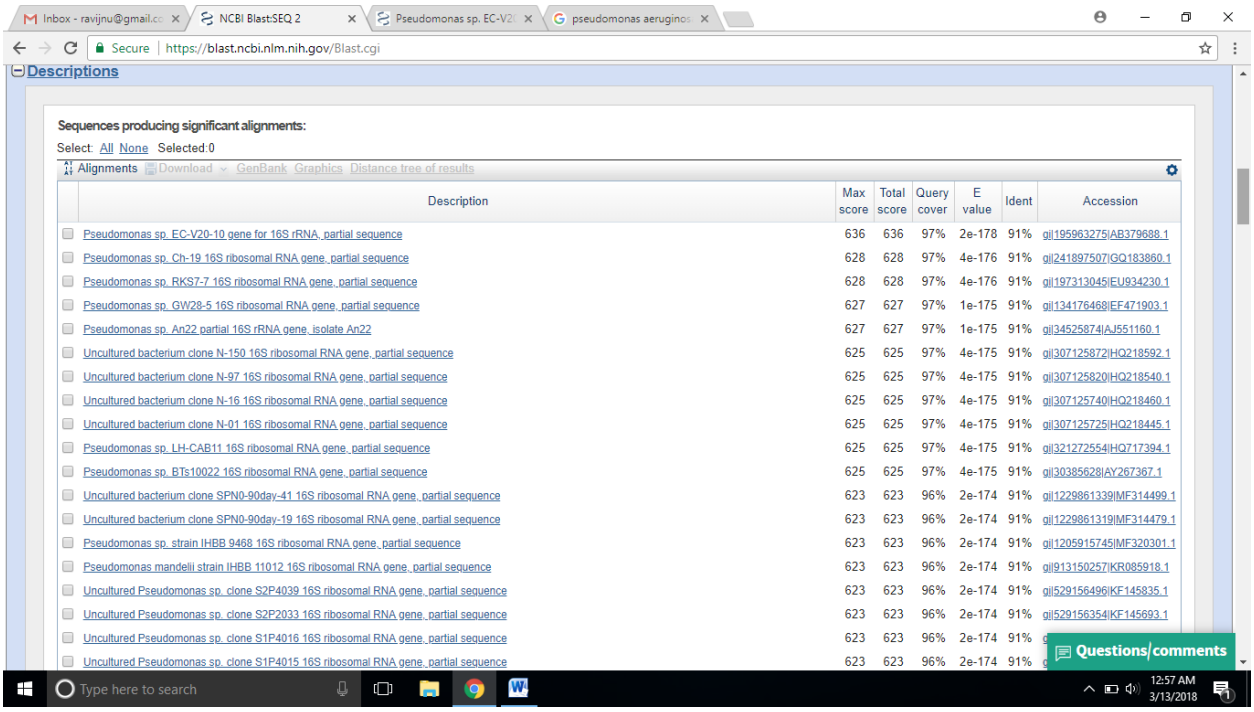
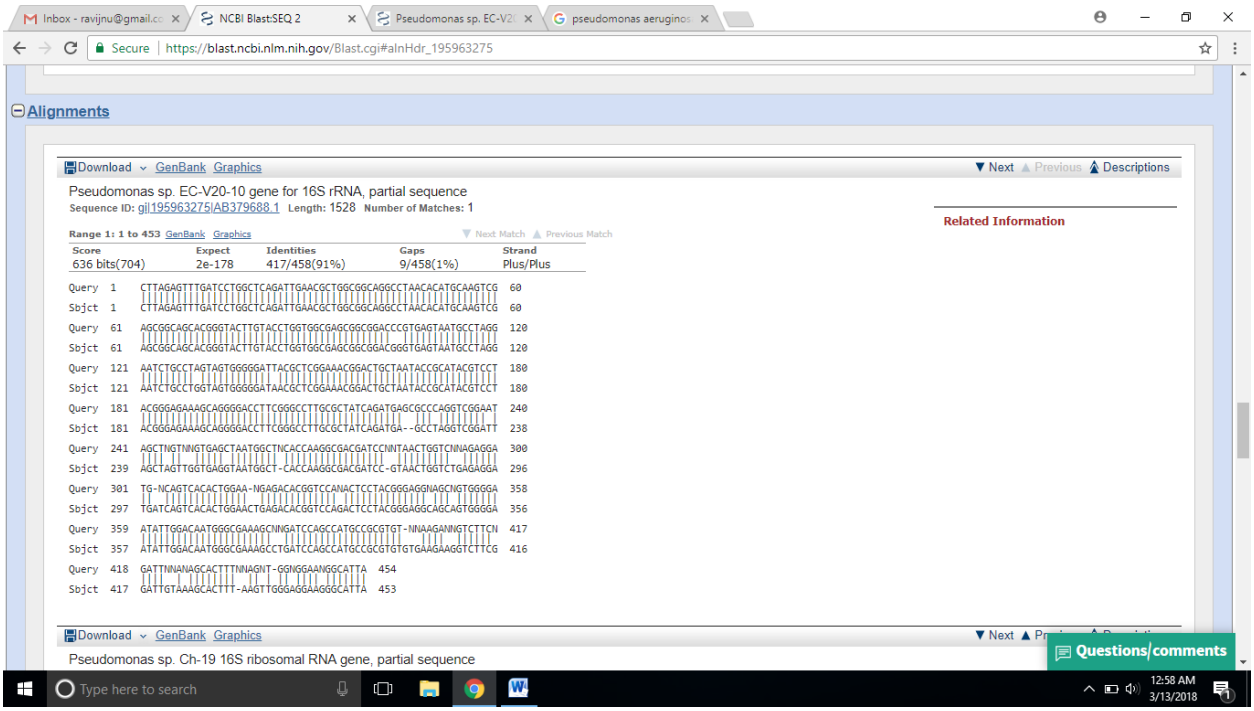
NCBI BLAST







Homology analysis/alignment



Figs. 4.68 Blast results of strain LR_2

The BLAST results indicate a match with DNA database with over 97% homology and an e-Value of 2e-178. The microbe in the present study was found to be from the *Pseudomonas* sp.

4.7.6.3 Strain LR_3

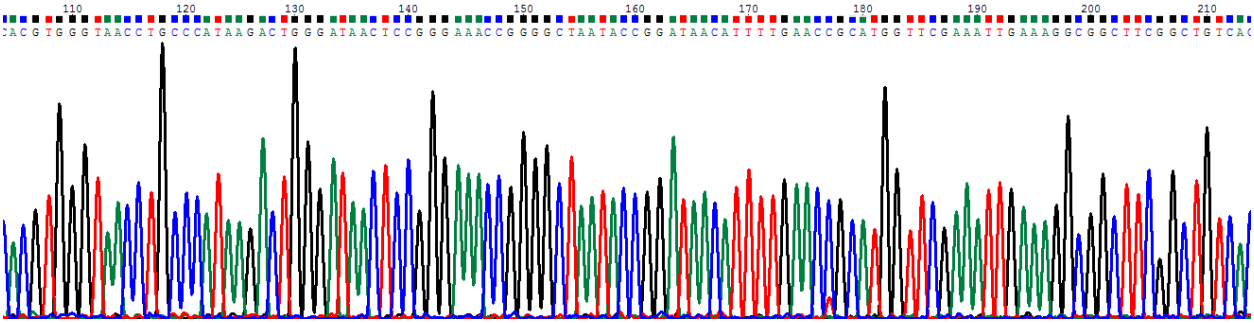


Fig. 4.69 DNA sequence chromatogram of strain LR_3

DNA Sequence in FASTA format

>CACATTCCCTCTACGTTTTTTTCTAAGGAGGGGCGCAACCATGCAGCCGAGCGGTAGAGATCTTTCGG
GATCTTGAGAGCGGCGTACGGGTGCGGAACACGTGTGCAACCTGCCTTTATCTGGGGGATAGCCTTTC
GAAAGGAAGATTAATACCCCATAATATACTGAATGGCATCATTCGGTATTGAAAACTCCGGTGGATAG
AGATGGGCACGCGCAAGATTAGATAGTTGGTGAGGTAACGGCTCACCAAGTCTGCGATCTTTAGGGGG
CCTGAGAGGGTGATCCCCCACACTGGTACTGAGACACGGACCAGACTCCTACGGGAGGCAGCAGTGA
GGAATATTGGACAATGGGTGAGAGCCTGATCCAGCCATCCCGCGTGAAGGACGACGGCCCTATGGGTT
GTAAACTTCTTTTGTATAGGGATAAACCTACCCTCGTGAGGGTAGCTGAAGGTACTATACGAATAAGC
ACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTATCCGGATTTATTGGGT
TTAAAGGGTCCGTAGGCGGATCTGTAAGTCAGTGGTGAAATCTCACAGCTTAACTGTGAAACTGCCAT
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GGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGCTAACTCGTTTTTGGAATG

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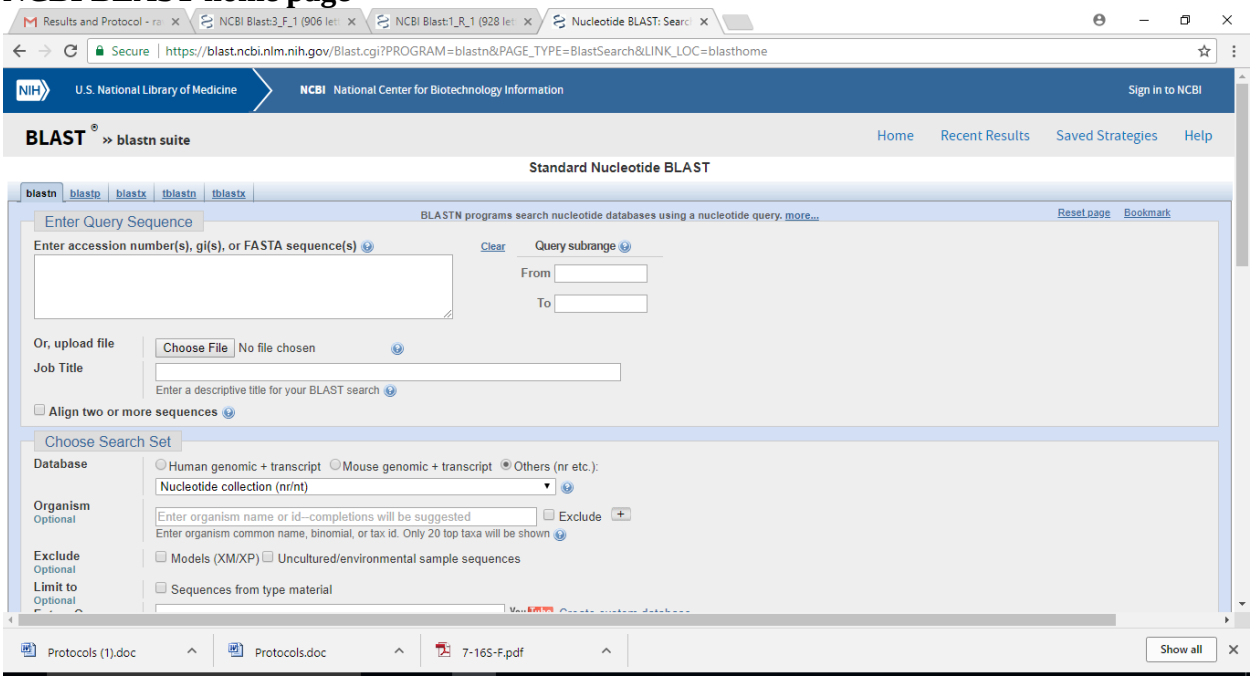
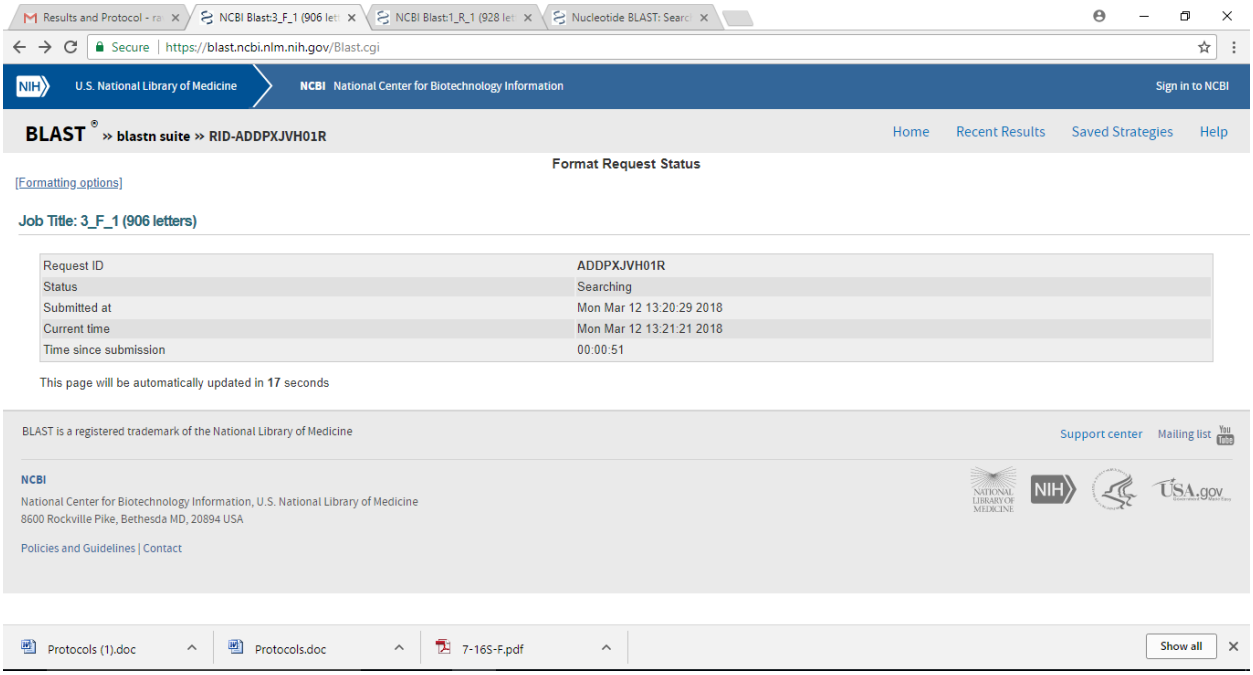
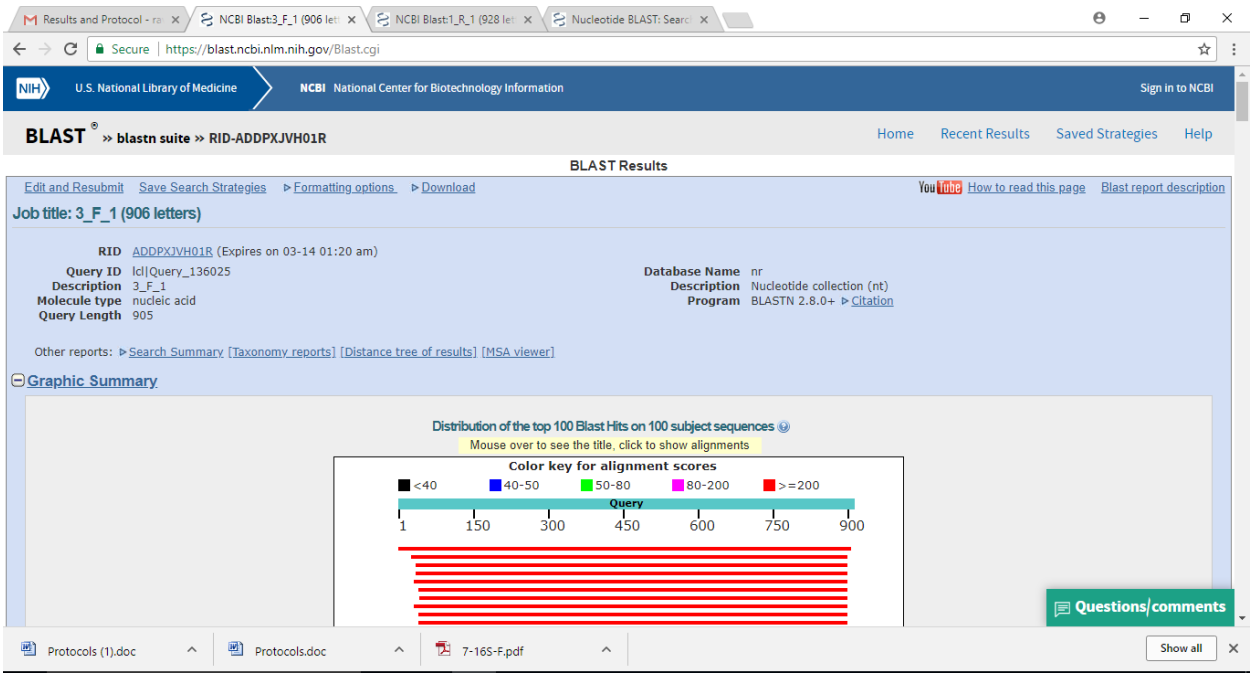
NCBI BLAST home page



BLAST result




Results and Protocol - ra
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Nucleotide BLAST: Searc
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Home
Recent Results
Saved Strategies
Help
BLAST Results
Edit and Resubmit
Save Search Strategies
Formatting options
Download
How to read this page
Blast report description
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Molecule type nucleic acid
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Description Nucleotide collection (nt)
Program BLASTN 2.8.0+ Citation
Other reports: Search Summary [Taxonomy reports] [Distance tree of results] [MSA viewer]
Graphic Summary
Distribution of the top 100 Blast Hits on 100 subject sequences
Mouse over to see the title, click to show alignments
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Questions/comments
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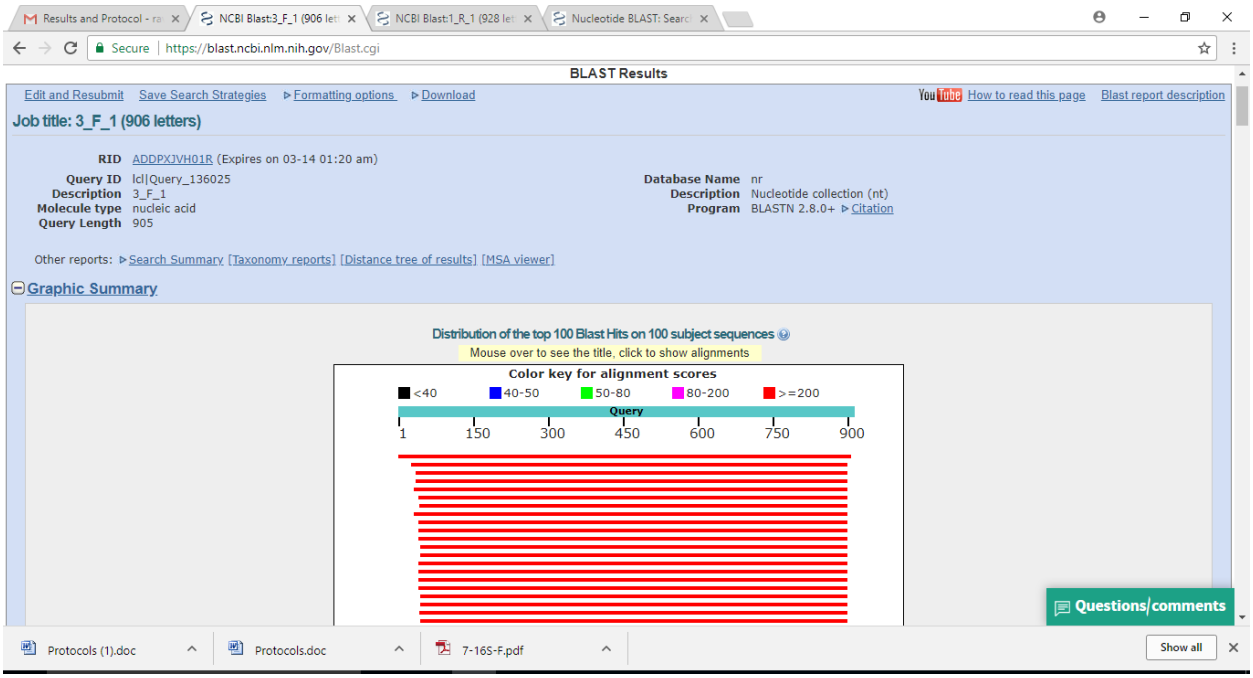
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Edit and Resubmit
Save Search Strategies
Formatting options
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How to read this page
Blast report description
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Molecule type nucleic acid
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Database Name nr
Description Nucleotide collection (nt)
Program BLASTN 2.8.0+ Citation
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Graphic Summary
Distribution of the top 100 Blast Hits on 100 subject sequences
Mouse over to see the title, click to show alignments
Color key for alignment scores
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80-200
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Questions/comments
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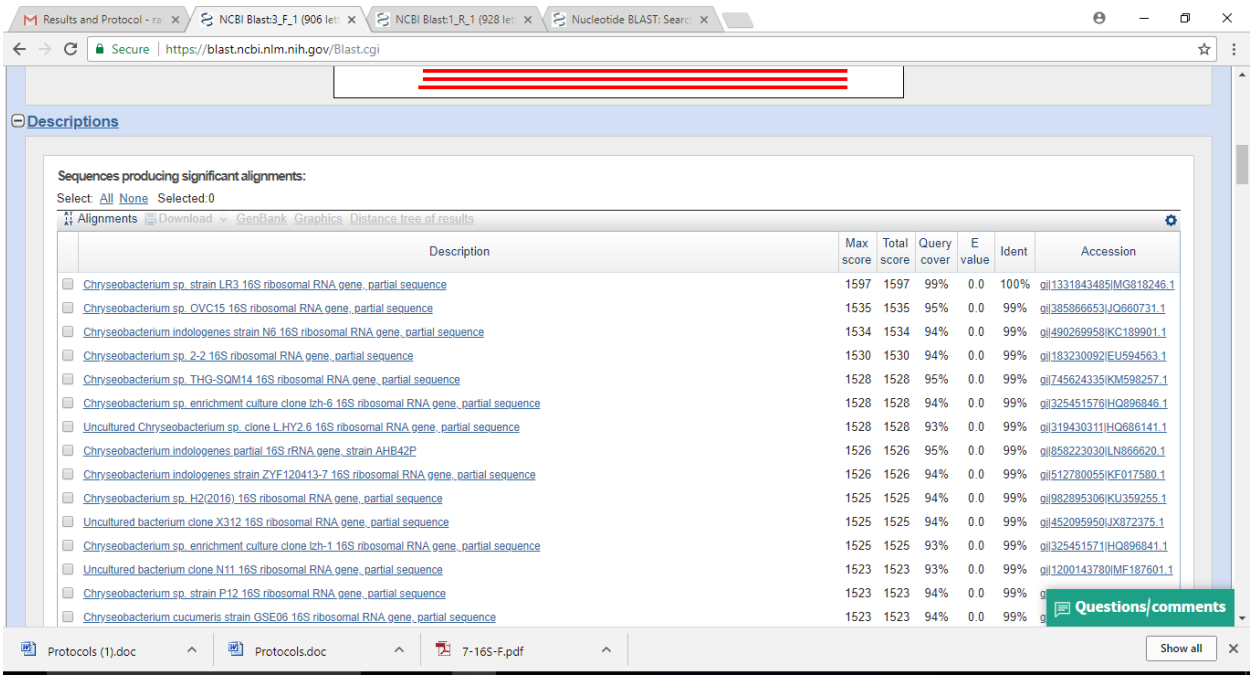

Homology Analysis/ Alignement:

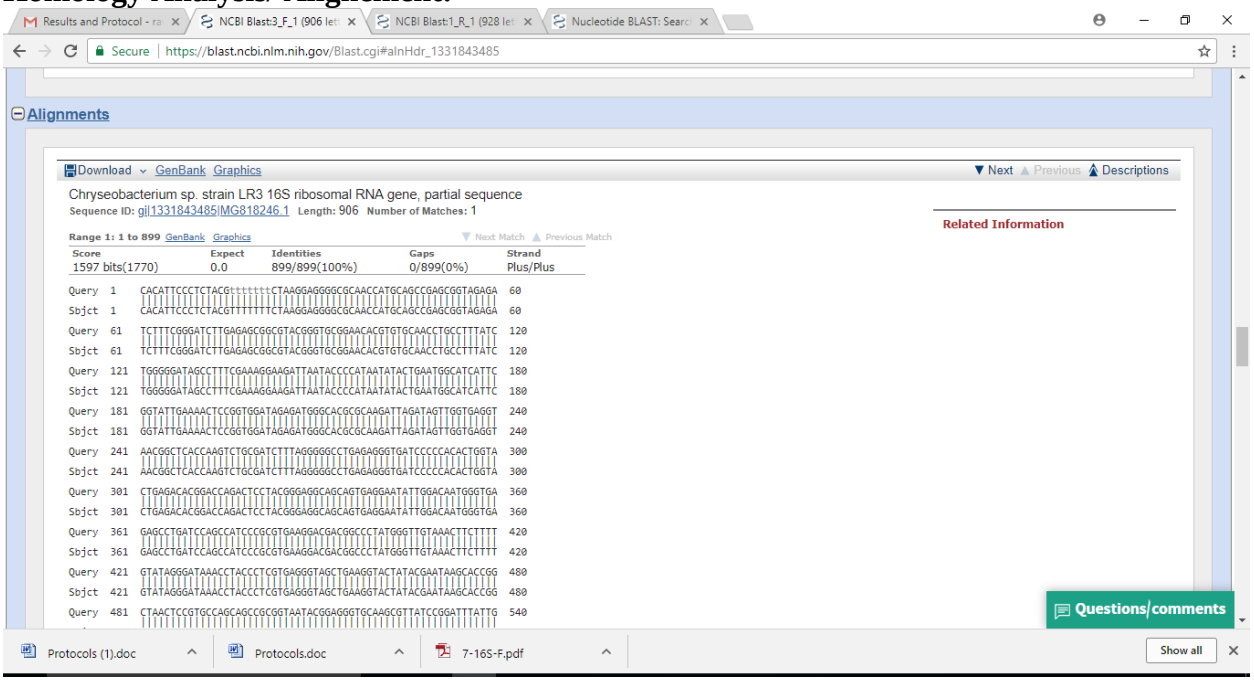

Fig. 4.70 Blast results of strain LR_3

The BLAST results indicate a good match with DNA database with over 99% homology and an e-Value of 0.0. The microbe in the present study was found to be from the *Chryseobacterium* sp.

4.7.6.4 Strain LR_4

Fig. 4.71 DNA sequence chromatogram of strainLR_4

DNA Sequence in FASTA Format:

AGGAATCTGCCTGGTAGAATGGGGGACAACGTTTCCCGAAAGGAACGCTAATACCGCATACGTCCTAC
GGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCANATGAGCCTAGGTCGGACTAGCTAGTTGGTG
GTTGGTAATGGCTCACCAAGGCGTGAACGATCCGTAACTGGTCTGAGAGGATGATCAGTCAGAACACT
GGAACTGGGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAANNATGGACAATGGGCGA
ANGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGTGCGTCTTCGGATTGTAAAGCACTTTAAGTTGG
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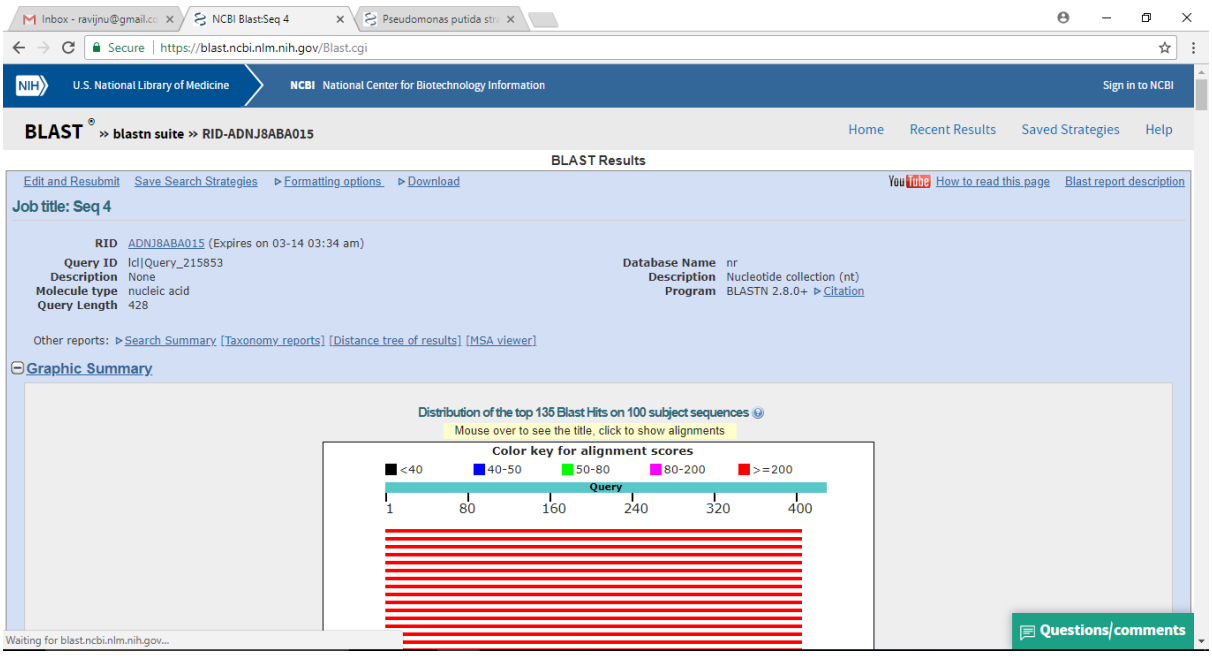
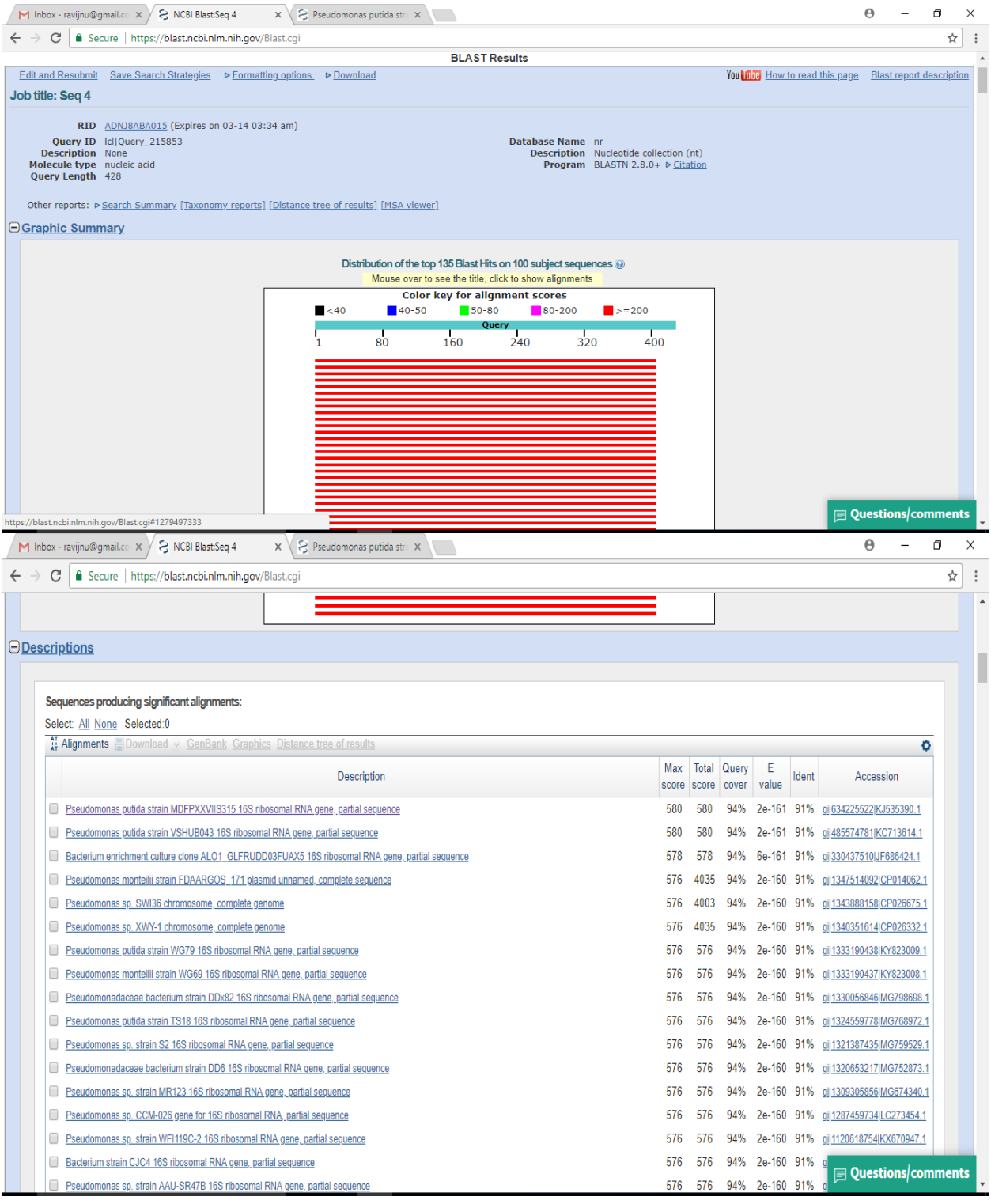
BLAST result





Homology analysis/alignment

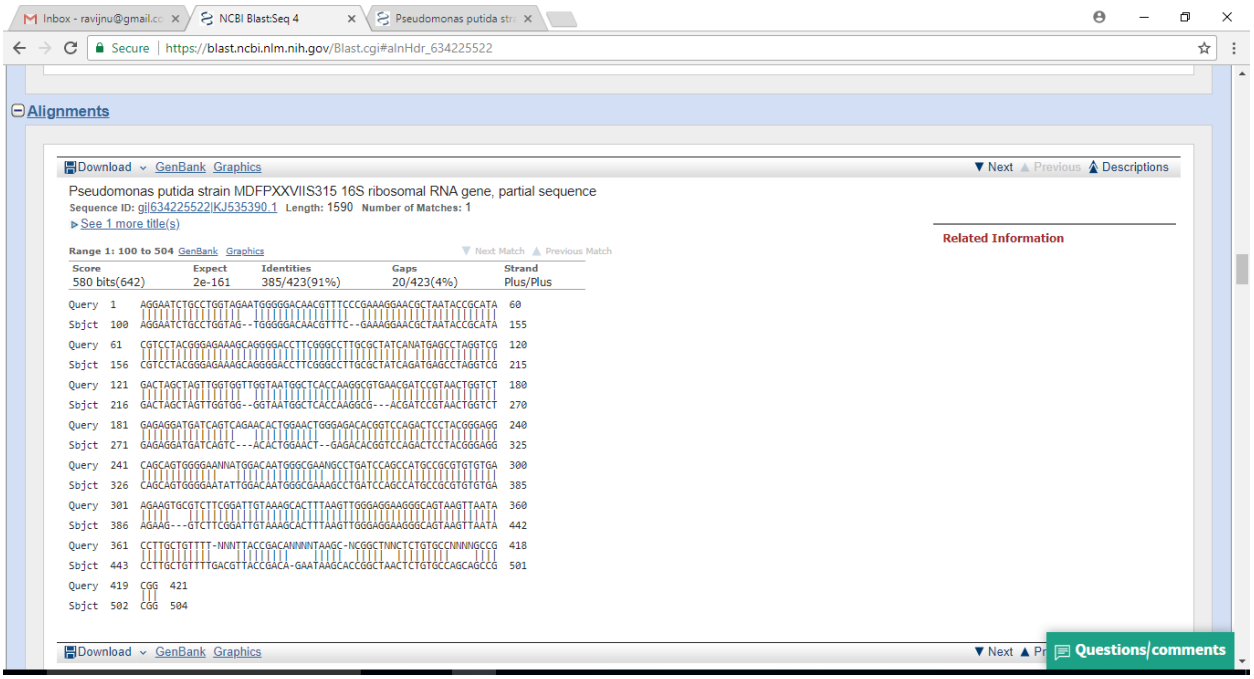


Fig. 4.72 Blast results of strain LR_4

The BLAST results indicate a good match with DNA database with over 91% homology and an e-Value of 2e-161 AND gaps of 4%. The microbe in the present study was found to be from the *Pseudomonas putida.*

On the basis of morphological study, biochemical testing and molecular characterization, we confirmed the four bacterial strains with the following identities:

1. LR_1 / LRN_1 - *Bacillus cereus*
2. LR_2 / LRN_2 - *Pseudomonas* sp
3. LR_3 / LRN_3 - *Chryseobacterium* sp
4. LR_4 / LRN_4 - *Pseudomonas putida*

5. Summary and conclusion

- A decline in response (germination & seedling) was seen at higher concentration (100 ppm) of malathion that inhibited the growth and development of wheat.
- In green gram 400 ppm of malathion inhibited the seed germination and growth, and development of seedling.
- A decline in response (germination & seedling) was seen at increasing concentration of lindane, a drastic decrease in percentage germination occurred at higher concentration (50 ppm) of lindane as compared to control in both wheat and green gram.
- At the end of 9 months of enrichment by using commercial formulations of organophosphates and pyrethroids mixture (500 ppm), morphologically 47 distinct bacteria were obtained on nutrient agar plates.
- All the 47 isolates were again mixed in equal quantities (0.01 OD) to reconstitute the consortium (undefined) and used for screening.
- This consortium showed 90.45 % - 55.6% degradation of 5-30 ppm lindane by 6 days.
- The temperature and pH optima were found to be 30^{o}C and 6 respectively.
- When this consortium was induced with lindane in broth only four cultures survived while 24 isolates showed the ability to clear lindane film on a nutrient agar plate.
- These four isolates constituted as a defined consortium for the degradation of lindane.
- The individual bacterial strain (LR_4) exhibited 50% degradation of 20 ppm lindane by 6 days
- In binary culture system, maximum degradation was observed by $LR_3 + LR_4$
- In ternary culture system, maximum degradation was observed by $LR_2 + LR_3 + LR_4$
- In presence of glucose, the maximum (80%) degradation by LR_4 bacterial isolates of 20 ppm lindane in 5 days.

- In presence of peptone, the maximum (50%) degradation by LR_3 bacterial isolates of 20 ppm lindane in 5 days.
- In presence of Tween 80, the maximum (45%) degradation by LR_4 bacterial isolates of 20 ppm lindane in 5 days.
- In presence of Triton X-100, the maximum (80%) degradation by LR_4 bacterial isolates of 20 ppm lindane in 5 days.

In conclusion, we can say that the pesticides have negative effect on the seed germination of wheat and green gram. While malathion inhibited at higher concentrations (25 ppm to 400 ppm) with lowering production of primary metabolites, photosynthetic pigments. Lindane, as compared to malathion, was able to impose inhibition on seed germination at low concentrations (20 ppm).
A microbial population was enriched completely on mixtures of commercial formulations of organophosphate and pyrethroid classes of pesticides that was used for degrading lindane. This consortium could degrade 5 ppm through 30 ppm in a short period of 6 days. The optimum pH was 6 while optimum temperature was found to be 30 ^{o}C. A response surface model was generated to know the degradation at various conditions of initial concentration of lindane, pH, temperature, initial inoculum size, and incubation period. Cometabolic studies reveled that efficient degradation could be effected in presence of glucose with individual member isolate LR_4. The consortiuim can be applied for remediation of contaminated water bodies; of course, after further vigorous training (acclimation) of the consortium with lindane in presence of the organophosphates and pyrethroids.

This is the first report where the consortium was enriched on non-organochlorine pesticides and used to study its potential to degrade an organochlorine pesticide, lindane. This probably is the beginning of new generation bioremediation practices to combat the pesticide hazards to living organisms and initiate bioremediation strategies to clean our environment for the upcoming generations.

5.1 Future perspectives

With the advancement in synthetic chemistry, more and more pollutants are being added in to the environment causing concern. To create a healthy and clean environment for the future generation , our consortium can be a useful tool. It can be used used for environment reclamation by

remediating various organochlorines, organophosphates, carbamates, pyrethroids and other organic pollutants. This might demand more vigorous training for the consortium to act efficiently on these various compounds of health and environmental hazard. Since most of the pollutants pose danger to humans and other animals bby entering the food chain through agriculture practices, the crops and other vegetations can be protected from the entry of pollutants with the help of bioremediation effected by our consortium.

For every problem created by man, there is a hidden solution in nature. Our consortium is also a product of nature, except that we trained it for use in bioremediation of lindane. The way it was visualized to be of future prospect, could take it a long way in dealing with the environmental hazards.

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List of publications

A. Research papers

1. Bidlan, R., **Kumar, S.** and Sharma, J.G. 2017. Effect of Mixed Commercial Formulations of Pesticides on the Germination of Mono and Dicotyledonous Crop Seeds. International Journal for Research in Applied Science & Engineering Technology. 5 (XII): 1952-1960 ISSN: 2321-9653
2. **Kumar S.** and Sharma J.G. 2017. Effect of malathion on seed germination and photosynthetic pigments in *Triticum astivum* L. Asian Journal of Applied Science and Technology. 1(7): 158-167
3. Raju, S. M., Bashambu, D., Bidlan, R., Sharma, J. G. and **Kumar, S.** 2017. Enhancement in Seed Germination through Simultaneous Degradation of Organochlorine Pesticides (Lindane and DDT) by A Novel Microbial Consortium. *Int.J.Adv.Res.Sci.Eng 6*(7): 707- 713, ISSN: 2319-8354 (Online).
4. **Kumar, S.** and Sharma, J.G. 2017. Isolation and Characterization of Lindane Degrading Bacteria from Enrichment on Multiple Pesticides. *Int.J.Adv.Res.Sci.Eng 6*(7): 699- 706, ISSN: 2319-8354 (Online)
5. **Kumar, S.,** Bidlan, R. and Sharma, J.G. 2016. Degradation of Lindane by Sludge Enriched on Mixed Commercial Formulations of Organophosphate and Pyrethroid Pesticides. *Int.J.Curr.Microbiol.App.Sci 5*(5): 138- 152, ISSN: 2319-7706 (Online).

B. Posters presentationed

- **Satish Kumar**, Rajkumar Bidlan, Shilpi Ahluwalia, Puneet and Jai Gopal Sharma 2018. Effect of Malathion on Seed Germination, Growth of Seedling and Photosynthetic Pigments in Green Gram (*Vigna radiata* L.) Presented in National Conference on Challenges and Strategies to Improve Crop Productivity in Changing Environment: An Integrated Approach organized by Zakir Husain Delhi College held on Jan 12, 2018.

- **Kumar, S.,** Bidlan, R. and Sharma, J.G. 2017. Co-metabolic degradation of Lindane by Bacteria Enriched on Organophosphates and Pyrethroids Pesticides Mixture Presented in International Conference and Outreach Program *"Environment & Ecology: Sustainability and Challenges"* organized by Sri Venkateswara College held on 4^{th}- 6^{th} Jan 2017

- **Kumar, S.,** Bidlan, R. and Sharma, J.G. 2016. Sludge novel bacteria for pesticide (lindane) degradation. Presented in *India International Science Festival (IISF)* 07- 11 Dec 200 at National Physical Laboratory (NPL) Delhi, India organized by CSIR, Ministry of Science and Technology & Ministry of Earth Science, Govt. of India.